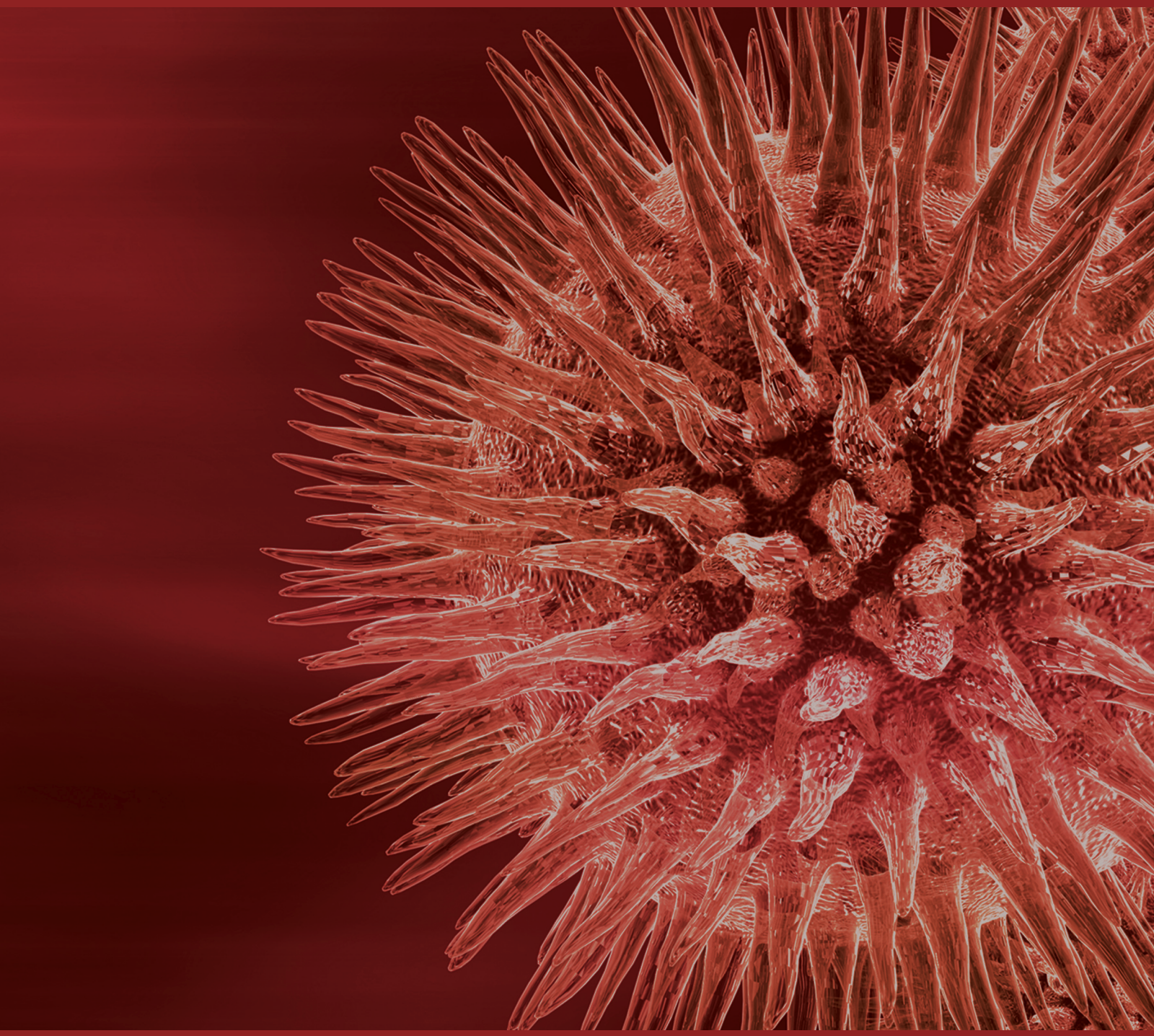
BioMed Research International

Laboratory Genetic Testing in Clinical Practice

Guest Editors: Ozgur Cogulu, Yasemin Alanay, and Gokce A. Toruner



Laboratory Genetic Testing in Clinical Practice

BioMed Research International

Laboratory Genetic Testing in Clinical Practice

Guest Editors: Ozgur Cogulu, Yasemin Alanay,
and Gokce A. Toruner

Contents

Laboratory Genetic Testing in Clinical Practice, Ozgur Cogulu, Yasemin Alanay, and Gokce A. Toruner
Volume 2013, Article ID 532897, 1 page

Identification and Characterization of DM1 Patients by a NewDiagnostic Certified Assay: Neuromuscular and Cardiac Assessments, Rea Valaperta, Valeria Sansone, Fortunata Lombardi, Chiara Verdelli, Alessio Colombo, Massimiliano Valisi, Elisa Brigonzi, Elena Costa, and Giovanni Meola
Volume 2013, Article ID 958510, 6 pages

Ultradeep Pyrosequencing of Hepatitis C Virus Hypervariable Region 1 in Quasispecies Analysis, Kamila Caraballo Cortés, Osvaldo Zagordi, Tomasz Laskus, Rafal Ploski, Iwona Bukowska-Ośko, Agnieszka Pawelczyk, Hanna Berak, and Marek Radkowski
Volume 2013, Article ID 626083, 10 pages

Feasibility of a Microarray-Based Point-of-Care *CYP2C19* Genotyping Test for Predicting Clopidogrel On-Treatment Platelet Reactivity, Hyojin Chae, Myungshin Kim, Yoon-Seok Koh, Byung-Hee Hwang, Min-Kyu Kang, Yonggoo Kim, Hae-il Park, and Kiyuk Chang
Volume 2013, Article ID 154073, 5 pages

FISH Detection of *PML-RARA* Fusion in ins(15;17) Acute Promyelocytic Leukaemia Depends on Probe Size, Lynda J. Campbell, Paul Oei, Ross Brookwell, Jake Shortt, Nicola Eaddy, Ashley Ng, Edward Chew, and Peter Browett
Volume 2013, Article ID 164501, 4 pages

Molecular Genetics and Genetic Testing in Myotonic Dystrophy Type 1, Dušanka Savić Pavićević, Jelena Miladinović, Miloš Brkušanin, Saša Šviković, Svetlana Djurica, Goran Brajušković, and Stanka Romac
Volume 2013, Article ID 391821, 13 pages

***TNNT2* Gene Polymorphisms Are Associated with Susceptibility to Idiopathic Dilated Cardiomyopathy in the Han Chinese Population**, Xiaoping Li, Huan Wang, Rong Luo, Haiyong Gu, Channa Zhang, Yu Zhang, Rutai Hui, Xiushan Wu, and Wei Hua
Volume 2013, Article ID 201372, 6 pages

Molecular and Survival Differences between Familial and Sporadic Gastric Cancers, Wen-Liang Fang, Shih-Ching Chang, Yuan-Tzu Lan, Kuo-Hung Huang, Su-Shun Lo, Anna Fen-Yau Li, Chin-Wen Chi, Chew-Wun Wu, and Shih-Hwa Chiou
Volume 2013, Article ID 396272, 10 pages

Implementation of High Resolution Whole Genome Array CGH in the Prenatal Clinical Setting: Advantages, Challenges, and Review of the Literature, Paola Evangelidou, Angelos Alexandrou, Maria Moutafi, Marios Ioannides, Pavlos Antoniou, George Koumbaris, Ioannis Kallikas, Voula Velissariou, Carolina Sismani, and Philippos C. Patsalis
Volume 2013, Article ID 346762, 14 pages

Detection of *C. trachomatis* in the Serum of the Patients with Urogenital Chlamydiosis, Naylia A. Zigangirova, Yulia P. Rumyantseva, Elena Y. Morgunova, Lidia N. Kapotina, Lubov V. Didenko, Elena A. Kost, Ekaterina A. Koroleva, Yuriy K. Bashmakov, and Ivan M. Petyaev
Volume 2013, Article ID 489489, 7 pages

Diagnosis of Familial Wolf-Hirschhorn Syndrome due to a Paternal Cryptic Chromosomal Rearrangement by Conventional and Molecular Cytogenetic Techniques, Carlos A. Venegas-Vega, Fernando Fernández-Ramírez, Luis M. Zepeda, Karem Nieto-Martínez, Laura Gómez-Laguna, Luz M. Garduño-Zarazúa, Jaime Berumen, Susana Kofman, and Alicia Cervantes
Volume 2013, Article ID 209204, 8 pages

The Italian National External Quality Assessment Program in Molecular Genetic Testing: Results of the VII Round (2010-2011), F. Censi, F. Tosto, G. Floridia, M. Marra, M. Salvatore, A. M. Baffico, M. Grasso, M. A. Melis, E. Pelo, P. Radice, A. Ravani, C. Rosatelli, N. Resta, S. Russo, M. Seia, L. Varesco, V. Falbo, and D. Taruscio
Volume 2013, Article ID 739010, 8 pages

Targeting the Immunogenetic Diseases with the Appropriate HLA Molecular Typing: Critical Appraisal on 2666 Patients Typed in One Single Centre, M. Guarene, C. Capittini, A. De Silvestri, A. Pasi, C. Badulli, I. Sbarsi, A. L. Cremaschi, F. Garlaschelli, C. Pizzochero, M. C. Monti, C. Montecucco, G. R. Corazza, D. Larizza, P. E. Bianchi, L. Salvaneschi, and M. Martinetti
Volume 2013, Article ID 904247, 7 pages

Clinical Genetic Testing of Periodic Fever Syndromes, Annalisa Marcuzzi, Elisa Piscianz, Giulio Kleiner, Alberto Tommasini, Giovanni Maria Severini, Lorenzo Monasta, and Sergio Crovella
Volume 2013, Article ID 501305, 8 pages

***HOXA4* Gene Promoter Hypermethylation as an Epigenetic Mechanism Mediating Resistance to Imatinib Mesylate in Chronic Myeloid Leukemia Patients**, Marjanu Hikmah Elias, Abdul Aziz Baba, Azlan Husin, Sarina Sulong, Rosline Hassan, Goh Ai Sim, S. Fadilah Abdul Wahid, and Ravindran Ankathil
Volume 2013, Article ID 129715, 7 pages

IROme, a New High-Throughput Molecular Tool for the Diagnosis of Inherited Retinal Dystrophies, Daniel F. Schorderet, Alexandra Iouranova, Tatiana Favez, Leila Tiab, and Pascal Escher
Volume 2013, Article ID 198089, 9 pages

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 532897, 1 page
http://dx.doi.org/10.1155/2013/532897

Editorial

Laboratory Genetic Testing in Clinical Practice

Ozgur Cogulu,[1,2] Yasemin Alanay,[3] and Gokce A. Toruner[4]

[1] *Division of Genetics, Department of Pediatrics, Faculty of Medicine, Ege University, 35100 Izmir, Turkey*
[2] *Department of Medical Genetics, Faculty of Medicine, Ege University, Izmir, 35100 Bornova, Turkey*
[3] *Pediatric Genetics Unit, Department of Pediatrics, Acibadem University School of Medicine, 34457 Istanbul, Turkey*
[4] *Cytogenetics & Molecular Genetics Laboratories, Institute of Genomic Medicine, UMDNJ-NJ Medical School, Newark, NJ 07109, USA*

Correspondence should be addressed to Ozgur Cogulu; ozgur.cogulu@ege.edu.tr

Received 18 June 2013; Accepted 18 June 2013

With the advances in genetic technology, the pages of the book of human biology and related diseases are turning over and over. Every new page is bringing a different vision into the life of humans not only limited to the scientific area but also to the daily life as well. The material used in genetic methods has found its place in this film with the discovery of a weak acid in the nuclei of white blood cells that we call DNA today. Unraveling the structure of DNA and chromosomes enabled us to find out the causes of many genetic diseases by cytogenetic and molecular genetic techniques. In situ hybridization with DNA probes followed these developments, and genome technology has advanced to the point of high-throughput sequencing nowadays. Throughout this period, the Human Genome Project provided a rapid increase of our insight into the field of human biology. The data obtained from this project allowed us to understand the underlying mechanisms of many genetic diseases and led to great interest among the scientific community.

On the other hand, the need for updated information in relation to the use of genetic techniques in the clinical practice of the clinicians increased in parallel with advances in genome technology. The practical use of the aforementioned genetic testing methods and proper interpretation of the generated test results have become a necessity not only for medical geneticists but also for the other specialists. Furthermore, physicians need to know how to order the most appropriate genetic test in the right time for the right indication to prevent under- or overutilization of those tests. That is why this special issue aimed to provide information about genetic techniques and their use in different research areas to improve knowledge, attitudes, and practices of physicians and researchers.

In this special issue, we would like to highlight both conventional and novel genetic methods, namely, classical cytogenetics, fluorescence in situ hybridization, PCR, RT-PCR, methylationspecific PCR, pyrosequencing, DNA sequencing, fully automated microarray-based assay, whole genome array CGH, MALDI-TOF mass spectrometry, and Southern blot analysis in a number of clinical conditions such as cancer, periodic fever syndromes, cardiomyopathy, myotonic dystrophy, retinal dystrophies, Wolf-Hirschhorn syndrome, and immunogenetic diseases.

Unquestionably those techniques do not comprise all of the methods in genomic studies; however they may give clues to understanding their potential use in clinical practice. Because genetic tests are unique and require robust quality assurance, quality assessment of genetic testing is undoubtedly necessary; therefore a study, which focused on the role of external quality assessments, was also featured in this issue as well.

Ozgur Cogulu
Yasemin Alanay
Gokce A. Toruner

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 958510, 6 pages
http://dx.doi.org/10.1155/2013/958510

Research Article

Identification and Characterization of DM1 Patients by a New Diagnostic Certified Assay: Neuromuscular and Cardiac Assessments

Rea Valaperta,[1] Valeria Sansone,[2] Fortunata Lombardi,[1] Chiara Verdelli,[1] Alessio Colombo,[3] Massimiliano Valisi,[3] Elisa Brigonzi,[2] Elena Costa,[1,4] and Giovanni Meola[2]

[1] *Research Laboratories-Molecular Biology, IRCCS Policlinico San Donato, Piazza E. Malan 2, San Donato Milanese, 20097 Milan, Italy*
[2] *Department of Neurology, Stroke Unit and Centre for Neuromuscular Disease, IRCCS Policlinico San Donato, Milan, Italy*
[3] *Service Lab, Fleming Research, Milan, Italy*
[4] *Service of Laboratory Medicine, IRCCS Policlinico San Donato, Milan, Italy*

Correspondence should be addressed to Rea Valaperta; rea.valaperta@grupposandonato.it

Received 26 October 2012; Accepted 19 February 2013

Academic Editor: Yasemin Alanay

The expansion of the specific trinucleotide sequence, [CTG], is the molecular pathological mechanism responsible for the clinical manifestations of DM1. Many studies have described different molecular genetic techniques to detect DM1, but as yet there is no data on the analytical performances of techniques used so far in this disease. We therefore developed and validated a molecular method, "*Myotonic Dystrophy SB kit,*" to better characterize our DM1 population. 113 patients were examined: 20 DM1-positive, 11 DM1/DM2-negative, and13 DM1-negative/DM2-positive, who had a previous molecular diagnosis, while 69 were new cases. This assay correctly identified 113/113 patients, and all were confirmed by different homemade assays. Comparative analysis revealed that the sensitivity and the specificity of the new kit were very high (>99%). Same results were obtained using several extraction procedures and different concentrations of DNA. The distribution of pathologic alleles showed a prevalence of the "classical" form, while of the 96 nonexpanded alleles 19 different allelic types were observed. Cardiac and neuromuscular parameters were used to clinically characterize our patients and support the new genetic analysis. Our findings suggest that this assay appears to be a very robust and reliable molecular test, showing high reproducibility and giving an unambiguous interpretation of results.

1. Introduction

In 1992, Myotonic Dystrophy type 1 (DM1) was shown to be caused by an expanded [CTG]*n* repeat in the 3′-untranslated region of the DMPK gene (dystrophia myotonica-protein kinase) in the chromosomal region 19q13.3 [1–3]. DM1 is the most common adult form of muscular dystrophy with a prevalence of 1 in 8000, characterized by progressive muscle weakness and atrophy, myotonia, early-onset cataracts and multiple organ involvement [4, 5]. The cardiac conduction system is selectively involved in DM1, and severe cardiac arrhythmias and respiratory insufficiency are the most frequent causes of death in these patients [6]. Currently, there are three known forms of DM1: "Mild", "Classic", and "Congenital." The "Mild" form has its onset after 50 years, only manifesting cataracts, myotonia, and a mild degree of muscle weakness. "Classic" myotonic dystrophy has onset between 10 and 60 years, presenting with myotonia, muscle weakness, cataracts, smooth muscle and cardiac muscle involvement, and multiple organ involvement. "Congenital" myotonic dystrophy (CDM) is symptomatic at birth or within the first year of life, presenting with respiratory and feeding difficulties and severe developmental delay. This form is almost always maternally transmitted. The number of [CTG] repeats is highly polymorphic, in both healthy individuals and DM1 patients. According to the DNA testing guidelines of

the EMQN (European Molecular Genetics Quality Network) [7], healthy individuals have alleles with between 5 and 37 [CTG] repeats [8–10], whereas in patients with clinical manifestations of DM1, the number of repeats varies from 51 to several thousands. Intermediate alleles with 38–50 triplets are not disease causing but they are considered as "pre-mutations"; repeats with alleles 51–100 are protomutations, both of which show increased instability towards expansions. Carriers of premutations or protomutations present no or few mild symptoms, such as cataracts [11, 12]. Anticipation is a specific event, where the number of repeats tends to increase as the disease is passed from one generation to the next, leading to increasing severity of symptoms and decreasing age of onset [13, 14]. Although there are studies demonstrating that the severity of phenotype, age of onset of myotonia and muscle wasting, and cardiac conduction abnormalities appear to be associated with an increase in the number of leukocyte [CTG] repeats [15–19], the relationship between phenotype and genotype is still controversial [20]. These conflicting results may be explained by the instability of [CTG] repeats and tissue specificity expression so that the expansion in leukocytes may underestimate the actual expansion in the specific tissues studied in the correlation analysis.

Another feature of DM1 is the incomplete penetrance, characterized by variable clinical signs within individuals having the same expansion sizes or within families. Symptoms can be overlapping with other dominant noncoding expansion disorders, such as DM2 or spinocerebellar ataxias (SCAs) [21], and differential diagnosis based on clinical examination can be uncertain; therefore, genetic testing plays an important role in making an accurate diagnosis of DM1 disease because it allows direct detection of the [CTG] expansion. In fact, the direct DNA tests have reduced the number of invasive (muscle biopsy) and noninvasive, but relatively painful (electromyography) diagnostic techniques for the diagnosis of DM1 [22, 23]. The genetic tests are often used for symptomatic confirmatory diagnostic testing and predictive testing, after the finding of the mutation in an affected family member. It is also useful for prenatal diagnosis, in both amniotic fluid cells and chorionic villus samples (CVS) [24], for at-risk pregnancies after evidence of fetal hypotonia and reduced fetal movements, considering a possible maternal cell contamination. On one hand the testing is extremely helpful in identifying individuals who are asymptomatic or exhibit equivocal symptoms, such as cataracts. Many studies have described different molecular genetic techniques to detect DM1, but as yet there is no data on the sensitivity, specificity, and reproducibility of the techniques used so far in this disease. For this reason, in this work we developed a new molecular diagnostic assay, Myotonic Dystrophy SB kit, a standardized and certified method, based on the combination of Long-Polymerase Chain Reaction and Southern Blot Analysis (SBA), to better characterize the DM1 mutation in a cohort of clinically well-defined DM1 patients attending the Neuromuscular Clinic at IRCCS Policlinico San Donato.

The principal aims of this study were to evaluate (i) the analytical performances of the Myotonic Dystrophy SB kit and (ii) the distribution of pathologic and normal [CTG] repeats in a population of northern Italy.

2. Materials and Methods

Informed consent was obtained from all patients in our study.

2.1. Subjects. From May 2010 to May 2012 a cohort of 113 patients attending the Neuromuscular Clinic at IRCCS Policlinico San Donato were subjected to the molecular genetics analysis for determination of DM1. All subjects were of Italian nationality and evenly distributed by sex and age: 39 female 34.5% (average age 40aa ± 19) and 74 male 65.5% (mean age 45aa ± 16). Patients were selected as follows: 20 DM1-positive (17.7%), 11 DM1/DM2-negative (9.7%), and 13 DM1-negative/DM2-positive (11.5%) from a previous molecular diagnosis with different "homemade" tests, including: Gold Standard Assay, TP-PCR, and Extra-Long-PCR with Southern Blot Analysis. The inclusion of patients with DM2 has allowed a better evaluation of the specificity of the new commercial kit. Sixty-nine subjects (61.1%) were new cases with unknown genotype but enrolled because they presented one or more of the following diagnostic criteria: positive family history, cataracts, myotonia, and proximal or distal weakness.

2.2. Muscle Strength Assessments. Muscle strength was evaluated using the modified Medical Research Council (MRC). 15 muscles on the left and right were included, adding up to a total of 150 for normal muscle strength.

2.3. Cardiac Evaluation. All patients were subjected to standard ECG, 24-hour ECG-Holter monitoring, and 2D echocardiograms.

2.4. DNA Isolation. The extraction of genomic DNA from peripheral blood in EDTA is that we performed with the commercial kit "High Pure PCR preparation kit Template" Roche. The quality and quantity of the extracted DNAs were determined by a spectrophotometer (NanoDrop).

2.5. Components of the "Myotonic Dystrophy SB Kit" (Experteam s.r.l, Venezia, Italy). Inside the kit there are a ready-to-use Master Mix (DM Master MIX), an Extra-Long Polymerase (DM DNA Polymerase), digoxigenin-labeled probe, DNA Molecular Weight Markers VII and VIII, DIG labeled (Roche Diagnostics), and step-by-step instructions and suggestions for optimization the analysis.

2.6. Long-PCR and MethaPhore Analysis. one μg of genomic DNA of each patient was amplified in a reaction volume of 100 μL, containing 55 μL of "DM Master MIX" and 4 μL of "DM DNA Polymerase." Forward primer was labeled at the 5′ end with fluorescent tag 6-FAM. PCR conditions were one cycle of 1 min at 94°C; 28 cycles of 20 sec at 94°C and 7 min at 62°C; and finally 10 min at 72°C. The amplifications were performed by MyCycler instrument (BioRad). After the

amplification 20 μL of each PCR product was run on 3.5% MethaPhore agarose gel at 100 V and stained with ethidium bromide. Alleles with less than 100 repeats were analyzed by capillary electrophoresis on 3500 Genetic Analyzer (Applied Biosystems) using LIZ600 as size standard. The analysis of results was performed using GeneMapper v4.1 (Applied Biosystems). For alleles with more than 100 repeats Southern Blot hybridization was performed using the DIG-labeled probe as described in the next section.

2.7. Southern Blot Analysis. Thirty-five microliters of PCR products were separated by electrophoresis on 1% agarose gels, transferred to Nylon Membranes (Roche Diagnostics) and hybridized overnight with a nonradioactive Digoxigenin-based probe, 5′DIG-labeled $[CTG]_{10}$. After being washed, the blots were incubated with antidigoxigenin alkaline phosphatase conjugate (AP) (Roche Diagnostics), and this one was detected by the addition of ready-to-use CDP Star (Roche Diagnostics). The chemiluminescence signal was visualized on the ChemiDoc Instrument (BioRad) after several exposures. finally we compared the bands obtained with two DNA molecular weight markers, DIG labeled.

2.8. Molecular Diagnosis. The genetic diagnosis was based on the guidelines of the EMQN; the technical validation of each analytical run was subjected to internal quality controls evaluation. Positive (high and low) and negative controls were patients with a previous molecular diagnosis. In addition after the genetic test, each patient was retrospectively reviewed for the distribution of pathologic and normal alleles, containing [CTG] repeats. The [CTG] repeats size in each allele was determined by capillary electrophoresis or by Southern Blot Analysis.

2.9. TP-PCR. TP-PCR was performed as discussed elsewhere [25, 26], using 500 ng of genomic DNA, after the amplicons were analyzed by capillary electrophoresis.

2.10. Statistical Analysis. The [CTG] expansion of each group was expressed as mean ± standard deviation and range. Statistical analyses were evaluated by Student's t-test. Probability values $P < 0.05$ were considered statistically significant.

3. Results

3.1. Assay Performance Characteristics. 113 patients (74 males and 39 females) were subjected to molecular analysis using the new commercial assay Myotonic Dystrophy SB kit, for detection of DM1 disease, including those in whom genetic test results were previously known from other laboratories using previous nonstandardized techniques. Out of 113 individuals, sixty-five patients (57.5%) were diagnosed genetically as DM1 patients and forty-eight (42.5%) as DM1-negative patients. The assay confirmed the diagnosis in the 20 patients in whom previous testing had shown [CTG] expansions consistent with DM1 and it also confirmed normal [CTG] expansions in the 11 patients in whom DM1 or DM2 had been previously ruled out. All patients with the genetic diagnosis of DM1 fulfilled clinical and laboratory criteria for the disease. None of the 13 patients with DM2, who had been included in this study to increase the specificity, had [CTG] expansion size consistent with DM1. Sixty-nine new cases, with unknown genotype, were correctly identified by new molecular assay and divided as follows: forty-five were DM1-positive and twenty-four were DM1-negative. Results of the new molecular assay were compared with "homemade assays" on 113 DNA samples (Table 1). Comparative analysis revealed that the sensitivity, the specificity, and the accuracy of Myotonic Dystrophy SB kit were very high (>99%). No false-negative results or failed amplifications were observed. Furthermore, we obtained the same results using several procedures for extraction of genomic DNA, from fresh or frozen blood samples, with different concentrations of the same (300–1000 ng of genomic DNA). In addition, we checked interrater reliability by checking results from three different lab technicians, and we found that diagnostic performances of different operators with different degrees of experience were similar. Another feature of this molecular diagnostic test was the best resolution of larger expansions, which appear as single and well-defined bands especially if we consider that expanded alleles often appear as a smeared signal due to the somatic instability of the mutation (Figure 1).

3.2. Pathologic [CTG] Distribution. We investigated the distribution of [CTG] repeats in all 113 individuals. In our cohort of 65 of 113 affected patients, the expanded alleles ranged from 70 to 2500 [CTG] repeats (mean ± SD size of the [CTG] repeat expansion; 385 ± 396 repeats, range 70–2500). According to EMQN 2008 classification for DM1 (Table 2), DM1 is classified into premutation (range 38–50 [CTG]), "Mild" form (range 51–149 [CTG]), "Classic" form (range 150–2000 [CTG]), and "Congenital" form (range > 2001 [CTG]). Because the range of [CTG] repeats in the "Classic" form is very large and may include patients with borderline expansions in the mild range and patients with expansions close to those in the "congenital" range, in this work we separated this range in two specific classes: *E2a* class ranging from 150 to 450 [CTG] and *E2b* class ranging from 451 to 2000 [CTG]. The majority of pathologic alleles, about 83.1% (54/65), were in the range of "Classic" phenotype. In particular 42/65 patients (64.6%) showed *E2a* -genotype and 12/65 patients (18.5%) presented *E2b* -genotype. The remaining 13.9% (9/65) had the "Mild" form, between 51 and 149 repeats. Only two cases were found with the [CTG] expansions over 2000 repeats (3.1%). The verification of the expanded size was obtained by automated capillary electrophoresis, because the amplicons were labeled. There was no significant difference ($P > 0.05$) between males (mean ± SD, 381 ± 385 repeats, range 70–2500) and females (mean ± SD, 326 ± 141 repeats, range 105–570).

3.3. Normal [CTG] Distribution. Instead, the histogram presented in Figure 2 describes the distribution of nonexpanded normal [CTG] repeats length in 96 alleles from 48 normal subjects. The preponderance of normal individuals

Table 1: Analytical performances of Myotonic Dystrophy SB kit compared to different "homemade" assays.

Total $n = 113$	*Myotonic Dystrophy SB kit		"Homemade" assays	
	DM1$^+$	DM1$^-$	DM1$^+$	DM1$^-$
DM1-positive ($n = 65$)	65	0	65	0
DM1/DM2-negative ($n = 32$)	0	48	0	48
DM1-negative/DM2-positive ($n = 13$)	0	13	0	13

*Sensitivity = >99%, specificity = >99%.

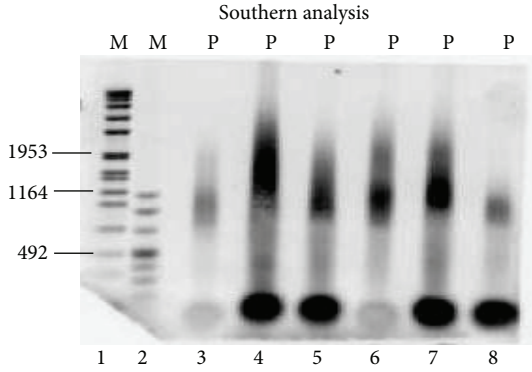


Figure 1: Expansion detection by Southern Blot Analysis. Lanes 1 and 2 are DNA molecular weight marker, (VII and VIII, resp.; Roche Diagnostics). Results for DM1 affected individuals are shown in lanes 3 to 8.

Table 2: Distribution of [CTG] expansions in 65 DM1 patients.

Molecular diagnosis	Clinical phenotype	[CTG] repeats	Number of patients
Premutation		38–50	0
DM1	"Mild"	51–149	9
	"Classic"	*E2a* 150–450	42
		E2b 451–2000	12
	"Congenital"	>2000	2
		Total DM1 patients	**65**

was heterozygous; only five patients presented homozygosity with five [CTG] repeats. After capillary electrophoresis, we observed nineteen different types of alleles and the size of [CTG] repeats ranged from 5 to 32. The allele most frequently observed presented 12 [CTG] expansions (24/96; 25%), followed by 13 (14/96; 14.6%), and finally by 14 (19/96; 19.8%) repeats. Thirteen patients presented the large normal alleles between 30–32 repeats (13/96; 13.5%), and none of the subjects had numbers of repeats near cut-off area (38 < [CTG] < 50).

3.4. Pitfalls of Molecular Analysis. Our experience demonstrates that, although conventional Long-PCR associated to Southern Blot Analysis proves to be accurate enough to detect large DM1 expansions, it is unsuitable for the identification of premutated or protomutated alleles and alleles with small

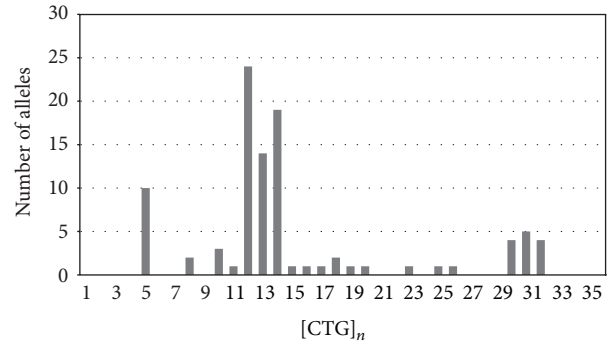


Figure 2: Distribution of [CTG] expansions in 96 normal alleles.

[CTG] size. Figure 3 shows three of the five homozygous-normal alleles (lines n° 4, 7, and 9) that our assay detects with a single band, while usually the heterozygous-normal alleles occur in two distinct bands (lines n° 6 and 11). As suggested by the guidelines, we went further with the analysis and confirmed these homozygous normal patients by TP-PCR: all homozygous patients analyzed were healthy.

3.5. Cardiac and Neuromuscular Assessments. To see whether [CTG] size correlated to muscle and cardiac impairment muscle strength, myotonia and ECG abnormalities were correlated to expansion size. Myotonia was the most frequent symptom at onset (about 70%), followed by distal muscle weakness (56%). There was no correlation between symptom at onset and [CTG] size. One quarter of our patients had abnormal ECGs (PR intervals > 200 msec; QRS duration > 120 msec) but had no symptoms suggestive of cardiac involvement. [CTG] expansion size did not correlate to the presence or absence of ECG abnormalities or degree of muscle weakness as assessed by MRC values.

4. Discussion

Myotonic Dystrophy type 1 belongs to a group of repeat disorders where an aberrant expansion of normally short tandem repeats in specific genes, known as "dynamic mutations," causes the disease. Molecular analysis represents, in these types of diseases, an essential tool to confirm the symptomatic manifestations, but it is also a predictive test. Currently, Southern Blotting of genomic DNA, digested with an appropriate restriction enzyme, has been the gold standard for the detection of DMPK alleles, with the use of several different probes for hybridization [27]. However,

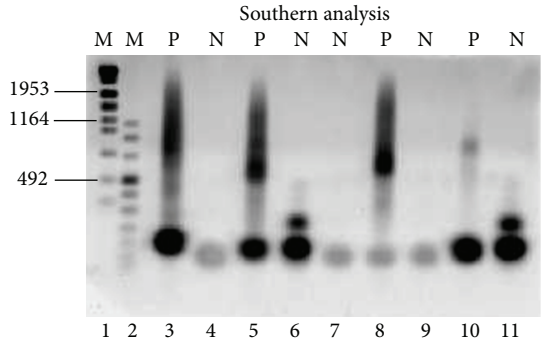


Figure 3: Expansion detection by Southern Blot Analysis. Lanes 1 and 2 are DNA Molecular Weight Marker, (VII and VIII, resp., Roche Diagnostics). Results for DM1 affected individuals are shown in lanes 3, 5, 8, and 10. Results for DM1 unaffected individuals are shown in lanes 4, 6, 7, 9, and 11, but three of these are homozygous normal (lanes 4, 7, and 9), confirmed by TP-PCR.

this procedure has a small false-negative rate because of the reduced sensitivity in cases of extreme somatic heterogeneity. For this reason, a Long-PCR associated with Southern Blot Analysis is still widely used and recommended for this type of disease. In this study, we developed and validated a molecular diagnostic method Myotonic Dystrophy SB kit, based on this type of methodology. The strong point of this assay is that all reagents are pre-packaged and ready to use. The analytical results, evaluated on a total of 113 DNA samples, in terms of sensitivity, specificity and accuracy were very high (>99%), and both prospective and retrospective analysis gave no false positives or false negatives. The opportunity, for molecular biology laboratories, to have CE-IVD marked product available, greatly reduces the probability of failures during PCR amplification or Southern Blot Analysis. This may reach 10% in some cases. On the other hand, the limitations related to the identification of premutated alleles and alleles with small [CTG] size can be overcome by checking the homozygous normal patients by TP-PCR. TP-PCR, for DM1detection, represents a robust and reliable PCR method that can rapidly identify the presence of expanded alleles for any disorder caused by repeat expansions. Although it can distinguish between healthy homozygous and affected heterozygous samples with no length restriction, it is not able to determine the exact size of the repeats over a certain threshold, that is, it is very important to allow correlation studies. The association of two molecular methods as a Long-PCR and Southern transfer, together with Triplet-repeat Primed (TP)-PCR [28, 29], is strongly recommended because they should be able to detect a wide range of mutations.

The analysis of distribution of large normal alleles can help to study the prevalence of DM1 in northern Italy. The different frequency of alleles with more than 20 [CTG] repeats also depends on ethnic groups [30] belonging and are not a pathologic cause in the individual but have been considered as a danger in the successive generation. In our study, the retrospective analysis showed that the most frequent normal allele presented 12 [CTG] repeats, and no individual carried a premutation allele. While the predominant pathologic [CTG] expansion size, in our population, was in the "*E2a*" range. The fact that no correlation was found between expansion size and muscle strength and ECG abnormalities should not be considered a limitation of molecular analysis and thus of the method, but it should be interpreted in the light of the instability of the expansion size and of tissue mosaicism. Where possible, correlations should be made between size of [CTG] expansion of the tissue involved and symptoms related to that tissue or system. In conclusion 20 years have passed since the [CTG]*n* repeat expansion mutation was discovered in patients with Myotonic Dystrophy type 1. Although much has been learned within this period, an identification and characterization of a biomolecular DM1 testing, such as that described in this paper, could be very helpful in clinical practice.

Acknowledgments

This study was financed by IRCCS Policlinico San Donato. All authors have contributed significantly to the work and have read and approved the paper. The authors are grateful to Dr. Federica Schiavon of Experteam s.r.l (Venezia, Italy), who provided "Myotonic Dystrophy SB kit."

References

[1] J. D. Brook, M. E. McCurrach, H. G. Harley et al., "Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member," *Cell*, vol. 68, no. 4, pp. 799–808, 1992.

[2] M. Mahadevan, C. Tsilfidis, L. Sabourin et al., "Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene," *Science*, vol. 255, no. 5049, pp. 1253–1255, 1992.

[3] Y. H. Fu, A. Pizzuti Jr., R. G. Fenwick et al., "An unstable triplet repeat in a gene related to myotonic muscular dystrophy," *Science*, vol. 255, no. 5049, pp. 1256–1258, 1992.

[4] T. Kurihara, "New classification and treatment for myotonic disorders," *Internal Medicine*, vol. 44, no. 10, pp. 1027–1032, 2005.

[5] G. Meola and E. Bugiardini, "Myotonic dystrophy," in *Medlink Neurology*, 2011.

[6] S. P. Chaudhry and W. H. Frishman, "Myotonic dystrophies and the heart," *Cardiology in Review*, vol. 20, no. 1, pp. 1–3, 2011.

[7] E. J. Kamsteeg, W. Kress, C. Catalli et al., "Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2," *European Journal of Human Genetics*, vol. 20, no. 12, pp. 1203–1208, 2012.

[8] L. Martorell, D. G. Monckton, A. Sanchez, A. Lopez de Munain, and M. Baiget, "Frequency and stability of the myotonic dystrophy type 1 premutation," *Neurology*, vol. 56, no. 3, pp. 328–335, 2001.

[9] The International Myotonic Dystrophy Consortium (IDMC), "New nomenclature and DNA testing guidelines for myotonic dystrophy type 1 (DM1)," *Neurology*, vol. 54, no. 6, pp. 1218–1221, 2000.

[10] T. Ashizawa and P. S. Sarkar, "Myotonic dystrophy types 1 and 2," *Handbook of Clinical Neurology*, vol. 101, pp. 193–237, 2011.

[11] C. J. Höweler, H. F. Busch, J. P. Geraedts, M. F. Niermeijer, and A. Staal, "Anticipation in myotonic dystrophy: fact or fiction?" *Brain*, vol. 112, pp. 3779–797, 1989.

[12] H. G. Harley, S. A. Rundle, J. C. MacMillan et al., "Size of the unstable CTG repeat sequence in relation to phenotype and parental transmission in myotonic dystrophy," *American Journal of Human Genetics*, vol. 52, no. 6, pp. 1164–1174, 1993.

[13] N. De Temmerman, K. Sermon, S. Seneca et al., "Intergenerational instability of the expanded CTG repeat in the DMPK gene: studies in human gametes and preimplantation embryos," *American Journal of Human Genetics*, vol. 75, no. 2, pp. 325–329, 2004.

[14] V. Rakocevic-Stojanovic, D. Savić, S. Pavlović et al., "Intergenerational changes of CTG repeat depending on the sex of the transmitting parent in myotonic dystrophy type 1," *European Journal of Neurology*, vol. 12, no. 3, pp. 236–237, 2005.

[15] M. F. Phillips and P. S. Harper, "Cardiac disease in myotonic dystrophy," *Cardiovascular Research*, vol. 33, no. 1, pp. 13–22, 1997.

[16] P. Melacini, C. Villanova, E. Menegazzo et al., "Correlation between cardiac involvement and CTG trinucleotide repeat length in myotonic dystrophy," *Journal of the American College of Cardiology*, vol. 25, no. 1, pp. 239–245, 1995.

[17] D. Brisson, M. Tremblay, C. Prévost, C. Laberge, J. Puymirat, and J. Mathieu, "Sibship stability of genotype and phenotype in myotonic dystrophy," *Clinical Genetics*, vol. 62, no. 3, pp. 220–225, 2002.

[18] A. Hunter, C. Tsilfidis, G. Mettler et al., "The correlation of age of onset with CTG trinucleotide repeat amplification in myotonic dystrophy," *Journal of Medical Genetics*, vol. 29, no. 11, pp. 774–779, 1992.

[19] C. Marchini, R. Lonigro, L. Verriello, L. Pellizzari, P. Bergonzi, and G. Damante, "Correlations between individual clinical manifestations and CTG repeat amplification in myotonic dystrophy," *Clinical Genetics*, vol. 57, no. 1, pp. 74–82, 2000.

[20] B. Schoser and L. Timchenko, "Myotonic dystrophies 1 and 2: complex diseases with complex mechanisms," *Current Genomics*, vol. 11, no. 2, pp. 77–90, 2010.

[21] L. P. W. Ranum and T. A. Cooper, "RNA-mediated neuromuscular disorders," *Annual Review of Neuroscience*, vol. 29, pp. 259–277, 2006.

[22] S. Cheng, J. M. Barceló, and R. G. Korneluk, "Characterization of large CTG repeat expansions in myotonic dystrophy alleles using PCR," *Human Mutation*, vol. 7, no. 4, pp. 304–310, 1996.

[23] M. Gennarelli, M. Pavoni, P. Amicucci, G. Novelli, and B. Dallapiccola, "A single polymerase chain reaction-based protocol for detecting normal and expanded alleles in myotonic dystrophy," *Diagnostic Molecular Pathology*, vol. 7, no. 3, pp. 135–137, 1998.

[24] P. Amicucci, M. Gennarelli, G. Novelli, and B. Dallapiccola, "Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma," *Clinical Chemistry*, vol. 46, no. 2, pp. 301–302, 2000.

[25] J. P. Warner, L. H. Barron, D. Goudie et al., "A general method for the detection of large GAG repeat expansions by fluorescent PCR," *Journal of Medical Genetics*, vol. 33, no. 12, pp. 1022–1026, 1996.

[26] M. Falk, M. Vojtísková, Z. Lukás, I. Kroupová, and U. Froster, "Simple procedure for automatic detection of unstable alleles in the myotonic dystrophy and Huntington's disease loci," *Genetic Testing*, vol. 10, no. 2, pp. 85–97, 2006.

[27] N. L. Carson, "Analysis of repetitive regions in myotonic dystrophy type 1 and 2," in *Current Protocols in Human Genetics*, chapter 9, 2009, unit 9. 6.

[28] G. Kakourou, S. Dhanjal, T. Mamas, P. Serhal, J. D. Delhanty, and S. B. Sengupta, "Modification of the triplet repeat primed polymerase chain reaction method for detection of the CTG repeat expansion in myotonic dystrophy type 1: application in preimplantation genetic diagnosis," *Fertility and Sterility*, vol. 94, no. 5, pp. 1674–1679, 2010.

[29] C. Catalli, A. Morgante, R. Iraci, F. Rinaldi, A. Botta, and G. Novelli, "Validation of sensitivity and specificity of tetraplet-primed PCR (TP-PCR) in the molecular diagnosis of myotonic dystrophy type 2 (DM2)," *Journal of Molecular Diagnostics*, vol. 12, no. 5, pp. 601–606, 2010.

[30] R. Deka, P. P. Majumder, M. D. Shriver et al., "Distribution and evolution of CTG repeats at the myotonin protein kinase gene in human populations," *Genome Research*, vol. 6, no. 2, pp. 142–154, 1996.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 626083, 10 pages
http://dx.doi.org/10.1155/2013/626083

Research Article

Ultradeep Pyrosequencing of Hepatitis C Virus Hypervariable Region 1 in Quasispecies Analysis

Kamila Caraballo Cortés,[1,2] Osvaldo Zagordi,[3] Tomasz Laskus,[1] Rafał Płoski,[4] Iwona Bukowska-Ośko,[1] Agnieszka Pawełczyk,[1] Hanna Berak,[5] and Marek Radkowski[1]

[1] *Department of Immunopathology of Infectious and Parasitic Diseases, Medical University of Warsaw, 3c Pawińskiego Street, 02-106 Warsaw, Poland*
[2] *Postgraduate School of Molecular Medicine, Żwirki i Wigury 61 Street, 02-091 Warsaw, Poland*
[3] *Institute of Medical Virology, University of Zurich, Winterthurerstrasse, 190 8057 Zurich, Switzerland*
[4] *Department of Medical Genetics, Medical University of Warsaw, 3c Pawińskiego Street, 02-106 Warsaw, Poland*
[5] *Hospital for Infectious Diseases, 37 Wolska Street, 01-201 Warsaw, Poland*

Correspondence should be addressed to Kamila Caraballo Cortés; kcaraballo@wum.edu.pl

Received 22 October 2012; Accepted 12 February 2013

Academic Editor: Ozgur Cogulu

Genetic variability of hepatitis C virus (HCV) determines pathogenesis of infection, including viral persistence and resistance to treatment. The aim of the present study was to characterize HCV genetic heterogeneity within a hypervariable region 1 (HVR1) of a chronically infected patient by ultradeep 454 sequencing strategy. Three independent sequencing error correction methods were applied. First correction method (Method I) implemented cut-off for genetic variants present in less than 1%. In the second method (Method II), a condition to call a variant was bidirectional coverage of sequencing reads. Third method (Method III) used *Short Read Assembly into Haplotypes* (ShoRAH) program. After the application of these three different algorithms, HVR1 population consisted of 8, 40, and 186 genetic haplotypes. The most sensitive method was ShoRAH, allowing to reconstruct haplotypes constituting as little as 0.013% of the population. The most abundant genetic variant constituted only 10.5%. Seventeen haplotypes were present in a frequency above 1%, and there was wide dispersion of the population into very sparse haplotypes. Our results indicate that HCV HVR1 heterogeneity and *quasispecies* population structure may be reconstructed by ultradeep sequencing. However, credible analysis requires proper reconstruction methods, which would distinguish sequencing error from real variability *in vivo*.

1. Introduction

Genetic variability is a characteristic feature of hepatitis C virus (HCV), due to an absence of error correction mechanisms of the viral RNA-dependent RNA polymerase, fast replication, and recombination events [1–3]. As a consequence, HCV displays high intrahost population diversity, forming a pool of closely related but distinct genetic variants (*quasispecies*) [1]. The viral genetic variability is not evenly distributed through the entire genome; the highest variable regions include HVR1, HVR2, and HVR3 of the envelope E2 protein [4]. It is believed that HCV variability has significant clinical implications, since it may result in the generation of immune escape mutants, which may contribute to chronic infection and treatment resistance [5].

The detailed study of the minor variants within the *quasispecies* population is hampered by the absence of sensitive sequencing strategies which would allow for the detection of low-frequency genomes. The traditional method for studying viral *quasispecies* is based on Sanger sequencing of bacterially cloned viral sequences. However, this strategy requires extensive cloning to achieve the desired sensitivity for minor variants detection, a process, that is, costly and time consuming. Another limitation of the Sanger method is its difficulty in sequencing GC-rich regions.

Other studies employed single strand conformational polymorphism (SSCP), an electrophoretic method shown to detect variants constituting as little as 3% of the viral population [6]. However, SSCP it is not informative of the

nature of genetic changes or genetic distance between variants and therefore could not be used for some applications such as investigation of the drug resistance. In addition, in a mixture of heterogenous sequences, certain bands may overlap, underrating viral complexity.

With next-generation sequencing (NGS) platforms, it is now possible to investigate viral *quasispecies* at much greater detail. Their high throughput allows for generation of millions of reads in a single sequencing run, facilitating in-depth sequencing.

NGS can detect variants at low frequencies, which would go undetected by standard sequencing methods [7]. Nevertheless, in order to make reliable reconstruction of the viral *quasispecies* from the noisy, incomplete data obtained by NGS, a proper data analysis is required [8, 9].

In the present study we used ultradeep pyrosequencing (454/Roche) to characterize the complexity and heterogeneity of hypervariable region 1 (HVR1) in a patient persistently infected with HCV genotype 1b. This region was chosen as its protein product is under constant selection pressure of the host immune responses, especially of cytotoxic T cells and neutralizing antibodies [10, 11]. We sequenced this short region at very high coverage, aiming at detecting a large number of minority variants. We took into account sequencing errors in order to have a reliable reconstruction of the viral *quasispecies* on this region.

Reports taking advantage of deep sequencing to investigate HCV genetic diversity for clinical and epidemiological studies are currently available [12–15]. Likewise, there are also works reporting and comparing bioinformatic approaches to infer the viral population from clinical samples, mostly HIV [9, 16–19]. Our study contributes progress in the evaluation of reconstruction methods and extends it for HCV *quasispecies* phenomenon investigation.

2. Patient and Methods

2.1. Sample. A serum sample from a 66-year-old treatment-naive female patient with genotype 1b chronic HCV infection was used. The serum HCV viral load was 1.54×10^6 IU/mL. The patient provided informed consent and the study was approved by the Institutional Bioethical Committee.

2.2. HVR1 Amplification. Viral RNA was extracted from 250 μL of serum by a modified guanidinium thiocyanate-phenol/chlorophorm method using a commercially available Trizol reagent (Invitrogen) and suspended in 20 μL of water. Five μL of the solution containing RNA was subjected to reverse transcription at 37°C for 30 minutes using AccuScript High Fidelity Reverse Transcriptase (Stratagene). HVR1 sequences were amplified in a two-step PCR using FastStart High Fidelity Taq DNA Polymerase (Roche) as described previously [20]. Primers used for reverse transcription (E2 AS) and first round HCV HVR1 amplification (E2 S) were as follows: 5′-CATTGCAGTTCAGGGCCGTGCTA-3′ and 5′-GGTGCTCACTGGGGAGTCCT-3′. Primers for the second round PCR (E2 NS and E2 NAS) were as follows: 5′-CGT ATC GCC TCC CTC GCG CCA TCAG TCC ATG GTG GGG AAC TGG GC-3′ and 5′-CTA TGC GCC TTG CCA GCC CGC TCAG TGC CAA CTG CCA TTG GTG TT-3′. The latter contained tags recognized by GS Junior Sequencing System (underlined).

2.3. SSCP Analysis of HVR1 Quasispecies. Second round PCR product was purified using Wizard SV Genomic DNA Purification System (Promega) and resuspended in 20 μL of water. Next, 2–5 μL of purified PCR product was subjected to SSCP assay as described previously [21]. Complexity of a population was reflected by the number of distinct bands.

2.4. Ultradeep Pyrosequencing. Pyrosequencing was carried out according to the manufacturer's protocol for amplicons using GS Junior System (454/Roche). In order to lower contamination with short sequences (i.e., primer residues), HVR1 product of the second round PCR was purified from agarose gel by QIAquick Gel Extraction Kit (Qiagen). The extracted product was measured fluorometrically using Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes), and the amount of DNA equivalent to 3×10^7 copies was subjected to emulsion PCR using GS Junior Titanium emPCR Kit (Lib-A). Pyrosequencing was performed according to the amplicon processing procedure for 100 cycles (recommended for amplicons up to 250 bp).

2.5. Data Analysis. Reads that did not match primer sequences or had undetermined bases (Ns) were excluded from further analysis. Retained sequences of 179 bp were visualized using GS Amplicon Variant Analyzer (Roche). Subsequently, primer sequences were trimmed from the target sequence and reads of 138 bp were aligned to the reference sequence for genotype 1b HCV (GenBank accession number AJ406073) and translated to amino acid sequences by (*Molecular Evolutionary Genetics Analysis)* MEGA, version 5.0 (http://www.megasoftware.net/) [22]. Phylogenetic analyses were conducted in MEGA5 using the Maximum Likelihood method based on the Tamura-Nei model [23] using MEGA 5.0 software. Genetic parameters such as genetic diversity and sequence polymorphisms within sequences were 5 assessed by DNA SP version (http://www.ub.edu/dnasp/). The program diri_sampler from the ShoRAH software was used to correct sequencing errors and infer haplotypes. Given the high number of reads obtained in the sequencing, the dataset was split equally in two, and the obtained sets were analyzed independently. Error correction included mismatches as well as insertions and deletions.

3. Results

3.1. Amplification and Sequencing Errors. As our experiment used RT-PCR-amplified material, we attempted to assess the error rate in the consecutive experimental steps taking into account error rates of employed enzymes. For reverse transcription, AccuScript High Fidelity Reverse Transcriptase (Stratagene) was used, which displays three times higher fidelity than commonly used MMLV reverse transcriptase [24]. The estimated AccuScript RT error rate is 2×10^{-5}

Figure 1: The SSCP image of HVR1 amplified from the serum of HCV-infected patient.

(manufacturers data). For PCR amplification, we used FastStart High Fidelity Taq DNA Polymerase (Roche), which has estimated error rate of 2×10^{-6} (three times lower than Taq DNA polymerase) [25]. Finally, the pyrosequencing error rate is estimated to be 1.07%, including mismatches (0.088%), insertions (0.541%), deletions (0.359%), and ambiguous base calls (0.085%) [26].

Studying clonal samples, or control samples where a set of clones are mixed in predetermined proportions are important to evaluate the error rate of the sequencing process and the performance of the haplotype reconstruction methods. Since these have already been reported elsewhere [16, 26], it seems not requisite to perform these experiments for every new study of the viral *quasispecies*.

3.2. Heterogeneity of HCV HVR1 Viral Variants Assessed by SCCP Analysis. Based on gel analysis, at least nine SSCP bands of HVR1 were observed (Figure 1). The frequency was not uniform across variants, as could be seen by the different intensities of the bands.

3.2.1. Heterogeneity of HCV HVR1 Assessed by Ultradeep Sequencing. To check the applicability of ultradeep pyrosequencing for HCV HVR1 heterogeneity analysis, the amplified product was sequenced by GS Junior System (454/Roche). Based on the data of GS Amplicon Variant Analyzer, the total number of sequenced nucleotides was 1.37×10^8. The system read 76 332 individual sequences, among them 73 236 (95.9%) (28 098 forward and 45 138 reverse) were aligned to the reference sequence AJ406073 of genotype 1b HCV. The GS Amplicon Variant Analyzer software detected 15 917 haplotypes. Mean coverage of each variant (expressed by the number of identical reads) was 4.6. The most abundant haplotype coverage was 4540 reads. The rarest haplotypes comprised single sequence reads (74,6% of detected haplotypes). Our results are summarized in Table 1.

Table 1: HVR1 HCV characteristics obtained by pyrosequencing using GS Junior System (454/Roche).

Number of sequenced nucleotides	1.37×10^8
Number of individual sequences that passed the quality control*	76 332
Number of individual sequences aligned to reference genome	73 236
Mean coverage per sequence	4.6
Identified haplotypes	15 917

*No undetermined bases, 100% match with primer sequences.

3.2.2. Error Correction in Haplotype Reconstruction. In order to reflect the HVR1 HCV population *in vivo* as accurately as possible, we explored the effect of different strategies to take the sequencing error rate into account. In a very conservative approach (Method I), we only considered variants detected at a frequency higher than 1%. This amounts to discard most variants, even if, given the high coverage, they appear in hundreds of reads. With this strategy we only retained 8 haplotypes.

A second strategy (Method II) consisted in requiring bidirectional coverage, that is, in only retaining variants supported by at least one forward and one reverse read. This method identified 40 HVR1 variants.

In the third approach we used the program diri_sampler from the software suite ShoRAH [16]. In this analysis, inference of the viral *quasispecies* is done in probabilistic manner using a Bayesian approach. It does not rely on the input of an error rate, rather, it estimates it from the sequencing data. Reads are clustered together and the consensus sequence of each cluster represents the original haplotype. Together with the frequency of each variant, the program enables assessments of the posterior probability of each haplotype, a confidence value for their existence. The number of diverse reads sequenced was higher than what the program can handle on a desktop computer with 4 GB of RAM. In order to face this limitation, we split the reads equally in two subsets, and performed haplotype reconstruction independently. Only haplotypes with confidence value >95% were retained. As an additional measure of reliability, only haplotypes supported by at least 5 reads were included. Since we are dealing with a coding sequence, frameshift inducing insertions/deletions were resolved correcting to the most common nucleotide for that position. As a result of two independent computations on raw data halves, two populations (A and B), consisting of 333 and 315 haplotypes respectively, were obtained. Their frequencies varied from 10.54% and 10.44% (the most abundant variants in population A and B, resp.) down to 0.013% and 0.014% (the least abundant variants in population A and B, resp.). 186 haplotypes were common to both populations and their frequencies were all above 0.02%. Seventeen haplotypes were present with a frequency >1%, constituting in total 58.6% of the entire population.

3.2.3. Characteristics of Inferred HVR1 Populations. After application of error correction methods, such parameters as

Table 2: The impact of haplotype reconstruction method on the variability parameters of HVR1.

Correction method	I Cut-off 1%	II Bidirectional coverage	III ShoRAH
Number of haplotypes	8	40	186
Number of nucleotide substitutions within HVR1	51	59	70
Percentage of mutated amino acid positions within HVR1 (%)	55.6	55.6	74.1
Genetic distance	3.874	0.065	0.110
Genetic diversity	0.923	0.998	0.984

percentage of mutated amino acid positions, genetic distance, genetic diversity as well as number of substitutions were calculated (Table 2). The highest genetic distance characterized population reconstructed by cut-off method (3.874) followed by ShoRAH method (0.110) and bi-directional coverage method (0.065), whereas genetic diversities were similar for all populations (0.923, 0.998 and 0.984 for method I, II and III, resp.). The highest number of nucleotide substitutions was detected in ShoRAH-reconstructed population (overall 70). 47 (67%) of them were present in genetic variants constituting more than 1% of the entire population.

HVR1 populations were also compared on amino acid level (Figure 2). Within 27 amino acid stretch of HVR1, only 15 (55.5%) positions were polymorphic after application of methods I and II, and 20 (74.1%) after ShoRAH computations. Based on ShoRAH computation results, the most variable was the fourth HVR1 position, where 11 amino acid substitutions were detected when compared to reference sequence (V/D, V/M, V/T, V/L, V/R, V/A, V/E, V/G, V/N, V/I, V/Q).

Viral populations were also analyzed phylogenetically. As shown in Figure 3, the general topology of three populations was similar. However, the tree topology based on ShoRAH computation was the most extensive.

4. Discussion

Pyrosequencing is a relatively novel technique which may help to decipher complex viral populations in terms of their diversity and structure. To date, it was successfully used in human immunodeficiency virus (HIV) research to identify minor drug resistant variants, analyze variable regions of heavy and light chains of neutralizing antibodies against HIV, as well as to determine HIV tropism, analyze superinfections and assess diversity of genital microbiota in HIV-infected women [27–31]. Ultradeep sequencing strategies also offers a new approach in HCV research. However, application of this method requires that several issues are taken into account. The foremost of these is the generation of mutations during reverse transcription and amplification reactions, due to enzyme errors [32]. Reverse transcriptase is the most error-prone, as it lacks a proofreading activity. For instance, error rate of common reverse transcriptases used *in vitro* to synthesize cDNA is at least 10^{-4} [24], and errors that occurred during this step are propagated during the subsequent PCR amplification. In the present study, in order to minimize errors, high fidelity enzymes were used in amplification reactions preceding sequencing (AccuScript High Fidelity Reverse Transcriptase and FastStart High Fidelity Taq DNA Polymerase). Nevertheless, the resulting hypothetical error rate of amplification is estimated to be lower than the sequencing error rate itself. The sequencing step introduces various types of errors related to the pyrosequencing chemistry and detection technology. The major contributor to errors is the ambiguity of homopolymer length, which results from the difficulty to resolve intensity of luminescence when a homopolymer is encountered. Moreover, insufficient flushing may lead to single base insertions. Overall, it was estimated that the mean error rate of pyrosequencing (defined as the number of errors such as miscalled bases or inserted or deleted bases divided by the total number of sequenced bases) was 1.07% [26]. This value may be considered as the experimentally confirmed resolution of the method. For the above reasons, the raw data obtained from sequencing should be additionally processed in order to remove low-quality reads and reads containing errors.

Three different error correction methods were applied to the raw sequencing data, which resulted in three HVR1 populations, differing in complexity and heterogeneity. The most sensitive was ShoRAH program reconstruction, which allowed to obtain the broadest spectrum of HVR1 sequences. This method has already been shown to reliably detect variants down to about 0.1% [33]. In this study we detected variants down to 0.02%, confirmed in two independent computations. The cut-off method, in which variants present in less than 1% of the population were discarded, was the least sensitive, as it allowed to detect only 8 haplotypes. Similar cut-off was applied in analysis of pyrosequencing reads of *pol/gag* of HIV population as well as *PePHD* E2 of HCV [14, 34]. It was reported that this method may result in inadequate haplotype reconstruction of low precision, low recall, or both, depending on the cut-off value. Too low cut-off value may result in low precision (fraction of true haplotypes among all called haplotypes) and conversely, high cut off may significantly lower recall (fraction of called haplotypes among all true haplotypes). For instance, based on the analysis of *gag/pol* HIV genes, it was shown that the cut-off of 50 read observations resulted in 80% precision but only 40% recall [33]. Application of the bi-directional coverage correction method II allowed us to determine the presence of 40 haplotypes, but such verification is laborious and raises concern regarding the acceptance of haplotypes characterized by high disproportion in forward and reverse strand counts. Among these forty sequences with bidirectional coverage, we could identify twenty that matched exactly one of the

```
                                      HVR1
#AJ406073                      KVLIVMLLFA GVDGGHTHVT GGAAAHAAYG FRHLFSPGSA QKLQLV
#CON_93_amplicon_Amp_1         .......... ....E-.RTI ...E.R..S. LSG..TR..Y ..I...
#CON_11189_amplicon_Amp_1      .......... ....E-.RTI ..-E.R..S. LSG..TR..Y ..I...
#CON_7239_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_7283_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_40_amplicon_Amp_1         .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_7319_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_7250_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_11507_amplicon_Amp_1      .......... ....E-..TI ..-V.RTTS. LSG..RA..H ..I...
```

(a)

```
                                      HVR1
#AJ406073                      KVLIVMLLFA GVDGGHTHVT GGAAAHAAYG FRHLFSPGSA QKLQLV
#CON_236_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1037_amplicon_Amp_1       .......... ....E-.RTI ...E.R..S. LSG..TR..Y ..I...
#CON_1051_amplicon_Amp_1       .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1080_amplicon_Amp_1       .......... ....E-..TI ...V.RTTS. LSS..TA..Q ..I...
#CON_1111_amplicon_Amp_1       .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1120_amplicon_Amp_1       .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1174_amplicon_Amp_1       .......... ....E-..TI ...V.RTTS. .SS..TA..Q ..I...
#CON_1179_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1241_amplicon_Amp_1       .......... ....E-.RTI ...E.R..S. LSG..TR..Y ..I...
#CON_125_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_126_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. .SG..RA..Q ..I...
#CON_1294_amplicon_Amp_1       .......... ....K-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1620_amplicon_Amp_1       .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1710_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1727_amplicon_Amp_1       .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_181_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_1832_amplicon_Amp_1       .......... ....E-.RTI ...E.R..S. LSG..TR..Y ..I...
#CON_184_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..RA..H ..I...
#CON_223_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_240_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..RA..H ..I...
#CON_243_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..RA..H ..I...
#CON_245_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..TA..Q ..I...
#CON_2667_amplicon_Amp_1       .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_3101_amplicon_Amp_1       .......... ....T-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_39_amplicon_Amp_1         .......... ....E-..TI ...V.RTTS. .SG..RA..H ..I...
#CON_397_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..RA..Q ..I...
#CON_40_amplicon_Amp_1         .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_307_amplicon_Amp_1        .......... ....E-..TI ...V..G.R. LSS..TA..Q ..I...
#CON_48_amplicon_Amp_1         .......... ....E-..TI ...V.RTTS. .SG..RA..H ..I...
#CON_491_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_493_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_508_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_54_amplicon_Amp_1         .......... ....E-..TI ...V.RTTS. LSG..RA..H ..I...
#CON_548_amplicon_Amp_1        .......... ....E-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_688_amplicon_Amp_1        .......... ....E-.RTI ..SV..G.R. LSS..TA..Q ..I...
#CON_692_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_724_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..RA..H ..I...
#CON_788_amplicon_Amp_1        .......... ....R-..TI ..SV..G.R. LSS..TA..Q ..I...
#CON_883_amplicon_Amp_1        .......... ....E-..TI ...V.RTTS. LSG..TR..Y ..I...
#CON_947_amplicon_Amp_1        .......... ....E-.RTI ...E.R..S. LSG..TR..Y ..I...
```

(b)

```
                                      HVR1
#AJ406073                      KVLIVMLLFA GVDGGHTHVT GGAAAHAAYG FRHLFSPGSA QKLQLV
#seq_1_frequency_10.496963     .......... .....DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_2_frequency_8.547829      .......... .....DS.MI ...E.R..S. LSG..TR..Y ..I...
#seq_3_frequency_7.419855      .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_4_frequency_6.384569      .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_5_frequency_4.296772      .......... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_6_frequency_3.850357      .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_7_frequency_3.171748      .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_8_frequency_2.787145      .......... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_9_frequency_2.250011      .......... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_10_frequency_1.504237     .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_11_frequency_1.251363     .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_12_frequency_1.187379     .......... ...-.E..TI ...V.RTTS. .SG..RA..H ..I...
#seq_13_frequency_1.178896     .......... .....DS.MI ...E.R..S. LSG..TR..Y ..I...
#seq_14_frequency_1.083473     .......... .....DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_15_frequency_1.075477     .......... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_16_frequency_1.038557     .......... .....DP.DR .-.V.RTTS. .SG..RA..H ..I...
#seq_17_frequency_1.000225     .......... .....DS.MI ...E.R..S. LSG..TR..Y ..I...
#seq_18_frequency_0.926960     .......... .....DP.DR .-.V..G.R. LSS..TA..Q ..I...
#seq_19_frequency_0.882146     .......... ..EW.DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_20_frequency_0.863415     .......... ....-E..TI ...V..G.R. LSS..TA..Q ..I...
#seq_21_frequency_0.803007     .......... .....DS.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_22_frequency_0.777475     .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_23_frequency_0.760876     .......... .....DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_24_frequency_0.755084     .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_25_frequency_0.701852     .......... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_26_frequency_0.672432     .......... .....DP.DR .-.V.RTTS. LSG..TR..Y ..I...
#seq_27_frequency_0.655984     .......... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_28_frequency_0.654307     .......... ..E.-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_29_frequency_0.651253     .......... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_30_frequency_0.650095     .......... ...-.E..TI ...V.RTTS. LSG..TR..Y ..I...
#seq_31_frequency_0.625705     .......... .....DP.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_32_frequency_0.612851     .......... ....-E..TI ...E.R..S. LSG..TR..Y ..I...
#seq_33_frequency_0.602512     .......... ...-.E..TI ...V.RTTS. .SG..RA..Q ..I...
```

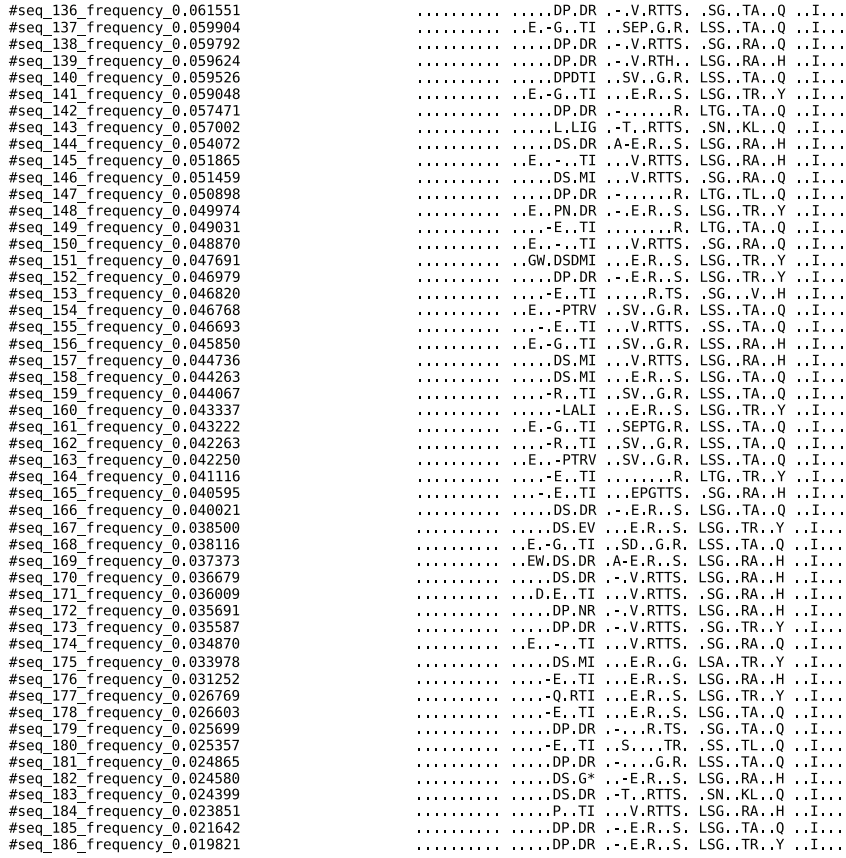
(c)

Figure 2: Continued.

```
#seq_34_frequency_0.566364              ..... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_35_frequency_0.539622              ..... .....DP.DR .-.V.RTTS. LSG..RA..Q ..I...
#seq_36_frequency_0.537195              ..... ...-.E..TI ...V.RTTS. .SG..RA..H ..I...
#seq_37_frequency_0.521421              ..... ...-.E..TI ...V.RTTS. LSG..RA..Q ..I...
#seq_38_frequency_0.511224              ..... .....DS.MI ..SV..G.R. LSS..TA..Q ..I...
#seq_39_frequency_0.506563              ..... .....DS.MI ...E.R..S. LSG..TR..Y ..I...
#seq_40_frequency_0.489579              ..... .....DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_41_frequency_0.477541              ..... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_42_frequency_0.475100              ..... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_43_frequency_0.416991              ..... .....DP.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_44_frequency_0.403574              ..... .....DS.DR .-.E.R..G. LSA..TR..Y ..I...
#seq_45_frequency_0.398722              ..... .....DS.DR ...E.R..S. LSG..TR..Y ..I...
#seq_46_frequency_0.386373              ..... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_47_frequency_0.375498              ..... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_48_frequency_0.368952              ..... ...-.E..TI ...V.RTTS. LSG..TA..Q ..I...
#seq_49_frequency_0.353843              ..... ...-.E..TI ...V.RTTS. .SS..RA..H ..I...
#seq_50_frequency_0.350268              ..... ..E..PN.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_51_frequency_0.346676              ..... ..E..PN.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_52_frequency_0.336838              ..... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_53_frequency_0.332207              ..... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_54_frequency_0.330468              ..... .....DP.DR .-.V.RTTS. .SG..RA..Q ..I...
#seq_55_frequency_0.325899              ..... .....DS.DR .-.E.R..S. LSG..TA..Q ..I...
#seq_56_frequency_0.324684              ..... .....DP.DR .-.V.RTTS. .SS..RA..H ..I...
#seq_57_frequency_0.323406              ..... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_58_frequency_0.322620              ..... .....DS.MS R..E.R..S. LSG..TR..Y ..I...
#seq_59_frequency_0.291277              ..... .....DS.MI ...E.R..S. LSG..TA..Q ..I...
#seq_60_frequency_0.290212              ..... .....DS.DR .A-E.R..S. LSG..RA..H ..I...
#seq_61_frequency_0.265723              ..... .....DS.MI ...E.R..G. LSA..TR..Y ..I...
#seq_62_frequency_0.256780              ..... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_63_frequency_0.243733              ..... .....DS.MI ...E.R..S. LSG..RA..H ..I...
#seq_64_frequency_0.232532              ..... .....DP.DR .-.V.RTTS. .SG..TA..Q ..I...
#seq_65_frequency_0.232330              ..... ...-.E..TI ...V.RTTS. .SS..TA..Q ..I...
#seq_66_frequency_0.231031              ..... ..E..PN.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_67_frequency_0.226299              ..... .....P..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_68_frequency_0.226212              ..... ...-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_69_frequency_0.226001              ..... ...-.E..TI ...V.RTTS. LSS..TA..Q ..I...
#seq_70_frequency_0.214432              ..... .....P..TI ..S....TR. .SS..TL..Q ..I...
#seq_71_frequency_0.198007              ..... ...-.E..TI ...V.RTTS. .SG..TA..Q ..I...
#seq_72_frequency_0.189106              ..... ..E..PN.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_73_frequency_0.171091              ..... .....DP.DR .-.V.RTTS. .SS..TA..Q ..I...
#seq_74_frequency_0.161615              ..... .....DP.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_75_frequency_0.158776              ..... ....-E.RAI ...E.R..S. LSG..TR..Y ..I...
#seq_76_frequency_0.156317              ..... ....-K..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_77_frequency_0.148586              ..... .....DS.DR ..RE.R..S. LSG..TR..Y ..I...
#seq_78_frequency_0.147589              ..... ..E..-LTLI ..SV..G.R. LSS..TA..Q ..I...
#seq_79_frequency_0.144746              ..... .....DP.DR .-.V.RTTS. LSS..TA..Q ..I...
#seq_80_frequency_0.143410              ..... ....-K..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_81_frequency_0.141619              ..... .....DS.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_82_frequency_0.140555              ..... .....R..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_83_frequency_0.138271              ..... .....DP.DR .-.V..G.R. LSS..TA..Q ..I...
#seq_84_frequency_0.132354              ..... ..E.-G..TI ..SEP.G.R. LSS..TA..Q ..I...
#seq_85_frequency_0.127754              ..... .....DP.DR .-.V.RTTS. LSG..TA..Q ..I...
#seq_86_frequency_0.123660              ..... ...-.E..TI ...V.RTH.. LSG..RA..H ..I...
#seq_87_frequency_0.121928              ..... ....-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_88_frequency_0.120898              ..... .....DS.MI ..SV..G.R. LSS..TA..Q ..I...
#seq_89_frequency_0.118086              ..... .....DS.EV ...E.R..S. LSG..TR..Y ..I...
#seq_90_frequency_0.117558              ..... ..E..-..TI ...V.RTTS. .SG..RA..H ..I...
#seq_91_frequency_0.117161              ..... .....R..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_92_frequency_0.112927              ..... .....P..TI ..S....TR. .SS..TA..Q ..I...
#seq_93_frequency_0.112631              ..... ....-.E..TI ...V.RTTS. LSG..RA..H ..I...
#seq_94_frequency_0.112472              ..... .....DS.MI ...V.RTTS. LSG..RA..H ..I...
#seq_95_frequency_0.112397              ..... ....-Q.RTI ...E.R..S. LSG..TR..Y ..I...
#seq_96_frequency_0.111663              ..... .....DP.DR ..RV.RTTS. .SG..RA..H ..I...
#seq_97_frequency_0.107630              ..... ..E..-..TI ...V.RTTS. LSG..RA..H ..I...
#seq_98_frequency_0.106809              ..... ....-.E..TI ...V.RTTS. S.G..RA..H ..I...
#seq_99_frequency_0.103442              ..... ..E..PN.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_100_frequency_0.100934             ..... ...-.E..TI ...V.RTTS. .SG..RA..Q ..I...
#seq_101_frequency_0.100065             ..... ..E..-PTRV ..SV..G.R. LSS..TA..Q ..I...
#seq_102_frequency_0.099957             ..... ..E..-PDTI ..SV..G.R. LSS..TA..Q ..I...
#seq_103_frequency_0.099954             ..... ...-.E..TI ...V.RTTS. LSG..TR..Y ..I...
#seq_104_frequency_0.097549             ..... ..E..PN.DR .-.V.RTTS. LSG..RA..H ..I...
#seq_105_frequency_0.095057             ..... ..EW.DS.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_106_frequency_0.095048             ..... ....-G..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_107_frequency_0.094927             ..... .....DS.DR .-.E..G.R. LSS..TA..Q ..I...
#seq_108_frequency_0.093505             ..... .....DP.DR .-SV..G.R. LSS..TA..Q ..I...
#seq_109_frequency_0.090986             ..... .....DP.DR .-.V.RTTS. LSG..TR..Y ..I...
#seq_110_frequency_0.090194             ..... ....-E..TI ...E.R..S. LSG..TR..Y ..I...
#seq_111_frequency_0.089734             ..... ..E.-G..TI ..SV..G.R. LSS..TR..Y ..I...
#seq_112_frequency_0.089089             ..... .....DP.DR .-.E.R..S. LSG..TR..Y ..I...
#seq_113_frequency_0.088302             ..... .....DS.MI ...E.R.G*. LSG..TR..Y ..I...
#seq_114_frequency_0.081532             ..... ....-K..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_115_frequency_0.081455             ..... ..E..-PTQI ..SV..G.R. LSS..TA..Q ..I...
#seq_116_frequency_0.079521             ..... ..GR.P..TI ...V.RTTS. .SG..RA..H ..I...
#seq_117_frequency_0.079391             ..... ....-E..TI ..SV..G.R. LSS..TA..Q ..I...
#seq_118_frequency_0.078571             ..... ...-.E..TI ..R*.RTTS. LSG..RA..H ..I...
#seq_119_frequency_0.076269             ..... .....DS.G* ..PE.R..S. LSG..TR..Y ..I...
#seq_120_frequency_0.074612             ..... .....DP.DR .-.V.RTTS. S.G..RA..H ..I...
#seq_121_frequency_0.074134             ..... .....DP.EV ..SV..G.R. LSS..TA..Q ..I...
#seq_122_frequency_0.074079             ..... ...-.E..TI ..R*PRTTS. LSG..RA..H ..I...
#seq_123_frequency_0.073492             ..... ..E.-G..TI ..SE..G.R. LSS..TA..Q ..I...
#seq_124_frequency_0.072251             ..... .....DS.G* ...E.R..S. LSG..TR..Y ..I...
#seq_125_frequency_0.071245             ..... ..E.-G..TI ..SE..G.R. LSS..TA..Q ..I...
#seq_126_frequency_0.070688             ..... ..E.-G..TI ..SE..G.R. LSS..TA..Q ..I...
#seq_127_frequency_0.070584             ..... .....DP.DR .-...R.TS. .SG...V..H ..I...
#seq_128_frequency_0.070491             ..... .....DS.DR .-.E.R..G. LSA..TR..Y ..I...
#seq_129_frequency_0.069469             ..... ....-E..TI ........R. LTG..TL..Q ..I...
#seq_130_frequency_0.065577             ..... ..E.-G..TI ..SV..G.R. LSS..TR..Y ..I...
#seq_131_frequency_0.065258             ..... ...-.E..TI ..RV.RTTS. LSG..RA..H ..I...
#seq_132_frequency_0.064382             ..... .....DS.MI ...E..G.R. LSS..TA..Q ..I...
#seq_133_frequency_0.064154             ..... ...-.E..TI ...V.RTTS. .SG..RA..H ..I...
#seq_134_frequency_0.063206             ..... ...-.E..TI ...EPRTTS. LSG..RA..H ..I...
#seq_135_frequency_0.061966             ..... ....-R..TI ..SV..G.R. LSS..TA..Q ..I...
```

(c)

Figure 2: Continued.



(c)

Figure 2: Amino acid sequences of HVR1 populations inferred after the application of three different error correction methods. (a) Cut-off method >1% (I), (b) bidirectional coverage (II), and (c) ShoRAH computation (III). Top sequence corresponds to reference sequence AJ406073 for genotype 1b HCV. Dots indicate consensus positions. Dashes indicate positions not present in the sequence. Asterisks indicate stop codons.

sequences obtained with ShoRAH. This is probably a very precise dataset, although, for example, some sequences might have a very biased forward/reverse read ratio and yet be included in it. Undoubtedly, using the strand information while reconstructing haplotypes is a strategy worth pursuing. A promising approach seems to be a proper statistical treatment of the strand bias, implemented together with the error correction of ShoRAH (McElroy, unpublished data).

In our study, using two independent ShoRAH computations, 186 haplotypes were reconstructed. In contrast, in the study of Bull et al. [13], 100 E2 variants were detected by ShoRAH. However, patients in that study were in an early phase of HCV infection and the study was performed along the whole genome, at much lower coverage, and thus HVR1 complexity could have been lower [13]. Based on ShoRAH reconstruction, we found that the most frequent HVR1 variants constitute a relatively small percentage of the entire population. Thus, the most abundant variant constituted 10.5%, and only 17 haplotypes were present in proportions higher than 1%. These data suggest that during the chronic phase of infection the *quasispecies* population is highly dispersed into minor variants, with no predominant sequences present. Similar haplotype frequency distribution was observed in foot-and-mouth virus population reconstructed by next generation genome sequencing [35]. In the only other HCV study investigating this issue, two to five variants were detected in frequency higher than 2.5%, whereas we detected eight such sequences. However, as already mentioned, viral samples in that study were drawn in the acute phase of infection [13]. The highest number of substitutions (70) within HVR1 was detected in population reconstructed by ShoRAH. Importantly, 47 (67%) of these were detected in variants constituting more than 1%. In contrast, during the acute phase of infection, less than 50% of substitutions were detected in variants present in more than 1% [13]. This suggests that, during the acute phase of infection, rare variants contribute more to the population diversity than during the chronic infection.

SSCP analysis, which has the sensitivity limit of 3%, revealed the presence of 9 bands. If the same cut-off value were applied in ShoRAH-based reconstruction, 7 haplotypes would have been identified. This fact suggests that the SSCP sensitivity might be higher than expected.

However, it must be stressed that the absolute SSCP band number may not reflect the haplotype number as it represents

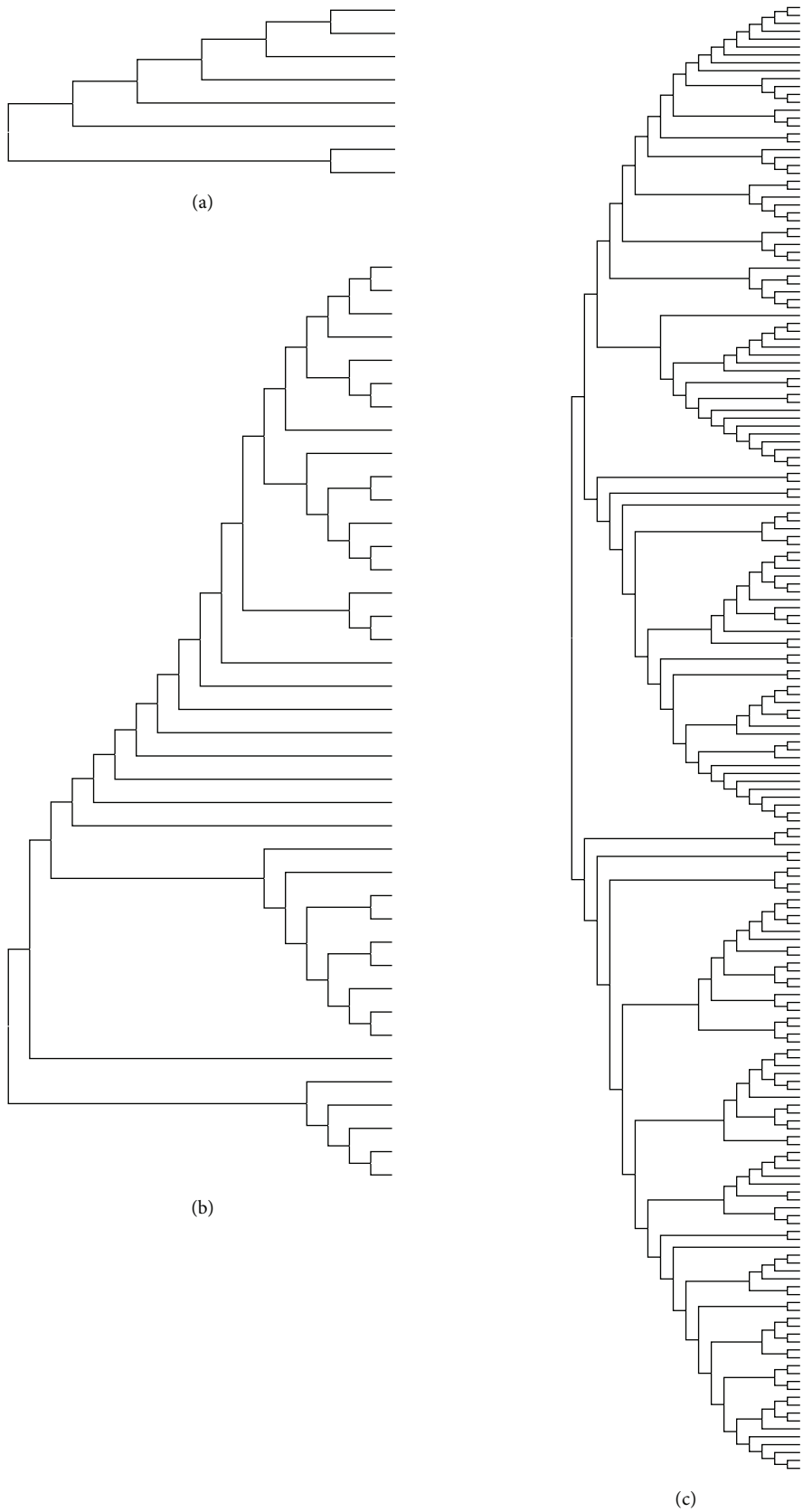


Figure 3: Molecular phylogenetic analysis of HVR1 populations inferred after the application of three different error correction methods. (a) Cut-off method >1% (I), (b) bidirectional coverage method (II), and (c) ShoRAH algorithm (III). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [23]. Evolutionary analyses were conducted using MEGA 5.0 [22].

resolved single DNA strands. Importantly, only sequencing provides information about the nucleotide sequence.

5. Conclusions

The newly available pyrosequencing technique opens a new approach to the analysis of complex viral genomes as it allows for detection of rare molecular variants. Better understanding of population genetics of complex viral populations seems crucial for understanding *quasispecies* phenomenon, viral evolution, and drug resistance.

In the evaluation presented here, we used ShoRAH to obtain the broadest spectrum of HVR1 variants while trying to preserve their reliability. The use of different sequencing platforms, the optimization of library preparation, and data analysis will further improve the reconstruction of viral *quasispecies*.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgment

This work was supported by Projects from Polish National Science Centre number NN 401 64 67 40 and 1 M24/PM12/12.

References

[1] M. Martell, J. I. Esteban, J. Quer et al., "Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution," *Journal of Virology*, vol. 66, no. 5, pp. 3225–3229, 1992.

[2] F. Kurbanov, Y. Tanaka, D. Avazova et al., "Detection of hepatitis C virus natural recombinant RF1_2k/1b strain among intravenous drug users in Uzbekistan," *Hepatology Research*, vol. 38, no. 5, pp. 457–464, 2008.

[3] P. Moreno, M. Alvarez, L. Lápez et al., "Evidence of recombination in Hepatitis C Virus populations infecting a hemophiliac patient," *Virology Journal*, vol. 6, article 203, 2009.

[4] J. M. Cuevas, M. Torres-Puente, N. Jiménez-Hernández et al., "Refined analysis of genetic variability parameters in hepatitis C virus and the ability to predict antiviral treatment response," *Journal of Viral Hepatitis*, vol. 15, no. 8, pp. 578–590, 2008.

[5] E. A. Duarte, I. S. Novella, S. C. Weaver et al., "RNA virus quasispecies: significance for viral disease and epidemiology," *Infectious Agents and Disease*, vol. 3, no. 4, pp. 201–214, 1994.

[6] T. Laskus, J. Wilkinson, J. F. Gallegos-Orozco et al., "Analysis of hepatitis C virus quasispecies transmission and evolution in patients infected through blood transfusion," *Gastroenterology*, vol. 127, no. 3, pp. 764–776, 2004.

[7] L. Barzon, E. Lavezzo, V. Militello, S. Toppo, and G. Palù, "Applications of next-generation sequencing technologies to diagnostic virology," *International Journal of Molecular Sciences*, vol. 12, no. 11, pp. 7861–7884, 2011.

[8] N. Beerenwinkel, H. F. Gunthard, V. Roth, and K. J. Metzner, "Challenges and opportunities in estimating viral genetic diversity from next-generation sequencing data," *Frontiers in Microbiology*, vol. 3, article 329, 2012.

[9] N. Beerenwinkel, "Ultra-deep sequencing for the analysis of viral populations," *Current Opinion in Virology*, vol. 1, no. 5, pp. 413–418, 2011.

[10] S. Guglietta, A. R. Garbuglia, V. Pacciani et al., "Positive selection of cytotoxic T lymphocytes escape variants during acute hepatitis C virus infection," *European Journal of Immunology*, vol. 35, no. 9, pp. 2627–2637, 2005.

[11] C. Di Lorenzo, A. G. Angus, and A. H. Patel, "Hepatitis C virus evasion mechanisms from neutralizing antibodies," *Viruses*, vol. 3, no. 11, pp. 2280–2300, 2011.

[12] A. Escobar-Gutierrez, M. Vazquez-Pichardo, M. Cruz-Rivera et al., "Identification of hepatitis C virus transmission using a next-generation sequencing approach," *Journal of Clinical Microbiology*, vol. 50, no. 4, pp. 1461–1463, 2012.

[13] R. A. Bull, F. Luciani, K. McElroy et al., "Sequential bottlenecks drive viral evolution in early acute hepatitis c virus infection," *PLoS Pathogens*, vol. 7, no. 9, Article ID e1002243, 2011.

[14] F. Bolcic, M. Sede, F. Moretti et al., "Analysis of the PKR-eIF2alpha phosphorylation homology domain (PePHD) of hepatitis C virus genotype 1 in HIV-coinfected patients by ultra-deep pyrosequencing and its relationship to responses to pegylated interferon-ribavirin treatment," *Archives of Virology*, vol. 157, no. 4, pp. 703–711, 2012.

[15] S. Fonseca-Coronado, A. Escobar-Gutierrez, K. Ruiz-Tovar et al., "Specific detection of naturally occurring hepatitis C virus mutants with resistance to telaprevir and boceprevir (protease inhibitors) among treatment-naive infected individuals," *Journal of Clinical Microbiology*, vol. 50, no. 2, pp. 281–287, 2012.

[16] O. Zagordi, A. Bhattacharya, N. Eriksson, and N. Beerenwinkel, "ShoRAH: estimating the genetic diversity of a mixed sample from next-generation sequencing data," *BMC Bioinformatics*, vol. 12, article 119, 2011.

[17] I. Astrovskaya, B. Tork, S. Mangul et al., "Inferring viral quasispecies spectra from 454 pyrosequencing reads," *BMC Bioinformatics*, vol. 12, supplement 6, 2011.

[18] C. Quince, A. Lanzen, R. J. Davenport, and P. J. Turnbaugh, "Removing noise from pyrosequenced amplicons," *BMC Bioinformatics*, vol. 12, article 38, 2011.

[19] P. Skums, Z. Dimitrova, D. S. Campo et al., "Efficient error correction for next-generation sequencing of viral amplicons," *BMC Bioinformatics*, vol. 13, Supplement 10, p. S6, 2012.

[20] M. Gerotto, F. Dal Pero, S. Loffreda et al., "A 385 insertion in the hypervariable region 1 of hepatitis C virus E2 envelope protein is found in some patients with mixed cryoglobulinemia type 2," *Blood*, vol. 98, no. 9, pp. 2657–2663, 2001.

[21] T. Laskus, M. Radkowski, L. F. Wang, M. Nowicki, and J. Rakela, "Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication," *Journal of Virology*, vol. 74, no. 2, pp. 1014–1017, 2000.

[22] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, "MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods," *Molecular Biology and Evolution*, vol. 28, no. 10, pp. 2731–2739, 2011.

[23] K. Tamura and M. Nei, "Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees," *Molecular Biology and Evolution*, vol. 10, no. 3, pp. 512–526, 1993.

[24] I. Malet, M. Belnard, H. Agut, and A. Cahour, "From RNA to quasispecies: a DNA polymerase with proofreading activity is

highly recommended for accurate assessment of viral diversity," *Journal of Virological Methods*, vol. 109, no. 2, pp. 161–170, 2003.

[25] J. Cline, J. C. Braman, and H. H. Hogrefe, "PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases," *Nucleic Acids Research*, vol. 24, no. 18, pp. 3546–3551, 1996.

[26] A. Gilles, E. Meglécz, N. Pech, S. Ferreira, T. Malausa, and J. F. Martin, "Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing," *BMC Genomics*, vol. 12, article 245, 2011.

[27] B. B. Simen, J. F. Simons, K. H. Hullsiek et al., "Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes," *Journal of Infectious Diseases*, vol. 199, no. 5, pp. 693–701, 2009.

[28] X. Wu, T. Zhou, J. Zhu et al., "Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing," *Science*, vol. 333, no. 6049, pp. 1593–1602, 2011.

[29] A. Gonzalez-Serna, R. A. McGovern, P. R. Harrigan et al., "Correlation of the virological response to short-term maraviroc monotherapy with standard and deep-sequencing-based genotypic tropism prediction methods," *Antimicrobial Agents and Chemotherapy*, vol. 56, no. 3, pp. 1202–1207, 2012.

[30] A. D. Redd, A. Collinson-Streng, C. Martens et al., "Identification of HIV superinfection in seroconcordant couples in Rakai, Uganda, by use of next-generation deep sequencing," *Journal of Clinical Microbiology*, vol. 49, no. 8, pp. 2859–2867, 2011.

[31] G. T. Spear, M. Sikaroodi, M. R. Zariffard, A. L. Landay, A. L. French, and P. M. Gillevet, "Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis," *Journal of Infectious Diseases*, vol. 198, no. 8, pp. 1131–1140, 2008.

[32] I. Vandenbroucke, H. Van Marck, P. Verhasselt et al., "Minor variant detection in amplicons using 454 massive parallel pyrosequencing: experiences and considerations for successful applications," *Biotechniques*, vol. 51, no. 3, pp. 167–177, 2011.

[33] O. Zagordi, R. Klein, M. Däumer, and N. Beerenwinkel, "Error correction of next-generation sequencing data and reliable estimation of HIV quasispecies," *Nucleic Acids Research*, vol. 38, no. 21, pp. 7400–7409, 2010.

[34] N. Eriksson, L. Pachter, Y. Mitsuya et al., "Viral population estimation using pyrosequencing," *PLoS Computational Biology*, vol. 4, no. 4, Article ID e1000074, 2008.

[35] C. F. Wright, M. J. Morelli, G. Thébaud et al., "Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing," *Journal of Virology*, vol. 85, no. 5, pp. 2266–2275, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 154073, 5 pages
http://dx.doi.org/10.1155/2013/154073

Research Article

Feasibility of a Microarray-Based Point-of-Care *CYP2C19* Genotyping Test for Predicting Clopidogrel On-Treatment Platelet Reactivity

Hyojin Chae,[1,2] Myungshin Kim,[1,2] Yoon-Seok Koh,[3] Byung-Hee Hwang,[4] Min-Kyu Kang,[4] Yonggoo Kim,[1,2] Hae-il Park,[1,2] and Kiyuk Chang[4]

[1] *Department of Laboratory Medicine, Bucheon St. Mary's Hospital, 2 Sosa-dong, Wonmi-gu, Gyeonggi-do, Bucheon-si 420-717, Republic of Korea*
[2] *Catholic Laboratory Development and Evaluation Center, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea*
[3] *Cardiovascular Center and Cardiology Division, Uijeongbu St. Mary's Hospital, Uijeongbu 480-717, Republic of Korea*
[4] *Cardiovascular Center and Cardiology Division, Seoul St. Mary's Hospital, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Republic of Korea*

Correspondence should be addressed to Hae-il Park; phi@catholic.ac.kr and Kiyuk Chang; kiyuk@catholic.ac.kr

Received 17 August 2012; Accepted 11 March 2013

Academic Editor: Yasemin Alanay

Clopidogrel is a prodrug which is converted into active metabolite by cytochrome P450 isoenzyme, *CYP2C19*. Numerous polymorphisms of *CYP2C19* are reported, and a strong link exists between loss-of-function (LOF) or gain-of-function polymorphisms, clopidogrel metabolism, and clinical outcome. Hence, a fully automated point-of-care CYP2C19 genotyping assay is more likely to bring personalized antiplatelet therapy into real practice. We assessed the feasibility of the Verigene 2C19/CBS Nucleic Acid Test, a fully automated microarray-based assay, compared to bidirectional sequencing, and performed VerifyNow P2Y12 assay to evaluate the effect of *CYP2C19* polymorphisms on on-treatment platelet reactivity in 57 Korean patients treated with clopidogrel after percutaneous coronary intervention. The Verigene 2C19/CBS assay identified *2, *3, and *17 polymorphisms with 100% concordance to bidirectional sequencing in 180 minutes with little hands-on time. Patients were classified into 4 groups: extensive (*1/*1; $n = 12$, 21.1%), intermediate (*1/*2, *1/*3; $n = 33$, 57.9%), poor (*2/*2, *2/*3, and *3/*3; $n = 11$, 19.3%), and ultrarapid metabolizers (*1/*17; $n = 1$, 1.8%). The prevalence of the *CYP2C19* *2, *3, and *17 alleles was 36.0%, 12.3%, and 0.9%. Platelet reactivity showed gene dose response according to the number of *CYP2C19* LOF allele. In conclusion, the Verigene 2C19/CBS assay gave accurate *CYP2C19* genotype results which were in well match with the differing on-treatment platelet reactivity.

1. Introduction

Clopidogrel is a thienopyridine prodrug, whose active liver metabolite covalently binds cysteine residues of the platelet surface P2Y12 receptor, irreversibly blocking the receptor, leading to inhibition of platelet aggregation in response to ADP and also to other agents through the inhibitory effect on released ADP.

The conversion of clopidogrel to its active metabolite relies on the CYP2C19 enzyme, a member of the hepatic cytochrome P450 family. Numerous polymorphisms in *CYP2C19* have been identified and individuals can be classified as the phenotype of extensive metabolizers (EMs), intermediate metabolizers (IMs), poor metabolizers (PMs), and ultrarapid metabolizers (UMs) according to the polymorphism of *CYP2C19*. Among the PM phenotypes, *CYP2C19* *2 and *3 polymorphisms are the most frequent and both alleles confer loss-of-function (LOF) leading to a complete loss of the enzyme activity. Indeed, in patients who carry the *CYP2C19* *2 or *3 allele, the conversion of clopidogrel to its active metabolite is reduced, resulting in decreased response of platelets to clopidogrel treatment and worse cardiovascular outcome.

Clopidogrel on top of aspirin has revolutionized the treatment of patients with acute coronary syndrome and undergoing percutaneous coronary intervention (PCI) [1, 2]. However, interindividual variation of platelet inhibition by clopidogrel has been noted, and from 5% to 11% of patients on clopidogrel treatment experience acute or subacute thrombosis after a coronary event or implantation of a coronary stent [1]. Accordingly, a black-box warning was added to the clopidogrel package insert indicating a significant clinical link between *CYP2C19* LOF genotypes (*2 and *3) and poor metabolism of clopidogrel [3]. Therefore, a *CYP2C19* genotyping assay with a rapid sample-to-result time could be beneficial in the appropriate dosing of clopidogrel based on the genotype of the patient and/or permitting change to other antiplatelet agents in a timely manner.

The aim of this study was to assess the performance of the Verigene 2C19/CBS Nucleic Acid Test (Nanosphere, Northbrook, IL, USA), a fully automated microarray-based assay that identifies 12 allelic variants of *CYP2C19* (*1–*10, *13, and *17) in a rapid turnaround time of approximately 3 hours [3], and to study the influence of the *CYP2C19* allelic variants derived from the Verigene test on on-treatment platelet reactivity as assessed by VerifyNow P2Y12 assay (Accumetrics, San Diego, CA, USA) in Korean patients treated with clopidogrel after PCI with the use of drug-eluting stents (DESs).

2. Materials and Methods

2.1. Patients. Peripheral blood samples were collected from 57 consecutive patients subjected to PCI. All patients were preliminarily treated with 100 mg/day of aspirin followed by coadministration of clopidogrel (loading dose, 600 mg; maintenance dose, 75 mg/day). Exclusion criteria were platelet count outside from 100 to 450×10^9/L range; hematocrit <25% or hemoglobin <8 g/dL; and chronic renal failure requiring dialysis. The study was conducted in accordance with the Declaration of Helsinki ethical guidelines and was approved by the Institutional Review Board at Catholic Medical Center.

2.2. Platelet Function Testing with VerifyNow P2Y12 Assay. The blood was drawn from the antecubital vein at 48 h after clopidogrel loading dose into a 3.2% sodium citrate tube for the VerifyNow P2Y12 assay. The time interval between blood sampling and VerifyNow P2Y12 testing did not exceed 2 hours. Platelet reactivity was assessed by the VerifyNow P2Y12 assay, and the VerifyNow P2Y12 assay was performed as previously described [4]. With this assay, higher P2Y12 reaction units (PRU) reflect greater ADP-mediated platelet reactivity. Cut-off value for high on-treatment residual ADP-inducible platelet reactivity (HRPR) was PRU > 235 for the VerifyNow P2Y12 assay according to the published consensus statement [5].

2.3. Genotyping with Verigene 2C19/CBS Nucleic Acid Test. The blood was drawn from the antecubital vein into an EDTA tube for genotyping. For the Verigene 2C19/CBS Nucleic Acid Testing, the EDTA-anticoagulated whole blood (EDTA-WB) samples could be stored at 2–8°C for up to 10 days before processing. Briefly, a single-use extraction tray containing all necessary reagents to lyse, extract, and purify DNA from WB was loaded into the Verigene Processor SP (Nanosphere). 1.0 mL of EDTA-WB was transferred to the sample well in the extraction tray. A single-use test cartridge containing the slide array and hybridization reagents was loaded into the Verigene Processor SP, and the assay was started. On completion of the assay, the test cartridge was removed from the processor, and the hybridization slide was inserted into the Verigene Reader.

2.4. Direct Sequencing. To evaluate the accuracy of the genotype results obtained with the Verigene 2C19/CBS Nucleic Acid Test we performed Sanger-based direct sequencing method in parallel. Briefly, the Genomic DNA was isolated from the peripheral leukocytes using the QIAmp DNA Mini Kit (Qiagen, Hamburg, Germany). PCR was carried out using previously published primer sets for *2 and *3 [6] and a newly designed primer set for *17. The PCR amplicons were sequenced using the Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The chromatograms were analyzed with the Sequencher software version 4.9 (Gene Codes).

2.5. Statistical Analysis. PRU values of VerifyNow P2Y12 assay were compared between the metabolizer statuses by one-way analysis of variance (ANOVA). MedCalc version 12.1.4 (Mariakerke, Belgium) was used for all statistical analyses; $P < 0.05$ was considered statistically significant.

3. Results

Clinical and laboratory characteristics of the total population are presented in Table 1. The average age was 67.0 years and about 65% of patients were men. According to the defined cut-off value, the frequency of clopidogrel resistance was 42.1%. Baseline demographics, clinical presentation, and treatment were well balanced between the *CYP2C19* LOF carrier/and noncarrier groups ($P > 0.05$).

3.1. Verigene 2C19/CBS Nucleic Acid Test for Identifying CYP2C19 Polymorphisms. An initial result was obtained for 53 of 57 specimens (93.0%) using the Verigene 2C19/CBS Nucleic Acid Test. On retesting, all 4 samples gave a definitive result. The total time-to-result was approximately 3 hours with about 15 minutes of hands-on time. The comparison of polymorphism results between bidirectional sequencing and the Verigene 2C19/CBS Nucleic Acid Test revealed 100% concordance rate for all 57 specimens that were analyzed.

3.2. Genotype Frequencies and Classification of Metabolizer Statuses. Distributions of the *CYP2C19* alleles, genotypes, and the predicted phenotypes in our study population are given in Table 2. The frequency of the LOF genotype was high (77%). The prevalence of the gain-of-function variant, *CYP2C19* *17 allele, was low (1%), and the prevalence of the *CYP2C19* *2 and *3 alleles were 36% and 12%, respectively. Of the 57 patients included in the study, 12 (21%) were classified as extensive (EM), 1 (2%) as ultrarapid (UM), 33 (58%) as intermediate (IM), and 11 (19%) as poor (PM) metabolizers.

Table 1: Baseline characteristics of the study population.

	Overall $N = 56$	LOF $N = 44$	No LOF $N = 12$	P value
Age	67.0	67.0	66.0	0.7
Male gender	36 (64.3)	29 (65.9)	7 (58.3)	0.7
Smoking	10 (17.9)	7 (15.9)	3 (25.0)	0.4
Hypertension	33 (58.9)	25 (56.8)	8 (66.7)	0.7
Diabetes mellitus	15 (26.8)	11 (25.0)	4 (33.3)	0.7
Dyslipidemia	12 (21.4)	9 (20.5)	3 (25.0)	0.7
Prior MI	3 (5.4)	1 (2.3)	2 (16.7)	0.1
Prior PCI	11 (19.6)	7 (15.9)	4 (33.3)	0.2
BMI	24.8	26.1	24.3	0.2
Statin	15 (26.8)	13 (29.5)	2 (16.7)	0.5
β-blocker	9 (16.1)	5 (11.4)	4 (33.3)	0.09
ACE inhibitor	4 (7.1)	3 (6.8)	1 (8.3)	1
CCBs	11 (19.6)	8 (18.2)	3 (25.0)	0.7
PPIs	2 (3.6)	1 (2.3)	1 (8.3)	0.4
Platelet count	215.5	221.0	215.5	1
C-reactive protein	0.1	0.1	0.1	0.2

The denotations are LOF: loss-of-function; MI: myocardial infarction; PCI: percutaneous coronary intervention; BMI: body mass index; ACE: angiotensin converting enzyme inhibitor; CCB: calcium channel blocker; and PPI: proton pump inhibitor.

3.3. Influence of Metabolizer Statuses on Platelet Reactivity. Platelet reactivity measured by the VerifyNow P2Y12 assay differed significantly according to metabolizer statuses when tested by analysis of variance (Figure 1). Higher on-clopidogrel platelet reactivity was observed as the number of *CYP2C19* LOF allele increased (UM 4.0; EM, 177.7; IM, 201.7; and PM 277.0). Student-Newman-Keuls test for pairwise comparison revealed a significant difference between UM and IM/PM using the VerifyNow P2Y12 assay.

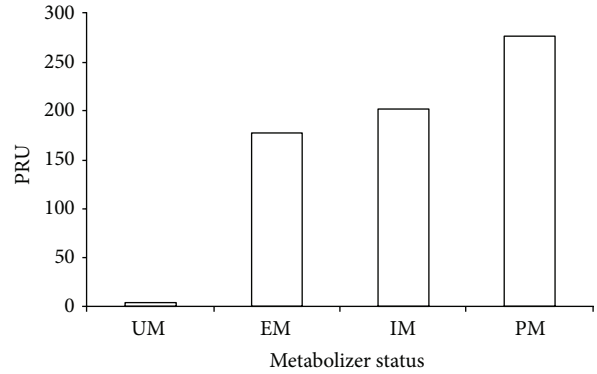


Figure 1: Result from the VerifyNow P2Y12 assay according to CYP2C19 metabolizer statuses. Data are shown as mean. The denotations are PRY: P2Y12 reaction units; UM: ultrarapid; EM: extensive; IM: intermediate; and PM: poor metabolizer.

4. Discussion

In this study, we found that the test reliability of the Verigene 2C19/CBS Nucleic Acid Test for the identification of *CYP2C19* polymorphisms was 100% accurate as compared with the bidirectional sequencing method. In addition, this Verigene test offered rapid detection time and enabled point-of-care diagnosis without conventional DNA extraction, PCR steps, and sequencing. Genotyping assays for *CYP2C19* can be performed with numerous molecular methods, such as real-time PCR, allele-specific PCR, PCR-RFLP, pyrosequencing, and bidirectional sequencing. Also a growing number of commercial analytical platforms are available, and these include the INFINITI (AutoGenomics, Inc., Vista, Carlsbad, CA, USA) [7], the Verigene, eSensor XT-8 (Genmark, Carlsbad, CA, USA) [8], Spartan RX (Spartan Biosciences, Ottawa, ON, Canada) [9], Invader (Hologic, Bedford, MA, USA) [10], and Luminex assays (Luminex, Austin, TX, USA) [8]. Although the comparison of different genotyping methods is beyond the scope of this paper, the Verigene assay utilizes signal amplification and not target amplification, and therefore it has a unique advantage of operating in a PCR-free environment. Also Verigene has the shortest TATs among commercial platforms that are capable of genotyping a certain number of polymorphisms and also the least complex to operate. And therefore the assay can be readily implemented in clinical laboratories without extensive experience in molecular techniques. The limitation of the Verigene assay is the no call rate of 7–10%. The rate of no call at first attempt was 7.3% in our study; however, in our experience, the no call errors were resolved with repeat testing.

The inhibitory effect of clopidogrel on platelet function shows marked interindividual variability. The prevalence of clopidogrel nonresponsiveness has been reported to be from 4% to 30% in patients with coronary artery disease [11]. Several factors including underdosing, inappropriate dosing, variable absorption of the prodrug, variable clearance of the active metabolite, potential drug-drug interactions, P2Y12 receptor variability, and genetic polymorphisms of cytochrome P450 isoenzymes are possible mechanisms of clopidogrel resistance [11]. In this respect, *CYP2C19* polymorphisms specifically addresses the issues associated with dosing, since *in vivo* transformation of the prodrug to its active metabolite is dependent on the hepatic cytochrome P450 isoenzyme CYP2C19. Today, at least 25 single nucleotide polymorphisms (SNPs) in the gene coding for CYP2C19 have been described. Of these, *CYP2C19* *2, *3 are the most frequent LOF polymorphisms and thus the main genetic determinants of clopidogrel response variability. In this study, we also observed that *CYP2C19* LOF alleles were significantly associated with reduced antiplatelet efficacy of clopidogrel.

There exists a marked interracial difference in the frequency of the *CYP2C19* polymorphisms. Asians have a higher prevalence of *CYP2C19* LOF alleles and PM phenotypes (from 13% to 23% in Asians and from 1% to 6% in Caucasians) [12, 13]. In this study, the prevalence of *CYP2C19* PM, *2, and *3 alleles were 19.3%, 36.0%, and 12.3%, respectively. These results are in agreement with the observed allele frequencies of 28.6% and 7.4% for *CYP2C19* *2 and *3 alleles in a previous report of 200 Korean individuals [14]. The carriage prevalence of the *CYP2C19* LOF variant is 77.2% in this study, which is

Table 2: Distributions of the *CYP2C19* alleles, genotypes, and the predicted phenotypes.

Allele	Frequency, *n* (%)	Genotype	Frequency, *n* (%)	Phenotype	Metabolizer status
*1	58 (51)	*17/wt	1 (2)	Rapid heterozygous	UM
*2	41 (36)	wt/wt	12 (21)	Extensive	EM
*3	14 (12)	*2/wt	24 (42)	Intermediate	IM
*17	1 (1)	*3/wt	9 (16)	Intermediate	IM
		*2/*2	6 (10)	Poor	PM
		*2/*3	5 (9)	Poor	PM

The denotations are wt: wild type; UM: ultrarapid metabolizer; EM: extensive metabolizer; IM: intermediate metabolizer; and PM: poor metabolizer.

also in line with the reported prevalence of 55% to 70% among Asians [12].

Multiple tests are available for the monitoring of antiplatelet therapy, especially aspirin and clopidogrel. Light transmission aggregometry (LTA) is the gold standard for assessing the platelet response to ADP [4], but this method is laborious and weakly standardized. In the present study, the VerifyNow P2Y12 assay, a method that shows the strongest correlation with the LTA among whole blood-based methods, was used to assess clopidogrel-mediated platelet inhibition. Platelet reactivity measured by the VerifyNow P2Y12 assay significantly differed according to metabolizer statuses when tested by analysis of variance in our population. Higher on-clopidogrel platelet reactivity was observed as the number of *CYP2C19* LOF allele increased (UM 4.0; EM, 177.7; IM, 201.7; and PM 277.0), but post hoc analysis did not reach statistical significance for linear trend, probably as a result of the small number of patients. Interestingly, a rare but well-recognized gain of function allelic variant *CYP2C19* *17 was identified in our study. Student-Newman-Keuls test for pairwise comparison revealed a significant difference between UM and both IM/PM platelets reactivity using the VerifyNow P2Y12 assay.

The Verigene 2C19/CBS Nucleic Acid Test is a fully automated microarray test that utilizes gold nanoparticle-conjugated oligonucleotide probes to detect nucleic acids captured by array probes, and this method eliminates the need for target amplification, namely, a PCR step, before array hybridization [3, 15]. Therefore the assay is less prone to errors introduced during the conventional nucleic acid extraction and target amplification processes and has a rapid turn-around-time. The initial call rate of this study was 93.0% which is similar to the reported initial call rate of 93.5% in a previous study [3], and the assay identified heterozygous and homozygous *2, *3, and *17 polymorphisms with 100% concordance to bidirectional sequence analysis in 57 patient samples. Most importantly, the total time to result was approximately 3 hours with less than 15 minutes of hands-on time.

There are a number of limitations of this study. Our cohort consisted of only 57 patient samples and the small sample size related to lack of statistical power. Also another limitation of our study was the use of PRU values as measured by VerifyNow P2Y12 assay as a measure of clinical efficacy and did not include clinical endpoints. However, the analytical validation of Verigene assay as well as clinical validation using PRU values as surrogate endpoints to clinical efficacy serves a crucial role in providing the link of the point-of-care microarray CYP2C19 genotyping assay towards pharmacogenetic dosing of clopidogrel in real clinical practice.

5. Conclusion

In conclusion, the concordance rate of the Verigene 2C19/CBS Nucleic Acid Test with Sanger's sequencing method was 100% in this study of Korean patients treated with clopidogrel after coronary stenting. In addition, the genetic test results of the *CYP2C19* polymorphisms highly predicted the on-treatment platelet reactivity as assessed by VerifyNow P2Y12 assay. The Verigene test offered several advantages for the detection of *CYP2C19* polymorphisms such as easiness of use, rapid detection time, and a lower test error rate and test failure rate. We anticipate that the rapid point-of-care *CYP2C19* genetic test will clarify the clinical utility of clopidogrel pharmacogenetic tests in patients treated with clopidogrel.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgments

This study was supported by a Grant from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (SN: A092258) and by a Grant of the National Project for Personalized Genomic Medicine, Ministry for Health & Welfare, Republic of Korea (A111218-PG02).

References

[1] I. Müller, F. Besta, C. Schulz, S. Massberg, A. Schönig, and M. Gawaz, "Prevalence of clopidogrel non-responders among patients with stable angina pectoris scheduled for elective coronary stent placement," *Thrombosis and Haemostasis*, vol. 89, no. 5, pp. 783–787, 2003.

[2] P. Savi and J. M. Herbert, "Clopidogrel and ticlopidine: $P2Y_{12}$ adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis," *Seminars in Thrombosis and Hemostasis*, vol. 31, no. 2, pp. 174–183, 2005.

[3] B. W. Buchan, J. F. Peterson, C. H. Cogbill et al., "Evaluation of a microarray-based genotyping assay for the rapid detection of cytochrome P450 2C19 *2 and *3 polymorphisms from whole

blood using nanoparticle probes," *American Journal of Clinical Pathology*, vol. 136, no. 4, pp. 604–608, 2011.

[4] T. Gremmel, S. Steiner, D. Seidinger, R. Koppensteiner, S. Panzer, and C. W. Kopp, "Comparison of methods to evaluate clopidogrel-mediated platelet inhibition after percutaneous intervention with stent implantation," *Thrombosis and Haemostasis*, vol. 101, no. 2, pp. 333–339, 2009.

[5] L. Bonello, U. S. Tantry, R. Marcucci et al., "Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate," *Journal of the American College of Cardiology*, vol. 56, no. 12, pp. 919–933, 2010.

[6] L. Chen, S. Qin, J. Xie et al., "Genetic polymorphism analysis of *CYP2C19* in Chinese Han populations from different geographic areas of mainland China," *Pharmacogenomics*, vol. 9, no. 6, pp. 691–702, 2008.

[7] P. Gladding, H. White, J. Voss et al., "Pharmacogenetic testing for clopidogrel using the rapid infiniti analyzer. A Dose-Escalation Study," *Cardiovascular Interventions*, vol. 2, no. 11, pp. 1095–1101, 2009.

[8] C. C. Lee, G. A. McMillin, N. Babic, R. Melis, and K. T. J. Yeo, "Evaluation of a *CYP2C19* genotype panel on the GenMark eSensor platform and the comparison to the Autogenomics InfinitiÛ and Luminex *CYP2C19* panels," *Clinica Chimica Acta*, vol. 412, no. 11-12, pp. 1133–1137, 2011.

[9] J. D. Roberts, G. A. Wells, M. R. Le May et al., "Point-of-care genetic testing for personalisation of antiplatelet treatmetn (RAPID GENE): a prospective, randomised, proof-of-concept trial," *The Lancet*, vol. 379, no. 9827, pp. 1705–1722, 2012.

[10] M. R. Langley, J. K. Booker, J. P. Evans, H. L. McLeod, and K. E. Weck, "Validation of clinical testing for warfarin sensitivity: comparison of CYP2C9-VKORC1 genotyping assays and warfarin-dosing algorithms," *Journal of Molecular Diagnostics*, vol. 11, no. 3, pp. 216–225, 2009.

[11] T. A. Nguyen, J. G. Diodati, and C. Pharand, "Resistance to clopidogrel: a review of the evidence," *Journal of the American College of Cardiology*, vol. 45, no. 8, pp. 1157–1164, 2005.

[12] X. P. Qin, H. G. Xie, W. Wang et al., "Effect of the gene dosage of CgammaP2C19 on diazepam metabolism in Chinese subjects," *Clinical Pharmacology and Therapeutics*, vol. 66, no. 6, pp. 642–646, 1999.

[13] Y. H. Jeong, U. S. Tantry, I. S. Kim et al., "Effect of *CYP2C19* *2 and *3 loss-of-function alleles on platelet reactivity and adverse clinical events in East Asian acute myocardial infarction survivors treated with clopidogrel and aspirin," *Circulation Cardiovascular Interventions*, vol. 4, no. 6, pp. 585–594, 2011.

[14] M. Man, M. Farmen, C. Dumaual et al., "Genetic variation in metabolizing enzyme and transporter genes: comprehensive assessment in 3 major East Asian subpopulations with comparison to Caucasians and Africans," *Journal of Clinical Pharmacology*, vol. 50, no. 8, pp. 929–940, 2010.

[15] J. J. Storhoff, S. S. Marla, P. Bao et al., "Gold nanoparticle-based detection of genomic DNA targets on microarrays using a novel optical detection system," *Biosensors and Bioelectronics*, vol. 19, no. 8, pp. 875–883, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 164501, 4 pages
http://dx.doi.org/10.1155/2013/164501

Research Article

FISH Detection of *PML-RARA* Fusion in ins(15;17) Acute Promyelocytic Leukaemia Depends on Probe Size

Lynda J. Campbell,[1,2] Paul Oei,[3] Ross Brookwell,[4] Jake Shortt,[5] Nicola Eaddy,[6] Ashley Ng,[7] Edward Chew,[7] and Peter Browett[3,6,8]

[1] *Victorian Cancer Cytogenetics Service, St Vincent's Hospital Melbourne, Fitzroy, VIC 3065, Australia*
[2] *Department of Medicine, St Vincent's Hospital, University of Melbourne, Fitzroy, VIC 3065, Australia*
[3] *LabPLUS, Auckland City Hospital, Auckland 1023, New Zealand*
[4] *Sullivan & Nicolaides Pathology, Indooroopilly, Brisbane, QLD 4068, Australia*
[5] *Department of Haematology, Alfred Hospital, Prahran, VIC 3004, Australia*
[6] *Department of Haematology, Auckland City Hospital, Auckland 1023, New Zealand*
[7] *Department of Haematology, Royal Melbourne Hospital, Parkville, VIC 3050, Australia*
[8] *Department of Molecular Medicine & Pathology, University of Auckland, Auckland 1142, New Zealand*

Correspondence should be addressed to Lynda J. Campbell; lynda.campbell@svhm.org.au

Received 22 October 2012; Accepted 4 March 2013

Academic Editor: Yasemin Alanay

The diagnosis of acute promyelocytic leukaemia (APL) is usually confirmed by cytogenetics showing the characteristic t(15;17), but a minority of patients have a masked *PML/RARA* fusion. We report ten patients with APL and no evidence of the t(15;17), in whom the insertion of *RARA* into *PML* could not be demonstrated by initial FISH studies using a standard dual fusion probe but was readily identified using smaller probes. Given the need for rapid diagnosis of APL, it is important to be aware of the false negative rate for large *PML/RARA* FISH probes in the setting of masked rearrangements.

1. Introduction

Acute promyelocytic leukaemia (APL) is characterised by the reciprocal 15;17 translocation involving the *PML* gene on 15q24, and *RARA* gene on 17q21 in more than 90% of cases. This translocation creates a *PML/RARA* fusion gene on the derivative chromosome 15 [1]. Occasional cases of complex translocations involve 15, 17 and other partner chromosomes, or insertions of 15 into 17 and vice versa, all resulting in a *PML/RARA* fusion [2]. There are also rare variant translocations involving *RARA* and other partner genes: *PLZF*, *NPM*, *NuMA*, *STAT5b, PRKAR1A, FIP1L1*, and *BCOR* [3–6]. In a series of APL cases without the standard t(15;17), most contained the *PML/RARA* fusion caused by an insertion and the fusions were usually demonstrated by both RT-PCR and fluorescence *in situ* hybridization (FISH) [7].

We present ten APL cases without cytogenetic evidence of t(15;17) in whom RT-PCR identified the PML/RARA fusion transcript, but initial FISH with standard probes showed no abnormality. Subsequent FISH revealed a small *PML/RARA* fusion signal in all cases on an apparently normal chromosome 15. Thus, all cases appeared to represent insertions of *RARA* into 15q24. Using the dual fusion probe from Abbott Molecular Inc., the *PML* signal swamped the tiny *RARA* signal. Careful examination showed a fusion signal in 3 cases, but, even in retrospect, there was no evidence of a fusion signal in 7 cases. Depending on which probe is used, a negative FISH result in APL does not, therefore, exclude the diagnosis.

2. Patients and Materials and Methods

Seven cases were identified from the records of 135 APL patients analysed by the Victorian Cancer Cytogenetics Service (VCCS) (cases 1, 3, 4, 5, 8, 9, and 10) over an 11-year period

from 2002, two cases were identified from 25 APLs analysed by the Cytogenetics Department of LabPLUS, Auckland City Hospital (cases 2, 7) over a 5-year period from 2002 and one case was analysed by the Cytogenetics Department of Sullivan and Nicolaides Pathology, Brisbane (case 6) in 2005.

Cytogenetic studies were performed using standard protocols and FISH was performed according to the manufacturers' instructions. Five FISH probes were used: LSI *PML/RARA* dual colour translocation probe (Abbott Molecular Inc., Des Plaines, IL, USA), LSI *PML/RARA* dual colour dual fusion translocation probe (Abbott), LSI *RARA* dual colour break apart rearrangement probe (Abbott), *PML/RARA* translocation probe (extra signal) and *PML/RARA* translocation dual fusion probe (Cytocell Technologies, Cambridge, UK).

FISH was performed on cytogenetic preparations fixed in 3 : 1 v : v methanol/glacial acetic acid, derived preferentially from short-term (usually less than 24 hour) cultures. Slide preparations were hybridized with the various locus-specific probes using codenaturation and overnight hybridization. Analysis was performed using a Zeiss Axioplan 2 Epifluorescence microscope and analysed by ISIS software (Metasystems, Altuβheim, Germany). A minimum of 200 cells were scored for each probe by two scorers. Cut-off values for false positive results (below which the result was regarded as normal) were <1% for the dual fusion probes, 3% for the RARA break apart probe, 10% for the Cytocell *PML/RARA* extra signal probe, and 10% for the *PML/RARA* single fusion probe. Karyotypes were described according to ISCN (2009) [8].

For quantitative t(15;17) *PML-RARA* gene analysis, RNA was purified using Trizol as per the manufacturer's instructions (Invitrogen), 1st-strand cDNA transcribed using SuperScript II (Invitrogen) and absolute quantitative PCR performed using Taqman assay on a Fast Real-Time ABI7500 PCR instrument (Applied Biosystems) using absolute quantitation standards.

3. Results and Discussion

APL is usually diagnosed on the bone marrow morphology and confirmed by the presence of the t(15;17) and detection of the PML/RARA fusion transcript via RT-PCR [3]. The t(15;17) is reported in 92% of APL cases, with 2% having simple or more complex variants, another 4% with insertions of *RARA* into *PML* or *PML* into *RARA*, and the rest with *RARA* fused to partner genes other than *PML* or, in 1%, with no RARA rearrangement [3]. We have identified ten cases of APL without a t(15;17) that appear to have produced a *PML/RARA* fusion gene by inserting a small segment of *RARA* into the *PML* gene on one cytogenetically normal chromosome 15. Seven of these cases were studied at the VCCS between 2002 and 2012, during which time 135 new cases of APL were diagnosed. Thus, the incidence of these cryptic insertions was 7/135 (5%), comparable to previously published series of cryptic abnormalities in APL [3, 9]. No specific morphological features distinguished this group as there were both classic hypergranular ($n = 8$) and variant hypogranular cases ($n = 2$). The RT-PCR results showed that there was no uniformity with regard to the PML breakpoint (Table 1), and immunophenotyping did not show any striking differences compared with the majority of APL cases (results not shown).

Clinical details of all patients are summarized in Table 1. The median age at diagnosis was 43 years (range 22–78 years) and there was an equal sex distribution. Survival data is available for 9/10 patients and 7/9 remain in complete remission 2–119 months after diagnosis. Two patients received modified Pethema protocols [11] and six were treated according to the Australian Leukaemia and Lymphoma Group studies APML3 [10] or APML4 [12]. All but two patients developed DIC and there were two early deaths—one of a presumed cerebral haemorrhage prior to the commencement of therapy (case 3) and one attributed to infection at 1 month post diagnosis (case 4).

The karyotypic, molecular, and FISH data are presented in Table 1. Seven of the ten patients had a normal karyotype; three contained additional abnormalities unrelated to the *PML-RARA* fusion. FISH in all cases using either the LSI *PML/RARA* t(15;17) dual colour dual fusion translocation probe (Abbott) in 9/10 cases or a single fusion translocation probe (Abbott) in one case failed to reveal a *PML-RARA* fusion signal despite RT-PCR identifying a PML/RARA transcript in all cases.

The Abbott dual fusion probe contains fluorescently labelled DNA that covers approximately 180 kb and 335 kb either side of the *PML* loci on chromosome 15 including all of the known BCR regions of *PML* and approximately 700 kb of chromosome 17 spanning all the breakpoint region of *RARA* (http://www.abbottmolecular.com/products/oncology/fish/hematology-probes.html). It was, therefore, puzzling that these probes were unable to detect a fusion signal in patients producing the PML/RARA transcript, whereas the Cytocell probes clearly revealed a fusion signal in all cases (Figure 1(a)).

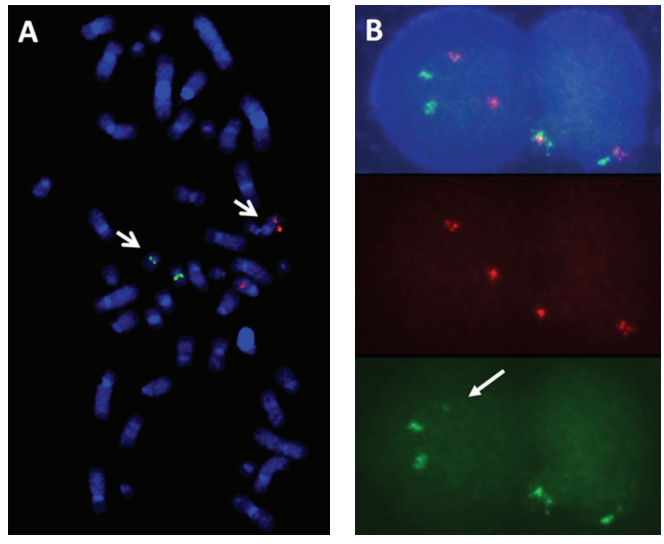
The major difference between the probes lies in the size of the respective probes. Both the *PML* and *RARA* segments of the extra signal probe from Cytocell are only approximately 40 kb in size, 100x smaller than their Abbott counterparts. The Cytocell *PML/RARA* translocation dual fusion probe is larger than the extra signal probe but the fluorescently labelled segments that span either side of the *PML* locus are only 151 kb and 174 kb and those spanning RARA are 167 kb and 164 kb in size. In three cases, a review of the Abbott dual fusion probe result revealed a tiny green (*RARA*) signal underlying one *PML* signal, only visualized using the single-colour Spectrum Green filter (Figure 1(b)). In the remaining 7 cases, despite careful examination, the extra *RARA* signal could not be seen. Apparently, the discrepancy between the size of the Abbott *PML* signal and very small *RARA* segment inserted into 15q24 allowed the *PML* signal intensity to quench the *RARA* signal, whereas the less disparate intensities of the two signals using the Cytocell probes allowed the fusion to be visualized.

Occasional cases of APL with normal cytogenetics and normal FISH studies have been reported previously [14, 15]. Indeed, Brockman et al., when reporting on the efficacy of the original dual fusion *PML/RARA* probe, noted the difficulty in identifying masked *PML/RARA* fusions. In their series

Table 1

Case no.	Age/sex	WBC $\times 10^9$/L	Dx	DIC	Karyotype	FISH probes*					PML-BCR#	Treatment protocol	Survival (months)
						SF (A)	DF (A)	BA (A)	ES (C)	DF (C)			
1	22/F	0.6	M3	No	46,XX	NT	−	NT	+	+	1	ALLG APML3 protocol [10]	2+
2	25/F	6.5	M3	Yes	46,XX	−	−/+	NT	+	NT	1	Modified Pethema protocol [11]	103+
3	30/M	161	M3V	Yes	46,XY,add(4)(q34), add(5)(q12)[4]/46,XY[17]	NT	−	NT	+	+	1	Nil	0
4	30/F	1.9	M3	Yes	46,XX,add(7)(q22)[10]/ 46,XX[20]	NT	−	−	+	+	1/2	ALLG APML4 protocol [12]	1
5	43/M	2.0	M3	Yes	46,XY	NT	−/+	−	+	+	3	ALLG APML3 protocol	119+
6	43/F	42	M3V	Yes	46,XX	NT	−	−	+	NT	3	NA	NA
7	50/F	11.5	M3	Yes	46,XX	−	−/+	NT	+	NT	3	Modified Pethema protocol	105+
8	57/M	1.1	M3	No	46,XY,t(2;13)(p25;q22) [18]/45,X,-Y[3]/46,XY[26]	NT	−	−	+	+	1	ALLG APML4 protocol	17+
9	59/M	6	M3	Yes	46,XY	NT	−	NT	+	+	1	ALLG APML4 protocol	41+
10	78/F	7.5	M3	Yes	46,XX	NT	−	NT	+	+	3	ALLG APLM3 protocol	55+

*FISH probes abbreviations: SF(A): single fusion probe—LSI PML/RARA dual colour translocation probe (Abbott Molecular Inc.); DF(A): dual fusion probe—LSI PML/RARA dual colour dual fusion translocation probe (Abbott); BA(A): break apart probe—LSI RARA dual colour break apart rearrangement probe (Abbott); ES(C): extra signal probe—PML/RARA translocation probe (Cytocell); DF(C): dual fusion probe—PML/RARA translocation dual colour probe (Cytocell); NT: not tested; NA: not available; −: not visible; −/+: only visible via single band-pass filters; +: observed; #PML/RARA RT-PCR identification of the variant transcripts: 1 refers to the *PML* bcr1 within intron 6 and 2 to the *PML* bcr2 with variable breakpoints within exon 6 and 3 to *PML* bcr3 within intron 3 [13].



(a) (b)

Figure 1: (a) Metaphase spread with the Cytocell *PML/RARA* extra signal probe showing a fusion signal on one chromosome 15 and a diminished *RARA* (green) signal on one chromosome 17. (b) Interphase cells with the Vysis dual colour dual fusion probe showing two red and two green signals in the top panel, two red signals in the middle panel, and two green signals plus a tiny third green signal (arrowed) in the bottom panel.

of 38 APL cases, two did not initially show a fusion signal and it was only by observing a tiny additional RARA signal, via the single-pass SpectrumGreen filter, located in the same position as one *PML* signal that the *PML/RARA* fusion was revealed [15]. This is the first report to consistently identify these cryptic rearrangements using alternate FISH probes.

Given the importance of a rapid and reliable test to confirm the diagnosis of APL, it is critical that the possibility of a false negative result using standard FISH probes is considered and that alternate probes are available for rare cases of insertions resulting in the *PML/RARA* fusion.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

The authors are grateful to Bruce Mercer for assistance with FISH testing and the development of the figure.

References

[1] A. Kakizuka, W. H. Miller, K. Umesono et al., "Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML," *Cell*, vol. 66, no. 4, pp. 663–674, 1991.

[2] D. Grimwade, A. Biondi, M. J. Mozziconacci et al., "Characterization of acute promyelocytic leukemia cases lacking the classic t(15;17): results of the European working party," *Blood*, vol. 96, no. 4, pp. 1297–1308, 2000.

[3] D. Grimwade and F. Lo Coco, "Acute promyelocytic leukemia: a model for the role of molecular diagnosis and residual disease monitoring in directing treatment approach in acute myeloid leukemia," *Leukemia*, vol. 16, no. 10, pp. 1959–1973, 2002.

[4] A. Catalano, M. A. Dawson, K. Somana et al., "Brief report: the PRKAR1A gene is fused to RARA in a new variant acute promyelocytic leukemia," *Blood*, vol. 110, no. 12, pp. 4073–4076, 2007.

[5] T. Kondo, A. Mori, S. Darmanin, S. Hashino, J. Tanaka, and M. Asaka, "The seventh pathogenic fusion gene FIP1L1-RARA was isolated from a t(4;17)-positive acute promyelocytic leukemia," *Haematologica*, vol. 93, no. 9, pp. 1414–1416, 2008.

[6] Y. Yamamoto, S. Tsuzuki, M. Tsuzuki, K. Handa, Y. Inaguma, and N. Emi, "BCOR as a novel fusion partner of retinoic acid receptor α in a t(X;17)(p11;q12) variant of acute promyelocytic leukemia," *Blood*, vol. 116, no. 20, pp. 4274–4283, 2010.

[7] D. Grimwade, P. Gorman, E. Duprez et al., "Characterization of cryptic rearrangements and variant translocations in acute promyelocytic leukemia," *Blood*, vol. 90, no. 12, pp. 4876–4885, 1997.

[8] L. G. Shaffer, M. L. Slovak, and L. J. Campbell, *ISCN (2009): An International System For Human Cytogenetic Nomenclature*, S. Karger, Basel, Switzerland, 2009.

[9] C. Schoch, S. Schnittger, W. Kern et al., "Rapid diagnostic approach to PML-RARα-positive acute promyelocytic leukemia," *The Hematology Journal*, vol. 3, no. 5, pp. 259–263, 2002.

[10] H. Iland, K. Bradstock, J. Seymour et al. et al., "Results of the APML3 trial incorporating all-*trans*-retinoic acid and idarubicin in both induction and consolidation as initial therapy for patients with acute promyelocytic leukemia," *Haematologica*, vol. 97, no. 2, pp. 227–234, 2012.

[11] M. A. Sanz, G. Martín, M. González et al., "Risk-adapted treatment of acute promyelocytic leukemia with all-trans-retinoic acid and anthracycline monochemotherapy: a multicenter study by the PETHEMA group," *Blood*, vol. 103, no. 4, pp. 1237–1243, 2004.

[12] H. J. Iland, K. Bradstock, S. G. Supple et al. et al., "All-*trans*-retinoic acid, idarubicin, and IV arsenic trioxide as initial therapy in acute promyelocytic leukemia (APML4)," *Blood*, vol. 120, no. 8, pp. 1570–1580, 2012.

[13] P. P. Pandolfi, M. Alcalay, M. Fagioli et al., "Genomic variability and alternative splicing generate multiple PML/RARα transcripts that encode aberrant PML proteins and PML/RARα isoforms in acute promyelocytic leukaemia," *EMBO Journal*, vol. 11, no. 4, pp. 1397–1407, 1992.

[14] K. Yamamoto, H. Hamaguchi, M. Kobayashi, Y. Tsurukubo, and K. Nagata, "Terminal deletion of the long arm of chromosome 9 in acute promyelocytic leukemia with a cryptic PML/RARα rearrangement," *Cancer Genetics and Cytogenetics*, vol. 113, no. 2, pp. 120–125, 1999.

[15] S. R. Brockman, S. F. Paternoster, R. P. Ketterling, and G. W. Dewald, "New highly sensitive fluorescence in situ hybridization method to detect PML/RARA fusion in acute promyelocytic leukemia," *Cancer Genetics and Cytogenetics*, vol. 145, no. 2, pp. 144–151, 2003.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 391821, 13 pages
http://dx.doi.org/10.1155/2013/391821

Review Article

Molecular Genetics and Genetic Testing in Myotonic Dystrophy Type 1

Dušanka Savić Pavićević, Jelena Miladinović, Miloš Brkušanin, Saša Šviković, Svetlana Djurica, Goran Brajušković, and Stanka Romac

Center for Human Molecular Genetics, Faculty of Biology, University of Belgrade, Studentski trg 16, P.O. Box 52, 11000 Belgrade, Serbia

Correspondence should be addressed to Dušanka Savić Pavićević; duska@bio.bg.ac.rs

Received 26 October 2012; Accepted 5 February 2013

Academic Editor: Yasemin Alanay

Myotonic dystrophy type 1 (DM1) is the most common adult onset muscular dystrophy, presenting as a multisystemic disorder with extremely variable clinical manifestation, from asymptomatic adults to severely affected neonates. A striking anticipation and parental-gender effect upon transmission are distinguishing genetic features in DM1 pedigrees. It is an autosomal dominant hereditary disease associated with an unstable expansion of CTG repeats in the 3′-UTR of the *DMPK* gene, with the number of repeats ranging from 50 to several thousand. The number of CTG repeats broadly correlates with both the age-at-onset and overall severity of the disease. Expanded DM1 alleles are characterized by a remarkable expansion-biased and gender-specific germline instability, and tissue-specific, expansion-biased, age-dependent, and individual-specific somatic instability. Mutational dynamics in male and female germline account for observed anticipation and parental-gender effect in DM1 pedigrees, while mutational dynamics in somatic tissues contribute toward the tissue-specificity and progressive nature of the disease. Genetic test is routinely used in diagnostic procedure for DM1 for symptomatic, asymptomatic, and prenatal testing, accompanied with appropriate genetic counseling and, as recommended, without predictive information about the disease course. We review molecular genetics of DM1 with focus on those issues important for genetic testing and counseling.

1. Introduction

Myotonic dystrophy type 1 (DM1, MIM 160900) is the most frequent adult-onset muscular dystrophy. It was first clinically recognized by Steinert [1] and Batten and Gibb [2] in 1909. The main characteristics of DM1 are myotonia, progressive muscle weakness and wasting, and a broad spectrum of systemic symptoms [3]. Its clinical expression is unusual, characterized by a marked variability between and within pedigrees [3, 4] and a striking genetic anticipation [5] where the age-at-onset typically decreases by 25 to 35 years per generation [6]. Based on clinical ascertainment, worldwide prevalence is estimated to be 12.5/100000 [3], but it can be higher as many patients in older generation remain undiagnosed.

DM1 is inherited in an autosomal dominant pattern. and the underlying mutation is an unstable expansion of CTG repeats in the 3′ untranslated region (3′UTR) of the dystrophia myotonica protein kinase gene (*DMPK*, MIM* 605377) [7–9] and in the promoter of the downstream SIX homeobox 5 gene (*SIX5*, MIM* 600963) [10]. Based on the nature of the causing mutation, DM1 belongs to "disorders of unstable repeat expansion" [11, 12]. Being the first disease described with an RNA gain-of-function mutation effect [13], DM1 is now the paradigm for RNA toxicity model of the disease pathogenesis, as reviewed elsewhere [14–16].

We review the molecular genetics of DM1 with the focus on the unstable nature of the underlying mutation in germline and soma, its relationship to clinical presentation of the disease, and implications for genetic testing and counseling.

2. Clinical Characteristics of DM1

DM1 is one of the most variable inherited human disorders, as the phenotypic expression varies from asymptomatic adults to severely affected neonates with congenital onset of the disease [3]. According to the age-at-onset and severity

of symptoms, the main clinical forms of DM1 are late-onset/mild, adult-onset/classical, childhood-onset/juvenile, and congenital [3, 17]. Although each form presents specific clinical features, there is not absolute distinction between them, and they rather form a continuum.

Adult-onset form is the most prevalent with common age-at-onset in the second or third decade of life and clinical presentation typical for DM1 [3, 17]. Muscular symptoms include progressive muscle degeneration leading to weakness and wasting of facial, neck, and distal limb muscles, and extending more proximally in later stages. Myotonia, clinically presented as slowing relaxation of a normal muscle contraction, typically affects distal parts of the limbs as well as cranial and axial muscles. The most common systemic complications are cataract, cardiac conduction defects and arrhythmias, endocrine dysfunctions, gastrointestinal and respiratory involvement, frontal balding, axonal peripheral neuropathy, neurobehavioral manifestations, and cognitive impairment [3, 18]. Life expectancy is greatly reduced in adult-onset patients, particularly those with an early onset of the symptoms, and the most frequent causes of death are pneumonia and cardiac arrhythmias [19–21].

Congenital form is the most severe, often presenting *in utero* as polyhydramnios and with reduced fetal movement [22]. After delivery, the main features are severe generalized muscle weakness, hypotonia, and respiratory compromise. This severe muscle weakness is not caused by degenerative changes, as in the adult-onset form, but by developmental defects. Mortality in congenital form is usually caused by respiratory complications within the first year of life, with long-term ventilation having a poor prognosis [23]. Children without complicated neonatal course survive and have developmental delay [22]. Their hypotonia improves, they overcome motor delay and start to walk, but their intellectual development is poor and a majority of them are mentally retarded. Clinical myotonia and muscle degeneration usually appear late in childhood. Adults with congenital form have reduced survival by 50% beyond their mid-thirties [24]. The biphasic presenting of congenital form and etiology of the initial hypotonia with its associated problems are still poorly understood.

Childhood-onset form presents between one and ten years of age and is more clearly associated with cognitive and behavioral abnormalities, such as difficulties in learning and socialization at school [25]. In adolescence, myotonia is frequently present and distal muscular weakness may develop. In the second decade of life, individuals with childhood-onset form show many of the symptoms seen in the adult-onset form.

Late-onset form presents after the fifth decade of life with only mild symptoms, and individuals are often not aware of them [26]. Cataracts and baldness may be the sole symptoms. A late-onset muscle weakness may develop and myotonia may only be detectable by electromyography.

3. Genetic Studies in DM1 Pedigrees

Since 1918, it has been known that DM1 is inherited in an autosomal dominant pattern, but, interestingly, with an increased expressivity presenting as decreased age-at-onset and increased severity in subsequent generations of DM1 pedigrees, a phenomenon known as genetic anticipation [5]. This was in contrast to the fundamental principle of Mendelian genetics that mutation was stably transmitted between generations. The frequent observation in DM1 pedigrees is that grandparental generation shows cataracts with minimal or no neuromuscular involvement, the parental generation has more severe neuromuscular symptoms, while an affected child has early or congenital onset and is severely disabled [27]. Beside this, the influence of gender of the transmitting parent on disease course in child is also noticed. Children with the severe congenital form are born almost exclusively to affected mothers, at higher risk being women with neuromuscular involvement [28, 29]. An excess of mildly affected or asymptomatic fathers was found to transmit the disease in a clinically recognizable form [30–32]. When both parents of a DM1 patient are clinically normal, the odds are approximately 2 : 1 that the father is the transmitting parent [32].

4. Molecular Genetics of DM1

Understanding the puzzling genetic features in DM1 pedigrees and an extreme clinical variation of the disease became possible when the underlying mutation was revealed [7–9]. It turned out to belong, at that time, to a newly discovered type of mutation, referred to as dynamic mutation. Dynamic mutations are unstable changes (mostly increases/expansions) in the copy number of simple DNA repeats [33]. They are associated with at least 22 hereditary neurological diseases (e.g., Huntington disease, fragile X syndrome, spinal and bulbar muscular atrophy), known as "disorders of unstable repeat expansion", as reviewed elsewhere [11, 12, 34].

Simple DNA repeats vary in copy number in normal individuals and are stably transmitted with an average mutation rate of $\sim 10^{-3}$ per locus per gamete per generation [35]. For the loci undergoing dynamic mutations, expansions begin when the length of simple DNA repeats exceeds a threshold of ~100–150 bases. After this threshold is overcome, further expansions become progressively more likely to occur, leading to the accumulation of dozens to thousands of repeats in just a few generations [33, 36]. The mutation rate is related to an initial repeat copy number and can even reach the value of 1 per locus per gamete per generation, meaning that the repeat copy number is changed upon every intergenerational transmission.

A considerable number of "disorders of unstable repeat expansion" are characterized by a specific genotype-phenotype correlation, such that the longer repeat arrays are associated with an earlier age-at-onset and with more severe symptoms [37]. The progressive nature of expansion process across generations, together with the characteristic genotype-phenotype correlation, provides a biological basis for a long-debated phenomenon of genetic anticipation, seen in a majority of the "disorders of unstable repeat expansion" [33, 37].

CTG repeat copy number in the *DMPK* gene is polymorphic in a general population, ranging from 5 to 35, and undergoes a pronounced expansion in DM1 individuals,

ranging from 50 to several thousand [38]. The size of the unstable CTG repeats is roughly correlated with both the age-at-onset and overall severity of the disease [7, 39, 40]. Commonly, asymptomatic or late-onset DM1 individuals have from ~50 to 80 CTG repeats and these relatively small expansions are termed protomutations [41]. The upper limit of expansion size in the late-onset DM1 individuals is ~150 CTG repeats [38, 40]. Adult-onset DM1 individuals have a broad range of CTG repeat number, roughly between 100 and 1000 (mean size ~650 repeats), while congenital and childhood-onset DM1 individuals have more than 1000 repeats (mean size ~1200 repeats), [38, 40]. The expansions of more than 80 CTG repeats are known as disease-associated (full) mutations. *DMPK* alleles, which are between the normal and protomutation range (from 35 up to ~50 repeats) are very rare. They are usually not associated with the disease and are termed premutations [42].

Until recently the CTG array in the *DMPK* gene was assumed to be a pure tract (without interruptions/variant repeats), in contrast to a majority of other simple DNA repeats associated with "disorders of unstable repeat expansion." However, 4-5% DM1 individuals carry interrupted expanded alleles with interruptions being multiple CCG triplets, CCGCTG hexamers or CTC triplets, all located at the $3'$ end of the CTG array [43, 44]. Variant repeats seem not to be to present in normal DM1 alleles.

4.1. Intergenerational Change in Repeat Copy Number and Parental-Gender Effect in DM1. In DM1 pedigrees intergenerational change in repeat copy number is biased toward further expansion [32, 39, 40, 45, 46], but less frequently contraction [47, 48], and extremely rare reversion can occur [49]. The direction and extent of intergenerational change in repeat copy number depend on both parental expansion size and gender of the transmitting parent. There is a wide correlation between the size of an expanded allele in parent and the change in the expansion size when it is transmitted to the offspring.

Premutation and protomutation are inherited stably or with smaller changes in repeat copy number for several generations if transmitted by female. When transmitted by men, premutation shows increased instability toward expansion, even reaching the full mutation in a single generation, while protomutation almost invariably results in a large increase in repeat copy number [32, 41, 50–52]. For example, average intergenerational expansion in DM1 alleles with less than 100 repeats was 310 repeats in male transmission versus 105 repeats in female transmission, and the expansions with more than 100 repeats occurred in 92% in paternal transmissions compared to 44% in maternal transmissions [32]. A marked expansion-biased instability of premutation and protomutation upon male transmission is the molecular basis for an excess of males in the last asymptomatic generation in DM1 pedigrees [30–32].

Disease-associated DM1 alleles are almost always unstably transmitted by both genders. For alleles with repeat copy number ranging from 200 to 600 the most frequent event is further increased in repeat copy number [39, 40, 45], but contraction [47, 48] and extremely rare reversion can occur [49]. The absolute increase in repeat copy number is greater in female transmission (mean size ~500–600 repeats) than in male transmission (mean size ~260–280 repeats) [39, 40]. This difference may be the result of an expanded allele size in parent, which is, on average, larger in mothers than in fathers, and could be due to a sampling bias, since the families with the more severe forms, often inherited from the mother, are more likely to be recruited [40]. When the intergenerational increase is expressed as a proportion of the expansion size in parent, the gender difference is not longer seen [39]. Still, a study on the largest cohort of DM1 individuals (~1500) with the expansion size in the range from 200 to 800 repeats, showed that 66% of maternal transmissions resulted in expansions, whereas the majority (69%) of paternal transmissions resulted in stable transmissions or contractions [53].

The largest expansions, associated with congenital form of disease, are almost exclusively maternally transmitted [40, 45], though a few cases of paternally transmitted congenital form have been reported [54, 55]. Mothers of congenital offspring have an expanded allele size significantly greater than mothers of noncongenital offspring (mean size ~600 repeats versus ~250 repeats, resp.) [39, 40, 45]. However, less than 10% of affected mothers give birth to congenitally affected children [56]. In almost all cases affected sisters have children affected with the same DM1 form (either adult-onset or congenital), and the affected sibships, although with variable expansion size, present the same form of the disease [40]. These indicate the existence of still unknown familiar (genetic) factors which increase the risk of having a congenitally affected child. The extremely rare paternal transmission of congenital form can be associated with the observation that fathers with ~650 repeats occasionally pass a larger expansion to their offspring [39].

The estimated frequency of repeat contractions in DM1-pedigrees is 4.2–6.4% [48, 53]. They are usually associated with the change toward less severe or even asymptomatic DM1 form, but in some parent-child pairs anticipation is still present [48, 57]. Contractions are more frequently transmitted by males, and the occurrence of contraction in a family member increases the probability of another contraction in that DM1 pedigree.

For alleles with similar expansion size, the parental-gender effect is comparable across different "disorders of unstable repeat expansion." For example, expansions from premutation to full mutation in Huntington disease (HD) and a large expansions (up to ~100 repeat copy number) associated with a severe juvenile HD occur primarily upon male transmission [58–60], similarly to male-biased instability of DM1 premutations and protomutations. The large expansions in noncoding regions, associated with fragile X syndrome [61] and spinocerebellar ataxia type 8 [62], as well as with congenital form of DM1, are transmitted by females.

Intergenerational changes in repeat copy number are a cumulative result of a pronounced germline and somatic instability of the CTG array in *DMPK* gene [57, 63, 64].

4.2. Germline Instability of Expanded DM1 Alleles. Germline and somatic instability of DM1 alleles was studied by

small-pool PCR (SP-PCR), a method for detailed quantification of the degree of repeat size variation in a given sample [65]. SP-PCR analysis of sperm samples from DM1 individuals, who had either the adult-onset form or were asymptomatic, revealed a high level of repeat size heterogeneity in an individual, with mutation rate of almost 1 per gamete and a pronounced expansion bias [57, 66]. Allele size heterogeneity in sperm showed normal two-tailed distribution with a lower tail extending back down in the normal size range [57]. The degree of variation was highly variable between examined individuals and not obviously correlated with the progenitor allele size-one originally inherited by zygote and measured as the lower boundary of allele size distribution in blood [57, 66]. Two asymptomatic DM1 individuals with relatively stable protomutations in blood (75 and 90 repeats) showed the highest degree of repeat size heterogeneity in sperm (even exceeding 1000 repeats) and the most dramatic allele size increase (on average 180 repeats in one individual and, even, 700 in another). Four individuals with relatively small expansions in blood (average size from 200 to 300 repeats), and progenitor allele size from 150 to 200 repeats, had an average increase from 30 to 120 repeats in sperm. Two other individuals with similar progenitor allele size (210 and 390 repeats), but a higher average size in blood (630 and 700) showed an average increase of about 250 repeats in sperm. Finally, one individual with a highly variable blood distribution (ranging from ~190 to 700 repeats) showed a relatively low level of variation in sperm with the average size of 150 repeats. In all analyzed sperm samples alleles with more than 700–800 repeats were very rare [57, 63].

Among analyzed spermatozoa, left as unused preimplantation diagnostic material and taken from 10 DM1 individuals with progenitor allele size ranging from 62 to 256 repeats, 67% showed expansions with an average change of ~60 repeats, 14% showed contractions with an average change of ~40 repeats, whereas the repeats remained stable in ~10% of spermatozoa [67].

Studies on post-preimplatation diagnostic material, the unique opportunity to study instability in female gametes, revealed a significant increase in repeat length in immature and mature oocytes, which was about 10 times greater than in spermatozoa from DM1 individuals with similar allele size: 22 DM1 individuals with progenitor allele size ranging from 42 to 428 repeats showed an average increase of ~ 300 repeats in oocytes, while the minimal increase was ~ 100 repeats and the maximum one was 950 repeats [67]. In contrast to spermatozoa, contractions were not observed in oocytes.

Timing of germline instability of loci associated with "disorders of unstable repeat expansion" is not completely understood. In DM1 human oocyte increase in the repeat copy number was observed before completion of meiosis, either during premeiotic proliferation of oogonia or during prophase I [67]. In spinocerebellar ataxia type 1 (SCA1) transgenic mice, instability of the repeat copy number occurred while the oocytes were arrested in meiosis I after meiotic DNA replication [68]. In DM1 transgenic mice carrying more than 300 repeats in a large human genomic segment [69], expansions were present in spermatogonia and spermatozoa, indicating that they occurred at the beginning of spermatogenesis and that meiosis and postmeiotic mechanisms are probably not involved in germline expansions [70]. Similarly, expansions in the HD locus were observed in both premeiotic and postmeiotic human male germline cells [71], but in contrast experiments on HD transgenic mice revealed that expansion was a postmeiotic event occurring late in elongation of spermatids, as they become mature sperm cells [72].

Molecular mechanism of the germline instability of expanded alleles and, in general, of dynamic mutations are not well understood. The tendency of the repeat tract to expand or contract seems to be a function of its primary sequence, which enables formation of secondary hairpin-like structure [73, 74]. This intermediate can be formed during processes that involve the separation of DNA strands: DNA replication, repair, and recombination, and each of them has been implicated in repeat instability, as reviewed elsewhere [36, 75]. Several models support instability of expanded repeats during DNA replication [76, 77], while others suggest the appearance of errors in the DNA repair—mismatch repair system [78–80] or gap repair [72].

Different dynamics of DM1 instability in male and female germline is not influenced only by the absolute repeat size, but may also be governed by gender-specific factors that are included in DNA repair and/or replication and are important for mutational pathway. It was hypothesized that some kind of a selection operated to preclude expansions with more than 1000 repeats in spermatogenesis [40, 63], but it is still unclear why DM1 premutation and protomutation stay stable upon passage through female meioses, while full mutation in the same background undergoes dramatic expansion.

4.3. Somatic Instability of Expanded DM1 Alleles. DM1 repeat size heterogeneity in somatic tissues (somatic mosaicism) is tissue specific [63, 81, 82], biased toward further expansions and continuous throughout the life of an individual [57, 64, 83].

Somatic mosaicism has been observed among a number of different tissues, and regarding those relevant to DM1 pathology, much larger expansions (2- to 13-fold greater) were found in skeletal muscles [57, 81, 82, 84] and heart [63] than in peripheral blood, while the smallest expansions were present in frontal cortex and thalamus [63]. In DM1 human fetuses the largest expansions occurred in heart, skin, skeletal muscle, brain, and kidney, and the smallest one in blood [40, 63, 82, 85, 86]. Since the repeat size heterogeneity was observed between tissues from 16-week-old fetus [86] and no mosaicism was detected in any of the affected embryos obtained as post-preimplantation diagnostic material [67], it is thought that somatic mosaicism in affected fetuses starts during the second trimester of gestation [85]. As proposed by Wöhrle et al. [85], this timing correlates with the period of onset of rapid growth of the fetus and implies that during differentiation period in the first trimester, the number of repeats is somehow stabilized. As differences between tissues do not appear to reflect the number of cell divisions during development, the timing of somatic mosaicism in human fetuses might be due to a greater fidelity of DNA repair

mechanisms during differentiation period, which could not be sustained during the rapid growth phase and/or by the suppression of repeat expansion through methylation of the DM1 repeat region [85].

Somatic mosaicism of DM1-expanded alleles within one tissue is seen as a diffused band or smear on Southern blot analysis, or as discrete bands heterogeneous in size on SP-PCR analysis [57, 64]. Quantitative measurement of the repeat size heterogeneity in blood samples by SP-PCR showed a high level of variation, with the mutation frequency from 50% to over 90%, and an allele size distribution skewed towards larger alleles with a lower boundary, below which variant alleles are rare [57]. Data from population-based mathematical modeling of DM1 expanded alleles in blood suggest that bias towards the expansion is a cumulative effect of many expansion and contraction events, possibly as frequently as every other day [87]. The distribution skewed toward expansions is in agreement with the increase of the allele size heterogeneity and the mean allele size with the age of patient observed in the longitudinal studies [83], and together they indicate that somatic instability is continuous throughout life of an individual and expansion-biased. Different DM1 allele size distribution between blood and male germline implicates the distinct mutational dynamics in these tissues.

The major factors affecting the level of somatic instability throughout life are the progenitor allele size and age-at-sampling [83], which together account for 89% of the observed variation [88]. Residual variation is individual specific and heritable as quantitative trait, which implicates the role of transacting factors as modifiers of somatic instability [88]. The most obvious candidates for transacting modifiers are components of the DNA mismatch repair pathway, since mismatch repair genes have been shown to be critical for generating somatic CTG-CAG repeat expansions in mice [79, 89, 90]. Estimation that many expansion and contraction events could occur as frequently as every other day additionally supports the role of DNA repair or transcription, rather than DNA replication, in somatic instability [87].

4.4. Genotype-Phenotype Correlation in DM1. Significant correlations have been reported between genotype and age-at-onset of DM1 [39, 53, 91, 92]. Correlations with specific DM1 symptoms (e.g., neuromuscular involvement [93, 94], cardiac involvement [95–97], cognitive impairment [98, 99], mortality [100], and peripheral neuropathy [101]) are often poor or absent. However, the largest examined cohort of DM1 patients (2650 individuals) revealed the correlation between expansion size and overall DM1 clinical phenotype: (i) patients with an expansion up to 100 repeats have almost 100% probability to stay asymptomatic with normal or abnormal EMG (F0 phenotype) or to develop minimal signs of myotonia with EMG abnormalities (F1 phenotype); (ii) patients with 100–350 repeats have a ~95% probability to develop F1 phenotype or more severe F2 phenotype characterized by myotonia and distal weakness, ECG abnormalities, gonadal dysfunction, mild mental retardation, glucose intolerance, and cataract; (iii) patients with 450–1800 repeats have a 85–95% probability of developing F2 phenotype or the most severe F3 phenotype marked by proximal weakness, cardiac involvement, endocrine dysfunction, mental retardation and cataract; (iv) patients with more than 2000 have a ~90% probability of developing F3 phenotype [53].

Nevertheless, genotype-phenotype correlations in DM1 are compromised due to potential inaccuracy in phenotypic data [17] and by tissue-specific, expansion-biased somatic instability of mutant alleles over the life of a patient [57, 64, 83]. Namely, larger mutant alleles are present in the primarily affected skeletal muscle tissue rather than in blood [81, 84], and there is a difficulty in eliminating the effect of the patient age-at-sampling. Additionally, somatic expansions are assumed to contribute to tissue-specificity and progressive nature of the symptoms [83, 88, 102, 103]. Also, there are technical difficulties for precise assessment of the number of CTG repeats using Southern blot hybridization of genomic DNA, as well as a disagreement in published data as to which point of the diffuse smear on the blot (representing alleles of a different size due to somatic instability) is the appropriate allele size to be used in genotype-phenotype correlation [38–40, 94]. This can be overcome by applying SP-PCR analysis and using the lower boundary in the allele size distribution in blood as progenitor allele. This is a good estimate for the progenitor allele size, since the lower boundary is conserved between tissues and specific for a DM1 individual, and blood is apparently one of the most stable tissues [57, 66]. Even with this approach in some older individuals with larger alleles, it is possible that DM1 alleles in all cells may have expanded beyond the progenitor allele length [83], and it is likely that, in some younger individuals, the lower boundary of the distribution may drop below the progenitor allele due to contractions [87].

By defining DM1 expansion in blood by three parameters: progenitor, average, and largest allele size and by using the SP-PCR, there was reported a negative linear correlation of age-at-onset and average expansion size in juvenile-adult DM1 individuals whose progenitor allele is less than 245 repeats long [103]. This result favors the hypothesis about the existence of a threshold beyond which an increase in repeat length makes no additional contribution toward age-at-onset [91]. However, recent study on a large cohort of DM1 individuals showed that the estimated progenitor allele length was the major modifier of age-at-onset of the disease, accounting for 64% of the variation, without the threshold above which repeat length did not contribute toward age-at-onset [88]. Age-at-onset is further modified by the level of individual-specific somatic instability: patients in whom the number of repeats expands more rapidly have an earlier age-at-onset [88]. Somatic instability of expanded alleles over life has also been implied in the progression of neuromuscular symptoms in juvenile-adult DM1 individuals [103].

Somatic instability has also compromised attempts to precisely measure intergenerational repeat dynamics. Namely, intergenerational change of the repeat length determined by measuring the blood allele size in parent and offspring usually correlates quite well with the observed anticipation. However, a relatively high proportion of cases with apparent contraction in the repeat length still show the anticipation [48, 66]. In these cases progressive age-depended somatic instability

in blood masks the true germline expansion, and such intergenerational change is termed "pseudocontraction" [57].

4.5. Effects of the Interruptions on Repeat Stability and Phenotypic Manifestation in DM1. Interruptions (CCG, CTGCCG, or CTC repeats or even nonrepetitive DNA sequences) at the 3′ end of the CTG array in DM1 alleles with more than 35 repeats may have considerable consequences on mutational dynamics and may also affect phenotypic manifestations [43, 44, 104]. Upon transmission, the interruptions in DM1-expanded alleles show instability and substantial intrafamilial variability, in both their number and location among the relatives [43, 44]. The exception was one pedigree where a complex interruption was stably transmitted [44]. The interruptions may have stabilizing effect on somatic and germline instability, as relatively small maternal intergenerational expansions were observed, or could even predispose to germline contractions, since the frequency of the intergenerational contractions was higher than expected for DM1 and transmitted by females [43, 44]. This can explain the absence of congenital form in the examined families with interrupted DM1 alleles where the disease was maternally transmitted.

DM1-expanded alleles with interruptions may be associated with an atypical phenotype, though the family with cosegregating DM1, Charcot-Marie-Tooth neuropathy, encephalopathic attacks and an early hearing loss is the unique example [44]. In families studied by Musova et al. [43] phenotype of the patients did not differ significantly from the typical clinical picture of DM1. However, in some cases muscular dystrophy was absent and the later age-at-onset than expected solely from the expanded allele size was seen. Interestingly, two of the patients also presented with a polyneuropathy.

Intriguingly, the interruptions were observed in the extremely rare premutations, even in four males. The individuals with interrupted premutations should be unaffected based on their repeat length alone, but this was the case only in one male with 37 interrupted repeats [105]. Other two males, with 37 and 43 interrupted repeats, had a neuromuscular phenotype [43]. Although premutations tend to expand upon male transmission [50], allele with 43 interrupted repeats was stably transmitted. Analysis of a larger set of individuals is warranted to access the frequency of interrupted DM1 alleles and to determine their possible causal or modifying effect on DM1 phenotype.

Identification of a tissue-specific CTG-free configuration in expanded allele in one juvenile-adult DM1 individual further broadens the possible unusual configuration of the expanded DM1 alleles [104]. Described insertion led to a complete loss of the CTG array, retaining only the first CTG and the TG of the very last CTG repeat in cerebral cortex, skeletal muscles, and cerebellum. However, the clinical significance of this distinct configuration in the DM1-expanded allele requires further analysis.

5. Genetic Testing and Counseling in DM1

Identification of the causing DM1 mutation enabled an accurate and specific genetic test to be routinely used in diagnostic procedure. However, genetic counseling in DM1 is still very complex, due to a highly variable clinical presentation, in both in severity and age-at-onset, anticipation, and influence of gender of the transmitting parent.

5.1. Molecular Diagnostic Tests in DM1. Two-step molecular diagnostic procedure is used in DM1 genetic testing (Figure 1) [106]. The first step is to analyze whether an individual is heterozygous for alleles within normal size range by using PCR and fragment-length analysis. If only one normal allele is detected, one of subsequent techniques are used to detect or exclude possible DM1 expansions. For many years Southern blotting of genomic DNA [107] or Southern blotting of long-range PCR products [108] has been used. Recently, triplet-repeat primed PCR (TP-PCR) [109] has come into routine diagnostic procedure [110].

Southern blotting of genomic DNA is time-consuming procedure and requires a considerable amount of high quality DNA. On the other hand, it gives information about repeat copy number and has no limitation to detect even the largest expansions. Southern blotting of long-range PCR products, optimized for amplification of a long and GC rich template, requires less DNA, as small as 15 pg [108], even of lower quality, and gives reliable information about repeat copy number with using a few replicate of PCRs with 180–300 pg of DNA [88]. However, it is also time-consuming and may fail to amplify the largest expansions. TP-PCR is a faster technique, whose specificity and sensitivity is almost 100%, even with the DNA isolated from a single cell, making it usable in preimplantation diagnostics [111]. It is based on the use of locus-specific PCR primers in combination with a primer designed across the repeated sequence [109]. After PCR and fragment analysis, products of different sizes are visible as continuous ladder with a 3-base-pair periodicity. In the presence of DM1 expansion, a continuous ladder exceeds the normal size range. This method provides no size estimation at all, but rather a simple "present"/"absent" result for an expanded allele. If used with a primer located downstream of the CTG repeat, it can be useful for detection of variant repeats located at the 3′ end of CTG array, when gaps could be observed in the regular and contiguous peak pattern, but the reaction may sometimes fail, leading to false negative results [43, 112]. TP-PCR in opposite direction or alternative Southern blotting methods can overcome this situation. This is the reason why performance of TP-PCR in both directions is suggested in order to increase its reliability and accuracy for DM1 testing.

Used together, the aforementioned techniques provide high sensitivity and specificity. As some samples may show inconclusive findings with just one method, diagnostic laboratories should have facility to use more than one methodological approach (usually TP-PCR and one of the Southern blot methods).

5.2. Indications for DM1 Genetic Testing. Genetic testing can be confirmatory/symptomatic testing, preclinical/asymptomatic testing, prenatal testing, and preimplantation testing. The main indications for appropriate kind

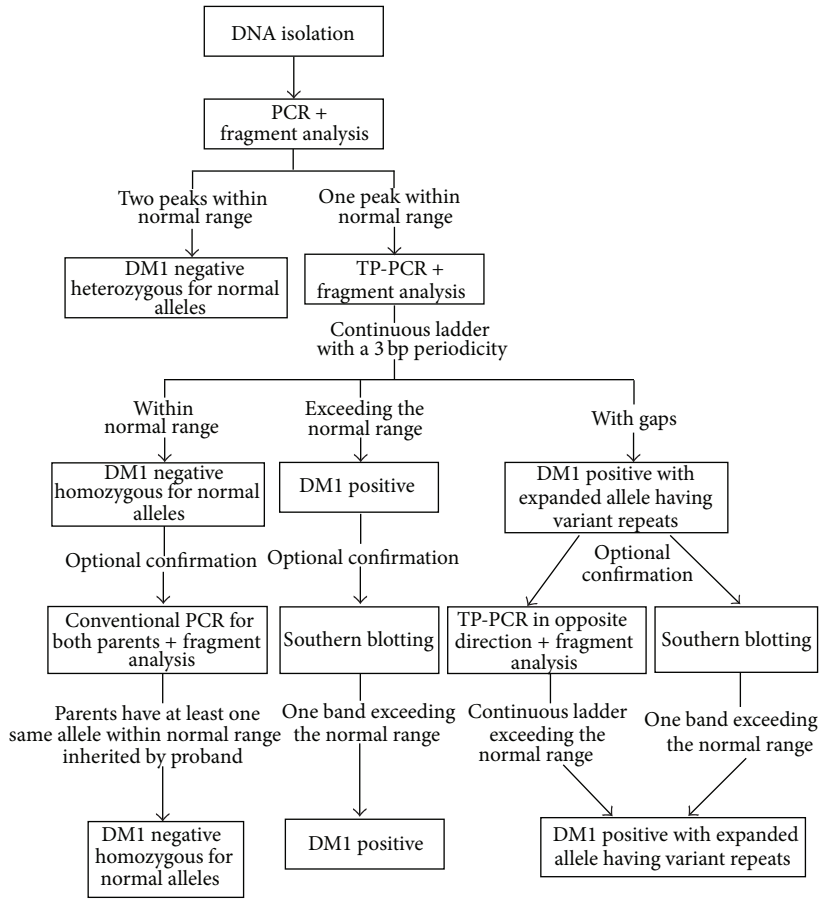


Figure 1: Flow diagram of a genetic test on myotonic dystrophy type 1 (DM1). A two-step procedure is used in DM1 genetic testing. The first step is PCR followed by fragment length analysis, which identifies and sizes alleles within normal range. The second step employs one of the techniques which differentiates between individuals who are homozygous for an allele within normal range and DM1 individuals carrying one allele within normal range and one unamplifiable expanded allele. The most widely used technique in the second step is the triplet-repeat primed PCR (TP-PCR), which utilizes locus-specific PCR primers in combination with a primer designed across the repeated sequence, and provides no size estimation, but rather a simple "present"/"absent" result for an expanded allele. After the fragment length analysis step, products of different sizes are visible as a continuous ladder with a 3-base-pair periodicity. In the presence of a DM1-expanded allele, a continuous ladder exceeds the normal size range. The lower part of the flow diagram shows optional methods used to confirm the obtained result of the two-step diagnostic procedure for DM1, employed when some samples show inconclusive findings. Applied together, PCR, TP-PCR, and Southern blotting methods provide high sensitivity and specificity, and diagnostic laboratories should have a facility to use more than only one methodological approach (usually TP-PCR and one of the Southern blot methods).

of genetic testing in DM1 were given by The International Myotonic Dystrophy Consortium (IDMC) [38] (Table 1).

Genetic testing should be accompanied with appropriate genetic counseling. The result of symptomatic genetic testing has direct implications for other family members (siblings and children), and genetic counseling should be available to tested person and to any other interested family members. Individuals who have asymptomatic testing should always have genetic counseling by a qualified counselor, including pretest counseling to assure that the tested person understands the risks and benefits of testing.

IDMC [38] recommendations for testing of minors are in agreement with many other policies regarding this issue [113]. Minors should not be tested unless there is a direct medical benefit, and this measure is to ensure that the tested person fully understands the risks and benefits of testing. Exceptions might be appropriate in the case of a symptomatic minor for whom confirmatory testing is necessary.

5.3. Reporting Guidelines. At the EMQN Best Practice Meeting, held in 2008 in Nijmegen (The Netherlands), a consensus for the optimal reporting guidelines in myotonic dystrophies was reached (Table 2), and subsequently published as "Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2" [106].

The predictive clinical use of the genetic test result in DM1 is not recommended [38] and may be misleading for several reasons: (i) the distribution of the expanded DM1 alleles is widely spread out, and the expansion sizes are overlapped with each other in different DM1 clinical forms; (ii) genotype-phenotype correlation is compromised by the age-dependent, expansion-biased somatic mosaicism, which also influences

Table 1: The main indications for genetic testing in DM1 given by The International Myotonic Dystrophy Consortium (IDMC) [38], complemented with the suggested indications for preimplantation genetic diagnosis [116].

Genetic testing	Indication for testing
Confirmatory or symptomatic	(i) To confirm the clinical diagnosis: the gene test will increase the physician's confidence in diagnosing a patient with typical symptoms. (ii) To clarify an uncertain/differential clinical diagnosis: the gene test will be useful for individuals in whom DM1 is part of a wider differential diagnosis.
Asymptomatic or preclinical	(i) To determine which progenitor has DM1 mutation, and this information is important in genetic counseling and carrier testing to the relevant side of the family. (ii) To modify *a priori* risk of inheriting the DM1 allele. (iii) To test asymptomatic parent who has 50% risk for DM1 and requires prenatal testing.*
Prenatal testing	(i) If a parent has already been diagnosed with DM1, genetic test can be used to assess fetal risk. (ii) If a parent is at 50% risk and asymptomatic, the best approach is a two-step process by which at-risk parent is tested first, and prenatal diagnosis is done subsequently (if still necessary). (iii) Prenatal diagnosis should not be considered if parents would have the child regardless the test result.
Preimplantation testing	(i) Alternative for prenatal testing. (ii) Couples with concomitant infertility. (iii) Couples unwilling to undergo termination of pregnancy.

*In addition to IDMC indications.

the severity of the disease; (iii) apart from depending on the expanded allele size and age-at-sampling, somatic instability also depends on individual-specific factors. Therefore, it is not appropriate to offer a prognosis based on the expansion size after symptomatic testing nor to give information about the age-at-onset, the kind of symptoms, their severity, nor the rate of progression based on the repeat size after asymptomatic or prenatal testing.

Estimating the risk of having congenitally affected offspring is complicated because of the fact that 18% of mothers with congenital offspring have a similar expansion size as mothers of noncongenital offspring (~less than 300 repeats). Moreover, there is the overlap in the range of expanded allele size between individuals with congenital and other DM1 forms [39, 40, 45]. However, the observations that affected sisters have children affected in almost all cases with the same DM1 clinical form [40] and that the affected sibships present with the same form of the disease could be useful in counseling.

5.4. Prenatal Diagnosis and Preimplantation Genetic Diagnosis in DM1. In families at risk to have a child with DM1, prenatal diagnosis (PND) can be offered. Prenatal samples are chorionic villi, taken between the 10th and 12th week of gestation, or amniotic fluid, taken between the 14th and 16th week of gestation. Analysis of DNA from the mother is also required to exclude maternal contamination in the fetal sample, especially if the chorionic villi sample is used. In some cases, usually when fetus is homozygous for DM1 alleles within normal range, analyses of DNA from the unaffected parent can be required to verify the PCR results. Indications for PND according to IDMC [38] are presented in Table 1.

Preimplantation genetic diagnosis (PGD) for DM1, an alternative to prenatal diagnosis for individuals at risk of transmitting DM1, was developed in 1995 [114] and is now offered routinely in several countries [115–117]. PGD involves the genetic testing of blastomeres from embryos obtained *in vitro*, followed by the transfer of only those diagnosed as healthy with regard to the disease under consideration [118]. So, unlike the PND which is followed by the termination of pregnancy in the case of an affected embryo, PGD circumvents the problem of therapeutic abortion. From the viewpoint of DNA analysis, prerequisite for a clinically applicable PGD for DM1 has been the development of a sensitive single-cell PCR assay. The first approach was based on the detection of embryos heterozygous for normal DM1 alleles, exploiting the feature that DM1 CTG repeats are highly polymorphic in a general population [38]. The originally applied DNA technique was a nested PCR [114] and was subsequently replaced by 1000 times more sensitive fluorescent PCR, which also reduced the rate of allelic drop out (failure to amplify one or two alleles in a heterozygous cell) from 21% to 5.2%, enabling a much smaller loss of embryos due to misdiagnosis [119]. As the expanded DM1 alleles were not amplified by aforementioned assays, the disadvantage of this approach was application only for informative couples—affected partner has a wild type allele clearly different in size from the unaffected one. The selected healthy embryos were always heterozygous, carrying the normal allele of the affected parent and one of the two normal alleles of the unaffected parent, while detecting only one DM1 allele from the unaffected parent (regardless of possible allelic drop out) indirectly meant that embryo was DM1 positive. From 1997 onwards, with the development of a sensitive TP-PCR for detection of DM1 expansions, PGD could also be offered for half-informative (couples with both partners sharing one normal allele of the same size) or noninformative couples (couples with three normal alleles identical in size) [111]. Further improvement of the accuracy of PGD for DM1, in terms of detection of contamination of the sample and allelic drop out,

Table 2: Reporting guidelines for DM1 genetic testing according to Kamsteeg et al. [106] complemented with the influence of gender of the transmitting parent.

Genetic test result	Recommended reporting
No expansion-homozygous or heterozygous for allele in the size range of 5–35 repeats (normal alleles)	DM1 diagnosis is excluded; when it concerns a fetus, it is not affected.
A heterozygous expansion in the size range of 36–50 repeats (premutation alleles)	(i) DM1 diagnosis is excluded; when it concerns a fetus, it is not affected. (ii) Premutations may or may not expand in next generations. Transmission by female mostly results in stable inheritance or small changes in repeat copy number, while when transmitted by men, they are more prone to expand, even reaching the disease-associated mutation in a single generation, thus raising the risk of having affected child. (iii) Relatives (including offspring) of the counselee may be at risk of developing DM1 and should be offered counseling. An offer of repeat-length analysis to those relatives is warranted.
A heterozygous expansion in the size range of 51–150 repeats	(i) When symptoms are evident, the diagnosis of DM1 is confirmed. (ii) When symptoms of DM1 are not evident (asymptomatic family member or fetus), the individual is at risk of developing DM1, although individuals with a repeat expansion of this size may also remain symptomless. (iii) Counselees in the reproductive age is warranted. Smaller repeat expansion of this size range can be stably transmitted by female, while larger repeat expansion of this size range raising the risk of having a child with even congenital form of DM1. When transmitted by male repeat expansion of this size range almost invariably results in a large increase into the disease-associated mutation, raising the risk of having affected offspring. (iv) Relatives (including offspring) of the counselee may be at risk of developing DM1. Due to anticipation in DM1, offspring may be more severely affected. Relatives should therefore be offered counseling. An offer of repeat-length analysis to those relatives is warranted.
A heterozygous expansion with a size over 150 repeats	(i) When symptoms are evident, the diagnosis of DM1 is confirmed. (ii) When symptoms of DM1 are not evident (asymptomatic family member), the individual is at risk of developing DM1, although individuals with a repeat expansion of this size range may rarely remain symptomless. (iii) When it concerns a fetus, it is very likely to be affected and has a high risk to be more severely affected than the affected parent. (iv) Counselees in the reproductive age is warranted. Women are, especially, at risk of having children with the congenital form of DM1. (v) Relatives (including offspring) of the counselee may be at risk of developing DM1. Due to anticipation in DM1, the offspring may be more severely affected. Therefore, relatives should be offered counseling. An offer of repeat-length analysis to those relatives is warranted.

was achieved by the use of multiplex PCR with combined DM1-linked markers and detection of the repeat fragments [116].

Report on a large cohort of DM1 patients undergoing PGD for DM1-showed that (i) it was safe in DM1-affected women after careful pretreatment assessment with regard to disease-specific complications (cardiological, anaesthetical, and obstetrical problems), (ii) delivery rate per treatment cycle was 20%, with at least one baby after two PGD cycles in almost half of the couples, and (iii) the children born were generally in good health up to 2 years of age and comparable to children born after intracytoplasmatic sperm injection for infertility and after PGD for other genetic conditions [116]. As pointed out by de Rademaeker et al. [116], PGD for DM1 is a well-established procedure resulting in the birth of unaffected and mostly healthy children and should be considered as an alternative to PND in couples with concomitant infertility and couples unwilling to undergo termination of pregnancy (Table 1).

6. Conclusions

Discovering that an expansion of the CTG repeats in the DMPK gene is underlying DM1 mutation has opened molecular genetic studies and has facilitated the understanding of underlying pathogenic mechanisms of this disease. Many of its puzzling features, such as a striking genetic anticipation, parental-gender effect in DM1 pedigrees, tissue-specificity, and progressive nature of the disease have been explained by a characteristic mutational dynamics in male and female germline as well as in somatic tissues. Also, a highly variable phenotypic expression, varying from asymptomatic adults to severely affected children with congenital onset of the disease, is broadly correlated with the repeat copy number in mutated allele. However, diverse mutational dynamics of different kinds of DM1-expanded alleles in male and female germline are not completely understood. In addition, it is clear that the repeat copy number is not the only factor determining the phenotypic manifestation of the disease or

the risk of having congenitally affected offspring. A new "next-generation" sequencing platform, single-molecule real-time (SMRT) sequencing, suited for sequencing of long, repetitive DNA sequence [120], is a promising approach for studying interruptions and epigenetic marks in the expanded DM1 alleles, as additional factors influencing germline and somatic repeat instability and phenotypic expression of the disease.

Acknowledgment

Research in Center for Human Molecular Genetics, Faculty of Biology, University of Belgrade has been supported by the Ministry of Education, Science and Technological Development, Serbia (Grant no. 173016).

References

[1] H. Steinert, "Uber das klinishe und anatomische bild des muskelschwundes der myotoniker," *Deutsche Zeitschrift für Nervenheilkunde*, vol. 37, p. 38, 1909.

[2] F. E. Batten and H. P. Gibb, "Myotonia atrophica," *Brain*, vol. 32, no. 2, pp. 187–205, 1909.

[3] P. S. Harper, *Myotonic Dystrophy*, WB Saunders, London, UK, 3rd edition, 2001.

[4] H. G. Harley, J. D. Brook, S. A. Rundle et al., "Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy," *Nature*, vol. 355, no. 6360, pp. 545–547, 1992.

[5] B. Fleischer, "Über myotonische dystrophie mit katarakt," *Albrecht von Græfes Archiv für Ophthalmologie*, vol. 96, no. 1-2, pp. 91–133, 1918.

[6] C. J. Höweler, H. F. Busch, J. P. Geraedts, M. F. Niermeijer, and A. Staal, "Anticipation in myotonic dystrophy: fact or fiction?" *Brain*, vol. 112, part 3, pp. 779–797, 1989.

[7] J. D. Brook, M. E. McCurrach, H. G. Harley et al. et al., "Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member," *Cell*, vol. 68, no. 2, pp. 799–808, 1992.

[8] M. Mahadevan, C. Tsilfidis, L. Sabourin et al., "Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene," *Science*, vol. 255, no. 5049, pp. 1253–1255, 1992.

[9] Y. H. Fu, A. Pizzuti, R. G. Fenwick et al., "An unstable triplet repeat in a gene related to myotonic muscular dystrophy," *Science*, vol. 255, no. 5049, pp. 1256–1258, 1992.

[10] C. A. Boucher, S. K. King, N. Carey et al., "A novel homeodomain-encoding gene is associated with a large CpG island interrupted by the myotonic dystrophy unstable (CTG)n repeat," *Human Molecular Genetics*, vol. 4, no. 10, pp. 1919–1925, 1995.

[11] J. R. Gatchel and H. Y. Zoghbi, "Diseases of unstable repeat expansion: mechanisms and common principles," *Nature Reviews Genetics*, vol. 6, no. 10, pp. 743–755, 2005.

[12] R. D. Wells and T. Ashizawa, *Genetic Instabilities and Hereditary Neurological Diseases*, Elsevier, 2nd edition, 2006.

[13] A. Mankodi, E. Logigian, L. Callahan et al., "Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat," *Science*, vol. 289, no. 5485, pp. 1769–1772, 2000.

[14] J. W. Day and L. P. W. Ranum, "RNA pathogenesis of the myotonic dystrophies," *Neuromuscular Disorders*, vol. 15, no. 1, pp. 5–16, 2005.

[15] D. H. Cho and S. J. Tapscott, "Myotonic dystrophy: emerging mechanisms for DM1 and DM2," *Biochimica et Biophysica Acta*, vol. 1772, no. 2, pp. 195–204, 2007.

[16] J. E. Lee and T. A. Cooper, "Pathogenic mechanisms of myotonic dystrophy," *Biochemical Society Transactions*, vol. 37, no. 6, pp. 1281–1286, 2009.

[17] B. Udd and R. Krahe, "The myotonic dystrophies: molecular, clinical, and therapeutic challenges," *Lancet Neurology*, vol. 11, no. 10, pp. 891–905, 2012.

[18] C. Delaporte, "Personality patterns in patients with myotonic dystrophy," *Archives of Neurology*, vol. 55, no. 5, pp. 635–640, 1998.

[19] C. E. M. de Die-Smulders, C. J. Höweler, C. Thijs et al., "Age and causes of death in adult-onset myotonic dystrophy," *Brain*, vol. 121, no. 8, pp. 1557–1563, 1998.

[20] J. Mathieu, P. Allard, L. Potvin, C. Prévost, and P. Begin, "A 10-year study of mortality in a cohort of patients with myotonic dystrophy," *Neurology*, vol. 52, no. 8, pp. 1658–1662, 1999.

[21] J. Mladenovic, T. Pekmezovic, S. Todorovic et al., "Survival and mortality of myotonic dystrophy type 1 (Steinert's disease) in the population of Belgrade," *European Journal of Neurology*, vol. 13, no. 5, pp. 451–454, 2006.

[22] P. S. Harper, "Congenital myotonic dystrophy in Britain. I. Clinical aspects," *Archives of Disease in Childhood*, vol. 50, no. 7, pp. 505–513, 1975.

[23] M. A. Rutherford, J. Z. Heckmatt, and V. Dubowitz, "Congenital myotonic dystrophy: respiratory function at birth determines survival," *Archives of Disease in Childhood*, vol. 64, no. 2, pp. 191–195, 1989.

[24] W. Reardon, R. Newcombe, I. Fenton, J. Sibert, and P. S. Harper, "The natural history of congenital myotonic dystrophy: mortality and long term clinical aspects," *Archives of Disease in Childhood*, vol. 68, no. 2, pp. 177–181, 1993.

[25] B. Echenne, A. Rideau, A. Roubertie, G. Sébire, F. Rivier, and B. Lemieux, "Myotonic dystrophy type I in childhood. Long-term evolution in patients surviving the neonatal period," *European Journal of Paediatric Neurology*, vol. 12, no. 3, pp. 210–223, 2008.

[26] M. E. Arsenault, C. Prévost, A. Lescault, C. Laberge, J. Puymirat, and J. Mathieu, "Clinical characteristics of myotonic dystrophy type 1 patients with small CTG expansions," *Neurology*, vol. 66, no. 8, pp. 1248–1250, 2006.

[27] P. S. Harper and R. Rudel, "Myotonic dystrophy," in *Myology, Engel and Franzini-Armstrong*, vol. 2, chapter 43, pp. 1192–1218, McGraw-Hill, New York, NY, USA, 1994.

[28] P. S. Harper and P. R. Dyken, "Early-onset dystrophia myotonica. Evidence supporting a maternal environmental factor," *Lancet*, vol. 2, no. 7767, pp. 53–55, 1972.

[29] P. R. Dyken and P. S. Harper, "Congenital dystrophia myotonica," *Neurology*, vol. 23, no. 5, pp. 465–473, 1973.

[30] J. Bell, "Dystrophia myotonica and allied diseases," *Treasury of Human Inheritance*, vol. 4, pp. 342–410, 1947.

[31] D. Klein, "La dystrophie myotonique (Steinert) et la myotonie congenitale [Thomsen] en Suisse: etude clinique, genetique, et demographique," *Journal De Génétique Humaine*, vol. 7, no. 20–41, pp. 320–326, 1958.

[32] H. G. Brunner, H. T. Bruggenwirth, W. Nillesen et al., "Influence of sex of the transmitting parent as well as of parental allele size on the CTG expansion in myotonic dystrophy (DM)," *American Journal of Human Genetics*, vol. 53, no. 5, pp. 1016–1023, 1993.

[33] R. I. Richards and G. R. Sutherland, "Dynamic mutations: a new class of mutations causing human disease," *Cell*, vol. 70, no. 5, pp. 709–712, 1992.

[34] A. R. La Spada and J. P. Taylor, "Repeat expansion disease: progress and puzzles in disease pathogenesis," *Nature Reviews Genetics*, vol. 11, no. 4, pp. 247–258, 2010.

[35] B. Brinkmann, M. Klintschar, F. Neuhuber, J. Hühne, and B. Rolf, "Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat," *American Journal of Human Genetics*, vol. 62, no. 6, pp. 1408–1415, 1998.

[36] S. M. Mirkin, "Expandable DNA repeats and human disease," *Nature*, vol. 447, no. 7147, pp. 932–940, 2007.

[37] R. I. Richards, "Dynamic mutations: a decade of unstable expanded repeats in human genetic disease," *Human Molecular Genetics*, vol. 10, no. 20, pp. 2187–2194, 2001.

[38] The International Myotonic Dystrophy Consortium (IDMC), "New nomenclature and DNA testing guidelines for myotonic dystrophy type 1 (DM1)," *Neurology*, vol. 54, no. 6, pp. 1218–1221, 2000.

[39] H. G. Harley, S. A. Rundle, J. C. MacMillan et al., "Size of the unstable CTG repeat sequence in relation to phenotype and parental transmission in myotonic dystrophy," *American Journal of Human Genetics*, vol. 52, no. 6, pp. 1164–1174, 1993.

[40] C. Lavedan, H. Hofmann-Radvanyi, P. Shelbourne et al., "Myotonic dystrophy: size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism," *American Journal of Human Genetics*, vol. 52, no. 5, pp. 875–883, 1993.

[41] J. M. Barcelo, M. S. Mahadevan, C. Tsilfidis, A. E. MacKenzie, and R. G. Korneluk, "Intergenerational stability of the myotonic dystrophy protomutation," *Human Molecular Genetics*, vol. 2, no. 6, pp. 705–709, 1993.

[42] H. Yamagata, T. Miki, S. I. Sakoda et al., "Detection of a pre-mutation in Japanese myotonic dystrophy," *Human Molecular Genetics*, vol. 3, no. 5, pp. 819–820, 1994.

[43] Z. Musova, R. Mazanec, A. Krepelova et al., "Highly unstable sequence interruptions of the CTG repeat in the myotonic dystrophy gene," *American Journal of Medical Genetics A*, vol. 149, no. 7, pp. 1365–1369, 2009.

[44] C. Braida, R. K. A. Stefanatos, B. Adam et al., "Variant CCG and GGC repeats within the CTG expansion dramatically modify mutational dynamics and likely contribute toward unusual symptoms in some myotonic dystrophy type 1 patients," *Human Molecular Genetics*, vol. 19, no. 8, Article ID ddq015, pp. 1399–1412, 2010.

[45] C. Tsilfidis, A. E. MacKenzie, G. Mettler, J. Barceló, and R. G. Korneluk, "Correlation between CTG trinucleotide repeat length and frequency of severe congenital myotonic dystrophy," *Nature Genetics*, vol. 1, no. 3, pp. 192–195, 1992.

[46] T. Ashizawa, P. W. Dunne, P. A. Ward, W. K. Seltzer, and C. S. Richards, "Effects of the sex of myotonic dystrophy patients on the unstable triplet repeat in their affected offspring," *Neurology*, vol. 44, no. 1, pp. 120–122, 1994.

[47] K. L. O'Hoy, C. Tsilfidis, M. S. Mahadevan et al., "Reduction in size of the myotonic dystrophy trinucleotide repeat mutation during transmission," *Science*, vol. 259, no. 5096, pp. 809–812, 1993.

[48] T. Ashizawa, M. Anvret, M. Baiget et al., "Characteristics of intergenerational contractions of the CTG repeat in myotonic dystrophy," *American Journal of Human Genetics*, vol. 54, no. 3, pp. 414–423, 1994.

[49] H. G. Brunner, G. Jansen, W. Nillesen et al., "Brief report: reverse mutation in myotonic dystrophy," *New England Journal of Medicine*, vol. 328, no. 7, pp. 476–480, 1993.

[50] L. Martorell, D. G. Monckton, A. Sanchez, A. Lopez de Munain, and M. Baiget, "Frequency and stability of the myotonic dystrophy type 1 premutation," *Neurology*, vol. 56, no. 3, pp. 328–335, 2001.

[51] Z. Simmons, C. A. Thornton, W. K. Seltzer, and C. Sue Richards, "Relative stability of a minimal CTG repeat expansion in a large kindred with myotonic dystrophy," *Neurology*, vol. 50, no. 5, pp. 1501–1504, 1998.

[52] D. Savić, D. Keckarević, V. Branković-Srećković, S. Apostolski, S. Todorović, and S. Romac, "Clinical case report: atypical myopathy in a young girl with 91 CTG repeats in DM1 locus and a positive DM1 family history," *International Journal of Neuroscience*, vol. 116, no. 12, pp. 1509–1518, 2006.

[53] L. B. Salehi, E. Bonifazi, E. Di Stasio et al., "Risk prediction for clinical phenotype in myotonic dystrophy type 1: data from 2,650 patients," *Genetic Testing*, vol. 11, no. 1, pp. 84–90, 2007.

[54] J. Bergoffen, J. Kant, J. Sladky, D. McDonald-McGinn, E. H. Zackai, and K. H. Fischbeck, "Paternal transmission of congenital myotonic dystrophy," *Journal of Medical Genetics*, vol. 31, no. 7, pp. 518–520, 1994.

[55] C. E. M. de Die-Smulders, H. J. M. Smeets, W. Loots et al., "Paternal transmission of congenital myotonic dystrophy," *Journal of Medical Genetics*, vol. 34, no. 11, pp. 930–933, 1997.

[56] M. C. Koch, T. Grimm, H. G. Harley, and P. S. Harper, "Genetic risks for children of women with myotonic dystrophy," *American Journal of Human Genetics*, vol. 48, no. 6, pp. 1084–1091, 1991.

[57] D. G. Monckton, L. J. C. Wong, T. Ashizawa, and C. T. Caskey, "Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses," *Human Molecular Genetics*, vol. 4, no. 1, pp. 1–8, 1995.

[58] Y. P. Goldberg, B. Kremer, S. E. Andrew et al., "Molecular analysis of new mutations for Huntington's disease: intermediate alleles and sex of origin effects," *Nature Genetics*, vol. 5, no. 2, pp. 174–179, 1993.

[59] Y. Trottier, V. Biancalana, and J. L. Mandel, "Instability of CAG repeats in Huntington's disease: relation to parental transmission and age of onset," *Journal of Medical Genetics*, vol. 31, no. 5, pp. 377–382, 1994.

[60] S. S. Chong, E. Almqvist, H. Telenius et al., "Contribution of DNA sequence and CAG size to mutation frequencies of intermediate alleles for Huntington disease: evidence from single sperm analyses," *Human Molecular Genetics*, vol. 6, no. 2, pp. 301–309, 1997.

[61] H. E. Malter, J. C. Iber, R. Willemsen et al., "Characterization of the full fragile X syndrome mutation in fetal gametes," *Nature Genetics*, vol. 15, no. 2, pp. 165–169, 1997.

[62] M. D. Koob, M. L. Moseley, L. J. Schut et al., "An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8)," *Nature Genetics*, vol. 21, no. 4, pp. 379–384, 1999.

[63] G. Jansen, P. Willems, M. Coerwinkel et al., "Gonosomal mosaicism in myotonic dystrophy patients: involvement of mitotic events in (CTG)(n) repeat variation and selection against extreme expansion in sperm," *American Journal of Human Genetics*, vol. 54, no. 4, pp. 575–585, 1994.

[64] L. J. C. Wong, T. Ashizawa, D. G. Monckton, C. T. Caskey, and C. S. Richards, "Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent," *American Journal of Human Genetics*, vol. 56, no. 1, pp. 114–122, 1995.

[65] A. J. Jeffreys, K. Tamaki, A. MacLeod, D. G. Monckton, D. L. Neil, and J. A. L. Armour, "Complex gene conversion events

in germline mutation at human minisatellites," *Nature Genetics*, vol. 6, no. 2, pp. 136–145, 1994.

[66] L. Martorell, D. G. Monckton, J. Gamez, and M. Baiget, "Complex patterns of male germline instability and somatic mosaicism in myotonic dystrophy type 1," *European Journal of Human Genetics*, vol. 8, no. 6, pp. 423–430, 2000.

[67] N. de Temmerman, K. Sermon, S. Seneca et al., "Intergenerational instability of the expanded CTG repeat in the DMPK gene: studies in human gametes and preimplantation embryos," *American Journal of Human Genetics*, vol. 75, no. 2, pp. 325–329, 2004.

[68] M. D. Kaytor, E. N. Burright, L. A. Duvick, H. Y. Zoghbi, and H. T. Orr, "Increased trinucleotide repeat instability with advanced maternal age," *Human Molecular Genetics*, vol. 6, no. 12, pp. 2135–2139, 1997.

[69] H. Seznec, A. S. Lia-Baldini, C. Duros et al., "Transgenic mice carrying large human genomic sequences with expanded CTG repeat mimic closely the DM CTG repeat intergenerational and somatic instability," *Human Molecular Genetics*, vol. 9, no. 8, pp. 1185–1194, 2000.

[70] C. Savouret, C. Garcia-Cordier, J. Megret, H. T. Riele, C. Junien, and G. Gourdon, "MSH2-dependent germinal CTG repeat expansions are produced continuously in spermatogonia from DM1 transgenic mice," *Molecular and Cellular Biology*, vol. 24, no. 2, pp. 629–637, 2004.

[71] S. R. Yoon, L. Dubeau, M. de Young, N. S. Wexler, and N. Arnheim, "Huntington disease expansion mutations in humans can occur before meiosis is completed," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8834–8838, 2003.

[72] I. V. Kovtun and C. T. McMurray, "Trinucleotide expansion in haploid germ cells by gap repair," *Nature Genetics*, vol. 27, no. 4, pp. 407–411, 2001.

[73] A. M. Gacy, G. Goellner, N. Juranic, S. Macura, and C. T. McMurray, "Trinucleotide repeats that expand in human disease form hairpin structures in vitro," *Cell*, vol. 81, no. 4, pp. 533–540, 1995.

[74] C. T. McMurray, "DNA secondary structure: a common and causative factor for expansion in human disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 1823–1825, 1999.

[75] C. E. Pearson, K. N. Edamura, and J. D. Cleary, "Repeat instability: mechanisms of dynamic mutations," *Nature Reviews Genetics*, vol. 6, no. 10, pp. 729–742, 2005.

[76] J. D. Cleary, K. Nichol, Y. H. Wang, and C. E. Pearson, "Evidence of cis-acting factors in replication-mediated trinucleotide repeat instability in primate cells," *Nature Genetics*, vol. 31, no. 1, pp. 37–46, 2002.

[77] Z. Yang, R. Lau, J. L. Marcadier, D. Chitayat, and C. E. Pearson, "Replication inhibitors modulate instability of an expanded trinucleotide repeat at the myotonic dystrophy type 1 disease locus in human cells," *American Journal of Human Genetics*, vol. 73, no. 5, pp. 1092–1105, 2003.

[78] C. E. Pearson, A. Ewel, S. Acharya, R. A. Fishel, and R. R. Sinden, "Human MSH2 binds to trinucleotide repeat DNA structures associated with neurodegenerative diseases," *Human Molecular Genetics*, vol. 6, no. 7, pp. 1117–1123, 1997.

[79] W. J. A. A. van den Broek, M. R. Nelen, D. G. Wansink et al., "Somatic expansion behaviour of the (CTG)n repeat in myotonic dystrophy knock-in mice is differentially affected by Msh3 and Msh6 mismatch-repair proteins," *Human Molecular Genetics*, vol. 11, no. 2, pp. 191–198, 2002.

[80] C. Savouret, E. Brisson, J. Essers et al., "CTG repeat instability and size variation timing in DNA repair-deficient mice," *EMBO Journal*, vol. 22, no. 9, pp. 2264–2273, 2003.

[81] M. Anvret, G. Ahlberg, U. Grandell, B. Hedberg, K. Johnson, and L. Edstrom, "Larger expansions of the CTG repeat in muscle compared to lymphocytes from patients with myotonic dystrophy," *Human Molecular Genetics*, vol. 2, no. 9, pp. 1397–1400, 1993.

[82] T. Ashizawa, J. R. Dubel, and Y. Harati, "Somatic instability of CTG repeat in myotonic dystrophy," *Neurology*, vol. 43, no. 12 I, pp. 2674–2678, 1993.

[83] L. Martorell, D. G. Monckton, J. Gamez et al., "Progression of somatic CTG repeat length heterogeneity in the blood cells of myotonic dystrophy patients," *Human Molecular Genetics*, vol. 7, no. 2, pp. 307–312, 1998.

[84] C. A. Thornton, K. Johnson, and R. T. Moxley III, "Myotonic dystrophy patients have larger CTG expansions in skeletal muscle than in leukocytes," *Annals of Neurology*, vol. 35, no. 1, pp. 104–107, 1994.

[85] D. Wöhrle, I. Kennerknecht, M. Wolf, H. Enders, S. Schwemmle, and P. Steinbach, "Heterogeneity of DM kinase repeat expansion in different fetal tissues and further expansion during cell proliferation in vitro: evidence for a causal involvement of methyl-directed DNA mismatch repair in triplet repeat stability," *Human Molecular Genetics*, vol. 4, no. 7, pp. 1147–1153, 1995.

[86] L. Martorell, K. Johnson, C. A. Boucher, and M. Baiget, "Somatic instability of the myotonic dystrophy (CTG)(n) repeat during human fetal development," *Human Molecular Genetics*, vol. 6, no. 6, pp. 877–880, 1997.

[87] C. F. Higham, F. Morales, C. A. Cobbold, D. T. Haydon, and D. G. Monckton, "High levels of somatic DNA diversity at the myotonic dystrophy type 1 locus are driven by ultra-frequent expansion and contraction mutations," *Human Molecular Genetics*, vol. 21, no. 11, pp. 2450–2463, 2012.

[88] F. Morales, J. M. Couto, C. F. Higham et al., "Somatic instability of the expanded CTG triplet repeat in myotonic dystrophy type 1 is a heritable quantitative trait and modifier of disease severity," *Human Molecular Genetics*, vol. 21, no. 16, pp. 3558–3567, 2012.

[89] K. Manley, T. L. Shirley, L. Flaherty, and A. Messer, "Msh2 deficiency prevents in vivo somatic instability of the CAG repeat in Huntington disease transgenic mice," *Nature Genetics*, vol. 23, no. 4, pp. 471–473, 1999.

[90] M. Gomes-Pereira, M. T. Fortune, L. Ingram, J. P. McAbney, and D. G. Monckton, "Pms2 is a genetic enhancer of trinucleotide CAG-CTG repeat somatic mosaicism: implications for the mechanism of triplet repeat expansion," *Human Molecular Genetics*, vol. 13, no. 16, pp. 1815–1825, 2004.

[91] M. G. Hamshere, H. Harley, P. Harper, J. D. Brook, and J. F. Y. Brookfield, "Myotonic dystrophy: the correlation of (CTG) repeat length in leucocytes with age at onset is significant only for patients with small expansions," *Journal of Medical Genetics*, vol. 36, no. 1, pp. 59–61, 1999.

[92] K. M. Hsiao, S. S. Chen, S. Y. Li et al., "Epidemiological and genetic studies of myotonic dystrophy type 1 in Taiwan," *Neuroepidemiology*, vol. 22, no. 5, pp. 283–289, 2003.

[93] M. Gennarelli, G. Novelli, F. Andreasi Bassi et al., "Prediction of myotonic dystrophy clinical severity based on the number of intragenic [CTG]n trinucleotide repeats," *American Journal of Medical Genetics*, vol. 65, no. 4, pp. 342–347, 1996.

[94] C. Marchini, R. Lonigro, L. Verriello, L. Pellizzari, P. Bergonzi, and G. Damante, "Correlations between individual clinical

manifestations and CTG repeat amplification in myotonic dystrophy," *Clinical Genetics*, vol. 57, no. 1, pp. 74–82, 2000.

[95] J. Finsterer, E. Gharehbaghi-Schnell, C. Stöllberger, K. Fheodoroff, and A. Seiser, "Relation of cardiac abnormalities and CTG-repeat size in myotonic dystrophy," *Clinical Genetics*, vol. 59, no. 5, pp. 350–355, 2001.

[96] K. Merlevede, D. Vermander, P. Theys, E. Legius, H. Ector, and W. Robberecht, "Cardiac involvement and CTG expansion in myotonic dystrophy," *Journal of Neurology*, vol. 249, no. 6, pp. 693–698, 2002.

[97] V. Rakočević-Stojanović, D. Savić, S. Pavlović, D. Lavrnić, S. Romac, and S. Apostolski, "Correlation between cardiac involvement and CTG repeat amplification in myotonic dystrophy type 1," *Acta Myologica*, vol. 22, no. 1, pp. 26–27, 2003.

[98] A. Modoni, G. Silvestri, M. G. Pomponi, F. Mangiola, P. A. Tonali, and C. Marra, "Characterization of the pattern of cognitive impairment in myotonic dystrophy type 1," *Archives of Neurology*, vol. 61, no. 12, pp. 1943–1947, 2004.

[99] S. Winblad, C. Lindberg, and S. Hansen, "Cognitive deficits and CTG repeat expansion size in classical myotonic dystrophy type 1 (DMI)," *Behavioral and Brain Functions*, vol. 2, article 16, 2006.

[100] W. J. Groh, M. R. Groh, C. Shen, D. G. Monckton, C. L. Bodkin, and R. M. Pascuzzi, "Survival and CTG repeat expansion in adults with myotonic dystrophy type 1," *Muscle and Nerve*, vol. 43, no. 5, pp. 648–651, 2011.

[101] V. Rakočević-Stojanović, S. Pavlović, D. Lavrinić et al., "Peripheral neuropathy in patients with myotonic dystrophy," *Acta Myologica*, vol. 21, no. 1, pp. 36–37, 2002.

[102] M. T. Fortune, C. Vassilopoulos, M. I. Coolbaugh, M. J. Siciliano, and D. G. Monckton, "Dramatic, expansion-biased, age-dependent, tissue-specific somatic mosaicism in a transgenic mouse model of triplet repeat instability," *Human Molecular Genetics*, vol. 9, no. 3, pp. 439–445, 2000.

[103] D. Savić, V. Rakočević-Stojanović, D. Keckarević et al., "250 CTG repeats in DMPK is a threshold for correlation of expansion size and age at onset of juvenile-adult DM1," *Human Mutation*, vol. 19, no. 2, pp. 131–139, 2002.

[104] M. M. Axford, A. López-Castel, M. Nakamori, C. A. Thornton, and C. E. Pearson, "Replacement of the myotonic dystrophy type 1 CTG repeat with 'non-CTG repeat' insertions in specific tissues," *Journal of Medical Genetics*, vol. 48, no. 7, pp. 438–443, 2011.

[105] E. P. Leeflang and N. Arnheim, "A novel repeat structure at the myotonic dystrophy locus in a 37 repeat allele with unexpectedly high stability," *Human Molecular Genetics*, vol. 4, no. 1, pp. 135–136, 1995.

[106] E. J. Kamsteeg, W. Kress, C. Catalli et al., "Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2," *European Journal of Human Genetics*, vol. 20, pp. 1203–1208, 2012.

[107] J. Buxton, P. Shelbourne, J. Davies et al., "Detection of an unstable fragment of DNA specific to individuals with myotonic dystrophy," *Nature*, vol. 355, no. 6360, pp. 547–548, 1992.

[108] M. Gennarelli, M. Pavoni, P. Amicucci, G. Novelli, and B. Dallapiccola, "A single polymerase chain reaction-based protocol for detecting normal and expanded alleles in myotonic dystrophy," *Diagnostic Molecular Pathology*, vol. 7, no. 3, pp. 135–137, 1998.

[109] J. P. Warner, L. H. Barron, D. Goudie et al., "A general method for the detection of large GAG repeat expansions by fluorescent PCR," *Journal of Medical Genetics*, vol. 33, no. 12, pp. 1022–1026, 1996.

[110] J. Radvansky, A. Ficek, and L. Kadasi, "Upgrading molecular diagnostics of myotonic dystrophies: multiplexing for simultaneous characterization of the DMPK and ZNF9 repeat motifs," *Molecular and Cellular Probes*, vol. 25, no. 4, pp. 182–185, 2011.

[111] K. Sermon, S. Seneca, M. de Rycke et al., "PGD in the lab for triplet repeat diseases—myotonic dystrophy, Huntington's disease and Fragile-X syndrome," *Molecular and Cellular Endocrinology*, vol. 183, supplement 1, pp. S77–S85, 2001.

[112] J. Radvansky, A. Ficek, G. Minarik, R. Palffy, and L. Kadasi, "Effect of unexpected sequence interruptions to conventional PCR and repeat primed PCR in myotonic dystrophy type 1 testing," *Diagnostic Molecular Pathology*, vol. 20, no. 1, pp. 48–51, 2011.

[113] P. Borry, L. Stultiens, H. Nys, J. J. Cassiman, and K. Dierickx, "Presymptomatic and predictive genetic testing in minors: a systematic review of guidelines and position papers," *Clinical Genetics*, vol. 70, no. 5, pp. 374–381, 2006.

[114] K. Sermon, W. Lissens, H. Joris et al., "Clinical application of preimplantation diagnosis for myotonic dystrophy," *Prenatal Diagnosis*, vol. 17, no. 10, pp. 925–932, 1997.

[115] G. Kakourou, S. Dhanjal, T. Mamas et al., "Preimplantation genetic diagnosis for myotonic dystrophy type 1 in the UK," *Neuromuscular Disorders*, vol. 18, no. 2, pp. 131–136, 2008.

[116] M. de Rademaeker, W. Verpoest, M. de Rycke et al., "Preimplantation genetic diagnosis for myotonic dystrophy type 1: upon request to child," *European Journal of Human Genetics*, vol. 17, no. 11, pp. 1403–1410, 2009.

[117] C. Dechanet, C. Castelli, L. Reyftmann et al., "Myotonic dystrophy type 1 and PGD: ovarian stimulation response and correlation analysis between ovarian reserve and genotype," *Reproductive BioMedicine Online*, vol. 20, no. 5, pp. 610–618, 2010.

[118] K. Sermon, A. van Steirteghem, and I. Liebaers, "Preimplantation genetic diagnosis," *Lancet*, vol. 363, no. 9421, pp. 1633–1641, 2004.

[119] K. Sermon, A. de Vos, H. van De Velde et al., "Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease)," *Molecular Human Reproduction*, vol. 4, no. 8, pp. 791–796, 1998.

[120] E. W. Loomis, J. S. Eid, P. Peluso et al., "Sequencing the unsequenceable: expanded CGG-repeat alleles of the fragile X gene," *Genome Research*, vol. 23, no. 1, pp. 121–128, 2013.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 201372, 6 pages
http://dx.doi.org/10.1155/2013/201372

Research Article

TNNT2 Gene Polymorphisms Are Associated with Susceptibility to Idiopathic Dilated Cardiomyopathy in the Han Chinese Population

Xiaoping Li,[1] Huan Wang,[1] Rong Luo,[2] Haiyong Gu,[3] Channa Zhang,[4] Yu Zhang,[4] Rutai Hui,[4] Xiushan Wu,[2] and Wei Hua[1]

[1] *Cardiac Arrhythmia Center, Cardiovascular Institute and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, China*
[2] *The Center of Heart Development, Key Lab of MOE for Development Biology and Protein Chemistry, College of Life Science, Hunan Normal University, Changsha, Hunan 410081, China*
[3] *Department of Cardiothoracic Surgery, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212000, China*
[4] *Sino-German Laboratory for Molecular Medicine, Key Laboratory for Clinical Cardiovascular Genetics, Ministry of Education, Cardiovascular Institute and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100037, China*

Correspondence should be addressed to Wei Hua; drhua@yahoo.cn

Received 27 September 2012; Accepted 11 January 2013

Academic Editor: Yasemin Alanay

Background. Idiopathic dilated cardiomyopathy (DCM) is characterized by ventricular chamber enlargement and systolic dysfunction. The pathogenesis of DCM remains uncertain, and the *TNNT2* gene is potentially associated with DCM. To assess the role of *TNNT2* in DCM, we examined 10 tagging single nucleotide polymorphisms (SNPs) in the patients. *Methods*. A total of 97 DCM patients and 189 control subjects were included in the study, and all SNPs were genotyped by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Results*. In the *TNNT2* gene, there was a significant association between DCM and genotype for the tagging SNPs rs3729547 ($\chi^2 = 6.63$, $P = 0.036$, OR = 0.650, and 95% CI = 0.453–0.934) and rs3729843 ($\chi^2 = 9.787$, $P = 0.008$, OR = 1.912, and 95% CI = 1.265–2.890) in the Chinese Han population. Linkage disequilibrium (LD) analysis showed that the SNPs rs7521796, rs2275862, rs3729547, rs10800775, and rs1892028, which are approximately 6 kb apart, were in high LD ($D' > 0.80$) in the DCM patients. *Conclusion*. These results suggest that the *TNNT2* polymorphisms might play an important role in susceptibility to DCM in the Chinese Han population.

1. Introduction

Idiopathic dilated cardiomyopathy (DCM) is a cardiac muscle disease of unknown origin that is characterized by ventricular chamber enlargement and systolic dysfunction with thinning of the left ventricular wall. DCM leads to progressive heart failure and a decline in left ventricular contractile function, conduction system abnormalities, thromboembolism, and sudden or heart failure-related death; only 50% of DCM patients survive more than 5 years beyond their initial diagnosis [1, 2]. Coronary artery disease, viral myocarditis, thyroid disease, immunologic processes, and toxins are known causes of DCM; however, the underlying pathology is not known in most cases [3–5]. In a population-based study, the prevalence of DCM was estimated to be 36.5 cases per 100,000, and 20–50% of these cases are familial [6–8]. Candidate gene analysis revealed that the cardiac actin encoding gene *ACTC1* mutations were the first sarcomeric gene mutations that caused DCM [9]. To date, mutations have been found in at least six genes encoding sarcomeric proteins: β-myosin heavy chain, cardiac myosin binding protein C, titin, cardiac actin, α-tropomyosin, cTnT, and cTnC [9–15].

The *TNNT2* gene (OMIM number *191045) encodes the protein cardiac TnT, which contains 15 exons and spans 25 kb

on chromosome 1q32 [16]. Mutations in the *TNNT2* gene can cause three phenotypically distinct cardiomyopathies: hypertrophic, restrictive, and dilated [10, 17–19]. *TNNT2* mutations are responsible for approximately 15% of all cases of familial hypertrophic cardiomyopathy (HCM) [20–22]. Recent data indicated that *TNNT2* mutations are also associated with DCM, and the overall frequency of *TNNT2* mutations in familial DCM is approximately 3–6% [23, 24].

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation in the human genome, and two recent large-scale SNP screens in European patients with DCM showed that SNPs in several genes were associated with DCM [25, 26]. Based on the above findings, we hypothesized that some cases of DCM are associated with specific polymorphisms in the *TNNT2* gene. To test this hypothesis and further understand the pathogenesis of DCM, we investigated 10 tagging SNPs in the *TNNT2* gene in DCM patients and normal control subjects from a Chinese Han population using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) techniques. Our results indicated that the SNPs rs3729547 and rs3729843 in the *TNNT2* gene were associated with DCM in the Chinese population, suggesting that the *TNNT2* polymorphisms may play an important role in susceptibility to DCM in the Chinese population.

2. Materials and Methods

2.1. Subjects and Selection of Tagging SNPs. This case-control study enrolled 97 unrelated DCM patients from the Fuwai Hospital. The clinical diagnosis was made in accordance with the revised criteria [1]. A total of 189 healthy unrelated individuals from a routine health survey were enrolled as controls. Patients with a history of hypertension, coronary heart disease, cardiac valve disease, diabetes, acute viral myocarditis, systemic diseases of putative autoimmune origin, and family history of DCM were intentionally excluded. This study was approved by the Ethics Committee of our hospital; the subjects involved were all of Han nation in the North of China and were informed of the study aims and provided written informed consent prior to participating.

Genotype data on the *TNNT2* gene from the Han Chinese in Beijing (CHB) population were downloaded from the phase 2 HapMap SNP database (available at http://www.hapmap.org/), and tagging SNPs were selected in the Haploview software (available at http://www.broadinstitute.org/haploview) using a minor allele frequency (MAF) cutoff of 0.05 and a correlation coefficient (r^2) threshold of 0.8.

2.2. Isolation of DNA and Genotyping by MALDI-TOF-MS. Blood samples were collected from patients using tubes containing ethylenediaminetetraacetic acid. Genomic DNA was isolated from whole blood with a QIAamp DNA Blood Mini Kit (Qiagen, Germany). Genotyping was performed by MALDI-TOF-MS as described previously [27]. SNP genotyping was performed using a MassARRAY system (Sequenom, San Diego, CA, USA) based on the MALDI-TOF-MS method, according to the manufacturer's instructions. Completed genotyping reactions were spotted onto spectroCHIP (Sequenom) using a MassARRAY Nanodispenser (Sequenom), and the genotype was determined by MALDI-TOF-MS. Genotype calling was performed in real time with MassARRAY RT software version 3.1 (Sequenom) and analyzed using MassARRAY Typer software version 4.0 (Sequenom) (Table 1).

2.3. Statistical Analyses. Differences in the distributions of selected variables and *TNNT2* genotypes between the cases and controls were evaluated using the χ^2 test. The correlations between the *TNNT2* genotype and the risk of DCM were estimated by computing the odds ratios (ORs) and the 95% confidence intervals (CIs) using logistic regression analysis. The χ^2 test was used to test for the Hardy-Weinberg equilibrium to compare the observed and expected genotype frequencies among the control subjects. All statistical analyses were performed with SPSS 13.0. All tests were two-tailed, and the significance was set at $P < 0.05$.

3. Results

The gender and age distributions of the DCM patients and the control subjects were compared with the Pearson's chi-square test and Student's t-test, respectively, and no significant differences were detected (control: $n = 189, 54.0 \pm 3.6$ years, male/female = 150/39; DCM: $n = 97, 51.6 \pm 12.0$ years, male/female = 75/22, $P > 0.05$).

The observed and expected genotype frequencies of each SNP were compared with the chi-squared test in DCM patients and the control subjects separately, and no significance was detected in either group. These results indicate that the samples fit the assumption of the Hardy-Weinberg equilibrium. The DNA variants and the Hardy-Weinberg equilibrium test of the 10 tagging SNPs in the DCM patients and control subjects were shown in Table 2.

Using the chi-squared test, we compared the genotype and allele frequencies in the *TNNT2* gene between the DCM patients and control subjects. Our results showed that the allele frequencies of the tagging SNPs rs3729547 ($\chi^2 = 5.474$, $P = 0.019$), rs1892028 ($\chi^2 = 5.855$, $P = 0.016$), rs3729843 ($\chi^2 = 9.620$, $P = 0.002$), rs12564445 ($\chi^2 = 4.351$, $P = 0.037$), and rs10800775 ($\chi^2 = 4.252$, $P = 0.039$) were significantly correlated with DCM. However, among the genotypes, only those of the tagging SNPs rs3729547 ($\chi^2 = 6.63$, $P = 0.036$, OR = 0.650, 95% CI = 0.453–0.934) and rs3729843 ($\chi^2 = 9.787$, $P = 0.008$, OR = 1.912, 95% CI = 1.265–2.890) had a significant correlation with DCM in the Chinese population. The allele and genotype frequencies of the ten tagging SNPs in the DCM patients and control subjects and the statistical analysis results were shown in Table 3 and Figure 1.

Because the great majority of DCM patients are male [1–5], we compared the frequencies of the genotypes of SNPs rs3729547, rs3729843, and rs10927875 in *TNNT2* between the DCM patients and control subjects stratified by gender. In

Table 1: Sequences of the PCR primers used to genotype SNPs in the DCM patients and control subjects.

Markers	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)	Temp. (°C)	GC (%)
rs7521796	TGCCAACAGAGAGGTGCTTC	CTTGAGGCTCAGCCTAATTG	93	46.5	56.3
rs2275862	AATATGAGGTGGGCCGCCAT	TATTACCGGACCCAGTGAAC	99	48.4	52.9
rs3729547	GAAGGACCTGAATGAGTTGC	AGAAACGAGCTCCTCCTCCT	99	50.2	60
rs10800775	AATCCCCTCCCAGGTCTTTG	TCATGTCATCAGCTTCTGCC	98	50.7	47.4
rs1892028	AGAGGGGACCATTGTCCAG	TCTAGGAGCTTCATGTGTGG	100	48	62.5
rs3729843	TCAAGGTCCTTGTTCTGAGC	TCTTGGCTAGGGCTTATCTG	99	47.1	44.4
rs3729842	TCAACGTTTGTTGATTGGGC	AGAACAGGCTTTCCCATGTG	99	46.7	31.8
rs12563114	TGGAAGGGCAGAGTAGGAGA	AATTCTCAGAGGAACCGTGC	100	45.2	44.4
rs12564445	AACTCGGAGACTGTTTCTAC	CTCTCTGACTTCAGTTAACC	95	47.7	47.1
rs4915232	CAATCTCGCTATTCTCTGCC	AGAAGAGTTTGAGGACTGGG	95	48.6	62.5

Table 2: Identified DNA variants and the Hardy-Weinberg equilibrium of 10 SNPs in the *TNNT2* gene in the DCM patients and control subjects.

Markers	Location of nucleotide change	Amino acid change	Note	Obs HET	Expt HET	HWE (*P*)	MAF
rs7521796	Intron 201330019 A>G	Noncoding	Novel noncoding SNP	0.115	0.115	1	0.061
rs2275862	Intron 201330366 C>G	Non-coding	Novel non-coding SNP	0.329	0.319	0.7683	0.199
rs3729547	201334382 T>C	Synonymous Ile [I]	Reported synonymous	0.504	0.484	1	0.41
rs10800775	Intron 201336386 C>T	Non-coding	Novel non-coding SNP	0.474	0.448	0.771	0.339
rs1892028	Intron 201336641 A>G	Non-coding	Novel non-coding SNP	0.489	0.498	0.7396	0.47
rs3729843	Intron 201336984 G>A	Non-coding	Reported non-coding SNP	0.285	0.331	0.5949	0.21
rs3729842	Intron 201337170 C>T	Non-coding	Reported non-coding SNP	0.225	0.241	0.3594	0.14
rs12563114	Intron 201344908 C>T	Non-coding	Novel non-coding SNP	0.081	0.084	0.5638	0.044
rs12564445	Intron 201345487 G>A	Non-coding	Novel non-coding SNP	0.435	0.427	0.7891	0.309
rs4915232	5′ near gene 201347946 A>G	Non-coding	Novel non-coding SNP	0.535	0.499	0.7407	0.482

Note: Obs HET: observed heterozygosity, Expt HET: expected heterozygosity, HWE (*P*): *P* value from the Hardy-Weinberg equilibrium test, and MAF: minor allele frequency.

males, the distributions of the SNP rs3729547 genotypes were not significantly different between the DCM patients and control subjects, but the distributions of the SNP rs3729843 genotype were significantly different in the DCM patients and control subjects ($\chi^2 = 8.102, P = 0.017$). In females, the distributions of the genotypes of rs3729843 and rs3729547 were not significantly different in the DCM patients and control subjects.

The nonrandom associations between polymorphic variants at different loci on the *TNNT2* gene were then measured by the degree of linkage disequilibrium (LD). LD analysis showed that the SNPs rs7521796, rs2275862, rs3729547, rs10800775, and rs1892028 in the *TNNT2* gene, which are approximately 6 kb apart (block 3, Figure 2), were in high LD in the DCM patients (Figure 2, $D' > 0.80$). The haplotype analysis showed that ACTCA ($\chi^2 = 6.66, P = 0.0099$) and AGCTG ($\chi^2 = 4.003, P = 0.0454$) in block 3 and AG ($\chi^2 = 3.988, P = 0.0458$) in block 4 (rs12564445 and rs4915232) of the *TNNT*2 gene correlated significantly with DCM (Figure 2, Table 4).

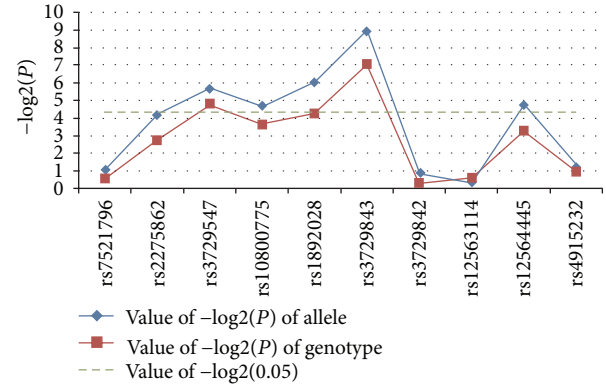


Figure 1: Mapping of the significance of each tagging SNP in the *TNNT2* gene. The *x*-axis shows the genomic position, and the *y*-axis shows the negative logarithm of the *P* value for each allele or genotype of each SNP.

4. Discussion

To our knowledge, this is the first study to show an association between DCM and SNPs in the *TNNT2* gene in the Chinese population. DCM is regarded as a heterogeneous disease. The present study shows that, in at least a subgroup of DCM patients, the SNPs in the *TNNT2* (rs3729547 and rs3729843) gene may be involved in the pathogenesis of DCM.

Table 3: Genotype and allele frequencies of the SNPs from the *TNNT2* gene in the DCM patients and control subjects.

Marker		Genotype		χ^2, *P* value		Allele	χ^2, *P* value	OR (95% CI)
rs7521796	A/A	A/G	G/G		A	G		1.303 (0.613–2.772)
Patients	87 (0.897)	10 (0.103)	0 (0)	$\chi^2 = 0.747$	184 (0.948)	10 (0.052)	$\chi^2 = 0.4751$	
Controls	165 (0.873)	23 (0.122)	1 (0.005)	$P = 0.688$	353 (0.934)	25 (0.066)	$P = 0.4906$	
rs2275862	C/C	C/G	G/G		C	G		1.562 (0.987–2.471)
Patients	69 (0.711)	26 (0.268)	2 (0.021)	$\chi^2 = 3.802$	164 (0.845)	30 (0.155)	$\chi^2 = 3.669$	
Controls	113 (0.598)	68 (0.360)	8 (0.042)	$P = 0.149$	294 (0.778)	84 (0.222)	$P = 0.055$	
rs3729547	C/C	T/C	T/T		C	T		0.650 (0.453–0.934)
Patients	8 (0.084)	49 (0.516)	38 (0.4)	$\chi^2 = 6.63$	65 (0.342)	125 (0.658)	$\chi^2 = 5.474$	
Controls	37 (0.196)	94 (0.497)	58 (0.307)	**P = 0.036**	168 (0.444)	210 (0.556)	**P = 0.019**	
rs10800775	C/C	C/T	T/T		C	T		1.486 (1.019–2.169)
Patients	47 (0.49)	44 (0.458)	5 (0.052)	$\chi^2 = 5.024$	138 (0.719)	54 (0.281)	$\chi^2 = 4.252$	
Controls	74 (0.392)	91 (0.481)	24 (0.127)	$P = 0.081$	239 (0.632)	139 (0.368)	**P = 0.039**	
rs1892028	A/A	A/G	G/G		A	G		1.578 (1.090–2.291)
Patients	30 (0.357)	42 (0.5)	12 (0.143)	$\chi^2 = 5.947$	102 (0.607)	66 (0.393)	$\chi^2 = 5.855$	
Controls	46 (0.253)	88 (0.484)	48 (0.264)	$P = 0.051$	180 (0.495)	184 (0.505)	**P = 0.016**	
rs3729843	A/A	A/G	G/G		A	G		1.912 (1.265–2.890)
Patients	12 (0.126)	30 (0.316)	53 (0.558)	$\chi^2 = 9.787$	54 (0.284)	136 (0.716)	$\chi^2 = 9.620$	
Controls	7 (0.037)	51 (0.270)	131 (0.693)	**P = 0.008**	65 (0.172)	313 (0.828)	**P = 0.002**	
rs3729842	C/C	C/T	T/T		C	T		1.158 (0.697–1.925)
Patients	74 (0.763)	21 (0.216)	2 (0.021)	$\chi^2 = 0.381$	169 (0.871)	25 (0.129)	$\chi^2 = 0.322$	
Controls	139 (0.739)	43 (0.229)	6 (0.032)	$P = 0.827$	321 (0.854)	55 (0.146)	$P = 0.571$	
rs12563114	C/C	C/T	T/T		C	T		0.899 (0.390–2.07)
Patients	87 (0.906)	9 (0.094)	0 (0)	$\chi^2 = 0.828$	183 (0.953)	9 (0.047)	$\chi^2 = 0.063$	
Controls	174 (0.921)	14 (0.074)	1 (0.005)	$P = 0.661$	362 (0.958)	16 (0.042)	$P = 0.8022$	
rs12564445	A/A	A/G	G/G		A	G		0.663 (0.450–0.977)
Patients	6 (0.062)	37 (0.381)	54 (0.557)	$\chi^2 = 4.503$	49 (0.253)	145 (0.747)	$\chi^2 = 4.351$	
Controls	20 (0.106)	87 (0.463)	81 (0.431)	$P = 0.105$	127 (0.338)	249 (0.662)	**P = 0.037**	
rs4915232	A/A	A/G	G/G		A	G		1.155 (0.816–1.635)
Patients	25 (0.258)	55 (0.567)	17 (0.175)	$\chi^2 = 1.385$	105 (0.541)	89 (0.459)	$\chi^2 = 0.6590$	
Controls	46 (0.246)	97 (0.519)	44 (0.235)	$P = 0.500$	189 (0.505)	185 (0.495)	$P = 0.4170$	

Table 4: Haplotype analysis of SNPs in *TNNT2* gene between the DCM patients and control subjects.

	Haplotype	Frequency (DCM patients)	Frequency (control subjects)	χ^2	*P* value
Block 1	ACTCA	0.604	0.489	6.66	**0.0099**
	AGCTG	0.145	0.215	4.003	**0.0454**
	ACCTG	0.136	0.150	0.208	0.6485
	ACCCG	0.058	0.070	0.312	0.5762
	GCTCG	0.052	0.066	0.436	0.5089
Block 2	GA	0.541	0.498	0.937	0.333
	AG	0.252	0.333	3.988	**0.0458**
	GG	0.207	0.163	1.699	0.1925

DCM represents the third most frequent cause of heart failure and the most frequent cause of heart transplantation. Among patients with the so-called idiopathic DCM, 20–50% of cases are of genetic origin [6, 7]. Over the past decade, de novo mutations have been found in more than 30 genes encoding essential sarcomeric, cytoskeletal, and nuclear proteins in DCM patients [28], and mutations in the *TNNT2* gene have been found to be associated with familial HCM and DCM [10, 17–19, 23, 24].

Recent studies have suggested that cardiac TnT is essential not only for the structural integrity of the troponin complex but also for sarcomere assembly and cardiac contractility [22]. The troponin complex is a calcium sensor that regulates the contraction of striated muscle, and TnT is important in

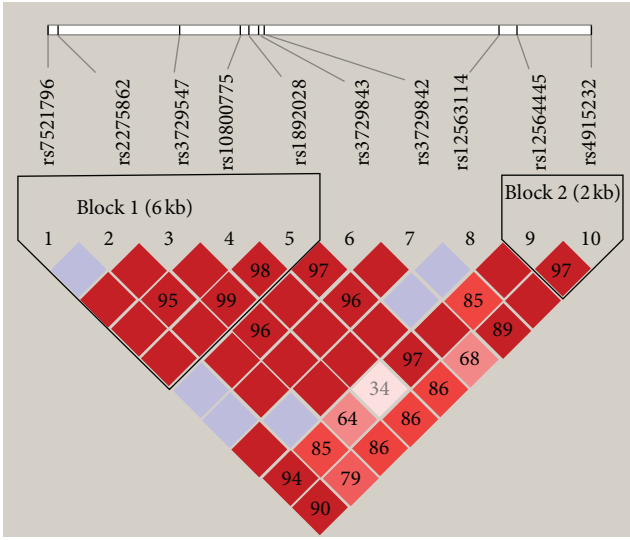


Figure 2: Pairwise linkage disequilibrium (LD) values calculated between tagging SNPs in the *TNNT2* gene. The value within each diamond represents the pairwise correlation between tagging SNPs (measured as D'), defined by the upper left and the upper right sides of the diamond. The diamond without a number corresponds to $D' = 1$. Shading represents the magnitude and significance of the pairwise LD with darker red reflecting higher LD values and white indicating lower LD values.

mediating the interaction between tropomyosin and actin and the rest of the troponin complex, which appears to modulate the activation of actomyosin ATPase activity and force [29]. Countless studies in reconstituted systems have provided valuable information on the functional effects of disease-associated mutations in TnT. The most extensively studied DCM-associated TnT mutation to date is ΔK210; functional studies of the ΔK210 mutation showed that the mutated protein reduced the Ca^{2+} sensitivity of actomyosin ATPase activity, which resulted in a decreased maximum speed of muscle contraction [30, 31]. Thus, DCM mutations in the troponin complex may induce a profound reduction in force generation, leading to impaired systolic function and cardiac dilation.

In this study, we assessed whether polymorphism within the *TNNT2* gene might affect DCM susceptibility by comparing ten tagging SNP loci in DCM patients and normal control subjects. The representative SNP in a region of the genome with high linkage disequilibrium is called a tagging SNP. Among the ten tagging SNPs in the *TNNT2* gene, we found a significant association between the genotypes of rs3729547 (synonymous variant) and rs3729843 (noncoding SNP) and DCM. Although the allele frequencies of five tagging SNPs (rs3729547, rs3729843, rs1892028, rs1256445, and rs10800775) were significantly associated with DCM, the genotypes of rs1892028, rs1256445, and rs10800775 were not significantly associated with DCM, possibly because of the limited number of patients enrolled in the present study. LD analysis of the polymorphic SNPs observed in our study revealed a group of five SNPs, rs7521796, rs2275862, rs3729547, rs10800775, and rs1892028, located 6 kb apart; these alleles were in high LD and associated with DCM risk. As the majority of SNPs are likely to be allelic variants that do not affect expression or function of a protein, such SNPs are commonly used as genetic markers to localize nearby disease-causing variations in linkage and association analyses. SNPs that directly influence phenotype may be located within coding or regulatory regions of genes. SNPs within regulatory regions tend to have more quantitative effects, for example, by altering the expression level of a receptor or signaling protein, and result in a more subtle variation in the associated phenotype [32]. Recently, study showed that polymorphism in intron 3 of *TNNT2* significantly affected the mRNA expression pattern by skipping exon 4 during splicing in cardiomyopathy patients [33]. Missing exon 4 in cardiac troponin T is corresponding to isoforms cTnT2 and cTnT4, and the two isoforms increase might be related to hemodynamic stress [34]. These results in our study suggest that *TNNT2* gene polymorphism, as like genetic markers to localize nearby disease-causing variations in linkage and association analyses, may play an important role in DCM susceptibility in the Chinese Han population. However, further functional analyses are needed to confirm the role of these polymorphisms in the pathogenesis of DCM.

In the present study, we have provided the evidence that shows that SNPs in the *TNNT2* gene may be implicated in the pathogenesis of DCM in a Chinese population. However, because the frequencies of genetic polymorphisms vary greatly among ethnic populations, further studies in other populations are needed to exclude a population-oriented association. In addition, the outcomes of the present study may be influenced by the limited sample size; larger studies are therefore required to investigate the potential associations between the SNPs in the *TNNT2* gene and the DCM susceptibility.

Authors' Contribution

X. Li and H. Wang contributed equally to this work.

Acknowledgments

This study was supported by Grants from the National Natural Science Foundation of China (no. 81000104 and no. 81160141) and the Postdoctoral Fellows Foundation of Chinese Academy of Medical Sciences (2011-XH-9).

References

[1] B. J. Maron, J. A. Towbin, G. Thiene et al., "Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention," *Circulation*, vol. 113, no. 14, pp. 1807–1816, 2006.

[2] J. A. Towbin and N. E. Bowles, "The failing heart," *Nature*, vol. 415, no. 6868, pp. 227–233, 2002.

[3] W. M. Franz, O. J. Müller, and H. A. Katus, "Cardiomyopathies: from genetics to the prospect of treatment," *Lancet*, vol. 358, no. 9293, pp. 1627–1637, 2001.

[4] J. Schönberger and C. E. Seidman, "Many roads lead to a broken heart: the genetics of dilated cardiomyopathy," *American Journal of Human Genetics*, vol. 69, no. 2, pp. 249–260, 2001.

[5] J. G. Seidman and C. Seidman, "The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms," *Cell*, vol. 104, no. 4, pp. 557–567, 2001.

[6] V. V. Michels, P. P. Moll, F. A. Miller et al., "The frequency of familial dilated cardiomyopathy in a series of patients with idiopathic dilated cardiomyopathy," *New England Journal of Medicine*, vol. 326, no. 2, pp. 77–82, 1991.

[7] E. L. Burkett and R. E. Hershberger, "Clinical and genetic issues in familial dilated cardiomyopathy," *Journal of the American College of Cardiology*, vol. 45, no. 7, pp. 969–981, 2005.

[8] L. Mestroni, C. Rocco, D. Gregori et al., "Familial dilated cardiomyopathy: evidence for genetic and phenotypic heterogeneity," *Journal of the American College of Cardiology*, vol. 34, no. 1, pp. 181–190, 1999.

[9] T. M. Olson, V. V. Michels, S. N. Thibodeau, Y. S. Tai, and M. T. Keating, "Actin mutations in dilated cardiomyopathy, a heritable form of heart failure," *Science*, vol. 280, no. 5364, pp. 750–752, 1998.

[10] M. Kamisago, S. D. Sharma, S. R. DePalma et al., "Mutations in sarcomere protein genes as a cause of dilated cardiomyopathy," *New England Journal of Medicine*, vol. 343, no. 23, pp. 1688–1696, 2000.

[11] M. Shimizu, H. Ino, T. Yasuda et al., "Gene mutations in adult Japanese patients with dilated cardiomyopathy," *Circulation Journal*, vol. 69, no. 2, pp. 150–153, 2005.

[12] B. Gerull, M. Gramlich, J. Atherton et al., "Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy," *Nature Genetics*, vol. 30, no. 2, pp. 201–204, 2002.

[13] T. M. Olson, N. Y. Kishimoto, F. G. Whitby, and V. V. Michels, "Mutations that alter the surface charge of alpha-tropomyosin are associated with dilated cardiomyopathy," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 4, pp. 723–732, 2001.

[14] D. Li, G. Z. Czernuszewicz, O. Gonzalez et al., "Novel cardiac troponin T mutation as a cause of familial dilated cardiomyopathy," *Circulation*, vol. 104, no. 18, pp. 2188–2193, 2001.

[15] J. Mogensen, R. T. Murphy, T. Shaw et al., "Severe disease expression of cardiac troponin C and T mutations in patients with idiopathic dilated cardiomyopathy," *Journal of the American College of Cardiology*, vol. 44, no. 10, pp. 2033–2040, 2004.

[16] A. V. Gomes, J. A. Barnes, K. Harada, and J. D. Potter, "Role of troponin T in disease," *Molecular and Cellular Biochemistry*, vol. 263, no. 1, pp. 115–129, 2004.

[17] J. Mogensen, T. Kubo, M. Duque et al., "Idiopathic restrictive cardiomyopathy is part of the clinical expression of cardiac troponin I mutations," *Journal of Clinical Investigation*, vol. 111, no. 2, pp. 209–216, 2003.

[18] L. Thierfelder, H. Watkins, C. MacRae et al., "α-Tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere," *Cell*, vol. 77, no. 5, pp. 701–712, 1994.

[19] P. J. Townsend, H. Farza, C. MacGeoch et al., "Human cardiac troponin T: identification of fetal isoforms and assignment of the *TNNT2* locus to chromosome 1q," *Genomics*, vol. 21, no. 2, pp. 311–316, 1994.

[20] T. Palm, S. Graboski, S. E. Hitchcock-DeGregori, and N. J. Greenfield, "Disease-causing mutations in cardiac troponin T: identification of a critical tropomyosin-binding region," *Biophysical Journal*, vol. 81, no. 5, pp. 2827–2837, 2001.

[21] M. García-Castro, J. R. Reguero, A. Batalla et al., "Hypertrophic cardiomyopathy: low frequency of mutations in the β-myosin heavy chain (*MYH7*) and cardiac troponin T (*TNNT2*) genes among Spanish patients," *Clinical Chemistry*, vol. 49, no. 8, pp. 1279–1285, 2003.

[22] A. J. Sehnert, A. Huq, B. M. Weinstein, C. Walker, M. Fishman, and D. Y. R. Stainier, "Cardiac troponin T is essential in sarcomere assembly and cardiac contractility," *Nature Genetics*, vol. 31, no. 1, pp. 106–110, 2002.

[23] A. N. Chang, M. S. Parvatiyar, and J. D. Potter, "Troponin and cardiomyopathy," *Biochemical and Biophysical Research Communications*, vol. 369, no. 1, pp. 74–81, 2008.

[24] R. E. Hershberger, J. R. Pinto, S. B. Parks et al., "Clinical and functional Characterization of *TNNT2* mutations identified in patients with dilated cardiomyopathy," *Circulation*, vol. 2, no. 4, pp. 306–313, 2009.

[25] E. Villard, C. Perret, F. Gary, C. Proust, G. Dilanian, and C. Hengstenberg, "A genome-wide association study identifies two loci associated with heart failure due to dilated cardiomyopathy," *European Heart Journal*, vol. 32, pp. 1065–1076, 2011.

[26] K. Stark, U. B. Esslinger, W. Reinhard et al., "Genetic association study identifies HSPB7 as a risk gene for idiopathic dilated cardiomyopathy," *PLoS Genetics*, vol. 6, no. 10, Article ID e1001167, 2010.

[27] E. Schaeffeler, U. M. Zanger, M. Eichelbaum, S. Asante-Poku, J. G. Shin, and M. Schwab, "Highly multiplexed genotyping of thiopurine S-methyltransferase variants using MALDI-TOF mass spectrometry: reliable genotyping in different ethnic groups," *Clinical Chemistry*, vol. 54, no. 10, pp. 1637–1647, 2008.

[28] L. Dellefave and E. M. McNally, "The genetics of dilated cardiomyopathy," *Current Opinion in Cardiology*, vol. 25, no. 3, pp. 198–204, 2010.

[29] J. D. Potter, Z. Sheng, B. S. Pan, and J. Zhao, "A direct regulatory role for troponin T and a dual role for troponin C in the Ca^{2+} regulation of muscle contraction," *Journal of Biological Chemistry*, vol. 270, no. 6, pp. 2557–2562, 1995.

[30] S. Morimoto, Q. W. Lu, K. Harada et al., "Ca^{2+}-desensitizing effect of a deletion mutation ΔK210 in cardiac troponin T that causes familial dilated cardiomyopathy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 2, pp. 913–918, 2002.

[31] P. Robinson, M. Mirza, A. Knott et al., "Alterations in thin filament regulation induced by a human cardiac troponin T mutant that causes dilated cardiomyopathy are distinct from those induced by troponin T mutants that cause hypertrophic cardiomyopathy," *Journal of Biological Chemistry*, vol. 277, no. 43, pp. 40710–40716, 2002.

[32] E. Ho, R. Bhindi, E. A. Ashley, and G. A. Figtree, "Genetic analysis in cardiovascular disease: a clinical perspective," *Cardiology in Review*, vol. 19, no. 2, pp. 81–89, 2011.

[33] K. Komamura, N. Iwai, K. Kokame et al., "The role of a common *TNNT2* polymorphism in cardiac hypertrophy," *Journal of Human Genetics*, vol. 49, no. 3, pp. 129–133, 2004.

[34] L. Mesnard-Rouiller, J. J. Mercadier, G. Butler-Browne et al., "Troponin T mRNA and protein isoforms in the human left ventricle: pattern of expression in failing and control hearts," *Journal of Molecular and Cellular Cardiology*, vol. 29, no. 11, pp. 3043–3055, 1997.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 396272, 10 pages
http://dx.doi.org/10.1155/2013/396272

Research Article

Molecular and Survival Differences between Familial and Sporadic Gastric Cancers

Wen-Liang Fang,[1,2] Shih-Ching Chang,[3,4] Yuan-Tzu Lan,[2,3] Kuo-Hung Huang,[1,2] Su-Shun Lo,[4,5] Anna Fen-Yau Li,[4,6] Chin-Wen Chi,[7,8] Chew-Wun Wu,[1,4] and Shih-Hwa Chiou[2,7,8]

[1] *Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital, 201 Section 2, Shih-Pai Road, Taipei 11217, Taiwan*
[2] *Institute of Clinical Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[3] *Division of Colorectal Surgery, Department of Surgery, Taipei Veterans General Hospital, 201 Section 2, Shih-Pai Road, Taipei 11217, Taiwan*
[4] *National Yang-Ming University, Taipei, Taiwan*
[5] *National Yang-Ming University Hospital, 152 Xin-Min Road, Yilan 26042, Taiwan*
[6] *Department of Pathology, Taipei Veterans General Hospital, 201 Section 2, Shih-Pai Road, Taipei 11217, Taiwan*
[7] *Department of Medical Research and Education, Taipei Veterans General Hospital, 201 Section 2, Shih-Pai Road, Taipei 11217, Taiwan*
[8] *Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan*

Correspondence should be addressed to Shih-Hwa Chiou; shchiou@vghtpe.gov.tw

Received 11 October 2012; Revised 27 December 2012; Accepted 24 January 2013

Academic Editor: Ozgur Cogulu

Mismatch repair (MMR) and germline E-cadherin (CDH1) mutations are two of the major pathways of carcinogenesis in familial gastric cancer (GC). A total of 260 sporadic and 66 familial GC patients were enrolled and molecular and survival differences were compared. Familial GC patients had earlier onset and were diagnosed at an earlier stage and had both a better 5-year overall survival rate and 3-year disease-free survival rate compared with sporadic GC patients. Only in diffuse type GC, the MSI-H phenotype and abnormal MMR protein expression were significantly higher in familial GC than in sporadic GC. In MSI-H GC, MLH1 promoter methylation was slightly higher in sporadic GC than familial GC (50% versus 23.1%), while the frequency of MMR gene mutation was slightly higher in familial GC than in sporadic GC (15.4% versus 3.1%). All of the patients with MMR gene mutation had diffuse type GC. Among familial GC patients with CDH1 mutation, most patients (72.3%) had diffuse type GC. In summary, for familial GC patients, we recommend screening of MSI status and CDH1 mutation especially for diffuse type GC. Because of the low incidence, mutation analysis of MMR gene might be considered in MSI-H familial GC with diffuse type only.

1. Introduction

Despite the decreasing incidence worldwide, gastric cancer (GC) is still one of the leading causes of cancer deaths [1]. Two major genomic instability pathways were involved in the pathogenesis of GC: (i) the chromosomal instability (CIN) pathway, which is characterized by gross copy number changes and alterations in chromosomal regions, occurs in at least 60% of cases [2], and (ii) the microsatellite instability (MSI) pathway, which is characterized by alterations in the length of repetitive microsatellite sequences, accounts for 10%–20% of cases [3–5].

In the MSI pathway of gastric carcinogenesis, mutations of hMLH1 were reported in approximately 0%–7.3% of MSI-H GC [6, 7]. However, hMLH1 silencing due to promoter methylation has been reported to be associated with the development of more than 50% of MSI-H GC [8–12].

The incidence of hereditary diffuse gastric cancer (HDGC) in the general population has not yet been clearly defined, but HDGC most likely accounts for only 1%–3% of GCs. Direct evidence shows that hereditary GC with germline E-cadherin (CDH1) mutations is an autosomal dominant inheritance [13]. However, CDH1 mutations account for only 1%–3% of all GCs. Only one-third to one-half of families with a strong history of diffuse GC are associated with CDH1 mutations. The definition of HDGC in 1999 by the International GC Linkage Consortium (IGCLC) was as follows: (i) in the first- or second-degree relatives, two or more cases of DGC diagnosed before 50 years old or (ii) at any age, three or more cases diagnosed as DGC [14, 15]. Nearly 30%–46% of the patients fulfilling the previous criteria carry the CDH1 mutations. The IGCLC criteria have been modified, and patients who were diagnosed with signet-ring carcinoma of the colon or lobular breast cancer were included [16]. However, only 11% of the patients fulfilling the modified criteria carry the CDH1 mutations.

A family history was reported in only 10% of GC cases [17]. Familial GC was reported to be associated with a worse prognosis than sporadic GC [8, 18]. The definition of familial GC includes familial diffuse GC (the so-called HDGC) as well as familial intestinal GC (FIGC) [14]. In countries with high incidence of GC, such as Japan and Portugal, the diagnostic criteria of FIGC were as follows: (i) at least three relatives have intestinal GC and one of them is a first-degree relative of the other two; (ii) at least two generations have GC; or (iii) in one of the relatives, GC should be diagnosed before 50 years old. In countries with low incidence of FIGC, such as USA and UK, the definition was as follows: (i) at least two first/second-degree relatives have intestinal GC, one diagnosed before 50 years old, or (ii) three or more relatives with intestinal GC at any age.

To our knowledge, only a few studies have investigated the MSI status and MLH1 methylation in GC patients with a family history [5, 7, 19–24]. Leite et al. [7] reported that the MSI status and MLH1 methylation were similar between sporadic and familial GCs; however, no MMR gene mutation could be identified in their studies. In Taiwan, there has been no report regarding genetic mutations (including MMR and CDH1 mutations) in GC patients with a family history. The aim of this study is to compare the clinicopathological characteristics, MSI phenotype, immunohistochemical (IHC) stains of MMR proteins, MLH1 promoter methylation, and genetic mutations between familial and sporadic GCs.

2. Materials and Methods

A total of 326 GC patients who received surgery between May 1988 and December 2004 were collected from Taipei Veterans General Hospital and included in this study. The information of family history of GC was obtained from the records of the patients and their families. The study was approved by the Institutional Review Board at the Taipei Veterans General Hospital. The written informed consent was obtained from all patients enrolled. The exclusion criteria include (i) patients with a history of gastric surgery or a pathological diagnosis other than adenocarcinoma and (ii) patients belonging to families of hereditary nonpolyposis colorectal cancer (HNPCC).

Patients enrolled in this study were classified and grouped as having either sporadic GC or familial GC. The definition of familial GC in the present study was (i) two or more cases of GC in the first- or second-degree relatives, including at least one patient of GC diagnosed before 50 years old or (ii) three or more cases of GC in first- or second-degree relatives diagnosed at any age. The definition of sporadic GC was patients without a family history of GC.

The pathological staging of cancer was according to the 7th AJCC/UICC TNM classification [25]. The data were collected prospectively and recorded using a computer. The patients were regularly followed up, and the database was updated regularly.

Microsatellite instability analysis and IHC stains for MMR protein were performed for all the 326 patients enrolled. Patients with MSI-H tumors (32 sporadic GC and 13 familial GC) were analyzed for MLH1 methylation and genetic mutations of MLH1 and MSH2. CDH1 mutations were performed for 66 familial GC patients (Figure 1).

2.1. Microsatellite Instability Analysis. The DNA of normal and tumor tissues was extracted from the formalin-fixed, paraffin-embedded (FFPE) tissues or from fresh frozen tissues stored at −80°C or liquid nitrogen. After the DNA was purified by the QIAamp Tissue Kit (QIAGEN GmbH, Germany), the quantitative DNA analysis was performed by measuring the optical density (OD) at wavelengths of 260 nm and 280 nm. The DNA quality was confirmed by the ratio of OD260/280.

The purified DNA was amplified by using a fluorescent polymerase chain reaction (PCR). Five reference microsatellite markers, including D5S345, D2S123, D17S250, BAT25, and BAT26, were used for the determination of MSI [26]. PCR products were denatured and analyzed by electrophoresis on 5% denatured polyacrylamide gels. The results were analyzed by an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). As reported in a previous study [27], the presence of novel alleles observed among the PCR products from tumor DNA that were not seen among the PCR products from the corresponding normal DNA was scored as MSI at that particular locus. Samples with ≥2 loci of instability with 5 markers were defined as MSI-H. Samples with one MSI or without MSI were defined as MSI-L/S.

2.2. Immunohistochemical Stains. IHC stains for MLH1, MSH2, MSH6, and PMS2 were performed for paraffin-embedded tissue. Paraffin-embedded tissue sections (4 mm thick) were stained with antibodies for MLH1 (1 : 10 dilution; Pharmingen, San Diego, CA, USA), MSH2 (1 : 200; Oncogene Research Products, La Jolla, CA, USA), MSH6 (1 : 300; Transduction Laboratories, San Diego, CA, USA), and PMS2 (C20; 1 : 400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control slides were made without the primary antibody.

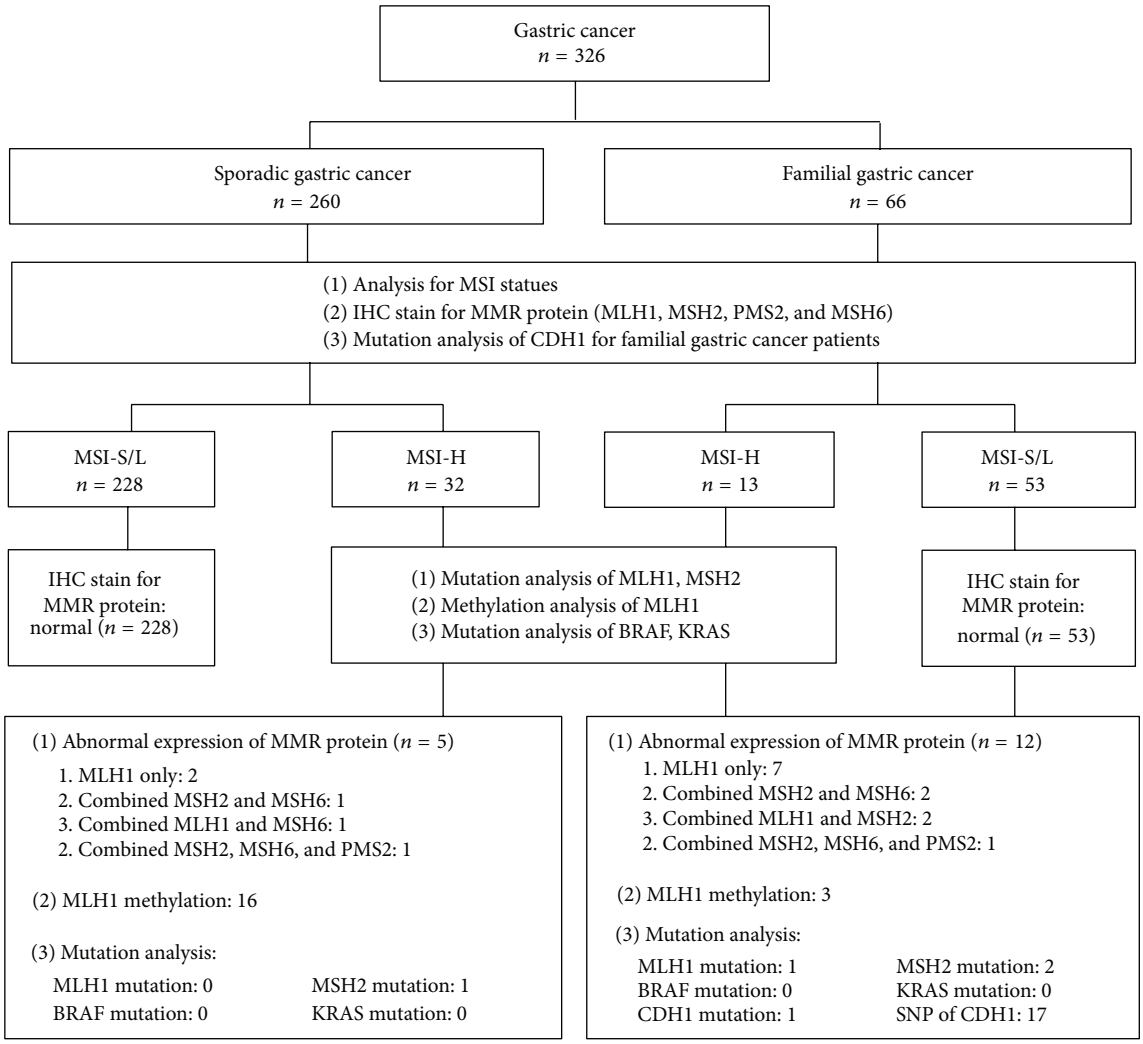


Figure 1: The flowchart of this study.

2.3. Methylation Analysis of MLH1. Methylation of the hMLH1 promoter was analyzed from GC tumor tissues by using a methylation-specific PCR method. The genomic DNA was modified by sodium bisulfite [28], and the sequences were amplified with different methylated and unmethylated primers [29].

2.4. Detection of Mutations for MLH1 and MSH2. Analysis of mutations of MLH1 and MSH2 genes was performed for MSI-H GC. The DNA was extracted from the normal tissue and amplified by PCR and sequenced with primers that have already been applied in the previous studies [30, 31]. For each round of PCR amplification, a negative control template containing no DNA was included. The PCR products were analyzed by the automated sequencer (ABI Prism 3100 Genetic Analyzer). Each sample was sequenced on both of the sense and antisense strands. A second sequencing procedure with new PCR products confirms each mutation.

Nonsense, missense, and frameshift mutations were identified by comparing the obtained sequence with the known sequence. Nonsense and frameshift mutations were considered as pathogenic. Missense mutations in the MMR genes that did not result in abnormal expression of MMR proteins were considered to be polymorphisms [32, 33].

2.5. Detection of Mutations for BRAF and KRAS. Mutation analysis for BRAF (V599E) and KRAS was performed for MSI-H GC (Figure 1). PCR reactions took place in a volume of 25 μL containing 20 ng genomic DNA template, 0.2 μM of each PCR primer, 0.2 mM dNTPs, PCR buffer, and 1U Taq DNA polymerase. Thirty-five cycles of 30 s at 95°C, 30 s at a primer pair annealing temperature of 55°C, and 60 s at 72°C were performed in programmable thermocyclers (GeneAmp PCR System 2700, ABI). A 3 μL aliquot of each PCR reaction was carried out on a 2% agarose gel. The remaining 17 μL of the PCR product was submitted to purification using a Favor-Prep GEL/PCR Purification Mini Kit (FAVORGEN), and the products were eluted in 30 μL Elution Buffer. Sequencing was performed using the BigDye Terminator V3.0 (ABI), data collection mode on an ABI 3730 capillary sequencer.

2.6. Detection of Mutations for CDH1. The DNA was extracted from the normal tissue of the familial GC tissue and further amplified by PCR reactions. The sequences of all the primers used in this study and their annealing temperatures are listed in Supplementary Table 1 available online at http://dx.doi.org/10.1155/2013/396272 PCR reactions was took placed in a volume of 25 μL containing 20 ng of genomic DNA template, 0.2 μM of each PCR primer, 0.2 mM dNTPs, PCR buffer, and 1U Taq DNA polymerase. Thirty-five cycles of 30 s at 94°C, 30 s at a primer pair specific annealing temperature of 50–55°C, and 30 s–90 s at 72°C were performed in programmable thermocyclers (GeneAmp PCR System 2700, ABI). A 3 μL aliquot of each PCR reaction was carried out on a 2% agarose gel, and the size, purity, and quantity of each PCR product were confirmed. The remaining 17 μL of the PCR products was submitted to purification using FavorPrep GEL/PCR Purification Mini Kit (FAVORGEN), and the products were eluted in 30 μL Elution Buffer. Sequencing was performed using the BigDye Terminator V3.0 (ABI), data collection mode on an ABI 3730 capillary sequencer.

2.7. Statistical Analysis. The results in the tables are shown as the mean values ± standard deviation. A chi-squared test with Yates' correction was used to analyze categorical variables. Student's t-test was used to compare quantitative variables between groups. SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

The overall survival was measured from the operation date to the date of death or the final followup. The disease-free survival was defined as the length of time after surgery for gastric cancer during which a patient survives without tumor recurrence. The distributions of overall survival and disease-free survival were estimated using Kaplan-Meier method. The differences between the curves were tested using a two-tailed log-rank test. Cox-proportional hazards models were used to explore the association of clinical parameters with overall survival. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Clinicopathological Characteristics. Because the biological behaviors are different between diffuse type and intestinal type in GC, we separate diffuse type and intestinal type for analyzing the difference between sporadic and familial GCs.

As shown in Table 1, for diffuse type GC, familial GC was associated with younger age, less male predominance, smaller tumor size, more well-defined gross appearance, an earlier tumor stage, and a significantly higher frequency of MSI-H tumors as compared to sporadic GC (28% versus 6.5%).

In Table 2, for intestinal type GC, familial GC was associated with younger age, less male predominance, smaller tumor size, more medullary stromal reaction types and an earlier tumor stage as compared to sporadic GC. The frequency of MSI-H tumors was similar between sporadic and familial GCs (16.3% versus 14.6%).

Table 1: Comparison of clinicopathological characteristics between diffuse type GC cases of sporadic and familial GCs.

	Sporadic GC (n = 107) n (%)	Familial GC (n = 25) n (%)	P value
Age (years)	65.4 ± 12.8	54.1 ± 15.0	<0.001
Gender (M/F)	77/30	12/13	0.032
Tumor maximal size (cm)	7.9 ± 3.1	5.8 ± 2.4	<0.001
Gross appearance			
Well-defined	16 (15)	12 (48)	
Ill-defined	91 (85)	13 (52)	0.001
Lymphovascular invasion			
Absent/present	10/97	11/14	<0.001
Stromal reaction type			
Medullary	18 (16.8)	7 (28)	
Intermediate	36 (33.6)	7 (28)	
Scirrhous	53 (49.5)	11 (44)	0.435
Pathological T category			
T1/T2/T3/T4	4/6/26/71	9/2/1/13	<0.001
Pathological N category			
N0/N1/N2/N3	15/15/17/60	9/2/4/10	0.074
Pathological TNM stage			
Stage I	5 (4.7)	9 (36)	
Stage II	11 (10.3)	2 (8)	
Stage III	46 (43)	7 (28)	
Stage IV	45 (42)	7 (28)	<0.001
MSI status			
MSI-L/S	100 (93.5)	18 (72)	
MSI-H	7 (6.5)	7 (28)	0.005
IHC stain for MMR protein			
Normal	105 (98.1)	22 (88)	
Abnormal	2 (1.9)	3 (12)	0.047

The overall survival rate was analyzed for GC patients after curative resection. Familial GC patients had a better 5-year overall survival rate than sporadic GC patients (65.3% versus 45.4%, P = 0.001, Figure 2(a)). Furthermore, familial GC patients also had a better 3-year disease-free survival rate than sporadic GC patients (71.1% versus 52.9%, P = 0.002, Figure 2(b)).

Univariate analysis showed that age, gender, tumor size, lymphovascular invasion, stromal reaction type, family history, MSI status, pathological T category, N category, and TNM stage were associated with survival. Multivariate Cox proportional-hazards model using the forward logistics regression stepwise procedure for the analysis of overall survival showed that gender, pathological TNM stage, and MSI status were independent prognostic factors (Table 3).

3.2. Analysis of MSI Status. Of the total 326 patients, 45 patients (13.8%) had MSI-H GC. MSI-H GC was associated with more tumors located over the distal third of the stomach compared with MSI-L/S GC (68.9% versus 50.2%, P = 0.019).

Table 2: Comparison of clinicopathological characteristics between intestinal type GC cases of sporadic and familial GCs.

	Sporadic GC (n = 153) n (%)	Familial GC (n = 41) n (%)	P value
Age (years)	70.8 ± 9.8	60.2 ± 13.4	<0.001
Gender (M/F)	124/29	24/17	0.006
Tumor maximal size (cm)	6.3 ± 2.3	4.9 ± 3.3	<0.001
Gross appearance			
Well-defined	64 (41.8)	21 (51.2)	
Ill-defined	89 (58.2)	20 (48.8)	0.293
Lymphovascular invasion			
Absent/present	23/130	19/22	<0.001
Stromal reaction type			
Medullary	16 (10.5)	14 (34.2)	
Intermediate	104 (68)	19 (46.3)	
Scirrhous	33 (21.5)	8 (19.5)	0.001
Pathological T category			
T1/T2/T3/T4	15/22/36/80	16/2/7/16	<0.001
Pathological N category			
N0/N1/N2/N3	39/25/29/60	19/5/3/14	0.047
Pathological TNM stage			
Stage I	24 (15.7)	16 (39)	
Stage II	33 (21.6)	7 (17.1)	
Stage III	46 (30.1)	8 (19.5)	
Stage IV	50 (32.7)	10 (24.4)	0.012
MSI status			
MSI-L/S	128 (83.7)	35 (85.4)	
MSI-H	25 (16.3)	6 (14.6)	1.000
IHC stain for MMR protein			
Normal	143 (93.5)	38 (92.7)	
Abnormal	10 (6.5)	3 (7.3)	1.000

The 5-year overall survival rate of the MSI-H patients was better than that of the MSI-L/S patients after curative surgery (68% versus 47.6%, P = 0.032).

3.3. Immunohistochemical Stains for MMR Proteins. IHC stains for MLH1, MSH2, MLH6, and PMS2 proteins were performed for all the 326 patients enrolled (Figure 1). None of the MSI-L/S tumors had abnormal IHC stains for MMR proteins.

As shown in Figure 1, among the 260 sporadic GC patients, 12 (4.6%) patients had abnormalities on IHC analysis of the MMR protein. Among them, seven patients had abnormal MLH1 stains only; two patients had combined abnormal stains of MLH1 and MSH2; three patients had combined abnormal stains of MSH2, and MSH6.

Among the 66 familial GC patients, six patients (9.1%) had abnormalities on IHC analysis for the MMR protein. Among them, 2 patients had abnormal stains of MLH1 only; one patient had combined abnormal stains of MLH1 and MSH2; one patient had combined abnormal stains of MSH2 and MSH6; one patient had combined MLH1 and MSH6; one patient had combined abnormal stains of MLH1, MSH2, MSH6 and PMS2.

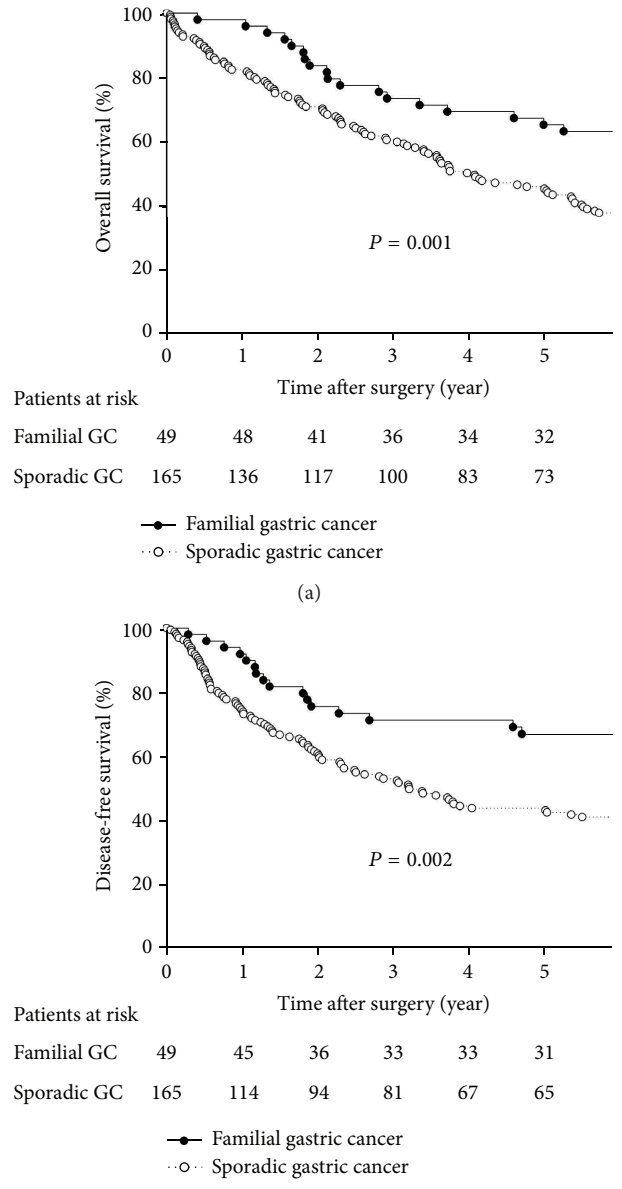


Figure 2: (a) Familial GC was associated with a better 5-year overall survival rate than sporadic GC (65.3% versus 45.4%, P = 0.001). (b) Familial GC was associated with a better 3-year disease-free survival rate than sporadic GC (71.1% versus 52.9%, P = 0.002).

As shown in Table 1, for diffuse type GC patients, abnormal IHC stains for MMR protein were significantly higher in familial GC than in sporadic GC (12% versus 1.9%, P = 0.047). In Table 2, for intestinal type GC, the frequency of abnormal IHC stains for MMR protein was similar between familial GC and sporadic GC (7.3% versus 6.5%, P = 1.000).

3.4. Methylation of the MLH1 Promoter. Methylation of the MLH1 promoter was performed for the 45 MSI-H GC

Table 3: Univariate analysis and multivariate analysis of factors affecting overall survival of GC patients after curative surgery.

	Univariate analysis		Multivariate analysis	
	OR (CI)	*P* value	OR (CI)	*P* value
Age (<65, ≥65 years)	1.52 (1.04–2.23)	0.031		
Gender (male, female)	0.62 (0.42–0.93)	0.020	0.66 (0.44–0.98)	0.038
Tumor size (<5 cm, ≥5 cm)	2.15 (1.47–3.13)	<0.001		
Lauren's classification (intestinal/diffuse)	0.85 (0.60–1.20)	0.347		
Lymphovascular invasion (−, +)	3.21 (1.99–5.17)	<0.001		
Stromal reaction type (medullary, intermediate, and scirrhous)	1.47 (1.15–1.87)	0.002		
Family history (−, +)	0.43 (0.27–0.71)	0.001		
MSI status (MSI-L/S, MSI-H)	0.52 (0.28–0.96)	0.035	0.50 (0.27–0.93)	0.029
TNM stage (I, II, III)	2.43 (1.89–3.13)	<0.001	2.44 (1.89–3.14)	<0.001

OR: odds ratio, CI: confidence interval.

patients, including 32 MSI-H sporadic GC and 13 MSI-H familial GC. Among the 45 MSI-H GC, methylation of the MLH1 promoter was identified in 50% (16/32) of sporadic GC and 23.1% (3/13) of familial GC ($P = 0.182$).

3.5. Mutation Analyses for MLH1 and MSH2. Mutation analyses for MLH1 and MSH2 were performed for the 45 MSI-H GC patients. A total of 3 patients were identified as having MMR gene mutations, including one familial GC patient with both MLH1 and MSH2 mutations, one familial GC patient with an MSH2 mutation, and one sporadic GC patient with an MSH2 mutation. All the three patients with MMR gene mutation had diffuse type GC. None of the patients with MLH1 methylation had mutations of MLH1 or MSH2. Among the 45 MSI-H GC patients, the frequency of MMR gene mutations was higher in familial GC (2/13, 15.4%) than sporadic GC (1/32, 3.1%).

3.6. The Correlation between MLH1 Expression, Promoter Methylation, and Mutation. As shown in Table 4, abnormal expression of IHC stain for MLH1 was observed in 12 patients, including 3 sporadic GC and 9 familial GC. Among the 3 sporadic GC patients, all had MLH1 methylation and none had MLH1 mutation. Among the 9 familial GC patients, 5 patients had MLH1 methylation and one had MLH1 mutation. The only one familial GC patient with MLH1 mutation did not have MLH1 methylation. MLH1 promoter methylation and MLH1 mutation play a different role in the cause of abnormal MLH1 expression.

3.7. Mutation Analyses for BRAF and KRAS. Mutation analyses for BRAF and KRAS were performed for the 28 MSI-H GC patients. However, no BRAF or KRAS mutation was identified.

3.8. Analysis of CDH1 Mutations for Familial GC Patients. Analysis of CDH1 mutations was performed for the 66 familial GC patients. Among them, 18 (27.3%) had CDH1 germline sequence alterations, including 9 patients with rs1801552 (2076T>C, exon 13) alterations, 3 patients with rs33964119 (2253C>T, exon 14) alterations, 1 patient with an exon 3 mutation, and 5 patients with both rs1801552 (exon 13) and rs33964119 (exon 14) alterations. Of the 18 patients with CDH1 germline sequence alterations, only one patient (5.6%) with an exon 3 mutation in codon 90 (268C>T) had an amino acid mutation (Thr to Met, Figure 3); single nucleotide polymorphism was observed in the other 17 patients. Among the 18 patients with CDH1 germline sequence alterations, 13 (72.3%) had diffuse type GC.

Table 4: The frequency of MLH1 promoter methylation and MLH1 mutation in GC patients with abnormal MLH1 expression.

	Sporadic GC ($n = 3$) *n* (%)	Familial GC ($n = 9$) *n* (%)
MLH1 promoter		
Methylation	3 (100)	5 (55.6)
Unmethylation	0	4 (44.4)
MLH1 mutation		
Yes	0	1 (11.1)
No	3 (100)	8 (88.9)

Table 5: The frequency of MSI status and CDH1 mutation in familial GC patients.

	MSI-H ($n = 13$) *n* (%)	MSI-L/S ($n = 53$) *n* (%)	*P* value
CDH1 mutation			
Present	4 (30.8)	14 (26.4)	
Absent	9 (69.2)	39 (73.6)	0.739

3.9. The Correlation between CDH1 Mutation and MSI Status in Familial GC. As shown in Table 5, among the 13 MSI-H familial GC patients, 4 (30.8%) patients had CDH1 mutation, while 14 (26.4%) out of the 53 MSI-L/S familial GC patients had CDH1 mutation. There was no significant difference between the frequency of CDH1 mutation in MSI-H and MSI-L/S familial GC patients ($P = 0.739$).

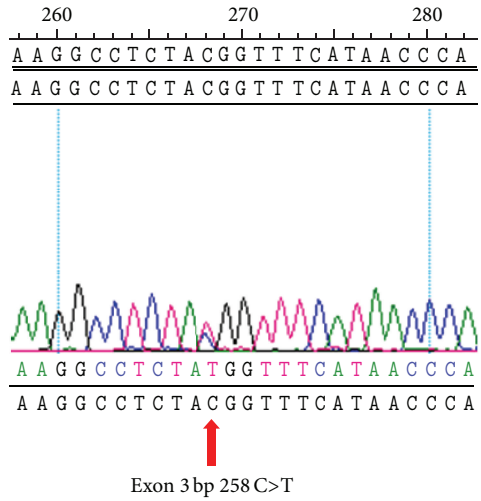


Figure 3: Chromatograms of one patient with pathogenic exon 3 mutation. An arrow indicates the position of the mutation.

4. Discussion

Our data showed that familial GC patients were diagnosed earlier and associated with a better 5-year overall survival rate (65.3% versus 45.4%, $P = 0.001$) and a better 3-year disease-free survival rate (71.1% versus 49.1%, $P = 0.002$) compared with sporadic GC patients. Only in diffuse type GC, the MSI-H phenotype and abnormal MMR protein expression were significantly higher in familial GC than in sporadic GC. MSI status was one of the independent risk factors affecting survival. For MSI-H GC, MLH1 promoter methylation was slightly higher in sporadic GC than familial GC (50% versus 23.1%). Because this is a retrospective study, selection bias might happen and affect the results. To our knowledge, this study is the first investigating the MSI status, mutation analysis of the MMR gene, and the CDH1 gene in familial GC in Taiwan.

MLH1 promoter methylation was reported to be responsible for more than 50% of MSI-H GC [4, 7, 23]. Leite et al. [7] reported that the frequency of MLH1 promoter methylation is similar between familial and sporadic GCs with MSI-H (71.4% versus 79.3%). Although not statistically significant, our results showed higher frequency of MLH1 promoter methylation in sporadic GC than in familial GC with MSI (50% versus 23.1%). It seems that MLH1 promoter methylation plays a less important role in the cause of MSI-H in our familial GC compared to the sporadic GC. However, it seems that the frequency of MLH1 methylation was lower in our series than the results in the study of Leite et al. [7]. Racial and environmental factors might have an impact on the frequency of MLH1 promoter methylation. Furthermore, we also analyzed the correlation between MLH1 expression, methylation, and mutation (Table 4). We found that the abnormal expression of MLH1 in the three sporadic GC patients was all related to MLH1 methylation. In contrast, the cause of abnormal expression of MLH1 was 55% by MLH1 methylation and 11.1% by MLH1 mutation in familial GC. Consequently, the mechanism of inactivation of MLH1 was mainly epigenetic in both sporadic and familial GCs. A larger sample size may be needed to compare the difference in MLH1 promoter methylation between familial and sporadic GCs.

Our results showed that the frequency of MLH1 or MSH2 gene mutation was slightly higher in familial GC than in sporadic GC (15.4% versus 3.1%) in MSI-H GC. All the three patients with MMR gene mutation had diffuse type GC. In the series of Leite et al. [7], analysis of MMR gene mutation was performed for two patients with simultaneous loss of MLH1 and PMS2 protein in IHC stain; however, no alteration of MLH1 or MSH2 gene was detected. Moreover, Leite et al. [7] also reported no significant difference of MSI status between familial and sporadic GC. In diffuse type GC, the frequency of MSI-H was zero in familial GC and 13.4% in sporadic GC. However, Pedrazzani et al. [5] and Kanemitsu et al. [22] reported significantly higher frequency of MSI-H in familial GC than in sporadic GC. Compared to other series, the novel findings of our results were significantly more MSI-H tumors in familial GC than sporadic GC only in diffuse type GC. Although MSI-H tumors were reported to be more frequent in intestinal type GC, our results showed that MMR gene mutation among the MSI-H tumors mainly occurred in the diffuse type GC. These results are interesting and might be a clue for future investigation of the MMR gene mutation in familial GC. Because the mutation rate of MMR gene is relatively low and the cost for mutation analysis is high, focusing on the diffuse type GC might be more cost-effective than mass screening for all MSI-H GC. According to our results, we recommend screening of MSI status in diffuse type familial GC. Because of the low incidence, analysis of MMR gene mutation might be considered for MSI-H familial GC with diffuse type only.

Ye et al. [8] matched their sporadic GC patients with their familial GC patients for age and TNM stage, and they concluded that familial GC was associated with a worse prognosis than sporadic GC. However, early onset and early diagnosis of familial cancer were ignored simultaneously, which might also cause selection bias. Our data showed that familial GC patients were diagnosed at an earlier tumor stage and had a younger age, which might be the reason why our familial GC patients had a better prognosis. It seems that GC patients with a family history tend to pay attention to the symptoms and seek medical help earlier than those without a family history, which was also observed in colorectal cancer [34]. Our results showed that family history is associated with a better prognosis only by univariate analysis. The possible reason might be that patients with a family history are usually diagnosed in the early stage, and the importance of family history in the prognosis might be replaced by the TNM stage in the multivariate analysis. Furthermore, as shown in Table 1, about 25.8% of our familial GC patients were diagnosed at stage IV, for whom curative surgery was impossible; consequently, this group of patients had a worse prognosis. We should make effort to strengthen the health education and perform extensive screening for the relatives of GC patients, especially for the first- and second-degree relatives, in order to detect GC earlier for them.

Our results demonstrated that MSI status was an independent risk factor of GC after curative surgery, which was also mentioned in our previous study [35] and similar to the results of some series [36, 37]. However, other authors [19, 38] reported that MSI was not associated with survival. Because the frequency of MSI was as low as 8%–25% in these series and a relative small number of patients with MSI tumor were analyzed, a larger sample size is needed to clarify the role of MSI in survival.

Some authors reported that the MSI status was associated with a family history of GC [5, 19, 22], but others reported the opposite [20, 21, 24]. Our data showed a higher percentage of MSI-H tumors in familial GC than in sporadic GC, which was only observed in diffuse type GC. In diffuse type GC, our data showed that familial GC was associated with a better 5-year overall survival rate than sporadic GC (44% versus 28%, $P = 0.028$). The reason might be due to a higher percentage of MSI-H tumors in our familial GC with diffuse type, and MSI-H tumors were associated with a better prognosis than MSI-L/S tumors. This divergence between each study is most likely due to the different criteria used for the definition of family history and the small size of patients with a family history. Furthermore, some studies included both intestinal and diffuse type GCs in their analysis, which might cause different results. Further meta-analysis of a larger number of patients and separating the diffuse and intestinal type GC for analyzing the association between the family history and MSI status might provide more reliable results.

BRAF and KRAS mutations were reported in only 2% of GC patients and, specifically, only in advanced GC [39]. BRAF mutation in GC could exclude germline mutations of MMR. Screening for BRAF in MSI-H GC could decrease the waste for an expensive mutation analysis. As a result, we also analyzed the BRAF and KRAS mutations in our patients. However, we detected neither BRAF nor KRAS mutations in our patients. BRAF and KRAS mutations may not play an important role in our GC patients.

Our data showed that the majority (94.4%, 17/18) of CDH1 gene alterations were silent mutations, or the so-called synonymous SNPs, including rs1801552 and rs33964119, which have been reported in some series [9, 10]. A silent, single nucleotide polymorphism (SNP) in a gene is one that creates a codon that is synonymous to the wild-type codon. However, this synonymous codon substitution may lead to different kinetics of mRNA translation, thus yielding a protein with different final structure and function [40]. *In silico* analysis suggests that these sequence alterations may affect splicing and protein conformations [41]. Moreover, we identified one patient with an exon 3 mutation at codon 90 (268C>T), which had never been reported in the literature. Further study of this newly found mutation in the CDH1 gene is required. Our results showed that among our familial GC patients with CDH1 mutation, 72.3% patients had diffuse type GC. As a result, routine screening of CDH1 mutation is recommended in diffuse type familial GC.

The two major genetic instability pathways of the carcinogenesis of gastric cancer were CIN and MSI. CDH1 mutation was reported to be involved in the pathogenesis in familial GC. In this study, we also analyzed the correlation between MSI status and CDH1 mutation in familial GC. However, there was no difference in the frequency of CDH1 mutation between MSI-H and MSI-L/S GC. It seems that there is no relationship between CDH1 mutation and MSI in familial GC. The result is reasonable because CDH1 mutation and MSI are involved in different pathways for carcinogenesis of GC.

As shown in Figure 1, we identified the MLH1 promoter methylation, MMR gene mutation, and CDH1 mutation in sporadic and familial GCs. However, these mutations can only explain the carcinogenesis of some of our patients, especially in familial GC. There are still unknown genes involved in the pathogenesis of GC, and additional studies are necessary to identify and characterize these genes. As a matter of course, there is still a lot of space for us to explore GC in the future.

5. Conclusion

In conclusion, familial GC was associated with an early stage at diagnosis and a better prognosis compared with sporadic GCs. Our results display the molecular and survival differences between sporadic and familial GC. Because of the relatively higher accumulation of GC in patients with a family history, annual upper gastrointestinal endoscopic examinations are recommended in the relatives of familial GC patients. For GC patients with mutations of CDH1 or MMR genes, genetic screening of their relatives is recommended, especially for the first- and second-degree relatives.

Acknowledgments

This work was supported by the Center of Excellence for Cancer Research at Taipei Veterans General Hospital (DOH102-TD-C-111-007), and the National Science Council (99-2314-B-075-009, 100-2314-B-075-011-MY3).

References

[1] D. M. Parkin, F. I. Bray, and S. S. Devesa, "Cancer burden in the year 2000. The global picture," *European Journal of Cancer*, vol. 37, no. 8, pp. S4–S66, 2001.

[2] L. Ottini, M. Falchetti, R. Lupi et al., "Patterns of genomic instability in gastric cancer: clinical implications and perspectives," *Annals of Oncology*, vol. 17, no. 7, pp. vii97–vii102, 2006.

[3] C. Oliveira, R. Seruca, M. Seixas, and M. Sobrinho-Simões, "The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different "target genes": a study of the TGFβ RII, IGFII R, and BAX genes," *American Journal of Pathology*, vol. 153, no. 4, pp. 1211–1219, 1998.

[4] M. Gu, D. Kim, Y. Bae, J. Choi, S. Kim, and S. Song, "Analysis of microsatellite instability, protein expression and methylation status of hMLH1 and hMSH2 genes in gastric carcinomas," *Hepato-Gastroenterology*, vol. 56, no. 91-92, pp. 899–904, 2009.

[5] C. Pedrazzani, G. Corso, S. Velho et al., "Evidence of tumor microsatellite instability in gastric cancer with familial aggregation," *Familial Cancer*, vol. 8, no. 3, pp. 215–220, 2009.

[6] J. Bacani, R. Zwingerman, N. Di Nicola et al., "Tumor microsatellite instability in early onset gastric cancer," *Journal of Molecular Diagnostics*, vol. 7, no. 4, pp. 465–477, 2005.

[7] M. Leite, G. Corso, S. Sousa et al., "MSI phenotype and MMR alterations in familial and sporadic gastric cancer," *International Journal of Cancer*, vol. 128, no. 7, pp. 1606–1613, 2011.

[8] Y. W. Ye, R. Z. Dong, Y. Zhou et al., "Prognostic analysis of familial gastric cancer in Chinese population," *Journal of Surgical Oncology*, vol. 104, no. 1, pp. 76–82, 2011.

[9] C. Oliveira, P. Ferreira, S. Nabais et al., "E-Cadherin (CDH1) and p53 rather than SMAD4 and Caspase-10 germline mutations contribute to genetic predisposition in Portuguese gastric cancer patients," *European Journal of Cancer*, vol. 40, no. 12, pp. 1897–1903, 2004.

[10] F. M. Richards, S. A. McKee, M. H. Rajpar et al., "Germline E-cadherin gene (CDH1) mutations predispose to familial gastric cancer and colorectal cancer," *Human Molecular Genetics*, vol. 8, no. 4, pp. 607–610, 1999.

[11] I. Kluijt, E. J. Siemerink, M. G. Ausems et al., "CDH1-related hereditary diffuse gastric cancer syndrome: clinical variations and implications for counseling," *International Journal of Cancer*, vol. 131, no. 2, pp. 367–376, 2012.

[12] N. A. Ottenhof, R. F. de Wilde, F. H. Morsink et al., "Pancreatic ductal adenocarcinoma in hereditary diffuse gastric cancer. A case report," *Human Pathology*, vol. 43, no. 3, pp. 457–461, 2012.

[13] P. Guilford, J. Hopkins, J. Harraway et al., "E-cadherin germline mutations in familial gastric cancer," *Nature*, vol. 392, no. 6674, pp. 402–405, 1998.

[14] C. Caldas, F. Carneiro, H. T. Lynch et al., "Familial gastric cancer: overview and guidelines for management," *Journal of Medical Genetics*, vol. 36, no. 12, pp. 873–880, 1999.

[15] M. Svrcek, "Case n(o) 6—signet ring cell intramucosal carcinoma in hereditary diffuse gastric cancer with mutated CDH1 gene," *Annales De Pathologie*, vol. 31, no. 5, pp. 381–384, 2011.

[16] A. R. Brooks-Wilson, P. Kaurah, G. Suriano et al., "Germline E-cadherin mutations in hereditary diffuse gastric cancer: assessment of 42 new families and review of genetic screening criteria," *Journal of Medical Genetics*, vol. 41, no. 7, pp. 508–517, 2004.

[17] C. Oliveira, R. Seruca, and F. Carneiro, "Hereditary gastric cancer," *Best Practice and Research*, vol. 23, no. 2, pp. 147–157, 2009.

[18] Z. H. Ma, Q. Z. Ren, Y. F. Zhao et al., "Comparison of clinicopathological features and prognosis in familial and sporadic gastric cancer," *Zhonghua Wei Chang Wai Ke Za Zhi*, vol. 14, no. 10, pp. 793–795, 2011.

[19] L. Ottini, D. Palli, M. Falchetti et al., "Microsatellite instability in gastric cancer is associated with tumor location and family history in a high-risk population from Tuscany," *Cancer Research*, vol. 57, no. 20, pp. 4523–4529, 1997.

[20] K. Shinmura, W. Yin, J. Isogaki et al., "Stage-dependent evaluation of microsatellite instability in gastric carcinoma with familial clustering," *Cancer Epidemiology Biomarkers and Prevention*, vol. 6, no. 9, pp. 693–697, 1997.

[21] G. Keller, M. Rudelius, H. Vogelsang et al., "Microsatellite instability and loss of heterozygosity in gastric carcinoma in comparison to family history," *American Journal of Pathology*, vol. 152, no. 5, pp. 1281–1289, 1998.

[22] K. Kanemitsu, K. Kawasaki, M. Nakamura et al., "MSI is frequently recognized among gastric cancer patients with a family history of cancer," *Hepato-Gastroenterology*, vol. 54, no. 80, pp. 2410–2414, 2007.

[23] Y. Yanagisawa, Y. Akiyama, S. Iida et al., "Methylation of the hMLH1 promoter in familial gastric cancer with microsatellite instability," *International Journal of Cancer*, vol. 85, no. 1, pp. 50–53, 2000.

[24] J. M. Chong, M. Fukayama, Y. Hayashi et al., "Microsatellite instability in the progression of gastric carcinoma," *Cancer Research*, vol. 54, no. 17, pp. 4595–4597, 1994.

[25] "TNM classification of malignant tumours," in *International Union AgaInst Cancer (UICC)*, L. H. Sobin and C. Wittekind, Eds., Wiley, New York, NY, USA, 7th edition, 2009.

[26] C. R. Boland, S. N. Thibodeau, S. R. Hamilton et al., "A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer," *Cancer Research*, vol. 58, no. 22, pp. 5248–5257, 1998.

[27] S. C. Chang, J. K. Lin, S. H. Yang, H. S. Wang, A. F. Y. Li, and C. W. Chi, "Relationship between genetic alterations and prognosis in sporadic colorectal cancer," *International Journal of Cancer*, vol. 118, no. 7, pp. 1721–1727, 2006.

[28] J. G. Herman, J. R. Graff, S. Myöhänen, B. D. Nelkin, and S. B. Baylin, "Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 18, pp. 9821–9826, 1996.

[29] W. M. Grady, A. Rajput, J. D. Lutterbaugh, and S. D. Markowitz, "Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer," *Cancer Research*, vol. 61, no. 3, pp. 900–902, 2001.

[30] L. A. Aaltonen, R. Salovaara, P. Kristo et al., "Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease," *The New England Journal of Medicine*, vol. 338, no. 21, pp. 1481–1487, 1998.

[31] R. B. Chadwick, R. E. Pyatt, T. H. Niemann et al., "Hereditary and somatic DNA mismatch repair gene mutations in sporadic endometrial carcinoma," *Journal of Medical Genetics*, vol. 38, no. 7, pp. 461–466, 2001.

[32] R. G. Cotton and C. R. Scriver, "Proof of "disease causing" mutation," *Human Mutation*, vol. 12, no. 1, pp. 1–3, 1998.

[33] W. S. Samowitz, K. Curtin, H. H. Lin et al., "The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer," *Gastroenterology*, vol. 121, no. 4, pp. 830–838, 2001.

[34] P. S. Kao, J. K. Lin, H. S. Wang et al., "The impact of family history on the outcome of patients with colorectal cancer in a veterans' hospital," *International Journal of Colorectal Disease*, vol. 24, no. 11, pp. 1249–1254, 2009.

[35] W. L. Fang, S. C. Chang, Y. T. Lan et al., "Microsatellite instability is associated with a better prognosis for gastric cancer patients after curative surgery," *World Journal of Surgery*, vol. 36, no. 9, pp. 2131–2138, 2012.

[36] S. Beghelli, G. de Manzoni, S. Barbi et al., "Microsatellite instability in gastric cancer is associated with better prognosis in only stage II cancers," *Surgery*, vol. 139, no. 3, pp. 347–356, 2006.

[37] G. Corso, C. Pedrazzani, D. Marrelli, V. Pascale, E. Pinto, and F. Roviello, "Correlation of microsatellite instability at multiple loci with long-term survival in advanced gastric carcinoma," *Archives of Surgery*, vol. 144, no. 8, pp. 722–727, 2009.

[38] J. Y. An, H. Kim, J. H. Cheong et al., "Microsatellite instability in sporadic gastric cancer: its prognostic role and guidance for 5-FU based chemotherapy after R0 resection," *International Journal of Cancer*, vol. 131, no. 2, pp. 505–511, 2012.

[39] S. H. Lee, J. W. Lee, Y. H. Soung et al., "BRAF and KRAS mutations in stomach cancer," *Oncogene*, vol. 22, no. 44, pp. 6942–6945, 2003.

[40] A. A. Komar, "Genetics. SNPs, silent but not invisible," *Science*, vol. 315, no. 5811, pp. 466–467, 2007.

[41] Y. Chen, K. Kingham, J. M. Ford et al., "A prospective study of total gastrectomy for CDH1-positive hereditary diffuse gastric cancer," *Annals of Surgical Oncology*, vol. 18, no. 9, pp. 2594–2598, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 346762, 14 pages
http://dx.doi.org/10.1155/2013/346762

Review Article

Implementation of High Resolution Whole Genome Array CGH in the Prenatal Clinical Setting: Advantages, Challenges, and Review of the Literature

Paola Evangelidou,[1] Angelos Alexandrou,[1] Maria Moutafi,[1] Marios Ioannides,[2] Pavlos Antoniou,[1] George Koumbaris,[2] Ioannis Kallikas,[3] Voula Velissariou,[4] Carolina Sismani,[1] and Philippos C. Patsalis[2]

[1] *Department of Cytogenetics and Genomics, The Cyprus Institute of Neurology and Genetics, 2370 Nicosia, Cyprus*
[2] *Professor Patsalis Research Team, The Cyprus Institute of Neurology and Genetics, 2370 Nicosia, Cyprus*
[3] *Ultrasound and Fetal Medicine Centre, 2025 Nicosia, Cyprus*
[4] *Department of Genetics and Molecular Biology, Gynecological, and Children's Hospital, Mitera Maternity, 15123 Athens, Cyprus*

Correspondence should be addressed to Carolina Sismani; csismani@cing.ac.cy

Received 26 October 2012; Accepted 17 January 2013

Academic Editor: Yasemin Alanay

Array Comparative Genomic Hybridization analysis is replacing postnatal chromosomal analysis in cases of intellectual disabilities, and it has been postulated that it might also become the first-tier test in prenatal diagnosis. In this study, array CGH was applied in 64 prenatal samples with whole genome oligonucleotide arrays (BlueGnome, Ltd.) on DNA extracted from chorionic villi, amniotic fluid, foetal blood, and skin samples. Results were confirmed with Fluorescence In Situ Hybridization or Real-Time PCR. Fifty-three cases had normal karyotype and abnormal ultrasound findings, and seven samples had balanced rearrangements, five of which also had ultrasound findings. The value of array CGH in the characterization of previously known aberrations in five samples is also presented. Seventeen out of 64 samples carried copy number alterations giving a detection rate of 26.5%. Ten of these represent benign or variables of unknown significance, giving a diagnostic capacity of the method to be 10.9%. If karyotype is performed the additional diagnostic capacity of the method is 5.1% (3/59). This study indicates the ability of array CGH to identify chromosomal abnormalities which cannot be detected during routine prenatal cytogenetic analysis, therefore increasing the overall detection rate. In addition a thorough review of the literature is presented.

1. Introduction

Since the 1970s that chromosomal analysis became available in prenatal diagnosis; it has proven to be a robust technique in detecting the majority of chromosomal abnormalities. With the use of amniocytes, starting in the second trimester of pregnancy [1], as well as cells isolated from chorionic villus samples in the first trimester of pregnancy [2], it was demonstrated that foetal material could be cultured to obtain sufficient metaphase cells to determine the karyotype of the foetus. These methods have been used extensively until today with many improvements over the years. A full karyotype analysed from either cultured amniocytes or chorionic villus samples can be obtained within 10 to 21 days. Furthermore chromosomal analysis can detect aneuploidy, structural rearrangements, and deletions/duplications of at least 3–10 Mb. Rapid aneuploidy tests being offered today like MLPA (Multiplex Ligation-Dependent Probe Amplification), QF PCR (Quantitative Fluorescent Polymerase Chain Reaction) are high throughput and provide rapid aneuploidy detection for certain chromosomes. They cannot, however, replace chromosomal analysis in all cases requiring invasive prenatal diagnosis, as there is a residual risk of 0.9% for a clinically significant chromosomal abnormality for all indications of invasive prenatal diagnosis [3]. As in the majority of cases with ultrasound abnormalities the karyotype in the

foetus is normal, thus demonstrating the need for additional diagnostic tests with higher diagnostic capacity [4].

Array CGH is a high throughput method which can be applied and detect copy number changes to a resolution of even as low as 1 Kb. Genome-wide arrays are rapidly replacing conventional karyotyping in postnatal diagnostics, as they are increasingly performed for the evaluation of individuals with birth defects, dysmorphic features, and mental retardation. ISCA (International Standard Cytogenomic Array) Consortium [5] supports the use of array CGH as a first-line test and suggests reserving chromosomal G-banding analysis for specific cases like patients with obvious chromosomal syndromes such as Down syndrome and family history of chromosomal rearrangements.

Its introduction, however, in prenatal diagnosis is still limited but will definitely increase in the near future. Many groups have demonstrated that by applying array CGH in prenatal diagnosis in conjunction with chromosomal analysis, there was an additional detection of clinically significant genomic imbalances [5–9], proving its usefulness, as well as its limitations, in using this technique in prenatal diagnosis. The question remains though as to whether it can be fully integrated in prenatal diagnosis, solely or in conjunction with other assays, and replace conventional cytogenetics.

There are, however, several issues that need to be addressed before implementing array CGH in prenatal diagnosis such as (1) for which pregnancies array CGH should be carried out, whether for all pregnancies or for pregnancies with ultrasound abnormalities, (2) which array platform to use, (3) the need to set the appropriate calling criteria, (4) which confirmatory methods to use for the array CGH findings, and (5) pretest counselling.

Pretest counselling is especially important in the prenatal setting, and it should be carried out to inform parents of the possibility of the fortuitous discovery of a copy number variation (CNV) unrelated to the phenotype during array CGH analysis. It should be explained to the parents that there may be asymptomatic/presymptomatic results with array CGH analysis, and they should be allowed to decide whether they wish to be informed of these findings or not [10].

In the current study, we present our experience of using whole genome oligonucleotide array CGH during prenatal diagnosis in cases with a normal karyotype with abnormal ultrasound findings or an apparently balanced structural aberration and provide a summary of our results; in addition we present the value of array CGH in the characterization of previously known aberrations. The role of whole genome oligonucleotide array CGH in prenatal diagnosis will be further evaluated in an attempt to gain more insight on its use in the prenatal setting.

2. Materials and Methods

2.1. Patients and Samples. Samples included in this study were received between May 2010, and October 2012 for prenatal diagnosis using G-banded karyotype and whole genome array CGH methodology. Among the 1414 prenatal samples received within the above period in 65 cases both chromosomal and array CGH analyses were carried out. Included in this cohort of patients were 42 amniotic fluid samples, 20 chorionic villus samples, 2 foetal blood samples, and 1 skin sample. Gestational age varied from 12.2 to 33 weeks. Ultrasound screening was carried out during the first trimester of pregnancies, and the findings include increased nuchal translucency, hypoplastic nasal bone, talipes, intrauterine growth retardation, hydronephrosis, choroid plexus cyst, tetralogy of fallot, hydrops, cardiac anomalies, ventriculomegaly, micrognathia, and skeletal abnormalities of the extremities.

These samples were further subcategorized into 5 categories (A–E) according to the chromosomal analysis results and the presence or absence of ultrasound findings (Table 1).

2.2. Conventional Cytogenetics and FISH Analyses. Conventional cytogenetic G-Banding analysis was carried out on all samples included in this study (CV, amniotic fluid, foetal blood, and skin) using standard cytogenetic methodologies [11]. Fluorescence In Situ Hybridization (FISH) was performed, where needed, using commercially available probes according to the manufacturer's protocol (VYSIS Co., Downers Grove, IL, and Cytocell, Co., UK).

2.3. Microarray Comparative Genomic Hybridization (Array CGH). DNA was extracted from CV/AF/Skin uncultured cells and from uncultured foetal blood using the Qiagen Mini and Midi Kits, respectively, according to the manufacturer's protocol (Qiagen, Valencia, CA), and concentration and purity of the extracted DNA were measured with the NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). Following DNA extraction, the test and reference DNA of the same gender were cohybridized to the array of choice, as previously described [12]. Briefly, 500 ng of patient and reference DNA were labelled by random priming using Bio Prime labelling kit (Invitrogen, Carlsbad, CA, USA) with Cyanine 3 and Cyanine 5 (Amersham Biosciences, UK) fluorescent dyes, respectively. Pooled genomic DNA from peripheral blood leukocytes of phenotypically normal males or females from Promega (Promega, Madison, WI, USA) was used as reference. DNA was then hybridized on the arrays (CytoChip, BlueGnome Ltd, UK) using an automated slide processor (HS 4800, Tecan Inc., Mannedorf, Switzerland). Array images were then acquired using an Agilent laser scanner G2565B, and image files were quantified using Agilent's feature extraction software (V9.5.3.1) and analysed with the BlueFuse for microarrays software package (BlueGnome, Ltd., UK). In the current study two different oligonucleotide arrays were used with 105,000 or 180,000 probes (BlueGnome, Ltd., UK). These arrays can detect copy number changes >50 kb in 138 targeted regions (microdeletion/duplication loci) and >150 kb in the remainder of the genome. CytoChip ISCA arrays report the gene content of over 500 recognized disease regions, while they have genome-wide coverage, including subtelomeres and pericentromeres, and support the detection of imbalances as small as 60 Kb.

2.4. Array Data and Confirmatory Analysis. Array data was analysed using BlueFuse software analysis (BlueGnome Ltd., UK), and the reporting threshold was set at 200 kb. Called

Table 1: Subcategories of samples based on the reason for referral.

Category	Karyotype	Ultrasound findings	Number of samples
A	Normal	YES	53
B	Balanced rearrangement	YES	5
C	Balanced rearrangement	No	2
D	Abnormal	YES	1
E	Abnormal	No	4

imbalances were further aligned with the in-house database as well as to known aberrations listed in publically available databases, such as the DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources http://decipher.sanger.ac.uk) and the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/) using NCBI136/hg18 UCSC or GRCh37/hg19 assemblies. Parental samples were analysed by array CGH only when needed. All copy number variations found were confirmed by FISH or Real-Time Polymerase Chain Reaction (RT-PCR) which were performed using previously described standard procedures [13, 14].

For a copy number change (CNC) to be considered as clinically significant/pathogenic the following criteria were applied:

(1) the aberration had to be *de novo* or inherited from an affected parent;

(2) the region contained genes and/or overlapped with a known syndrome or with a DECIPHER entry;

(3) the region was not listed as polymorphic in DGV;

(4) it was not previously found in the in-house database.

If an aberration met criteria 2 and 3 but was found in a normal parent and was not previously reported as a recurrent syndrome with variable phenotype due to incomplete penetrance, it was classified as a CNV of unclear significance.

All prospective parents were offered genetic counselling by the referring clinician and consented prior to the testing.

3. Results and Discussion

3.1. Findings. A total of 65 samples/cases were included in this study out of which 40 and 25 were investigated using 105 K and 180 K oligonucleotide arrays, respectively. Out of the 65, one sample gave inconclusive results (failure rate 1/65, 1.53%). Consequently a total of 64 cases will be presented in this study.

A total of seventeen cases (17/64, 26.5%) with CNVs were determined by array CGH analysis, and the findings are listed in Table 2. Four out of the seventeen cases with CNVs detected in array CGH were abnormalities previously detected by other methods (G-banding and MLPA). Thirteen out of the seventeen CNVs detected were from pregnancies with a normal karyotype and ultrasound abnormalities out of which three (Cases 12, 34, and 38, 3/64, 4.7%) were pathogenic, while the remaining ten (10/64, 15.6%) were initially categorized as variables of unknown significance (VOUS). Following parental analysis seven out of the ten VOUS were determined to represent familial CNVs which were unrelated to the reason for referral. For three out of ten of those VOUS (Cases 52, 61, and 63) parental investigation is still on going. The diagnostic capacity of array CGH in the current cohort of prenatal cases is 10.93% (7/64) for clinically significant changes. From a total of five cases with abnormal findings previously identified by other methods (Groups D and E in Table 1), the aberration was confirmed and further characterized by array CGH in four cases (Cases 5, 9, 29, and 31). In one case, Case 7, in which chromosomal analysis determined an abnormal mosaic female karyotype with a supernumerary marker chromosome, array CGH, failed to determine the origin of the marker chromosome, suggesting that it most probably did not contain any euchromatic material.

3.2. Selected Case Presentations

3.2.1. Pathogenic De Novo CNVs. Case 12 was a CVS sample from an 18-week pregnancy which was referred, initially, for chromosomal analysis, due to increased nuchal translucency. QF PCR analysis was carried out and revealed normal results. The sample was also treated as usually to establish cultures for chromosomal analysis, but after 14 days in culture there were no signs of growth. After obtaining consent from the patient and the physician, array CGH was carried out using 105 K oligonucleotide array on both the foetus and the parents. Array CGH revealed a duplication of 2.1 Mb in size on the short arm of chromosome 5, inherited from the healthy father, and a *de novo* deletion of 2.4 Mb in size on the long arm of chromosome 15 (Figure 1). The duplication on chromosome 5 was classified as likely benign, as it was inherited from the normal father, consequently stressing the necessity of confirming the presence/absence of CNVs in the parents to further categorize them. The deletion on chromosome 15 was reported as likely pathogenic, as it was relatively large in size, and it was *de novo*; the deleted region contained many genes and was not listed as polymorphic in the publicly available databases. Such single segmental imbalance even though it was determined by array CGH to be *de novo*, it could be the consequence of the unbalanced transmission of a derivative chromosome involved in an insertional balanced translocation (IT) in the parents [15]. Nowakowska et al. demonstrated that ITs underlie ~2.1% of apparently *de novo* interstitial CNVs. Such information may not be important to further evaluate the risk for the current foetus, but it is important for the accurate estimation of the recurrence risk to family members. Therefore chromosome visualization after microarray analysis is essential for delineating the rearrangement and assessing for further potential imbalance (in the immediate or even in the extended family). In the current case chromosomal analysis carried out in the parents did not detect an insertional translocation.

The deletion, however, was rather small in size for chromosomal analysis to detect (2.5 Mb); therefore FISH analysis would have been necessary to visualize exactly the nature of the imbalance. If FISH analysis cannot be performed in time

Table 2: CNVs identified during array CGH analysis using CytoChip oligonucleotide arrays.

Case	Sample	GA	Reason for referral	Result	Status	Inh.	Clinical significance	Array type	Karyotype	Cat	Genome build
5	AF	17	Investigation of abnormal karyotype	mos 47,XY,+mar. arr 21q11.2q21.1(13,539,832-15,716,987)x3~4,21q21.3 (27,787,566-28,368,946)x3	Dup (2.1 Mb), Dup (0.5 Mb)	*De novo*	Significant	105 K	47,XY,+mar	E	NCBI36/hg18
9	CVS	13	Ultrasound abnormalities/hypoplastic nasal bone	arr 7q34q35(139,107,925-145,455,647x1)dn	Del (6.3 Mb)	*De novo*	Significant	105 K	46,XX,del(7)(q34q35)	D	NCBI36/hg18
12	AF	18	U/S Findings/NT thickness	arr 5p14.3p14.2(22,344,207-24,523,053)x3 pat,15q25.2q25.3(81,011,096-83,478,823)x1 dn	Del (2.4 Mb), Dup (2.2 Mb)	Pat, *de novo*	Significant	105 K	46,XY	A	NCBI36/hg18
29	CVS	12.2	Investigation of abnormal karyotype	47,XX,+mar/46,XX. arr 16p11.2p11.1(29,727,747-35,004,980)x2~3 dn	Dup (5.2 Mb)	*De novo*	Significant	105 K	47,XX,+mar/46,XX	E	NCBI36/hg18
31	AF	17	Investigation of abnormal results with MLPA	arr 22q11.21(17,274,865-19,891,492)x3 mat	Dup (2.6 Mb)	Mat	Significant	105 K	46,XY.mlpa 22q11.2(P023)x3 mat	E	NCBI36/hg18
34	CVS	13.2	U/S Findings	arr 9q34.3(139,754,208-141,102,496)x1 mat,arr 17p13.3(48,569-2,002,395)x3 mat	Del (1.35 Mb), Dup (1.95)	Mat	Significant	105 K	46,XY	A	GRCh37/hg19
36	CVS	12	U/S Findings/NT thickness	arr 7q31.1(112,763,119-113,252,118)x3 mat	Dup (0.5 Mb)	Mat	Unrelated to the RFR	180 K	46,XY	A	GRCh37/hg19
38	AF		U/S Findings/tetralogy of Fallot	arr 9q34.3(139,754,208-141,102,496)X3,17p13.3(48,569-2,002,395)X1 mat	Dup (1.35 Mb), Del (1.95)	Mat	Significant	180 K	46,XX	A	GRCh37/hg19
42	AF	25	U/S Findings/foetal anomaly, extremities artrogryposis	arr 10p15.3(1,011,902-1,396,788)x3 pat,15q21.1(49,491,651-49,809,467)x1 mat	Dup (0.38 Mb), Del (0.32 Mb)	Pat, mat	Unrelated to the RFR	180 K	46,XX	A	GRCh37/hg19
44	AF	21	U/S Findings/foetal abnormality, cardiac anomaly	arr 5q15(95,655,383-96,003,162)x1 pat	Del (0.35 Mb)	Pat	Unrelated to the RFR	180 K	46,XX	A	GRCh37/hg19
47	AF	16	U/S findings/increased NT= 3,7 mm	arr Xp22.33(716,598-1,224,238)x3 pat	Dup (0.5 Mb)	Pat	Unrelated to the RFR	180 K	46,XY	A	GRCh37/hg19
48	CVS		U/S Findings/small-asymmetric embryo	arr 7p22.2(4,137,938-4,677,493)x3 mat	Dup (0.53 Mb)	Mat	Unrelated to the RFR	180 K	46,XY	A	GRCh37/hg19

Table 2: Continued.

Case	Sample	GA	Reason for referral	Result	Status	Inh.	Clinical significance	Array type	Karyotype	Cat	Genome build
49	AF	23.2	U/S findings/aortic arch abnormality	arr Xp22.33(2,039,059-2,275,983)x3 mat	Dup (0.24 Mb)	Mat	Unrelated to the RFR	180 K	46,XY	A	GRCh37/hg19
65	AF	25.1	U/S findings/ ventriculomegaly	arr 4q35.1(185,787,238-186,132,543)x3 mat	Dup (0.35 Mb)	Mat	Unrelated to the RFR	105 K	46,XY	A	GRCh37/hg19
52	AF	20	U/S findings/bilateral hands polydactyly	Request family analysis before final report (array CGH), pending parental testing	VOUS	N/A	N/A	180 K	46,XX	A	GRCh37/hg19
61	AF	23.4	U/S findings/cataracts, limb abnormalities/IUD	Request family analysis before final report (array CGH), pending parental testing	VOUS	N/A	N/A	105 K	46,XX	A	GRCh37/hg19
63	AF	18	U/S findings/absence of nasal bone, hypoplastic	Request family analysis before final report (array CGH), pending parental testing	VOUS	N/A	N/A	105 K	46,XX	A	GRCh37/hg19

AF: amniotic fluid, CVS: chorionic villus sample, NT: nuchal translucency, IUGR: intrauterine growth retardation, Inh.: inheritance status, U/S findings: ultrasound findings, N/A: not applicable, GA: gestational age, Cat: category; Mat: maternal, Pat: paternal, RFR: reason for referral, VOUS: variable of unclear significance, and IUD: intrauterine death.

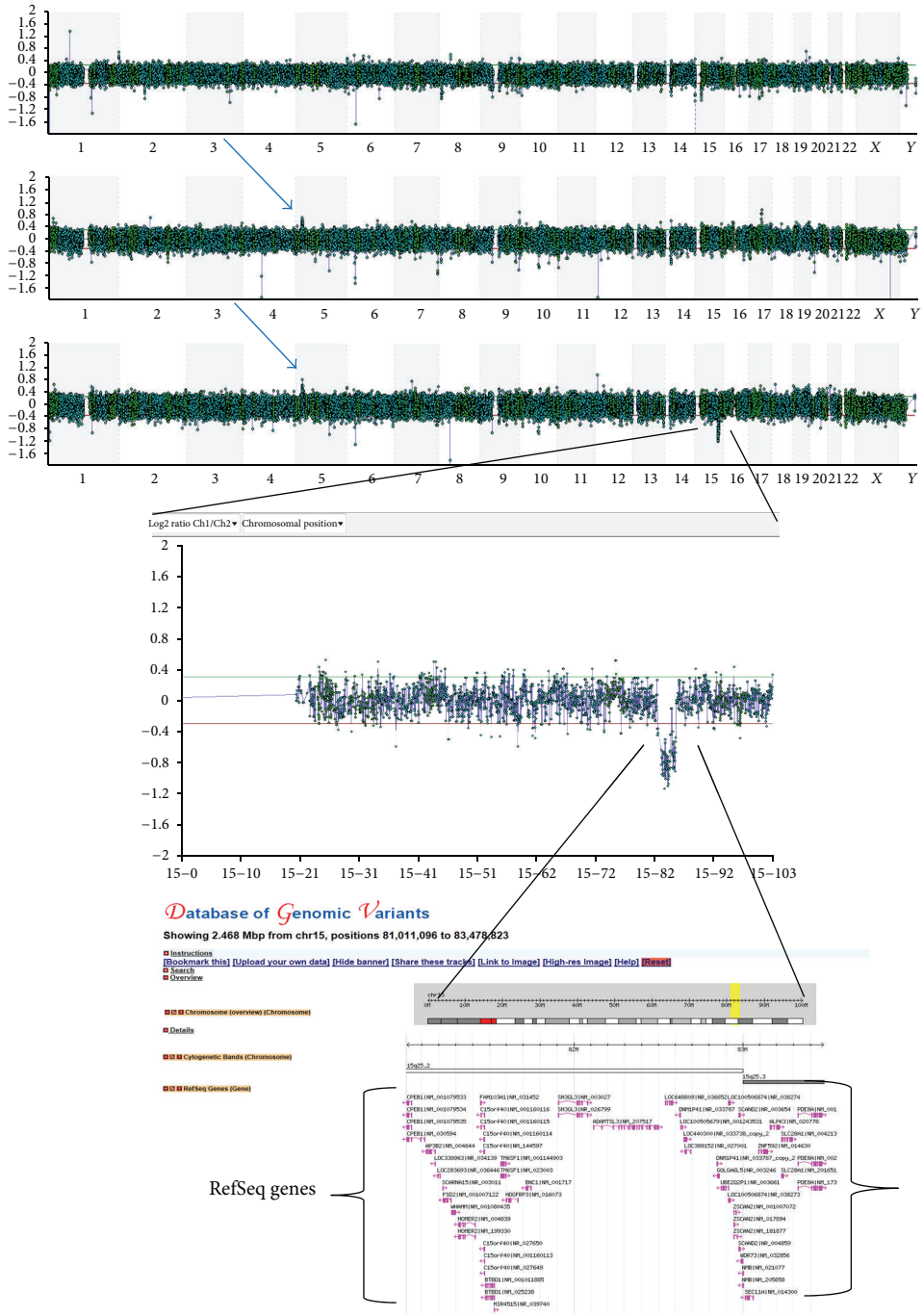


Figure 1: Case 12 showing a copy number gain on the short arm of chromosome 5 inherited from the healthy father and a *de novo* copy number loss on the long arm of chromosome 15. Representation of the chromosomal and genomic location region on chromosome 15 that has the copy number change in the Database of Genomic Variants. A loss of 2.4 Mb in size, which encompasses several RefSeq genes (shown in brackets); the region is not covered by any CNVs determining that it is not polymorphic.

for the prenatal case, a disclaimer should be written on the report regarding this point.

It is important to point out that in the current case, had chromosomal analysis been carried, out this aberration would have been missed.

3.2.2. Pathogenic Familial CNVs. Case 34, a 12-week pregnancy, was referred for chromosomal analysis and array CGH due to increased nuchal translucency (7.1 mm). Chromosomal analysis was normal (46,XY), but array CGH revealed double segmental imbalance which is usually an indication for the presence of an unbalanced translocation. Array CGH carried out with 105 K oligonucleotide array showed a terminal deletion on the long arm of chromosome 9, approximately 1.35 Mb in size, and a terminal duplication on the short arm of chromosome 17, approximately 1.95 Mb

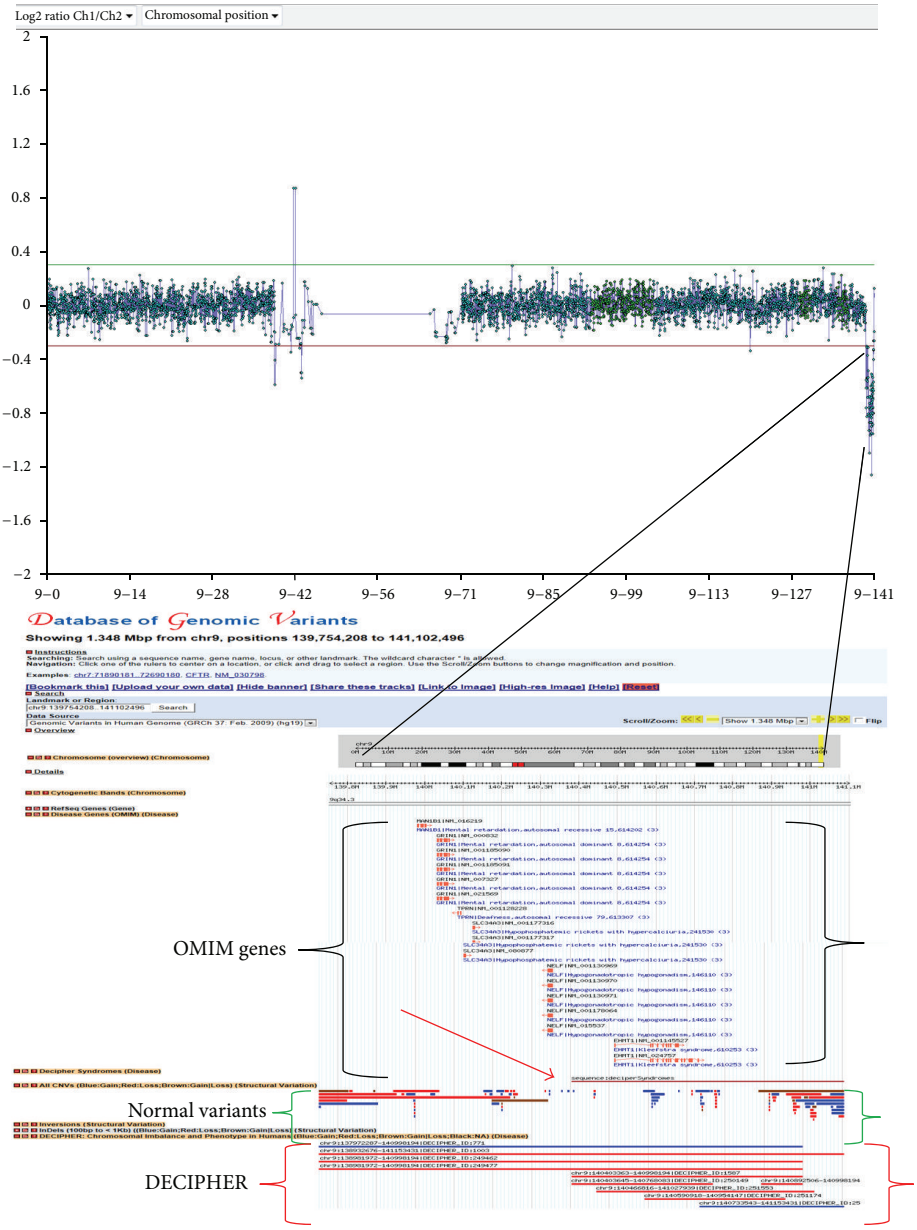


Figure 2: Case 34 showing a copy number loss on the long arm terminal of chromosome 9. Representation of the chromosomal and genomic location region on chromosome 9 that has the copy number change in the Database of Genomic Variants. A loss of 1.35 Mb in size which encompasses several OMIM genes (shown in brackets) and overlaps with a DECIPHER syndrome (the 9q microdeletion syndrome- shown by the red arrow). The area is not covered by a significant number of CNVs determining that it is not polymorphic.

in size (Figures 2 and 3). FISH analysis, using subtelomeric specific probes for chromosomes 9 and 17, was then performed which confirmed the array CGH results and determined the presence of an unbalanced translocation (Figure 4). As expected, retrospective analysis of the foetus's karyotype could not detect any of the abnormalities, since the imbalances (1.35 Mb and 1.95 Mb) were beyond the resolution of the karyotype. Chromosomal and FISH analyses carried out in the parents revealed the presence of a submicroscopic apparently balanced translocation in the mother between the long-arm terminus of chromosome 9 and the short-arm terminus of chromosome 17. The subtelomeric 9 deletion found in the foetus includes many genes, several of which are OMIM genes. In addition, the duplicated region on chromosome 17 contained many genes including two OMIM genes and partially overlapped with the Miller-Dieker syndrome region. The couple went through counselling for further explanation of the implications of the findings for the current pregnancy, as well as for future pregnancies; the couple was elected to terminate the pregnancy.

The usefulness of the additional information array CGH provided in the diagnosis in this case is obvious; without it the copy number change would have remained undetected. Furthermore, the information acquired from this case will

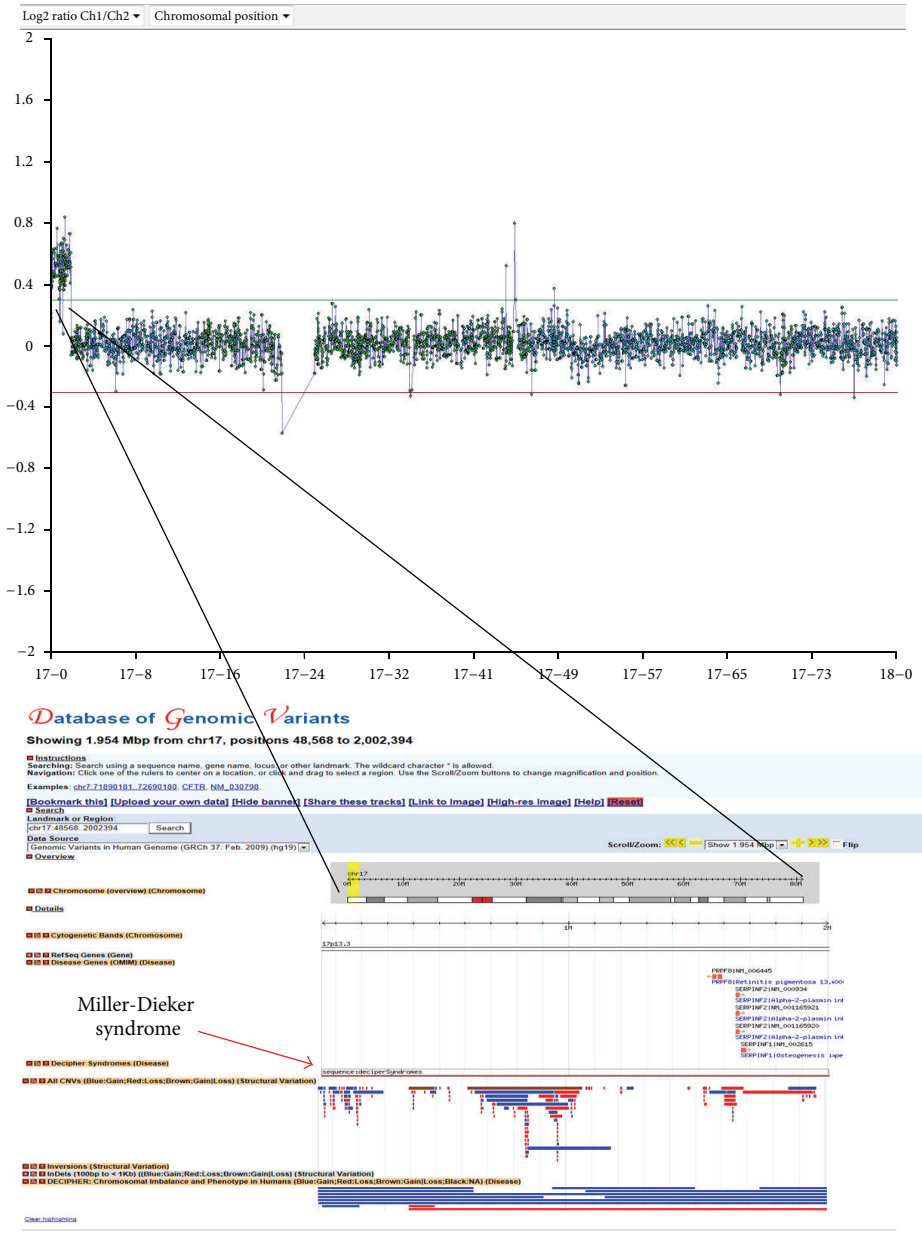
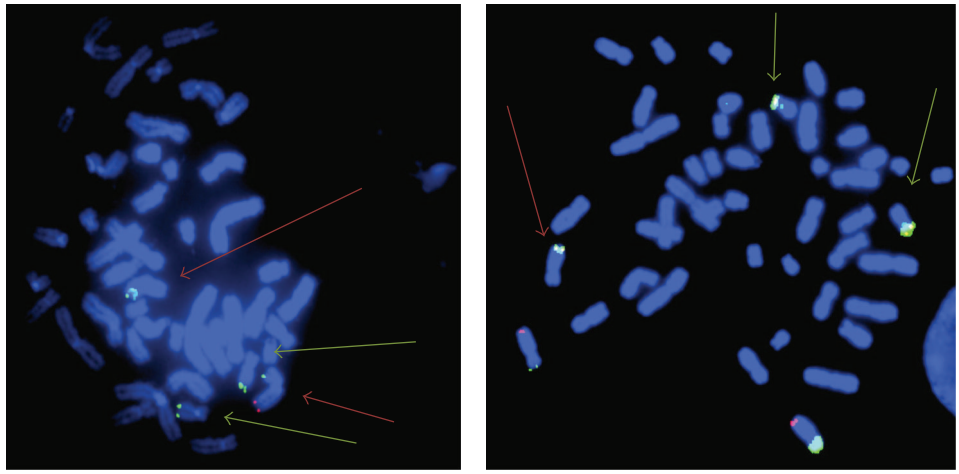


Figure 3: Case 34 showing a copy number gain on the short arm of chromosome 17. Representation of the chromosomal and genomic location region on chromosome 17 that has the copy number change in the Database of Genomic Variants. A gain of 1.95 Mb in size which encompasses some OMIM genes and overlaps with a DECIPHER syndrome (the Miller-Dieker syndrome—shown by the red arrow). The area is not covered by a significant number of CNVs determining that it is not polymorphic.

be used from the family for the better management of their pregnancies in the future. After careful evaluation of this couple's reproductive and medical history, it was revealed that they had a previous pregnancy (Case 38) which was terminated due to multiple severe ultrasound findings (tetralogy of Fallot, talipes, and other). In addition the couple also had an affected child. Both the previous pregnancy, and the child were previously karyotyped by our laboratory, and the results were normal. As expected, retrospective G-banding analysis of both the child and the previous pregnancy did not detect the abnormalities, and the parents consented to perform array CGH on stored genetic material from their previous pregnancy and their affected child. Array CGH analysis revealed related findings to the current case and contributed to the diagnosis for their affected child who had the same unbalanced karyotype as the analysed foetus. The importance of having the pedigree of a family being investigated is paramount as shown in this case. Had the parents informed the clinicians during the previous pregnancy that they already had an affected child; the management of the first pregnancy might have been different. The first pregnancy was investigated by chromosomal analysis on amniotic fluid sample



(a) (b)

Figure 4: FISH analysis showing the confirmation of the unbalanced translocation in Case 34, using subtelomeric probes for chromosomes 9 and 17. Chromosome 8p and q probes (b) are also included in the probe mixture used (VYSIS, ToTelVysion probes). (a) Probes used: subtelomeric 9p and 17q, the top red arrow points at the derivative chromosome 9 (showing the deletion of 9q) and the green arrows point at chromosome 17. (b) Probes used: subtelomeric 17p, 8p and 8q, the red arrow points at the derivative chromosome 9 (showing the duplication of 17p) the green arrows point at chromosome17p.

on the 16th week and revealed normal karyotype. It was terminated based on the ultrasound findings despite the fact that the karyotype was apparently normal. Had the parents known at the time that their born child had a chromosomal abnormality which was inherited from the mother; they would have opted for an earlier prenatal diagnosis on their first pregnancy perhaps by chorionic villus sampling. This would have lessened their anxiety.

3.2.3. Likely Benign CNVs/VOUS. The importance of carrying out confirmatory tests to the parents as well as the foetuses can also be seen in two other prenatal cases; CNVs found in the foetuses were classified as benign, after parental testing, as they were also present in healthy parents. Case 36, a 12-week pregnancy, was referred for chromosomal and array CGH analyses because of increased nuchal translucency. Array CGH analysis revealed a duplication of 0.5 Mb in size on the long arm of chromosome 7 which was classified to be benign, as it was also present in the healthy mother. Case 42, a 25-week pregnancy was referred for chromosomal analysis due to ultrasound findings (artrogryposis). Array CGH analysis revealed a duplication of 0.38 Mb in size on the short arm of chromosome 10 and a deletion of 0.32 Mb in size on the long arm of chromosome 15. Array CGH analyses carried out in the parents determined that the duplication was of paternal origin and the deletion was of maternal origin, determining that both CNVs were likely benign as each one was present in each one of the healthy parents. In Cases 52, 61, and 63 the CNVs found are considered variable of unknown significance (VOUS) as the abnormality still needs to be investigated through parental testing in order to determine if they represent clinically significant or benign CNVs.

It has to be pointed out that in the previous two cases array CGH analyses were carried out in the parents after extensive review of the publicly available databases (DGV, DECIPHER) as well as our own dataset. These databases did not show the CNVs found in these two cases to be common variants and that is why parental array CGH was subsequently carried out and showed that those CNVs were specific to that family.

3.3. Characterization of Previously Known Aberrations. Array CGH was able to characterize previously known abnormalities in four out of five cases. In three cases with marker chromosomes it confirmed the presence of additional genomic material and determined its size (Cases 5 and 29), but failed to confirm copy number gain in one case. Furthermore, array CGH delineated a deletion on the long arm of chromosome 7, in Case 9, which was identified by chromosomal analysis. The deletion was clinically significant, and it was determined to be approximately 6.3 Mb in size.

3.4. Array CGH Detection Rate in Prenatal Diagnosis. Many groups (Table 3) have demonstrated that by applying array CGH there was an additional detection of clinically significant genomic imbalances of approximately 3.6% (average from all studies) when the karyotype was normal, regardless of the indication of the referral for chromosomal analysis. This detection rate increased to 5.2% when the pregnancy had a structural malformation on ultrasound [5, 7, 16–20, 23, 24]. In these studies the overall detection of array CGH over chromosomal analysis was 12%. When benign CNVs were removed and considered as normal results the detection rate dropped to 3.6% [24]; this percentage included the pathogenic CNVs as well as the variants of unknown significance (VOUS) with a potential of being pathogenic. The presence of VOUS was found in 1.1% of cases [24].

The ultrasound findings included cardiac abnormalities, increased nuchal translucencies, cystic hygromata or

Table 3: Comparison between various studies which used array CGH in prenatal diagnosis.

Study	Array type	Karyotype/reason for referral	Results	Clinical significance of results
Kleeman et al., 2009 [16]	Signature prenatal targeted BAC chip V, signature whole genome chip	Normal karyotype/sonographic anomalies	4/50 abnormal	2% clinically significant, 6% inherited or benign variant
Vialard et al., 2009 [17]	Targeted genosensor BAC/PAC array	Normal karyotype/multiple congenital abnormalities	4/37 abnormal	10.8% clinically significant
Bi et al., 2008 [18]	BCM V6 oligonucleotide array	Normal karyotype/maternal age, sonographic anomalies, family history, and miscarriages	3/15 abnormal	13% clinically significant, 7% inherited or benign variant
Shaffer et al., 2008 [19]	Prenatal targeted BAC array	149/151 normal karyotype/maternal age, sonographic anomalies, family history, and parental anxiety	15/151 abnormal	1.3% clinically significant, 8% benign, and 0.5% unclear significance
Sahoo et al., 2006 [20]	BCM V4 targeted BAC array	93/98 normal karyotype/maternal age, sonographic anomalies, and family history	5/98 abnormal of which one had additional abnormalities	5% clinically significant
Tyreman et al., 2009 [7]	GeneChip SNP whole genome oligonucleotide array	Sonogramphic abnormalities	35/106 abnormal	9% likely pathogenic, 12% likely benign, and 13% unclear significance
Coppinger et al., 2009 [5]	Signature V 4.0, prenatal targeted BAC array, and whole genome array	Normal karyotype/maternal age, sonographic anomalies, family history, and anxiety	*Whole genome*: 22/180 abnormal *Targeted*: 7/62 abnormal	*Whole genome*: 2.7% clinically significant, 0.5% unclear significance, and 8.8% benign variants *Targeted*: 0.9% clinically significant, 0.5 unclear significance, and 8% benign variants
Fiorentino et al., 2011 [21]	Whole genome CytoChip focus BAC array	Maternal age, sonographic anomalies, family history, and anxiety	34/1037 abnormal	3.3% clinically significant, 13% benign variants.
Wapner et al., 2012 [22]	Agilent 44 K targeted array Affymetrix Genome-Wide Human SNP Array 6.0	Normal karyotype/maternal age, sonographic anomalies, abnormal serum biochemistry, family history, anxiety, and previous pregnancy with abnormality	1399/3822 (36.6%)	2.5% pathogenic and likely to be pathogenic, 32.3% common benign CNVs (34.1% if the likely to be benign VOUS is added), and 3.4% unclear significance (1.8% likely to be benign and 1.6% potential for clinical significance)
Our study	Whole genome105K or 180 K CytoChip oligo arrays	Normal karyotype and sonographic anomalies, balanced rearrangements with or without sonographic anomalies, abnormal karyotype, or MLPA	17/64 abnormal	4.7%clinically significant, 10.9% inherited or benign variants, and 4.6% unclear significance

hydrops, or central nervous system abnormalities. Most of these studies used targeted BAC arrays [5, 16–20, 23], and some used both targeted and whole genome arrays [5, 16, 18]. The resolution for the arrays varied from 287 to 4685 BAC probes and from 44,000 to 946,000 oligonucleotide probes.

Tyreman et al. conducted a retrospective analysis of 106 karyotypically normal referrals with ultrasound findings using the GeneChip 6.0 SNP array from Affymetrix. This platform provides uniquely high resolution coverage of the genome with over 1.8 million probes, using oligonucleotide targets that provide copy number information only and single nucleotide polymorphisms (SNPs) oligonucleotide targets which provide genotyping as well as copy number information. In this study a total of 35 rare CNVs were identified, 10 (9%) of which were considered to be pathogenic, 12 were likely to be benign (11%), and 13 were VOUS (12%). The percentage of VOUS is slightly higher than the other studies because parental testing was not used in this study for their clarification. In addition in this study a case with a cryptic mosaic trisomy for chromosome 10 was identified as well as a case with loss of heterozygosity (LOH). The same platform can detect triploidy as well which is a major advantage; one of the limitations of array CGH is its inability to detect triploidies [7]. Table 3 shows the comparison between these studies.

In another study completed by Fiorentino et al. [21] pregnant women were referred for chromosomal and array CGH analyses. Both methods were carried out concurrently in order to compare results. A total of 1037 prenatal samples were studied, and the reason for referral of these samples included advanced maternal age, ultrasound findings, parental anxiety, and family history of a genetic condition or chromosome abnormality. Array CGH was carried out using whole genome BAC array with a resolution of 1 Mb across the genome and ~100 kb resolution in 139 regions associated with constitutional disorders. From the analysis it was determined that 13% of the samples had likely benign and of no clinical significance CNVs. Furthermore, array CGH revealed clinically significant chromosome alterations in 3.3% of the samples. In 0.9% of the samples array CGH provided diagnosis of clinically significant chromosomal abnormality which was not detected by chromosomal analysis and would have otherwise gone undetected. Clinically significant results were also identified by conventional cytogenetics as well in 73.5% of the total abnormalities also detected by array CGH (25/34) and in 2.4% of the total number of samples.

Finally, in the largest prenatal study published to date by Wapner et al. which includes over 4000 cases, microarray analysis provided additional clinically relevant information in 1.7% of pregnancies with standard indications for prenatal diagnosis and in 6.0% of pregnancies with an anomaly on ultrasonography. In addition, uncertain findings (VOUS) occurred in 1.5% of all karyotypically normal cases. In total out of the 3822 normal karyotypes, 1234 common benign CNVs were identified (32.3%), 35 pathogenic CNVs (0.9%), and 130 VOUS (3.4%). Out of the 130 VOUS the 69 were likely to be benign, and the 61 were likely to be pathogenic. If we add the likely to be benign VOUS to the common CNVs, then the total of benign CNVs raises to 1303 (34.1%). If the likely to be pathogenic VOUS are added to the known pathogenic CNVs, then a total of 96 (2.5%) is reached. The authors do comment, however, that the number of VOUS is expected to fall, as additional experience is acquired. They also point out that for the interpretation of uncertain results, close collaboration between laboratory directors, clinical geneticists, counsellors and practitioners is necessary. This study also suggests that SNP arrays are used in prenatal testing to reliably identify triploidy which is missed with the use of standard arrays [22].

3.5. Can Array CGH Analysis Fully Replace Karyotyping? Arrays CGH analysis is being introduced in prenatal diagnosis in conjunction to chromosomal analysis, but it cannot yet fully replace karyotyping for the following reasons: (a) it cannot detect balanced rearrangements such as translocations, balanced insertions, and inversions. This is especially important in Robertsonian translocations, as carriers of such are at high risk for uniparental disomy (UPD) [25] and the risks UPD imply. Even in the case were SNP arrays are used which can detect isodisomy [26], they cannot detect heterodisomy which is the most common form of UPD. In addition to Robertsonian translocations, balanced rearrangements especially *de novo* reciprocal translocations or insertions are important to be detected, as they can sometimes lead to abnormal phenotypes. Furthermore knowing the presence of a balanced rearrangement can provide the couple future risk assessments for an unbalanced offspring and information useful for reproductive planning, (b) it cannot detect low level mosaicism, a finding that we often see in prenatal diagnosis. Mosaicism is detected in 1-2% of CVS samples and in 0.2% of amniotic fluid samples [27]. Even though in about 84% of mosaic cases in CVS, the mosaicism is confined to the placenta [28], the remaining cases would have remained undetected if array CGH was the only method applied, and (c) it cannot always detect the presence of marker chromosomes, as was the case in one of our samples (Case 7), even in the nonmosaic state. Marker chromosomes are encountered in about 0.1% of prenatal diagnoses [27] and very often in the mosaic form. Depending on which chromosome they were derived from, their size, their inheritance mode and whether they are euchromatic or heterochromatic the phenotypic risk can be determined. In a study of 55 cases with marker chromosome it was demonstrated that out of the 26 nonmosaic markers only 14 were detected leaving 46% of array results normal. Even if this percentage reflects that the markers are mainly heterochromatic, the lack of detection does not completely exclude a possible phenotypic effect [29], and finally (d) it cannot visualize the type of rearrangement in the event where deletion or duplication detected by array CGH is proven to be *de novo* after parental testing [15].

3.6. Genetic Counselling. As genome-wide analysis is being introduced into prenatal diagnosis pretest counselling is of paramount importance due to the nature of the test and the findings emerging from the analysis. Information should be offered by counsellors, and everything should be explained clearly and in a nondirective way, so that prospective parents

can make their own decision having their future child's best interest in mind.

It is imperative that the following information is given by the prospective parents:

(i) medical history of both parents;

(ii) medical history of the pregnancy which should include any ultrasound findings;

(iii) family pedigree of both parents up to three generations.

Counsellors should be aware of the state of mind parents-to-be are in, right after an ultrasound abnormality has been detected. Parents may not be able to absorb any information given to them at the time, so it is good practice to have everything written down as well, so that it is available for them to read later on. Following this, parental consent should be obtained. Prospective parents should be informed of the test, and its limitations should be further explained. They should know that the array technique cannot detect every single disease or well-known syndrome. In a study of 141 foetuses with ultrasound abnormalities and normal array results, there was a diagnosis in 15% of them when they were reviewed postnatally [30].

If, in the course of testing the foetus, whole genome array analysis is needed to be carried out for the parents, they should be counselled appropriately including informed consent on what information they want to receive.

The parents should be aware of all the possible outcomes of the array testing which could either be normal or abnormal. It should be explained to them that if CNVs are detected they could (a) explain the foetal ultrasound abnormalities, (b) be *de novo* and of unknown clinical significance, (c) be inherited and of unknown clinical significance, and (d) be an unsolicited finding unrelated to the ultrasound findings.

Variables of unknown significance and incidental findings are the most challenging for counsellors. This is why it is of prime importance to inform parents of such possible findings; an example is a late-onset inherited disease either *de novo* or inherited in the family. Its implications should be explained, and a distinction should be made between treatable (hereditary cancer) and nontreatable (Huntington's disease) late-on-set diseases. There is no straight forward guideline on how this should be carried out, but, for example, in Europe the current tendency is to ask parents whether they want to be informed about treatable late-onset diseases. Some laboratories even have a policy of not reporting unsolicited CNVs to nontreatable diseases [30]. There are many ethical questions arising from all these, one of them being the extent to which pregnant women and their partners should be allowed to determine the range of possible outcomes that will or will not be reported back to them [31]. National guidelines in the use of array CGH in prenatal diagnosis remain to be established.

4. Conclusions

Karyotyping has been the golden standard method for prenatal diagnosis for decades, being able to sufficiently diagnose numerical and large structural abnormalities (<3–10 Mb). With the introduction of array CGH analysis in postnatal analysis and its use as a first-tier test in cases of intellectual disabilities, it has been postulated that this method might someday actually replace conventional cytogenetics in prenatal diagnosis as well. Array CGH in a postnatal setting has been demonstrated to be a high throughput, comprehensive, and fast to detect copy number changes that can go undetected by light microscopy.

The current study has demonstrated that the usefulness of array CGH in prenatal diagnosis depends on the selection of the appropriate platform. More importantly, it has clearly shown that array CGH is a valuable tool in prenatal diagnosis, both in cases with foetal malformations and normal karyotype as well as in cases where an abnormality was detected with another method and further investigated with array CGH. Array CGH provided valuable information for phenotype-genotype correlation and provided more accurate information regarding the clinical significance and the risk in the current and future pregnancy of the respective patient. Another critical factor for accurate CNV classification is parental testing to determine between familial and *de novo* CNVs. Appropriate pre- and posttest genetic counsellings offer the prospective parents tools to decide on the management of their pregnancy. However, one of the problems posing dilemmas to genetic counsellors and something that array CGH has to overcome is the fact that it can detect coincidental findings, variants of unknown significance and variants with variable expressivity.

Currently the ideal setting to advance prenatal diagnosis and increase its resolution would be to apply array CGH in high risk pregnancies in conjunction with chromosomal analysis with a microarray designed especially for prenatal diagnosis. As we have seen, this increases the detection rate for likely pathogenic CNVs up to 5%. To avoid interpretation problems (previously discussed) these arrays should cover all known pathogenic CNVs and have a low-resolution backbone for the detection of relatively large CNVs thus keeping the detection of CNVs of unclear significance to the minimum. A shared database specifically dedicated to prenatal diagnosis coupled with the growing amount of data regarding CNVs and dosage sensitive genes could make it easier to interpret genomic arrays.

Conflict of Interests

The authors declare that they no conflict of interests.

Consent

The authors declare that the experiments were conducted with the understanding and the consent of the human subjects used in these experiments.

References

[1] M. W. Steele and W. R. Breg Jr., "Chromosome analysis of human amniotic-fluid cells," *The Lancet*, vol. 1, no. 7434, pp. 383–385, 1966.

[2] J. Mohr, "Foetal genetic diagnosis: development of techniques for early sampling of foetal cells," *Acta pathologica et microbiologica Scandinavica*, vol. 73, no. 1, pp. 73–77, 1968.

[3] W. C. Leung and T. T. Lao, "Rapid aneuploidy testing, traditional karyotyping, or both?" *The Lancet*, vol. 366, no. 9480, pp. 97–98, 2005.

[4] K. D. Lichtenbelt, N. V. Knoers, and G. H. Schuring-Blom, "From karyotyping to array-CGH in prenatal diagnosis," *Cytogenetics and Genome Research*, vol. 135, no. 3-4, pp. 241–250, 2011.

[5] J. Coppinger, S. Alliman, A. N. Lamb, B. S. Torchia, B. A. Bejjani, and L. G. Shaffer, "Whole-genome microarray analysis in prenatal specimens identifies clinically significant chromosome alterations without increase in results of unclear significance compared to targeted microarray," *Prenatal Diagnosis*, vol. 29, no. 12, pp. 1156–1166, 2009.

[6] P. Evangelidou, C. Sismani, M. Ioannides et al., "Clinical application of whole-genome array CGH during prenatal diagnosis: study of 25 selected pregnancies with abnormal ultrasound findings or apparently balanced structural aberrations," *Molecular Cytogenetics*, vol. 3, no. 1, article 24, 2010.

[7] M. Tyreman, K. M. Abbott, L. R. Willatt et al., "High resolution array analysis: diagnosing pregnancies with abnormal ultrasound findings," *Journal of Medical Genetics*, vol. 46, no. 8, pp. 531–541, 2009.

[8] M. Valduga, C. Philippe, P. B. Segura et al., "A retrospective study by oligonucleotide array-CGH analysis in 50 fetuses with multiple malformations," *Prenatal Diagnosis*, vol. 30, no. 4, pp. 333–341, 2010.

[9] I. B. Van Den Veyver, A. Patel, C. A. Shaw et al., "Clinical use of array comparative genomic hybridization (aCGH) for prenatal diagnosis in 300 cases," *Prenatal Diagnosis*, vol. 29, no. 1, pp. 29–39, 2009.

[10] J. R. Vermeesch, P. D. Brady, D. Sanlaville, K. Kok, and R. J. Hastings, "Genome-wide arrays: quality criteria and platforms to be used in routine diagnostics," *Human Mutation*, vol. 33, no. 6, pp. 906–915, 2012.

[11] M. E. Chandler and J. J. Yunis, "A high resolution in situ hybridization technique for the direct visualization of labeled G-banded early metaphase and prophase chromosomes," *Cytogenetics and Cell Genetics*, vol. 22, no. 1, pp. 352–356, 1978.

[12] H. Fiegler, R. Redon, D. Andrews et al., "Accurate and reliable high-throughput detection of copy number variation in the human genome," *Genome Research*, vol. 16, no. 12, pp. 1566–1574, 2006.

[13] P. C. Patsalis, P. Evangelidou, S. Charalambous, and C. Sismani, "Flourescence in situ hybridization characterization of apparently balanced translocation reveals cryptic complex chromosomal rearrangements with unexpected level of complexity," *European Journal of Human Genetics*, vol. 12, no. 8, pp. 647–653, 2004.

[14] R. Weksberg, S. Hughes, L. Moldovan, A. S. Bassett, E. W. C. Chow, and J. A. Squire, "A method for accurate detection of genomic microdeletions using real-time quantitative PCR," *BMC Genomics*, vol. 6, article 180, 2005.

[15] B. A. Nowakowska, N. de Leeuw, C. A. Ruivenkamp et al., "Parental insertional balanced translocations are an important cause of apparently de novo CNVs in patients with developmental anomalies," *European Journal of Human Genetics*, vol. 20, no. 2, pp. 166–170, 2012.

[16] L. Kleeman, D. W. Bianchi, L. G. Shaffer et al., "Use of array comparative genomic hybridization for prenatal diagnosis of fetuses with sonographic anomalies and normal metaphase karyotype," *Prenatal Diagnosis*, vol. 29, no. 13, pp. 1213–1217, 2009.

[17] F. Vialard, D. Molina Gomes, B. Leroy et al., "Array comparative genomic hybridization in prenatal diagnosis: another experience," *Fetal Diagnosis and Therapy*, vol. 25, no. 2, pp. 277–284, 2009.

[18] W. Bi, A. M. Breman, S. F. Venable et al., "Rapid prenatal diagnosis using uncultured amniocytes and oligonucleotide array CGH," *Prenatal Diagnosis*, vol. 28, no. 10, pp. 943–949, 2008.

[19] L. G. Shaffer, J. Coppinger, S. Alliman et al., "Comparison of microarray-based detection rates for cytogenetic abnormalities in prenatal and neonatal specimens," *Prenatal Diagnosis*, vol. 28, no. 9, pp. 789–795, 2008.

[20] T. Sahoo, S. W. Cheung, P. Ward et al., "Prenatal diagnosis of chromosomal abnormalities using array-based comparative genomic hybridization," *Genetics in Medicine*, vol. 8, no. 11, pp. 719–727, 2006.

[21] F. Fiorentino, F. Caiazzo, S. Napolitano et al., "Introducing array comparative genomic hybridization into routine prenatal diagnosis practice: a prospective study on over 1000 consecutive clinical cases," *Prenatal Diagnosis*, vol. 31, no. 13, pp. 1270–1282, 2011.

[22] R. J. Wapner, C. L. Martin, B. Levy et al., "Chromosomal microarray versus karyotyping for prenatal diagnosis," *The New England Journal of Medicine*, vol. 367, no. 23, pp. 2175–2184, 2012.

[23] C. Le Caignec, M. Boceno, P. Saugier-Veber et al., "Detection of genomic imbalances by array based comparative genomic hybridisation in fetuses with multiple malformations," *Journal of Medical Genetics*, vol. 42, no. 2, pp. 121–128, 2005.

[24] S. C. Hillman, S. Pretlove, A. Coomarasamy et al., "Additional information from array comparative genomic hybridization technology over conventional karyotyping in prenatal diagnosis: a systematic review and meta-analysis," *Ultrasound in Obstetrics and Gynecology*, vol. 37, no. 1, pp. 6–14, 2011.

[25] L. G. Shaffer, "Risk estimates for uniparental disomy following prenatal detection of a nonhomologous Robertsonian translocation," *Prenatal Diagnosis*, vol. 26, no. 4, pp. 303–307, 2006.

[26] B. H. W. Faas, I. Van Der Burgt, A. J. A. Kooper et al., "Identification of clinically significant, submicroscopic chromosome alterations and UPD in fetuses with ultrasound anomalies using genome-wide 250k SNP array analysis," *Journal of Medical Genetics*, vol. 47, no. 9, pp. 586–594, 2010.

[27] M. R. J. Gardner and G. R. Sutherland, *Chromosome Abnormalities and Genetic Counceling*, 2004.

[28] J. M. Hahnemann and L. O. Vejerslev, "European collaborative research on mosaicism in CVS (EUCROMIC)—fetal and extrafetal cell lineages in 192 gestations with CVS mosaicism involving single autosomal trisomy," *American Journal of Medical Genetics*, vol. 70, no. 2, pp. 179–187, 1997.

[29] T. H. Bui, A. Vetro, O. Zuffardi, and L. G. Shaffer, "Current controversies in prenatal diagnosis 3: is conventional chromosome analysis necessary in the post-array CGH era?" *Prenatal Diagnosis*, vol. 31, no. 3, pp. 235–243, 2011.

[30] A. Vetro, K. Bouman, R. Hastings et al., "The introduction of arrays in prenatal diagnosis: a special challenge," *Human Mutation*, vol. 33, no. 6, pp. 923–929, 2012.

[31] W. Dondorp, B. Sikkema-Raddatz, C. de Die-Smulders, and G. de Wert, "Arrays in postnatal and prenatal diagnosis: an exploration of the ethics of consent," *Human Mutation*, vol. 33, no. 6, pp. 916–922, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 489489, 7 pages
http://dx.doi.org/10.1155/2013/489489

Research Article

Detection of *C. trachomatis* in the Serum of the Patients with Urogenital Chlamydiosis

Naylia A. Zigangirova,[1] Yulia P. Rumyantseva,[1] Elena Y. Morgunova,[1] Lidia N. Kapotina,[1] Lubov V. Didenko,[1] Elena A. Kost,[1] Ekaterina A. Koroleva,[1] Yuriy K. Bashmakov,[2] and Ivan M. Petyaev[2]

[1] *Department of Medical Microbiology, Gamaleya Institute of Epidemiology and Microbiology, Ministry of Health of the Russian Federation, Gamaleya Street 18, Moscow 123098, Russia*
[2] *Lycotec Ltd., St John's Innovation Park, Cowley Road, Cambridge CB4 0WS, UK*

Correspondence should be addressed to Naylia A. Zigangirova; zigangirova@mail.ru

Received 22 October 2012; Accepted 4 January 2013

Academic Editor: Gokce A. Toruner

Extragenital chlamydial complications may be associated with systemic spread of infection, but haematogenous route for *C. trachomatis* dissemination has not been clearly demonstrated. Here we report that serum specimens obtained from patients with chlamydiosis contain elementary bodies of *C. trachomatis* shown by culture and immunogold electron microscopy. We have found that 31 of the 52 patients had serum precipitates which were infective to McCoy cells. Immunostaining revealed very small inclusions resembling those reported during persistent *C. trachomatis* infection *in vitro*. DNA specimens from 49 (out of 52) patients with chlamydiosis gave positive PCR readings. The viability of the pathogen present in the sera was confirmed by chlamydial RNA detection in the cell monolayer inoculated by the serum precipitates. By using DNA isolation protocol from 1 mL of serum and quantitative TaqMan PCR, it was estimated that bacterial load in patients' sera was 2×10^2-10^3 GE/mL. These findings for the first time demonstrated that *C. trachomatis* can be disseminated directly by the plasma, independently from blood cell, which may represent a new possible pathway of the chronic infection development. Therefore, new methodological approaches for detection of *C. trachomatis* in the serum of patients with complicated and chronic chlamydiosis could be important in the diagnosis of the infection regardless of its anatomical localization.

1. Introduction

Chlamydia trachomatis is an obligate gram-negative intracellular bacteria which belongs to genus *Chlamydia*. Three major biovars of *C. trachomatis* are believed to induce different diseases in humans trachoma (serovars A, B, Ba, or C), urethritis, epididymitis, cervicitis, salpingitis and pelvic inflammatory disease (serovars D–K), and lymphogranuloma venereum (LGV, serovars L1, L2, and L3) [1]. Cellular paradigm of chlamydial infection emerges from the fact that epitheliocytes are the main primary target of *C. trachomatis* in the human body [2]. Chemokines released from epithelial cells can recruit neutrophils and a modest number of monocytes which in turn can infiltrate submucosal zone within 24–48 hours after initiation of the infection [3]. Epitheliocytes provide perfect support for full developmental cycle of *C. trachomatis* which concludes in cells lysis and exposure of adjacent epithelial cells to the *de novo* infectious progeny [4]. Horizontal spread of the pathogen within epithelium and further cytokine release from migrated innate immune cells culminate in the appearance of lymphocytes in the site of infection [5]. Viable forms of *C. trachomatis* can be found within monocytes, neutrophils, lymphocytes in epithelial lesions, and as far as in regional lymph nodes [6, 7]. Phagocytosis, pinocytosis, and receptor-mediated uptake are responsible for chlamydial entry into innate immunity cells [8]. According to their ability to spread, *C. trachomatis* serovars are divided into invasive and noninvasive. Serotypes L1, L2, and L3 cause invasive infections, whereas types D, E, F, G, and K restrict their propagation to the site of primary

chlamydial insult [9]. Nevertheless, in many cases of *C. trachomatis* infection the pathogen and clinical manifestation of the disease spread far beyond originally infected area. Canicular spread of the pathogen is traditionally believed to be a major way of *C. trachomatis* generalization [2, 3] though this mechanism is not relevant when pathogen is detected in tissues with no anatomical connectivity to urogenital system. Extragenital complications of *C. trachomatis* infection include sexually acquired arthritis (SARA), perihepatitis, and conjunctivitis [10]. Isolation and/or detection of *C. trachomatis* has been reported from synovial exudate [11], ascitic fluid [12, 13], liver biopsy material [14, 15], and respiratory secretion fluids [16]. However, the mechanism of systemic generalisation of chlamydial disease remains yet to be understood. Lymphogenic dissemination is likely to be implicated since *C. trachomatis* propagates in lymphocytes and can be isolated from regional lymph nodes [7, 17].

Recently, we showed that *C. pneumoniae* and *C. trachomatis* can be detected in serum specimens obtained from patients [18, 19]. Here we report new data on isolation and identification of *C. trachomatis* viable infectious forms from serum of patients with urogenital chlamydial infection.

2. Material and Methods

2.1. Patients. The study was conducted in the outpatient clinic NearMedic at Gamaleya Institute of Epidemiology and Microbiology RAMN (Moscow, Russia). The study protocol was approved by the local ethical committee. All patients were informed about the purpose of the study and have given a written consent regarding participation in the study. The major group of the study included 52 patients with newly diagnosed symptomatic chlamydial infection having complaints of abnormal vaginal discharge, genital ulcer, lower abdominal pain (women), or urethral discharge (men). Patients with pelvic inflammatory diseases or epididymitis and other infections (*N. gonorrhoeae*, tuberculosis, HIV, Hepatitis B or C, and syphilis) or patients admitting antibiotic use within 4 weeks before enrollment were excluded from the study. Thirty women (range 19–38 years) and 22 men (range 24–45 years) with diagnosis of urogenital symptomatic *C. trachomatis* infection confirmed by cell culture and PCR were eligible for the study. Control group included 20 age-matched healthy volunteers (9 males range 19–30 years, 11 females 23–40 years) with no history or current evidence of STD and with negative PCR results of swab specimens. Groups of 36 patients with chronic pelvic inflammatory diseases (range 28–41 years), 33 patients with Reiter's disease (range 31–46 years), and 20 control subjects (range 26–50 years) were examined for chlamydial DNA presence in serum and urogenital swabs. The study neither interfered with diagnostic and therapeutic options nor had impact on treatment options chosen by physicians for each consented individual.

2.2. Specimen Handling

2.2.1. Serum. Blood was collected in the morning hours (8–11 AM) under sterile conditions from fasted patients by venipuncture. After clotting the blood for 1 hour at room temperature, the tubes were centrifuged at 1600 g for 15 min at 4°C. Serum was separated immediately and 3 mL was subjected to the centrifugation on Beckman centrifuge AN (Beckman Coulter, Inc., USA) at 16000 g for 60 min at 4°C. Obtained sediments were gently resuspended with micropipette in 0.5 mL of SPG and stored at −80°C. Vials were opened only in the laminar air flow safety cabinet for culture test and PCR analysis after confirmation of diagnosis of chlamydial infection.

2.2.2. Urogenital Swabs. Urethral and cervical swabs for bacteriological culture and DNA amplification were collected by qualified professionals using commercial swabs (Aptaca, China). Swabs were placed in the tubes containing RPMI-1640 medium with 5% FCS, amphotericin B (5 μg/mL), and gentamycin (4 μg/mL) and were frozen at −80°C until cultivation.

2.3. Reagents and Bacteria. All reagents were purchased from Sigma-Aldrich unless specifically mentioned otherwise. *C. trachomatis* strain L2/Bu434 kindly provided by Dr. P. Saikku (University of Oulu, Finland) was used as reference culture. The pathogen was propagated in *Mycoplasma*-free McCoy cells grown in RPMI-1640 medium supplemented with 2 mM L-glutamine (Invitrogen), 5% fetal bovine serum, 50 μg/mL gentamycin sulfate, and 1 mg cycloheximide/mL. Infectious elementary bodies were isolated from McCoy cells by sonication, washed in phosphate-buffered saline, purified by Renografin gradient centrifugation, and kept frozen at −80°C in SPG buffer (pH 7.2; 250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid).

2.4. Cell Culture Test

2.4.1. Urogenital Swabs. Thawed swabs were vortexed vigorously and centrifuged at 300 g for 3 min. Resulting supernatant was added to 24 well plates with subconfluent McCoy cells. The plates were centrifuged at 1600 g for 1 hour at 30°C and incubated for 2 h at 37°C in 5% CO_2. The medium was removed and replaced with fresh RPMI-1640 and plates were cultivated for 48 hours at 37°C in 5% CO_2. Infected cells were visualized by direct immunofluorescence with FITC-labeled antibodies against *C. trachomatis* MOMP (NearMedic Plus, Russia). The identity of propagating bacteria was confirmed by TaqMan—PCR conducted with DNA extracted from infected McCoy cells monolayers.

2.4.2. Serum. Tubes containing 0.5 mL of frozen serum sediments in SPG were thawed on ice, then 2.5 mL of RPMI-1640 supplemented with FCS, amphotericin B and, gentamycin was added to each tube. Resulting suspension was transferred to 3 wells of subconfluent monolayers of McCoy cells grown in 24 well plates. Plates were incubated and processed as described above for urogenital swabs. Remaining serum was used for DNA extraction and further PCR analysis.

C. trachomatis-specific IgG antibodies were evaluated by using *Chlamydia trachomatis*-IgG-ELISA plus medac commercial kit (Medac, Hamburg, Germany).

2.5. Immunofluorescence Staining. Infected McCoy cell monolayers were grown on coverslips in 24 well plates. After fixating with methanol and permeabilization with Triton X-100, cells were stained by direct immunofluorescence with FITC—conjugated monoclonal antibody against *C. trachomatis* MOMP (NearMedic Plus, Russia). Inclusion-containing cells were examined and photographed under Nikon Eclipse 50i fluorescent microscope at 1350x magnification.

2.6. Electron Microscopy. Thawed serum samples (10 mL) were spun at 16000 g for 60 min. Resulting pellets were analyzed by TaqMan PCR for *C. trachomatis* 16S rRNA. Positive specimens were fixed for 4 hours in phosphate buffer (pH 7.8) containing 5% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 hour, dehydrated in ethanol, and embedded in LR White resin (EMS, USA). Stained ultrathin sections (200–300 Å) were evaluated by electron microscopy using JEM-100B microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Purified EBs of *C. trachomatis* reference strain were used as positive control for electron microscopy studies. PCR-negative sediments of serum obtained from healthy volunteers served as negative control.

2.7. DNA Isolation. Extraction of total nucleic acids was conducted with IVD-labeled automated system NucliSENS easyMAG (BioMérieux Inc., The Netherlands). DNA was isolated from urogenital swaps, serum specimens, and McCoy cells' infected serum aliquots. Up to 24 samples were analyzed in one BioMérieux automated system run. All specimens with the adjusted volume of 1.0 mL were treated with 1 mL of BioMérieux lysis buffer. Loadings of samples, reagents, and disposables were the only manual steps during DNA extraction procedure using NucliSENS easyMAG platform. DNA was eluted from the cartridges with 50 μL of BioMérieux elution buffer.

2.8. Quantitative TaqMan PCR. For quantification purpose, real-time PCR for 16S rRNA and cryptic plasmid of *C. trachomatis* was conducted. PCR primers and TaqMan probe for 16S rRNA of *C. trachomatis* (GenBank accession number AM884176) and cryptic plasmid of *C. trachomatis* (GenBank accession number X06707.3) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). Designed primers and TaqMan probe were as follows: for 16S rRNA of *C. trachomatis* forward primer, 5′-GGC GTA TTT GGG CAT CCG AGT AAC G-3′; reverse primer, 5′-TCA AAT CCA GCG GGT ATT AAC CGC CT-3′; and TaqMan probe R6G-TGG CGG CCA ATC TCT CAA TCC GCC TAG A-BHQ2, and for cryptic plasmid of *C. trachomatis* forward primer, 5′-GGG ATT CCT GTA ACA ACA AGT CAG G-3′; reverse primer, 5′-CCT CTT CCC CAG AAC AAT AAG AAC AC-3′; and TaqMan probe ROX-CTC CCA GAG TAC TTC GTG CAA GCG CTT TGA-BHQ2. An additional BLAST search analysis was conducted to unsure specificity of the primers and probe. Real-time PCR was performed with the iCycler IQ system (Bio-Rad, USA). All samples were analyzed in triplicates.

Bacterial load in serum specimens and urogenital swabs is shown below in genome equivalents of per mL of serum or in genome equivalents of the pathogens referred to 10^5 copies of eukaryotic β-actin (swabs). Calibrator standards were prepared using amplified fragments of 16S rRNA and pLGV440 of *C. trachomatis*, or eukaryotic β-actin and cloning them into the pGEM-T plasmid vector (pVU56) using the TA cloning kit (Invitrogen, San Diego, CA, USA) similarly to Broccolo's protocol patients [20].

2.9. RNA Isolation. RNA was isolated from infected McCoy cells using Trizol protocol (Invitrogen, USA). RNA preparations were treated with RNase-free DNase RQ1 (Promega Corporation, USA) and resuspended in DEPC-treated water. RNA samples were confirmed to be DNA free by performing a PCR for selected *C. trachomatis* genes. The concentration of isolated RNA in samples was established by spectrophotometry.

2.10. Reverse Transcription and PCR. cDNA was synthesized using "Reverse Transcription System" (Promega Corporation, USA) with random primer. PCR has been performed using sets of primers for 4 *C. trachomatis* genes as follows:

groEL-forward 5′-TCTGCGAACGAAGGATATGA-3′ and reverse 5′-ATAGTCCATTCCTGCGCCAGG-3′;

ompl-forward 5′-CGTTCGTTGCAGACTTACCA-3′ and reverse 5′-GTTCCTCGCATACCGAATGT-3′;

pmpD-forward 5′-GTTAGACCAAATTCGAGATC-3′ and reverse 5′-AAGATTCTCCGTCACGAGGA-3′;

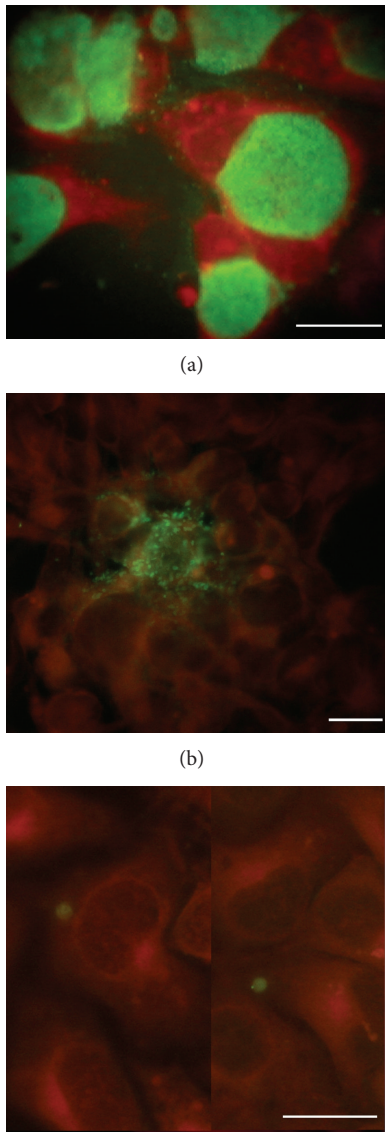
omcB-forward 5′-CTGCAACAGTATGCGCTTGT-3′ and reverse 5′-CACGCTGTCCAGAAGAATGA-3′.

PCR products were separated on 1.5% agarose gels using ethidium bromide staining. *C. trachomatis* L2/434/Bu was used as positive control.

3. Results and Discussion

As can be seen from Figure 1(a), 48-hour inclusions of *C. trachomatis* reference strain had irregular shape with granular or punctuate IF signal. Some inclusions were stained heterogeneously and contained distinct "empty" pockets that appeared to be deficient in immune-reactive substance. There were many small extracellular particles spread around individual cells which may be indicative of chlamydial inclusion burst.

Inoculation of urogenital swabs obtained from the patients with confirmed chlamydiosis into McCoy cell monolayers led to appearance of inclusions with very variable morphology. Besides typical inclusions, there were many inclusions with irregular borders and reduced intensity of IF



(a)

(b)

(c)

Figure 1: Chlamydial inclusion bodies in McCoy cells infected with reference strain (a), urogenital (b), and serum (c) isolates of *C. trachomatis*. 48 hpi. Bar marker 5 mkm.

signal. In some cells, inclusions retained granular and punctuate IF prototype even at 48 h of postinfection period (Figure 1(b)). Despite variable inclusion morphology, all urogenital swabs from 52 patients with chlamydiosis were culture positive. No specific IF staining has been seen with swab specimens collected in control group except some background signals originated apparently by contaminating blood constituents.

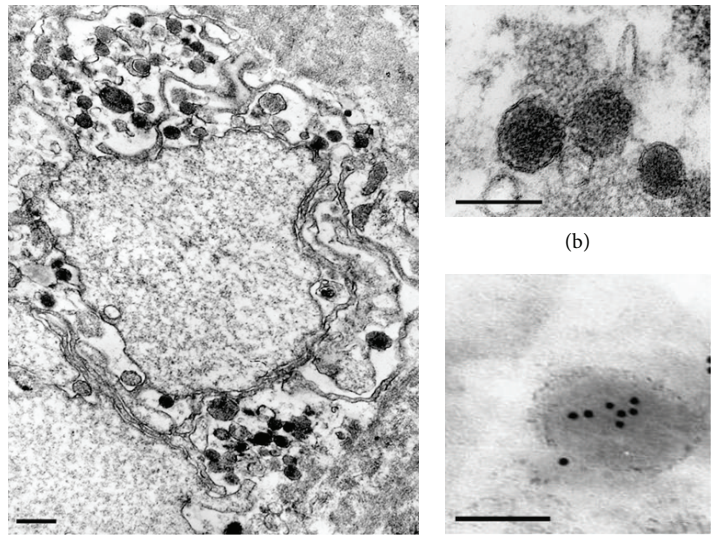
Next, we determined if serum sediments obtained from the same patients with confirmed chlamydiosis might contain infectious forms of *C. trachomatis*. We have found that thirty-one of the 52 patients had serum sediments infective to McCoy cells under conditions used. Immunostaining seen in 48 h of postinoculation had remarkably distinctive pattern (Figure 1(b)). Chlamydial inclusions were fewer in number, smaller, rounder, and more uniformly stained if compared to those seen in McCoy cells infected with reference strain or urogenital swabs (Figure 1). IF signal was commonly weaker and was best seen at 72 h of postinoculation. Most of the inclusions were intact with no extracellular particles extruded suggesting that infectious cycle was not lytic. Overall appearance of IF staining resembled that reported during persistent *C. trachomatis* infection *in vitro* [21].

Ten primary serum isolates sustained at least three serial passages in cell culture. In 9 cases, inclusion morphology remained principally unchanged indicating that these serum isolates had a stable phenotype. Only one serum isolate reversed morphological appearance by showing multiple punctuate pattern of inclusions with extracellular particles on the third passage.

The identity of infectants propagating in McCoy cells was also examined with nucleic acid amplification protocol. Genetic markers of *C. trachomatis* were detected in all 52 DNA specimens from McCoy cells infected with urogenital swabs of patients with chlamydiosis. However, when matching serum sediments were inoculated to McCoy cells, only 49 DNA specimens (out of 52) gave positive PCR readings. Therefore, nucleic acid amplification protocol showed higher detection rate (94.2%) of *C. trachomatis* in McCoy cell infected with serum sediments when compared to IF culture test (59.6%). Simultaneous detection of the pathogen in serum sediments by two methods took place in 30 patients only. Noteworthy that 1 serum specimen inoculated to McCoy cells gave positive IF signal whilst it was negative in PCR. On the other hand, 19 serum sediments which were negative by culture test had PCR-detectable chlamydial markers in the cell monolayers. Positive PCR findings were reproducible for any particular DNA specimen. No positive results were obtained in the control group. Amplicons from two PCR positive samples were sequenced. Full homology was established for chosen region of 16S rRNA gene among two isolates and two reference strains of *C. trachomatis* (L2/434/Bu and A/HAR-13).

The identity of serum isolates from urogenital patients was further evaluated with immunogold electron microscopy using *C. trachomatis* specific antibody. Duplicates of two serum specimens tested positively in culture test and PCR were chosen randomly for retrospective microscopic analysis. Both serum specimens from the patients with urogenital *C. trachomatis* infection contained typical elementary bodies with attached immunogold particles disclosing the identity of the pathogen (Figure 2).

Next, we decided to assess the viability of *C. trachomatis* primary serum isolates by direct comparison of bacterial DNA in inoculums and infected host cells after 48 hours of cultivation of the same volume. Five serum specimens yielding positive identification in culture test and PCR were selected for this purpose. As can be seen from Table 1, there is a significant difference in values of bacterial load measured in inoculums and McCoy cells in 48 hours of postinfection period suggesting effective pathogen propagation in the host cells.



(a) (b) (c)

Figure 2: Electron-microscopic images of *C. trachomatis* elementary bodies obtained from serum centrifugates ((a) and (b)). Bar marker 0.5 mkm. Immunogold labeling of *C. trachomatis* elementary bodies after preincubation with monoclonal antibody against chlamydial MOMP (c). Bar marker 0.1 mkm.

Table 1: Comparison of amounts of *C. trachomatis* genome equivalents (GEs), detected by direct isolation from the serum and after cultivation in cell culture using PCR.

Patient	*C. trachomatis* GEs in 1 mL of serum	*C. trachomatis* GEs isolated from cells after cultivation of 1 mL serum inoculum for 48 h
2	$2.54 * 10^2$	$1.36 * 10^4$
6	$8.21 * 10^2$	$2.54 * 10^4$
9	$4.21 * 10^2$	$4.41 * 10^4$
15	$2.78 * 10^2$	$3.27 * 10^4$
19	$3.05 * 10^2$	$9.21 * 10^4$

In order to reconfirm the viability of pathogen present in serum specimens, we also have been trying to identify chlamydial RNA transcripts in cell monolayer inoculated with serum sediments of the patients with urogenital infections and cultivated for 48 hours. Quantification attempts of *C. trachomatis* RNAs in these specimens were unsuccessful due to extremely high C_t values in TaqMan PCR which is attributable to low bacterial load. We examined the presence of 4 chlamydial transcripts by conventional PCR. Genetic markers of the early (*groEL*), middle (*omp1* and *pmpD*), and late (*omcB*) developmental cycle were chosen. RNA transcripts for *groEL*, *omp1*, and *pmpD* were detectable in total RNA obtained from McCoy cells after inoculation with serum sediments (Figure 3). However, RNA for *omcB* was undetectable in cultured serum isolates suggesting prolonged developmental cycle of serum-derived pathogen.

Next, we determined the possibility of direct detection of chlamydial DNA in patients serum. Using DNA isolation protocol from 1 mL of serum and quantitative TaqMan PCR, it has been shown that bacterial load in patients sera was 2×10^2-10^3 GE/mL.

We used the developed protocol for DNA detection in serum to examine the prevalence of *C. trachomatis* infection in patients with chronic urogenital and extragenital complications. Thirty-six patients with chronic pelvic inflammatory diseases, 33 patients with Reiter's disease, and 20 control subjects were examined by simultaneous detection of *C. trachomatis* DNA in urogenital swab and sera. In patients with chronic pelvic inflammatory diseases and a history of chlamydial infection, *C. trachomatis* DNA was detected in the serum of 61% of cases, while in the swabs pathogen was detected in only 17%. In patients with Reiter's disease, *C. trachomatis* DNA was detected in the serum of 64% and 30% in the swabs. In control group chlamydial DNA was found in 7% serum and in 3% swabs (Figure 4).

C. trachomatis is an obligate intracellular human pathogen accounting for most of the cases of STDs and preventable blindness in the world. Mucosal surfaces of urogenital system and conjunctivae are the primary target of the pathogen in the human body [2]. However, there is a growing body of evidence that chlamydial infection can spread far beyond mucosal membranes affecting some abdominal organs and joints [10]. Pathophysiological mechanisms of *C. trachomatis* infection generalisation remain obscure [3, 10]. In this paper, we report that viable elementary bodies of *C. trachomatis* can be detected in serum of the patients with newly diagnosed cases of urogenital chlamydial disease.

Firstly, immunogold electron microscopy and cell culture analysis revealed that 14000 g pellet fraction of serum specimens obtained from the patients contains elementary bodies of *C. trachomatis*.

Secondly, 59.6% of serum specimens were tested positively in IF culture test suggesting viability of bacteria.

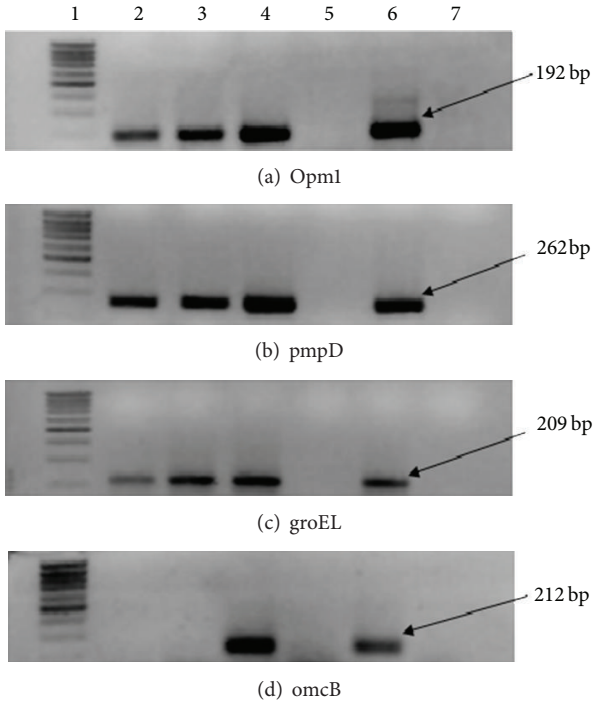


Figure 3: *C. trachomatis*-specific amplicons recovered in RT-PCR reactions with RNA isolated from serum isolates of the patients with urogenital chlamydiosis. RNA was isolated from infected McCoy and RT-PCR reaction performed as described in Section 2. A percentage of 1.2% agarose gel contains the following lanes: 1: size marker; 2 and 3: amplification products obtained in PCR with RNA isolated from serum of patient no. 1 and patient no. 2; 4: amplification product using RNA from reference strain L2/434/Bu; 5: RNA extraction control; 6: positive control; 7: negative control.

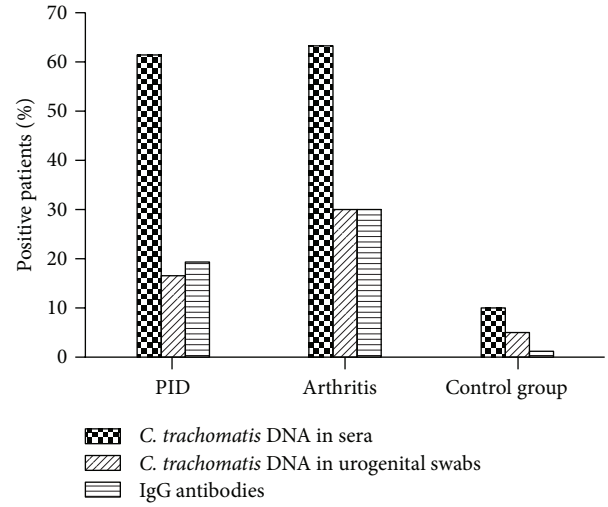


Figure 4: Patients with PID and Reiter's disease and control subjects were examined for presence of *C. trachomatis* DNA in serum and urogenital swab, and *C. trachomatis*-specific IgG antibodies as described in Section 2.

Inclusions originated in McCoy cells by inoculation of serum specimens were remarkably atypical. Inclusions were fewer in number, smaller, round-shaped and had reduced intensity of IF staining when compared side by side with reference strain of *C. trachomatis* propagating in host cells. However, culture test alone seems to underestimate the bacteraemia rate in urogenital patients. When PCR for 16S rRNA and cryptic plasmid of *C. trachomatis* was applied for culture test, estimated detection rate of the pathogen in serum was 94.2%. This finding itself reveals presence of viable pathogen in serum since extrinsic chlamydial DNA is unstable. Coincident detection of *C. trachomatis* in serum specimens by both methods (culturing with further PCR) took place in 57.7% of the patients with urogenital chlamydiosis. No specific IF staining or valid PCR readings with $C_t < 40$ were seen in serum specimens from control patients. Amplicon sequencing confirmed the identity of the pathogen propagating in McCoy cells after inoculation of serum sediments.

Thirdly, *C. trachomatis* serum isolates display reasonable replicative capacities. There was net increase of bacterial copy number in infected McCoy cells over incubation period when postincubation bacterial load values were compared to PCR readings in the inoculums. It is clear that *de novo* formed chlamydial particles rather than the carryover from the inoculum are responsible for such an increase. Convincing evidence comes from RNA analysis. Genetic markers of chlamydial developmental cycle (*groEL*, *omp1*, and *pmpD*) were expressed in the cultured cells after inoculation of serum specimens.

Direct analytical quantification of *C. trachomatis* genetic markers in serum specimens by nucleic acid amplification reactions needs to be addressed in further studies. Although there are detectable chlamydial target sequences in DNA specimens from plasma of the patients with urogenital chlamydiosis, high C_t readings undermine analytical value of the assay. In search for another target sequence, multiplexed format with two calibrators might be useful.

Therefore our data above suggests that serum specimens of the patients with urogenital chlamydiosis may contain viable forms of *C. trachomatis*. Although diagnostic evaluation of this finding needs to be assessed in further larger trials, our data already indicates a high level of prevalence of the detection of this bacteria in patients with chlamydial diseases. Moreover, we have recently published that another chlamydial species, *C. pneumonia*, can be detected in serum specimens of the patients with acute coronary syndrome [18]. It seems that the detectability, that is, presence of chlamydial pathogens in the serum, and their circulation in the blood, is quite a common feature attributable to different chlamydial diseases, indicating similarity in mechanisms of dissemination of these two pathogens. Epitheliotropism as well as ability to infect mononuclear cells and lymphocytes is another common feature shared by both pathogens in terms of pathogenesis of chlamydial disease.

Our study did not reveal precisely whether pathogen present in serum has originated from mononuclear cells or plasma. However, it becomes clear from our work that even early stages of chlamydial urogenital disease are accompanied by presence of culturally retrievable *C. trachomatis* in blood

predisposing to systemic dissemination of the pathogen in the human body.

4. Conclusions

It was demonstrated for the first time that *C. trachomatis* can, independently from blood cells, be disseminated by circulation which may lead to the development of chronic infection. Taken together, our data suggest that serum specimens of the patients with urogenital chlamydiosis contain viable forms, elementary bodies of *C. trachomatis*, capable of initiation of new infectious cycle in the host cells. The new methodological approach of detection of *C. trachomatis* in the serum of patients with complicated chlamydiosis could be important in the direct diagnosis of the infection regardless of its localization and for evaluation of the effectiveness of treatment based on quantitative estimation of bacterial load.

References

[1] G. I. Byrne, "*Chlamydia trachomatis* strains and virulence: rethinking links to infection prevalence and disease severity," *Journal of Infectious Diseases*, vol. 201, supplement 2, pp. S126–S133, 2010.

[2] R. S. Stephens, "The cellular paradigm of chlamydial pathogenesis," *Trends in Microbiology*, vol. 11, no. 1, pp. 44–51, 2003.

[3] T. Darville and T. J. Hiltke, "Pathogenesis of genital tract disease due to *Chlamydia trachomatis*," *Journal of Infectious Diseases*, vol. 201, supplement 2, pp. S114–S125, 2010.

[4] S. J. Rasmussen, L. Eckmann, A. J. Quayle et al., "Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis," *Journal of Clinical Investigation*, vol. 99, no. 1, pp. 77–87, 1997.

[5] K. A. Kelly, D. Wiley, E. Wiesmeier, M. Briskin, A. Butch, and T. Darville, "The combination of the gastrointestinal integrin ($\alpha 4\beta 7$) and selectin ligand enhances T-cell migration to the reproductive tract during infection with *Chlamydia trachomatis*," *American Journal of Reproductive Immunology*, vol. 61, no. 6, pp. 446–452, 2009.

[6] E. Manor and I. Sarov, "Fate of *C. trachomatis* in human monocytes and monocyte-derived macrophages," *Infection and Immunity*, vol. 54, no. 1, pp. 90–95, 1986.

[7] M. Maurin and D. Raoult, "Isolation in endothelial cell cultures of *Chlamydia trachomatis* LGV (Serovar L2) from a lymph node of a patient with suspected cat scratch disease," *Journal of Clinical Microbiology*, vol. 38, no. 6, pp. 2062–2064, 2000.

[8] A. Dautry-Varsat, A. Subtil, and T. Hackstadt, "Recent insights into the mechanisms of Chlamydia entry," *Cellular Microbiology*, vol. 7, no. 12, pp. 1714–1722, 2005.

[9] L. N. Pedersen, B. Herrmann, and J. K. Møller, "Typing *Chlamydia trachomatis*: from egg yolk to nanotechnology," *FEMS Immunology and Medical Microbiology*, vol. 55, no. 2, pp. 120–130, 2009.

[10] S. Baguley and P. Greenhouse, "Non-genital manifestations of *Chlamydia trachomatis*," *Clinical Medicine*, vol. 3, no. 3, pp. 206–208, 2003.

[11] M. Rihl, L. Köhler, A. Klos, and H. Zeidler, "Persistent infection of Chlamydia in reactive arthritis," *Annals of the Rheumatic Diseases*, vol. 65, no. 3, pp. 281–284, 2006.

[12] J. M. Shabot, G. D. Roark, and A. L. Truant, "*Chlamydia trachomatis* in the ascitic fluid of patients with chronic liver disease," *American Journal of Gastroenterology*, vol. 78, no. 5, pp. 291–294, 1983.

[13] J. M. Shabot, "*Chlamydia trachomatis* and ascites: going with the flow?" *Hepatology*, vol. 9, no. 3, pp. 505–506, 1989.

[14] M. Dan, L. D. J. Tyrrell, and G. Goldsand, "Isolation of *Chlamydia trachomatis* from the liver of a patient with prolonged fever," *Gut*, vol. 28, no. 11, pp. 1514–1516, 1987.

[15] B. Mesurolle, F. Mignon, J. H. Gagnon, and P. J. Pickhardt, "Fitz-hugh-curtis syndrome caused by *Chlamydia trachomatis*: atypical CT findings," *American Journal of Roentgenology*, vol. 182, no. 3, pp. 822–824, 2004.

[16] C. J. Chen, K. G. Wu, R. B. Tang, H. C. Yuan, W. J. Soong, and B. T. Hwang, "Characteristics of *Chlamydia trachomatis* infection in hospitalized infants with lower respiratory tract infection," *Journal of Microbiology, Immunology and Infection*, vol. 40, no. 3, pp. 255–259, 2007.

[17] J. B. Dureux, Canton Ph., and G. Roche, "Lymphogranuloma venereum affecting simultaneously cervical and inguinal lymph nodes," *Annales de Dermatologie et de Venereologie*, vol. 108, no. 6-7, pp. 523–529, 1981.

[18] I. M. Petyaev, N. A. Zigangirova, A. M. Petyaev et al., "Isolation of *Chlamydia pneumoniae* from serum samples of the patients with acute coronary syndrome," *International Journal of Medical Sciences*, vol. 7, no. 4, pp. 181–190, 2010.

[19] I. P. Pashko, N. A. Zigangirova, I. M. Petiaev et al., "Modern aspects of diagnostics of chronic chlamydiosis caused by persisting forms of Chlamydia," *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii*, vol. 4, pp. 89–93, 2009.

[20] F. Broccolo, G. Locatelli, L. Sarmati et al., "Calibrated real-time PCR assay for quantitation of human herpesvirus 8 DNA in biological fluids," *Journal of Clinical Microbiology*, vol. 40, no. 12, pp. 4652–4658, 2002.

[21] R. J. Hogan, S. A. Mathews, S. Mukhopadhyay, J. T. Summersgill, and P. Timms, "Chlamydial persistence: beyond the biphasic paradigm," *Infection and Immunity*, vol. 72, no. 4, pp. 1843–1855, 2004.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 209204, 8 pages
http://dx.doi.org/10.1155/2013/209204

Research Article

Diagnosis of Familial Wolf-Hirschhorn Syndrome due to a Paternal Cryptic Chromosomal Rearrangement by Conventional and Molecular Cytogenetic Techniques

Carlos A. Venegas-Vega,[1,2] Fernando Fernández-Ramírez,[1] Luis M. Zepeda,[1] Karem Nieto-Martínez,[2] Laura Gómez-Laguna,[1] Luz M. Garduño-Zarazúa,[1] Jaime Berumen,[2,3] Susana Kofman,[1,2] and Alicia Cervantes[1,2]

[1] *Servicio de Genética, Hospital General de México, Dr. Balmis No. 148, Colonia Doctores, 06726 México, DF, Mexico*
[2] *Facultad de Medicina, Universidad Nacional Autónoma de México, México, DF, Mexico*
[3] *Departamento de Medicina Genómica, Hospital General de México, Dr. Balmis No. 148, Colonia, Doctores, 06726 México, DF, Mexico*

Correspondence should be addressed to Alicia Cervantes; acervant@unam.mx

Received 26 October 2012; Accepted 13 December 2012

Academic Editor: Ozgur Cogulu

The use of conventional cytogenetic techniques in combination with fluorescent *in situ* hybridization (FISH) and single-nucleotide polymorphism (SNP) microarrays is necessary for the identification of cryptic rearrangements in the diagnosis of chromosomal syndromes. We report two siblings, a boy of 9 years and 9 months of age and his 7-years- and 5-month-old sister, with the classic Wolf-Hirschhorn syndrome (WHS) phenotype. Using high-resolution GTG- and NOR-banding karyotypes, as well as FISH analysis, we characterized a pure 4p deletion in both sibs and a balanced rearrangement in their father, consisting in an insertion of 4p material within a nucleolar organizing region of chromosome 15. Copy number variant (CNV) analysis using SNP arrays showed that both siblings have a similar size of 4p deletion (~6.5 Mb). Our results strongly support the need for conventional cytogenetic and FISH analysis, as well as high-density microarray mapping for the optimal characterization of the genetic imbalance in patients with WHS; parents must always be studied for recognizing cryptic balanced chromosomal rearrangements for an adequate genetic counseling.

1. Introduction

Current diagnosis of chromosomal syndromes should include a combination of conventional cytogenetic techniques with molecular cytogenetic methods, particularly fluorescent *in situ* hybridization (FISH), as well as modern genomic applications such as copy number variations (CNVs) analysis by single-nucleotide polymorphism (SNP) or comparative genomic hybridization (aCGH) microarray techniques. The laboratory methods employed to achieve an adequate diagnosis of a familial case of Wolf-Hirschhorn syndrome (WHS, OMIM194190) exemplifies how the conventional, molecular and genomic techniques are complementary and useful to provide an appropriate genetic counseling in chromosomal syndromes.

Wolf-Hirschhorn syndrome affects at least 1/50,000 newborns and presents a broad range of clinical manifestations. WHS is characterized by a typical craniofacial appearance, growth delay, mental retardation, hypotonia, and seizures [1]. In the majority of cases (50–60%), WHS is caused by "pure" *de novo* terminal or interstitial deletions in 4p16; unbalanced translocations (45%), either *de novo* or inherited from a balanced rearrangement (~15%), and other complex cytogenetic findings (>1%) such as a chromosome 4 ring, del(4p) mosaicism, or a duplication/deletion rearrangement derived from a chromosome 4 inversion [2, 3] have been observed as well. In a high proportion of the WHS patients (25–30%), the chromosomal abnormality is cryptic and not detectable by conventional cytogenetic techniques. In cases of clinical suspicion of WHS in a patient with normal karyotype,

Table 1: Phenotype traits of our patients with a 4p deletion of ~6.5 Mb compared with the frequencies of the main clinical features associated with 4p deletions of an average size between 5 and 18 Mb, from Zollino et al. [5].

	II-2	II-4	%
Sex	Male	Female	
Age at examination (years. Months)	9.9	7.5	
Preterm delivery (<38 weeks)	+	+	
Hypotonia	+	+	91
Mild/moderate mental retardation	−	−	24
Severe mental retardation	+	+	80
Seizures	+	+	80
Prenatal growth delay	+	+	84
Postnatal growth delay	+	+	91
Microcephaly	+	+	95
Typical facial dysmorphisms	+	+	100
Cranial asymmetry	+	+	
Round-broad face	+	+	
High-diffuse frontal hair line	+	+	
High forehead	+	+	
Prominent glabella	+	+	
Sparse eyebrows	+	+	
Long eyelashes	+	+	
Downslanting palpebral fissures	+	−	
Ptosis	$+^{L}$	−	
Exophthalmos	$+^{R}$	+	
Ocular coloboma	−	−	30
Strabismus	+	+	
Hypertelorism	+	+	
Broad nasal bridge	+	+	
Beaked nose	+	+	
Short nasal wings	+	+	
Short philtrum	+	+	
Prominent philtrum columns	+	+	
Downturned corners of mouth	+	+	
Cleft lip/palate	$+^{a}$	$+^{a}$	25
Oligodontia	+	+	
Micrognathia	+	+	
Prominent ears	+	+	
Low set and malformed ears	+	+	
Others			
Brain anomalies	$+^{b}$	$+^{b}$	
Hearing loss	+	+	
Congenital heart defects	$+^{c}$	$+^{d}$	52
Renal abnormalities	−	$+^{e}$	37
Hypospadias	−	NA	41
Skeletal anomalies	$+^{f}$	$+^{f}$	37
Sacral dimple	+	+	

Clinical findings: +: present; −: absent; R: right; L: left; NA: not applicable.
[a]Cleft palate.
[b]Cortical/subcortical atrophy, enlargement of lateral ventricles, and septum pellucidum agenesis.
[c]Ventricular septal defect and pulmonary stenosis.
[d]Atrial septal defect.
[e]Malrotation of left kidney.
[f]Hip dislocation.

additional FISH studies of the sub-telomeres and the WHS critical region (WHSCR) must be performed [4].

The high degree of variation in the clinical presentation of WHS has been attributed to differences in the size of the 4p deletion, the presence of a partial trisomy from the segregation of a chromosomal translocation or inversion, allelic differences or multifactorial inheritance [2–5]. The majority of familial cases have been associated with parental chromosomal balanced translocations, particularly t(4p; 8p), which represents a distinct genetic entity [2, 5, 6]. Chromosomes 7p, 11p, 12p, and Dp/Gp, have also frequently been implicated in 4p inherited or *de novo* rearrangements [2, 5, 7–10]. We describe two sibs with a classic WHS phenotype and 4p16.1-p16.3 deletions (~6.5 Mb) due to the segregation of a paternal balanced rearrangement, characterized by karyotyping, FISH, and microarray copy-number analysis.

2. Materials and Methods

2.1. Clinical Report. The family pedigree is shown in Figure 1. The parents were a healthy, young, non-consanguineous couple. II:1 is a healthy 11-year old girl. The propositus (II:2) is a 9 years and 9 months of age boy, born at 37.5 gestation weeks by cesarean section due to fetal distress; birth weight: 2,125 g, height 43 cm (both <3rd centile). Hypotonia was noted at birth. At 7 months of age, the patient developed generalized tonic-clonic seizures. Clinical examination at 9 years and 9 months of age revealed psychomotor retardation; height 124 cm, weight 15 kg, and OFC 45.3 (all <3rd centile). He displayed facial features typical of WHS (Figure 1(a)). Psychological examination by WISC-R revealed a global IQ of 25. II:4 is a 7-years and 5-month old girl, born at 37.2 weeks by cesarean section, birth weight 2,100 g and height 42 cm (both <3rd centile). She showed clinical findings similar to those of her brother (Figure 1(b)), and renal ultrasound reported left kidney malrotation. Her global IQ was 30. The clinical features of both patients are described in Table 1. Initial conventional cytogenetic analysis by GTG banding (400–700 bands) revealed a 4p16 deletion in both sibs, suggesting a parental chromosomal balanced rearrangement.

2.2. Cytogenetic and FISH Analysis. Chromosome analyses on lymphocytes by GTG (400–700 bands) and NOR banding were performed according to standard protocols. FISH was performed using LSI WHSCR1 Spectrum Orange and CEP 4 Spectrum Green probes and ToTelVysion Mixtures number 4 (4p Spectrum Green, 4q Spectrum Orange, 21q Spectrum Green/Orange, and LSI AML1 Spectrum Aqua) and number 10 (10p Spectrum Green, 10q Spectrum Red, 15q Spectrum Green/Orange and LSI PML Spectrum Aqua) from Vysis Abbot, Inc. (Abbot Park, IL, USA), according to the procedures described by the manufacturer.

2.3. Microarray Analysis. High purity genomic DNA was extracted from 3 mL whole blood using the Versagene DNA Purification kit (Gentra Systems Inc., Minneapolis, MN, USA). Genomic mapping was performed on the affected sibs and parents using the Genome-wide human SNP array 5.0 set (Affymetrix Inc., Santa Clara, CA, USA), according to the protocol supplied by the manufacturer. Genotyping Console 4.1 (Affymetrix Inc.) was used for quality assessment and genotyping of the data. The QC call rate by the BRLMM-P algorithm was over 93%. CNV analysis was performed using SNP & Variation Suite 7.5.6 software (Golden Helix Inc., Bozeman, MT, USA). Patients' data were normalized against a reference set generated in our laboratory, consisting of 71 healthy subjects including the patients' parents. The copy number analysis method (CNAM) was used to identify the CNV segments with a moving window of 10,000 markers in a univariate basis. Mapping was carried out based on the human genome assembly Feb 2009 (GRCh 37/hg19) (NCBI Reference Sequence (RefSeq) http://www.ncbi.nlm.nih.gov/RefSeq/).

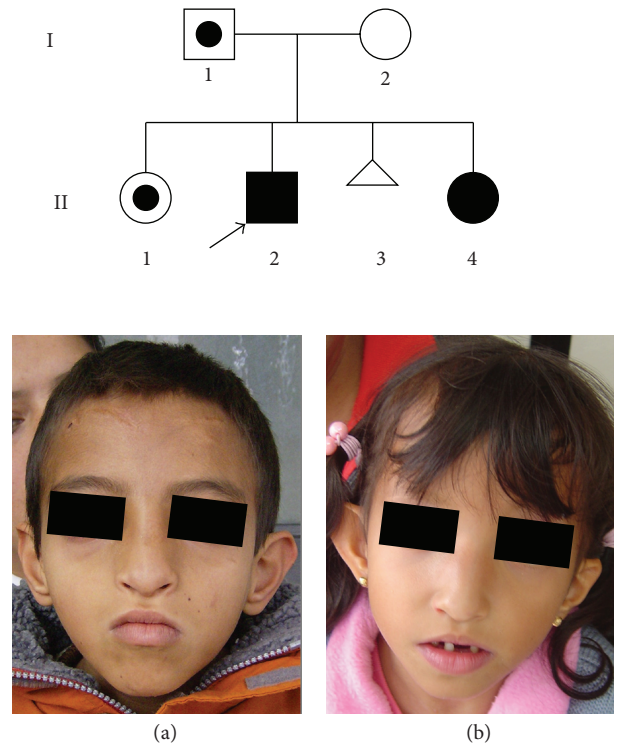


Figure 1: Family pedigree and patient profiles: (a) II.2 at the age of 9 years 9 months; (b) II.4 at the age of 7 years 5 months. Both patients exhibited typical WHS phenotypes.

3. Results

3.1. Cytogenetic and FISH Analysis. High-resolution GTG banding on the affected children revealed a 4p16.1 deletion (Figures 2(a) and 2(b)). FISH using WHSCR1 and 4p subtelomeric probes confirmed the loss of both sequences (Figures 2(g) and 2(h)). The mother's karyotype was normal, while the phenotypically normal father carries a derivative chromosome 4 and an apparent heteromorphism in both chromosomes 15 (Figure 2(c)). FISH using ToTelVysion Mixtures 4 and 10 showed that 4p subtelomeric signal was located on the short arm of one chromosome 15

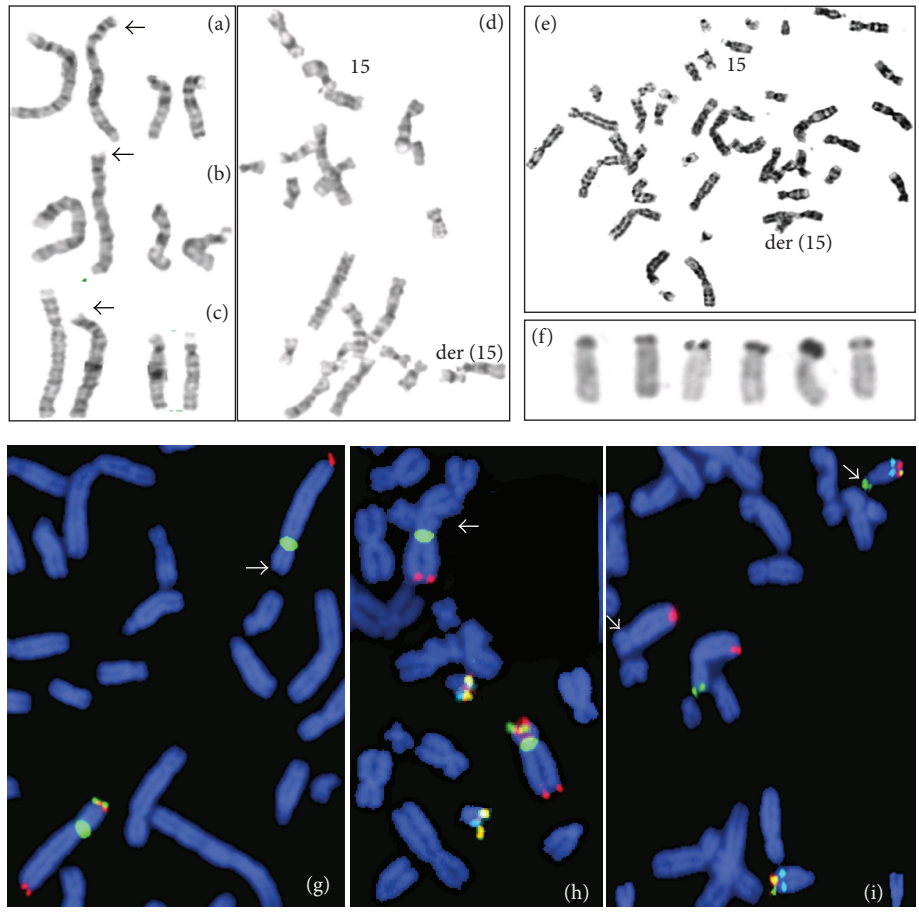


Figure 2: Partial karyotypes from the family. (a) II:2, (b) II:4, and (c) I:1: chromosomes 4 and 15 with GTG banding. (d) and (e) Partial metaphases from the father showing chromosome 15 and der(15) associated with acrocentric chromosomes. (f) Group D metaphase chromosomes from the father demonstrating active Ag-NOR in all chromosomes, including der(15). (g) II:2 and (h) II:4 FISH with LSI WHSCR1 (orange), subtelomeric 4p (green) probes and controls CEP 4 (green), 4q subtelomeric (orange), 21q (orange/green) and LSI AML1 (aqua), showing the absence of both 4p signals on one chromosome 4. (i) I:2 (father) FISH with ToTelVysion mixtures 4 and 10, showing a green 4p subtelomeric signal on 15p.

(Figure 2(i)). Ag-NOR banding was negative on 4p, and no association of der(4) with acrocentric chromosomes was observed; der(15) was positive for Ag-NOR and acrocentric association (Figures 2(d), 2(e) and 2(f)) confirming an insertion from 4p to 15p. The other chromosome 15 showed an increased stalk on its short arms (Figures 2(e) and 2(f)). Both affected children inherited this chromosome 15pstk+ (Figures 2(a) and 2(b)). The father's final karyotype was 46,XY,ins(15;4)(p12;p16.1p16.3).ish ins(15;4) (D4S3359+,PML+,D15S936+; D4S3359-,D4S2930+). II: 1 inherited the same balanced rearrangement from her father (data not shown).

3.2. Microarray Analysis. CNV analysis confirmed a similar 4p deletion in both siblings, 6.48 Mb in the propositus, and 6.50 Mb in his affected sister. The minimal deletion positions were from nt.69,535 to 6,546,304 and from nt.58,388 to 6,560,313, respectively (Figure 3(a)). These include WHSCR and WHSCR2. The telomeric break points affected the *ZNF718* and *ZNF595* genes in both sibs; however an 11.1 Kb difference was observed between these (Figure 3(b)). The distal region of 4p is highly variable (database of Genomic Variants http://projects.tcag.ca/variation/), and the children inherited a different chromosome 4 from their mother, as documented by the SNP genotyping analysis (data not shown). The centromeric break point differs by 14 Kb between sibs, and in both cases the gene *MAN2B2* maps outside the deletion, at least 15.2 Kb from its start point (Figure 3(c)). Acrocentric p arms are not represented in the 5.0 SNP array.

4. Discussion

Three clinical categories of WHS have been defined according to the size of the 4p deletion: (1) <3.5 Mb, linked to a mild form, (2) between 5 and 18 Mb, associated with the classical phenotype observed in our patients, and (3) >22 Mb, causing a severe form [5]. The pathogenesis of WHS is multigenic, and genotype-phenotype correlation studies may clarify the role of specific genes on 4p in the disease etiology [2, 4]. CNV analysis in our patients revealed a similar 4p

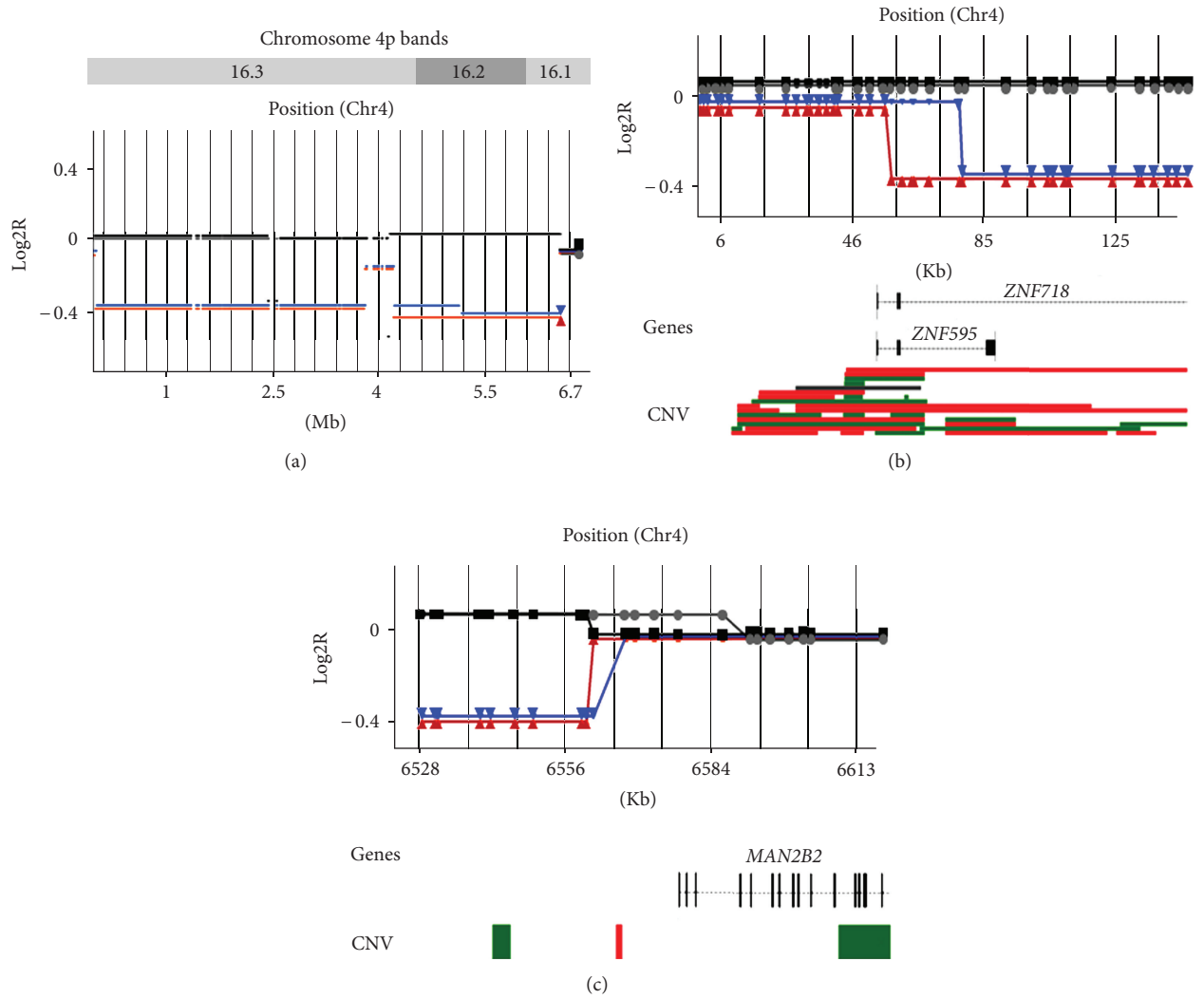


Figure 3: (a) Deletion involving chromosome bands 4p16.1-p16.3 was confirmed by microarray mapping of the propositus (▼), his younger sister (▲) and both parents (father (■) and mother (•)). (b) The affected patients display differential telomeric break points, which occur at a variable region including genes *ZNF718* and *ZNF595*. (c) The centromeric break points in both patients were located >15 kb upstream of the *MAN2B2* transcriptional start site (pos. 6576902). Gene (RefSeq) and CNV (DGV) annotation maps are shown below. CNV gain regions are indicated in red, losses in green, and gain/losses in gray. Log2R, logarithmic value of the sample to reference ratio.

deletion of ~6.5 Mb, with the common deleted segment spanning from 69,535 Kb to 6,546,304 Mb, that is, 4p16.1 to 4p16.3 (Figure 3(a)). The deletion affects at least 70 genes, including the 200 kb critical region for the typical WHS phenotype [11], and the candidate genes *LETM1, FGFRL1* and *WHSC1*, which have been associated with seizures, some facial findings, distinctive facial features and growth delay, respectively [4, 12]. The genes *ATP5I, FGFR3, HTT, MSX1* and *PPP2R2C* are likely haploinsufficient (Decipher database http://decipher.sanger.ac.uk), and could also be relevant to the WHS phenotype.

Different types of chromosomal rearrangements are associated with WHS; among these, inherited unbalanced translocations are frequently maternal, while *de novo* unbalanced translocations are usually paternal, with the exception of the t(4;8) [2, 5, 13]. In our patients, we identified an isolated 4p deletion due to a paternal balanced insertion (Figure 4). To our knowledge, this rearrangement has not been previously reported in WHS.

Recently, it has been suggested that chromosomal insertions are more frequent (1 : 500) [14–16] than previously reported (1 : 80000) [17]. These rearrangements involve three chromosome breakage events that can be intra- or interchromosomal. The use of FISH to confirm deletions and/or duplications detected by microarrays showed that these genomic imbalances resulted from the segregation of a parentally balanced insertion [14, 15]. Interestingly, the short arms of acrocentric chromosomes are frequently involved in these rearrangements, especially the NOR of chromosomes 15 and 22, and they are often cryptic when present in an unbalanced form [14]. Genomic studies have demonstrated that different gene families and certain satellite repeats, like the olfactory

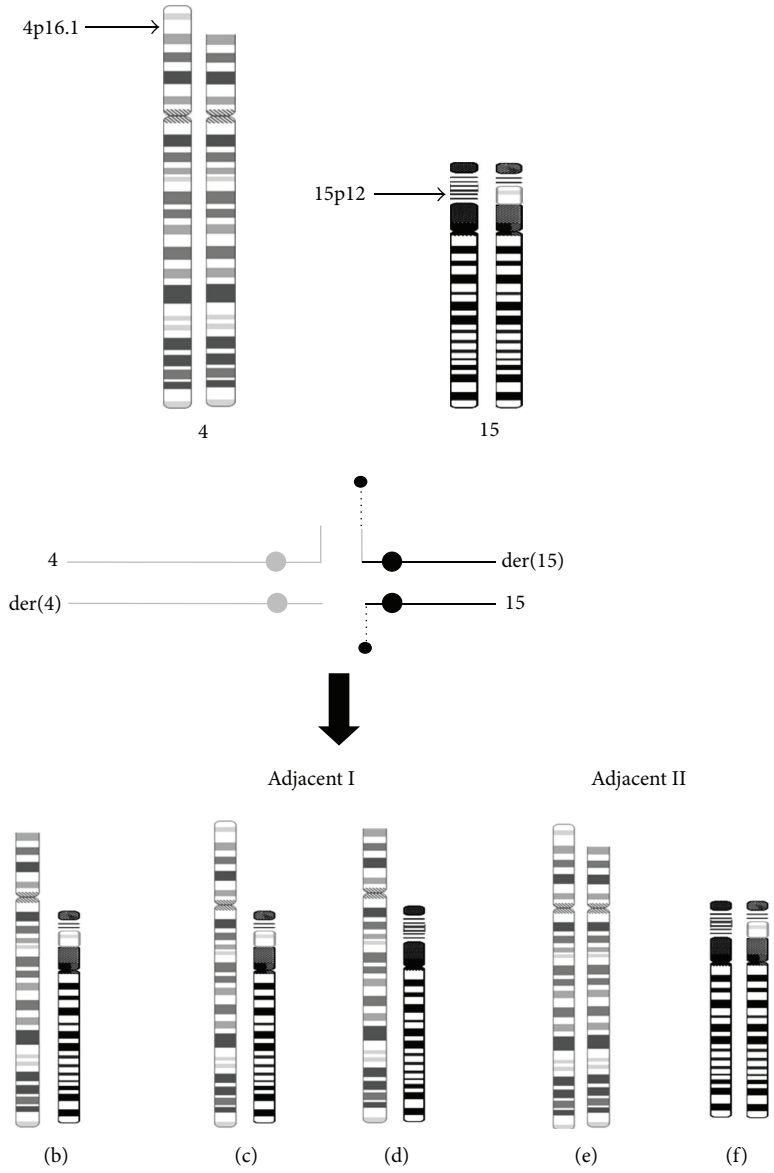


Figure 4: Chromosomes 4 and 15 ideograms showing the paternal insertion and its meiotic segregation. The affected siblings were the product of an adjacent I segregation.

receptor gene family and the terminal 4p repeats, constitute nucleolus-associated chromatin domains that interact with the satellite repeats and rDNA of acrocentric chromosomes [18, 19]. This could explain the high frequency of acrocentric chromosomal rearrangements with different partners. Recently some WHS rearrangements have been recognized to involve a translocation between the NOR of an acrocentric chromosome and chromosome 4, producing a satellited 4p chromosome. Some of these cases are sporadic and other familial [2, 7, 10, 20]. Our patients are the product of an adjacent I segregation from the paternal insertion, and the nonaffected girl received both derivative chromosomes by alternate segregation (Figure 4). Wu et al. [10] reported a family with coexisting sibs, which are the products of both types of gametes from an adjacent I segregation: one with a 4p deletion of 5 Mb and classical WHS and the other with a pure duplication of the same region of 4p.

Only few cases of familiar recurrence of WHS have been described and they are usually associated with a balanced chromosomal translocation in one parent. Nevertheless, the WHS phenotype is modified by the trisomy of other chromosomal region [5, 13, 21, 22]. One instance of a familial recurrence of a 4p pure deletion was due to a meiotic amplification of a maternal 1.5 Mb deletion. The mother had mild WHS, while her two affected sons displayed a typical phenotype. One of the sons was studied and revealed a 2.8 Mb deletion [23]. Another case of two sibs showing a mild form of WHS were reported to have a pure 4p deletion of 2.8

Mb, from a mother with a karyotype 46,XX,t(4;14)(p16.3;p12) [20]. Our patients also have a pure 4p terminal deletion of 6.5 Mb associated with classical WHS phenotype due to a father ins(15;4)(p12;p16.1p16.3); however, only minor phenotype differences were observed between sibs in both families. The differences in the severity of the phenotypes in these two familiar cases could be result of the size of the chromosomal deleted region as has been suggested [5].

The small difference in the size of the deleted material, 25 Kb, among our propositus and his affected sister could be attributed to a maternal polymorphism, recombination aneusomy, or microarray data normalization. The most striking clinical differences between sibs were the type of cardiac defect, the presence of downslanting palpebral fissures and ptosis only in the boy, and kidney malrotation present only in the girl. Comparing the clinical data of our patients with the data reported by Zollino et al. [5] in patients with classical WHS phenotype and deletions between 5 and 18 Mb (Table 1), the only major differences were the absence of ocular coloboma and hypospadias. Until now, few WHS patients had been studied by genomic high-resolution methods [1, 2, 4, 10, 14, 20]. As the number of these studies increases, a better determination of the exact size of deletions will be achieved, improving the definition of the regions and genes implicated in each phenotypic trait associated with the classical WHS.

5. Conclusions

The clinical variability in our classical WHS patients could be explained by polymorphisms in the 4p alleles present plus multifactorial inheritance patterns.

Our results reinforce the importance of thorough clinical diagnosis, as well as conventional and molecular karyotyping of patients and their parents for proper genetic diagnosis and counseling. Particularly, the use of high-density SNP arrays for CNV analysis in the patients enables the determination of the size of the deletion with higher precision and can detect cryptic partial trisomies. In order to give an adequate genetic counseling, the parents of a child with a 4p deletion should always be studied by FISH with subtelomeric 4p and WHSCR1 specific probes, to corroborate if they are carriers of a cryptic balanced rearrangement. In conclusion, we identified a novel type of chromosome rearrangement involved in sibs recurrent classical WHS, and its mechanism is apparently more frequent than previously thought. This case demonstrates the importance of the combined application of classical and molecular techniques to clarify chromosomal structural rearrangements.

Conflict of Interest

The authors declare no conflict of interests and state that none of them have any financial relation with the commercial identities mentioned in this work.

Acknowledgment

The authors thank the family for their participation in the study and CONACyT (2006-C01-13947).

References

[1] A. Battaglia, T. Filippi, and J. C. Carey, "Update on the clinical features and natural history of Wolf-Hirschhorn (4p-) syndrome: experience with 87 patients and recommendations for routine health supervision," *American Journal of Medical Genetics C*, vol. 148, no. 4, pp. 246–251, 2008.

[2] S. T. South, H. Whitby, A. Battaglia, J. C. Carey, and A. R. Brothman, "Comprehensive analysis of Wolf-Hirschhorn syndrome using array CGH indicates a high prevalence of translocations," *European Journal of Human Genetics*, vol. 16, no. 1, pp. 45–52, 2008.

[3] A. Battaglia, J. C. Carey, S. T. South, and T. J. Wright, "Wolf Hirschhorn syndrome," in *GeneReviews [Internet]*, R. A. Pagon, T. D. Bird, C. R. Dolan, and K. Stephens, Eds., Seattle University of Washington, Seattle, Wash, USA, 2007.

[4] N. M. C. Maas, G. Van Buggenhout, F. Hannes et al., "Genotype–phenotype correlation in 21 patients with Wolf–Hirschhorn syndrome using high resolution array comparative genome hybridisation (CGH)," *Journal of Medical Genetics*, vol. 45, no. 2, pp. 71–80, 2008.

[5] M. Zollino, M. Murdolo, G. Marangi et al., "On the nosology and pathogenesis of Wolf-Hirschhorn syndrome: genotype-phenotype correlation analysis of 80 patients and literature review," *American Journal of Medical Genetics C*, vol. 148, no. 4, pp. 257–269, 2008.

[6] D. Wieczorek, M. Krause, F. Majewski et al., "Unexpected high frequency of de novo unbalanced translocations in patients with Wolf-Hirschhorn syndrome (WHS) [7]," *Journal of Medical Genetics*, vol. 37, no. 10, pp. 798–804, 2000.

[7] J. B. Ravnan, J. H. Tepperberg, P. Papenhausen et al., "Subtelomere FISH analysis of 11 688 cases: an evaluation of the frequency and pattern of subtelomere rearrangements in individuals with developmental disabilities," *Journal of Medical Genetics*, vol. 43, no. 6, pp. 478–489, 2006.

[8] S. T. South, H. Whitby, T. Maxwell, E. Aston, A. R. Brothman, and J. C. Carey, "Co-occurrence of 4p16.3 deletions with both paternal and maternal duplications of 11p15: modification of the Wolf-Hirschhorn syndrome phenotype by genetic alterations predicted to result in either a Beckwith-Wiedemann or Russell-Silver phenotype," *American Journal of Medical Genetics A*, vol. 146, no. 20, pp. 2691–2697, 2008.

[9] Z. Ou, P. Stankiewicz, Z. Xia et al., "Observation and prediction of recurrent human translocations mediated by NAHR between nonhomologous chromosomes," *Genome Research*, vol. 21, no. 1, pp. 33–46, 2011.

[10] L. Wu, D. Meng, Z. Zhou, J. Du, Z. Long, and D. Liang, "A family with partial duplication/deletion 4p due to a balanced t (4; 15) (p16.2; p11.2) translocation," *American Journal of Medical Genetics A*, vol. 155, no. 3, pp. 656–659, 2011.

[11] M. Zollino, R. Lecce, R. Fischetto et al., "Mapping the Wolf-Hirschhorn syndrome phenotype outside the currently accepted WHS critical region and defining a new critical region, WHSCR-2," *American Journal of Human Genetics*, vol. 72, no. 3, pp. 590–597, 2003.

[12] H. Engbers, J. J. van der Smagt, R. van't Slot, J. R. Vermeesch, R. Hochstenbach, and M. Poot, "Wolf-Hirschhorn syndrome facial dysmorphic features in a patient with a terminal 4p16.3 deletion telomeric to the WHSCR and WHSCR 2 regions," *European Journal of Human Genetics*, vol. 17, no. 1, pp. 129–132, 2009.

[13] M. Zollino, R. Lecce, A. Selicorni et al., "A double cryptic chromosome imbalance is an important factor to explain phenotypic variability in Wolf-Hirschhorn syndrome," *European Journal of Human Genetics*, vol. 12, no. 10, pp. 797–804, 2004.

[14] S. H. L. Kang, C. Shaw, Z. Ou et al., "Insertional translocation detected using FISH confirmation of array-comparative genomic hybridization (aCGH) results," *American Journal of Medical Genetics A*, vol. 152, no. 5, pp. 1111–1126, 2010.

[15] N. J. Neill, B. C. Ballif, A. N. Lamb et al., "Recurrence, submicroscopic complexity, and potential clinical relevance of copy gains detected by array CGH that are shown to be unbalanced insertions by FISH," *Genome Research*, vol. 21, no. 4, pp. 535–544, 2011.

[16] B. A. Nowakowska, N. de Leeuw, C. A. Ruivenkamp et al., "Parental insertional balanced translocations are an important cause of apparently de novo CNVs in patients with developmental anomalies," *European Journal of Human Genetics*, vol. 20, no. 2, pp. 166–170, 2012.

[17] J. O. Van Hemel and H. J. Eussen, "Interchromosomal insertions: identification of five cases and a review," *Human Genetics*, vol. 107, no. 5, pp. 415–432, 2000.

[18] A. Nmeth, A. Conesa, J. Santoyo-Lopez et al., "Initial genomics of the human nucleolus," *PloS Genetics*, vol. 6, no. 3, article e1000899, 2010.

[19] S. Van Koningsbruggen, M. Gierliński, P. Schofield et al., "High-resolution whole-genome sequencing reveals that specific chromatin domains from most human chromosomes associate with nucleoli," *Molecular Biology of the Cell*, vol. 21, no. 21, pp. 3735–3748, 2010.

[20] D. Liang, Z. Zhou, D. Meng et al., "Three patients with Wolf-Hirschhorn syndrome carrying a satellited chromosome 4p," *Birth Defects Research A*, vol. 94, no. 7, pp. 549–552, 2012.

[21] J. Goodship, A. Curtis, I. Cross et al., "A submicroscopic translocation, t(4;10), responsible for recurrent Wolf-Hirschhorn syndrome identified by allele loss and fluorescent in situ hybridisation," *Journal of Medical Genetics*, vol. 29, no. 7, pp. 451–454, 1992.

[22] E. Reid, N. Morrison, L. Barron et al., "Familial Wolf-Hirschhorn syndrome resulting from a cryptic translocation: a clinical and molecular study," *Journal of Medical Genetics*, vol. 33, no. 3, pp. 197–202, 1996.

[23] F. Faravelli, M. Murdolo, G. Marangi, F. D. Bricarelli, M. D. Rocco, and M. Zollino, "Mother to son amplification of a small subtelomeric deletion: a new mechanism of familial recurrence in microdeletion syndromes," *American Journal of Medical Genetics A*, vol. 143, no. 11, pp. 1169–1173, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 739010, 8 pages
http://dx.doi.org/10.1155/2013/739010

Research Article

The Italian National External Quality Assessment Program in Molecular Genetic Testing: Results of the VII Round (2010-2011)

F. Censi,[1] F. Tosto,[1] G. Floridia,[1] M. Marra,[1] M. Salvatore,[1] A. M. Baffico,[2] M. Grasso,[2] M. A. Melis,[3] E. Pelo,[4] P. Radice,[5] A. Ravani,[6] C. Rosatelli,[7] N. Resta,[8] S. Russo,[9] M. Seia,[10] L. Varesco,[11] V. Falbo,[1] and D. Taruscio[1]

[1] *National Centre of Rare Diseases, Istituto Superiore di Sanità, 00161 Rome, Italy*
[2] *E. O. Ospedali Galliera, S.C. Laboratorio di Genetica, 16128 Genova, Italy*
[3] *Dipartimento di Scienze Biomediche e Biotecnologie, Università di Cagliari, 09121 Cagliari, Italy*
[4] *SOD Diagnostica, AOU Careggi, 50134 Firenze, Italy*
[5] *Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Preventive and Predictive Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy*
[6] *Department of Reproduction and Growth, Operative Unit of Medical Genetics, University Hospital S. Anna, Ferrara, Italy*
[7] *Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, 09121 Cagliari, Italy*
[8] *Dipartimento di Scienze Biomediche ed Oncologia Umana, University of Bari, 70124 Bari, Italy*
[9] *Laboratory of Molecular Genetics, Istituto Auxologico Italiano, Cusano Milanino, 20135 Milano, Italy*
[10] *Laboratorio di Genetica Medica, Fondazione IRCCS Policlinico Ca' Granda Ospedale, Milano, Italy*
[11] *Unit of Hereditary Cancer, Department of Epidemiology, Prevention and Special Functions, Istituto Nazionale per la Ricerca sul Cancro (IST), Genova, Italy*

Correspondence should be addressed to D. Taruscio; domenica.taruscio@iss.it

Received 26 October 2012; Revised 21 December 2012; Accepted 28 December 2012

Academic Editor: Ozgur Cogulu

Since 2001 the Istituto Superiore di Sanità established a quality assurance programme for molecular genetic testing that covers four pathologies: Cystic Fibrosis (CF), Beta Thalassemia (BT), Fragile X Syndrome (FX), and Familial Adenomatous Polyposis Coli (APC). Since 2009 this activity is an institutional activity and participation is open to both public and private laboratories. Seven rounds have been performed until now and the eighth is in progress. Laboratories receive 4 DNA samples with mock clinical indications. They analyze the samples using their routine procedures. A panel of assessors review the raw data and the reports; all data are managed through a web utility. In 2010 the number of participants was 43, 17, 15, 5 for CF, BT, FX, APC schemes respectively. Genotyping results were correct in 96%, 98.5%, 100%, and 100% of CF, BT, FX, and APC samples, respectively. Interpretation was correct in 74%, 91%, 88%, and 60% of CF, BT, FX, and APC reports, respectively; however in most of them it was not complete but a referral to genetic counseling was given. Reports were satisfactory in more than 60% of samples in all schemes. This work presents the 2010 results in detail comparing our data with those from other European schemes.

1. Introduction

Since the human genome sequencing was completed, the number of diseases for which genetic tests are available has grown rapidly (2500 diseases for which genetic tests were available in 2011—http://www.ncbi.nlm.nih.gov/projects/GeneTests/static/whatsnew/labdirgrowth.shtml).

Genetic tests are unique in their kind; they are performed only once in the life of a patient because their outcome never changes. Therefore an error may have harmful consequences on the choice of clinical/therapeutic planning and can significantly affect the life choices of the patients and their family.

Laboratories that perform genetic tests are required to work to very high quality standards; monitoring such laboratories is an obligation for the National Health System as part of its mandate to protect the health and quality of life of citizens.

The role of External Quality Assessments (EQA) in ensuring good laboratory practice is recognized at national and international level [1–5]. EQA schemes are the main tools for measuring the quality of laboratory results, for maintaining confidence in molecular genetic tests, and for implementing the standards of quality assurance [6–9]. A number of initiatives were taken internationally to improve quality in genetic testing services, for example, Cystic Fibrosis Quality Network and EMQN in Europe, and CAP in the USA [10, 11].

In 2001 the Italian National Centre for Rare Diseases of the Istituto Superiore di Sanità (ISS, Rome) established EQA schemes for both molecular genetic testing and classical cytogenetic. In particular, for molecular genetic testing, it offers specific EQA schemes for 4 diseases: Cystic Fibrosis (*CFTR* gene) (CF), Beta Thalassemia (*HBB* gene) (BT), Fragile X-Syndrome (*FMR1* gene) (FX), and Familial Adenomatous Polyposis Coli (*APC* gene) (APC) [12].

The Italian EQA (IEQA) has primarily an educational role and it aims to improve the quality of genetic tests used in clinical practice [12].

Until now seven rounds have been completed and overall 91 different laboratories have been monitored in the context of the (IEQA). National experts have assessed laboratory performance on genotyping, interpretation, and reporting of test results for a total number of 3158 samples.

A web utility was developed in 2008 to support this activity; it represents a computer interface, among the ISS, the laboratories, and the assessors, that facilitates communication, simplifies data archiving, and minimizes paper usage.

In 2009 (VII round, 2010) the activity was published in the Official Bulletin of the Italian Republic [13]. Participation to IEQA is voluntary and open to both public and private laboratories; laboratories pay a fee to participate.

During the first six rounds the assessment focused in particular on genotyping results and on completeness of reports to evaluate technical ability and harmonize reports among Italian laboratories. Results show that genotyping is in general of good quality whereas reporting presents much larger variations between laboratories and generally a lack of information [14–17]. In the seventh round assessors focused their attention on the ability of laboratories to accurately detect mutations and, in particular, on the ability to interpret the results.

In this work we describe the results of the seventh round of IEQA.

2. Materials and Methods

2.1. Organization of the IEQA. IEQA is organized and coordinated by the National Centre of Rare Diseases of the ISS [12]. Participation is open to both public and private laboratories. Since 2008 the activity is supported by the web utility.

Schemes are strictly anonymous and the identity of laboratories is known only to the ISS. The IEQA scheme organizer and national experts provide advice on the scientific context of the scheme and take decisions and educational actions for the development of the program.

In every scheme ISS provides 4 validated samples of genomic DNA for each round; all samples are distributed with mock data identifications, mock clinical information, and technical data.

Laboratories are asked to test samples using their routine protocols and to provide results of genotyping (raw data) and a full interpretative report in their normal laboratory style by a given deadline: 60 days for CF, BT, and FX; 90 days for APC.

Laboratory results are evaluated by assessors according to established criteria and results are available to laboratories in the reserved area on the web utility.

2.2. Sample Collection and Validation. The genomic DNA samples were obtained from peripheral blood and lymphoblastoid cell lines collected, respectively, by a clinical hospital (Presidio Ospedaliero Microcitemico, Cagliari) (only for the BT scheme) and by biobanks (Galliera Genetic Bank, Genova, and Coriell Cell Repository, Camden, NJ). Two independent working units in the ISS were responsible for DNA samples processing and validation [12].

Mutations carried by samples are validated in the ISS by routine methods: (1) direct sequencing for APC [17]; (2) PCR and Southern Blot for FX [15]; (3) PCR, sequencing and Reverse Dot Blot (RDB) for BT [16]; (4) PCR and RDB Kit according to manufacturing protocols (INNO-LiPA *CFTR*19 and INNO-LiPA *CFTR*17+Tn, Innogenetics, Belgium) for CF. Each laboratory received four aliquots of 7 μg, 7 μg, 20 μg, and 40 μg of validated DNA for the CF, BT, APC, and FX scheme, respectively. Table 1 lists samples with mock clinical data and mutations submitted during the VII round.

2.3. Web Utility. The web utility was developed in 2008 and was designed to simplify communications and data sharing among ISS, laboratories, and assessors.

Laboratories receive an identification code (ID) and password (PW) to access the personal area (http://www.iss.it/site/cnmr/privato/cqtg/entry.asp) and the scheme area where they find samples data and instructions to participate in the EQA.

Upon completing the analysis of the samples, the laboratories upload the raw data (jpg format) and the reports (pdf format).

At deadline the data are made available to the assessors who access their reserved area; they assess the results of the laboratories and write their observations in a schedule that is forwarded to the ISS via the web utility.

Final results are uploaded in the reserved area of each laboratory which, however, is informed by e-mail. A report with a summary of all anonymous results is also included in the reserved area and published on the website (http://www.iss.it/cnmr/tege/qual/cont.php?id=90&lang=1&tipo=4).

2.4. Assessment. National experts evaluated the laboratory results twice: first online and then in a meeting at the ISS. Assessment took into account technical performance (raw data), genotyping, interpretation, and reports. All data were treated anonymously and the identity of each laboratory is unknown to the assessors.

Following the assessment, participating laboratories received a feedback with an evaluation of the results. Since

Table 1: List of proposed mutations and mock clinical information.

Scheme	Identification data	Gender	Clinical information	Proposed mutations
CF	Irene Pettorbi 12/01/1989	F	Female of Pakistani origin affected by Cystic Fibrosis; she manifests moderate respiratory symptomatology, pancreatic sufficiency, and normal values of sweat chloride	c.3717+12191C>T/c.3717+12191C>T (3849+10KbC>T/3849+10KbC>T)
	Manuela Statenti 13/01/1998	F	Female with positive sweat test, mild breathing symptoms. She asks for molecular characterization for Cystic Fibrosis.	c.579+1G>T/c.489+1G>T (711+1G>T/621+1G>T)
	Sara Ulmilefa 12/04/1966	F	Female clinically healthy with child affected by Cystic Fibrosis	c.3846G>A heterozygous (W1282X heterozygous)
	Anna Ellicine 05/05/1995	F	Female with positive sweat test; her brother is affected by Cystic Fibrosis. She asks for molecular characterization	c.1521_1523delCTT/c.1657C>T (F508del/R553X)
BT	Mario Tappenti 05/09/1982	M	Affected by Beta Thalassemia major	c.20delA/c.118C>T (Bcd6(-A)/Bcd39C>T)
	Antonio Aberuste 22/01/1988	M	Affected by Beta Thalassemia major	c.118C>T/c.118C>T (Bcd39C>T/Bcd39C>T)
	Giovanni Pormitou 17/07/1987	M	Affected by Beta Thalassemia intermediate	c.20delA/c.93-21G>A (Bcd6(-A)/IVS1-110G>A)
	Elio Smantico 01/06/1976	M	Carrier of Beta Thalassemia	c.93-21G>A heterozygous (IVS1-110G>A heterozygous)
FX	Dompinti Anna 20/02/1983	F	Female, with normal phenotype, has two children and a brother affected by Fragile X Syndrome	23/200 repeats (Premutation)
	Ornicapo Irene 12/03/1983	F	Female, with normal phenotype, has a nephew and an uncle affected by Fragile X Syndrome	29/90 repeats (Premutation)
	Quezzamo Nicola 13/03/1958	M	Male, with normal phenotype, has brother and nephew with Fragile X Syndrome; suspect carrier	100 repeats (Premutation)
	Ubbronti Mario 30/12/1970	M	Male with suspect on Fragile X Syndrome	30 repeats (Wild)
APC	Anuttifo Ennio 05/02/1969	M	No clinical indication	c.4012C>T heterozygous* c.4597A>C heterozygous#
	Piclilma Gianni 14/02/1957	M	No clinical indication	c.1629_1630delT heterozygous
	Ordectio Mario 17/03/1995	M	No clinical indication	c.1621C>T heterozygous
	Simpieti Aldo 11/03/1977	M	No clinical indication	c.3149delC heterozygous* c.7417C>T heterozygous#

CF: Cystic Fibrosis; BT: Beta Thalassemia; FX: Fragile X Syndrome; APC: Familial Polyposis Adenomatous Coli. *Pathogenic mutation; #additional gene variant.

2009 a marking system has been introduced and laboratories receive a mark for genotyping, interpretation, and reporting of results for each sample including comments or suggestions, if necessary. Until now poor performance has neither been assigned nor penalized.

2.5. Evaluation Criteria. Evaluation criteria have been established by the ISS and a panel of national experts taking into account the EMQN criteria, national and international best practice guidelines and publications [18–20].

For each scheme, three assessment topics are identified; a maximum score of 5 points is assigned to each topic for genotyping performance, and of 4 points for interpretation and for reporting. In accordance with the evaluation criteria, points are subtracted for errors and for lack of important information. The presence of raw data and reports and the correct identification of the genotype are necessary preconditions for making the assessment. If the genotyping result is not correct, assessors do not mark the interpretation and the report, but write a comment as feedback to help the laboratories to improve their performance.

The main common elements of the evaluation criteria for all schemes are listed in Table 2; specific topics were discussed by the assessors from time to time within the framework of each scheme.

3. Results

Fifty-six different laboratories participated in the VII round of the IEQA: 41 laboratories affiliated with the public health system and 15 private laboratories. In particular, 43 laboratories participated for CF, 17 for BT, 15 for FX, and 5 for APC.

Table 2: General evaluation criteria: items common to all schemes taken into account for assessment.

Genotyping
Quality of raw data
Lack of data legend
Lack of DNA variants detection
Correctness of the nomenclature
Completeness of technical information
Correctness/lack of detection rate
Problems with counting of triplets (X-Fra)
Lack of indication for advanced investigations when appropriate
Interpretation
Lack and/or inaccuracy of important information on the pathogenic role of mutation and/or reproductive risk (not for APC), and/or other important information.
Lack of information about test validity
Lack of genetic counseling indication if necessary
Reporting
General inadequacy of the report
Inadequate language
Lack of laboratory heading
Lack of identification of the patient
Clerical error in identification of the patient
Lack of gender indication
Lack of geographical origin of the patient where necessary
Lack of identification number of sample
Lack of report title
Lack of reason for testing
Lack of sample source
Lack of primary sample type
Lack of signature of the person releasing the report
Lack of date primary sample collection and release of the report
Lack of indication of certification/accreditation of laboratory
Lack of page numbering

22 laboratories participated in more than one scheme. During this round 320 samples were analyzed and results were examined by the assessors to evaluate their performance.

3.1. Genotyping Results

3.1.1. Cystic Fibrosis. Overall, 172 samples were sent to the laboratories by the ISS. Genotypes were correctly detected in 165/172 (96%) samples. Genotyping errors occurred in 4% of samples (Table 3). Four laboratories gave no information about the methods used to analyze 14 samples.

3.1.2. Beta Thalassemia. Overall, 68 samples were sent to the laboratories by the ISS. Genotypes were correctly detected in 67/68 (98.5%) of samples. Genotyping errors occurred in 1/68 (1.5%) samples (Table 3).

3.1.3. Fragile X Syndrome. Overall, 60 samples were sent to the laboratories by the ISS. Genotypes were correctly detected in 60/60 samples (100%), but errors occurred in $(CGG)_n$ repeat quantification (Table 3). Moreover in one sample the methylation test was not reported.

3.1.4. Familial Adenomatous Polyposis of the Colon (Gene APC). Overall, 20 samples were sent to the laboratories by the ISS. All samples were correctly genotyped for pathogenic mutations. However one laboratory did not identify an additional gene variant present in one sample (Table 3).

Raw data of 3 samples analyzed by the same laboratory did not have good quality.

3.2. Interpretation Results. Figure 1 shows detailed results on the assessment of genotype interpretation and includes also samples that were not assessed for incorrect genotype; Table 4 shows the information that is most commonly missing for all schemes.

3.2.1. Cystic Fibrosis, Beta Thalassemia, and Fragile X Syndrome. Correct interpretation was reported in 71%, 91%, and 88% of CF, BT, and FX cases, respectively, even though a lack of information was found in the majority of them (i.e., 86%, 64%, and 77% of CF, BT, and XF reports, resp.). It has to be underlined that a referral to genetic counseling was present in most reports not complete (i.e., 87%, 72%, and 80% of CF, BT, and XF reports, resp.).

There was not any interpretation of genotyping results in 25%, 6%, and 3% of CF, BT, and FX reports, respectively; however most CF and FX reports refer to genetic counseling (i.e., 27/43 CF and 2/2 FX reports).

3.2.2. Familial Adenomatous Polyposis of the Colon (Gene APC). Interpretation was correct in 12/20 (60%) of reports, even though it was not complete in the majority of them, that is, 8/12 (67%). It has to be underlined that, when the interpretation was lacking information, a referral to genetic counseling was indicated in most reports, that is, 4/8 (50%).

3.3. Report Results. Table 5 shows information most commonly missing in the reports for all schemes.

3.3.1. Cystic Fibrosis. 70% of reports assessed were correct and complete; 50 reports were not evaluated for lack of interpretation or for genotyping error. Few laboratories presented incomplete reports for lack of information such as clinical indication, and ethnic or geographic origin of the patient. Only one laboratory (4 samples) sent inadequate reports for lack or unclear reporting of important information.

3.3.2. Beta Thalassemia. 65% of reports assessed were correct and complete; 1 report was not assessed for incorrect genotype. Few laboratories presented incomplete reports for lack of information such as clinical indication and title of the report; only one laboratory sent inadequate reports on

Table 3: Errors performed by laboratories in genotyping detection and reporting.

CEQ scheme	*N* of laboratories performing errors	*N* of samples	Type of error	Error percentage/scheme
CF	2/43	6/172	Genotyping error: samples swap	4%
	1/43	1/172	Genotyping error: mutation correctly detected but not correctly reported (c.3484C>T instead of c.3846G>A)	
BT	1/17	1/68	Genotyping error: mutation correctly detected but not correctly reported (c.118C>T was reported in heterozygous instead of homozygous status)	1,5%
FX	5/15	17/60	Information about genotype not adequate: number of triplets absent or not clearly reported	28%
APC	1/5	1/20	Information about genotype not adequate: gene variant not reported	5%

CF: Cystic Fibrosis; BT: Beta Thalassemia; FX: Fragile X Syndrome; APC: Familial Polyposis Adenomatous Coli.

Table 4: Information most commonly missing in interpretation of results.

	Incomplete interpretation			
Not mentioned information	CF (105)	BT (39)	FX (41)	APC (8)
Analytical sensitivity and specificity of procedures	88%	89%	20%	100%
Detection rate absent or incorrect	60%	69%	93%	0%
Indication for genetic counseling	13%	28%	20%	50%
Reproductive risk or request to test the partner	52%	28%	0%	—
Request to test parents to confirm homozygous nature of mutation	0%	18%	—	—

CF: Cystic Fibrosis; BT: Beta Thalassemia; FX: Fragile X Syndrome; APC: Familial Polyposis Adenomatous Coli. The numbers of samples that were reported with incomplete interpretations are indicated in brackets.

all their samples for lack or unclear reporting of important information.

3.3.3. Fragile X Syndrome. Only 60% of reports were found to be correct and complete. Few laboratories presented incomplete reports for lack of information such as voice of clinical indication or indication of gender.

3.3.4. Familial Adenomatous Polyposis of the Colon (Gene APC). 60% of reports were found to be correct and complete. One laboratory (4 samples) did not report clinical indications; one laboratory (4 samples) did not indicate the title of the report.

4. Discussion

The EQA program focuses in particular on standardizing laboratory procedures. Participation in EQA schemes provides a measure of technical, analytical, and interpretative performance. It has an educational role for laboratories and gives the opportunity to review their internal standards and policies, and also to provide advice on the updating of best practice guidelines [1, 4, 21]. Moreover participation in EQA program is essential for laboratory accreditation with the international ISO 15189 standard [7, 21]. To date, EQA programs have been also used by laboratories as a tool for improving sample processing quality, hence the assessment takes into account not only genotyping and reporting, but it also looks at other aspects such as the interpretation of results [1, 21].

The Italian molecular EQA program was established in 2001; the VII round (2010) was reviewed versus previous rounds.

Fifty-six laboratories participated in the VII round and 320 samples were analyzed.

Genotyping was found to be quite satisfactory in general; only 4/56 laboratories in all schemes failed to correctly genotype samples: 3 for CF (4% of cases) and 1 for BT (1,5% of cases). In comparison, the error percentage for the CF scheme was higher in this round than the median error rate registered in previous rounds (0.2%) [14]. In the framework of the BT scheme, only the analysis of 1/68 samples was wrong (1.5%) and the error percentage was higher than the median error rate reported in previous rounds (0.33%) [16]. The analysis of these errors shows that techniques were generally well performed and raw data had good quality. In spite of previous rounds, where errors were mainly due to technical errors, during the VII round all errors were caused by suboptimal management of samples [14, 16].

No genotyping errors were performed by laboratories participating in the APC and FX schemes; this is a good result which reflects the outcome of the other rounds for the APC scheme and it represents an improvement in quality for the FX scheme [15, 17]. However, in the FX scheme, some

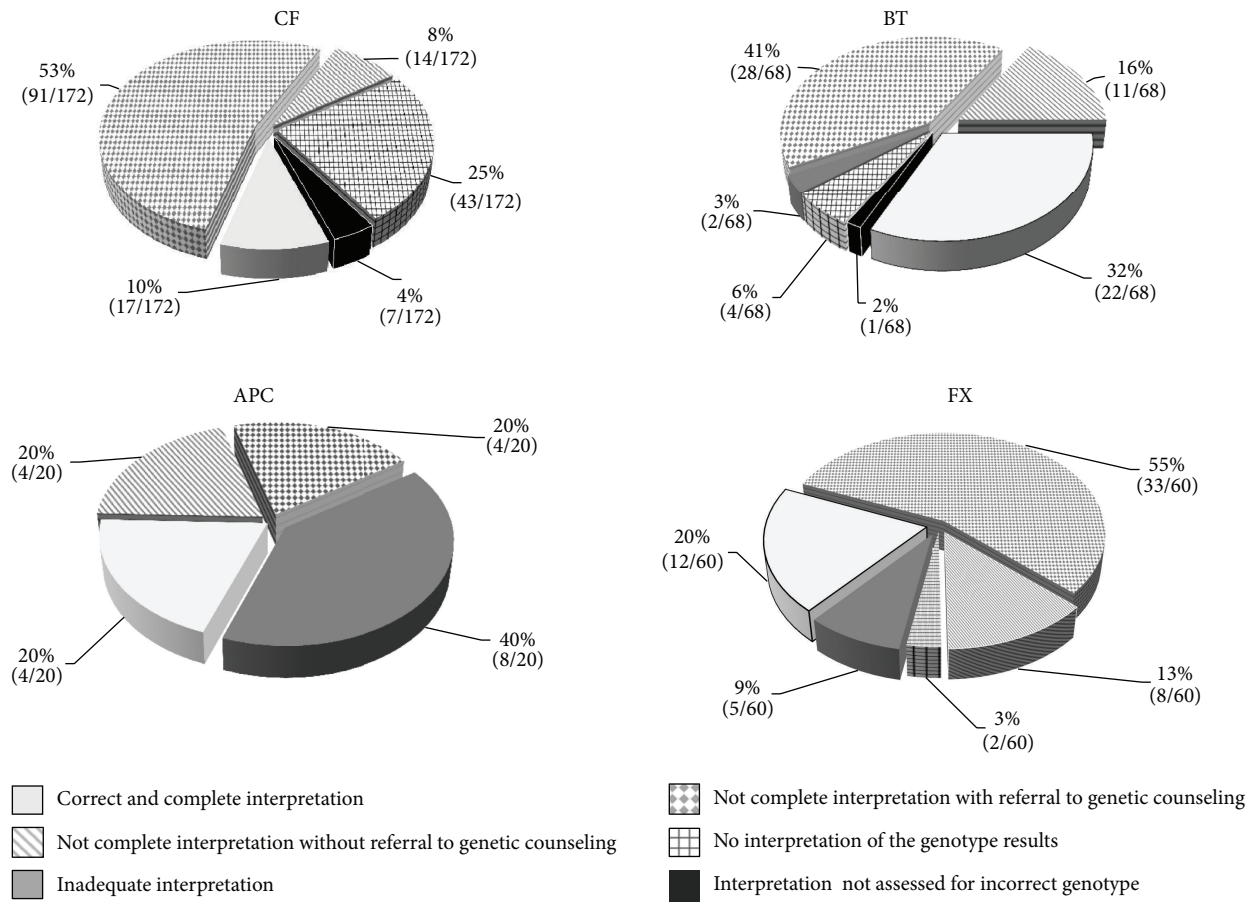


Figure 1: Interpretation of genotyping results.

genotypes were not completely defined because the number of triplets were not accurately reported in 28% of laboratory reports, and a variant, in one sample in APC scheme, was not detected by one laboratory.

Although laboratories returned acceptable analytical results in 97.5% of samples in all schemes, in the majority of reports, with variants within each schemes, the interpretation was correct but not complete. This data can be explained by the fact that during routine analysis, laboratories write out a technical report and leave it up to the genetic consultant to provide an accurate interpretation of genotype results in a separate report. In fact about 82% of laboratories who were penalized for lack of interpretation, or lack of necessary information, suggest and/or offer genetic counseling.

A comparison between our results and other published data shows a similar scenario during the first year of the interpretation survey [5, 22].

Like genotyping results, also report results were satisfactory for completeness of the information; during the VII round, in fact, 66% of reports on samples were good.

The focus on IEQA for the VII round shows satisfactory quality in genotyping and reports in comparison with previous published data, but we still observe room for improvement. A closer examination of the entire process of data reporting has highlighted the need to focus attention on the interpretation of results; this aspect was neglected in previous rounds because more attention was attached to the technical approach to sample analysis in order to ensure sound genotyping results.

All data collected within the framework of the IEQA and the European EQAs highlighted the need and the importance to carry on this activity in order to ensure adequate quality standards for the genetic tests performed in all laboratories.

At the present time the VIII round of the IEQA is under way; there has been an increase in the number of participating laboratories, and we expect some improvements in the laboratories that participated in the VII round.

Moreover in this round we are introducing a "poor performance" score for laboratories that make critical errors in genotype and/or interpretation and/or reports, that may significantly affect patient management. In this context our role is to contribute to improving laboratory quality through specific educational actions, improving the dialogue with laboratories and, if necessary, involving assessors and national experts in the process.

Acknowledgments

The authors thank the Galliera Genetic Bank—"Telethon Genetic Biobank Network," supported by Italian Telethon

Table 5: Details of lack of information/inadequacy in reporting results.

Reporting	FC (122)	BT (67)	XF (60)	APC (20)
General inadequacy of the report	3,30%	6%	6,60%	
Inadequate language		**13,40**%	6,60%	
Lack of laboratory heading				
Lack of identification of the patient	3,30%		6,60%	
Clerical error in identification of the patient		4,40%		
Lack of gender indication			**40**%	
Lack of geographical origin of the patient where necessary	**53,20**%			
Lack of identification number of sample		4,40%		
Lack of report title	11,40%	10,40%		20%
Lack of reason for testing	**14,70**%	**10,40**%	**22**%	**20**%
Lack of sample source	1,60%		6,60%	40%
Lack of primary sample type		6%	6,60%	
Lack of signature of the person releasing the report				
Lack of date primary sample collection and release of the report			6,60%	
Lack of indication of certification/accreditation of laboratory	36%		6%	40%
Lack of page numbering	36%			

CF: Cystic Fibrosis; BT: Beta Thalassemia; FX: Fragile X Syndrome; APC: Familial Polyposis Adenomatous Coli. The numbers of evaluated reports for pathologies are indicated in brackets.

grants (Project no. GTB07001A) for providing them with specimens. The authors are also grateful to the laboratories that participated in the IEQA for contributing their data to all schemes.

References

[1] J. C. Libeer, "Role of external quality assurance schemes in assessing and improving quality in medical laboratories," *Clinica Chimica Acta*, vol. 309, no. 2, pp. 173–177, 2001.

[2] Conferenza Stato-Regioni 15 luglio 2004.

[3] OECD, *Guidelines for Quality Assurance in Molecular Genetic Testing*, OECD, Paris, France, 2007.

[4] R. J. Hastings and R. T. Howell, "The importance and value of EQA for diagnostic genetic laboratories," *Journal of Community Genetics*, vol. 1, no. 1, pp. 11–17, 2010.

[5] S. Berwouts, E. Girodon, M. Schwarz et al., "Improvement of interpretation in cystic fibrosis clinical laboratory reports: longitudinal analysis of external quality assessment data," *European Journal of Human Genetics*, vol. 20, no. 12, pp. 1209–1215, 2012.

[6] Guidelines for Genetic Tests, 1999—Linee Guida per i test genetici, http://www.cnmr.iss.it/lgui, 1999.

[7] S. C. Ramsden, Z. Deans, D. O. Robinson et al., "Monitoring standards for molecular genetic testing in the United Kingdom, the Netherlands, and Ireland," *Genetic Testing*, vol. 10, no. 3, pp. 147–156, 2006.

[8] L. Sciacovelli, S. Secchiero, L. Zardo, M. Zaninotto, and M. Plebani, "External quality assessment: an effective tool for clinical governance in laboratory medicine," *Clinical Chemistry and Laboratory Medicine*, vol. 44, no. 6, pp. 740–749, 2006.

[9] B. Chen, M. Gagnon, S. Shahangian et al., "Good laboratory practices for molecular genetic testing for heritable diseases and conditions," *MMWR Recommendations and Reports*, vol. 58, no. RR-6, pp. 1–37, 2009.

[10] C. S. Richards and W. W. Grody, "Alternative approaches to proficiency testing in molecular genetics," *Clinical Chemistry*, vol. 49, no. 5, pp. 717–718, 2003.

[11] K. E. Weck, B. Zehnbauer, M. Datto et al., "Molecular genetic testing for fragile X syndrome: laboratory performance on the College of American Pathologists proficiency survey (2001–2009)," *Gentics in Medicine*, vol. 14, no. 3, pp. 306–312, 2012.

[12] D. Taruscio, V. Falbo, G. Floridia et al., "Quality assessment in cytogenetic and molecular genetic testing: the experience of the Italian Project on Standardisation and Quality Assurance," *Clinical Chemistry and Laboratory Medicine*, vol. 42, no. 8, pp. 915–921, 2004.

[13] Official Bulletin of the Italian Republic-G.U. n.199 del 28/08/2009.

[14] M. Salvatore, V. Falbo, G. Floridia et al., "The Italian External Quality Control Programme for cystic fibrosis molecular diagnosis: 4 years of activity," *Clinical Chemistry and Laboratory Medicine*, vol. 45, no. 2, pp. 254–260, 2007.

[15] V. Falbo, G. Floridia, F. Tosto et al., "The Italian External Quality Assessment scheme for fragile X syndrome: the results of a 5-year survey," *Genetic Testing*, vol. 12, no. 2, pp. 279–288, 2008.

[16] F. Tosto, M. Salvatore, V. Falbo et al., "The Italian scheme of External Quality Assessment for beta-thalassemia: genotyping and reporting results and testing strategies in a 5-year survey," *Genetic testing and molecular biomarkers*, vol. 13, no. 1, pp. 31–36, 2009.

[17] F. Censi, V. Falbo, G. Floridia et al., "The Italian external quality control program for familial adenomatous polyposis of the colon: five years of experience," *Genetic Testing and Molecular Biomarkers*, vol. 14, no. 2, pp. 175–181, 2010.

[18] C. Castellani, H. Cuppens, M. Macek et al., "Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice," *Journal of Cystic Fibrosis*, vol. 7, no. 3, pp. 179–196, 2008.

[19] E. Dequeker, M. Stuhrmann, M. A. Morris et al., "Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders - Updated European recommendations," *European Journal of Human Genetics*, vol. 17, no. 1, pp. 51–65, 2009.

[20] M. Grasso, M. A. Melis, A. Murgia et al., Proposta Linee guida per la diagnosi molecolare della Sindrome dell'X Fragile (Proposed Guideline for Fragile X Diagnosis), http://www.sigu.net/index.php?option=com_docman&task=cat_view&gid=46&limitstart=15, 2008.

[21] S. Berwouts, K. Fanning, M. A. Morris et al., "Quality assurance practices in Europe: a survey of molecular genetic testing laboratories," *European Journal of Human Genetics*, vol. 20, no. 11, pp. 1118–1126, 2012.

[22] I. Touitou, C. Rittore, L. Philibert, J. Yagüe, Y. Shinar, and I. Aksentijevich, "An international external quality assessment for molecular diagnosis of hereditary recurrent fevers: a 3-year scheme demonstrates the need for improvement," *European Journal of Human Genetics*, vol. 17, no. 7, pp. 890–896, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 904247, 7 pages
http://dx.doi.org/10.1155/2013/904247

Research Article

Targeting the Immunogenetic Diseases with the Appropriate HLA Molecular Typing: Critical Appraisal on 2666 Patients Typed in One Single Centre

M. Guarene,[1] C. Capittini,[1] A. De Silvestri,[2] A. Pasi,[1] C. Badulli,[1] I. Sbarsi,[1] A. L. Cremaschi,[1] F. Garlaschelli,[1] C. Pizzochero,[1] M. C. Monti,[1] C. Montecucco,[3] G. R. Corazza,[4] D. Larizza,[5] P. E. Bianchi,[6] L. Salvaneschi,[7] and M. Martinetti[1]

[1] *Laboratorio di Immunogenetica, Servizio di Immunoematologia e Medicina Trasfusionale, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy*
[2] *Unità di Biometria, Direzione Scientifica, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy*
[3] *Clinica Reumatologica, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*
[4] *Clinica Medica I, Centro per lo Studio e la Cura delle Malattie Infiammatorie Croniche Intestinali, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*
[5] *Clinica Pediatrica, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*
[6] *Clinica Oculistica, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*
[7] *Servizio di Immunoematologia e Medicina Trasfusionale, Fondazione IRCCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*

Correspondence should be addressed to M. Martinetti; m.martinetti@smatteo.pv.it

Received 26 October 2012; Accepted 20 December 2012

Academic Editor: Ozgur Cogulu

We compared the immunogenetic data from 2666 patients affected by HLA-related autoimmune diseases with those from 4389 ethnically matched controls (3157 cord blood donors CBD, 1232 adult bone marrow donors BMD), to verify the appropriateness of HLA typing requests received in the past decade. The frequency of HLA-B*27 phenotype was 10.50% in 724 ankylosing spondylitis, 16.80% in 125 uveitis (3.41% BMD, 4.24% CBD, $P < 0.0001$); HLA-B*51 allele was 15.57% in 212 Behçet's disease (12.91% BMD, 9.88% CBD, $P < 0.0001$); the HLA-DRB1-rheumatoid arthritis (RA) shared epitope was 13.72% in 554 RA (10.85% BMD, 13.48% CBD, $P = 0.016$); the carriers of almost one of HLA-DQB1 susceptibility alleles were 84.91% in 795 celiac disease (CD) and 59.37% in 256 insulin-dependent diabetes mellitus (IDDM) (46.06% in 875 CBD, 42.75% in 662 BMD $P < 0.0001$). Overall, our results show that the HLA marker frequencies were higher in patients than controls, but lower than expected from the literature data (excluding CD and IDDM) and demonstrate that, in complex immunogenetic conditions, a substantial number of genetic analyses are redundant and inappropriate, burdening to the public health costs. For this reason, we suggest the Italian Scientific Society of Immunogenetics to establish guidelines to improve the appropriateness of typing requests.

1. Introduction

An autoimmune disease arises when the immune system looses the ability to distinguish body's own cells from foreign cells (nonself), thus eliciting the attack of self-tissues [1]. From a genetic point of view, an autoimmune condition is the result of a tight interaction between environmental factors and specific predisposing genes. The first autoimmune disease found to be associated with an HLA-B marker was ankylosing spondylitis (AS), a chronic and progressive inflammation of the spine articulations, and less frequently the peripheral joints, leading to a forward-stooped posture which causes rigidity and severe pain [2, 3]. After almost four decades, AS is still tightly associated with HLA-B*27 with a phenotype frequency of 90% [4].

From then onwards, the susceptibility to develop autoimmune diseases has been found to be conditioned by several HLA molecules (Table 1). In ocular autoimmune conditions, diseases of the uvea show the strongest correlation with HLA markers. Uveitis is an inflammation of the uvea that destroys eye tissues, causes light sensitivity, and decreases visual acuity [5, 6]. Acute anterior uveitis is described as strictly associated to HLA-B*27 (phenotypic frequency 50%) [7]. Behçet's disease (BD) is a chronic vasculitis characterized by aphthosis, uveitis, and skin lesions, and sometimes affecting also the musculoskeletal, nervous, and gastrointestinal systems [8]. In Southern Europe, the HLA-B*51 allele accounts for a 30–50% of the genetic risk for BD development and is carried by one- to two-thirds of patients all over the world (Caucasian phenotypic frequency 60–70%) [9]. Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disease affecting the joints, and gradually leading to their destruction [10–12]. Since the Seventies, the RA risk has been associated to specific HLA-DRB1 molecules (HLA-DRB1*01, *04, *10) and, more recently, to few variants sharing an amino acid sequence (position 70–74) in the third hypervariable region of HLA-DR β chain known as Shared Epitope (SE) (Table 1) [13]. Celiac disease (CD) is a chronic enteropathy caused by gluten intake in people carrying the HLA predisposing variants coding for the DQ2 (HLA-DQB1*02:01 and HLA-DQB1*02:02) and DQ8 (HLA-DQB1*03:02) molecules [14–16]. The phenotype frequency of DQ2/8 is 20% in healthy people and more than 90% in CD patients [17]. Insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disorder in which the immune system attacks and destroys the beta-cells of pancreas, leading to insulin dependence [18]. The HLA markers of IDDM are HLA-DQB1*02:01 (part of the DR3 haplotype: DRB1*03-DQB1*02:01) and HLA-DQB1*03:02 (part of the DR4 haplotype: DRB1*04-DQB1*03:02) [19]. About 40–60% of patients with IDDM carry the DR3/4-DQ2/8 genotypes [20].

Since 1989, the Immunogenetics Laboratory of the IRCCS Policlinico San Matteo of Pavia (Italy) is involved in the HLA molecular typing of hematopoietic stem cell donors and patients affected by autoimmune diseases. In particular, the HLA typing requests for autoimmune diseases come from both medical specialists (inside and outside the IRCCS Policlinico San Matteo) and general practitioners, to support their clinical diagnosis. The collaboration between the physicians and the immunogenetists is fundamental to reach the most appropriate typing request. In fact, thanks to the tight correlation between specific HLA markers and the predisposition to certain autoimmune diseases, the HLA analysis has become a sensitive genetic tool to identify those individuals at high risk to develop these conditions [21].

A recent survey, performed by the Italian Society of Immunogenetics (AIBT), has shown that the HLA analyses, requested for disease association studies, are plethoric and often inappropriate (unpublished data). Moreover, HLA typing technologies adopted in the immunogenetics laboratories throughout Italy are heterogeneous. Trying to overcome the waste of economic resources for useless investigations, in 2010 the AIBT has proposed a long-distance course addressed to Italian operators in the immunogenetics field.

Taking into account these premises, we have retrospectively analyzed the HLA typing data of 2666 patients referred to our hospital for different autoimmune pathologies, and addressed to our Immunogenetics Laboratory in the last decade (2002–2011), to verify the appropriateness of the typing requests, comparing the frequency of HLA variants with those of 4389 ethnically matched controls typed in the same period with the same molecular techniques.

2. Materials and Methods

2.1. Patients. The Immunogenetics Laboratory of the IRCCS Policlinico San Matteo (Pavia) is accredited by the EFI (European Federation of Immunogenetics) since 1998 for stem cell transplantation and HLA disease association studies and daily receives HLA typing requests from physicians to support the suspected diagnosis. The well-recognized HLA susceptibility markers of ankylosing spondylitis, rheumatoid arthritis, uveitis, Behçet's disease, celiac disease, and insulin-dependent diabetes mellitus are listed in Table 1.

HLA typing requests also come from the other hospitals of Pavia and Province as well as from general practitioners. Thus, we could not verify the compliance of the classification criteria conventionally recognized for the diagnosis of the six autoimmune pathologies considered in the study (Table 1) [22–27]. In detail, Table 2 shows the percentage of HLA typing requests coming from medical specialists (inside and outside the Policlinico San Matteo of Pavia) and general practitioners.

We considered 724 HLA typing data for patients affected by ankylosing spondylitis (mean age 44.3 years, female/male rate 1.5), 554 with rheumatoid arthritis (mean age 52.2 years, female/male rate 2.8), 125 with uveitis (mean age 41.9 years, female/male rate 0.9), 212 with Behçet's disease (mean age 41.7 years, female/male rate 2.1), 795 affected by celiac disease (mean age 20.5 years, female/male rate 1.5), and 256 with type I diabetes (mean age 10.3 years, female/male rate 1.0). All these immunogenetic data were collected from 2002 to 2011.

2.2. Control Subjects. Two control groups were considered: 3157 cord blood (CB) donors belonging to the Pavia CB Bank (IRCCS Foundation Policlinico San Matteo, Pavia, Italy) and 1232 adult hematopoietic stem cell donors belonging to the PV01 Registry (Italian Bone Marrow Registry, IBMDR, Genoa, Italy). All the individuals were of Caucasian ancestry. The mothers of each CB donor signed an informed consent to participate in the CB banking program for unrelated stem cell transplantation. Following the international protocols, the physicians obtained a complete medical history of the newborn's family (mother, father, siblings, and grandparents). An exhaustive obstetric history (i.e., of previous pregnancies) was also obtained, so that we could exclude women with recurrent spontaneous abortions from donation. Furthermore, during each pregnancy, the health of both mother and fetus was carefully monitored, in order to exclude from donation any CB unit derived from pathological pregnancies, including preterm ones (<37 gestational weeks). All the adult hematopoietic stem cell donors signed an informed consent

to be enrolled in the Italian Bone Marrow Registry. The IBMDR criteria exclude from donation individuals affected by genetic, cardiovascular, hematologic, autoimmune and psychiatric conditions, malignancies, and infectious diseases.

2.3. HLA Classes I and II Genomic Typing. The entire data set of patients and controls is composed by molecular typing for both HLA class I and II genes. The PCR sequence-specific primer method (Olerup, Sweden) and/or the reverse PCR sequence specific oligonucleotide hybridization method (Innogenetics, Murex Biotech Limited, Belgium) were employed, as described elsewhere [28]. Ambiguous typing results were resolved by direct sequencing.

Patients affected by ankylosing spondylitis were typed for the presence of the HLA-B*27 allele, those affected by rheumatoid arthritis and uveitis for the HLA-B and HLA-DRB1 loci, and patients affected by Behçet's disease for the HLA-B locus. The polymorphisms at the HLA-DQA1 and HLA-DQB1 loci were defined at high resolution in individuals affected by celiac disease and type I diabetes.

According to the international policy of FACT-Netcord and Italian CB Banks, the CB units were typed for HLA-A and HLA-B polymorphisms at low-resolution level and for HLA-DRB1 at high-resolution level before banking. According to the policy of IBMDR, the adult hematopoietic stem cell donors were typed for HLA-A and HLA-B and, more recently, for HLA-C polymorphisms at low-resolution level and for HLA-DRB1 at high resolution. We analysed the HLA-B polymorphisms at low-resolution level and the HLA-DRB1 polymorphisms at high-resolution level for 3157 CB donors and 1232 adult hematopoietic stem cell donors. We analysed the HLA-DQA1 and HLA-DQB1 polymorphisms at high-resolution level for 875 CB donors and 662 adult hematopoietic stem cell donors.

2.4. Statistical Analysis. Differences between the groups were evaluated with Chi-squared statistics or Fisher's exact test, as appropriate. P values reported were two tailed; $P < 0.05$ was considered statistically significant.

We employed the Principal Coordinates Analysis (PCoA) statistical method to explore and visualize similarities or dissimilarities between patients and controls. PCoA starts with a similarity matrix (correlation) or dissimilarity matrix (distance matrix) and assigns for each population a location in a low-dimensional space, for example, as a 2D or a 3D graphics. PCoA involves projecting the points onto a space defined by a small number of principal axes (uncorrelated linear combinations of the variables that contain most of the variance), accounting for the greatest variability. The first axis accounts for the highest variance and the second axis for the lowest one. The samples are represented by points, and the proximity among them shows their similarity or dissimilarity [29].

3. Results and Discussion

In the past decade, our Immunogenetics Laboratory received a growing number of typing requests (500 in 2011) for patients in the setting of HLA-related autoimmune diseases. Aiming at verifying the appropriateness of all these genetic analyses in helping physicians to define the clinical diagnoses, we retrospectively analyzed the HLA typing data of 2666 autoimmune patients that we collected from 2002 to 2011, comparing the frequency of HLA markers with those of 4389 ethnically matched controls (3157 cord blood donors CBD and 1232 adult bone marrow donors BMD).

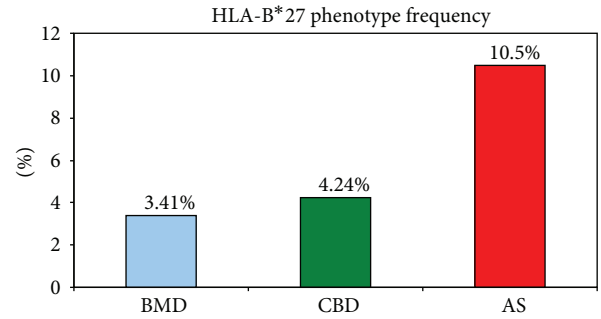


Figure 1: Phenotype frequency of HLA-B*27 in ankylosing spondylitis (AS) patients, adult bone marrow donors (BMD), and cord blood donors (CBD).

HLA-B*27 is the molecular marker of both ankylosing spondylitis (AS) and acute anterior uveitis (AAU) (Table 1). We received 724 HLA-B*27 typing requests to support the suspected diagnosis of AS based on clinical symptoms or for familial counseling. The literature reports that about 90% of truly diagnosed Caucasian AS patients carry the HLA-B*27 marker [24]. Comparing the phenotype frequency of HLA-B*27 between the patients and the two control groups, we found that 10.5% of suspected AS (sAS) patients were actually positive for HLA-B*27, whereas only 3.41% of BMD and 4.24% of CBD carried the HLA-B*27 marker (AS versus BMD $P < 0.0001$, AS versus CBD $P < 0.0001$; see Figure 1).

We also considered 125 HLA-B*27 typing requests for Uveitis (U) patients, finding a higher HLA-B*27 allele frequency with respect to controls (8.40% uveitis versus 1.70% BMD and 2.15% CBD, $P < 0.0001$) (Table 3), corresponding to 16.80% uveitis versus 3.41% BMD and 4.24% CBD phenotype frequencies. According to several association studies, the phenotypic frequency of the HLA-B*27 marker in classic acute anterior uveitis patients is about 50% [7] whilst in our sample is only 16.80%. This result may be due to the heterogeneity of patients referring to our laboratory with a generic definition of uveitis, but probably affected by anterior, intermediate, posterior uveitis, and panuveitis.

We considered 212 requests of HLA-B low-resolution typing for Behçet's disease (BD) patients. The allelic frequency of HLA-B*51 was 15.57% slightly higher than in BMD (12.91%) and significantly increased with respect to CBD (9.88%, $P < 0.0001$) (Table 3). Once more, in our patients with a suspect of BD, we found an HLA-B*51 allele frequency lower than the expected, suggesting an extreme caution of our physicians in giving a fast and definite diagnosis of such a complex syndrome.

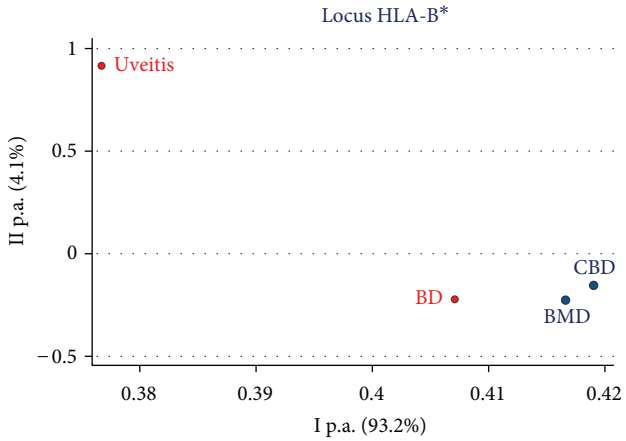


Figure 2: Principal component analysis of HLA-B in uveitis, Behçet's disease (BD), adult bone marrow donors (BMD), and cord blood donors (CBD).

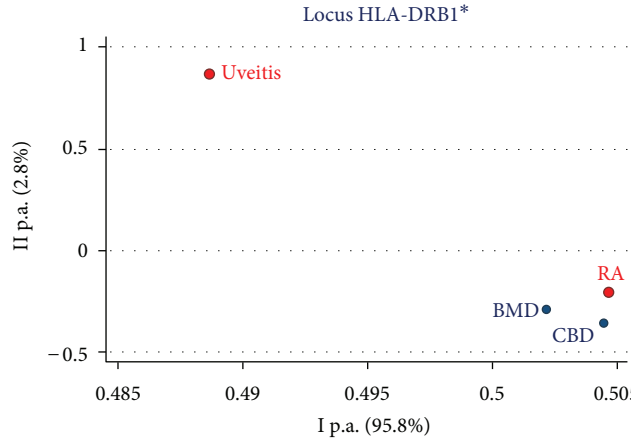


Figure 3: Principal component analysis of HLA-DRB1 in uveitis, rheumatoid arthritis (RA), adult bone marrow donors (BMD), and cord blood donors (CBD).

In order to visualize in a single graphic the genetic distances among groups (patients and controls), we performed the statistical test of the Principal Coordinates Analysis (PCoA): the closer the points, the more similar the samples. According to HLA-B typing, the BD patients were nearer the healthy controls than U patients (Figure 2), and this may be explained by the type of pathology, as uveitis is restricted to a single organ (the eye), whereas BD is a multiorgan syndrome affecting the nervous and gastrointestinal systems, the joints, the eye, and the skin. Interestingly, the phenotype frequency of HLA-B*51 carriers was 57.14% in the group of patients treated in neurological clinics, 28.77% in rheumatologic clinics, and 29.03% referring to general practitioners. Thus, the BD with a neurological component seems to be more clearly HLA targeted than the other clinical subtypes.

We reviewed 554 HLA-DRB1 typing requests asked to support the suspected diagnosis of rheumatoid arthritis (RA) based on early clinical manifestations. The cumulative frequency of the shared epitope (SE) alleles was 13.72%, whereas in BMD it was 10.85% (RA versus BMD $P = 0.016$) and in CBD 13.48% (Table 3). In particular, HLA-DRB1*01:01 represented the most frequent variant in RA patients (7.45%) compared to BMD (4.91%, RA versus BMD $P = 0.005$) and CBD (6.51%). In addition, HLA-DRB1*03:01 was found to be significantly more frequent in RA patients (10.05%) than in both BMD (7.47%, RA versus BMD $P = 0.018$) and CBD (7.71%, RA versus CBD $P = 0.019$). This last result is in line with the literature data, as HLA-DRB1*03:01 allele is part of the HLA-A1,B8,DR3 ancestral haplotype (AH8.1), which is the most cited haplotype in the literature for its correlation to a plethora of autoimmune diseases.

In the PCoA for the HLA-DRB1 alleles, U patients were far from both controls, while RA patients were close (Figure 3). The distance between U patients and controls highlighted the involvement of specific HLA-DRB1 markers. However, in 72 U patients we did not found a higher frequency of the well-known panuveitis markers [25], but a significant increase of HLA-DRB1*16 (12.50% U versus 7.23% BMD, $P = 0.03$; versus 6.74% CBD, $P = 0.01$). At our knowledge, this association has never been reported so far, thus it should be confirmed in a larger sample. The proximity between RA patients and healthy controls may be explained by two causes: first, the majority of our RA patients belong to a group of "early" RA with few disease symptoms which unambiguously characterize the overt RA; second, as RA is associated to a group of HLA-DRB1 alleles encoding the same epitope in the binding cleft, this subdivides the susceptibility frequency among different variants instead of just one. Moreover, the immunogenetic proximity of RA to CBD, rather than to BMD, might be the expression of the lifespan selection. This selective force has acted more on BMD than on CBD, as the formers were enrolled in adulthood, whereas the latters at birth. As a result of this selection, CBD group shows a higher frequency of disease-correlated HLA markers and a higher genetic variability compared to BMD, and this characteristic makes the choice of a CB unit more likely than adult donors in transplantation setting [26].

Finally, we revised the HLA-DQB1 typing requested for 795 celiac disease (CD) patients and 256 insulin-dependent diabetes mellitus (IDDM) patients. The frequencies of the HLA-DQB1 susceptibility alleles for CD and IDDM are listed in Table 3 for patients and controls. In CD patients the frequencies of HLA-DQB1*02:01, HLA-DQB1*02:02 susceptibility alleles were significantly higher than both control groups ($P < 0.0001$), whereas the frequency of HLA-DQB1*03:02 allele was significantly higher only versus the BMD ($P = 0.039$). The frequency of HLA-DQB1*02:01 HLA-DQB1*03:02 was significantly higher in IDDM patients than both BMD and CBD ($P < 0.0001$).

HLA-DQB1 data from CD and IDDM patients were considered in the last PCoA (Figure 4). Both CD and IDDM samples are very distant from the two healthy controls, underlying a high genetic diversity between patients and controls and highlighting the appropriateness of HLA typing requests. Carriers of almost one of the HLA-DQB1 susceptibility markers were 84.91% in our CD patients (expected 95%

Table 1: The HLA susceptibility alleles and classification criteria references for ankylosing spondylitis, rheumatoid arthritis, uveitis, behçet's disease, celiac disease, and insulin-dependent diabetes mellitus.

Autoimmune disease	HLA susceptibility alleles	Classification criteria according to
Ankylosing spondylitis	HLA-B*27	Sieper and Rudwaleit [22]
Uveitis	HLA-B*27 HLA-DRB1*01:02, *04:05, *15:01	Deschenes et al. [23]
Behçet's disease	HLA-B*51	International Team for the Revision of the International Criteria for Behcet's Disease [24]
Rheumatoid arthritis	HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01,*14:02	Silva-Fernández et al. [25]
Celiac disease	HLA-DQB1*02:01, *02:02, *03:02	Rostom et al. [26]
Insulin-dependent diabetes mellitus	HLA-DQB1*02:01, *03:02	American Diabetes Association [27]

Table 2: Percentage of HLA typing requests coming from medical specialists and general practitioners for each autoimmune pathology.

Autoimmune disease	*N*	HLA typing request	
		Medical specialists	General practitioners
Ankylosing spondylitis	724	45.17%	54.83%
Uveitis	125	73.60%	26.40%
Behçet's disease	212	58.49%	41.51%
Rheumatoid arthritis	554	93.86%	6.14%
Celiac disease	795	78.24%	21.76%
Insulin-dependent diabetes mellitus	256	98.44%	1.56%

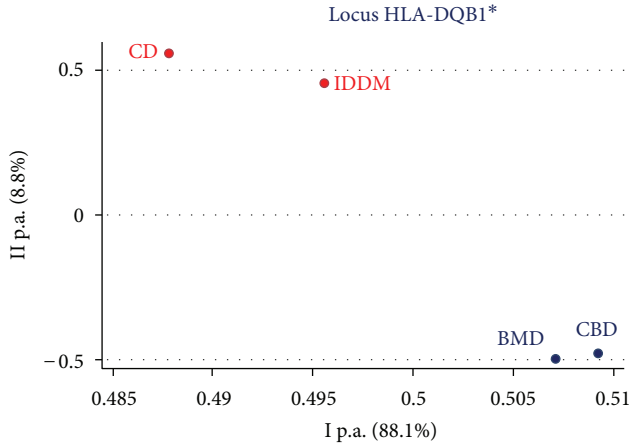


Figure 4: Principal component analysis of HLA-DQB1 in celiac disease (CD), insulin-dependent diabetes mellitus (IDDM), adult bone marrow donors (BMD), and cord blood donors (CBD).

reported in the literature) and 59.37% in IDDM (expected 60%). In both CD and IDDM, the frequency of susceptibility HLA-DQB1 alleles was in line with the literature data, and we think that the good results obtained in the analyses of HLA-DQB1-associated autoimmune pathologies may be a consequence of the correct knowledge of the marker-disease correlation for the CD and IDDM conditions.

4. Conclusions

Our study aims at giving a critical appraisal on the usefulness and appropriateness of the HLA typing requests in the diagnosis of HLA-associated diseases, after ten years of molecular assays typing in a single Laboratory (Immunogenetics Laboratory of the IRCCS Policlinico San Matteo of Pavia, Italy). Nevertheless, this is not an HLA-disease association study.

In this retrospective study we found that, except for HLA-DQB1 mediated pathologies (CD and IDDM), the typing requests gave an immunogenetic result less congruent than expected by the literature data. It is evident that the policy for proper typing requests needs to be improved.

To our knowledge, this is the first survey conducted with the aim to infer the proper management of the financial resources of public health for immunogenetic testing. This is not a weird point of view if we consider two fundamental characteristics of our Hospital. First, our Immunogenetics Laboratory receives a lot of typing requests from many sources outside the Policlinico San Matteo, such as general practitioners; thus it is difficult to verify the classification criteria considered by these physicians and also their actual expertise. Secondly, our Foundation is a research hospital where patients affected by spurious syndromes or unusual cases refer to our physicians who have to deal with atypical signs and difficult diagnosis; therefore we think that the exclusion of these cases from the survey would introduce a bias in considering the total health costs for genetic testing.

In synthesis, this is an unmanipulated survey with the mere exploratory aim to improve the management of healthcare financial resources. Therefore, taking into account our data, we suggest to invest in training courses to enhance the expertise of all physicians, in particular general practitioners. To this, it is imperative to ask the Italian Society of Immunogenetics (AIBT) to set up a series of learning courses, with continuous medical education credits, to give the clinicians a precise tool to increase the appropriateness of typing requests. We believe that the interplay between clinicians and immunogenetists must be strengthened to

Table 3: Allele frequencies of HLA genes correlated to autoimmune pathologies in adult bone marrow donors (BMD), cord blood donors (CBD), Behçet's disease (BD), rheumatoid arthritis (RA), celiac disease (CD), insulin-dependent diabetes mellitus (IDDM), and shared epitope (SE).

HLA susceptibility alleles	BMD	CBD	Uveitis	BD	RA	CD	IDDM
HLA-B*27	1.7%	2.15%	8.40%				
HLA-B*51	12.91%	9.88%		15.57%			
SE alleles (HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01, *14:02)	10.85%	13.48%			13.72%		
HLA-DQB1*02:01	9.37%	7.92%				21.76%	17.19%
HLA-DQB1*02:02	8.99%	9.98%				17.61%	
HLA-DQB1*03:02	4.38%	5.93%				6.10%	14.45%

reach a better definition of the role of HLA markers in the management of those autoimmune diseases with well-defined HLA associations.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors wish to thank the Working Group on HLA and Disease of the Italian Association for Immunogenetics (Associazione Italiana di Immunogenetica e Biologia dei Trapianti AIBT), in particular Dr. Luca Mascaretti, for the national survey "Italian recommendations for HLA typing and disease association studies" presented during the XXIV EFI Congress in 2010 and awarded with the Best Poster Session Prize. This work was partially financially supported by the current research projects numbers 556 and 657 of the IRCCS Foundation Policlinico San Matteo of Pavia, Italy.

References

[1] S. Caillat-Zucman, "Molecular mechanisms of HLA association with autoimmune diseases," *Tissue Antigens*, vol. 73, no. 1, pp. 1–8, 2009.

[2] D. A. Brewerton, F. D. Hart, A. Nicholls, M. Caffrey, D. C. James, and R. D. Sturrock, "Ankylosing spondylitis and HL-A 27," *Lancet*, vol. 1, no. 7809, pp. 904–907, 1973.

[3] L. Schlosstein, P. I. Terasaki, R. Bluestone, and C. M. Pearson, "High association of an HL-A antigen, W27, with ankylosing spondylitis," *The New England Journal of Medicine*, vol. 288, no. 14, pp. 704–706, 1973.

[4] A. El Maghraoui, "Extra-articular manifestations of ankylosing spondylitis: prevalence, characteristics and therapeutic implications," *European Journal of Internal Medicine*, vol. 22, no. 6, pp. 554–560, 2011.

[5] J. H. M. Chang and D. Wakefield, "Uveitis: a global perspective," *Ocular Immunology and Inflammation*, vol. 10, no. 4, pp. 263–279, 2002.

[6] E. Antoniazzi, R. Guagliano, V. Meroni, S. Pezzotta, and P. E. Bianchi, "Ocular impairment of toxoplasmosis," *Parassitologia*, vol. 50, no. 1-2, pp. 35–36, 2008.

[7] L. Du, A. Kijlstra, and P. Yang, "Immune response genes in uveitis," *Ocular Immunology and Inflammation*, vol. 17, no. 4, pp. 249–256, 2009.

[8] H. Yazici, I. Fresko, and S. Yurdakul, "Behçet's syndrome: disease manifestations, management, and advances in treatment," *Nature Clinical Practice Rheumatology*, vol. 3, no. 3, pp. 148–155, 2007.

[9] T. I. Kaya, "Genetics of Behçet's disease," *Pathology Research International*, vol. 2012, Article ID 912589, 6 pages, 2012.

[10] J. Worthington, "Investigating the genetic basis of susceptibility to rheumatoid arthritis," *Journal of Autoimmunity*, vol. 25, supplement, pp. 16–20, 2005.

[11] R. Caporali, F. B. Pallavicini, M. Filippini et al., "Treatment of rheumatoid arthritis with anti-TNF-alpha agents: a reappraisal," *Autoimmunity Reviews*, vol. 8, no. 3, pp. 274–280, 2009.

[12] E. G. Favalli, R. Caporali, L. Sinigaglia et al., "Recommendations for the use of biologic therapy in rheumatoid arthritis: update from the Italian Society for Rheumatology II. Safety," *Clinical and Experimental Rheumatology*, vol. 29, no. 3, supplement 66, pp. 15–27, 2011.

[13] D. Ou, L. A. Mitchell, and A. J. Tingle, "HLA-DR restrictive supertypes dominate promiscuous T cell recognition: association of multiple HLA-DR molecules with susceptibility to autoimmune diseases," *Journal of Rheumatology*, vol. 24, no. 2, pp. 253–261, 1997.

[14] J. S. Fraser and P. J. Ciclitira, "Pathogenesis of coeliac disease: implications for treatment," *World Journal of Gastroenterology*, vol. 7, no. 6, pp. 772–776, 2001.

[15] R. Ciccocioppo, A. Di Sabatino, and G. R. Corazza, "The immune recognition of gluten in coeliac disease," *Clinical and Experimental Immunology*, vol. 140, no. 3, pp. 408–416, 2005.

[16] A. Di Sabatino and G. R. Corazza, "Coeliac disease," *The Lancet*, vol. 373, no. 9673, pp. 1480–1493, 2009.

[17] A. S. Louka and L. M. Sollid, "HLA in coeliac disease: unravelling the complex genetics of a complex disorder," *Tissue Antigens*, vol. 61, no. 2, pp. 105–117, 2003.

[18] M. M. Jahromi and G. S. Eisenbarth, "Cellular and molecular pathogenesis of type 1A diabetes," *Cellular and Molecular Life Sciences*, vol. 64, no. 7-8, pp. 865–872, 2007.

[19] D. Larizza, V. Calcaterra, C. Klersy et al., "Common immunogenetic profile in children with multiple autoimmune diseases: the signature of HLA-DQ pleiotropic genes," *Autoimmunity*, vol. 45, no. 6, pp. 470–475, 2012.

[20] F. Pociot and M. F. McDermott, "Genetics of type 1 diabetes mellitus," *Genes and Immunity*, vol. 3, no. 5, pp. 235–249, 2002.

[21] E. Thorsby and B. A. Lie, "HLA associated genetic predisposition to autoimmune diseases: genes involved and possible mechanisms," *Transplant Immunology*, vol. 14, no. 3-4, pp. 175–182, 2005.

[22] J. Sieper and M. Rudwaleit, "Early referral recommendations for ankylosing spondylitis (including pre-radiographic and radiographic forms) in primary care," *Annals of the Rheumatic Diseases*, vol. 64, no. 5, pp. 659–663, 2005.

[23] J. Deschenes, P. I. Murray, N. A. Rao, and R. B. Nussenblatt, "International Uveitis Study Group (IUSG): clinical classification of uveitis," *Ocular Immunology and Inflammation*, vol. 16, no. 1-2, pp. 1–2, 2008.

[24] International Team for the Revision of the International Criteria for Behcet's Disease, "Evaluation of the International Criteria for Behcet's disease (ICBD)," *Clinical and Experimental Rheumatology*, vol. 24, supplement 42, p. 13, 2006.

[25] L. Silva-Fernández, I. Castrejón, C. Bombardier, and L. Carmona, "Diagnostic and prognostic value of genetics in undifferentiated peripheral inflammatory arthritis: a systematic review," *Journal of Rheumatology*, vol. 38, supplement 87, pp. 38–44, 2011.

[26] A. Rostom, J. A. Murray, and M. F. Kagnoff, "American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease," *Gastroenterology*, vol. 131, no. 6, pp. 1981–2002, 2006.

[27] American Diabetes Association, "Standards of medical care in diabetes—2010," *Diabetes Care*, vol. 33, supplement 1, pp. S11–S61, 2010.

[28] C. Capittini, C. Tinelli, M. Guarene et al., "Possible KIR-driven genetic pressure on the genesis and maintenance of specific HLA-A,B haplotypes as functional genetic blocks," *Genes and Immunity*, vol. 13, no. 6, pp. 452–457, 2012.

[29] D. Groth, S. Hartmann, S. Klie, and J. Selbig, "Principal components analysis," *Methods in Molecular Biology*, vol. 930, pp. 527–547, 2013.

[30] M. T. Fiorillo, C. Rückert, M. Hülsmeyer et al., "Allele-dependent similarity between viral and self-peptide presentation by HLA-B27 subtypes," *Journal of Biological Chemistry*, vol. 280, no. 4, pp. 2962–2971, 2005.

[31] F. Mackensen, F. David, V. Schwenger et al., "HLA-DRB1*0102 is associated with TINU syndrome and bilateral, sudden-onset anterior uveitis but not with interstitial nephritis alone," *British Journal of Ophthalmology*, vol. 95, no. 7, pp. 971–975, 2011.

[32] C. Capittini, A. Pasi, P. Bergamaschi et al., "HLA haplotypes and birth weight variation: is your future going to be light or heavy?" *Tissue Antigens*, vol. 74, no. 2, pp. 156–163, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 501305, 8 pages
http://dx.doi.org/10.1155/2013/501305

Review Article

Clinical Genetic Testing of Periodic Fever Syndromes

Annalisa Marcuzzi,[1] Elisa Piscianz,[1] Giulio Kleiner,[1] Alberto Tommasini,[1] Giovanni Maria Severini,[1] Lorenzo Monasta,[1] and Sergio Crovella[1,2]

[1] *Laboratory of Immunopathology, Institute for Maternal and Child Health (IRCCS) "Burlo Garofolo", 34137 Trieste, Italy*
[2] *Department of Medical Sciences, University of Trieste, 34100 Trieste, Italy*

Correspondence should be addressed to Annalisa Marcuzzi; marcuzzi@burlo.trieste.it

Received 25 October 2012; Accepted 12 December 2012

Academic Editor: Ozgur Cogulu

Periodic fever syndromes (PFSs) are a wide group of autoinflammatory diseases. Due to some clinical overlap between different PFSs, differential diagnosis can be a difficult challenge. Nowadays, there are no universally agreed recommendations for most PFSs, and near half of patients may remain without a genetic diagnosis even after performing multiple-gene analyses. Molecular analysis of periodic fevers' causative genes can improve patient quality of life by providing early and accurate diagnosis and allowing the administration of appropriate treatment. In this paper we focus our discussion on effective usefulness of genetic diagnosis of PFSs. The aim of this paper is to establish how much can the diagnostic system improve, in order to increase the success of PFS diagnosis. The mayor expectation in the near future will be addressed to the so-called next generation sequencing approach. Although the application of bioinformatics to high-throughput genetic analysis could allow the identification of complex genotypes, the complexity of this definition will hardly result in a clear contribution for the physician. In our opinion, however, to obtain the best from this new development a rule should always be kept well in mind: use genetics only to answer specific clinical questions.

1. Introduction

Periodic fever syndromes (PFSs) represent a wide group of diseases characterized by recurrent attacks of apparently unprovoked inflammation and are thus considered among the so-called autoinflammatory diseases.

For the clinician, the question whether a patient suffers from a PFS usually arises after the evaluation and exclusion of more common clinical problems associated with fevers and inflammation, such as chronic infections, systemic autoimmune diseases, and paraneoplastic inflammatory conditions [1]. However, the pattern of associated clinical manifestations, the age at disease onset and, above all, the stereotypic recurrence of attacks can induce the suspicion of a PFS. A key to diagnose PFSs appears to simply be the consideration of its evenience [2, 3]: this can diminish the delay in time to diagnosis, avoiding in some cases repeated invasive and unsuccessful investigations performed to exclude more common multifactorial disorders.

A definite diagnosis is made easier today thanks to improved feasibility of genetic analysis for most PFSs: Familial Mediterranean Fever (FMF), Mevalonate Kinase Deficiency (MKD), Tumor Necrosis Factor Receptor-Associated Periodic Syndrome (TRAPS), and Familial Cold Autoinflammatory Syndromes (FCAS). Indeed, each of these diseases can be identified and diagnosed by the detection of mutations in specific genes (*MEFV*, *MVK*, *TFRSF1A*, and *NLRP3/NLRP12*, resp.) [4] (Table 1).

A genetic cause is not yet known for another PFS, called PFAPA (Periodic Fever, Aphthous stomatitis, Pharyngitis, and cervical Adenitis). This is a common and benign condition that can be easily diagnosed from clinical data, not requiring specialized laboratory investigations [5].

All these autoinflammatory diseases are characterized by recurrent flares of systemic inflammation, presenting sudden fever episodes associated with elevation of acute phase reactants and with a number of clinical manifestations that might include inflammation of serosal surfaces and joints,

Table 1: Genetic and clinical features of PFSs and correlated treatments.

Disease	*Gene* (Chromosome)	Transmission	Protein	Clinical manifestation	Treatment		
					Drugs	Biologics	Surgery
FMF	*MEVF* (16p13.3)	AR	Pyrin	Fever, serositis, sterile peritonitis, monoarthritis, pleuritis, and skin erythema	Colchicine	Anakinra Canakinumab Etanercept Infliximab Adalimumab	
MKD	*MVK* (12q24)	AR	Mevalonate kinase	Fever, lymphadenopathy, abdominal pain, and skin rash	Steroids	Etanercept Adalimumab Anakinra Canakinumab	
TRAPS	*TNFRSF1A* (12p13)	AD	p55 TNF-receptor	Prolonged fever, abdominal pain, erythematous macules, peritonitis, myalgias, arthralgias, periorbital oedema, and amyloidosis	High-dosage corticosteroids	Etanercept Infliximab Adalimumab Anakinra Tocilizumab	
NLRP-related diseases							
FCAS1	*NLRP3/CIAS1* (1q44)	AD	Cryopyrin	Fever, urticarial skin rash, arthralgia, and conjunctivitis		Anakinra Rilonacept, Canakinumab	
FCAS2	*NLRP12* (19q13)	AD	NLRP12	Fever, skin rash, lymphadenopathy, aphthous ulcers, and abdominal pain	Steroidal or nonsteroidal anti-inflammatory	Anakinra Rilonacept Canakinumab	
PFAPA	*ND*	ND	ND	Fever, pharyngitis, cervical adenitis, and aphthous stomatitis	Corticosteroids, colchicine		Tonsillectomy ± adenoidectomy

PFAPA: periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis; FMF: familial mediterranean fever; MKD: mevalonate kinase deficiency; NLRP: NOD-like receptor protein; CIAS: cold-induced autoinflammatory syndrome; FCAS: familial cold autoinflammatory syndrome; TRAPS: TNF-receptor-associated periodic syndrome; AR: autosomal recessive; AD: autosomal dominant; ND: not defined.

skin rashes of unknown origin, lymphadenopathy, arthritis, as well as the involvement of other organs such as muscles and the central nervous system [6]. Rheumatic manifestations are extremely common and highly variable in their presentation and course in PFSs. In most cases, however, the typical recurrence of attacks with symptoms-free intervals can help in differentiating the disorder from a chronic rheumatic disease. Furthermore, the pattern of musculoskeletal involvement and concomitant manifestations can help to formulate the correct diagnosis, which can be confirmed by genetic testing [1]. A family history of similar problems, when present, enforces the idea of a genetic origin of the disease.

Due to some clinical overlap between different PFSs, differential diagnosis can be a difficult challenge that can require sequential or simultaneous analysis of different genes. In fact, there are no universally agreed recommendations for most PFSs. Various attempts have been made in order to develop clinical criteria or guidelines to identify which of these patients should be considered for genetic testing [7, 8]. Databases such as INFEVERS (http://fmf.igh.cnrs.fr/ISSAID/infevers/) and the Eurofever Project (http://www.printo.it/eurofever/autoinflammatory_diseases.asp) have been developed to collect clinical and genetic information on patients with all monogenic autoinflammatory syndromes, including HPFs. This has led to improved recognition of the spectrum of clinical problems associated with these conditions as well as development of targeted treatment strategies [9].

In this paper, we focus our discussion on the effective usefulness of genetic diagnosis. Even if a molecular diagnosis is not always fundamental for the choice of an effective treatment, it is of great utility in the followup of patients, avoiding inadequate diagnostic procedures and focusing on the risk of specific complications, such as amyloidosis. The aim of this paper is to establish how much can the diagnostic system improve, in order to increase the success of PFS diagnosis.

2. Clinical Considerations and Treatment

Hereditary PFSs encompass a rare group of diseases that share lifelong recurrent episodes of inflammatory symptoms and an acute phase response. Periodic fevers are typically present in the pediatric population, but a disease onset in adult age is not a rare occurrence for some conditions [10].

This paper will focus on four hereditary PFSs, which include two autosomal recessive conditions, FMF and MKD, and a group of autosomal dominant diseases, including TRAPS and FCAS [11]. Moreover, in pediatrics, these pathologies have been distinguished from the more common and usually self-limiting PFAPA [11]. Other autoinflammatory syndromes, associated with fever, show a continuous inflammatory phenotype but not necessarily with a periodic feature. This category includes diseases such as MWS (Muckle-Wells Syndrome), CINCA (Chronic Infantile Neurologic Cutaneous and Arthritis) syndrome, PAPA (Pyogenic Arthritis, Pyoderma gangrenosum, and Acne), Behçet's disease, adult Still's disease, and SoJIA (Systemic-onset Juvenile Idiopathic Arthritis) syndrome.

Not all inherited PFSs are rare diseases and for most of them the prevalence can vary depending on the ethnology [8, 12]. As an example, FMF has a high prevalence, from 1 : 500 to 1 : 256 in different ethnic groups of non-Ashkenazi Jews [13], while MKD is a rare disease, and it is found predominantly in north-western Europe, with a carriage rate of MVK V377I of 1 : 350 [14]. Among the autosomal dominant PFSs, TRAPS is thought to be the most common form of dominant PFS in Europe [14].

The onset of symptoms occurs generally in childhood, usually in the first year of life for MKD and with more variability for other PFSs. Besides, the diagnosis of PFSs can be formulated later due to mild or unclear symptoms that overlap among different diseases and sometimes remain for long time misinterpreted or ignored [15]. In fact, the current approach for the diagnosis of PFSs in children may not be adequate for adults, in which the diseases are often underdiagnosed, and there is the need for specific diagnostic algorithms [16, 17].

The diagnosis of most periodic fevers in children is based on clinical history and physical examination. The management of the acute attacks is nonspecific and poorly standardized in PFSs. Similarly, long treatments are specifically designed to reduce the number and severity of the inflammatory attacks, as well as to prevent the development of amyloidosis.

2.1. Familial Mediterranean Fever (FMF). The FMF (MIM 249100) is the most frequent hereditary recessive PFS [18]. It predominantly affects populations from Mediterranean descent in which the frequency of carriers is high [19, 20]. In the vast majority of patients FMF becomes clinically apparent before the age of 20. Signs of painful serositis accompanying fever are the hallmark of the disease [21].

Acute attacks are characterized by a high-grade fever and inflammation of one or more serous membranes (peritoneum in 90% of cases, pleura, synovial membrane, tunica vaginalis, and pericardium). Fever usually lasts less than three days and in most cases a single pick is observed. There are usually no symptoms between acute attacks, although the joint involvement and myalgia may persist for several weeks.

Colchicine is the first-choice treatment for FMF, but a small percentage (5–10%) of patients does not show a complete response [22, 23] to this drug and needs additional treatments such as interleukin-1beta (IL-1β) inhibitors [24].

2.2. Mevalonate Kinase Deficiency (MKD). MKD (MIM 260920) is a PFS identified in 1984 [25]. The first clinical episode usually begins before the end of the first year of life. Attacks start with chills, followed by a sharp rise in body temperature accompanied by cervical lymphadenopathy and abdominal pain. Hepatomegaly, splenomegaly, arthralgia, skin rash, diarrhea, and vomiting are common symptoms [26]. Mean attack duration is 5–7 days. Intervals between attacks usually range from 4 to 8 weeks and tend to increase with age after adolescence.

Suspect of MKD is usually confirmed by elevated concentrations of mevalonic acid in urines during the flares and/or

by a decreased enzymatic activity of mevalonate kinase during asymptomatic intervals. The diagnosis is consequently defined by genetic testing of the *MVK* gene. A marked elevation of polyclonal immunoglobulin D is found in the serum, so that the disease is also called Hyper-IgD Syndrome (HIDS), but this is neither specific, as it can also be found in some patients with other PFSs, nor sensitive, as younger patients may have normal IgD values.

In MKD, steroids are administered during febrile attacks, but for some patients with long-lasting flares, the treatment becomes continuative. Other treatments (colchicine, cyclosporine, thalidomide, and statins) are of little benefits and biological drugs (anakinra and etanercept) have been used with some success as steroid-sparing agents [24]. The heterogeneous results obtained by novel biologic treatments [27, 28] remain unclear since the molecular events leading to inflammatory phenotype are still unknown, and a role of cell apoptosis has been recently proposed in the MKD pathogenesis [29, 30].

2.3. Tumor-Necrosis-Factor- (TNF-) Receptor-Associated Periodic Syndrome (TRAPS). TRAPS was first described in 1982 in a family of Irish and Scottish descent and was initially called "familial Hibernian fever" [31]. Since then, cases have been identified in other populations [32]. Mean age at onset is approximately 10 years. Each attack lasts few days to few weeks. Sometimes, in addition to fever, the attacks are characterized by severe abdominal pain, which may mimic a surgical emergency. Cutaneous manifestations are present in 87% of patients during attacks [33]. Most patients exhibit localized painful erythematous macules and patches that tend to migrate to the distal parts of the extremities.

High-dosage corticosteroids are administered during febrile attacks. Colchicine and immunosuppressant are ineffective. Etanercept, to prevent febrile attacks and to avoid long-term renal complications, is effective in a subgroup of patients, while IL-1β inhibitors are effective in most. On the contrary anti-TNF monoclonal antibodies (infliximab or adalimumab) have been shown to worsen the inflammatory condition [16].

2.4. NOD-Like-Receptor-Protein (NLRP-) Related Diseases. NLRP-related disease comprises phenotypically distinct autosomal dominant syndromes, such as NLRP3-associated Familial Cold Autoinflammatory Syndrome (also named FCAS1) and NLRP12 associated periodic fever (also named FCAS2).

2.4.1. Familial Cold Autoinflammatory Syndromes-1 (FCAS1). FCAS1, formerly known as familial cold urticaria, was first reported in 1940 [34], and, since then, only 20 families have been described worldwide. It is a rare autosomal dominant syndrome caused by a mutation in the NLRP3 gene, located on chromosome 1p44 that encodes a pyrin-like protein, known as cryopyrin [6]. There are three distinct clinical disorders related to NLRP3 mutations (FCAS1, MWS, and CINCA) that can now be seen as a single disorder with variable phenotypic expression [35–37].

FCAS1 is characterized by attacks of fever, urticarial skin rash, arthralgia, and conjunctivitis brought on by exposure to cold. An episode starts 2-3 hours after exposure and generally subsides within 24 hours. Attacks are accompanied by an intense acute phase response, as evidenced by high leukocyte counts in peripheral blood [38, 39].

In patients with FCAS1, treatment with IL-1β antagonists (i.e., anakinra) allows a complete response for almost all patients, but it has limited effects on hearing loss, bone dysplasia, and mental retardation [24].

2.4.2. Familial Cold Autoinflammatory Syndrome-2 (FCAS2). FCAS2 is a more recently characterized condition, firstly described in two families from Guadeloupe [37]. The disease is secondary to mutations in the *NLRP12* gene with an autosomal dominant inheritance. It was the first example of an NLRP capable of negatively regulating NF-κB activation [40], and it could be classified as a NF-κB activation disorder [41]. The phenotype is characterized by recurrent episodes of fever, secondary to cold exposure, associated with other signs, as skin rash, lymphadenopathy, aphthous ulcers, and abdominal pain [42]. A few patients, so far, have been presenting a good response to anti-inflammatory drugs, given at occurrence, while treatment with anakinra induced only an initial good response, that decreased over time [24].

2.5. Periodic Fever, Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis (PFAPA). PFAPA syndrome was firstly described in 1987 by Marshall et al. [43] and is characterized by episodes of fever, generally higher than 39°C, lasting for 3–6 days with recurrences every 3–8 weeks. Fever attack is associated with at least one of three main signs: aphthous stomatitis, cervical adenitis, and pharyngitis. The onset of the disease usually occurs before the age of 5 years and generally resolves by adolescence. Diagnosis is established on the basis of clinical criteria by which cardinal signs and symptoms must be carefully observed for a differential diagnosis [44, 45]. Periodic fever with tonsillitis can be part of the clinical phenotype also in some of the previous mentioned monogenic PFSs, even if these diagnoses should be considered only in the presence of atypical signs, such as clinical relapse after tonsillectomy, complaint of severe symptoms not referred to ear-nose and throat, and absent response to glucocorticoids [5].

Corticosteroids (prednisone, betamethasone) are successfully used in PFAPA syndrome during the febrile flares, as they can dramatically switch off fever in few hours. Other accompanying symptoms, however, take longer to resolve, and corticosteroid therapy sometimes can lead to shorten the interval between attacks. Colchicine, used as prevention of febrile attacks, can induce an increased interval between fever attacks but not a complete remission. Cimetidine, a histamine type 2 receptor blocker with immunomodulating properties, was shown to be effective in only one-third of patients [45]. Although the role of tonsillectomy is still controversial, it remains the most effective intervention for long-term resolution of PFAPA syndrome symptoms [44, 46].

3. Molecular Pathophysiology of PFSs

Recently, Masters et al. [41] proposed a classification scheme for the autoinflammatory disorder based on molecular mechanisms rather than on clinical classification. According to this scheme, PFSs were classified into six categories: (1) IL-1β activation disorders (inflammasomopathies), (2) nuclear factor (NF)-kappaB (NF-κB) activation syndromes, (3) protein misfolding disorders, (4) complement regulatory diseases, (5) disturbances of cytokine signaling, (6) macrophage activation syndromes. The PFSs share the dysregulation of NLR and TLR family genes in the development of the diseases.

The inflammasomopathies include intrinsic inflammasomopathies (such as FCAS1 [47, 48]), extrinsic inflammasomopathies (such as FMF [49, 50] and MKD [51, 52]), and Complex/Acquired Inflammasomopathies. This category was characterized by an activation of NLRP3 (originally denoted cryopyrin or CIAS1) that processes pro-IL-1β into an active form, mature IL-1β. The difference between intrinsic and extrinsic forms depends on whether the mutation is on NLRP3 constituents complex or on proteins that regulate the production of IL-1β, respectively.

The NF-κB activation syndromes are mediated predominantly by improper regulation of NF-κB within the innate immune system (i.e., FCAS2). In NF-κB/FCAS2, although clinically similar to FCAS1, the mutational screening in NLRP3 and other known periodic fever genes is negative [37]. A subsequent examination of NLRP12 (NALP12, PYPAF7, and MONARCH-1), chosen because of its similarity to NLRP3 and because of its expression in myelomonocytic cells, revealed dominantly inherited nonsense and splice-site mutations in the two families. Moreover, NLRP12 was the first example of an NLR protein capable of negatively regulating NF-κB activation [40]. It is not known whether NLRP12 can participate in an inflammasome complex regulating IL-1β production.

The TRAPS syndrome instead is an example of a protein misfolding disorder. This kind of disorder is caused by multiple mechanisms leading to several problems, that can vary from misfolding of proteins to production of proinflammatory cytokines by innate immune cells [53].

Finally, there are a number of disorders that appear clearly to be autoinflammatory, but for which there are insufficient data to place them into one of the six categories. An example is the PFAPA, which is not inherited as a mendelian trait, but does exhibit some familial tendency [54, 55]. While the exact cellular determinants and pathogenic molecular mediators remain elusive, the clinical picture and cytokine profile of PFAPA outline a very complex, heterogeneous, but nevertheless autoinflammatory, disease [55].

4. Genetic Testing

Molecular analysis of periodic fevers' causative genes can dramatically improve patient quality of life by allowing early and accurate diagnosis and the administration of appropriate treatments.

However, the molecular genetic analysis of these diseases based solely on the candidate gene has low efficiency (close to 20%) and is time consuming and expensive. Moreover, since most of the causative mutations are located in specific regions of the genes mainly involved in these disorders, only some exons are analyzed for genetic diagnosis [56, 57] (Figure 1). The greatest difficulty is to decide which gene should be first screened, based on patient's clinical features. This is true in particular for patients with low penetrance mutations in whom the clinical manifestations of the disorders can show wide overlapping. Aimed at ameliorating the efficiency of the genetic diagnosis, a set of clinical parameters that can predict the probability of carrying mutations in one of the genes associated with hereditary autoinflammatory syndromes, has been proposed by Gattorno et al. [58]. In particular, the study allowed to identify some clinical variables (family history, age of onset, presence of abdominal, and chest pain, diarrhea) that are strongly associated with the probability of detecting relevant mutations in known PFS genes, thus also suggesting the order in which the genes should be screened. This diagnostic score revealed high sensitivity (87%) and good specificity (72%) for the identification of genetically positive and negative patients. Moreover, Federici et al. [59] performed a similar study in a large group of adult patients screened for three genes (*MEFV*, *MVK*, and *TNFRSF1A*) and obtained results comparable with the study carried out on children.

Unfortunately, despite the application of this validated diagnostic system, literature data support the evidence that PFSs are underdiagnosed or not correctly classified. As reported by Lainka et al. [60], in Germany FMF is the most frequent PFS, but its incidence is low and, at present, genetic testing should be considered after an accurate clinical history including ethnic ancestry and family history [60, 61]. Besides, the overlap and the wide range of clinical signs shared by different PFSs and/or other diseases may cause a delayed or incorrect diagnosis [62]. In these cases, clinicians can be supported in a faster and correct identification by appropriate genetic tests.

An ordinary medium-throughput genotyping method based on a 96-well sequencing plate can also be developed to analyze the most frequently PFS causing genes by automating several processes required for direct sequencing, using a commercially available robotic systems and routinely used instruments [63]. This medium-throughput genotyping method may improve diagnostic success in patients with overlapping phenotype but cannot completely solve all the lacks of correct evaluation that still exist in PFS diagnostics.

High-throughput DNA sequencing (HTS) could be also used for sequencing patients' whole genomes or targeted regions such as all exonic regions (i.e., the exome) [64]. The objective is the identification of genetic variants such as single nucleotide polymorphisms (SNPs) that can influence the clinical phenotype. The extraction of SNPs from the raw genetic sequences involves many processing steps and the application of a diverse set of tools, and thus its use is mainly limited to research [65]. These second-generation sequencing technologies, including resequencing microarray, allow for multiple gene tests in a single experiment and thus hold considerable promise for routine molecular diagnosis of heterogeneous disorders [66]. Recent data confirmed the key

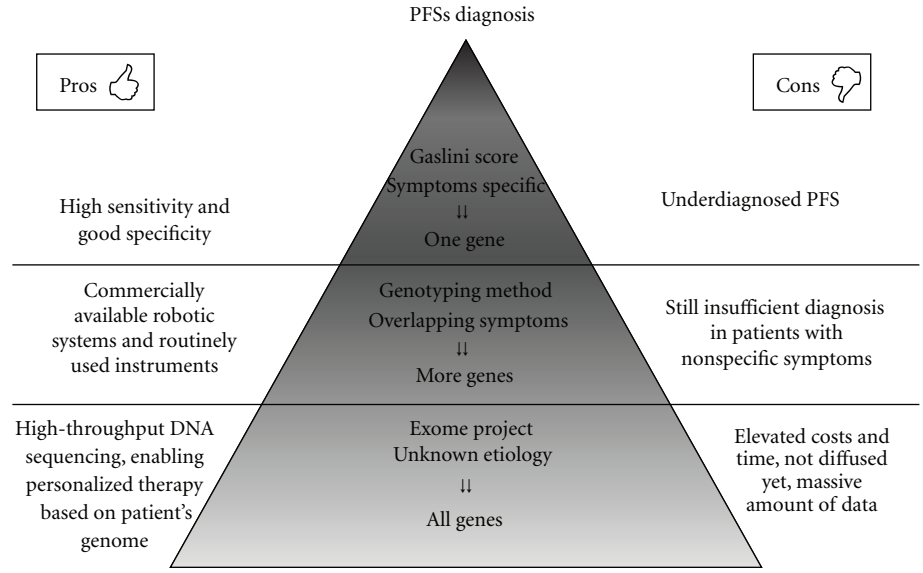


Figure 1: Schematic representation of the possible diagnostic methods for periodic fever syndromes.

role of this technology in the diagnosis of autoinflammatory diseases [67].

The expectation is that, in the very near future, this technology will enable us to identify all the variants in an individual's personal genome and, in particular, clinically relevant alleles. Beyond this, whole genome sequencing is also expected to bring a major shift in clinical practice in terms of diagnosis and understanding of diseases, ultimately enabling personalized medicine based on one's genome [68]. However, increasing the potential of genetics can bring to the attention new problems linked to the right interpretation of a huge amount of data.

5. Conclusions

PFSs are increasingly recognized in current clinical practice. This is probably due to a diminished clinical impact of infections at present times and to a wider awareness of the problem among physicians. PFAPA is by far the most common of PFSs and in most cases it can be easily diagnosed on the basis of the typical clinical presentation, usually in young children and with complaints mainly limited to pharynx, mouth, and neck. On the contrary, atypical PFAPA and other kinds of periodic fever can be a diagnostic challenge, even for experienced physicians. In some cases, the clinical picture may suggest a specific PFS, and the diagnosis can be confirmed on the basis of specific clinical features, response to drugs, and ultimately molecular analyses. However, patients may present incomplete phenotypes that can be compatible with different PFSs, making it difficult for the choice of the correct analysis to perform. In such cases, the analysis of a set of different genes, thanks to the availability of new technological platforms, can help the clinician. Near half of patients may remain without a genetic diagnosis even after performing multiple-gene analysis. In few of these cases, high-throughput genetic analysis will probably allow to identify novel monogenic disorders, but it is reasonable to hypothesize that a consistent percentage of patients will still remain without a definite diagnosis. The application of bioinformatics to high throughput genetic analysis could allow the identification of complex genotypes, but the complexity of this definition will hardly result in a clear contribution for the physician.

In conclusion, great improvements have been obtained in recent years in the field of PFSs thanks to the identification of a genetic cause for different disorders, and novel knowledge, including novel doubts, will come from high-throughput molecular analysis. In our opinion, however, to obtain the best from these new developments, a rule should always be kept in mind: use genetics only to answer specific clinical questions.

Authors' Contribution

The first two authors equally contributed to this study.

References

[1] I. Touitou and I. Koné-Paut, "Autoinflammatory diseases," *Best Practice and Research*, vol. 22, no. 5, pp. 811–829, 2008.

[2] A. Soriano and R. Manna, "Familial Mediterranean fever: new phenotypes," *Autoimmunity Reviews*, vol. 12, no. 1, pp. 31–37, 2012.

[3] L. Cantarini, O. M. Lucherini, B. Frediani et al., "Bridging the gap between the clinician and the patient with cryopyrin-associated periodic syndromes," *International Journal of Immunopathology and Pharmacology*, vol. 24, no. 4, pp. 827–836, 2011.

[4] P. J. Hashkes and O. Toker, "Autoinflammatory syndromes," *Pediatric Clinics of North America*, vol. 59, no. 2, pp. 447–470, 2012.

[5] K. T. Thomas, H. M. Feder Jr., A. R. Lawton, and K. M. Edwards, "Periodic fever syndrome in children," *Journal of Pediatrics*, vol. 135, no. 1, pp. 15–21, 1999.

[6] S. Padeh, "Periodic fever syndromes," *Pediatric Clinics of North America*, vol. 52, no. 2, pp. 577–609, 2005.

[7] M. Piram, J. Frenkel, M. Gattorno et al., "A preliminary score for the assessment of disease activity in hereditary recurrent fevers: results from the AIDAI (Auto-Inflammatory Diseases Activity Index) consensus conference," *Annals of the Rheumatic Diseases*, vol. 70, no. 2, pp. 309–314, 2011.

[8] N. Toplak, J. Frenkel, S. Ozen et al., "An international registry on autoinflammatory diseases: the Eurofever experience," *Annals of the Rheumatic Diseases*, vol. 71, no. 7, pp. 1177–1182, 2012.

[9] S. Savic, L. J. Dickie, M. Wittmann, and M. F. McDermott, "Autoinflammatory syndromes and cellular responses to stress: pathophysiology, diagnosis and new treatment perspectives," *Best Practice & Research Clinical Rheumatology*, vol. 26, no. 4, pp. 505–533, 2012.

[10] L. J. Dickie, S. Savic, A. Aziz, M. Sprakes, and M. F. McDermott, "Periodic fever syndrome and autoinflammatory diseases," *F1000 Medicine Reports*, vol. 2, no. 1, article 3, 2010.

[11] M. Gattorno, R. Caorsi, A. Meini et al., "Differentiating PFAPA syndrome from monogenic periodic fevers," *Pediatrics*, vol. 124, no. 4, pp. e721–e728, 2009.

[12] E. Ben-Chetrit and I. Touitou, "Familial mediterranean fever in the world," *Arthritis Care and Research*, vol. 61, no. 10, pp. 1447–1453, 2009.

[13] E. Ben-Chetrit and M. Levy, "Familial Mediterranean fever," *The Lancet*, vol. 351, no. 9103, pp. 659–664, 1998.

[14] H. J. Lachmann, "Clinical immunology review series: an approach to the patient with a periodic fever syndrome," *Clinical and Experimental Immunology*, vol. 165, no. 3, pp. 301–309, 2011.

[15] M. Sayarlioglu, A. Cefle, M. Inanc et al., "Characteristics of patients with adult-onset familial Mediterranean fever in Turkey: analysis of 401 cases," *International Journal of Clinical Practice*, vol. 59, no. 2, pp. 202–205, 2005.

[16] I. Muscari, F. Iacoponi, L. Cantarini et al., "The diagnostic evaluation of patients with potential adult-onset autoinflammatory disorders: our experience and review of the literature," *Autoimmunity Reviews*, vol. 12, no. 1, pp. 10–13, 2012.

[17] L. Cantarini, F. Iacoponi, O. M. Lucherini et al., "Validation of a diagnostic score for the diagnosis of autoinflammatory diseases in adults," *International Journal of Immunopathology and Pharmacology*, vol. 24, no. 3, pp. 695–702, 2011.

[18] D. L. Kastner, "Familial mediterranean fever: the genetics of inflammation," *Hospital Practice*, vol. 33, no. 4, pp. 131–134, 139–140, 143–146, 1998.

[19] I. Touitou, T. Sarkisian, M. Medlej-Hashim et al., "Country as the primary risk factor for renal amyloidosis in familial Mediterranean fever," *Arthritis & Rheumatism*, vol. 56, no. 5, pp. 1706–1712, 2007.

[20] M. La Regina, G. Nucera, M. Diaco et al., "Familial Mediterranean fever is no longer a rare disease in Italy," *European Journal of Human Genetics*, vol. 11, no. 1, pp. 50–56, 2003.

[21] J. C. H. van der Hilst, A. Simon, and J. P. H. Drenth, "Hereditary periodic fever and reactive amyloidosis," *Clinical and Experimental Medicine*, vol. 5, no. 3, pp. 87–98, 2005.

[22] M. Lidar, J. M. Scherrmann, Y. Shinar et al., "Colchicine nonresponsiveness in familial Mediterranean fever: clinical, genetic, pharmacokinetic, and socioeconomic characterization," *Seminars in Arthritis and Rheumatism*, vol. 33, no. 4, pp. 273–282, 2004.

[23] G. Guz, M. Kanbay, and M. A. Ozturk, "Current perspectives on familial mediterranean fever," *Current Opinion in Infectious Diseases*, vol. 22, no. 3, pp. 309–315, 2009.

[24] R. Caorsi, S. Federici, and M. Gattorno, "Biologic drugs in autoinflammatory syndromes," *Autoimmunity Reviews*, vol. 12, no. 1, pp. 81–86, 2012.

[25] J. W. M. van der Meer, J. M. Vossen, and J. Radl, "Hyperimmunoglobulinemia D and periodic fever: a new syndrome," *The Lancet*, vol. 1, no. 8386, pp. 1087–1090, 1984.

[26] J. P. H. Drenth, C. J. Haagsma, J. W. M. van der Meer et al., "Hyperimmunoglobulinemia D and periodic fever syndrome. The clinical spectrum in a series of 50 patients," *Medicine*, vol. 73, no. 3, pp. 133–144, 1994.

[27] M. Cailliez, F. Garaix, C. Rousset-Rouvière et al., "Anakinra is safe and effective in controlling hyperimmunoglobulinaemia D syndrome-associated febrile crisis," *Journal of Inherited Metabolic Disease*, vol. 29, no. 6, p. 763, 2006.

[28] J. C. H. van der Hilst, E. J. Bodar, K. S. Barron et al., "Long-term follow-up, clinical features, and quality of life in a series of 103 patients with hyperimmunoglobulinemia D syndrome," *Medicine*, vol. 87, no. 6, pp. 301–310, 2008.

[29] A. Marcuzzi, E. Piscianz, M. Girardelli, S. Crovella, and A. Pontillo, "Defect in mevalonate pathway induces pyroptosis in Raw 264.7 murine monocytes," *Apoptosis*, vol. 16, no. 9, pp. 882–888, 2011.

[30] A. Marcuzzi, V. Zanin, E. Piscianz et al., "Lovastatin-induced apoptosis is modulated by geranylgeraniol in a neuroblastoma cell line," *International Journal of Developmental Neuroscience*, vol. 30, no. 6, pp. 451–456, 2012.

[31] L. M. Williamson, D. Hull, R. Mehta, W. G. Reeves, B. H. Robinson, and P. J. Toghill, "Familial hibernian fever," *The Quarterly Journal of Medicine*, vol. 51, no. 204, pp. 469–480, 1982.

[32] M. F. McDermott, I. Aksentijevich, J. Galon et al., "Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes," *Cell*, vol. 97, no. 1, pp. 133–144, 1999.

[33] J. R. Toro, I. Aksentijevich, K. Hull, J. Dean, and D. L. Kastner, "Tumor necrosis factor receptor-associated periodic syndrome: a novel syndrome with cutaneous manifestations," *Archives of Dermatology*, vol. 136, no. 12, pp. 1487–1494, 2000.

[34] V. J. Derbes and W. P. Coleman, "Familial cold urticaria," *Annals of Allergy*, vol. 30, no. 6, pp. 335–341, 1972.

[35] H. M. Hoffman, J. L. Mueller, D. H. Broide, A. A. Wanderer, and R. D. Kolodner, "Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome," *Nature Genetics*, vol. 29, no. 3, pp. 301–305, 2001.

[36] J. Feldmann, A. M. Prieur, P. Quartier et al., "Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in *CIAS1*, a gene highly expressed in polymorphonuclear cells and chondrocytes," *American Journal of Human Genetics*, vol. 71, no. 1, pp. 198–203, 2002.

[37] I. Jéru, P. Duquesnoy, T. Fernandes-Alnemri et al., "Mutations in *NALP12* cause hereditary periodic fever syndromes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 5, pp. 1614–1619, 2008.

[38] H. M. Doeglas and E. Bleumink, "Familial cold urticaria. Clinical findings," *Archives of Dermatology*, vol. 110, no. 3, pp. 382–388, 1974.

[39] H. M. Hoffman, A. A. Wanderer, and D. H. Broide, "Familial cold autoinflammatory syndrome: phenotype and genotype of an autosomal dominant periodic fever," *Journal of Allergy and Clinical Immunology*, vol. 108, no. 4, pp. 615–620, 2001.

[40] K. L. Williams, J. D. Lich, J. A. Duncan et al., "The CATERPILLER protein Monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor α-, and *Mycobacterium tuberculosis*-induced pro-inflammatory signals," *Journal of Biological Chemistry*, vol. 280, no. 48, pp. 39914–39924, 2005.

[41] S. L. Masters, A. Simon, I. Aksentijevich, and D. L. Kastner, "Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease," *Annual Review of Immunology*, vol. 27, pp. 621–668, 2009.

[42] S. Borghini, S. Tassi, S. Chiesa et al., "Clinical presentation and pathogenesis of cold-induced autoinflammatory disease in a family with recurrence of an NLRP12 mutation," *Arthritis & Rheumatism*, vol. 63, no. 3, pp. 830–839, 2011.

[43] G. S. Marshall, K. M. Edwards, J. Butler, and A. R. Lawton, "Syndrome of periodic fever, pharyngitis, and aphthous stomatitis," *Journal of Pediatrics*, vol. 110, no. 1, pp. 43–46, 1987.

[44] S. Peridis, G. Pilgrim, E. Koudoumnakis, I. Athanasopoulos, M. Houlakis, and K. Parpounas, "PFAPA syndrome in children: a meta-analysis on surgical versus medical treatment," *International Journal of Pediatric Otorhinolaryngology*, vol. 74, no. 11, pp. 1203–1208, 2010.

[45] G. Vigo and F. Zulian, "Periodic fevers with aphthous stomatitis, pharyngitis, and adenitis (PFAPA)," *Autoimmunity Reviews*, vol. 12, no. 1, pp. 52–55, 2012.

[46] W. Garavello, M. Romagnoli, and R. M. Gaini, "Effectiveness of adenotonsillectomy in PFAPA syndrome: a randomized study," *Journal of Pediatrics*, vol. 155, no. 2, pp. 250–253, 2009.

[47] F. S. Sutterwala, Y. Ogura, M. Szczepanik et al., "Critical role for NALP3/CIAS1/cryopyrin in innate and adaptive immunity through its regulation of caspase-1," *Immunity*, vol. 24, no. 3, pp. 317–327, 2006.

[48] M. Saito, R. Nishikomori, N. Kambe et al., "Disease-associated *CIAS1* mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients," *Blood*, vol. 111, no. 4, pp. 2132–2141, 2008.

[49] J. W. Yu, J. Wu, Z. Zhang et al., "Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization," *Cell Death and Differentiation*, vol. 13, no. 2, pp. 236–249, 2006.

[50] E. Mansfield, J. J. Chae, H. D. Komarow et al., "The familial Mediterranean fever protein, pyrin, associates with microtubules and colocalizes with actin filaments," *Blood*, vol. 98, no. 3, pp. 851–859, 2001.

[51] A. Marcuzzi, S. Crovella, L. Monasta, L. V. Brumatti, M. Gattorno, and J. Frenkel, "Mevalonate kinase deficiency: disclosing the role of mevalonate pathway modulation in inflammation," *Current Pharmaceutical Design*, vol. 18, no. 35, pp. 5746–5752, 2012.

[52] V. Zanin, A. Marcuzzi, E. Piscianz et al., "The effect of clodronate on a mevalonate kinase deficiency cellular model," *Inflammation Research*, vol. 61, no. 12, pp. 1363–1367, 2012.

[53] K. M. Hull, E. Drewe, I. Aksentijevich et al., "The TNF receptor-associated periodic syndrome (TRAPS): emerging concepts of an autoinflammatory disorder," *Medicine*, vol. 81, no. 5, pp. 349–368, 2002.

[54] P. Anton-Martin, R. O. Movilla, S. G. Martin et al., "PFAPA syndrome in siblings. Is there a genetic background?" *European Journal of Pediatrics*, vol. 170, no. 12, pp. 1563–1568, 2011.

[55] L. Kolly, N. Busso, A. von Scheven-Gete et al., "Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis syndrome is linked to dysregulated monocyte IL-1beta production ," *The Journal of Allergy and Clinical Immunology*. In press.

[56] A. D'Osualdo, P. Picco, F. Caroli et al., "MVK mutations and associated clinical features in Italian patients affected with autoinflammatory disorders and recurrent fever," *European Journal of Human Genetics*, vol. 13, no. 3, pp. 314–320, 2005.

[57] A. D'Osualdo, F. Ferlito, I. Prigione et al., "Neutrophils from patients with *TNFRSF1A* mutations display resistance to tumor necrosis factor-induced apoptosis: pathogenetic and clinical implications," *Arthritis & Rheumatism*, vol. 54, no. 3, pp. 998–1008, 2006.

[58] M. Gattorno, M. P. Sormani, A. D'Osualdo et al., "A diagnostic score for molecular analysis of hereditary autoinflammatory syndromes with periodic fever in children," *Arthritis & Rheumatism*, vol. 58, no. 6, pp. 1823–1832, 2008.

[59] L. Federici, C. Rittore-Domingo, I. Koné-Paut et al., "A decision tree for genetic diagnosis of hereditary periodic fever in unselected patients," *Annals of the Rheumatic Diseases*, vol. 65, no. 11, pp. 1427–1432, 2006.

[60] E. Lainka, U. Neudorf, P. Lohse et al., "Familial Mediterranean fever in Germany: epidemiological, clinical, and genetic characteristics of a pediatric population," *European Journal of Pedaitrics*, vol. 171, no. 12, pp. 1775–1785, 2012.

[61] D. Tchernitchko, S. Moutereau, M. Legendre et al., "MEFV analysis is of particularly weak diagnostic value for recurrent fevers in western European Caucasian patients," *Arthritis & Rheumatism*, vol. 52, no. 11, pp. 3603–3605, 2005.

[62] K. Krause, C. E. Grattan, C. Bindslev-Jensen et al., "How not to miss autoinflammatory diseases masquerading as urticaria," *Allergy*, vol. 67, no. 12, pp. 1465–1474, 2012.

[63] B. Bortot, E. Athanasakis, F. Brun et al., "High-throughput genotyping robot-assisted method for mutation detection in patients with hypertrophic cardiomyopathy," *Diagnostic Molecular Pathology*, vol. 20, no. 3, pp. 175–179, 2011.

[64] T. J. Dixon-Salazar, J. L. Silhavy, N. Udpa et al., "Exome sequencing can improve diagnosis and alter patient management," *Science Translational Medicine*, vol. 4, no. 138, Article ID 138ra178, 2012.

[65] A. Altmann, P. Weber, D. Bader, M. Preuss, E. B. Binder, and B. Muller-Myhsok, "A beginners guide to SNP calling from high-throughput DNA-sequencing data," *Human Genetics*, vol. 131, no. 10, pp. 1541–1554, 2012.

[66] P. Kothiyal, S. Cox, J. Ebert, B. J. Aronow, J. H. Greinwald, and H. L. Rehm, "An overview of custom array sequencing," in *Current Protocols in Human Genetics*, Unit 7, chapter 7, p. 17, 2009.

[67] Q. Zhou, G. S. Lee, J. Brady et al., "A hypermorphic missense mutation in PLCG2, encoding phospholipase Cγ2, causes a dominantly inherited autoinflammatory disease with immunodeficiency," *American Journal of Human Genetics*, vol. 91, no. 4, pp. 713–720, 2012.

[68] R. Thompson, C. J. Drew, and R. H. Thomas, "Next generation sequencing in the clinical domain: clinical advantages, practical, and ethical challenges," *Advances in Protein Chemistry and Structural Biology*, vol. 89, pp. 27–63, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 129715, 7 pages
http://dx.doi.org/10.1155/2013/129715

Research Article

HOXA4 Gene Promoter Hypermethylation as an Epigenetic Mechanism Mediating Resistance to Imatinib Mesylate in Chronic Myeloid Leukemia Patients

Marjanu Hikmah Elias,[1] Abdul Aziz Baba,[2] Azlan Husin,[2] Sarina Sulong,[1] Rosline Hassan,[3] Goh Ai Sim,[4] S. Fadilah Abdul Wahid,[5] and Ravindran Ankathil[1]

[1] *Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Health Campus 16150 Kubang Kerian, Kelantan, Malaysia*
[2] *Haemato-Oncology Unit, Department of Internal Medicine, School of Medical Sciences, Universiti Sains Malaysia, Health Campus 16150 Kubang Kerian, Kelantan, Malaysia*
[3] *Hematology Department, School of Medical Sciences, Universiti Sains Malaysia, Health Campus 16150 Kubang Kerian, Kelantan, Malaysia*
[4] *Hospital Pulau Pinang, Malaysia*
[5] *Medicine Department & Cell Therapy Centre, UKM Medical Centre, Malaysia*

Correspondence should be addressed to Ravindran Ankathil; rankathil@hotmail.com

Received 4 October 2012; Accepted 26 November 2012

Academic Editor: Gokce A. Toruner

Development of resistance to imatinib mesylate (IM) in chronic myeloid leukemia (CML) patients has emerged as a significant clinical problem. The observation that increased epigenetic silencing of potential tumor suppressor genes correlates with disease progression in some CML patients treated with IM suggests a relationship between epigenetic silencing and resistance development. We hypothesize that promoter hypermethylation of *HOXA4* could be an epigenetic mechanism mediating IM resistance in CML patients. Thus a study was undertaken to investigate the promoter hypermethylation status of *HOXA4* in CML patients on IM treatment and to determine its role in mediating resistance to IM. Genomic DNA was extracted from peripheral blood samples of 95 CML patients (38 good responders and 57 resistant) and 12 normal controls. All samples were bisulfite treated and analysed by methylation-specific high-resolution melt analysis. Compared to the good responders, the *HOXA4* hypermethylation level was significantly higher ($P = 0.002$) in IM-resistant CML patients. On comparing the risk, *HOXA4* hypermethylation was associated with a higher risk for IM resistance (OR 4.658; 95% CI, 1.673–12.971; $P = 0.003$). Thus, it is reasonable to suggest that promoter hypermethylation of *HOXA4* gene could be an epigenetic mechanism mediating IM resistance in CML patients.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that comprises 14% of all leukemias. The molecular pathogenesis of CML involves the clonal expansion of pluripotent haematopoietic stem cells containing the *BCR-ABL* fusion oncogene. *BCR-ABL* gene results from a reciprocal translocation between chromosome 9 and 22 to form the Philadelphia chromosome [1]. This *BCR-ABL* fusion gene codes for a p210 kD protein with increased tyrosine kinase activity. Imatinib mesylate (IM) or Glivec (NOVARTIS Pharma) is a selective molecular inhibitor of the BCR-ABL oncogene protein and permits long term disease control in about two thirds of chronic phase CML patients [2]. IM has dramatically improved the treatment of CML and is generally considered as frontline therapy for CML patients. Despite its striking efficacy, development of resistance in significant proportion of CML patients on IM therapy has emerged as a major clinical problem affecting both patients and treating physicians.

Various mechanisms of resistance and suboptimal response to IM have been described, involving *BCR-ABL1*-dependent and *BCR-ABL1*-independent pathways [3, 4]. *BCR-ABL1*-dependent mechanism usually involves point mutations in the tyrosine kinase domain (TKD) and amplification of *BCR-ABL* gene, with mutations in the *BCR-ABL* tyrosine kinase domain being better characterized [5]. Our previous study on *BCR-ABL* TKD mutation analysis showed that *BCR-ABL* mutations accounted for IM resistance in only 21.7% of Malaysian CML patients on IM therapy (communicated separately; in Press). This indicated that *BCR-ABL* mutations are not the only cause for relapse and resistance. It is presumed that the mechanisms of IM resistance in CML patients who do not have TKD mutation might be mediated through *BCR-ABL*-independent pathways. However, the exact mechanism in *BCR-ABL*-independent pathway still remains unclear, despite several genetic and epigenetic mechanisms postulated to be involved in the *BCR-ABL*-independent pathway.

It is largely known that DNA in cancer cells is very unstable. Epigenetic silencing is a phenomenon whereby gene transcript maybe suppressed through DNA methylation. Gene expression can be strongly modified through epigenetic alteration such as DNA hypo or hypermethylation. DNA methylation at cytosine residues in gene promoter CpG sequences is known to inhibit gene transcription, resulting in decreased protein expression. Genomic instability and DNA modifications certainly confer to the cancer cells, a higher capability of becoming resistant [6]. The human Homeobox (*HOX*) gene network encodes master regulators in haematopoiesis and DNA methylation has been implicated to have an important role in aberrant control of *HOX* gene expression [7]. Inappropriate expression of *HOX* gene has been implicated in development of hematopoietic malignancies. Methylation of a *HOX* gene, *HOXA4* has been strongly associated with progression to blast crisis and poor response to treatment in other types of leukemia patients [7].

In CML, increased epigenetic silencing of potential tumor suppressor genes has been found to be correlated with disease progression in a small proportion of patients treated with Imatinib [8]. This suggests the possibility of a relationship between epigenetic silencing and development of IM resistance. Few studies have suggested that hypermethylation might play a role in disease progression in CML. It could be plausible that changes in gene silencing by DNA methylation might play a role in developing alternative routes for cells to circumvent the effects of IM. We hypothesized that promoter hypermethylation of *HOXA4* could be an epigenetic mechanism which mediate resistance to IM in CML patients. This study was designed to test this hypothesis.

2. Methodology

2.1. Patient Samples and Control. The study was undertaken at Hospital Universiti Sains Malaysia, after getting approval from the Research and Ethics Committee of University Sains Malaysia and Ministry of Health (MOH), Malaysia (NMRR-10-1206-7127). A total of 95 Malaysian CML patients during their treatment with IM were enrolled. The patients selected were Philadelphia chromosome positive CML patients in chronic, accelerated, or blast phase, treated for at least 12 months, with IM (400 mg and 600 mg, resp.) on frontline treatment. These CML patients were categorized into IM resistant and IM good responders based on their molecular and/or cytogenetic response. IM-resistant patients were defined as those CML patients showing less than complete cytogenetic response by 12 months and/or lack of attainment of major molecular response by 18 months after initiation of therapy. Secondary resistance was defined as loss of complete cytogenetic response and/or loss of major molecular response.

Three millilitres of peripheral blood from each patient was collected in EDTA tube. Additionally, blood samples from 12 normal healthy controls were also collected and included for analysis. Universal methylated DNA and unmethylated DNA (ZYMO research, USA) were used as 100% and 0% methylation DNA control, respectively. Both types of the DNA were modified with bisulfite treatment and was subsequently mixed according to the ratio of 10%, 25%, 50%, and 75%. This serial methylation percentage was included in each experimental run.

2.2. Genomic DNA Extraction and Sodium Bisulfite Treatment. The genomic DNA of all patients and controls was isolated using the GENTRA PUREGENE Blood Kit (Qiagen, Germany) according to the supplier's recommendation. DNA quantity was identified spectrophotometrically by using NanoQuant Infinite M200 (Tecan, Switzerland) and the quality of the DNA was confirmed by agarose gel electrophoresis using 1% agarose gel.

After extraction of genomic DNA, 500 ng of the DNA was subjected to bisulfite treatment utilizing the EZ DNA Methylation-Gold Kit (ZYMO Research, USA) following manufacturer's recommendation. Besides the patient samples, universal methylated DNA and unmethylated DNA were also treated with bisulfite using the same kit. Before mixing the methylated and unmethylated controls into 10%, 25%, 50%, and 75% percentages, the concentration of bisulfite treated DNA control samples were carefully measured at a value of 40 μg/mL for Ab_{260} = 1.0 (the wavelength used corresponds to RNA wavelength as the recovered bisulfite-treated DNA was single stranded with limited non-specific base-pairing at room temperature) (ZYMO research, USA). The concentration of the eluted bisulfite treated DNA samples of all patients was also measured and the final concentration used was 20 ng for MS-HRM analysis.

2.3. Primer Design. Primers were designed based on criteria stated by Wojdacz et al. [9] with some additional modifications using the Methyl Primer Express v1.0 Software (Applied Biosystem, USA). While designing the primers, the following points were considered. The primers should amplify 100 to 150 bp PCR product with only one CpG dinucleotides each, as more CpG dinucleotide in the primer sequence was found to promote bias amplification towards the methylated template. The primers also should amplify both methylated and unmethylated sequence simultaneously

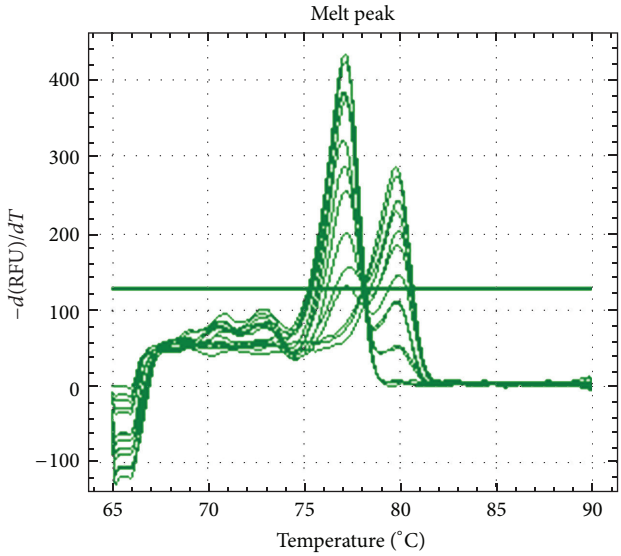


FIGURE 1: Derivative Melt peak of the serial percentage of methylation control produced two specific peaks which represent the unmethylated (approximately 77°C) and methylated (approximately 80°C) PCR product. Fully unmethylated sample produced only unmethylated peak, 100% methylated sample produced only methylated peak and samples with mixture of unmethylated, and methylated displayed both peaks.

(Figure 1). The CpG in the primers should not be at 3′-end, and preferably, it should be placed as close as possible to the 5′-end of the primer. However, CpG situated in the middle (at least 5th nucleotide from the 3′-end) could also be acceptable provided it could produce good melt curve differentiation [10]. To ensure that the bisulfite converted DNA specific amplification and to prevent amplification of unconverted DNA template, the 3′-end of primers should contain one or more Ts derived from the non-CpG after bisulfite treatment. Accordingly the primers sequences designed for our study was 5′-TTTTGAAGGATACGAAGTTTGA-3′ (forward primer) and 5′-TCCTCTCGAAAACCCTCTAC-3′ (reverse primer) for *HOXA4* promoter.

2.4. Validation of Designed Primer. Subsequent to primer design, the forward and reverse primer sequences were tested for their possible secondary structure, self dimer and hetero dimer formation using OligoAnalyzer 3.1 Software (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). As ΔG less than −7 may form a very stable primer dimer, primer sequence with ΔG higher than −7 was chosen. The higher the ΔG (more than −3.5) the better it seemed, as it could subordinate the primer dimer problem.

The computational prediction of the melting curve as well as the derivative melting curve shape was also derived on the sequence of the PCR product generated, using algorithm like the uMelt v2.0.2 (http://www.dna.utah.edu/umelt/um.php). By using this algorithm, the expected melting temperature of the PCR product was of help in forecasting the melting curve temperature adjustment during the optimization of the laboratory work. Care was taken to see that the derivative melting peak also had only one specific peak without any shoulder at the adjacent slope. PCR amplicon with several melting peaks would be showing the presence of multiple melting domains and may produce complex melting profile that maybe hard to interpret.

A sequence similarity search program designed to explore in silico bisulfite modified DNA (either methylated or not at its CpG dinucleotides) was used to confirm the amplification specificity of the designed primer. The primers were blast before synthesised, using the methBLAST software (http://medgen.ugent.be/methBLAST/).

2.5. High-Resolution Melt Analysis. PCR amplification and MS-HRM analysis were performed using CFX Real Time PCR Detection System (Bio-Rad Laboratories, USA). The PCR amplifications were performed and monitored using the CFX Manager Software and the HRM data was analysed with the Bio-Rad Precision Melt Analysis. PCR amplification was performed in a total volume of 10 μL, containing 1x Precision Melt Supermix (Bio-Rad Laboratories, USA), 200 nM of each designed primer, and 20 ng of bisulfite treated DNA template. All samples and DNA percentage controls were performed in triplicate. The PCR condition was started at 95°C for 2 minutes for initial denaturation, followed by 50 cycles of 10 seconds at 95°C for denaturation, 30 seconds at 50°C for annealing and another 30 seconds at 72°C for extension. The PCR amplification was then followed by heteroduplex formation at 95°C for 30 seconds and subsequently 60°C for 1 minute. The high-resolution melting analysis was performed immediately afterwards by increasing the temperature from 65°C to 95°C for 10 seconds at each step with the 0.2°C increments. For each run, a no template control (NTC) and serial percentage control (0%, 10%, 25%, 50%, 75%, and 100%) in triplicate were included (Figure 2).

The annealing temperature during the PCR amplification was gradiently optimized as it could create amplification bias in MS-HRM. Higher annealing temperature could introduce bias towards the amplification of methylated template. The most preferable annealing temperature would be the one that could differentiate between the serial percentage controls. Hence, multiple annealing temperatures with mixtures of methylated controls were tested and the best fit standard melting curve was selected.

2.6. Statistical Analysis. Unconditional logistic regression analysis was used to assess the relationship between *HOXA4* promoter methylation percentage and the response of CML patients to IM by calculating the Odd Ratios (ORs) and 95% Confidence Interval (CI). The test was conducted by SPSS software with all P values as two-sided.

3. Results

A total of 95 samples including both IM resistant (n = 57) and IM good response (n = 38) CML patients and 12 samples from normal control donors were tested for methylation percentage employing the methylation-specific

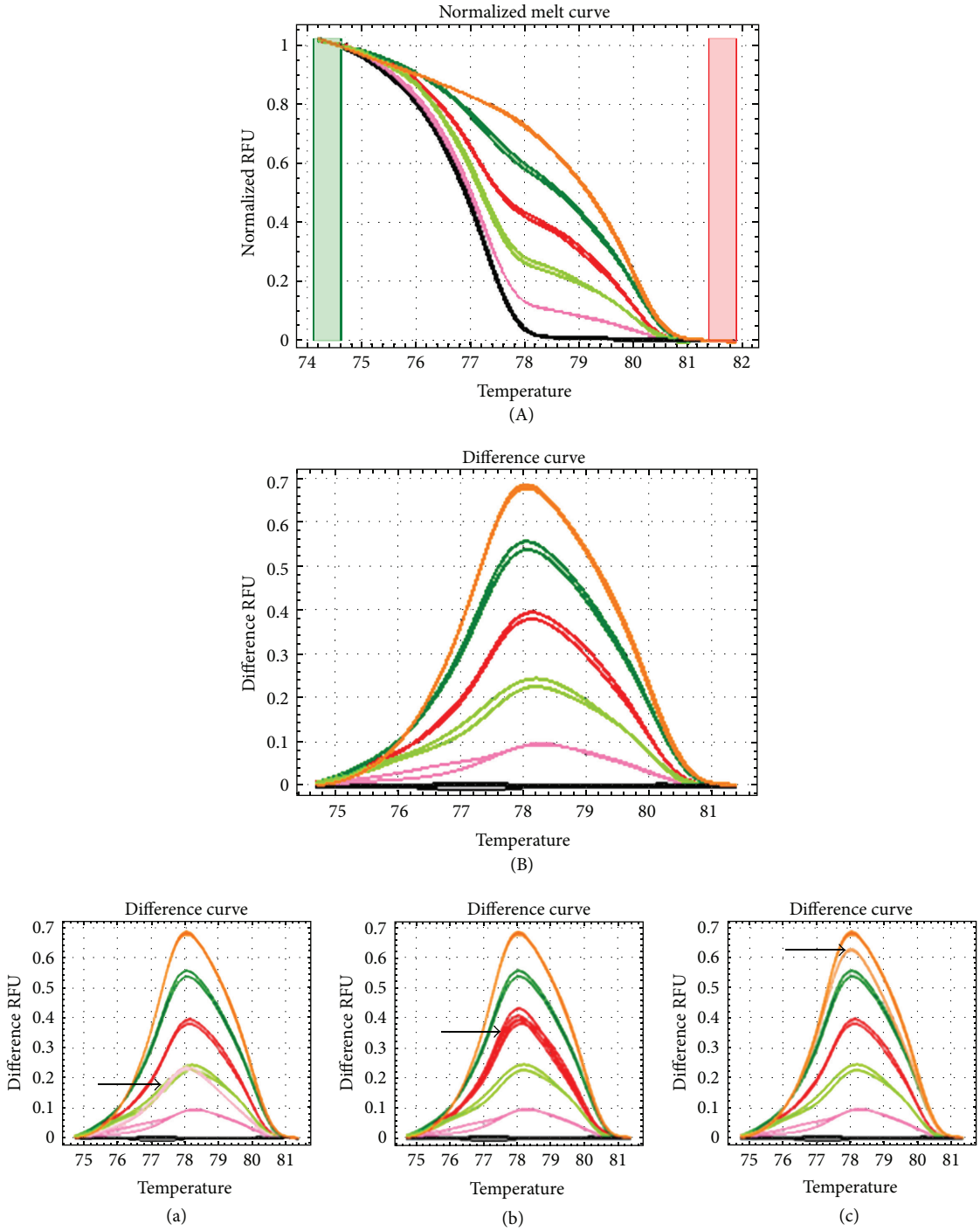


Figure 2: HRM curves for HOXA4 methylation standard. (A) Normalized melt curve of HOXA4 methylation standards in the form of serial methylation percentage (0% black lines, 10% pink line, 25% green line, 50% red line, 75% dark green line, and 100% orange line. (B) Melting curves were normalized to the 0% methylation standards and the standard melt curve was used as the marker for identifying the methylation percentage of samples. For example: (a) ~25% methylated, (b) ~50% methylated, and (c) 75%–100% methylated samples.

high-resolution melt analysis (MS-HRM analysis). All IM-resistant CML patients were initially screened for *BCR-ABL* TKD mutations and those who showed mutations were excluded from MS-HRM analysis. Fifty seven (57) IM-resistant CML patients without *BCR-ABL* mutations were subjected to *HOXA4* methylation analysis. For comparison, 38 CML patients showing good response to IM and 12 normal controls were also subjected to *HOXA4* methylation analysis. Thus, in this report, IM-resistant CML patients are relatively higher than good response CML patients (57 versus 38). Out of 57 IM-resistant CML patients, 22 were males and 35 were females with mean age of 45 years. In the case of

Table 1: Methylation percentage frequencies of *HOXA4* gene in IM resistant and good response CML patients.

HOXA4 methylation (%)	Normal Control	CML Patients	*P* value	CML Patients		
				Good Response	Resistance	*P* value
0–24	6	6	0.000*	4	2	0.213
25–49	6	16	0.016*	11	5	0.010*
50–74	0	43	0.001*	18	25	0.736
75–100	0	30	0.018*	5	25	0.002*

*Chi-Square test, $P < 0.05$ significant at 95% CI.

Table 2: Risk association between *HOXA4* methylation status and IM response among CML patients.

HOXA4 methylation (%)	CML Patients on IM therapy			*P* value	OR (95% CI)
	Good Response	Resistance	Patients Total		
0–49	15	7	22	—	Reference
50–100	23	50	73	0.003*	4.658 (1.673–12.971)

*Chi-Square test, $P < 0.05$ significant at 95% CI.

38 IM good response CML patients, 20 were males and 18 were females with mean age of 36 years. Among the IM-resistant CML patients, 48 patients were categorized into primary resistance group and 9 patients were categorized into secondary resistance group.

Methylation percentage of the promoter region of *HOXA4* gene in the normal controls was in the range of 10% to 49%. In the case of whole group of 95 CML patients, the *HOXA4* promoter methylation was in the range of 10% to 100% with most of them showing dense range of more than 50% methylation. Table 1 shows the methylation percentage frequencies of *HOXA4* gene promoter in normal controls and CML patients, in which the methylation percentages were subdivided into four categories. Except for the low level category (0–24%), the percentages of methylation levels in other 3 categories were significantly higher in CML cases in comparison to control.

When the *HOXA4* methylation profile among CML patients showing good response and resistance to IM was evaluated separately, hypermethylation was found to be significantly less dense in IM good response CML patients, compared to IM-resistant CML patients. However, when the methylation percentages of *HOXA4* were categorized into two classes, 1–49% as methylated and 50–100% as hypermethylated and the values were compared, *HOXA4* hypermethylation was significantly higher among IM-resistant CML patients ($P = 0.002$) than IM good response CML patients. Furthermore, when the risk association of the two methylation categories (methylated and hypermethylated) with IM resistance was evaluated, *HOXA4* hypermethylation was found to be associated with a significantly higher risk for IM resistance with OR, 4.658 (95% CI, 1.673–12.971; $P = 0.003$) as shown in Table 2.

4. Discussion

DNA promoter hypermethylation is a powerful mechanism of tumor-suppressor gene silencing that mediates neoplastic transformation [11]. Despite CML starts as a genetically homogeneous disease, it has been hypothesized that disease progression and clinical heterogeneity in CML are related to epigenetic factors including DNA hypermethylation. Hypermethylation in several tumor-suppressor genes (i.e., *TFAP2A* and *EBF2*) had been reported in CML patients on disease progression [12]. Recently, Jelinek et al., [8] observed a higher frequency of hypermethylation in *OSCP1* and *NPM2* genes among CML patients who were resistant or intolerant to IM. However, there are still no reports available on the involvement of *HOX* gene family hypermethylation in mediating resistance to IM.

The *HOX* gene family consisting of 39 genes are a large family of homeodomain containing transcription factors which regulate developmental process, haematopoietic differentiation, and leukemogenesis. *HOX* gene translocations are observed frequently in leukemia. Majority of the *HOX* genes have CpG islands at their transcription start site (TSS) regions. Silencing of *HOX* genes by DNA methylation are thought to disrupt normal development of blood cells and thus to be involved in leukemic transformation [13]. Hence, compared to other protooncogenes, hypermethylation of *HOX* genes might affect the CML transformation.

By utilizing MS-HRM, the *HOXA4* promoter methylation quantification showed a distribution profile of 10% to 100% methylation, with none of the samples showing 0% methylation. Samples from normal individuals showed methylation of 10% to 49% whereas samples from CML patients showed methylation of 10% up to 100%.

However, based on our experience as well as from the literature, designing of the primer was found to be the most crucial part in ensuring that the methylation percentage be clearly differentiated [10]. Our experience prompt us to suggest that, in order to amplify *HOXA4* promoter for methylation profiling, the methylation-specific primers should have only one CpG site in the forward and reverse primer, respectively. By considering this kind of factor in primer designing, methylation percentage ranging from 0%

to 75% could be clearly differentiated. However, samples that show 75%–100% hypermethylation cannot be clearly differentiated among themselves.

In leukemia-free normal population, few researchers showed absence of methylation, whereas few other studies showed a very low/absence of methylation [14]. However, in the present study, normal samples showed a range of 10% to 49% methylation level at the promoter region of *HOXA4*. Because of this, samples that showed methylation level of 50%–100% only were considered as *HOXA4* hypermethylated samples.

There are reports correlating hypermethylation of *HOXA4* with the development of leukemia. Zangenberg et al. [15] reported that 77% of their acute myeloid leukemia (AML) patients exhibited hypermethylation of *HOXA4* promoter region. Apart from AML, another study had demonstrated the contribution of *HOXA4* promoter hypermethylation in chronic lymphoid leukemia (CLL) [7]. Furthermore, *HOXA4* hypermethylation has been demonstrated to be usually associated with the progression of CML to blast phase and play an important role in the development of leukemia [7].

In the current study, we further evaluated whether *HOXA4* hypermethylation induced gene silencing could be an alternative mechanism of CML cells to circumvent the effects of IM and thereby develop resistance to IM. To the best of our knowledge, no previous reports are available on the involvement of *HOXA4* in mediating IM resistance among CML patients, ours being the first of its kind. Interestingly, in our study, *HOXA4* hypermethylation level of 50–100% was significantly higher ($P = 0.002$) among IM-resistant CML patients compared to IM good response CML patients. When the association between *HOXA4* hypermethylation and IM resistance was examined, patients with *HOXA4* promoter hypermethylation level in 50–100% showed a significantly higher risk for IM resistance (OR = 4.658; 95% CI, 1.673–12.971; *P* value, 0.003). Thus, hypermethylation of *HOXA4* may be a marker of resistance to IM. However, mechanistic studies are still needed to confirm if hypermethylation of *HOXA4* is indeed causing poor response to IM.

The treatment mechanism of IM involves the arrest of *BCR-ABL* dynamic activity. No known mechanism of IM activity towards *HOXA4* has been literally reported so far. Hypermethylation of *HOXA4* has been found to promote inactivation of gene expression [15, 16]. As HOXA4 protein is a DNA-binding transcription factor which may regulate gene expression, morphogenesis, and differentiation, it is reasonable to suggest that the suppression of HOXA4 protein production by hypermethylation induced gene silencing could be one of the potential mechanisms in *BCR-ABL*-independent pathway that promote IM resistance in CML patients. Moreover, Fournier et al. demonstrated the potential of *HOXA4* retrovirus-mediated over expression of haematopoietic stem cell to give rise to mature myeloid progeny [17]. In ovarian cancer, several studies reported that *HOXA4* plays an important role in cell motility, spreading and cell-cell adhesion [18, 19]. Based on those reports, it is reasonable to suggest that suppression of HOXA4 protein might be impairing the normal development as well as proliferation of myeloid progeny and could be a potential epigenetic mechanism in BCR-ABL-independent pathway in promoting IM resistance among CML patients.

It is reasonable to suggest that hypermethylation of *HOXA4* gene might be circumventing the clinical response to IM and thus playing an important role as inhibitor to normal leukemogenesis. This data contributes to a new understanding of epigenetic mechanism also as a mediator in resistance development to IM in CML patients. Inhibition of this process may have potential as better therapy and warrants the need of utilizing hypomethylating agents for CML patients showing this epigenetic mechanism of resistance. Thus, hypermethylation profile of *HOXA4* gene also could be considered as an epigenetic biomarker, in addition to the *BCR-ABL* gene mutations analysis, for prediction of response to IM treatment among CML patients.

Acknowledgments

This study was supported by Universiti Sains Malaysia—Research University Grant (USM-RU) 1001/PPSP/812070. M. H. Elias is Universiti Sains Malaysia Fellowship holder. Exceptional appreciation to Dr. Hoh Boon Peng and Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi MARA, for permitting to use the facilities used in this study. The cooperation and support of staffs, Human Genome Center, Universiti Sains Malaysia, and all the patients who have participated in this study are gratefully acknowledged.

References

[1] J. V. Melo and C. Chuah, "Resistance to imatinib mesylate in chronic myeloid leukaemia," *Cancer Letters*, vol. 249, no. 2, pp. 121–132, 2007.

[2] B. J. Druker, F. Guilhot, and G. Stephen, "Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia," *New England Journal of Medicine*, vol. 355, no. 23, pp. 2408–2417, 2006.

[3] A. Hochhaus, "Chronic myelogenous leukemia (CML): resistance to tyrosine kinase inhibitors," *Annals of Oncology*, vol. 17, no. 10, supplement, pp. x274–x279, 2006.

[4] M. J. Mauro, "Defining and managing imatinib resistance," *Hematology*, pp. 219–225, 2006.

[5] P. La Rosée and M. W. Deininger, "Resistance to imatinib: mutations and beyond," *Seminars in Hematology*, vol. 47, no. 4, pp. 335–343, 2010.

[6] B. Rochat, "Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents," *Current Cancer Drug Targets*, vol. 9, no. 5, pp. 652–674, 2009.

[7] G. Strathdee, T. L. Holyoake, A. Sim et al., "Inactivation of *HOXA* genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis," *Clinical Cancer Research*, vol. 13, no. 17, pp. 5048–5055, 2007.

[8] J. Jelinek, V. Gharibyan, M. R. H. Estecio et al., "Aberrant DNA methylation is associated with disease progression, resistance to imatinib and shortened survival in chronic myelogenous leukemia," *PLoS ONE*, vol. 6, no. 7, Article ID e22110, 2011.

[9] T. K. Wojdacz, A. Dobrovic, and L. L. Hansen, "Methylation-sensitive high-resolution melting," *Nature Protocols*, vol. 3, no. 12, pp. 1903–1908, 2008.

[10] T. K. Wojdacz, T. Borgbo, and L. L. Hansen, "Primer design versus PCR bias in methylation independent PCR amplifications," *Epigenetics*, vol. 4, no. 4, pp. 231–234, 2009.

[11] M. Toyota and J. P. J. Issa, "Epigenetic changes in solid and hematopoietic tumors," *Seminars in Oncology*, vol. 32, no. 5, pp. 521–531, 2005.

[12] T. Dunwell, L. Hesson, T. A. Rauch et al., "A Genome-wide screen identifies frequently methylated genes in haematological and epithelial cancers," *Molecular Cancer*, vol. 9, article 44, 2010.

[13] J. P. J. Issa, "DNA methylation as a therapeutic target in cancer," *Clinical Cancer Research*, vol. 13, no. 6, pp. 1634–1637, 2007.

[14] L. Irving, T. Mainou-Fowler, A. Parker, R. E. Ibbotson, D. G. Oscier, and G. Strathdee, "Methylation markers identify high risk patients in IGHV mutated chronic lymphocytic leukemia," *Epigenetics*, vol. 6, no. 3, pp. 300–306, 2011.

[15] M. Zangenberg, L. Grubach, A. Aggerholm et al., "The combined expression of *HOXA4* and MEIS1 is an independent prognostic factor in patients with AML," *European Journal of Haematology*, vol. 83, no. 5, pp. 439–448, 2009.

[16] G. Strathdee, A. Sim, A. Parker, D. Oscier, and R. Brown, "Promoter hypermethylation silences expression of the *HoxA4* gene and correlates with IgVh mutational status in CLL," *Leukemia*, vol. 20, no. 7, pp. 1326–1329, 2006.

[17] M. Fournier, C. E. Lebert-Ghali, G. Krosl, and J. J. Bijl, "*HOXA4* induces expansion of hematopoietic stem cells in vitro and confers enhancement of pro-B-cells in vivo," *Stem Cells and Development*, vol. 21, no. 1, pp. 133–142, 2011.

[18] Z. L. Kelly, A. Michael, S. Butler-Manuel, H. S. Pandha, and R. G. Morgan, "HOX genes in ovarian cancer," *Journal of Ovarian Research*, vol. 4, article 16, 2011.

[19] C. Klausen, P. C. K. Leung, and N. Auersperg, "Cell motility and spreading are suppressed by HOXA4 in ovarian cancer cells: possible involvement of β1 integrin," *Molecular Cancer Research*, vol. 7, no. 9, pp. 1425–1437, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 198089, 9 pages
http://dx.doi.org/10.1155/2013/198089

Research Article

IROme, a New High-Throughput Molecular Tool for the Diagnosis of Inherited Retinal Dystrophies

Daniel F. Schorderet,[1,2,3] Alexandra Iouranova,[3] Tatiana Favez,[1] Leila Tiab,[1] and Pascal Escher[1,2]

[1] *Institute for Research in Ophthalmology (IRO), Grand-Champsec 64, 1950 Sion, Switzerland*
[2] *Department of Ophthalmology, University of Lausanne, 1004 Lausanne, Switzerland*
[3] *Ecole Polytechnique Fédérale (EPFL), 1015 Lausanne, Switzerland*

Correspondence should be addressed to Daniel F. Schorderet; daniel.schorderet@irovision.ch

Received 4 November 2012; Accepted 27 November 2012

Academic Editor: Gokce A. Toruner

The molecular diagnosis of retinal dystrophies is difficult because of the very important number of genes implicated and is rarely helped by genotype-phenotype correlations. This prompted us to develop IROme, a custom designed in solution-based targeted exon capture assay (SeqCap EZ Choice library, Roche NimbleGen) for 60 retinitis pigmentosa-linked genes and three candidate genes (942 exons). Pyrosequencing was performed on a Roche 454 GS Junior benchtop high-throughput sequencing platform. In total, 23 patients affected by retinitis pigmentosa were analyzed. Per patient, 39.6 Mb were generated, and 1111 sequence variants were detected on average, at a median coverage of 17-fold. After data filtering and sequence variant prioritization, disease-causing mutations were identified in *ABCA4*, *CNGB1*, *GUCY2D*, *PROM1*, *PRPF8*, *PRPF31*, *PRPH2*, *RHO*, *RP2*, and *TULP1* for twelve patients (55%), ten mutations having never been reported previously. Potential mutations were identified in 5 additional patients, and in only 6 patients no molecular diagnosis could be established (26%). In conclusion, targeted exon capture and next-generation sequencing are a valuable and efficient approach to identify disease-causing sequence variants in retinal dystrophies.

1. Introduction

Retinitis pigmentosa (RP) (MIM number 268000) is a group of genetically highly heterogeneous-inherited retinal dystrophies [1]. Typically, night blindness starts during adolescence, and patients progressively loose the rod photoreceptor-mediated peripheral vision. At later stages, the cone photoreceptors also become affected, constricting vision over time to the most central fovea and eventually resulting in complete blindness. To date, more than fifty genes have been linked to nonsyndromic RP (RetNet; http://www.sph.uth.tmc.edu/RetNet/). Inheritance can be autosomal dominant (AD), autosomal recessive (AR) or X-linked, and, rarely, mitochondrial or digenic [2]. Sporadic or simplex cases account for about 30% [3].

The molecular diagnosis of RP is difficult because (i) there is no genotype/phenotype correlation in a vast majority of patients, (ii) a high intra- and interfamilial variability of clinical phenotypes is observed in patients carrying the same causative mutation, (iii) different mutations in a same disease-linked gene cause highly variable clinical phenotypes if not clinically distinct retinal degenerations, and (iv) overlapping clinical phenotypes and disease-linked genes exist with additional retinal degenerations, that is, early-onset Leber congenital amaurosis (LCA), congenital stationary night blindness (CSNB), cone-rod dystrophies (CRD), enhanced S-cone syndrome (ESCS), or syndromic RP in Bardet-Biedl and Usher syndrome [2]. However, identification of RP-linked sequence variants is important for genetic counseling and patient management.

Similar to other Mendelian disorders, mutations in RP patients were identified until recently by linkage mapping and subsequent Sanger sequencing of candidate genes [4]. For molecular diagnosis, the validated RP mutations could be

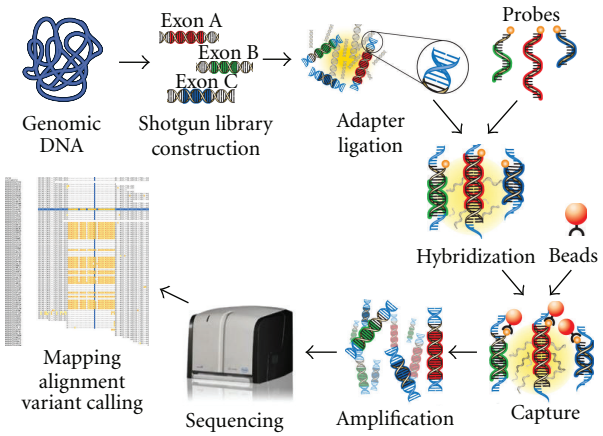


Figure 1: Workflow of the custom-designed targeted exome liquid hybridization capture assay IROme. Genomic DNA from patients was fragmented by nebulization and used for shotgun library construction (454 Roche GS Titanium Rapid Library). Upon adapter ligation, target enrichment is achieved by hybridizing the processed genomic DNA to biotinylated probes (Roche NimbleGen SeqCap EZ Choice). After biotin-streptavidin-based capture and washing, DNA was amplified by emulsion PCR and sequenced on a454 Roche GS Junior Sequencer. Sequencing data was aligned and mapped with the Roche 454 Reference Mapper program. Figure adapted from Roche NimbleGen technical information (http://www.nimblegen.com/products/seqcap/index.html).

detected by arrayed primer extension (APEX) chip technology [5]. However, a low success rate in detecting mutations by APEX was inherent to the genetic heterogeneity of RP patients, and in a cohort of 272 Spanish families affected by ARRP, causative mutations were identified in only 11% of them [6].

The development of next-generation sequencing (NGS) tools in recent years has allowed the production of an enormous volume of sequencing data at low costs [7]. Whole genome sequencing and downstream data handling remains cost and labor intensive, limiting its use in routine mutation detection [8]. Targeted capture of the about 30 Mb of protein-coding regions in the human genome, the so-called exome, reduced the sequencing and data handling effort by a factor of 100 and allowed the identification of mutations in unrelated patients affected by the same syndrome [9]. Exome sequencing has since been widely used as a tool for Mendelian disease gene discovery [10, 11]. Initially array-based, targeted sequence capture has become easy-to-use, thanks to the development of in-solution capture methods [12]. Finally, benchtop high-throughput sequencers made exome sequencing available to small-size diagnostic laboratories [13].

These technological advances prompted us to develop a custom designed in solution-based targeted capture assay, called IROme, for the detection of mutations located in the exons, including complete 3′-untranslated regions (UTR), intron-exon boundaries and potential promoter, and 5′-UTR regions of 63 genes on a 454 GS Junior sequencing platform.

2. Material and Methods

2.1. Patients and DNA Samples. These studies were approved by the Swiss Federal Department of Health (authorization number 035.0003-48) and followed the principles of the Declaration of Helsinki. The 23 patients analyzed in this study were of Swiss, Algerian, and Tunisian origin. Blood samples were collected after informed consent. Genomic DNA was extracted from peripheral blood using a Nucleon BACC2 genomic DNA extraction kit (GE Healthcare, Glattbrugg, Switzerland). Four patients had been previously analyzed at Asper Biotech for known RP-linked mutations by APEX technology [5].

2.2. Design of Solution-Based Capture Assay for Retinitis Pigmentosa-Linked Genes. Exons of targeted genes were identified in the reference human genome version hg19 (http://www.ensembl.org/) (Table 1). For each exon 50 bp were added in both 5′ and 3′ of the exon, including the complete 3′UTR for each gene. Potential alternative transcripts were also considered in the design. To include potential proximal promoters, an additional 1000 bp in 5′ of the first exon of each gene, containing the complete 5′-UTR, were added. The resulting custom-designed SeqCap EZ Choice library (NimbleGen, Roche) was called IROme, version 1.

2.3. GS Junior Sequencing. The workflow for GS Junior sequencing is summarized in Figure 1. DNA concentrations were measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). 500 ng of gDNA were fragmented by nebulization, and size selected by Agencourt AMPure XP beads (Beckman-Coulter, Beverly, MA) to obtain fragments between 500 and 1200 bp. Adaptors provided in the GS Titanium Rapid Library Preparation Kit (Roche, Basel, Switzerland) were ligated to the fragmented DNA and then quantified by fluorometry (QuantiFluor, Promega, Madison, WI). This library was amplified by ligation-mediated (LM)-PCR using specific 454 primers. Then, 1 μg of the PCR amplification product was dried down with COT-DNA (Roche) and 454-Hybridization Enhancing Primer in a Speedvac. The pellet was resuspended in NimbleGen's hybridization buffer and hybridized to the custom-designed SeqCap EZ Choice library (NimbleGen, Roche), called IROme v1, for 70 h at 47°C in a thermocycler. The captured DNA was bound to Streptavidin M-270 Beads (Invitrogen Dynal, Oslo, Norway) for 45 min at 47°C and, using a magnet support, washed with the 4 different NimbleGen buffers provided according to the manufacturer's instructions. The captured DNA-Beads were amplified by LM-PCR using the same specific 454 primers as before. Captured and noncaptured DNA was subjected to quantitative PCR on a Lightcycler480II (Roche, Basel, Switzerland) to measure the relative fold enrichment of the targeted sequences. Postcapture samples with an enrichment higher than 200-fold were further processed. According to the 454 GS Junior protocol (Roche), an emulsion PCR was done on 2 molecules per beads. After PCR, the beads were collected, washed, and bound to the Enrichment Beads. The enriched

Table 1: List of genes enriched by targeted sequence capture (IROme).

Gene	Alias	Chr	Chr location	Exons	Pathology
ABCA4	RP19, STGD1, CORD3, and ARMD2	1	94458391-94586688 (rs)	50	ADRP, ARRP, ARCRD, and ARMD
AIPL1	LCA4	17	6327057-6338519 (rs)	6	ARLCA, ADCRD
BEST1	RP50, BMD, and VMD2	11	61717293-61732987 (fs)	11	ADRP, ARRP, and ADMD
C2ORF71	RP54	2	29284556-29297127 (rs)	2	ARRP
CA4	RP17	17	58227302-58236902 (fs)	8	ADRP
CABP4	CSNB2B	11	67219877-67226699 (fs)	7	ARLCA, ARCSNB
CEP290	LCA10, BBS14, and NPHP6	12	88442794-88535993 (rs)	53	ARLCA, ARBBS
CERKL	RP26	2	182401403-182545392 (rs)	14	ARRP, ARCRD
CLRN1	RP61, USH3A	3	150643950-150690786 (rs)	3	ARRP
CNGA1	RP49	4	47937994-48018689 (rs)	13	ARRP
CNGA2		X	150906923-150913776 (fs)	6	
CNGB1	RP45	16	57917847-58005020 (rs)	33	ARRP
CRB1	LCA8, RP12	1	197170592-197447585 (fs)	12	ARRP, ARLCA
CRX	LCA7, CORD2	19	48325097-48364769 (fs)	4	ADRP, ADLCA, ARLCA, and ADCRD
DHDDS	RP59	1	26758773-26797785 (fs)	9	ARRP
EYS	RP25	6	64429876-66417118 (rs)	43	ARRP
FAM161A	RP28	2	62051989-62081278 (rs)	6	ARRP
FSCN2	RP30	17	79495422-79504156 (fs)	5	ADRP, ADMD
GUCA1B	RP48, GCAP2	6	42152139-42162694 (rs)	4	ADRP, ADMD
GUCY2D	LCA1, CORD6	17	7905988-7923658 (fs)	20	ARLCA, ADCRD
IMPDH1	LCA11, RP10	7	128032331-128050306 (rs)	17	ADRP, ADLCA
IMPG2	RP56, sparcan	3	100945570-101039404 (rs)	20	ARRP
IQCB1	NPHP5	3	121488610-121553926 (rs)	15	ARLCA
KLHL7	RP42	7	23145353-23215040 (fs)	12	ADRP
LCA5	Lebercilin	6	80194708-80247175 (rs)	8	ARLCA
LPCAT1	AYTL2	5	1456595-1524092 (rs)	14	ARLCA
LRAT	LCA14	4	155548097-155674270 (fs)	4	ARRP, ARLCA
MERTK	RP38	2	112656056-112787138 (fs)	19	ARRP
NR2E3	RP37, PNR	15	72084977-72110559 (fs)	8	ADRP, ARRP, and ARESCS
NRL	RP27	14	24549316-24584223 (rs)	3	ADRP, ARRP, and ARESCS
OFD1	RP23	X	13752832-13787480 (fs)	23	XRP
OTX2		14	57267426-57277197 (rs)	5	ADLCA
PDE6A	RP43	5	149237519-149324356 (rs)	22	ARRP
PDE6B	RP40, CSNBAD2	4	619373-664571 (fs)	23	ARRP, ADCSNB
PDE6G	RP57	17	79617489-79623607 (rs)	4	ARRP
PRCD	RP36	17	74523871-74541458 (fs)	5	ARRP
PROM1	RP41, STGD4, CORD12, and MCDR2	4	15964699-16086001 (rs)	28	ARRP, ADCRD, and ADMD
PRPF3	RP18	1	150293925-150325671 (fs)	16	ADRP
PRPF6	RP60	20	62612488-62664453 (fs)	21	ADRP
PRPF8	RP13	17	1553923-1588154 (rs)	43	ADRP
PRPF31	RP11	19	54618837-54635140 (fs)	14	ADRP
PRPH2	RDS, RP7	6	42664340-42690312 (rs)	3	ADRP, ADMD, ADCRD, and digenic
RBP3	IRBP	10	48381487-48390991 (rs)	4	ARRP
RDH12	LCA13, RP53	14	68168603-68201169 (fs)	8	ADRP, ARLCA
RGR	RP44	10	86004809-86019716 (fs)	7	ADRP, ARRP, and ADCA
RHO	RP4, CSNBAD1	3	129247483-129254012 (fs)	5	ADRP, ARRP, and ADCSNB
RLBP1	CRALBP	15	89753098-89764922 (rs)	9	ARRP
ROM1		11	62379194-62382592 (fs)	3	ADRP, digenic
RP1		8	55471729-55682531 (fs)	4	ADRP, ARRP
RP2		X	46696375-46741793 (fs)	5	XRP
RP9	PAP1	7	33134409-33149013 (rs)	7	ADRP

Table 1: Continued.

Gene	Alias	Chr	Chr location	Exons	Pathology
RPE65	LCA2, RP20	1	68894505-68915642 (rs)	14	ARRP, ARLCA
RPGR	RP3, CORDX1	X	38128424-38186817 (rs)	19	XRP, XCRD, XMD
RPGRIP1	LCA6, CORD13	14	21756098-21819460 (fs)	24	ARLCA, ARCRD
SAG	RP47, Arrestin	2	234216309-234255701 (fs)	16	ARRP, ARCSNB
SEMA4A	RP35, CORD10	1	156117157-156147543 (fs)	16	ADRP, ARRP, and ADCRD
SNRNP200	RP33	2	96940074-96971297 (rs)	45	ADRP
SPATA7	LCA3	14	88851268-88936694 (fs)	12	ARLCA
TOPORS	RP31	9	32540542-32552551 (rs)	3	ADRP
TUB		11	8040791-8127659 (fs)	13	
TULP1	LCA15, RP14	6	35465651-35480715 (rs)	15	ARRP, ARLCA
USH2A	RP39	1	215796236-216596738 (rs)	73	ARRP
ZNF513	RP58	2	27600098-27603657 (rs)	4	ARRP

Genes are listed alphabetically according to their official gene symbol, and, in addition, gene aliases commonly used in ophthalmic research provided. Chromosomal (chr) location is based on the Homo sapiens high-coverage assembly GRCh37, yielding in the UCSC hg19 database (fs: forward strand; rs: reverse strand). For each gene the number of exons is listed. Targeted sequence capture was directed against genes causing autosomal dominant (AD), autosomal recessive (AR) X-linked (X), retinitis pigmentosa (RP), and Leber congenital amaurosis (LCA). Other retinopathies caused by a given gene are also indicated: cone or cone-rod dystrophy (CRD), macular degeneration (MD), congenital stationary night blindness (CSNB), Bardet-Biedl syndrome (BBS), enhanced S-cone syndrome (ARESCS), and chorioretinal atrophy (CA). Heterozygote ROM1 and PRPH2 mutations cause digenic disease. ORF15 of RPGR was not included in the assay.

DNA was then eluted and quantified with the provided bead counter. Sequencing was performed following the 454 GS Junior protocol. Briefly, 500′000 enriched DNA beads were mixed with Packing Beads. Then, the PicoTiterPlate (PTP) was sequentially loaded with Prelayer Beads, DNA-Packing Beads, Postlayer Beads, and PPiase Beads. Finally, the PTP was mounted in the 454 GS Junior Sequencer, and the program was run in full processing for shotgun sequencing.

2.4. Data Analysis. The workflow for data analysis and data validation is summarized in Figure 2. Sequencing data (.sff file) were analyzed with Roche 454 Reference Mapper program. Reference text (ref.txt) for gene annotations and the snp131 version of the single nucleotide polymorphism database (snp131.txt) were downloaded from the Golden Path database (http://www.genome.ucsc.edu/). The sequence variants provided by the 454HCDiffs.txt file were filtered for known SNPs (http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/), type of amino acid changes (http://genetics.bwh.harvard.edu/pph2/), and repetitive sequences. An additional in-house developed program was used to check the remaining SNPs against reference sequences obtained in Ensembl. Sequence variants were further prioritized according to inheritance, if family information was available, and to the percentage of reads containing a given sequence variant (threshold at 20%). To analyze the coverage, scripts were written to extract global coverage data from the 454AlignmentInfo.tsv file (unique depth, column 5) and the quality of coverage at each targeted nucleotide (column 4). Part of the sequencing data was analyzed by Sequence Pilot version 3.5 (JSI Medicals, Kippenheim, Germany).

2.5. Data Validation. Sanger sequencing validated all potential pathogenic sequence variants. Briefly, 20-bp primers flanking the given region and yielding amplicons of 300–600 bp were designed (primer sequences available on request). The polymerase chain reaction (PCR) was performed in a total volume of 20 μL, containing 20 ng genomic DNA, 1 mM of each primer (Eurogentec, Liège, Belgium), and 10 μL FastStart PCR Master Mix (Roche, Basel, Switzerland). Amplification was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, CA, USA) with the following conditions: 1 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C, and, a final elongation step at 72°C for 10 min. PCR-amplified products were purified with an Invitek MSB Spin PCRapace kit (STRATEC Molecular GmbH, Berlin, Germany). Sanger sequencing was done in a final reaction volume of 10 μL, using BigDye Terminator v3.1 (Applied Biosystems) with forward and reverse primers. Fragments were separated on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sequences were analyzed using Chromas 2.23 software (Technelysium, Tewantin, QLD, Australia).

3. Results and Discussion

3.1. IROme: Design and Validation of the Assay. The vast genetic heterogeneity of RP prompted us to develop a custom-designed hybridization-based targeted exon capture assay, called IROme. Enrichment was targeted towards a total of 63 genes (942 exons), of which 60 genes were linked to RP, LCA, and related retinal dystrophies (Table 1). The exon ORF15 of *RPGR* was not included in the assay because of the presence of repetitive sequences. Two RP- or LCA-linked genes, *IDH3B* and *RD3*, had been reported only in a single family so far and were not included in this version of IROme. Conversely, two candidate genes that were linked to retinal degeneration in mice, but not humans, were added to the assay (*TUB* and *LPCAT1*). A third candidate gene

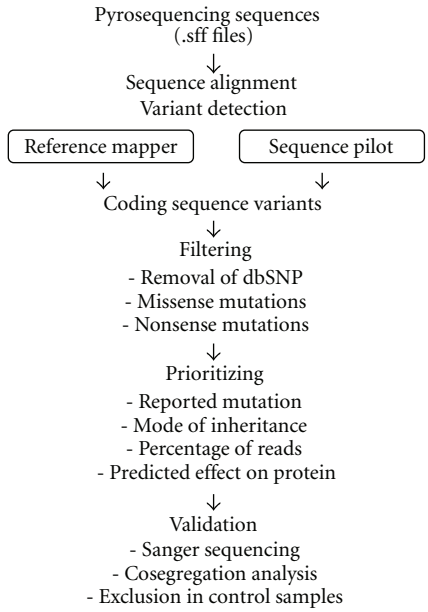


Figure 2: Workflow of data analysis and filtering. The sff (sequence file format) files generated by 454 Roche GS Junior sequencing were imported either into Reference Mapper or Sequence Pilot software. The coding sequence variants were selected from the 454_HCDiffs.txt files that contained all sequence variants. During filtering, coding sequence variants reported in dbSNP were removed, and missense and nonsense mutations kept. The remaining coding sequence variants were prioritized according to known reported mutations, the mode of inheritance, the percentage of sequence reads reporting the variant (threshold of 20%), and the predicted effect on the protein (PolyPhen score).

located on chromosome X, *CNGA2*, was included because of its homology to *CNGA1*. The total of targeted regions spans 394′758 bp.

Of note, after the design of IROme was completed, *TTC8* (*BBS8/RP51*), *C8ORF37,* and *MAK* were linked to RP, and *KCNJ13* and *NMNAT1* to LCA. These latter genes, as well as *IDH3B* and *RD3*, will be included in a future version of IROme.

Patients 1–4 had previously been investigated by APEX technology for known RP-linked mutations [5]. All nucleotides tested by APEX were correctly detected by IROme, with a 98.9% accuracy of the sequence reads for nucleotides at a homozygous state (Table 2). A p.USH2A-V2562A mutation had been detected by APEX in patient 2 in a heterozygous state, and this was correctly validated by IROme (46.8% of the sequence reads at 47-fold coverage).

As an additional control, the IROme assay was tested on genomic DNA of a previously described family of Algerian origin, affected by LCA or early onset retinal degeneration [14]. The causative 6-base in-frame duplication c. *TULP1*-1593_1598dupTTCGCC was readily detected in exon 15 (Table 3, patient 5).

3.2. IROme: Variant Detection, Coverage, and Data Filtering. A total of 23 RP patients were analyzed by IROme (Table 3). Pyrosequencing generated an average of 39.6 ± 14.1 Mb per patient, with an average read length of 408 ± 48 bp. These long read lengths are comparable to published analyses, where the Roche 454 GS Junior generated the longest read lengths, in comparison to the other benchtop high-throughput sequencing platforms, MiSeq (Illumina) and Ion Torrent PGM (Life Technologies) [13].

Table 2: Validation of IROme by APEX.

Pat number	nt tested by APEX	nt detected by IROme	Mean cvg	% reads homo
1	557	100%	25	98.9
2	558	100%	26	99.4
3	558	100%	22	99.2
4	547	100%	20	98.3

The nucleotides (nt) tested by APEX represent validated RP-linked mutations or variants. The mean coverage (cvg) refers to the average of the coverage of all exons where the mutations are located. The percentage of sequence reads generated by IROme and correctly calling the nucleotides at homozygous state are indicated.

On average per patient, 1′111.7 ± 222.2 sequence variants were found (range: 736–1′826). Among these, 90.1 ± 10.0 were located in coding sequences, and a further 42.1 ± 4.7 were changing the amino acid sequence. By considering all patients, the median coverage was 17-fold, with a maximal 112-fold coverage in one exon of patient 16 (Figure 3). No coverage was observed for four exons (0.3%): exons 1 of *RP9*, *IMPDH1,* and *LPCAT1* and an alternative exon 2 of *CNGA2*. These exons contained GC-rich and/or repetitive sequences impeding efficient probe design and targeting [15]. Another 15 exons were not covered in all patients (1.6%). Because these exons were not restricted to the 5′ regions, absence of coverage was attributed to technical limitations or, as observed for patient 9, to a deletion (see below).

For patients 20 and 21, two potential heterozygote mutations had been detected at 22.6% (53-fold coverage) and 21.3% (61-fold coverage), respectively. However, these two sequence variants could not be validated by Sanger sequencing. For further patient analyses, a more stringent threshold up to 35% of sequence reads might be used for prioritization of sequence variants. Alternatively, a dynamic threshold could be implemented, starting at a high stringency and going down until one or two mutations are identified.

In conclusion, the design of IROme resulted in an over 98% coverage of the targeted exons. The variant detection workflow could be improved by further increasing the quality of the sequencing data, that is, by using a benchtop sequencer less prone to homopolymer-associated insertion/deletion errors (e.g., MiSeq, Illumina) [13] and high-fidelity DNA polymerases [16].

3.3. IROme: Molecular Diagnosis on RP Patients. IROme analysis yielded in definite diagnosis for 55% of the RP patients, that is, 12 out of 23 patients (Patients 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 19, and 23). This was in line with the approximately 60% success rate reported for exome

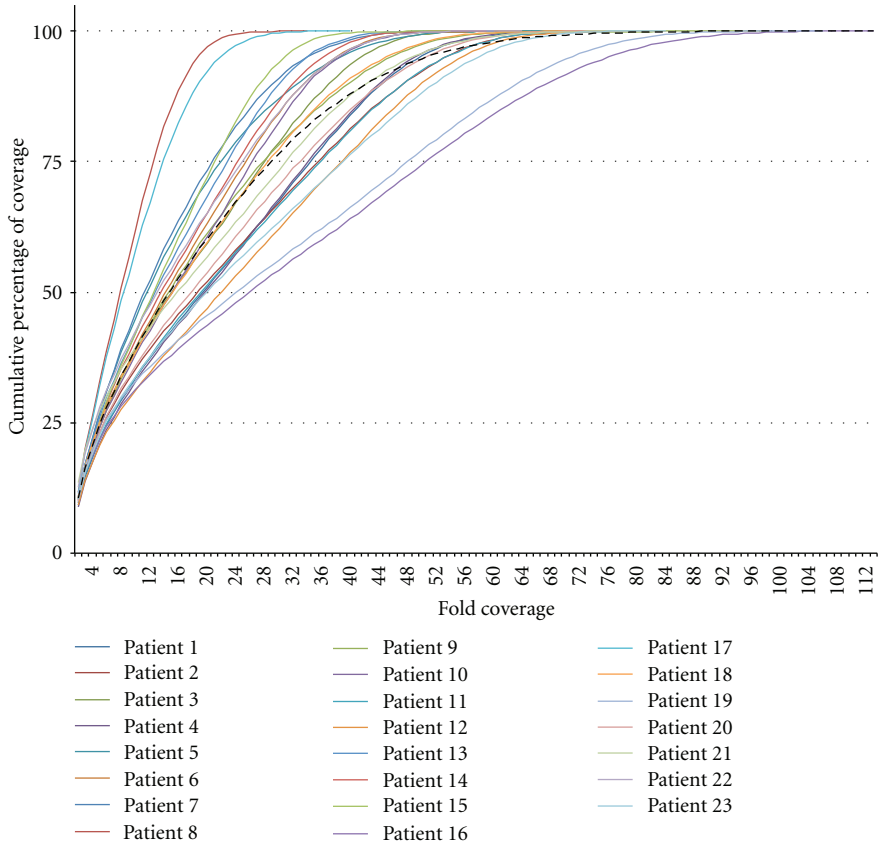


Figure 3: Fold coverage of targeted sequences. For each patient the unique depth data provided by column 5 of the 454_AlignmentInfo.tsv file was used to estimate the coverage per targeted bp. The onefold coverage data corresponding to reference genome sequences used for alignment purposes, but not targeted by IROme, were removed. The coverage data is represented as cumulative percentage; that is, indicating what percentage of targeted bp has a minimal coverage of x-fold (x axis represents the fold coverage). The average coverage for all patients is represented as a black dashed line, and the median coverage for all patients is 17-fold.

capture strategies to identify Mendelian disease genes [4], but represented a 5-fold increase in mutation detection as compared to the APEX assay [6]. A solution-based targeted exon capture assay similar to IROme had also identified disease-causing mutations in 11 out of 17 families affected by various retinal degenerations (65%) [17]. In contrast, in a cohort of 100 RP patients, array-based targeted exon capture resulted in the identification of pathogenic mutations in 36 individuals (36%) [15]. Amplicon-based approaches identified potential mutations in 24% of patients affected by retinal degenerations (5/21) [18], in 79% of ADRP patients (15/19) [19], and 24% of LCA patients (4/17) [20].

In addition to the control (patient 5), only the p.PROM1-R373C mutation identified in patient 10 had been previously described [21], further underscoring the importance of screening RP-linked genes for the presence of new mutations.

The workflow for variant detection was not immediately successful for two patients. For patient 9, a deletion of exons 45–47 in *ABCA4* was only found by analyzing the coverage data. For patient 16, the 33 bp insertion in *PRPF31* was detected by Sequence Pilot, but not Reference Mapper software.

Potential mutations were found in three patients (13%). Patient 1 inherited from her healthy mother a heterozygous p.C2ORF71-R571delRTVVPP mutation and from her healthy father a heterozygous p.FSCN2-P231S mutation. Digenic RP has been linked so far to heterozygous PRPH2 and ROM1 mutations [2], and further analyses will be necessary to validate this molecular diagnostic. Patient 2 and 20 had, respectively, two and one potential mutation, but no family members were available to confirm the result.

Results were questionable for two additional patients. Patient 6 carried a p.RHO-R252P mutation that had been previously reported [22]. However, unaffected family members were not available to confirm this dominant mutation. Also, a heterozygous p.CRX-Q105X sequence variant was detected in patient 14, but his healthy mother was also carrying it.

Finally, no molecular diagnostic could be established for six patients (26%): in patients 18 and 21 no potential mutations were found by IROme analysis, in patients 7 and 15 the potential mutation did not segregate with disease in the family, and in patients 3 and 22 heterozygous mutations were found in genes only reported for recessive inheritance (*CLRN1, EYS*).

Table 3: Synopsis of molecular diagnostic on RP patients by IROme.

Pat number	Total seq Mb	Read length bp	Median fold cvg	Total seq var	cds seq var	filt. seq var	prio. seq var	Test/val seq var	Potential mutation	cvg pot mut	mut reads %	Cosegregate family
1	47	453	21.3	1206	114	51	8	2/2	p.C2ORF71-R571_P576del p.FSCN2-P231S	38 25	55.3 44	M het norm F het norm
2	47.6	433	20.9	1217	98	44	7	2/2	p.PDE6B-H337R p.OTX2-G222R	21 52	100 48	? ?
3	42.2	416	17.0	1085	78	39	6	1/1	p.CLRN1-P134L	19	68.4	?
4	44.6	395	21.6	1173	95	42	5	3/3	p.RHO-Y191C	39	38.5	yes
5	24.4	429	13.8	894	104	45	1	1/1	p.TULP1-F529_A530dup	6	100	yes
6	31.7	422	16.2	1039	85	47	1	1/1	p.RHO-R252P	22	54.5	?
7	20.1	281	13.3	789	77	38	4	2/2	p.SAG-E11K p.IMPG2-G684R	30 34	56.7 38.2	no no
8	13.9	445	9.1	736	70	33	2	1/1	p.RP2-D161Y	22	45.5	yes
9	29	297	17.3	832	80	39	9	1/1	g.ABCA4-ex45-47del	0	0	yes
10	37.3	443	16.7	1247	93	46	3	3/3	p.PROM1-R373C	32	50	yes
11	50.2	440	21.5	1151	92	46	2	1/1	p.RP2-E20X	28	67.8	yes
12	49.1	394	23.8	1116	94	39	9	4/4	p.CNGB1-R765C	30	100	yes
13	33	436	14.6	1017	85	33	3	2/2	p.GUCY2D-V887G	18	94.4	yes
14	32.6	443	15.3	1205	93	42	3	1/1	p.CRX-Q105X	17	58.8	M het norm
15	32.7	442	14.2	1026	86	40	3	1/1	p.USH2A-P2630R	25	40	no
16	69.4	434	28.4	1246	87	41	1	1/1	p.PRPF31-E183_ins33bp	74	40	yes
17	16.7	452	9.8	861	82	43	2	2/2	p.PRPH2-L39P	18	50	yes
18	39.6	429	17.2	1826	85	35	3	1/1				
19	66.5	449	26.4	1298	103	45	5	2/2	p.PRPH2-S217_dup16bp	71	39.4	yes
20	47.1	358	19.8	1171	91	47	3	2/1	p.C2ORF71-L889P	23	39.1	?
21	47	363	17.3	1197	102	48	3	1/0				
22	36.6	354	14.7	1072	86	45	2	2/1	p.EYS-D2930G	38	60.5	?
23	53.3	393	22.4	1164	92	40	7	5/5	p.PRPF8-E2331X	38	44.7	yes

For each patient, the total number of Mb (10^6 bp) sequenced on the Roche 454 GS Junior (total seq Mb) and the average read length (read length bp) are indicated. The median fold coverage (cvg) was extracted from the unique depth information. From all the sequence variants (total seq var), first only the sequence variants located in coding sequences were analyzed (cds seq var), with filtering (filt seq var) and prioritizing (prio seq var) according to Figure 2. The sequence variants eventually tested and validated by Sanger sequencing (test/val seq var) are also indicated. For each potential mutation, the coverage (cvg pot mut) and the percentage of sequence reads reporting the potential mutation (mut reads %) are indicated. For cosegregation analysis, "?" indicates absence of available family members and/or simplex cases. For patients 1 and 14, the mother (M) and/or the father (F) are healthy heterozygous carriers (het norm).

Of note, all these patients carry novel sequence variants in noncoding regions. To prioritize for potential disease-causing sequence variants in these regions, systematic annotation should not only cover splicing sites, 5′- and 3′-UTRs, but also implement detailed information about transcription factor binding sites and regulatory elements located in the potential proximal promoter regions. Promoter sequence variants could then be tested by reporter transactivation assays (e.g., luciferase reporter assays), but this time-consuming approach cannot be implemented in a routine molecular diagnostic lab.

4. Conclusions

The custom designed in solution-based targeted exon capture assay IROme efficiently detected disease-causing mutations in 55% of RP patients (12/23). A 99.7% coverage of the targeted regions was obtained. The first translated exon often contains sequences with a high GC content in its 5′-UTR that hinders an efficient capture [23]. Remarkably, more than 95% of exons 1 (60/63) were successfully enriched by IROme.In comparison, a pilot study carried out in our

laboratory on 25 patients using whole exome sequencing (SureSelect, Agilent) resulted in no coverage of promoter regions, highly variable coverage of 3′-UTRs, and several genes had their first translated exon very poorly covered. For instance, the first exons of the following RP-linked genes could not be correctly analyzed: *C2ORF71*, *CA4*, *CABP4*, *CERKL*, *CNGA1*, *FAM161A*, *FSCN2*, *GUCY2D*, *IMPDH1*, *LPCAT1*, *MERTK*, *RDH12*, *RP9,* and *RPGR* (D. F. Schorderet, unpublished results). It is tempting to speculate that the additional sequences upstream of exon 1 included in IROme further enhanced the performance of the NimbleGen exome capture technology, that reportedly has more specific targeting and a higher percentage of on-target reads than competing products [23, 24]. However, because the costs for whole exome sequencing have dramatically decreased to about 1000 $ per patient, this method may in the future replace target enrichment and resequencing, providing that a new line of "whole exome" kits covering effectively all exons, including the first one, of all genes, will become commercially available [24].

Meanwhile, custom-designed target enrichment and subsequent next-generation sequencing are a cost-efficient approach for the molecular diagnosis of retinal dystrophies, also with respect to the relative ease of data handling and analysis [25]. Finally, the median global coverage of 17-fold observed with the IROme assay also indicated the possibility to include additional retinal degeneration-linked genes, newly discovered ones or candidate genes.

Acknowledgments

The authors thank Etienne Bagnoud for technical support in informatics. This paper is supported by Swiss National Science Foundation Grants 31003A-122269 (to P. Escher and D. F. Schorderet) and 31003A_138492 (to P. Escher).

References

[1] D. T. Hartong, E. L. Berson, and T. P. Dryja, "Retinitis pigmentosa," *The Lancet*, vol. 368, no. 9549, pp. 1795–1809, 2006.

[2] W. Berger, B. Kloeckener-Gruissem, and J. Neidhardt, "The molecular basis of human retinal and vitreoretinal diseases," *Progress in Retinal and Eye Research*, vol. 29, no. 5, pp. 335–375, 2010.

[3] S. Ferrari, E. Di Iorio, V. Barbaro, D. Ponzin, F. S. Sorrentino, and F. Parmeggiani, "Retinitis pigmentosa: genes and disease mechanisms," *Current Genomics*, vol. 12, no. 4, pp. 238–249, 2011.

[4] C. Gilissen, A. Hoischen, H. G. Brunner, and J. A. Veltman, "Disease gene identification strategies for exome sequencing," *European Journal of Human Genetics*, vol. 20, no. 5, pp. 490–497, 2012.

[5] J. Zernant, M. Külm, S. Dharmaraj et al., "Genotyping microarray (disease chip) for leber congenital amaurosis: detection of modifier alleles," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 9, pp. 3052–3059, 2005.

[6] A. Ávila-Fernández, D. Cantalapiedra, E. Aller et al., "Mutation analysis of 272 Spanish families affected by autosomal recessive retinitis pigmentosa using a genotyping microarray," *Molecular Vision*, vol. 16, pp. 2550–2558, 2010.

[7] M. L. Metzker, "Sequencing technologies—the next generation," *Nature Reviews Genetics*, vol. 11, no. 1, pp. 31–46, 2010.

[8] L. G. Biesecker, "Exome sequencing makes medical genomics a reality," *Nature Genetics*, vol. 42, no. 1, pp. 13–14, 2010.

[9] S. B. Ng, E. H. Turner, P. D. Robertson et al., "Targeted capture and massively parallel sequencing of 12 human exomes," *Nature*, vol. 461, no. 7261, pp. 272–276, 2009.

[10] S. B. Ng, K. J. Buckingham, C. Lee et al., "Exome sequencing identifies the cause of a mendelian disorder," *Nature Genetics*, vol. 42, no. 1, pp. 30–35, 2010.

[11] M. J. Bamshad, S. B. Ng, A. W. Bigham et al., "Exome sequencing as a tool for Mendelian disease gene discovery," *Nature Reviews Genetics*, vol. 12, no. 11, pp. 745–755, 2011.

[12] L. Mamanova, A. J. Coffey, C. E. Scott et al., "Target-enrichment strategies for next-generation sequencing," *Nature Methods*, vol. 7, no. 2, pp. 111–118, 2010.

[13] N. J. Loman, R. V. Misra, T. J. Dallman et al., "Performance comparison of benchtop high-throughput sequencing platforms," *Nature Biotechnology*, vol. 30, no. 5, pp. 434–439, 2012.

[14] A. Mataftsi, D. F. Schorderet, L. Chachoua et al., "Novel TULP1 mutation causing leber congenital amaurosis or early onset retinal degeneration," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 11, pp. 5160–5167, 2007.

[15] K. Neveling, R. W. Collin, C. Gilissen et al., "Next-generation genetic testing for retinitis pigmentosa," *Human Mutation*, vol. 33, no. 6, pp. 963–972, 2012.

[16] I. Vandenbroucke, H. Van Marck, P. Verhasselt et al., "Minor variant detection in amplicons using 454 massive parallel pyrosequencing: experiences and considerations for successful applications," *Biotechniques*, vol. 51, no. 3, pp. 167–177, 2011.

[17] I. Audo, K. M. Bujakowska, T. Leveillard et al., "Development and application of a next-generation-sequencing (NGS) approach to detect known and novel gene defects underlying retinal diseases," *Orphanet Journal of Rare Diseases*, vol. 7, no. 1, article 8, 2012.

[18] S. J. Bowne, L. S. Sullivan, D. C. Koboldt et al., "Identification of disease-causing mutations in autosomal dominant retinitis pigmentosa (adRP) using next-generation DNA sequencing," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 1, pp. 494–503, 2011.

[19] J. Song, N. Smaoui, R. Ayyagari et al., "High-throughput retina-array for screening 93 genes involved in inherited retinal dystrophy," *Investigative Ophthalmology and Visual Science*, vol. 52, no. 12, pp. 9053–9060, 2011.

[20] F. Coppieters, B. de Wilde, S. Lefever et al., "Massively parallel sequencing for early molecular diagnosis in Leber congenital amaurosis," *Genetics in Medicine*, vol. 14, no. 6, pp. 576–585, 2012.

[21] Z. Yang, Y. Chen, C. Lillo et al., "Mutant prominin 1 found in patients with macular degeneration disrupts photoreceptor disk morphogenesis in mice," *Journal of Clinical Investigation*, vol. 118, no. 8, pp. 2908–2916, 2008.

[22] E. R. Grinberg, L. I. Dzhemileva, and E. K. Khusnutdinova, "The novel R252P Mutation of the RHO gene in patients with retinitis pigmentosa from Bashkortostan," *Molecular Biology*, vol. 41, no. 4, pp. 746–748, 2007.

[23] P. Frommolt, A. T. Abdallah, J. Altmüller et al., "Assessing the enrichment performance in targeted resequencing experiments," *Human Mutation*, vol. 33, no. 4, pp. 635–641, 2012.

[24] A. M. Sulonen, P. Ellonen, H. Almusa et al., "Comparison of solution-based exome capture methods for next generation sequencing," *Genome Biology*, vol. 12, no. 9, article R94, 2011.

[25] M. Fromer, J. L. Moran, K. Chambert et al., "nd statistical genotyping of copy-number variation from whole-exome sequencing depth," *The American Journal of Human Genetics*, vol. 91, no. 4, pp. 597–607, 2012.