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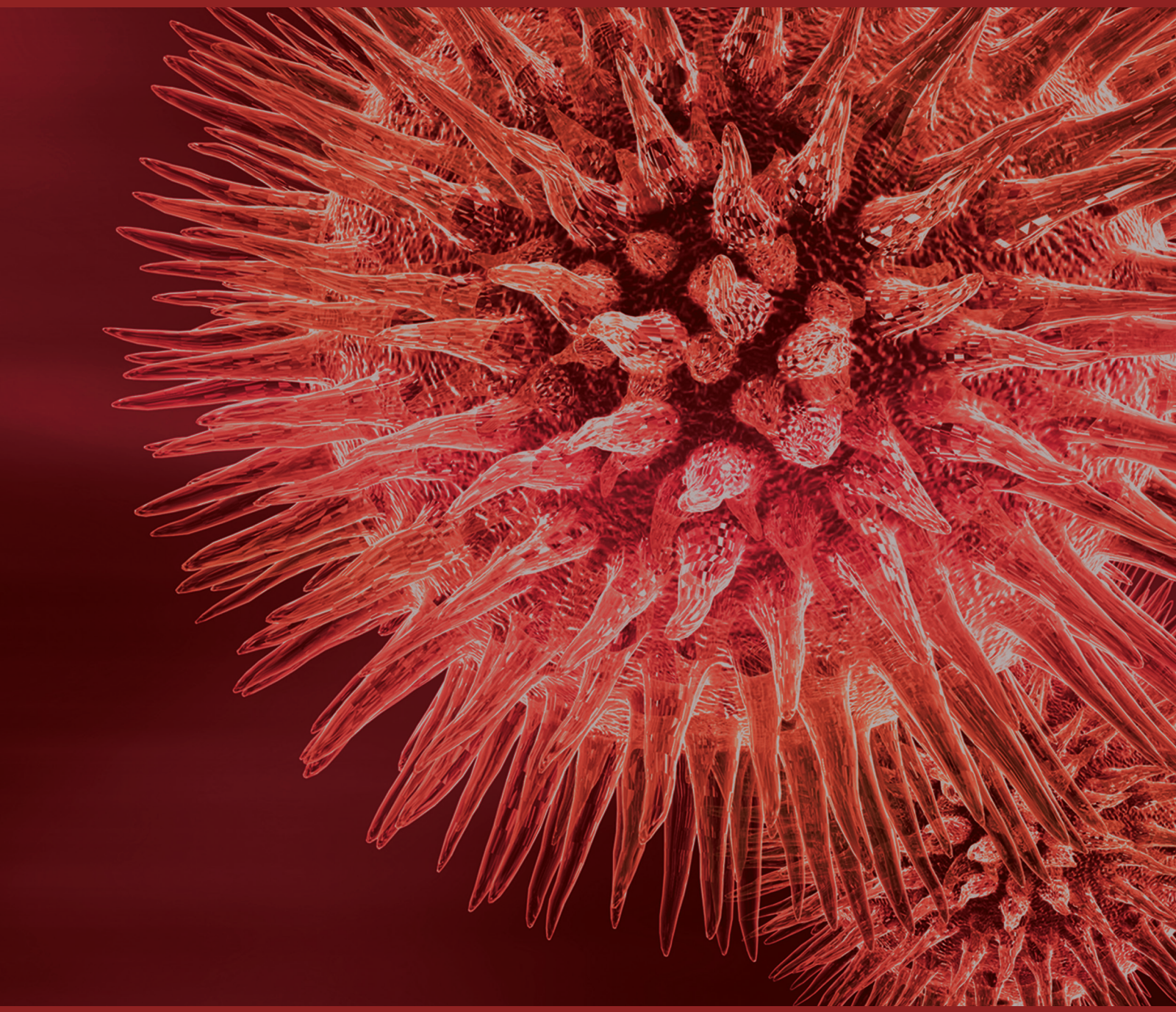
Laboratory Genetic Testing in Clinical Practice 2014

Guest Editors: Ozgur Cogulu, Jacqueline Schoumans, Gokce Toruner, Urzsula Demkow, Emin Karaca, and Asude Alpman Durmaz



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Editorial

Laboratory Genetic Testing in Clinical Practice 2014

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The laboratory genetic testing is rapidly emerging in clinical practice. Recently, in parallel with the report of human genome project, next generation sequencing techniques are widely applied to study human genome providing a basis for integrating information about genome, exome, and transcriptome of a living organism. The future applications of genetic testing are discussed in popular media everyday. Accordingly, this special issue was prepared to provide a basis for integrating information from this emerging field. Among them, we have given place to research articles as well as clinical studies. Although polymorphism studies have lost their significance compared to previous years when genetic studies gained popularity, some polymorphisms in the genome have been reported to play considerable role in development of pathological conditions. Their importance in nasopharyngeal carcinoma, osteosarcoma, and pediatric tuberculosis is reported in 3 research articles. Integrated diagnostic approach including clinical and biochemical analysis followed by genetic tests was discussed in children with neurodevelopmental and neurometabolic diseases. Notwithstanding a great deal of advanced genetic testing that has become available, cytogenetics never lost its importance. One of the studies points out that patients benefit from the information that could be provided by cytogenetics in clinical decision-making, followup, and prognosis in myelodysplastic syndrome. On the other hand, fragile X, which is the most common genetic cause in hereditary intellectual disability syndromes, was discussed on a basis of a large number of enrolled subjects. Genetic variant in hepatitis C virus and its effect in the course of antiviral treatment were also evaluated in chronic hepatitis C patients and has made an important contribution to the knowledge of patients' management. Finally and undoubtedly, next generation studies as a robust and cost-effective genetic technology have found place in this special issue in 3 clinical studies relating to the breast and ovarian cancer, Charcot-Marie-Tooth disease, and retinitis pigmentosa.

In summary we aimed to combine reports relating to various genetic technologies in a number of disease conditions. However, as mentioned in the review article by A. A. Durmaz et al., novel technologies in the field of genetics have noticeably accelerated in the last few decades. Even though Moore's law will continuously apply to the developmental speed in computer technologies, the cost of sequencing has declined and the data obtained from NGS platforms has increased at a rate that outpaced Moore's law. Recently a new era has clearly begun with genetics to be one of the major players in human life. In the near future, it will be possible to estimate the risks and prepare prevention strategies for a number of genetically based conditions just by analyzing a few drops of blood. Genetic cloning, genetic engineered organisms, extreme longevity, artificial intelligence, and personalized treatment will become a reality.

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Review Article

Evolution of Genetic Techniques: Past, Present, and Beyond

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Genetics is the study of heredity, which means the study of genes and factors related to all aspects of genes. The scientific history of genetics began with the works of Gregor Mendel in the mid-19th century. Prior to Mendel, genetics was primarily theoretical whilst, after Mendel, the science of genetics was broadened to include experimental genetics. Developments in all fields of genetics and genetic technology in the first half of the 20th century provided a basis for the later developments. In the second half of the 20th century, the molecular background of genetics has become more understandable. Rapid technological advancements, followed by the completion of Human Genome Project, have contributed a great deal to the knowledge of genetic factors and their impact on human life and diseases. Currently, more than 1800 disease genes have been identified, more than 2000 genetic tests have become available, and in conjunction with this at least 350 biotechnology-based products have been released onto the market. Novel technologies, particularly next generation sequencing, have dramatically accelerated the pace of biological research, while at the same time increasing expectations. In this paper, a brief summary of genetic history with short explanations of most popular genetic techniques is given.

1. Introduction

Due to rapid advances in genomic technologies, genetics analyses have become essential in clinical practice and research. During the past decade, a great stride has been made to unravel underlying mechanisms of genetic-related disorders. Landmarks in genetic history are summarized in Figure 1. Moreover, genetic testing methods have become widely accessible and feasible to perform even for small size laboratories in particular after the completion of Human Genome Project, which coincided with developments in computer technology. With the application of genetic testing for personalized medicine, we are at the beginning of an era that will provide new horizons in human health.

2. History of Genetic Techniques and Properties of Methods

2.1. Conventional Cytogenetic Techniques. Looking at the history in brief, genetics is the term introduced for the study of genes in organisms. Many early discoveries contributed as important milestones to evolve the study of genomes as it is applied today. One of the crucial steps that enabled visualizing intracellular structures was the invention of the single-lens optical microscope by Janssen in 1595 [1]. After many researchers started to make observations with the newly invented microscope, Hooke has proposed the description of the "cell" in 1665 [2]. A Swiss botanist Nageli first described thread-like structures in the nuclei of plant cells in 1840s, what he called "transitory cytoblasts" which would be defined

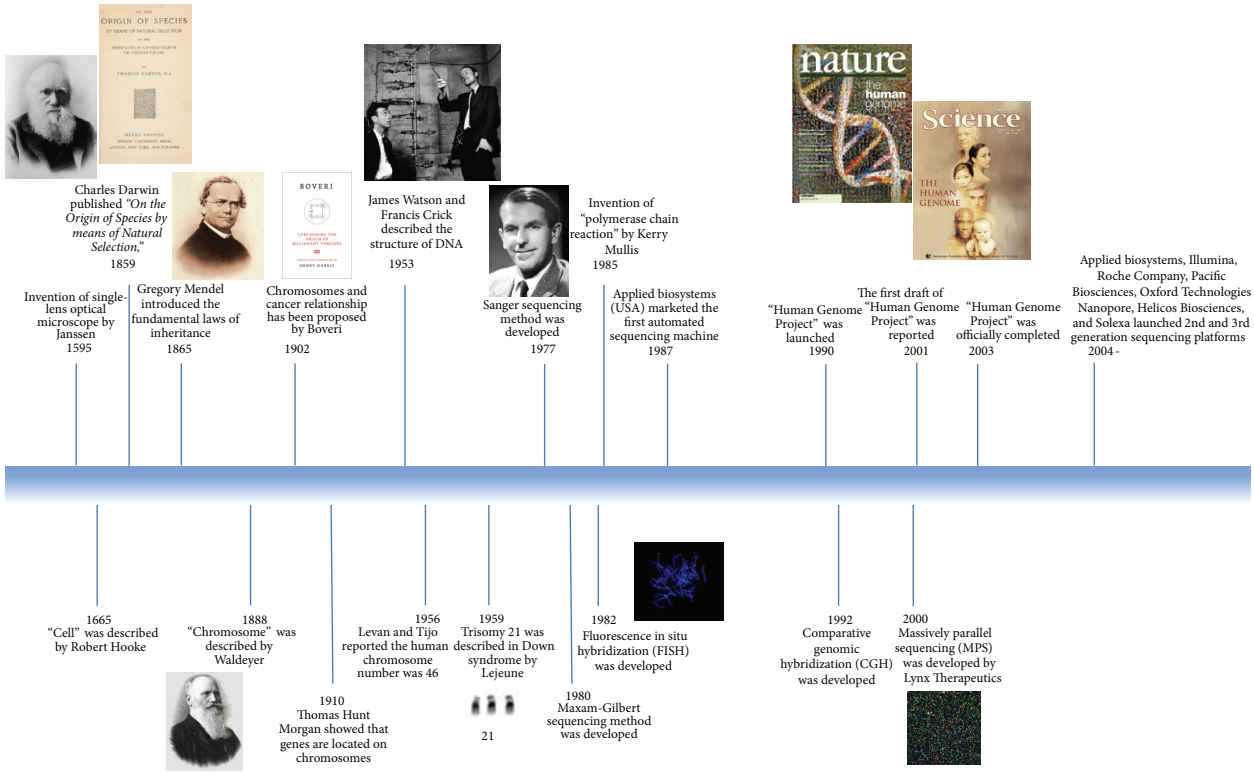

Figure 1: Landmarks in the history of genetics.

as chromosomes later on by Waldeyer in 1888 [3]. After Charles Darwin published *On the Origin of Species by means of Natural Selection* in 1859, Gregor Mendel introduced the fundamental laws of inheritance in 1865. This was crucial for understanding that "some characteristics of the organisms are inherited through genes." Mendel's rules were improved by the experiments of Thomas Hunt Morgan in 1910 who discovered that genes were responsible for the appearance of a specific phenotype located on chromosomes. In 1911, the first genetic map was achieved by mapping the fruit fly genes. From the 1800s until the middle of the 1900s, it was not understood that the structure of hereditary genetic material was responsible for the inheritance of traits from one generation to the next. In 1953, Watson and Crick described the double-stranded, helical, complementary, and antiparallel model for DNA [4]. They shared Nobel Prize in Medicine for this major discovery of the structure of DNA in 1962. In 1966, the genetic code in the DNA was finally discovered by defining that a codon which is a sequence of adjacent 3 nucleotides codes for the amino acids. The discovery of DNA and chromosomes paved the way for the rapid improvement in genetics and establishment of new technologies that have taken place over the last 50 years.

The first genetic analysis was performed in the field of cytogenetics. Although it was published that normal human chromosome number was 48, Tjio and Levan reported in 1956 that the correct number was 46 [5]. After the establishment of the peripheral leucocyte culture method incorporation with the fixation and staining methods, it became possible to identify human chromosome abnormalities associated with specific congenital defects [6]. Just a few years later, when Tjio and Levan reported the correct human chromosome number, several reports identifying numerical chromosome abnormalities such as trisomy 21 in Down syndrome, monosomy X and XXY in two frequent sex chromosomal disorders, Turner and Klinefelter syndromes, respectively, were published in 1959 [7–9]. After 1966, genetic techniques were performed not only in the postnatal samples but also in the prenatal samples, as it was shown that fetal cells derived from amniotic fluid could be obtained by using an invasive procedure which is termed as amniocentesis. Steele and Breg reported that cells cultured from amniotic fluid could be used to determine the chromosome aberrations in the fetus [10]. However, the resolution of the chromosomes was not high enough to determine the structural aberrations in cultured cells which was improved by the high resolution banding techniques using synchronized lymphocyte cultures established by Yunis in 1976 [11]. This novel banding technique allowed identifying the genetic etiology of clinically well-known syndromes such as Cri-du-Chat and Wolf-Hirschhorn syndromes. Besides unbalanced aberrations in patients, underlying chromosomal anomalies in those cases having balanced translocations with a history of recurrent miscarriages or having a deceased child with multiple congenital anomalies, have also been described by the use of the high resolution banding techniques. The relation between chromosomes and cancer was also established by Boveri in 1902. According to Boveri's somatic mutation theory, cancer is caused by at least one mutation in

the cells which causes defect in control of the cell proliferation and division [12]. He emphasized that the underlying main reason was abnormal chromosomal changes in a cancer cell.

2.2. Molecular Cytogenetic Techniques. Despite the establishment of high-resolution techniques which enabled revealing many known or unknown genetic syndromes, several cases having submicroscopic aberrations that were not visible at resolution between 500 and 1000 bands remained undiagnosed. A new technique called fluorescence in situ hybridization (FISH) was developed in the field of molecular cytogenetics in 1982 [13]. In this method, cytogenetics and molecular genetics were bridged. FISH identifies specific nucleic acid sequences from interphase nuclei or applied on metaphase chromosomes of [14, 15]. While this technique has advanced significantly today, it was previously based on the radioactively labeled ribosomal RNA hybridized to acrocentric chromosomes followed-up visualizing hybridization by autoradiography. Several techniques before fluorescence based techniques such as enzyme-based and gold-based probe systems were also used in the past [16]. The first application of FISH was established in 1980, when RNA that was directly labeled with fluorophore was used as a probe for specific DNA sequences [17]. Langer et al. developed a new technique involving the use of a nonradioactive probe for indirect labeling through nick translation [18]. In later times, by the development of new fluorescent molecules, which led to direct and indirect fluorescent labeled probe, binding to DNA bases improved the protocols of FISH. Chromosome rearrangements could be detected more easily with increased resolution of the FISH in both metaphases and interphases nuclei that could be used for both clinical diagnosis and research. FISH also provided the option for the simultaneous use of one or more DNA probes by labeling different colors or color combinations. Several types of probes can be used for FISH. Whole-chromosome painting probes, chromosome-arm painting probes, and centromeric, subtelomeric, and locus-specific probes are some of the examples which are available for the detection of specific constitutional and acquired chromosomal abnormalities. Consequently, a large number of sophisticated approaches were established based on the FISH-methods, for example, SKY (spectral karyotyping FISH) [19], Q-FISH (quantitative FISH) [20], fiber-FISH [21], heterochromatin-M-FISH [22] (M-FISH multicolor FISH) [23], COBRA-FISH (combined binary ratio labeling FISH) [24], cenM-FISH (centromere-specific M-FISH) [25], and other modified FISH approaches. The most advanced FISH-based approaches for whole-chromosome analysis are COBRA-FISH, M-FISH, and SKY FISH. Hybridizing all 24 different human chromosomes with whole-chromosome painting probes labeled with a combination of 5 different fluorophores enables visualizing each chromosome with a specific color. FISH also enabled showing that chromosomes are compartmentalized into discrete territories in the nucleus [26]. A correlation between the location and the size and the gene content of the chromosomes was described. Smaller and gene-rich chromosomes are generally situated towards the interior whereas larger and gene-poor chromosomes are generally situated towards the periphery of the nucleus [27, 28]. With the accurate map of the human genome obtained by the Human Genome Project, more and more probes from cloned and mapped segments (cosmids, PACs, BACs, and YACs) have become available for diagnostic purposes [29]. In light of these studies, several clinical diagnostic FISH tests have become commercially available. The most remarkable one is the test for deletion or duplication of the subtelomeric regions leading to a clinical picture mostly characterized by multiple congenital anomalies and intellectual disability. Subtelomeric aberrations are found in approximately 5% of patients [30–32] and this finding suggested that submicroscopic aberrations (i.e., deletions and duplications) might be present across the genome, particularly in patients demonstrating similar phenotypes with normal cytogenetic investigations.

Being time-consuming and expensive to evaluate chromosomal rearrangements in the whole genome by FISH led to the development of new techniques such as array-based comparative genomic hybridization [33–35]. Comparative genomic hybridization (CGH) which is based on competitive hybridization of amplified tumor DNA and normal DNA hybridized on normal metaphase slide was first developed by Kallioniemi et al. in 1992 to detect genomic imbalances in tumor cells [36]. If we look at the evolution of genetic technologies, the emergence of new technologies has always been inevitable due to the necessities revealed by the previous technologies. Although hybridization-based methods, which allow screening RNA, DNA, or protein such as northern blot, southern blot, or western blot, respectively, are widely used, innovative and powerful microarray hybridization methods were developed in the 1990s. Instead of hybridizing a labeled probe to targeted DNA on a slide, with array-CGH, the patient's DNA is hybridized to a large number of well-characterized probes immobilized on a slide. To summarize briefly, in this method, DNA of the patient is labeled with a specific color (green) and mixed with exactly the same amount of DNA of a normal control, which is labeled with a different color (red). This DNA mixture is then hybridized to the denaturated probe DNA on the glass and signal intensity ratios of test over reference are measured. Yellow dye appears when both patient and reference DNA are equal in proportion because of the presence of the same amount of red and green dyes, while regions with copy number losses are visualized as red and gains are green. This technique permits the detection of whole genome copy number variation (CNV) (duplications and deletions) at high resolution [36].

Array-CGH failed to detect the recessive disease genes, mosaic aneuploidy, uniparental disomy (UPD), or heterochromatic rearrangements. Because of this disadvantage, it is thought to combine with SNP arrays to improve the resolution of array-CGH [37]. These arrays have the highest resolution of all of the available array-based platforms. They have 10–15 times higher resolution when compared with FISH analysis (5–10 kb) [38]. Combination of array-CGH and SNP genotyping in a single platform increases the clinical diagnostic capability and uncovers the detection of small copy number variants [39]. In addition, array-based CGH has the advantage over FISH in the fact that living cells are not needed to obtain metaphase chromosomes because only DNA is

needed for analysis. The major drawback of array-based CGH is that it can only detect unbalanced rearrangements and is unable to detect balanced aberrations such as chromosome translocations, inversions, and insertions. However, recently, a modified array protocol, called translocation CGH (tCGH), was developed to address recurrent translocation breakpoints [40].

2.3. Molecular Genetics. Larger genomic changes such as deletions, duplications, and translocations can be detected by conventional karyotyping, FISH, or array-CGH methods but single nucleotide changes cannot be detected by these techniques. Molecular genetic techniques were rapidly developed after the establishment of polymerase chain reactions that enabled generating thousands to millions of copies of a particular DNA sequence [41]. Mullis and Smith have shared the 1993 Nobel Prize in Chemistry for their discovery of the polymerase chain reaction (PCR) technique. Although PCR was developed only a few decades ago, it has found numerous basic and clinical applications and is indispensable in today's science.

Taq DNA polymerase that was selected the first "Molecule of the Year" by Journal of Science was a major advantage in PCR technology [42]. Automation of PCR was greatly facilitated and simplified the detection of genomic mutations. PCR was previously used by the following techniques such as restriction fragment length polymorphism (RFLP), single-strand confirmation polymorphism (SSCP), and sequencing based methods. SSCP and RFLP, the most widely used techniques for mutation screening method in genetic diagnostic laboratories, were not able to detect every mutation, so development of new methods was needed. If the sequence of the gene of interest is not known, it may be difficult to interpret the results of these techniques. The determination of DNA sequencing enabled identifying the definite nucleotide changes in the targeted genes. This necessity was overcome by Maxam and Gilbert introducing Maxam-Gilbert chemical sequencing technology based on chemical modification of DNA followed by cleavage at specific bases [43]. Despite the efficiency of Maxam-Gilbert sequencing method, the use of hazardous chemical and inability to read long PCR fragments made this method replaced by Sanger sequencing that was based on dideoxynucleotide chain termination [44]. Manual Sanger sequencing method has been improved by the introduction of first generation of automated DNA sequencers [45]. Automatization of DNA sequencing enabled sequencing human genome in a fast and accurate way.

With the advances in the field of molecular genetics, it became possible to launch the Human Genome Project to reveal the complete human genome. The programme was launched in the USA with an effort of the Department of Energy and the National Institutes of Health in collaboration involving 20 groups in 1990. The first draft of human genome was published in 2001 by The International Human Genome Sequencing Consortium [46]. This first report covering 94% of the human genome announced that the human genome had 30,000–40,000 protein-coding genes and more than 1.4 million single nucleotide polymorphisms (SNPs). One day later, in parallel with HGP, Craig Venter who launched a human genome sequencing project by Celera Genomics using shot-gun sequencing method published the whole human genome sequence in Science [47]. The project was declared to be finished two and a half years ahead of scheduled time in 2003 coinciding with the 50th anniversary of the paper in which Watson and Crick reported DNA's double helix [48]. It was reported that 20,000–25,000 genes were present in the human genome covering 93% of the euchromatic region. Human Genome Project not only revealed the complete sequence of the human genome but also led to a huge improvement in the sequencing technology. Amplification of the gene of interest in the affected individual(s) enabled revealing mutations associated with specific monogenic disorders. Although automation of traditional dideoxy DNA sequencing Sanger method increases the efficiency of DNA sequencing, it was still not cost- and time-effective. A new technology called massively parallel sequencing (MPS) erasing these disadvantages was developed by Lynx Therapeutics [49]. This technology using reads of multiple reactions simultaneously and generating large amounts of sequence data in parallel provided a large impetus for exome sequencing, whole genome sequencing, and transcriptome and methylation profiling. This high-throughput technology that is called next generation sequencing (NGS) technology reduced the cost of sequencing of a human genome to less than $1.000. This technology is projected to sequence a human genome in 1 hour for $100 after new technological improvements in the near future. NGS technology is widely used for a variety of clinical and research applications, such as detection of rare genomic variants by whole genome resequencing or targeted sequencing, transcriptome profiling of cells, tissues, and organisms, and identification of epigenetic markers for disease diagnosis. One of the most successful applications of NGS technology is genome-wide discovery of causal variants in single gene disorders and complex genomic landscapes of many diseases. While whole genome or whole exome sequencing is the most comprehensive strategy in the diagnosis of unknown diseases and identification of new disease genes, targeted sequencing using selected panels of genes can reduce the sequencing time and cost by combining the diseases in the same group or pathway genes in known clinical pictures such as intellectual disability, neurometabolic disorders, or malignancies [50].

In addition to cost-effective advantages, sequencing the small part of the genome allows reducing the number of variations that in turn reduce the cost and time needed for data interpretation [51]. Targeted sequencing opened a new window in the diagnosis of several diseases with unknown etiology. For instance, it is possible to analyze more than 4800 genes which were identified in the Human Gene Mutation Database (HGMD Professional) and the Online Mendelian Inheritance in Man (OMIM) catalog in a single run for patients having unknown genetic disorders. Following the rapid advances in NGS technologies, the role of NGS in routine clinical practice will increase exponentially. Noninvasive prenatal diagnosis by using NGS is another application of this new technology. The most important step in the prenatal diagnostic procedures is obtaining fetal material to evaluate genetic condition. For years, invasive and noninvasive tests

have been used to assess the fetal health, particularly chromosomal abnormalities, during the pregnancy. Noninvasive tests measure epiphenomena, which does not analyze the pathology underlying the clinical picture of interest. Their sensitivity and specificity have not also reached the expected level despite several studies. On the other hand, invasive tests have been found to be associated with significant risks for both the mother and fetus. The identification of cell-free fetal DNA in maternal circulation and analyzing this fetal material by using NGS opened up a new horizon in the field of reproductive medical care. Despite main advantages of NGS technology, the researchers and clinicians still have many concerns about the implementation of NGS in practice [52]. The interpretation of huge amount of data obtained by NGS technology, billing and insurance issues, duration and content of consent process, and disclosure of incidental findings and variants of unknown significance were the main challenges related to offering this technology [53].

Approximately 10 years ago, karyotyping was the gold standard in patients with intellectual disability but array-CGH analysis has become the first line diagnostic test replacing karyotyping and FISH nowadays. As evident from this example, approaches to the genetic-related diseases could change in parallel with the advances in technology and science. "Philadelphia chromosome" is one of the oldest evolutionary examples of personalized medicine by revealing the etiological factor and the treatment options of a disease step by step by using the genetic analysis as the improvements in the genetic techniques were established. In 1960, a small chromosome called Philadelphia chromosome was identified to be the cause of the chronic myeloid leukaemia (CML). It was shown in 1973 by the chromosome banding technique that this chromosome was a result of a translocation between chromosomes 9 and 22 [54, 55]. It took more than 10 years to identify the fusion gene *BCR/ABL* (breakpoint cluster region and v-abl Abelson murine leukaemia viral oncogene homologue) through the improvements of the molecular techniques [56]. The following studies revealed that this fusion gene resulted in activation of a tyrosine kinase, which led to the discovery of a tyrosine kinase inhibitor drug Gleevec that was shown to be a highly successful treatment for CML [57].

Genetic test that will be used in the diagnosis should be chosen very carefully, which might not be the newest or the most sophisticated one. Sometimes only a karyotype could be enough to identify the genetic condition in the patient instead of more complicated array-CGH or NGS methods. As the technology in genetics rapidly evolves, new insights in terms of data interpretation and genetic counseling including pretest counseling, return of results, and posttest counseling need to be considered. Databases and consortium reports regarding the experiences of the clinicians and geneticists are crucial for integration of genomics into clinical practice.

3. Conclusion

If the developments in genetics and computer technologies continue to progress at their current speed, history has shown us we can look forward to some amazing developments in human life in the very near future. Some realistic scenarios of human life in the future could even see us carrying identity cards, which include our genome characteristics, rather than the format we are currently using. Gene corrections, cloned individuals and organs, and even genetic-based techniques as a primary laboratory analysis in almost all human diseases for a clinician will no longer be a dream. We have come to the point nowadays where genetic testing is commercially available; the individual now has the possible means to access this delicate information named as direct to consumer (DTC) genetic testing. Contrary to the traditional hospital or physician based testing, accessing an individual's genetic information without medical or specialized interpretation has gradually been finding a place in our daily lives. Today, more than 25 companies, from all over the world, offer DTC service to the public. Serious concerns, however, regarding the use of this kind of service, have been raised in terms of misleading and incidental results derived from unproven or invalidated data. Moreover, there is also a significant risk for unauthorized use of sensitive genetic information by big business, particularly in the fields such as health insurance. On the other hand, DTC does provide early awareness of genetic diseases and thereby offers individuals the opportunity to play an active role in their own health care. The issue at stake here leads us to the same difficult medical ethics dilemma: patient autonomy and right to know one's genetic composition versus nonmaleficence.

To conclude, in parallel with the rapid developments in the field of genetic technologies, ethical and legal issues regarding the implementation of those technologies need to be addressed. Because use of personal genetic information looks certain to directly impact our daily lives in the near future, protocols need to be discussed in detail, with guidelines provided and updated regularly as part of a regulated multidisciplinary approach.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Cytogenetic as an Important Tool for Diagnosis and Prognosis for Patients with Hypocellular Primary Myelodysplastic Syndrome

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We analyzed cytogenetically 105 patients with hypocellular primary MDS and their clinical implications. The main chromosomal abnormalities found were del(5q)/−5, del(6q)/+6, del(7q)/−7, del(11q), and del(17p). Pediatric patients had a higher frequency of abnormal karyotypes compared with adult patients ($P < 0{,}05$). From our patients, 18% showed evolution of the disease. The chromosomal abnormalities presented in the diagnosis of patients who evolved to AML included numerical (−7, +8) and structural del(6q), del(7q), i(7q), t(7;9), i(9q), and del(11q) abnormalities and complex karyotypes. Although the frequency of evolution from hypocellular MDS to AML is low, our results suggest that some chromosomal alterations may play a critical role during this process. We applied the IPSS in our patients because this score system has been proved to be useful for predicting evolution of disease. When we considered the patients according to group 1 (intermediate-1) and group 2 (intermediate-2 and high risk), we showed that group 2 had a high association with respect to the frequency of abnormal karyotypes ($P < 0{,}0001$), evolution of disease ($P < 0{,}0001$), and mortality ($P < 0{,}001$). In fact, the cytogenetic analysis for patients with hypocellular primary MDS is an important tool for diagnosis, prognosis, in clinical decision-making and in follow-up.

1. Introduction

Myelodysplastic syndrome (MDS) comprises a heterogeneous group of clonal bone marrow disorders characterized by various degrees of pancytopenia and morphological and functional abnormalities of hematopoietic cells and an increased risk of transformation into acute myeloid leukemia (AML) [1]. MDS is known as a disease of adults, particularly the elderly. Pediatric MDS is an uncommon disorder, accounting for less than 5% of hematopoietic malignancies [2, 3]. The bone marrow in primary MDS patients is usually hypercellular or normocellular; however between 10% and 20% of patients can present hypocellular bone marrow [4–6].

The remarkable progress in understanding the leukemogenesis was sustained by methodological developments in the cytogenetic field. The cytogenetic abnormalities have provided molecular basis for the discovery of the genes involved into the leukemogenesis mechanisms. In several studies, the cytogenetic turned out to be one of the most important prognostic parameters and it was incorporated into statistical models aiming for a better prediction of the individual prognosis [7]. In primary MDS, the discovery of nonrandom chromosomal abnormalities confirmed the clonality,

providing a way to identify the malignant clone and point out some oncogenes and tumor suppressor genes, possibly involved in the development and in the leukemic transformation. The cytogenetic evaluation of a bone marrow sample from patients with MDS has become an integral part of clinical care [8, 9]. The clonal cytogenetic alterations can be detected in 30–50% of adult patients with primary MDS. In pediatric patients, this incidence is 50–70% of the cases. The most frequent chromosomal abnormalities in MDS are del(5q), del(7q)/−7, +8, del(11q), del(12p), del(17p), del(20q), and loss of Y chromosome [7, 10].

Reviewing the literature, we can notice that there are few studies in hypocellular primary MDS relating the frequency of abnormal karyotypes, their correlation with the subtypes and leukemic transformation [6, 11, 12]. Some authors suggested that the frequency of abnormal karyotypes in hypocellular primary MDS is less than in normo-/hypercellular MDS [11, 12]. However, Yue and colleagues suggested there is no difference in the frequency of abnormal karyotypes between hypocellular and normo-/hypercellular MDS [6]. As we can notice, these studies showed controversies about the frequency of abnormal karyotypes in hypocellular MDS. Several studies have demonstrated the prognosis value of cytogenetic analysis in MDS [10, 11, 13, 14]. So, the karyotype was incorporated in prognostic scores, allowing risk group stratification and helping to choose the therapy like the International Scoring System for Evaluating Prognosis (IPSS). The IPSS divides MDS patients in four risk groups: low, intermediate-1, intermediate-2, and high risk. The parameters used to stratify the patients according to risk groups are percentage of bone marrow blasts, cytogenetic pattern, and number of cytopenias [15]. This score system is the most used in MDS, but it is not well known if it can be used in patients with hypocellular MDS. The hypocellularity in MDS is considered an independent factor which predicts a favorable outcome. However, about 10–16% of the cases showed evolution to AML [6, 12]. In spite of these studies, the chromosomal abnormalities involved for the leukemic transformation in the cases of hypocellular primary MDS are still unknown. In this study, we analyzed the chromosomal pattern of hypocellular primary MDS in pediatric and adult patients, the frequency of chromosomal alterations, its correlation with the different subtypes and with the disease evolution. We analyzed the chromosomal abnormalities during leukemic transformation and we suggested the involvement of genes associated with these cytogenetic abnormalities. We also discussed the application of the IPSS for patients with hypocellular primary MDS.

2. Material and Methods

2.1. Patients. Bone marrow cells were obtained from a total of 105 patients with hypocellular primary MDS. These patients were studied between 1991 and 2013. Chromosomal and clinical studies were carried out in all cases. The patients were diagnosed at the Hematology/Oncology Centers of some hospitals in Rio de Janeiro, Brazil: Bone Marrow Transplantation Center (CEMO-INCA), Hematology Service (INCA), Arthur Siqueira Cavalcanti Hematology Institute (HEMORIO), and Martagão Gesteira Pediatric and Puericulture Institute (IPPMG). From 105 patients, there were 56 males and 49 female, and the mean age was 31 years, with a range from 1 to 84 years. None of these patients were previously treated for a malignancy. Diagnosis was based on morphological, cytochemical studies and immunophenotypic and cytogenetic analyses. The adult patients were classified according to FAB criteria [16] and the pediatric patients were classified according to Hasle and colleagues [17]. This study was approved by Ethics Committee of National Cancer Institute and all procedures performed followed the bioethics standard, according to resolution 196/96 of Health National Committee.

2.2. Conventional and Molecular Cytogenetic Analysis. Karyotypes of bone marrow cells were obtained from cultures in RPMI 1640, with 20% fetal calf serum (GIBCO) at 37°C for 24 hours. Cell cultures were pulsed with colcemid to a final concentration of 0.05 μg/mL for the final hour of incubation. Cells were subsequently harvested by standard procedures (hypotonic shock: 0,075 M) and fixed in methanol: acetic acid (3 : 1). GTG banding was performed. Chromosomes were identified and arranged according to the International System for Cytogenetic Nomenclature, 2013 [18].

Fluorescence "*in situ*" hybridization (FISH) analysis was performed using the following probes: dual color probe for chromosome 11 (LSI MLL dual color break apart rearrangement probe) and probe to *TP53* gene (LSI p53, spectrum orange). The probes were from Vysis, Abbott Laboratories, USA. Slide pretreatment, probe hybridization, posthybridization washing, and signal detection were done according to manufactured protocols. FISH analyses were done to confirm some chromosomal deletions as 11q23 and 17p and to characterize the breakpoint and the gene involved in the chromosomal abnormality. We used the samples of cytogenetic cultures.

2.3. Statistical Analysis. We studied the correlation between the karyotype and the clinical features. All the statistical analyses were done using the χ^2 test. A value of $P < 0{,}05$ was considered significant in all analyses. We studied statistically the following variables: age (≤18 years and >18 years), sex (male and female), MDS subtypes (RA/CR, RAEB, and RAEB-t), and risk groups with the frequency of normal versus abnormal karyotypes. We analyzed the association of frequency of abnormal karyotypes with the evolution of disease. Considering MDS subtypes, we analyzed the association of mortality with the frequency of abnormal karyotypes. For this, we classified our patients in two groups: RA/CR (initial stage of MDS) and RAEB or RAEB-t (later stages of MDS). We also studied the association between risk groups, according to the IPSS, and the frequency of abnormal karyotypes, evolution of disease, and mortality. In our study there were no patients classified as low risk IPSS subgroup. Although our patients were classified in three risk groups (intermediate-1, intermediate-2, and high risk), for the statistical analyses we considered two groups: group 1 and group 2. The elements in group 1 are the patients classified as

intermediate-1 and the elements in group 2 were the patients classified as intermediate-2 or high risk. We also calculated the P value to study the evolution of disease and mortality comparing group 1 with group 2.

3. Results

3.1. Clonal Chromosomal Abnormalities in Patients with Hypocellular Primary MDS. From a total of 482 patients, 105 patients showed hypocellular primary MDS, representing 21,8% of all cases. Among a total of 105 hypocellular primary MDS patients, clonal chromosomal abnormalities were detected in fifty-eight patients (55%). The distribution of abnormal karyotypes in each FAB subgroup was 42% in RA, 100% in RAEB, and 100% in RAEB-t (Figure 1). The frequency of abnormal karyotypes was significantly higher in later stages of disease (RAEB and RAEB-t) compared to the initial stage (RA) ($P < 0{,}0001$). Cytogenetic results showed that patients with RA presented normal karyotypes or single abnormalities as del(1q), del(3q), inv(3q), del(4q), del(5q), del(6q), del(7q), −7, del(9p), del(11q), del(12p), del(17p), hyperdiploid karyotype, biclonal chromosomal abnormality, and a marker chromosome. In the RAEB and RAEB-t group, single chromosomal abnormalities were observed such as −5, del(6q), +6, del(7q), −7, i(7q), t(7;9), +8, i(9q), del(11q), del(17p), del(20q), and complex karyotypes. As we can see in Table 1, there was no specific chromosomal abnormality associated with a subtype of hypocellular primary MDS. We analyzed 105 patients with hypocellular primary MDS. Among 39 pediatric patients with hypocellular primary MDS, 27 (69%) had abnormal karyotypes and among 66 adult patients, 31 (47%) showed abnormal karyotypes. The pediatric MDS has different frequencies of chromosomal abnormalities comparing to adult MDS. In pediatric patients, the chromosomal abnormalities that had higher frequencies were del(7q)/−7, del(11)(q23), and del(17p) and in adult patients were del(5q), +8, and del(17p). All cases of del(11)(q23) and del(17p) were confirmed by FISH analyses. These analyses showed the deletion of one allele of *MLL* gene in 11q23 and of *TP53* gene in 17p, confirming the results of conventional cytogenetic, the G-banding.

From 105 patients, 19 (18%) showed progression from MDS to AML. The median time for AML transformation was 2,5 months. The median and mean of overall survival in 105 patients were 35 and 52 months, respectively. The chromosomal abnormalities presented in the diagnosis of patients, who showed evolution of disease, included the numerical chromosomal abnormalities −7, +8, and the structural abnormalities del(6q), del(7q), i(7q), t(7;9), i(9q), del(11q), and complex karyotypes. During the evolution of the disease, we observed the gain of the chromosomal abnormalities del(7p), i(9q), dup(1q), del(11)(q23), and +8.

3.2. Correlation of Karyotypes and Clinical Features in Patients with Hypocellular Primary MDS. We analyzed different variables in patients with primary MDS as age, sex, MDS subtypes, and distribution of risk groups according to IPSS with the presence of normal versus abnormal karyotypes (Table 2).

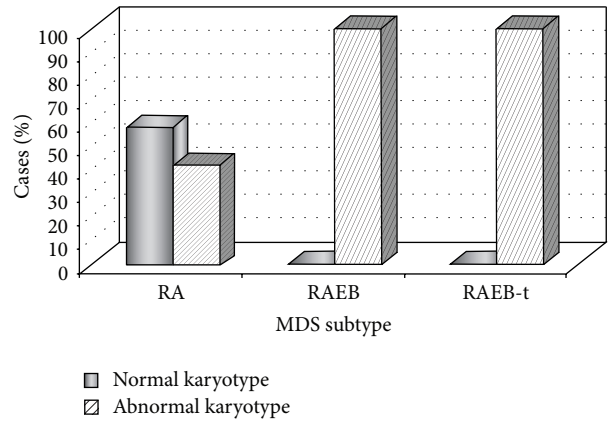

Figure 1: Frequency of normal versus abnormal chromosomal findings in subtypes of hypocellular primary MDS.

In our study, we observed some extremely significant results. One is that advanced stages (RAEB and RAEB-t) had a highly significant association with the frequency of abnormal karyotypes ($P < 0{,}0002$), evolution of disease ($P < 0{,}0001$), and mortality ($P < 0{,}0005$). Considering the patients according to group 1 (intermediate-1) and group 2 (intermediate-2 and high risk), we also showed that group 2 had a high association with respect to the frequency of abnormal karyotypes ($P < 0{,}0001$), evolution of disease ($P < 0{,}0001$), and mortality ($P < 0{,}001$). Our patients received different treatments: chemotherapy, supportive care, immunosuppressive therapy (ATG/cyclosporine), allogeneic hematopoietic stem cell transplantation, and others (thalidomide, lenalidomide). In our study, we verified that patients treated with allogeneic stem cell transplantation achieved a better treatment response.

4. Discussion

The hypocellular primary MDS is a rare neoplastic disease. In this study, we analyzed the chromosomal abnormalities of 105 patients with hypocellular primary MDS and the clinical features. In our study, the frequency of abnormal karyotypes was 55%. This result was similar to the studies of Yue and colleagues [6] and Huang and colleagues [12]. In the first study, it analyzed 163 patients and it found 47,5% of abnormal karyotypes [6]. In the second study, in a total of 33 patients, it found 42,2% of cases with abnormal karyotypes [12]. However, Marisavljević and colleagues showed a small frequency of cases with abnormal karyotypes (12,5%) [11]. We think this result may be associated with the low number of patients studied: 24 patients. Although the authors of these studies showed the frequency of abnormal karyotypes in hypocellular MDS, they did not discuss specific chromosomal abnormalities and their correlation with leukemic transformation.

The main chromosomal abnormalities found in our patients were del(5q), del(7q), −7, +8, del(11q), del(17p), and complex karyotypes. These chromosomal alterations are similar to those found in normo-/hypercellular. So, in primary MDS, independently of the cellularity in the bone marrow,

Table 1: Cytogenetic analysis in 105 patients with hypocellular primary MDS.

Case	Age (years)	FAB	Karyotype	IPSS	Evolution from MDS to AML/time to AML transformation (months)
1	7	RA	46, XX[25]	Int-1	No
2	16	RA	46, XY[33]	Int-1	No
3	53	RA	46, XY[42]	Int-1	No
4	61	RA	46, XX[20]	Int-1	No
5	17	RA	46, XX[20]	Int-1	No
6	12	RA	46, XX[49]	Int-1	No
7	27	RA	46, XY, del(17)(p12)[5]/46, XY[23]	Int-1	No
8	26	RA	46, XX[30]	Int-1	No
9	22	RA	46, XY[25]	Int-1	No
10	7	RA	46, XX[31]	Int-1	No
11	22	RA	46, XX[22]	Int-1	No
12	32	RA	46, XY[28]	Int-1	No
13	9	RA	46, XX, del(12)(p12)[5]/46, XX[15]	Int-1	No
14	36	RA	46, XX[23]	Int-1	No
15	43	RA	46, XX[25]	Int-1	No
16	23	RA	46, XX[30]	Int-1	No
17	32	RA	46, XY, del(6)(q21)[3]/46, XY[21]	Int-1	No
18	31	RA	46, XY, del(17)(p12)[6]/46, XY[24]	Int-1	No
19	51	RA	46, XY, del(17)(p12)[8]/46, XY[20]	Int-1	No
20	14	RA	46, XY, del(17)(p12)[13]/46, XY[36]	Int-1	No
21	15	RA	46, XY, del(17)(p12)[4]/46, XY[18]	Int-1	No
22	37	RA	46, XX[30]	Int-1	No
23	16	RA	51, XX, +4, +6, +8, +14, +20[3]/46, XX[41]	Int-1	No
24	12	RA	46, XY, del(17)(p12)[4]/46, XY[17]	Int-1	No
25	16	RA	46, XX, inv(3)(q21q26)[5]/46, XX[15]	Int-1	No
26	84	RA	46, XY, del(17)(p12)[4]/46, XY[16]	Int-1	No
27	19	RA	46, XY[32]	Int-1	No
28	29	RA	46, XY, del(17)(p12)[6]/46, XY[19]	Int-1	No
29	62	RA	46, XX[29]	Int-1	No
30	18	RA	46, XY, del(1)(q32)[8]/46, XY[16]	Int-1	No
31	51	RA	46, XY, del(11)(q23)[8]/46, XY[17]	Int-1	No
32	30	RA	46, XX, del(17)(p12)[5]/46, XX[16]	Int-1	No
33	31	RA	46, XY, del(11)(q23)[4]/46, XY[17]	Int-1	No
34	13	RA	46, XY, del(7)(q22)[7]/46, XX[14]	Int-2	Yes/6
35	13	RA	46, XY, del(12)(p12)[4]/46, XY[36]	Int-1	No
36	49	RA	46, XX[20]	Int-1	No
37	9	RA	46, XY, del(3)(q23)[4]/46, XY[13]	Int-1	No
38	10	RA	46, XX[34]	Int-1	No
39	42	RA	46, XY, del(17)(p12)[6]/46, XY[16]	Int-1	No
40	32	RA	46, XY[27]	Int-1	No
41	48	RA	46, XY[20]	Int-1	No
42	41	RA	46, XX[20]	Int-1	No
43	56	RA	46, XX[22]	Int-1	No
44	34	RA	46, XY[22]	Int-1	No

Table 1: Continued.

Case	Age (years)	FAB	Karyotype	IPSS	Evolution from MDS to AML/time to AML transformation (months)
45	10	RA	46, XY, del(17)(p12)[5]/46, XY[16]	Int-1	No
46	24	RA	46, XX, del(17)(p12)[3]/46, XX[20]	Int-1	No
47	19	RA	46, XY[30]	Int-1	No
48	29	RA	46, XX[34]	Int-1	No
49	11	RA	46, XY[20]	Int-1	No
50	4	RA	47, XY, +mar[3]/46, XY[25]	Int-1	No
51	38	RA	46, XX[20]	Int-1	No
52	26	RA	46, XX[42]	Int-1	No
53	27	RA	46, XX[20]	Int-1	No
54	7	RA	46, XY, del(12)(p12)[3]/46, XX[27]	Int-1	No
55	13	RA	46, XY, del(17)(p12)[9]/46, XY, del(17)(p12), del(12)(p13)[5]/46, XY, del(11)(q23)[3]/46, XY[34]	Int-1	No
56	45	RA	46, XX[20]	Int-1	No
57	18	RA	46, XX, del(9)(p21)[4]/46, XX[17]	Int-1	No
58	44	RA	46, XY[22]	Int-1	No
59	48	RA	46, XX, del(5)(q13q33)[7]/46, XX[13]	Int-1	No
60	32	RA	46, XY[30]	Int-1	No
61	72	RA	46, XX[21]	Int-1	No
62	56	RA	46, XX[30]	Int-1	No
63	42	RA	46, XY[32]	Int-1	No
64	7	RA	46, XY, del(11)(q23)[4]/46, XY[48]	Int-1	No
65	2	RA	46, XX, del(4)(q22)[4]/46, XX[28]	Int-1	No
66	14	RA	46, XY[50]	Int-1	No
67	61	RA	46, XX, del(5)(q13q33)[6]/46, XX[21]	Int-1	No
68	36	RA	46, XY[22]	Int-1	No
69	12	RA	46, XY[30]	Int-1	No
70	29	RA	46, XX[21]	Int-1	No
71	11	RA	46, XX[36]	Int-1	No
72	52	RA	46, XY[25]	Int-1	No
73	58	RA	46, XX, del(5)(q13q33)[16]/46, XX[7]	Int-1	No
74	36	RA	46, XY[28]	Int-1	No
75	12	RA	46, XY[30]	Int-1	No
76	49	RA	46, XY[27]	Int-1	No
77	10	RA	45, XY, −7[16]/46, XY[6]	Int-2	Yes/4
78	13	RA	45, XY, −7[8]/46, XY[12]	Int-2	No
79	37	RA	46, XX[24]	Int-1	No
80	10	RA	46, XX, del(6)(q24)[3]/46, XX[15]	Int-1	No
81	17	RA	46, XX[20]	Int-1	No
82	11	RAEB	46, XY, del(11)(q23)[7]/46, XY[13]	Int-2	Yes/4.5
83	6	RAEB	46, XX, i(9)(q10)[15]/46, XX[7]	Int-2	Yes/1
84	56	RAEB	46, XX, dup(1)(q12)[18]/45, XX, dup(1)(q12), del(3)(q23), −5[3]/46, XX[42]	Int-2	Yes/2
85	44	RAEB	46, XX, del(17)(p12)[8]/46, XX[14]	Int-2	No
86	63	RAEB	46, XX, del(17)(p12)[5]/46, XX[45]	Int-2	No

Table 1: Continued.

Case	Age (years)	FAB	Karyotype	IPSS	Evolution from MDS to AML/time to AML transformation (months)
87	58	RAEB	46, XY, del(20)(q11)[29]/46, XY[2]	Int-2	No
88	17	RAEB	46, XY, del(11)(q23)[9]/46, XY[13]	Int-2	Yes/4
89	45	RAEB	46, XY, del(17)(p12)[8]/46, XY[14]	Int-2	No
90	41	RAEB	46, XY, del(11)(q23)[8]/46, XY[16]	Int-2	No
91	15	RAEB	46, XX, del(11)(q23)[8]/46, XX[16]	Int-2	Yes/3
92	42	RAEB	46, XX, del(5)(q21), del(8)(q22)[14]/46, XX, del(5)(q21), del(8)(q22), i(9)(q10)[9]	High	Yes/1
93	60	RAEB	47, XY, +8[32]/46, XY[23]	Int-2	Yes/2.5
94	58	RAEB	46, XX, t(7; 9)(q32; q34)[3]/46, XX[29]	High	Yes/3
95	19	RAEB	46, XX, i(7)(q10)[4]/46, XX[17]	High	Yes/1
96	61	RAEB	46, XX, del(6)(q21)[13]/46, XX[9]	Int-2	Yes/2
97	58	RAEB	45, XY, del(7)(q22)[16]/46, XY[5]	High	Yes/3
98	11	RAEB	45, XY, −7[25]/46, XY[3]	High	Yes/1
99	49	RAEB	47, XY, +6[8]/46, XX[20]	Int-2	No
100	55	RAEB	45, XX, −5[6]/46, XX[18]	Int-2	No
101	1	RAEB-t	45, XX, −7[24]/46, XX[2]	High	Yes/1
102	42	RAEB-t	46, XY, del(6)(q21)[5]/46, XY[18]	High	Yes/5
103	64	RAEB-t	46, XY, t(1; 6)(p23; p25)[16]/46, XY, t(1; 6)(p23; p25), del(7)(p25), i(9)(q10)[2]/46, XY[11]	High	Yes/1
104	57	RAEB-t	47, XY, +8[14]/46, XY[8]	High	Yes/2
105	7	RAEB-t	46, XY, del(11)(q23)[12]/46, XY[13]	High	Yes/3.5

cytogenetic pattern is characterized mainly by losses of partial or total chromosomes, suggesting that the main class of genes involved in the pathogenesis of MDS is the tumor suppressor genes. In the context of tumor suppressor genes, both alleles usually must be inactivated according to Knudson's two-hit hypothesis, wherein one allele is often deleted, and the other allele is inactivated either by deletion, mutation, or epigenetic modification. However, chromosome deletions, such as del(5q) in MDS, have introduced the concept of haploinsufficiency where there is a monoallelic inactivation, for example, the *RPS4* gene that is involved in the development of del(5q) MDS [19]. The most frequent chromosomal alteration found in our study was del(17p) associated with the deletion of one allele of the tumor suppressor gene *TP53*. The *TP53* gene has been described as "the guardian of the genome," because of its role in maintaining the chromosomal stability. Deletion 17p has been reported in approximately 15% of the *novo* MDS cases. Alterations in this gene have been reported to be a later genetic event in the carcinogenesis model established in colorectal cancer and in chronic myeloid leukemia. However, in primary MDS alterations in *TP53*, like chromosomal deletions, may be detected in early stages. So, in those patients with a *TP53* alteration, a careful follow-up seems to be necessary because of the risk of early leukemic transformation. And a more intensive treatment may have to be considered for these patients [20–22]. In our study, most patients with del(17p) were at the early MDS stage, RA/RC. Most of these patients were pediatric and they were treated with hematopoietic stem cell transplantation. Silveira and colleagues suggested that *TP53* deletion in MDS represents a clinically relevant biomarker, which could be used to define *de novo* pediatric MDS [21].

Although the hypocellular MDS shows a low frequency of disease evolution [6, 11, 12], it is important to elucidate the chromosomal alterations associated with leukemic transformation, because the numerical and structural chromosomal abnormalities involved for the leukemic transformation in the cases of hypocellular primary MDS are still unknown. In our study, from 105 patients analyzed, 19 (18%) showed evolution of disease. The chromosomal abnormalities presented in the diagnosis of patients who showed evolution from MDS to AML included the numerical chromosomal abnormalities −7, +8 and the structural abnormalities del(6q), del(7q), i(7q), t(7;9), i(9q), del(11q), and complex karyotypes. During the evolution of the disease, we observed the gain of the chromosomal abnormalities del(7p), i(9q), dup(1q), del(11)(q23), and +8. Some of these chromosomal alterations, such as dup(1q), +8, and del(11)(q23), were previously described by our group as involved in the evolution from MDS to AML [10]. It is interesting to observe that important genes involved in the hematopoietic process, cell cycle control, and epigenetic control are in the regions of these chromosomal alterations.

Table 2: Correlation of karyotypes and the clinical features in patients with hypocellular primary MDS.

Patient's variables	Number of patients/frequency (%)	Karyotypes (%)		*P* value	Evolution from MDS to AML	*P* value	Mortality	*P* value
		Normal	Abnormal					
Age				$P < 0,05$		NS*		$P < 0,05$
≤18 years (pediatric patients)	39 (37%)	12 (31%)	27 (69%)		39/9		39/12	
>18 years (adult patients)	66 (63%)	35 (53%)	31 (47%)		66/10		66/36	
Sex				NS*		NS*		NS*
Male	56 (53%)	20 (36%)	36 (64%)		56/11		56/26	
Female	49 (47%)	27 (55%)	22 (45%)		49/8		49/22	
MDS subtypes/% of bone marrow blasts				$P < 0,0002$		$P < 0,0001$		$P < 0,0005$
Initial stage RA/<5%	81 (77%)	47 (58%)	34 (42%)		81/2		81/27	
Advantage stages RAEB and RAEB-t ≥5%	24 (23%)	0	24 (100%)		24/17		24/21	
IPSS				$P < 0,0001$		$P < 0,0001$		$P < 0,001$
Low	0 (0%)	—	—		—		—	
Int-1	78 (74%)	47 (60%)	31 (40%)		78/0		78/25	
Int-2	17 (16%)	0 (0%)	17 (100%)		17/9		17/13	
High	10 (10%)	0 (0%)	10 (100%)		10/10		10/10	

*No significance.

The members of *Hox* gene family, *HoxA9* and *HoxA10*, are localized in 7p15. These genes are expressed in hematopoietic precursors, with preferential expression in selfrenewing hematopoietic stem cells (HSC) and downregulation during terminal differentiation. The dysregulation of *Hox* genes is associated with a number of malignancies including the AML [23]. Deletions of 9p21 have been detected in various tumor types. The p15*INK4B* and p16*INK4A* genes are members of the cyclin-dependent kinase (CDK) inhibitor family, which control progression of the cell cycle from G1 to S phase. These genes are located in the 9p21 region. It also has been found that methylation in the promoter region of these genes is involved in evolution from MDS to AML [24]. Another chromosomal abnormality observed during leukemic transformation was the duplication of the long arm of chromosome 1. The dup(1q) has been associated with leukemic transformation in MDS as the unique cytogenetic event or associated with other chromosomal abnormalities [25]. In the long arm of chromosome 1, there are important genes that may be involved in the leukemic transformation. However, the *BCL9* gene, mapped in the 1q21, plays an important role in the *Wnt* signaling pathway and it is associated with tumor progression. This pathway is evolutionary conserved. At cellular level, this pathway regulates morphology, proliferation, and cell fate [26]. We also observed the del(11)(q23) involved in cases of evolution of disease. The *MLL* gene is mapped in the 11q23 region. The *MLL* gene is associated with various hematologic malignancies but is particularly common in infant [27]. Trisomy 8 was another chromosomal abnormality observed in the evolution of disease. Our group had already suggested that the gene probably involved in this leukemic transformation is the c-myc mapped in 8q24 [28]. It is interesting to observe that the hypocellular primary MDS has as chromosome alterations involved in leukemic evolution as hyper- and normocellular MDS. Our results suggest that probably the pathways of leukemic transformation may be the same.

We applied the IPSS in our patients with hypocellular MDS. According to the risk group stratification our patients were distributed in intermediate-1, intermediate-2, and high risk group. We did not have patients classified as low risk group, because in our sample all the patients had at least two cytopenias, receiving the classification of intermediate-1. This score system has been proved to be useful for predicting evolution of disease. When we considered the patients according to group 1 (intermediate-1) and group 2 (intermediate-2 and high risk), we showed that group 2 had a high association with respect to the frequency of abnormal karyotypes ($P < 0,0001$), evolution of disease ($P < 0,0001$), and mortality ($P < 0,001$). The IPSS is an important standard for assessing prognosis of primary MDS. And recently, this score system was revised and multiple statistically weighted clinical features were used to generate a prognostic categorization model. But bone marrow cytogenetics, marrow blasts percentage, and cytopenias remained the basis of the revised system [29].

Rare cytogenetic abnormalities, considered as intermediate group in the IPSS, as hyperdiploidy and cytogenetic biclonality, already described by our group, may be reported to help to elucidate its clinical implications in hypocellular primary MDS [30, 31]. In these studies we showed the importance of cytogenetic abnormality for the diagnosis of hypocellular primary MDS and to indicate the patients to stem cell transplantation. It is important to note that, in some

cases, the hypocellular bone marrow makes the diagnosis between MDS and aplastic anemia a difficult process, and the cytogenetic, in these cases, is considered an important tool for diagnosis characterizing a clonal chromosomal abnormality and indicating the diagnosis of MDS [30, 32].

In our patients, the majority of mortality was not associated with evolution of disease, but with the cytopenias associated with infections, anemia, and hemorragie. So, in hypocellular primary MDS, it is important to analyze the frequency of transfusions and the life quality of the patients. Regarding this point, recently Tong and collaborators showed that patients with hypocellular MDS presented more frequently with thrombocytopenia, neutropenia, increased transfusion dependency and intermediate-2/high risk disease compared with patients with hyper-/normocellular MDS [33]. However, in our study, the most of patients were classified in intermediate-1; they were in the initial stage of MDS (RA/RC).

The rates of allogeneic stem cell transplantation (SCT) to treat MDS are continually increasing [34]. The allogeneic SCT is the only treatment modality that has been demonstrated to cure patients with MDS [35]. However, given the variety of therapeutic options in parallel to the heterogeneity of MDS, determining the indications for SCT in MDS is considered a difficult task [34]. Based on cytogenetic and clinical studies our patients were indicated for bone marrow transplantation at initial stages of MDS, where the cytogenetic abnormalities play an important role aiding to indicate and select these patients for this treatment, specially the pediatric patients. So, the cytogenetic gives more precision in deciding the treatment with bone marrow transplantation. It has been suggested that allogeneic HSCT offers optimal survival benefits when the procedure is performed before MDS patients progress to advanced disease stages [36]. Our study provides new information into the role of the chromosomal abnormalities in hypocellular primary MDS with important clinical implications. The cytogenetic analysis is an important laboratory tool for diagnosis, prognosis, in clinical decision-making and in follow-up for pediatric and adult patients with hypocellular primary MDS.

New cytogenetic methods like FISH, array-CGH (comparative genomic hybridization), SKY (spectral karyotype), and MCB (multicolor banding) are considered complementary analyses for conventional cytogenetic. The diagnostic workup for MDS now frequently includes FISH panels using multiple probes for most balanced chromosomal defects. Since FISH can be performed on interphase nuclei, these panels allow for target detection of specific chromosomal abnormalities even when metaphase cytogenetics is not possible because of no mitosis [37]. The molecular cytogenetic methods as MCB allowed the characterization and provided the ability to identify candidate genes involved in the leukemogenesis process in MDS [38].

As clonal chromosomal abnormalities were observed in about 50% of MDS patients, the necessity of new additional biomarkers that aid the diagnosis and prognosis for MDS is clear [39]. The new karyotyping and molecular tests, such as chromosomal microarray analysis, next generation sequencing (NGS), have increased the detection of genetic abnormalities in MDS and increased our understanding on the MDS biology. But these new genetic methods are being used mainly in basic research. Although new methods are potentially diagnostic tools, they still have not replaced the traditional laboratory techniques such as conventional cytogenetic and FISH analyses. Several studies point that cytogenetic analysis is still the gold standard genetic laboratory testing for diagnosis and prognosis in myelodysplastic syndrome [7, 15, 29]. Another important point is that chromosome banding remains the only low-cost genome screening technique, allowing the identification of balanced as well as unbalanced genomic rearrangements in single cells [40].

5. Conclusions

The remarkable progress in understanding the leukemogenesis was sustained by methodological developments in the cytogenetic field. In several studies, the cytogenetic turned out to be one of the most important prognostic parameters and it was incorporated into statistical models aiming a prognostic scoring system, like the International Prognostic Scoring System (IPSS) for myelodysplastic syndrome (MDS). Because MDS is a very heterogeneous disease, the diagnosis and the prognosis are generally considered a difficult clinical practice. In this study, we analyzed the chromosomal abnormalities of 105 patients with hypocellular primary MDS and the clinical features. The main chromosomal abnormalities found in our patients were del(5q), del(7q), −7, +8, del(11q), del(17p), and complex karyotypes. In our study, from 105 patients analyzed, 19 (18%) showed evolution of disease. The chromosomal abnormalities presented in the diagnosis of patients who showed evolution from MDS to AML included the numerical chromosomal abnormalities −7, +8 and the structural abnormalities del(6q), del(7q), i(7q), t(7;9), i(9q), del(11q), and complex karyotypes. During the evolution of the disease, we observed the gain of the chromosomal abnormalities del(7p), i(9q), dup(1q), del(11)(q23), and +8. It is interesting to observe that important genes involved in the hematopoietic process, cell cycle control, and epigenetic control are in the regions of these chromosomal alterations. Our study provides new information into the role of the chromosomal abnormalities in hypocellular primary MDS with important clinical implications. The cytogenetic analysis is an important laboratory tool for diagnosis, prognosis, in clinical decision-making and in follow-up for pediatric and adult patients with hypocellular primary MDS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Evaluation and Integration of Genetic Signature for Prediction Risk of Nasopharyngeal Carcinoma in Southern China

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Genetic factors, as well as environmental factors, play a role in development of nasopharyngeal carcinoma (NPC). A number of single nucleotide polymorphisms (SNPs) have been reported to be associated with NPC. To confirm these genetic associations with NPC, two independent case-control studies from Southern China comprising 1166 NPC cases and 2340 controls were conducted. Seven SNPs in *ITGA9* at 3p21.3 and 9 SNPs within the 6p21.3 *HLA* region were genotyped. To explore the potential clinical application of these genetic markers in NPC, we further evaluate the predictive/diagnostic role of significant SNPs by calculating the area under the curve (AUC). *Results.* The reported associations between *ITGA9* variants and NPC were not replicated. Multiple loci of *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* were statistically significant in both cohorts (P_{combined} range from 5.96×10^{-17} to 0.02). We show for the first time that these factors influence NPC development independent of environmental risk factors. This study also indicated that the SNP alone cannot serve as a predictive/diagnostic marker for NPC. Integrating the most significant SNP with IgA antibodies status to EBV, which is presently used as screening/diagnostic marker for NPC in Chinese populations, did not improve the AUC estimate for diagnosis of NPC.

1. Background

Nasopharyngeal carcinoma (NPC) is rare in most regions of the world; however, it is a common cancer in Southern China, especially in the Guangdong and Guangxi Provinces. The incidence rate of NPC for males in the Southern Chinese provinces of Guangdong and Guangxi is more than 20 per 100,000 person-years and up to 25–40 per 100,000 person-years in some areas bordering the Xijiang River and Pearl River drainages in these two provinces [1, 2]. It has been well established that Epstein-Barr virus (EBV) is strongly associated with NPC [3–5]. The association of EBV antibodies and NPC were first reported in 1966 [6]. Later, the presence of IgA antibodies to EBV in serum was found to serve as a predictive marker for NPC in Chinese populations [7–9]. IgA antibody titers to the EBV viral capsid antigen (EBV/IgA/VCA) and to the EBV early antigen (EBV/IgA/EA) have been used for the screening and diagnosis of NPC

for many years in Southern Chinese populations [7, 9–11]. Epidemiological studies have pointed to other environmental factors (including consumption of salt-preserved fish, exposure to domestic wood-cooking fires, and exposure to occupational solvents) as having a role in development of NPC [5, 12].

Evidence for genetic modulation of NPC risk has accumulated recently. Familial aggregation of NPC cases has been observed in both high- and low-risk populations in different geographic regions [5, 13–15]. Several studies have shown associations between *HLA* genes and NPC [16–21]. The results from our phase I cohort confirm and extend previously reported *HLA* and NPC associations in Southern Chinese populations [22]. Two genome-wide association studies (GWAS) have identified multiple gene association with risk of NPC in Chinese ancestry cohorts [23, 24]. The first GWAS comprised 111 unrelated NPC cases and 260 controls and a replication sample set of 168 cases and 252 controls from the Malaysian Chinese population [23] reported evidence of association with *ITGA9* on Chr 3p21.31-21.2. The second GWAS was conducted in 277 Taiwanese NPC cases and 285 controls and included two independent replication sets. This group found associations with variants on Chr 6p21.3 in or near *HCG9*, *HLA-A*, *HLA-F*, and *GABBR1* genes [24].

To investigate whether genetic variants can improve the EBV IgA antibodies test method for NPC diagnosis, we extended previously reported GWAS associations with NPC to Han Chinese from Southern China—the highest NPC incidence region. Here, two independent case-control studies were conducted—phase I cohort with 350 NPC cases and 619 controls and phase II cohort with 816 NPC cases and 1721 controls to determine if the polymorphisms of *ITGA9*, *HLA-A*, *HLA-F*, *GABBR1*, and *HCG9* were associated with NPC development or can be potential genetic markers for onset of NPC in a Southern Chinese population.

2. Materials and Methods

2.1. Cases and Controls (Table 1). The NPC cohorts were recruited from areas along the Xijiang River in Guangdong and Guangxi Provinces of Southern China in two collection phases [5, 25]. Phase I participants were recruited from April 2000 to June 2001. NPC cases were either incident or prevalent biopsy-confirmed NPC cases. The controls were the case's spouse or geographically matched residents who were NPC-free at the time of study enrollment. Phase II study participants were recruited from November 2004 to October 2005. Cases were incident or prevalent, biopsy-confirmed NPC. Controls were NPC-free at the time of study enrollment and matched to NPC cases on age and district/township of residence. NPC cases were patients at Wuzhou Red Cross Hospital in Wuzhou City and outpatients at Cangwu Institute for NPC Control and Prevention in Cangwu County. All participants self-identified as Han Chinese and reported at least three generations of residency in Guangdong or Guangxi Province, China.

IgA antibodies to EBV capsid antigen (EBV/IgA/VCA) and IgA antibodies to EBV early antigen (EBV/IgA/EA) were determined by serological testing at the time of study enrollment. The cutoff titer for the seropositive status was at least 1 : 10 and 1 : 5 for IgA/VCA and IgA/EA, respectively, based on local standard. Blood samples were obtained from 350 NPC cases (66.6% male) and 619 controls (42.8% male) for phase I; the mean age was 45 years ± 11 and 46 years ± 10 for NPC cases and controls, respectively. For phase II, blood samples were collected from 816 NPC cases (73.2% male) and 1721 controls (61.4% male); the mean age was 45 years ± 11 and 46 years ± 12 for NPC cases and controls, respectively. Family history of NPC, parental ancestry for three generations, dietary and smoking habits, household exposures to wood fires, and occupational exposures to solvents were also captured by questionnaire in the phase II cohort [5]. Participants were asked if there was a family history of NPC in first- (children, siblings, or parents), second- (aunts or uncles, nieces or nephews, and grandparents), or third-degree relatives (first cousins). Information was also collected on the frequency of consumption per month (≥3 times/month, and <3 times/month) of salty fish and preserved meat. Questions on cigarette smoking included current and past smoking habits and number of cigarettes smoked per day. Questions on household and occupational exposures captured data on domestic exposure to wood fires for cooking and occupational exposures to solvents (e.g., formaldehyde, acetone, toluene, or xylene) and duration of exposure (>10 years or ≤10 years). Responses were recorded by double-entry and verification of all data was performed to avoid data entry errors. We excluded persons of minority ethnicity and those who had blood relatives in either the case or control group. We also did not allow overlap in participation between phase I and phase II; the cohorts were independent. Institutional review board approval was obtained from all participating institutions and informed consent was obtained from each study participant.

2.2. Genomic DNA Extraction. In phase I participants, DNA was extracted from whole blood or lymphoblastoid cell lines using QIAamp DNA blood maxi kit (Qiagen, Valencia, CA, catalog number 51194). More than 80% of the genotypes were determined from DNA directly extracted from whole blood. In phase II participants, DNA was extracted from whole blood by traditional phenol/chloroform method with Phase Lock Gel tube (Qiagen, MaXtract High Density, catalog number 129065).

2.3. Genotyping. In both phases I and II, 7 SNPs of *ITGA9* on 3p21.3 and 9 SNPs within the *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* genes on chromosome 6p21.3 were genotyped by using commercially available TaqMan SNP genotyping assays and GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), in accordance with the manufacturer's instructions. The sequence detection software was used for allelic discrimination. For quality control, 8 to 16 template-free controls, one family sample [25], and 5% to 10% of duplicate samples were included in each 384-well plate.

Table 1: Characteristics of participants in a study of nasopharyngeal carcinoma (NPC) in southern China.

	Phase I		Phase II	
	Cases	Controls	Cases	Controls
Age (years)	45 ± 11.4 (SD)	46 ± 9.7 (SD)	45 ± 11.0 (SD)	46 ± 11.7 (SD)
Male, %	66.6 (233/350)	42.8 (265/619)	73.2 (597/816)	61.4 (1056/1721)
IgA/VCA+*, %	95.4 (334/350)	44.7 (277/619)	95.8 (782/816)	42.3 (731/1721)
IgA/EA+**, %	59.5 (201/348)	2.3 (14/619)	72.4 (591/816)	2.6 (44/1721)
Total	350	619	816	1721

Age: the age at diagnosis of NPC for cases and age of enrollment for controls.
SD: standard deviation.
*Positive for IgA antibodies to Epstein-Barr virus capsid antigen.
**Positive for IgA antibodies to Epstein-Barr virus early antigen.
The cutoff value for seropositive status is 1 : 10 for IgA/VCA and 1 : 5 for IgA/EA.

Table 2: Association between alleles of SNPs at 6p21.3 and NPC in phase I and phase II combined.

Gene-SNP	Risk allele	Phase I and phase II		Phase I and phase II		Phase II	
		OR (95% CI)*	P^*	OR (95% CI)**	P^{**}	OR (95% CI)***	P^{***}
GABBR1-rs2267633	A	1.61 (1.41–1.84)	$1.02E-12$	1.48 (1.17–1.87)	0.001	1.41 (1.02–1.95)	0.03
GABBR1-rs29230	T	1.64 (1.45–1.89)	$1.36E-13$	1.61 (1.28–2.04)	$6.14E-05$	1.61 (1.16–2.22)	0.004
GABBR1-rs29232	A	1.35 (1.21–1.49)	$1.85E-08$	1.41 (1.16–1.71)	0.0006	1.33 (1.01–1.76)	0.05
HLA-F-rs3129055	G	1.14 (1.02–1.28)	0.02	1.33 (1.09–1.64)	0.008	1.47 (1.10–1.20)	0.01
HLA-A-rs2517713	T	1.61 (1.43–1.82)	$2.44E-16$	1.69 (1.35–2.08)	$2.58E-06$	1.64 (1.20–2.22)	0.003
HCG9-rs9260734	G	1.67 (1.47–1.87)	$5.96E-17$	1.75 (1.41–2.17)	$6.48E-07$	1.75 (1.28–2.44)	0.0005
HCG9-rs3869062	A	1.60 (1.42–1.81)	$3.4E-14$	1.63 (1.30–2.04)	$1.97E-05$	1.60 (1.16–2.19)	0.004
HCG9-rs5009448	C	1.62 (1.45–1.82)	$1.89E-16$	1.66 (1.33–2.06)	$3.46E-06$	1.64 (1.20–2.26)	0.002
HCG9-rs16896923	T	1.54 (1.35–1.75)	$4.56E-11$	1.69 (1.33–2.13)	$2.19E-05$	1.64 (1.18–2.27)	0.005

OR: odds ratio. CI: confidence interval.
*Adjusted for sex and age.
**Additionally adjusted for EBV/IgA/VCA and EBV/IgA/EA titers.
***Additionally adjusted for EBV/IgA/VCA and EBV/IgA/EA antibody titers and other environmental factors including family history with NPC, consumption of salt-preserved fish, exposure to domestic wood cooking fires, and exposure to occupational solvents.

2.4. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) assumptions were independently tested for each SNP in cases and controls for each phase group as well as the two phases combined as a quality control measure. For allele association (Table 2, Supplementary Tables 1 and 2; Supplementary Material available online at http://dx.doi.org/10.1155/2014/434072), the Armitage's trend test was used to calculate the P value for additive allele effects on the disease penetrance. ORs were calculated by Mantel-Haenszel estimate based on contingency tables of allele-by-trait counts. For controlling the confounding covariates (age, sex, etc.), the stratified case-control test was performed. All results shown were adjusted for age and sex. In order to exclude the influence of EBV, we analyzed the associations between polymorphisms and the occurrence of NPC using EBV/IgA/VCA and EBV/IgA/EA antibody titers as covariates. For phase II, environmental factors including family history with NPC, consumption of salt-preserved fish, exposure to domestic wood-cooking fires, and exposure to occupational solvents were used as covariates. The receiver operator characteristic (ROC) curve was used to assess the diagnostic performance of EBV/IgA/VCA or EBV/IgA/EA alone, SNP alone, and the integration of these risk factors. Statistics were calculated in the statistical package SAS and SAS Genetics version 9.1.3. Linkage disequilibrium (LD) maps, blocks, and haplotypes were generated by Haploview software [26].

3. Results

3.1. Association Results with SNPs on HLA Region at 6p21.3. As shown in Table 1, over 95% of NPC cases (titer 1 : 10 to 1 : 640) and 42%–45% of the controls (titer 1 : 10 to 1 : 160) were positive for EBV/IgA/VCA antibodies; about 60%–72% of NPC cases (titer 1 : 5 to 1 : 640) and 2%-3% of the controls (titer 1 : 5 to 1 : 80) were positive for EBV/IgA/EA antibodies in the two cohorts. EBV/IgA/EA positive serostatus was always concordant with IgA/VCA seropositive status. To replicate the results of the GWAS showing association between NPC and chromosome 6 [24], 9 SNPs within the *HLA* region previously found to be associated with NPC were genotyped (Table 2). The genotype frequencies for 9 polymorphisms conformed HWE expectations for two control groups; the call rate was 97.9%–99.3% for the 9 SNPs. Table 2 provides the risk alleles, the OR, 95% confidence intervals (CIs), and P values for phases I and II combined and phase II controlling for environmental factors. Eight SNPs in *GABBR1*, *HLA-A*, and *HCG9* were significantly associated

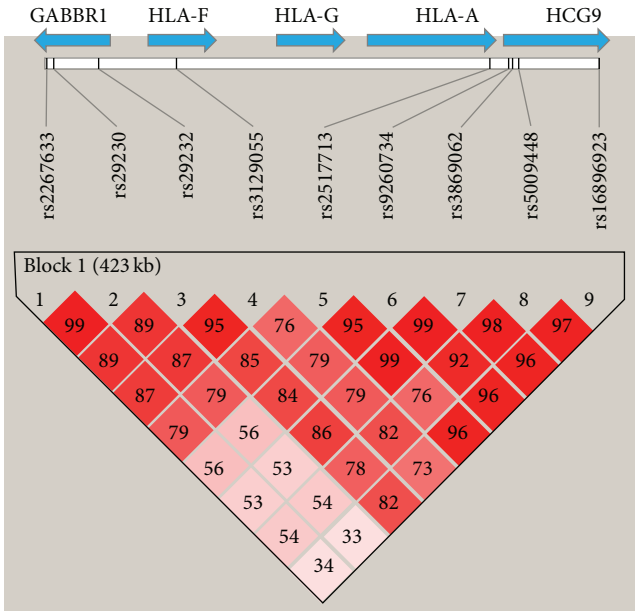

Figure 1: LD map based on D' was drawn using the genotype of the cases and controls.

with NPC in the phase I cohort (Supplementary Table 1: $P = 0.0001–0.02$), phase II cohort (Supplementary Table 1: $P = 3.09 \times 10^{-12}–1.13 \times 10^{-6}$), and combined phases I and II (Table 2: $P = 5.96 \times 10^{-17}–1.85 \times 10^{-8}$). The SNP on *HLA-F* was significant in phase II and in the combined cohort but not in the smaller phase I cohort, although the ORs were in the same direction. After additionally controlling for EBV/IgA/VCA and EBV/IgA/EA antibody titers, 9 SNPs were also significant (Table 2). For the phase II cohort, after adjusting for sex, age, and EBV antibodies titers, we further controlled for environmental factors, which were shown to be associated with NPC in this cohort [5], including family history with NPC, consumption of salt-preserved fish, exposure to domestic wood-cooking fires, and exposure to occupational solvents as covariates. The 9 SNPs remained significantly associated with the risk of NPC (Table 2, last two columns).

Based on the LD map all 9 SNPs are in the same block (Figure 1). The *HCG9*-rs9260734, the most significant SNP, was used for evaluating whether the genetic signature can serve as a diagnostic marker for NPC. Table 3 presented the sensitivity, specificity, and accuracy for EBV/IgA/VCA, EBV/IgA/EA, and SNP test. The specificity (9.3%) and accuracy (38%) of *HCG9*-rs9260734 are lower than 50%, which indicated that the SNP does not qualify as a diagnostic marker. To evaluate if the SNP can improve current IgA antibodies test for NPC diagnosis, we compared the receiver operating characteristic (ROC) curves between IgA/VCA or IgA/EA alone and integrated the SNP with status of IgA/VCA or IgA/EA. The area under the curve (AUC) for integrated markers did improve compared with IgA/VCA or IgA/EA alone (0.917 versus 0.915 for IgA/VCA; 0.833 versus 0.839 for IgA/EA; Figures 2(a) and 2(b)); however, this was not statistically significantly different.

Table 3: The diagnosis performance of IgA/VCA and IgA/EA and genetic signature.

Test	Sensitivity (%)	Specificity (%)	Accuracy (%)
EBV/IgA/VCA	95.7	56.9	69.8
EBV/IgA/EA	68.6	97.5	87.9
HCG9-rs9260734	95.6	9.3	38.0

Note: the threshold for IgA/VCA is 1 : 10 and for IgA/EA is 1 : 5.

3.2. Association Results with SNPs on ITGA9 at 3p21.3. To examine the influence of *ITGA9* gene variants on NPC [23], 7 SNPs in *ITGA9* were genotyped in phase I and phase II cohorts. Each of the 7 SNPs confirmed to HWE expectations in controls for both cohorts and the genotype call rate was 96.8%–99.4%. No evidence of association was seen between 6 of the SNPs and NPC in phase I or phase II or in the combined analysis (Supplementary Tables 1 and 2). SNP (rs169111) was modestly significant in the combined analysis (Supplementary Table 2: OR = 1.4, $P = 0.03$) but not after adjusting for EBV IgA antibodies titers.

4. Discussion

In our study, we have demonstrated the strong associations of 9 SNPs located within *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* with NPC. Our results, from two independent Han Chinese NPC cohorts, confirm the previous associations and effect sizes reported in the Taiwanese GWAS [24]. The etiology of nasopharyngeal carcinoma is influenced by both genetic and environmental factors. EBV IgA antibody status is a strong predictive marker and plays an important role in NPC development in Southern Chinese populations [9, 10, 27, 28]. Our results show that over 95% of NPC cases were EBV/IgA/VCA antibody positive and about 60%–72% of NPC cases were positive for EBV/IgA/EA antibodies. To exclude potential influence of EBV antibody status, we controlled EBV/IgA/VCA and EBV/IgA/EA antibody titers during the analysis. The results indicated that the variants of *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* were still associated with NPC. In a previous study we reported that family history with NPC, consumption of salt-preserved fish, exposure to domestic wood-cooking fires, and exposure to occupational solvents were risk factors of NPC [5]. These environmental exposure data were available in our phase II cohort. When we adjusted for these factors in our association analysis, the 95% confidence intervals overlapped indicating that variants on *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* association were independent of environmental factors with NPC onset; the reduced statistical significance in the analysis adjusted for environmental factors reflects the small sample size with environmental data. These results, from two independent cohorts, affirm the associations of *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* with NPC in the Han Chinese population. *HLA-A* and *HCG9* have been reported to be associated with EBV-positive Hodgkin lymphoma and infectious mononucleosis caused by EBV infection [29, 30]. This suggested that genetic variation chromosome 6p21.3 can influence the outcome of primary EBV infection and the level of viral persistence.

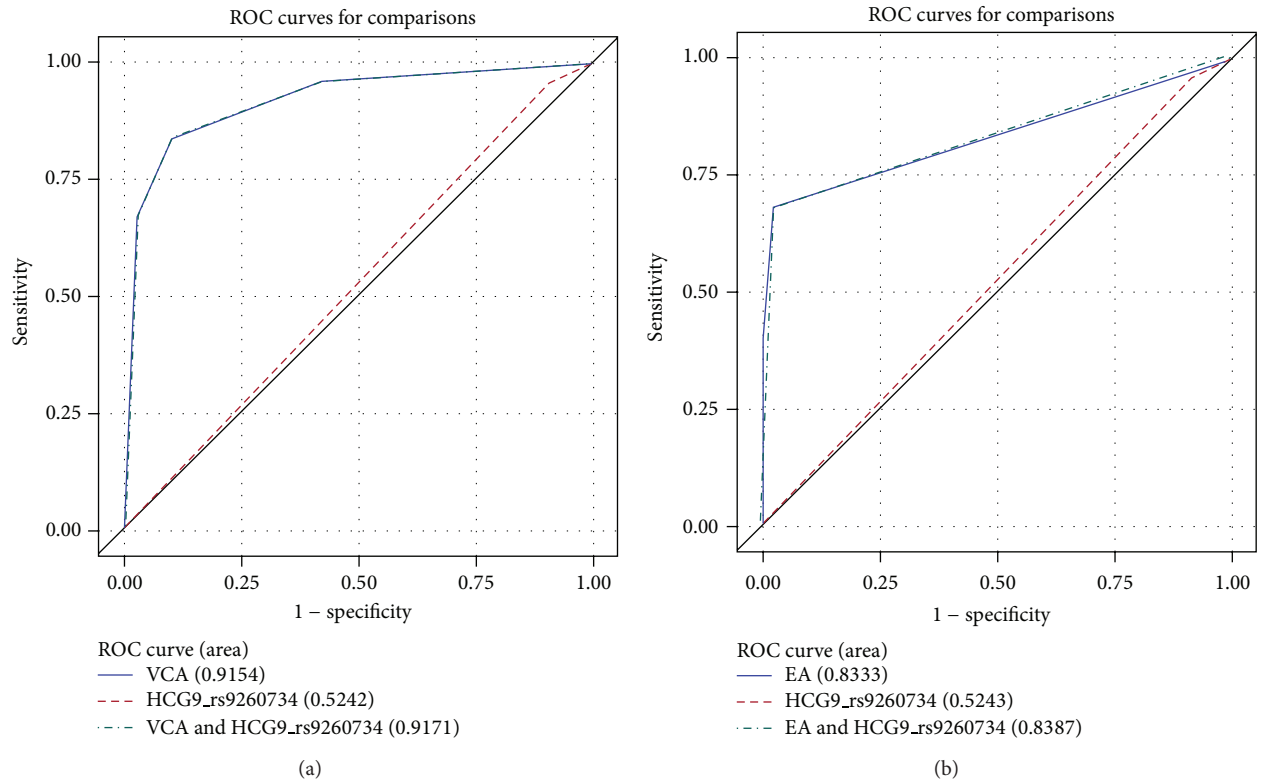

Figure 2: ROC curves.

A genome-wide expression profiling has revealed that increased EBV gene expression is strongly associated with inhibition of multiple *HLA* class I gene expression in NPC [31], further implicating these genes with NPC.

EBV/IgA/VCA and EBV/IgA/EA antibody titers, especially EBV/IgA/VCA, have been used for the screening and diagnosis of NPC for over 30 years in Southern Chinese populations [7, 9–11]. Along with the cancer genetic/genomic project development, new genetic variants associated with NPC are continuously being discovered. There is a growing need to evaluate the genetic markers for medical practice. Our interest is whether the significant SNPs can serve as a diagnostic marker or improve the IgA antibodies test for NPC prediction. Sensitivity, specificity, and accuracy are widely used statistics to quantify how good and reliable a test is. The receiver operating characteristic (ROC) curve is a graphic presentation of the relationship between both sensitivity and specificity and becomes the standard analytical tool for evaluating diagnostic tests. In this study, the specificity and accuracy of SNP was lower than 50%; the AUC of SNP was less than 0.6 indicating SNP alone cannot be a diagnostic marker for NPC. Considering the positive rate for EBV/IgA/VCA antibodies in the general population is about 3% [10], we randomly selected samples and made the control group that contained 3% IgA/VCA positive, then we repeated the ROC analysis; we obtained similar results. The AUC increased when IgA/VCA or IgA/EA was integrated with SNP; however, the effect was not statistically significant. To our knowledge, this is the first study to explore and incorporate the genetic variants to clinical use for NPC. Our results show that there is a strong association between variants of *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* and NPC, but they cannot be useful for individualized risk prediction/diagnosis of NPC. However, risk profile based on a combination of genetic and other risk factors leads to an appreciable increased risk of disease and there is potential for increased predictive power as more genetic risk variants are detected [32].

We were unable to confirm the reported association with NPC of 7 SNPs in *ITGA9* in our large Chinese Han population ($N = 3506$) from an NPC high incidence region of Southern China, although this study was well powered to detect a similar level of association. Based on our results, the minor allele frequencies (MAFs) for these 7 SNPs were lower (between 0.024 and 0.03). The previous study was carried out on a relatively small sample with 279 cases and 512 controls [23]; the few patients and a low MAF may have contributed to low statistical power for SNP association. However, two more recent NPC GWAS also failed to replicate the association of *ITGA9* with NPC [33, 34].

Our study is unique in that two critical EBV antibody titers, as well as environmental factors, are available for inclusion in the statistical modeling. We controlled for these risk factors to determine the genetic association with NPC. This study systematically replicates the association from two NPC GWAS [23, 24] performed in NPC high-risk Asian populations. We also explored potential clinical use for significant genetic makers. Further study should focus on

how these genetic variants impact function at the molecular and cellular levels to affect NPC development. Understanding the functional consequences of genetic variation will be critical to advancing our knowledge of the etiology of the disease and implementing rational medical strategies.

5. Conclusion

In summary, our results extend the association of the *GABBR1*, *HLA-F*, *HLA-A*, and *HCG9* locus with NPC to the Han Chinese population of Southern China. We show for the first time that these factors are independent of environmental risk factors previously shown to influence NPC development. This study also indicated that EBV IgA antibodies in combination with these genetic makers may not be useful for diagnostic prognosis of NPC.

Conflict of Interests

No potential conflict of interests was disclosed.

Acknowledgments

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Research Article

Rho GTPase-Activating Protein 35 rs1052667 Polymorphism and Osteosarcoma Risk and Prognosis

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The Rho GTPase-activating protein 35 (ARHGAP35), an important Rho family GTPase-activating protein, may be associated with tumorigenesis of some tumors. Here, we investigated the relationship between an important polymorphic variant at 3′-UTR of this gene (rs1052667) and osteosarcoma risk and prognosis. This hospital-based case-control study, including 247 osteosarcoma patients and 428 age-, sex-, and race-matched healthy controls, was conducted in Guangxi population. Genotypes were tested using TaqMan PCR technique. We found a significant difference in the frequency of rs1052667 genotypes between cases and controls. Compared with the homozygote of rs1052667 C alleles (rs1052667-CC), the genotypes with rs1052667 T alleles (namely, rs1052667-CT or -TT) increased osteosarcoma risk (odds ratios: 2.41 and 7.35, resp.). Moreover, rs1052667 polymorphism was correlated with such pathological features of osteosarcoma as tumor size, tumor grade, and tumor metastasis. Additionally, this polymorphism also modified the overall survival and recurrence-free survival of osteosarcoma cases. Like tumor grade, ARHGAP35 rs1052667 polymorphism was an independent prognostic factor influencing the survival of osteosarcoma. These results suggest that ARHGAP35 rs1052667 polymorphism may be associated with osteosarcoma risk and prognosis.

1. Instruction

Osteosarcoma is the most frequent primary malignant bone tumor and usually occurs in patients between 10 and 25 years of age [1, 2]. In the past several years, the 5-year survival of patients with osteosarcoma has significantly improved because of the combined treatment (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy) [2, 3]. However, about 80% of patients would eventually develop metastatic disease following surgical treatment, and outcome remains poor for these patients [2–4].

Therefore, a better understanding of its basic biology is urgently needed to identify its risk and prognostic markers. Several studies have reported potential associations of common genetic variants with osteosarcoma risk in biologically plausible pathways. This suggests that the genetic factors could play important roles in the pathogenesis of this malignant tumor [5, 6].

The Rho GTPase-activating protein 35 (ARHGAP35, also called GRLF1 and p190RhoGAP) is an important Rho family GTPase-activating protein, and is identified as a tyrosine-phosphorylated protein associated with p120RasGAP in v-Src transformed cells [7–14]. Functionally, it mainly plays a crucial role in regulating cytoskeletal rearrangements, cell spreading and migration, and endothelial barrier function [15–18]. Recent data have shown that this protein can regulate

cell proliferation and the dysregulation of ARHGAP35 may be associated with gliomas and breast cancer [19–21]. A common polymorphism at 3′-untranslated region (3′-UTR) of this gene, namely, rs1052667 C > T, has been identified. However, it is unclear whether this polymorphism correlates with tumor. Therefore, we specifically conducted a hospital-based case-control study to examine whether ARHGAP35 rs1052667 polymorphism modifies osteosarcoma risk and prognosis.

2. Materials and Methods

2.1. Study Population. The present study was approved by the ethics committees of the hospitals involved in this study. This hospital-based case-control study was conducted in Guangxi Zhuang Autonomous Region, China, a relatively high incident area of osteosarcoma. All osteosarcoma patients and control individuals were residents of Guangxi Zhuang Autonomous Region and were recruited from the affiliated hospitals of Guangxi Medical University between January 1996 and August 2005. All cases were histopathologically confirmed. During the same period, control subjects without a history of cancer were randomly selected from a pool of healthy volunteers who visited the general health check-up center of the same hospitals because of their routine scheduled physical exams [22, 23]. To control the effects of confounders, the controls were individually matched (1 : 1 or 2 : 1) to cases based on ethnicity (Han, Minority), sex, and age (±5 years). In this study, a total of 247 cases and 428 controls, representing 97% of eligible cases and 92% of eligible controls, were enrolled, interviewed, and included in the final analysis. After giving written consent, demographic information and clinical pathological data (including age, sex, race, smoking and drinking status, disease history, tumor history, tumor size, tumor grade, and tumor site, etc.) were collected using a standard interviewer administered questionnaire and/or medical records. At the same time, 2 mL of peripheral blood was obtained for analyzing the genotypes of ARHGAP35 rs1052667 polymorphism. All subjects did not have chemotherapy or radiotherapy history before samples collection.

Among 247 osteosarcoma cases, about 57 percent (140/247) of osteosarcomas located in femur. All cases received surgical resection of primary tumor; however, only fifty-seven cases underwent the radical treatment (including both curative resection and adjuvant chemotherapy). In this study, tumor grade was evaluated according to Broders' grading system [24]. Low grade was defined as tumor type with well or moderately differentiated tumor cells (less than 50% undifferentiated cells), whereas high grade was defined as tumor type with poorly or anaplastic/pleomorphic differentiated tumor cells (more than 50% undifferentiated cells) [25].

2.2. DNA Detraction. Leukocytes were isolated from peripheral venous blood samples from all tumor patients and control subjects by standard procedures. DNA was then extracted from leukocyte samples by standard phenol-chloroform extraction and ethanol precipitation. DNA samples were stored at −20°C until additional analysis.

2.3. Genotyping. The ARHGAP35 rs1052667 genotypes were analyzed by TaqMan polymerase chain reaction (PCR) on an iCycler iQ real-time PCR detection system (iQ5, Bio-Rad, Hercules, CA, USA). The corresponding TaqMan SNP Genotyping Assay Kit (cat# 4351379) was obtained from Applied Biosystems, Carlsbad, CA, USA. TaqMan PCR was performed in total volume of 25 μL consisting of 1 × TaqMAN Universal Master Mix II (cat# 4440041, Applied Biosystems), 1 × TaqMan SNP Genotyping Assay Mix (including both primers and probes, cat# C_16007053_10), and about 75 ng of genomic DNA. Cycling conditions were 95°C for 30 s, and 50 cycles of 95°C for 15 s, and 60°C for 1 min. For quality control, laboratory personnel were blinded to case and control status. Controls were included in each run, and repeated genotyping and sequencing of a random 20% subset yielded 100% identical genotypes.

2.4. Osteosarcoma Patients Follow-Up. For survival analysis, we followed all osteosarcoma cases. All patients underwent serial monitoring every 2 months for the first 2 years and semiannually thereafter for detection of any recurrence. In this study, the last follow-up day was December 31, 2013, and survival status was confirmed by clinic records and either patient or family contact. The duration of overall survival (OS) was defined as from the date of curative treatment to the date of death or last known date alive, whereas the recurrence-free survival (RFS) was defined as from the date of curative treatment to the date of tumor recurrence or last known date alive.

2.5. Statistical Analysis. All analyses were performed with the statistical package for social science (SPSS) version 18 (SPSS Institute, Chicago, IL, USA). Pearson's χ^2 test or Fisher's exact test was used to test the differences between osteosarcoma patients and control subjects in the distribution of gender, age, race, and ARHGAP35 rs1052667 genotypes. Because this study was based on an individually matched design, conditional logistic regression was used to evaluate odds ratios (ORs) and 95% confidence intervals (CIs) for risk of osteosarcoma. Kaplan-Meier survival analysis (with the log-rank test) was used to elucidate the relationship between ARHGAP35 rs1052667 polymorphism and osteosarcoma prognosis. Hazard ratios (HRs) and 95% CIs for ARHGAP35 genotypes were calculated from a multivariate Cox regression model (with stepwise forward selection based on the likelihood ratio test). In the present study, a P value of < 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinic Characteristics of the Subjects. In this study, 247 osteosarcoma cases and 428 controls were included in the final analysis. The demographic characteristics of all cases and controls are shown in Table 1. The mean age, gender ratio, smoking and drinking status, and

Table 1: Demographic and etiologic characteristics of osteosarcoma cases and controls.

Variable	Controls (n = 428)		Cases (n = 247)		P
	n	%	n	%	
Sex					
Male	265	61.9	154	62.3	0.934
Female	163	38.1	93	37.7	
Age (yrs)					
≤26	278	65.0	161	65.2	0.952
>26	150	35.0	86	34.8	
Race					
Han	279	65.2	159	64.4	0.555
Minority	149	34.8	88	35.6	
Smoking status					
No	398	93.0	231	93.5	0.792
Yes	30	7.0	16	6.5	
Drinking status					
No	402	93.9	233	94.3	0.829
Yes	26	6.1	14	5.7	
Paget's disease					
No	428	100.0	246	96.6	0.366
Yes	0	0.0	1	0.4	
Trauma					
No	411	96.0	228	92.3	0.038
Yes	17	4.0	19	7.7	
Radiation exposure					
No	415	97.0	236	95.5	0.339
Yes	13	3.0	11	4.5	
PBBL[b]					
No	428	100.0	246	96.6	0.366
Yes	0	0.0	1	0.4	

[a]The mean ± S.D. ages were 26.38 ± 15.51 and 26.31 ± 14.16 for cases and controls, respectively.
[b]PBBL refers to the preexisting benign bone lesions, including fibrous dysplasia, osteochondromatosis, and chondromatosis.

Table 2: The rs1052667 polymorphism of ARHGAP35 and osteosarcoma risk.

rs1052667	Controls		Cases		OR	P
	n	%	n	%		
Genotype						
CC	341	79.7	133	53.8	1	
CT	72	16.8	70	28.3	2.41 (1.64–3.55)[a]	9.00×10^{-6}
TT	15	3.5	44	17.8	7.35 (3.95–13.68)[a]	3.12×10^{-10}
CT/TT[b]	87	20.3	114	46.2	3.27 (2.31–4.61)[a]	1.92×10^{-11}
Allele						
C	754	88.1	336	68.0	1	
T	102	11.9	158	32.0	2.25 (1.64–3.09)	4.43×10^{-7}

[a]OR conditional on matched set adjusted by smoking and drinking status, radiation exposure history, trauma history, paget's disease history, and benign bone lesions.
[b]CT/TT represented the combination of rs1052667-CT genotype and rs1052667-TT genotype.

race distribution are of the same levels in both control and osteosarcoma groups ($P > 0.05$).

3.2. ARHGAP35 Polymorphism Increased Osteosarcoma Risk. Table 2 summarized the genotypic and allelic distribution of ARHGAP35 rs1052667 polymorphism for both tumor patients and controls. Genotype frequent distribution in controls fitted the Hardy-Weinberg equilibrium well. The heterozygous genotype with rs1052667 C and T allele (rs1052667-CT) and the variant homozygous genotype with rs1052667

Table 3: The rs1052667 polymorphism of ARHGAP35 and osteosarcoma risk stratified by race (Han and minority), gender (female and male), and age (≤26 yrs and >26 yrs).

Variable	Genotype	Control		Case		OR (95% CI)[a]	P
		n	%	n	%		
Race[b]	rs1052667						
Han	CC	218	78.1	82	51.6	1	
	CT/TT	61	21.9	77	48.4	3.40 (2.22–5.19)	1.61×10^{-8}
Minority	CC	123	82.6	51	58.0	1	
	CT/TT	26	17.4	37	42.0	3.39 (1.86–6.18)	6.67×10^{-5}
Gender[c]	rs1052667						
Female	CC	129	79.1	49	52.7	1	
	CT/TT	34	20.9	44	47.3	3.42 (1.96–5.98)	1.53×10^{-5}
Male	CC	212	80.0	84	54.5	1	
	CT/TT	53	20.0	70	45.5	3.29 (2.12–5.12)	1.08×10^{-7}
Age[d]	rs1052667						
≤26	CC	214	77.0	80	49.7	1	
	CT/TT	64	23.0	81	50.3	3.40 (2.23–5.17)	1.11×10^{-8}
>26	CC	127	84.7	53	61.6	1	
	CT/TT	23	15.3	33	38.4	3.35 (1.79–6.25)	1.50×10^{-4}

[a]OR conditional on matched set.
[b]Likelihood ratio test for interaction of the stratified variable (Han and Minority) and rs1052667 genotype was calculated as test for the heterogeneity of ORs across strata (interact term OR = 1.02, $P_{\text{interaction}} = 0.957$).
[c]Likelihood ratio test for interaction of the stratified variable (male and female) and rs1052667 genotype was calculated as test for the heterogeneity of ORs across strata (interact term OR = 0.99, $P_{\text{interaction}} = 0.983$).
[d]Likelihood ratio test for interaction of the stratified variable (age: ≤26 yrs and >26 yrs) and rs1052667 genotype was calculated as test for the heterogeneity of ORs across strata (interact term OR = 1.01, $P_{\text{interaction}} = 0.982$).

T allele (rs1052667-TT) were more frequent among cases than among the controls ($P < 0.01$), resulting in an Ser allele frequency of 32.0% in cases and 11.9% in controls. Logistic regression analysis exhibited that the adjusted OR for osteosarcoma for these individuals carrying rs1052667-CT compared with those exhibiting the homozygote for C alleles (rs1052667-CC) was 2.41 (95% CI, 1.64–3.55) and the corresponding OR for those featuring rs1052667-TT was 7.35 (95% CI, 3.95–13.68). These results showed that osteosarcoma risk was associated with the number of rs1052667 T alleles.

3.3. ARHGAP35 Polymorphism and Osteosarcoma Risk Stratified by Gender, Age, and Race. To evaluate possible interactive effects of matching factors (including gender, age, and race) and ARHGAP35 rs1052667 polymorphism on osteosarcoma risk, we performed a series of bivariate stratified analyses by matching factors (Table 3). Because of the small number of subjects with rs1052667-TT among different strata, genotypes rs1052667-CT and rs1052667-TT were combined into one stratum (also called rs1052667-CT/TT). Similar risk values for osteosarcoma were found among Han subjects and among minority participants (adjusted ORs were 3.40 and 3.39, resp.). Similar results were also found in the stratified analysis between rs1052667 polymorphism and other two matching variables. Likelihood ratio tests for interaction of the stratified variables and ARHGAP35 genotypes showed that these matching factors did not modulate the effects of this polymorphism on osteosarcoma risk ($P_{\text{interaction}} > 0.05$; Table 3). This suggested that these factors should be effectually manipulated and should not modify the association between this polymorphism and osteosarcoma risk.

3.4. ARHGAP35 Polymorphism Modified Osteosarcoma Prognosis. To investigate the effects of ARHGAP35 polymorphism on outcome of osteosarcoma patients, we followed all cases and analyzed the survival information of all osteosarcoma cases. During the follow-up period of these patients, 222 faced tumor recurrence with 15.9% of the 5-year RFS rate, and 238 died with 12.5% of the five-year OS rate. Kaplan-Meier survival analysis showed that patients with ARHGAP35 rs1052667 T alleles featured a significantly poorer prognosis than those with rs1052667-CC (P is 1.19×10^{-11} for OS and P is 2.04×10^{-17} for RFS, resp.; Figures 1(a) and 1(b)). Considering that some patients did not accomplish entire adjuvant chemotherapy because of poor economic conditions, we stratified the analysis of the correlation between ARHGAP35 genotypes and osteosarcoma outcome by the radical treatment status to explore whether this difference affected the results (Figure 2). Among these cases receiving the radical treatment (Figures 2(c) and 2(d)), shorter median overall survival time (MST) and shorter median tumor recurrence-free survival time (MRT) were found in cases having risk genotypes (including ARHGAP35 rs1052667-CT and -TT) than in those without risk genotypes. Similar results were observed in the nonradical treatment

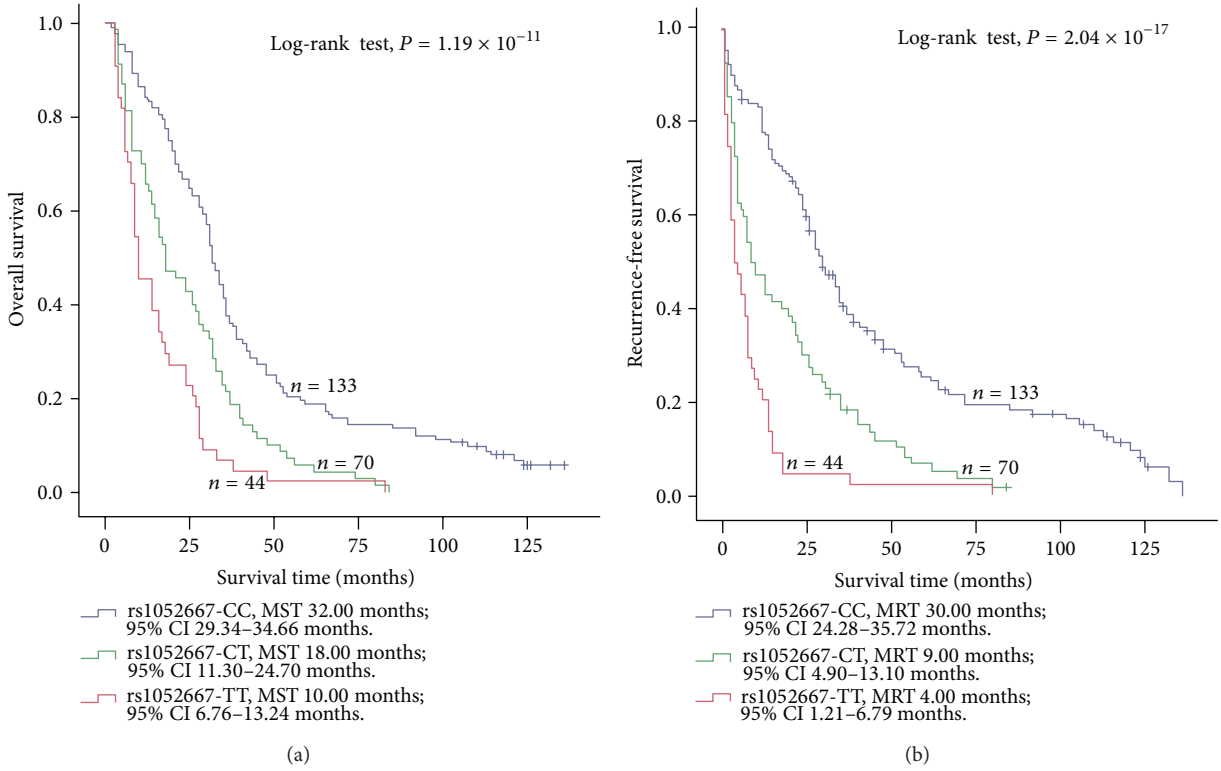

Figure 1: Association between ARHGAP35 rs1052667 polymorphism and osteosarcoma prognosis in 247 osteosarcoma patients. ARHGAP35 rs1052667 polymorphism was correlated with (a) the overall survival and (b) the recurrence-free survival of osteosarcoma. Cumulative hazard function was plotted by the Kaplan-Meier methodology and the *P* value was calculated with two-sided log-rank tests. MST, the median overall survival time; MRT, the median tumor recurrence-free survival time.

Table 4: The rs1052667 polymorphism of ARHGAP35 and the prognosis of osteosarcoma.

Rs1052667	Overall survival		Recurrence-free survival	
Genotype	HR (95% CI)	*P*	HR (95% CI)	*P*
CC	1		1	
CT	1.57 (1.16–2.12)	3.86×10^{-3}	1.82 (1.33–2.50)	2.12×10^{-4}
TT	1.91 (1.32–2.77)	5.95×10^{-4}	2.53 (1.73–3.70)	1.73×10^{-6}

stratum (Figures 2(a), and 2(b)). Multivariate cox regression analysis (with stepwise forward selection based on likelihood ratio test) was next performed to determine whether ARHGAP35 rs1052667 polymorphism was an independent predictor of osteosarcoma cases. The results exhibited that the genotypes with rs1052667 T alleles increased the dying risk of tumor patients compared with rs1052667-CC (HRs: 1.57 for rs1052667-CT and 1.91 for rs1052667-TT, resp.). Risk role was also found in the RFS analysis; the corresponding HRs were 1.82 for rs1052667-CT and 2.53 for rs1052667-TT, respectively (Table 4). Taken together, these results implied that this polymorphism could be used as an independent prognostic marker for osteosarcoma.

3.5. ARHGAP35 Polymorphism Correlated with the Clinic-Pathological Features of Osteosarcoma Patients. To explore whether ARHGAP35 rs1052667 polymorphism correlated with the clinical pathological features of osteosarcoma, an association analysis of the risk genotypes (rs1052667-CT/TT) or the nonrisk genotype (rs1052667-CC) and the clinical pathological characteristics of osteosarcoma was performed separately. Results showed that these osteosarcoma cases with risk genotypes of ARHGAP35, compared to those without risk genotypes, faced larger tumor size (OR is 4.85), lower tumor differentiation (OR is 4.07), and higher metastasis risk (OR is 2.78; Table 5). However, this polymorphism did not affect other features.

4. Discussion

To the best of our knowledge, no studies have investigated the role of ARHGAP35 rs1052667 polymorphism in the risk of osteosarcoma. In this study, we analyzed the association between aforementioned polymorphism and the risk of osteosarcoma among Guangxi population and found ARHGAP35 rs1052667 T alleles increased osteosarcoma risk (adjusted OR is 3.27). These results imply that this polymorphism may have functional significance in osteosarcoma carcinogenesis.

Osteosarcoma is one of major cancer types in the Guangxi Zhuang Autonomous Region; the possible risk factors of which include radiation exposure, foreign bodies, genetic predisposition, and so on. Increasing epidemiological evidence has shown that an individual susceptibility related

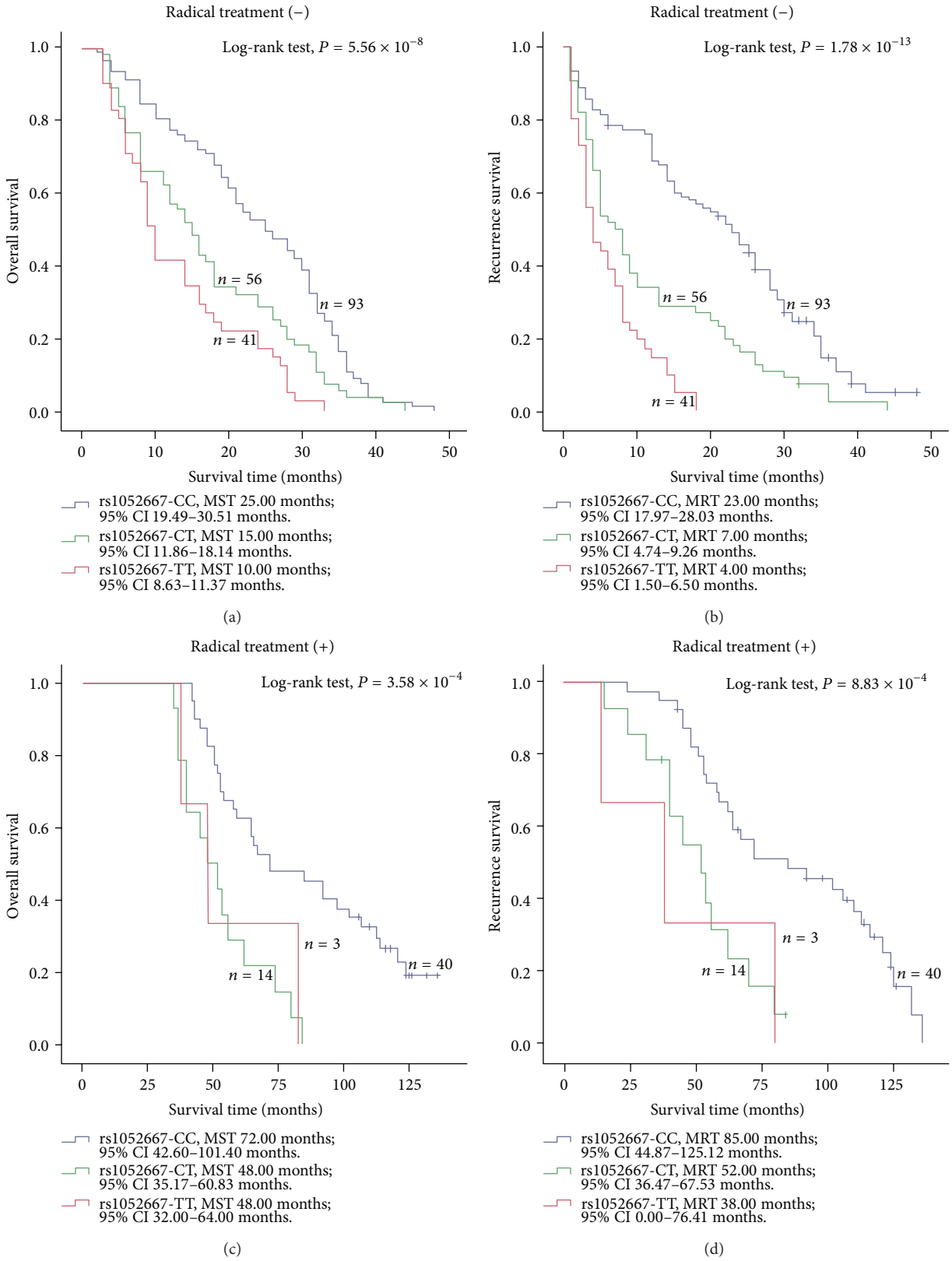

Figure 2: Survival analysis of ARHGAP35 rs1052667 polymorphism in strata of treatment status. According to whether cases received radical treatment (RT) including both surgical resection and adjuvant chemotherapy, 247 osteosarcoma cases were divided into two groups: RT (+) and RT (−). ((a), (c)) Overall survival and ARHGAP35 rs1052667 polymorphism in strata of RT status. ((b), (d)) Tumor recurrence-free survival and ARHGAP35 rs1052667 polymorphism in strata of status. Cumulative hazard function was plotted by Kaplan-Meier's methodology, and P value was calculated with two-sided log-rank tests. MST, the median overall survival time; MRT, the median tumor recurrence-free survival time.

Table 5: The rs1052667 polymorphism of ARHGAP35 and clinic pathological features of osteosarcoma.

Variable	rs1052667-CC		rs1052667-CT/TT		OS (95% CI)	P
	n	%	n	%		
Age (yrs)						
≤26	80	60.2	81	71.1	1	
>26	53	39.8	33	28.9	0.72 (0.39–1.35)	0.31
Gender						
Female	49	36.8	44	38.6	1	
Male	84	63.2	70	61.4	1.07 (0.61–1.89)	0.82
Race						
Han	82	61.7	77	67.5	1	
Minority	51	38.3	37	32.5	0.98 (0.54–1.77)	0.95
Tumor site						
Femur	72	54.1	68	59.6	1	
Tibia	30	22.6	21	18.4	0.66 (0.32–1.33)	0.24
Humeral bone	22	16.5	16	14	0.90 (0.40–2.01)	0.79
Others	9	6.8	9	7.9	0.91 (0.31–2.70)	0.86
Tumor size						
≤5 cm	57	42.9	15	13.2	1	
>5 cm	76	57.1	99	86.8	4.85 (2.51–9.37)	2.69×10^{-6}
Tumor grade						
Low	68	51.1	23	20.2	1	
High	65	48.9	91	79.8	4.07 (2.28–7.37)	2.14×10^{-6}
Metastasis						
No	87	65.4	45	39.5	1	
Yes	46	34.6	69	60.2	2.78 (1.63–4.76)	1.88×10^{-4}

to genetic factors might be associated with osteosarcoma carcinogenesis [5, 6].

While ARHGAP35 spans 87 kb on chromosome 19q13.3 and contains 7 exons and 6 introns (PubMed Databases). Its encoding protein is a 190 kDa protein consisting of three major functional domains: (1) an NH_2-terminal GTP-binding domain (GBD), (2) a middle domain (MD), and (3) a COOH-terminal GAP domain, which displays specificity for GTP-bound RhoA [9, 26]. Functionally, ARHGAP35 plays important roles in promoting cell spreading, membrane protrusion, and cell polarity [15, 27]. Recently, several reports have shown that ARHGAP35 plays an important role in cancer formation and metastasis [19, 20, 28]. In 2008, Shen et al. [19] investigated the role of ARHGAP35 in the breast tumor kinase (Brk) signal pathway and found that it is a Brk substrate both in vitro and in vivo. Through this signal pathway, ARHGAP35 is phosphorylated at the Y1105 residue by Brk and next associated with p120RasGAP. As a consequence, ARHGAP35 is stimulated and p120 functions are attenuated, leading to RhoA inactivation and Ras activation, respectively. Their results show ARHGAP35 activation promotes breast cancer growth, migration, and invasion, and provide important evidence for the crucial roles of this Brk-ARHGAP35 signaling pathway in promoting breast malignancy [19]. In accordance with these reports, our present study exhibited that ARHGAP35 might be involved in osteosarcoma tumorigenesis.

With the Human Genome Project developing, more than one hundred polymorphisms have been identified in ARHGAP35 (dbSNP in NCBI Database). In this study, we only analyzed ARHGAP35 rs1052667 polymorphism, primarily because this polymorphism is relatively common in most populations, whereas other polymorphisms are rare. In this study, we collected 247 osteosarcoma and 428 control samples from Guangxi Zhuang Autonomous Region, a relatively high incident area of osteosarcoma. we found that about 20 percent of control individuals had ARHGAP35 rs1052667 T alleles, similar to the data from the Human Genome Project (dbSNP Database, web: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs1052667). However, higher frequency was observed in the individuals with osteosarcoma, and following analysis showed this polymorphism increased osteosarcoma risk. These results suggested ARHGAP35 rs1052667 polymorphism might modify the risk of tumors such as osteosarcoma.

This risk role might be related to the posttranscriptional regulation of gene expression. Because rs1052667 polymorphism locates at the 3′-UTR of ARHGAP35 gene, this variant might be involved in the regulation of mRNA stability and the control of mRNA subcellular localization [29]. Consequently, it may be associated with the functional dysregulation of ARHGAP35 and play a role in the carcinogenesis. Supporting the aforementioned hypothesis, recent studies have shown that the dysregulation of ARHGAP35 expression and function is involved in the tumorigenesis of some tumors

such as lung cancer [30], melanoma [31], and breast cancer [19, 28, 32]. Thus, ARHGAP35 polymorphism might play an important role in the tumorigenesis of osteosarcoma, and this provided a new genetic insight into osteosarcoma tumorigenesis.

Additionally, we also investigated the association between ARHGAP35 rs1052667 polymorphism and osteosarcoma prognosis. We found that osteosarcoma patients having genotypes with ARHGAP35 rs1052667 T alleles had a significant poor RFS and OS compared to those without T alleles. Considering the difference of the treatment and to explore whether this difference affected the modifying role of ARHGAP35 rs1052667 polymorphism, we stratified the analysis of the effects of ARHGAP35 genotypes on osteosarcoma outcome by the treatment status. Results showed that this polymorphism modulated osteosarcoma prognosis, regardless of the radical or nonradical treatment status. Multivariate cox regression analysis next showed this polymorphism increased 1.53-times tumor reoccurrence risk and 0.91-times death risk; moreover, this risk did not depend on the clinical pathological change. This is possibly because it correlates with the fact that this polymorphism modifies tumor grade and differentiation and, consequently, might promote tumor proliferation and metastasis. Supporting our results, recent studies have exhibited that the dysregulation of ARHGAP35 promotes tumor growth, infiltration, and metastases and subsequently might result in poor prognosis of tumors [19, 20, 28, 30]. These data implied that ARHGAP35 rs1052667 polymorphism should be able to modify the prognosis of osteosarcoma and should be an important prognostic marker for this tumor.

In the present study, to control the effects of confounders such as age, gender, and race, we used an individually matched design. In the stratified analysis, no interactive effects were found, suggesting that these factors do not modify the correlation between ARHGAP35 rs1052667 polymorphism and osteosarcoma risk.

However, there were several limitations to our study. Potential selection bias might have occurred because the selection of control subjects in our study was hospital-based. Despite the analysis of ARHGAP35 rs1052667 polymorphism, we did not analyze other polymorphisms of this gene possibly able to modify the risk of osteosarcoma. Although this study is molecular epidemiological investigation based on clinic samples of osteosarcomas, it is deficient in functional analysis. Additionally, our findings were based on relatively small numbers and limited by small number of subjects in part of the genotype strata. Therefore, more genes deserve further elucidation based on a large sample and the combination of genes.

5. Conclusions

In summary, to the best of our knowledge, this is the first report investigating an association between ARHGAP35 rs1052667 polymorphism and osteosarcoma risk and prognosis in Guangxi patients. We have found evidence that the genotypes of ARHGAP35 rs1052667 T alleles may be correlated with increased risk and poor prognosis for osteosarcoma and that this polymorphism may be involved in the tumorigenesis of this type of tumor. Given that osteosarcoma is a highly fatal tumor, the finding of a genetic susceptibility (if confirmed) may have implications for screening and prevention.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jinmin Zhao and Hua Xu contributed equally to this work.

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Research Article

Analysis of Genotype 1b Hepatitis C Virus IRES in Serum and Peripheral Blood Mononuclear Cells in Patients Treated with Interferon and Ribavirin

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Hepatitis C virus (HCV) highly conserved IRES (internal ribosome entry site) sequence, localized within the 5′-untranslated region (5′UTR), may determine viral properties like replication efficiency and cell tropism. The aim of the present study was to characterize newly emerging 5′UTR variants in serum and peripheral blood mononuclear cells (PBMC) in chronic hepatitis C patients treated with interferon (IFN) and ribavirin and to identify their effect on IRES secondary structures. The study group consisted of 87 patients infected with genotype 1b from whom serum and PBMC samples were collected at 9 time points (before, during, and after treatment). New 5′UTR variants developed in 9 patients. Out of the overall 14 new variants, 9 (64%) were found in PBMC. HCV variants with decreased thermodynamic stability were identified only in PBMC and C183U mutation was the most common one in this compartment. In conclusion, antiviral treatment may favor emergence of new 5′UTR variants both in blood and in PBMC compartments. However, variants developing in the latter compartment were predicted to have lower thermodynamic stability of the IRES secondary structures compared to serum strains. C-U change in position 183, which has not been described previously, might indicate viral adaptation to lymphoid cells.

1. Introduction

Hepatitis C virus (HCV) displays significant molecular variability and circulates in the infected host as a heterogenous population referred to as *quasispecies* [1, 2]. This dynamic population of closely related but not identical variants could play a significant role in immune evasion, resistance to antiviral therapy, and adaptation to the cells of the immune system [1, 3, 4].

IRES (internal ribosome entry site) sequence is localized between nucleotide positions 40 and 372 and partly overlaps with both the 5′untraslated region (5′UTR) and the open reading frame [5]. It forms a secondary structure of high stability containing four domains [2, 5–7].

Domain II is crucial for RNA replication and translation [5]. Mutations within this region may decrease translation efficiency [5]. The most complex part of IRES is domain III that is composed of branched structures (hair pins) IIIabcde with inner loops inside some of them; however, it represents the most stable fragment of HCV genome with regard to nucleotide sequence and secondary structure. Domain III participates in the maintenance of the whole IRES secondary

structure stability [5]. It binds the 40S subunit and interacts with the eukaryotic initiation factor eIF3 and ribosomal proteins thus playing a critical role in translation [8].

The AUG start codon at position 342 and the first 11 nucleotides of the open reading frame (ORF) are localized in the last IRES domain IV [9, 10].

In addition to its role in the translation initiation, 5′UTR is likely to confer cellular tropism, as specific mutations are often identified in variant isolated from extrahepatic compartments such as PBMC, lymphoid system, brain, and bone marrow [1, 11–14].

The aim of the present study was to characterize polymorphism of IRES domains II and III in serum and peripheral blood mononuclear cells (PBMC) in chronic hepatitis C patients treated with interferon (IFN) and ribavirin and to identify their effect on IRES secondary structures.

2. Materials and Methods

The study group consisted of 87 patients monoinfected with HCV genotype 1b who were treated with PEG-IFNα (PEGASYS ROCHE or PEGINTRON SCHERING) and ribavirin (COPEGUS ROCHE or REBETOL SCHERING) for 48 weeks. The study was approved by the Internal Review Board at the Warsaw Medical University (reference number KBO/23/09), and each patient signed an informed consent form. There were 44 women and 43 men; their mean age was 44 years (range from 19 to 69). None of the patients has been previously treated for hepatitis C and none has had history of decompensated liver disease. Furthermore, all patients were negative for anti-HIV. The sustained virological response (SVR) rate among our patients was 67%.

Serum and PBMC samples were collected: before treatment (baseline), during treatment (weeks 4, 6, 8, 16, 24, and 48), and after treatment (weeks 60 and 72).

Sera were isolated 2 hours after blood drawing and PBMC were isolated by density gradient centrifugation [15]. Both sera and PBMC samples were immediately frozen and kept at −80°C until analysis. RNA was extracted from 3×10^6 to 1×10^7 cells and from 250 μL of serum by Chomczynski method. One-quarter (1/4) of this RNA solution was used for each RT-PCR reaction [16].

5′UTR HCV RNA was amplified as described elsewhere [12]. Amplified RT-PCR products were first screened by SSCP (single-strand conformation polymorphism) [17], and whenever band pattern indicated sequence change, the samples involved were sequenced after initial cloning.

PCR products were cloned using TA Cloning Kit (Invitrogen). Plasmids were purified with Quick Plasmid Miniprep Kit (Invitrogen) and sequenced using Applied Biosystems 3130 Genetic Analyzer. Sequences were analyzed using the MEGA 5.0 program [18].

Prediction of IRES domains II and III secondary structures and thermodynamic stability was performed separately for each domain using MFOLD 3.2 program http://mfold.rna.albany.edu [19].

3. Results

5′UTR viral sequences were amplified from sera and PBMC from all 87 patients and first analyzed by SSCP. In 9 patients, the SSCP band pattern changes during treatment, and the exact nature of these changes was further analyzed by cloning and sequencing.

The characteristics of patients with and without 5′UTR changes are summarized in Table 1.

Altogether, 14 newly emerging HCV variants were identified: 10 developed during treatment and four appeared only after the end of therapy. Seven of these emerged in PBMC (50%), five (36%) emerged in serum, and two (14%) emerged in both serum and PBMC (Table 2).

Within the 14 new variants there were 22 point mutations distributed in domains II and III. Most of the observed changes were substitutions 20/22 (91%); in the remaining two, there was one deletion and one insertion (Table 3, Figure 1).

Sixteen (73%) of the 22 mutations were localized in domain III including seven in domain IIId (G261U, U263G, U273G, C274A, U277G, U271G, and G271U), two within domain IIIa (one substitution A142G and one G deletion at position 146), four in domain IIIb (C183U, G188U, A233G, and one insertion 206A), and three in domain IIIc (A243G, G243A, and A244G). The remaining six mutations emerged in domain IIb (U104C, C104U, G107A, and A109C) and in ssRNA junction between domains II and III (A119U and C121U) (Table 3).

Analysis of the localization of these mutations in the predicted IRES secondary structure showed that 18/22 (82%) occurred in the paired regions of both analyzed domains, whereas two were observed in domains II and III loops at positions 109 and 183, respectively, and two were observed in ssRNA regions (Figure 1).

3.1. Compartmentalization of New Variants. Eleven IRES mutations were detected exclusively in variants amplified from PBMC (U104C, A109C, A142G, 146Gdel, G188U, 206Ains, A233G, A243G, A244G, G261U, and G271U), whereas three were identified only in serum (C104U, G107A, and C121U). Eight substitutions were found in both serum and PBMC (A119U, C183U, G243A, U263G, U271G, U273G, C274A, and U277G) (Table 3).

Viral variants appearing in PBMC have had more changes than those appearing in serum. Thus, 5 serum-derived variants (Pt. 1, 2, 7, and 9) contained only a single mutation when compared to the strains in baseline population. In contrast, 6 out of 7 newly emerging variants in PBMC (Pt. 3 and 6–9) contained 2 to 5 mutations per variant (Table 2). One quasispecies variant emerging in both compartments (Pt. 5) contained 5 changes with respect to the initial sequence.

The C183U substitution was the most common mutation as it was found in 6 variants present in four patients (Pt. 3 and 6–8) (Tables 2 and 3). The C183U substitution was identified in five PBMC-derived variants and in one sequence present in serum. Another change (A119U) was detected in two different variants (Table 3).

All new variants isolated from PBMC have had changes at positions 204 and 243: C204 and A243 were found in

Table 1: Some characteristics of the patients with and without 5′UTR changes during treatment.

	Patients with 5′UTR changes ($n = 9$)	Patients without 5′UTR changes ($n = 78$)
Number of SVR patients	2 (22%)	56 (72%)
HCV viral load at baseline IU/mL ($\times 10^4$)*	181,6 ± 151,9 (range 36,6–281,0)	121,6 ± 125,4 (range 1,7–409,0)
HCV viral load at 12 weeks IU/mL ($\times 10^2$)*	223,8 ± 669,9 (range 0–2010,0) ($n = 3$)	7,6 ± 28,7 (range 0–209,0) ($n = 15$)
Number of patients with HCV RNA in PBMC at week 72	7 (78%)	21 (30%)
ALT (IU/L)*	96,9 ± 63,4	107,5 ± 53,3

SVR, sustained virological response; PBMC, peripheral blood mononuclear cell; ALT, alanine transaminase; *n*, number of patients.
*Mean ± SD.

Table 2: Distribution of mutations developing in the 5′-untranslated region of HCV in serum and PBMC from patients treated with pegylated interferon and ribavirin.

Position	Mutation	Domain	Number of patients		Number of variants	
			SVR+	SVR−	PBMC	Serum
104	U-C	IIb		1	1	
104*	C-U	IIb	1			1
107*	G-A	IIb		1		1
109	A-C	IIb		1	1	
119	A-U			2	1	1
121	C-U			1		1
142	A-G	IIIa		1	1	
146	G del	IIIa		1	1	
183	C-U	IIIb	1	3	4	1
183*	C-U	IIIb	1		1	
188	G-U	IIIb		1	1	
206	A ins	IIIb		1	1	
233*	A-G	IIIb	1		1	
243	A-G	IIIc		1	1	
243*	G-A	IIIc		1	1	1
244	A-G	IIIc		1	1	
261	G-U	IIId		1	1	
263	U-G	IIId		1	1	1
271	G-U U-G	IIId		2	2	1
273	U-G	IIId		1	1	1
274	C-A	IIId		1	1	1
277	U-G	IIId		1	1	1

SVR+, sustained virological response; SVR−, no sustained virological response; PBMC, peripheral blood mononuclear cell.
*Mutations were detected after the end of therapy (weeks 60 and 72).

two variants, C204 and G243 were found in five variants, U204 was found in one variant, and G243 was found also in one variant (Table 3). U104C and A243G variants appeared during therapy, but after discontinuation of treatment the sequence reverted back to the original (C104U and G243A).

3.2. Thermodynamic Stability of the RNA Secondary Structure. The Gibbs minimum free energy values (ΔG), which characterize the stability of RNA secondary structures, were compared between newly appearing HCV variants and the baseline viral strains (Table 3). Changes in ΔG were observed

Table 3: Analysis of HCV 5′UTR mutations developing during treatment with pegylated interferon and ribavirin.

Patient	Time point of new variant appearance (weeks)	Mutation	Position	Region	SVR	Localization		Effect on ΔG (kcal/mol)**
						PBMC	Serum	
1*	60	C-U	104	IIb	+		1	Increased
2	8	A-U	119	ssRNA	−		1	No effect
	24	C-U	121	ssRNA	−		1	No effect
3	24	A-U	119	ssRNA	−	1		Increased
		C-U	183	IIIb				
		A-G	244	IIIc				
		G-U	261	IIId				
		G-U	271					
4	4	A-G	142	IIIa	−	1		Decreased
5	8	U-G	263	IIId	−	1	1	Increased
		U-G	271					
		U-G	273					
		C-A	274					
		U-G	277					
6*	60	C-U	183	IIIb	+	1		Decreased
		A-G	233	IIIc				
7	8	C-U	183	IIIb	−		1	Decreased
	8	C-U	183	IIIb		1		Increased
		G del	146	IIIa				
8	16	C-U	183	IIIb	−	1		Decreased
		A-G	243	IIIc				
	24	A-C	109	IIb		1		No effect
		C-U	183	IIIb				
		A-G	243	IIIc				Decreased
		U-C	104	IIb				
9*	16	G-U	188	IIIb	−	1		Increased
		A ins.	206	IIIb IIIc				
		A-G	233	IIIb				
		G-A	107	IIIc IIb				
		G-A	243	IIIc				
	60						1	No effect
	72					1	1	No effect

SVR, sustained virological response; PBMC, peripheral blood mononuclear cell.

*Novel variants emerging after treatment (weeks 60 and 72).

**Increased ΔG implies lower stability, whereas decreased ΔG entails higher stability.

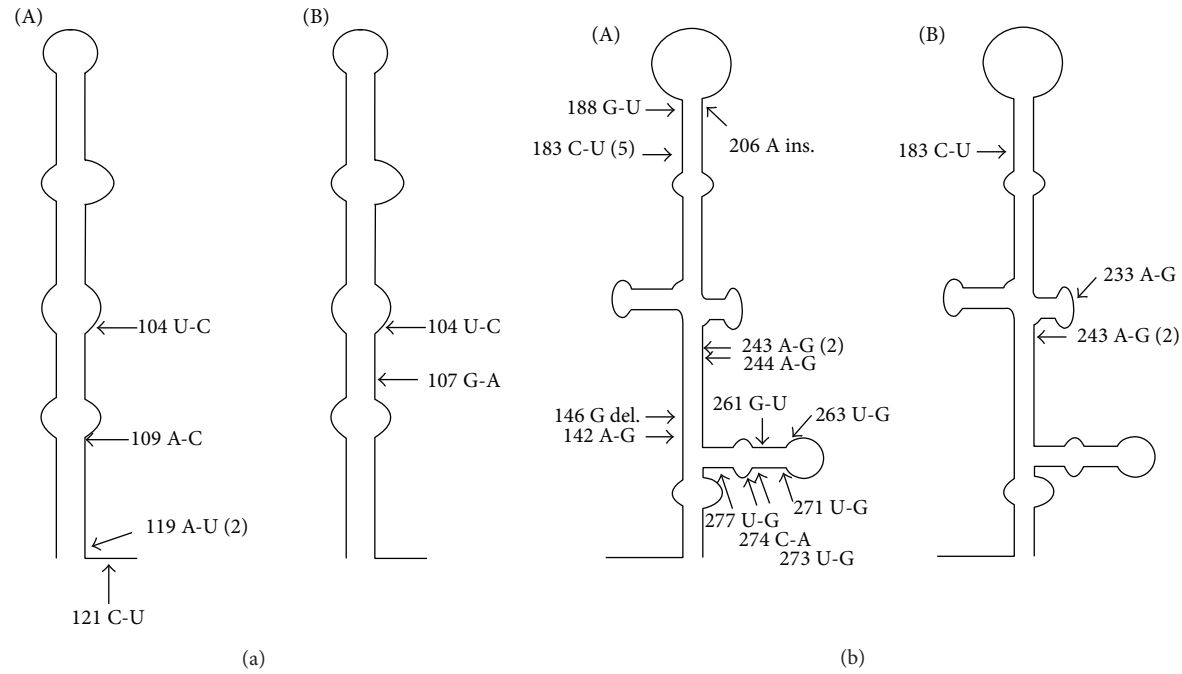

Figure 1: Distribution of detected mutations in IRES domains II (I) and III (II) secondary RNA structure in newly emerging variants appearing during treatment (a) and after completing therapy (b). Arrows indicate localization of nucleotide changes; number of variants is provided in brackets; del., deletion; ins., insertion.

in eight patients altogether. Variants with decreased stability of the II and/or III domains developed in three patients, increased stability variants appeared in another three, and in one patient variants with decreased and increased stability developed simultaneously. Furthermore, in one patient, opposite changes in domains II and III stability were present within the same viral variant.

New HCV variants manifesting increase in predicted IRES structure stability appeared in both serum (three variants) and PBMC (four variants). However, variants with decreased thermodynamic stability were identified exclusively in PBMC (three variants).

Mutations affecting IRES secondary structure stability were localized both in paired RNA regions and in loops (Figure 1). Changes within ssRNA segments (nt 119 and nt 121) did not affect ΔG value. The most significant impact on IRES secondary structure showed C-U substitution at nucleotide 104 and G deletion at nucleotide 146, which destabilized paired region of domains IIb and IIIa, respectively. Mutations G-U at position 188, 261, or 271 disrupted RNA pairing and induced additional loop formation. Additional connection in loop (nt 183) and substitutions U-G at positions 263, 271, 273-4, and 277 increased IRES stability.

4. Discussion

While HCV is mainly hepatotropic, cells of the lymphoid system constitute a secondary site of replication and lymphotropic variants often differ from those circulating in blood [7, 12, 13, 20, 21]. Several studies have shown that sequence changes within the 5′UTR affect the stability of secondary RNA structures and that they affect viral translation and replication efficiency; in addition, they are likely to determine viral tropism to particular cell compartments [2, 3, 5–7, 14, 22].

The aim of the present study was to identify and characterize newly emerging 5′UTR variants in PBMC and blood of patients undergoing antiviral treatment with pegylated interferon and ribavirin. In our study, the majority of the newly emerging variants (64%) were localized in PBMC, confirming this compartment as an independent site of replication and suggesting that it is under immune pressure related to treatment.

Interestingly, all newly emerging variants with decreased IRES stability were localized in PBMC. It was previously reported that "lymphotropic" HCV variants may demonstrate impaired translation efficiency, which could be an unintended consequence of viral adaptation mutations to different cells [14]. However, lower translation efficiency could confer its own benefits, particularly in the setting of treatment-related immune pressure, as it would lower the expression of viral proteins on infected cells and thus facilitate viral survival and latency. Whether the mutations developed *de novo* or were already present and simply became dominant once the major variants were suppressed by treatment is unclear.

The decrease in viral load during treatment could impede the detection of minor variant. However, we did not see any differences in viral loads between patients with and without changes in viral sequence.

Previous studies demonstrated that the apical part of domain III is essential for effective HCV translation. Due

to binding of the eukaryotic translation factor 3 (eIF3) and 40S ribosomal subunit, it positions viral RNA and enables 80S complex formation on the IRES [23, 24]. A recent study found that the competition between HCV domain III and eIF3 for binding with 40S subunit may result in the reduction of 43S complex formation and may thus favor translation of HCV mRNAs [25]. We did not find any variants with changes within the eIF3 binding sites in domain III. However, the relevance of identified mutations, especially at position 183, for eIF3 interaction and translation cannot be excluded.

5. Conclusion

In conclusion, HCV 5′UTR variants emerging in PBMC compartment during antiviral treatment are characterized by higher number of nucleotide changes and lower thermodynamic stability compared to serum strains. Nucleotide changes in position C183U might indicate viral lymphoid tropism.

Conflict of Interests

The authors declare no conflict of interests.

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Clinical Study

Whole Exome Sequencing Reveals Genetic Predisposition in a Large Family with Retinitis Pigmentosa

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Next-generation sequencing has become more widely used to reveal genetic defect in monogenic disorders. Retinitis pigmentosa (RP), the leading cause of hereditary blindness worldwide, has been attributed to more than 67 disease-causing genes. Due to the extreme genetic heterogeneity, using general molecular screening alone is inadequate for identifying genetic predispositions in susceptible individuals. In order to identify underlying mutation rapidly, we utilized next-generation sequencing in a four-generation Chinese family with RP. Two affected patients and an unaffected sibling were subjected to whole exome sequencing. Through bioinformatics analysis and direct sequencing confirmation, we identified p.R135W transition in the rhodopsin gene. The mutation was subsequently confirmed to cosegregate with the disease in the family. In this study, our results suggest that whole exome sequencing is a robust method in diagnosing familial hereditary disease.

1. Introduction

Retinitis pigmentosa (RP) is an inherited retinopathy with extreme clinical and genetic heterogeneity. Epidemiological study has indicated the prevalence of RP in China to be 1 in 3800 [1]. The common clinical manifestations usually start off as night blindness from adolescence followed by impaired visual fields and visual acuity. Eventually, RP patients suffer from tunnel vision and complete blindness in late stage of the disease. Bone spicule deposits and attenuated retinal vessels are usually observed in the fundus of RP patients. The severity and age of onset of this disease vary dramatically due to diverse genetic contributions [2]. Retinitis pigmentosa can be caused by various defects in many different genes and pathogenicity mechanisms; therefore, it is almost impossible to precisely diagnose this disease with clinical findings alone. RP can be inherited in all inheritance patterns, mainly including autosomal recessive, autosomal dominant, and X-linked recessive patterns [3]. Currently, mutations in 20 genes have been identified for adRP (RetNet). Due to the complex phenotype and genetic heterogeneity of RP, the process of precise molecular diagnosis is still quite difficult and time consuming.

Since the 1980s, tremendous technological advances have been made in identifying genetic mutations contributive to inherited retinal diseases. The continuous development of new techniques not only accelerates the process of identifying pathogenic genes of human genetic diseases, but also provides insights into the mechanisms involved in retinal pathologies [4]. However, most of these techniques are relatively inefficient, expensive, and labor intensive and most importantly do not improve diagnostic efficiency. Protein-coding genes constitute only approximately 1% of the human genome, but they account for 85% of the mutations linked to most genetic disorders [5]. Next-generation sequencing is capable of capturing all protein-coding sequences and therefore allowing the simultaneous analysis of multiple genes and the generation of massive amount of sequence data. These features of next-generation sequencing make it an attractive approach for investigation of coding variations [6].

On account of its remarkable characteristic, next-generation sequencing can contribute to more efficient and accurate molecular diagnosis, especially for those diseases that have no evident symptoms.

In this study, we attempted to identify the candidate pathogenic gene responsible for a Chinese family with RP using whole exome sequencing. In addition, we wanted to evaluate the diagnostic efficiency of using this technique and to establish a correlation between candidate genes and clinical phenotypes.

2. Methods

2.1. Patient Recruitment. This study complied with the Declaration of Helsinki and was approved by the Ethics Committee of The Eye Hospital of Wenzhou Medical College. Written informed consents were obtained from every patient. We collected information on their detailed family history of RP, such as age of onset and the progress of the disease. Then each participant underwent careful examinations including visual acuity testing using E decimal charts, slit-lamp biomicroscopy, fundus examination, visual field testing, and optical coherence tomography (OCT). The diagnosis of RP was based on the presence of night blindness, typical fundus findings, reduced peripheral visual field, abnormal OCT result, and family history.

We selected two affected participants (III-7 and III-10) and an unaffected sibling (III-4) for whole exome sequencing. Total genomic DNA was extracted from peripheral blood using a DNA Extraction Kit (TIANGEN, Beijing) according to the manufacturer's instructions. DNA was quantified with Nanodrop 2000 (Thermal Fisher Scientific, DE).

2.2. Exome Sequencing. For the Illumina HiSeq 2000 platform, Illumina libraries were generated according to the manufacturer's sample preparation protocol. In short, 3 ug of each patient's genomic DNA was fragmented to 100–300 base pairs. According to standard Illumina protocols, we prepared DNA libraries using procedures like end-repair, adenylation, and adapter ligation. DNA fragments were captured by hybridization to the capture panel by using the Exome Enrichment V5 Kit (Agilent Technologies, USA) and sequenced on Illumina HiSeq 2000 Analyzers for 90 cycles per read [7]. The PCR products were purified using SPRI beads (Beckman Coulter) according to manufacturer's protocol. Then the enrichment libraries were sequenced on Illumina Solexa HiSeq 2000 sequencer for paired read of 100–300 bp [8].

2.3. Data Filtering and Analysis. After the whole exome sequencing was complete, image analysis, error estimation, and base calling were processed using the Illumina Pipeline to obtain primary data. Firstly, the short paired-end reads were aligned to the reference human genome using SOAPaligner software [9]. Then the mutations in noncoding and intronic regions and the low quality reads were removed from the primary data [10]. SNPs and indels were identified using the SOAPsnp software and the GATK Indel Genotyper [11].

Table 1: The clinical features of patients in this study.

Subject	Age	Sex	BCVA (OD/OS)	Onset age of night blindness
III-2	50	F	HM/0.1	<10
III-7	40	M	0.05/0.15	<10
III-10	36	F	0.3/0.2	<10
III-13	46	F	0.3/0.3	<10
III-16	42	M	0.3/0.4	<10
IV-2	25	F	0.5/0.5	<10
IV-10	23	F	0.5/0.6	<10

M: male; F: female; BCVA: best corrected visual acuity; OD: right eye; OS: left eye.

Variants above 1% frequency were also removed and then the remaining variants were analyzed based on their predictive effect of amino acid change and on protein function using PolyPhen, SIFT, PANTHER, and Pmut [12]. Given that this is an inherited disorder in the family, we only kept common variants in the two affected individuals for further analysis.

2.4. Confirmation of the Potential Mutations. Once we obtained a list of candidate variants, we amplified the same site of each participant's DNA template and then sequenced the PCR products using Sanger sequencing to confirm the precision of the variants. Then we analyzed Sanger sequencing results by Mutation Surveyor (Softgenetics, PA) and estimated pathogenic effects of the mutations on protein function by Mutation Taster (http://www.mutationtaster.org).

3. Results

3.1. Phenotypic Determination. The four-generation Chinese family we recruited has 42 members including 14 affected individuals (Figure 1). The inheritance pattern of RP in this family was autosomal dominant. All the affected patients in this study began suffering from severe night blindness before the age of ten. Although they were diagnosed in their youth, most patients started to exhibit characteristic clinical symptoms after the age of thirty and developed total blindness in later life. As they reached the age of onset, their visual acuity reduced quite rapidly and their BCVA dropped to less than 0.3 in their worse eyes. Fundus examinations presented attenuation of the retinal vessels, bone spicule-like pigmentation in the inferior periphery, and retinal pigment epithelium (RPE) atrophy. OCT results clearly displayed severely thin and disorganized inner and outer segment of photoreceptors. Humphrey visual field testing evidently showed serious impairment of peripheral visual field (Figure 2). The unaffected sibling (III-4) has normal vision activity without pathological examination results. Detailed clinical information of the family was summarized in Table 1.

3.2. Whole Exome Sequencing Identified the Candidate Gene. The exomes of two affected individuals (III-7 and III-10) in the family were captured and sequenced. Millions of

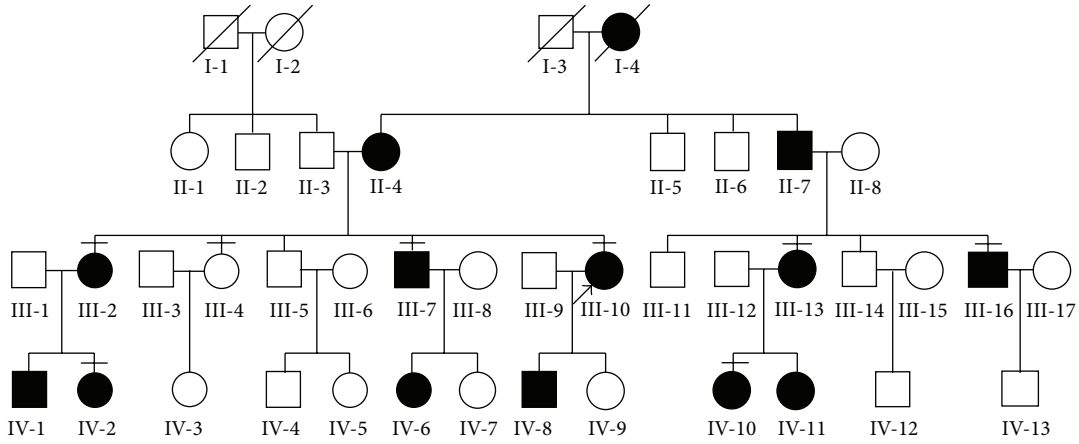

Figure 1: Pedigree of the Chinese family in this study. Filled symbols represent affected patients and unfilled symbols indicate unaffected subjects. The bars over the symbol indicate subjects enrolled in this study.

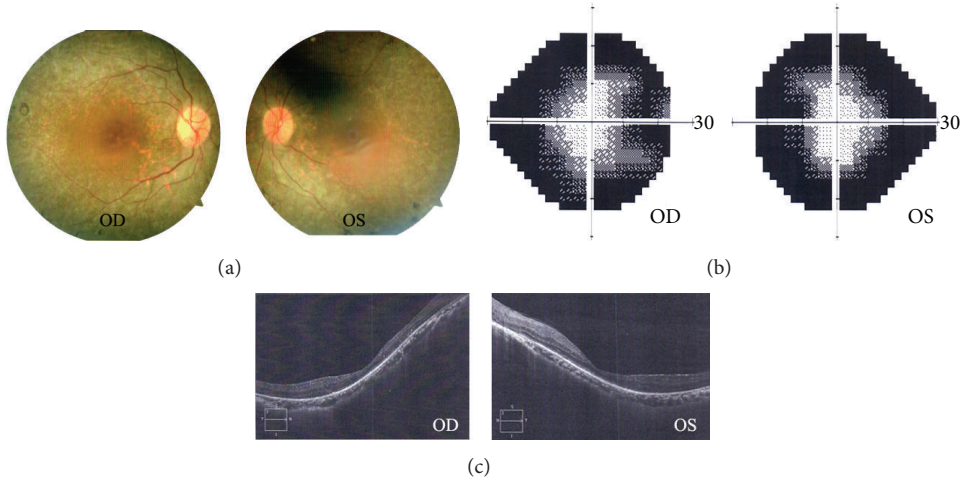

Figure 2: Representative clinical characteristics of the proband. (a) Fundus photographs show bone spicule-like pigmentation and retinal vascular attenuation. (b) Visual field test point locations show the loss of peripheral visual field. (c) Optical coherence tomographic (OCT) images show severe thinning of the photoreceptor inner/outer segment.

sequencing reads were generated from the two samples. Most of them were aligned to the human reference genome or mutations in noncoding and intronic regions. Mean read depth of target regions was 41.9 X, 44.6 X, and 34.0 X, respectively (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/302487). The remaining variants were then further evaluated by SOAPsnp and their impact on protein function was predicted. At last only one variant in *RHO* (p.R135W) was found among the RP related genes. This mutation and its effect on protein function were previously identified in RP patients by other researchers. We therefore supposed this variant as a candidate mutation responsible for RP.

3.3. Sanger Sequencing Confirmation. Candidate variant identified from whole exome sequencing was confirmed using conventional Sanger sequencing to exclude the possibility of false positive. We extracted DNA from the three participants (III-4, III-7, and III-10) and amplified the target fragments. We also amplified and sequenced DNA from five other individuals (III-2, III-13, III-16, IV-2, and IV-10) in the pedigree. Sequence analysis was performed with Mutation Surveyor (Softgenetics, PA). With the exception of the unaffected participant, all of the examined RP patients were confirmed to have the same mutation in their *RHO* gene. We are therefore confident that the mutation (p.R135W) is the disease-causing gene in this family (Figure 3).

3.4. Effect of the Mutation. The *RHO* gene, the first gene known to cause RP, encodes the protein rhodopsin, which plays an important role in capturing light and initiating the signal transduction cascade [13]. Mutations in *RHO* usually not only cause dominant RP but also are found in a small fraction of recessive RP [14]. The p.R135W mutation locates in a specific region of the *RHO* gene that impacts the putative second transmembrane segment of the protein, which leads

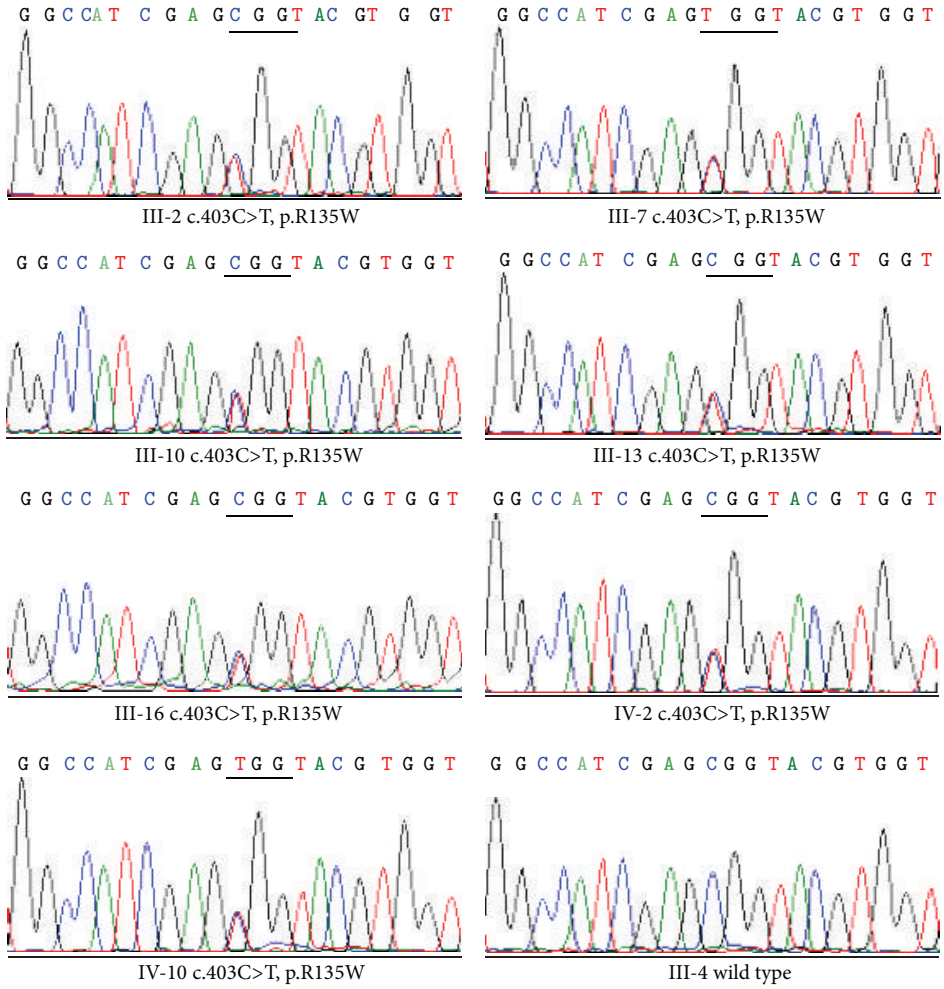

Figure 3: Chromatography of the identified mutation in each patient. Sanger sequencing results obtained from seven affected members (III-2, III-7, III-10, III-13, III-16, IV-2, and IV-10) and an unaffected member (III-4) in the Chinese family.

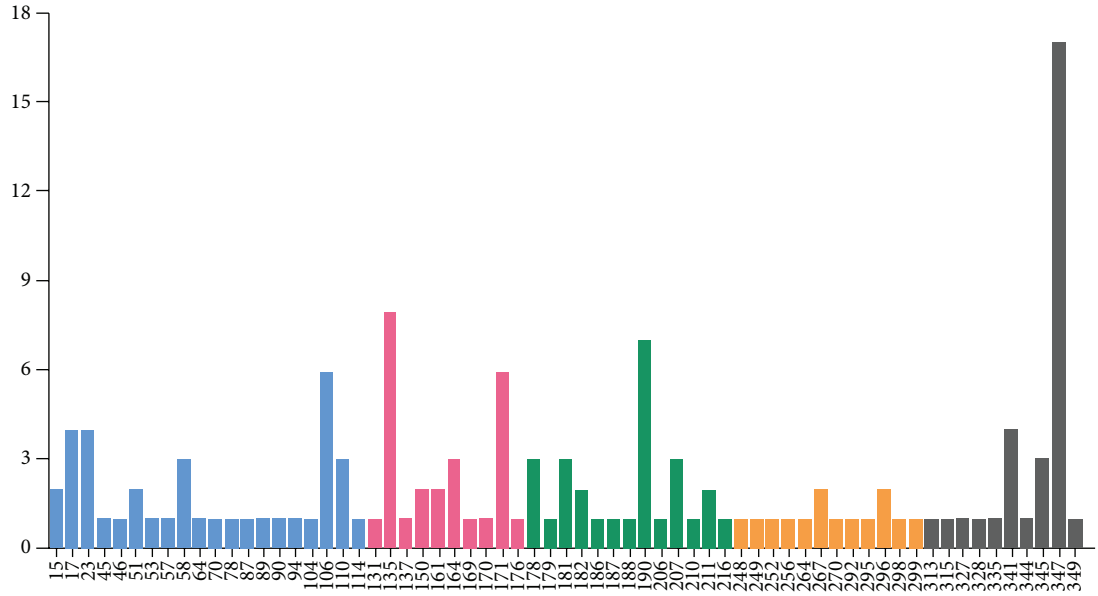

Figure 4: The mutation spectrum of *RHO* gene. The *x*-axis indicates the amino acid position and *y*-axis represents the number of reported mutations. Each color represents an exon: blue: exon 1; pink: exon 2; green: exon 3; orange: exon 4; grey: exon 5.

to impaired function of rod photoreceptors [15]. The severity of the disease seems to correlate with the disease stage of the patient and also the molecular basis of the disease. We believe that the p.R135W mutation causes a mild phenotype of night blindness in young age that worsens by middle age.

3.5. Mutation Spectrum of the RHO Gene. The mutation distribution in previously reported *RHO* mutations was summarized (Figure 4). Mutations in the *RHO* gene spread out over the entire length of the gene. According to our statistics, amino acid positions 135, 190, and 347 were the top three hot spots in the worldwide RP population.

4. Discussion

Due to the genetic and clinical heterogeneity of the inherited retinal diseases, efficient and accurate molecular diagnosis has proven to be quite difficult. Although typical clinical symptoms and information on family history can help promote the diagnosis process, we have yet to narrow down the disease-causing gene from more than 100 candidate genes linked to RP (RetNet).

Current technological advances have allowed once unaffordable techniques like whole exome sequencing to be used as a routine diagnostic tool [16–18]. This sequencing technique covers all of the coding sequences in the human genome and makes it possible to screen a number of genes simultaneously. One of the aims of this study is to evaluate the efficiency of using high-throughput sequencing technique in diagnosing monogenic disorders. We selected two affected patients and one healthy sibling to be subjected to the sequencing. Combining the sequencing data and Sanger sequencing results, we efficiently mapped the candidate mutation to the *RHO* gene at the position 403 on cDNA (c.403C>T), which converts arginine to tryptophan. *RHO* was the first reported gene associated with adRP and its incidence in Chinese individuals was estimated to be relatively high. In our study, the inheritance pattern of the four-generation family follows that of adRP and the clinical symptoms of the affected members are similar to each other. Hence, we propose possible correlations between clinical features of RP and the candidate mutation in *RHO*. Most of the patients in this family suffered night blindness at young age and their visual acuity and vision field worsen from middle age. We speculate that the mutation (p.R135W) may be responsible for these clinical symptoms common within this family.

In conclusion, we have successfully performed whole exome sequencing for screening mutations within a RP family and showed that this technology is an inexpensive and efficient tool for identifying disease-causing mutations. Using whole exome sequencing as part of the routine clinical examination not only will contribute to rapid diagnosis, but also might allow for the discovery of other underlying disease-causing mutations. Furthermore, identifying the disease-causing mutations also opens up the option of using gene therapy to treat the disease.

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Authors' Contribution

Juan Wu and Lijia Chen contributed equally to this study.

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Research Article

Genetic Testing in Hereditary Breast and Ovarian Cancer Using Massive Parallel Sequencing

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High throughput methods such as next generation sequencing are increasingly used in molecular diagnosis. The aim of this study was to develop a workflow for the detection of *BRCA1* and *BRCA2* mutations using massive parallel sequencing in a 454 GS Junior bench top sequencer. Our approach was first validated in a panel of 23 patients containing 62 unique variants that had been previously Sanger sequenced. Subsequently, 101 patients with familial breast and ovarian cancer were studied. *BRCA1* and *BRCA2* exon enrichment has been performed by PCR amplification using the BRCA MASTR kit (Multiplicom). Bioinformatic analysis of reads is performed with the AVA software v2.7 (Roche). In total, all 62 variants were detected resulting in a sensitivity of 100%. 71 false positives were called resulting in a specificity of 97.35%. All of them correspond to deletions located in homopolymeric stretches. The analysis of the homopolymers stretches of 6 bp or longer using the BRCA HP kit (Multiplicom) increased the specificity of the detection of *BRCA1* and *BRCA2* mutations to 99.99%. We show here that massive parallel pyrosequencing can be used as a diagnostic strategy to test for *BRCA1* and *BRCA2* mutations meeting very stringent sensitivity and specificity parameters replacing traditional Sanger sequencing with a lower cost.

1. Introduction

Germline mutations that inactivate *BRCA1* and *BRCA2* are responsible for breast and ovarian cancer susceptibility [1, 2]. The prevalence of *BRCA1* and *BRCA2* mutations where family history shows more than one occurrence of breast cancer under the age of 50 ranges from 8 to 21.2%. Mutation carriers are at an increased cumulative risk to the age of 70 of 36–70% and 10–65% for breast cancer and ovarian cancer, respectively [3, 4]. Moreover, *BRCA1* and *BRCA2* mutation carriers are also at increased risk of pancreatic, prostate, and endometrial cancer. Molecular diagnosis is an important factor in clinical decisions that include increased surveillance, chemoprevention, or prophylactic surgery [5, 6]. Predictive testing in family members allows the identification of other individuals at risk.

BRCA1 and *BRCA2* mutation screening is offered to patients from high risk families. Direct Sanger sequencing allows the identification of the sequence alteration and is considered the gold standard. Sequencing of *BRCA1* and *BRCA2* genes is time consuming and costly due to the large size of the genes and the equal distribution of mutations along the whole *BRCA1* and *BRCA2* sequence (5589 and 10254 nucleotides, resp.). A high level of allelic

heterogeneity has been described including single nucleotide variants (SNVs), short insertions and deletions (InDels), and large structural variants (see Breast Cancer Information Core database: http://www.research.nhgri.nih.gov/bic/). Currently, many laboratories include a scanning method that allows the detection of all different types of mutations with a sensitivity and specificity of 100% [7].

High throughput methods such as next generation sequencing are increasingly used in molecular diagnosis [8]. Massive parallel sequencing allows the generation of millions of DNA sequences in a single run with low cost per base [9]. The development of technologies to capture and enrich specific regions of the genome improves performance and reduces the cost allowing joint sample analysis of numerous individuals [10]. Several studies have demonstrated the potential of massive sequencing both in the field of research and in genetic diagnosis [11, 12]. Recently, next generation sequencing methods for the mutation analysis of the *BRCA1* and *BRCA2* genes in patients with breast and ovarian cancer have been described using both high capacity and bench top platforms [13–18]. Bench top sequencers are addressed to individual labs to suit the demand of midsize diagnostic laboratories.

Here, we developed a workflow using massive parallel pyrosequencing in a bench top 454 GS Junior sequencer together with homopolymer scanning to screen for mutations in the *BRCA1* and *BRCA2* genes. Our workflow was first validated in a panel of 23 patients previously Sanger sequenced. Subsequently, 101 patients with familial breast and ovarian cancer were studied. We found 18 pathogenic mutations and 10 variants with unknown clinical significant effect (VUS). We show here that our workflow performs as Sanger sequencing in terms of sensitivity and specificity with the advantage of taking less time and cost consuming being suitable for genetic diagnosis.

2. Methodology

2.1. Patients. A total of 23 samples containing 62 unique variants were used to evaluate the methodology. 49 variants corresponded to single nucleotide variants (SNV) while 13 corresponded to deletions (8), insertions (3), and combined insertions and deletions (2). Among the 62 variants tested 14 were pathogenic mutations (11 insertions/deletions, 1 missense mutation, 1 nonsense mutation, and 1 splice site mutation). DNA samples were obtained from the Hereditary Cancer Program at the Catalan Institute of Oncology (ICO-IDIBELL) and the Genetic Counselling Unit at the Hospital of Sabadell (Barcelona, Spain).

Then, 101 patients with breast and ovarian cancer were screened for mutations using our validated workflow. DNA samples were collected from patients referred to the Genetic Counselling Unit at the Hospital of Sabadell (Barcelona, Spain). Informed consent was obtained from all the patients included in our study. Genomic DNA was extracted from peripheral blood following standard procedures and using Gentra Puregene DNA reagents (Qiagen, Valencia, CA, USA).

2.2. Multiplex PCR Target Amplification, NGS Library Preparation, and Sequencing. *BRCA1* and *BRCA2* coding regions and exon intron boundaries were amplified using the BRCA MASTR kit (Multiplicom, Niel, Belgium). Samples used to evaluate the methodology performance were amplified using the BRCA MASTR kit v1.2 (7 samples) and v2.0 (16 samples) following manufacturer instructions. The samples screened for *BRCA1* and *BRCA2* mutations were amplified using the BRCA MASTR kit v2.1. The BRCA MASTR kit v1.2 amplifies *BRCA1* and *BRCA2* coding regions and exon intron boundaries in 169 amplicons while versions 2.0 and 2.1 amplify both genes in 94 and 93 amplicons, respectively. Briefly, 50 ng of genomic DNA was used in a two-step multiplex reaction to firstly amplify *BRCA1* and *BRCA2* coding regions followed by the incorporation of molecular barcodes (multiple identifiers, MIDs) and 454 adapters to each amplicon. A *BRCA1* and *BRCA2* amplicon library of each patient was generated and quantified using Quant-iT PicoGreen (Invitrogen, Life Technologies, San Diego, CA, USA). Equivalent amounts of the patient libraries were pooled to generate a unique sequencing library that is twice purified using Agencourt AMPure XP (Beckman Coulter, Beverly, MA, USA) and PicoGreen quantified. Emulsion PCR was performed using the GS Junior Titanium emPCR kit (Lib-A) and pyrosequenced in the sense and antisense strands with the GS Junior following manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

2.3. Bioinformatic Analysis. Data analysis was performed using the GS Amplicon Variant Analyzer version 2.7 (AVAv2.7) software (Roche). After sequence quality filtering, specific primers, MIDs, and adapter sequences are trimmed. Reads are then mapped to *BRCA1* and *BRCA2* genomic reference sequences NG_005905 and NG_012772, respectively. Coverage was obtained for all amplicons and analysed to detect low coverage amplicons. Variants are filtered using the AVAv2.7 software according to two parameters, the presence of the variant in both strands and the percentage of reads with the variant. Finally, variants are annotated according to the Human Genome Variation Society guidelines (http://www.hgvs.org/). Functional significance of variants is assigned by the authors following established criteria [19].

2.4. Homopolymer Analysis. *BRCA1* and *BRCA2* coding homopolymers of 6 bp or longer were analyzed using the BRCA HP v.2.0 (Multiplicom). Briefly, 50 ng of genomic DNA is amplified in two multiplex reactions resulting in 39 fragments that comprise all coding homopolymers. Fragment length is analysed on the ABI 3130 sequencer using the Gene Mapper software (Applied Biosystems, Foster City, CA, USA).

2.5. Multiple Amplicon Quantification (MAQ) Analysis. *BRCA1* and *BRCA2* large rearrangements were analysed using the BRCA MAQ kit (Multiplicom). It consists in the simultaneous amplification of several fluorescently labelled target amplicons (*BRCA1* and *BRCA2* exons) and reference sequences. Fragments are then size separated on an ABI 3130

Table 1: Summary of sequencing runs and coverage results of the validation set.

	Run 1 BRCAMASTRv1.2	Run 2 BRCAMASTRv2.0	Run 3 BRCAMASTRv2.0
Samples	7	8	8
Amplicons	169	94	94
Passed filter reads	118006	78777	100771
Mapped reads	113374	78698	100344
	Coverage (number of reads/amplicon)		
Minimum	19	13	17
Mean	96,98	102,7	132,27
Maximum	277	445	414
Standard deviation coverage	35,8	46,92	70,51
Amplicons <38 reads (%)	32 (2.7)	32 (4.2)	13 (1.72)

sequencer (Applied Biosystems). Comparison of the relative intensities of the target amplicons in the test individual and a control individual results in a dosage quotient, indicating the copy number of the CNV in the test sample.

3. Results

3.1. Validation of Next Generation Sequencing Performance for BRCA1 and BRCA2 Mutation Screening. In order to evaluate our massive parallel sequencing approach 23 patients previously Sanger sequenced were pyrosequenced in a 454 GS Junior platform.

In total three runs were performed. In the first run 7 samples were sequenced using the BRCA MASTR kit v1.2 while in the last two runs 8 samples were simultaneously sequenced using the BRCA MASTR kit v2.0. The number of reads was variable between the runs. The average coverage per amplicon was higher in the third run. The use of the BRCA MASTR kit v2.0 that amplifies the two BRCA genes in 94 amplicons instead of 169 increases the average number of reads per amplicon as well as decreasing the number of amplicons with less than 38 reads even though in the first run we sequenced seven samples instead of eight (Table 1).

Bioinformatic analysis of reads is performed with the AVA software v2.7 (Roche). First, adapter and MIDs are trimmed from the obtained reads. Then, reads are mapped to the references sequences and variants are called and reported. We considered true variants those found in both strands and present in at least 25% of reads. The list of variants reported by the AVA software was further filtered excluding those variants present in amplicons with less than 38x coverage. It has been described that a minimum coverage of 38x is required to obtain a Phred score of 30 (or $P = 99.9\%$) when using a variant detection filter of 25% [20]. The number of amplicons with less than 38x coverage ranged from 13 to 32 which represent less than 5% of the total number of amplicons sequenced. All 49 distinct substitutions were detected both in heterozygosity and homozygosity. Heterozygous substitutions were detected between 25% and 76.47% of the reads while homozygous substitutions were detected between 97.44% and 100% of the reads (Supplementary Table 1 availabe online at http://dx.doi.org/10.1155/2014/542541). As expected the variant detection is closer to 50% with high coverage. All the deletions and insertions, except from c.548-58delTT located in a homopolymeric stretch of 7 T in intron 7 of the *BRCA1* gene, were detected in both the forward and the reverse strands and between 26% and 82.14% of the reads. We detected the variant c.548-58delTT in all samples at high frequency even though it was not present in all samples resulting in a false positive. Deletion of c.6841+79delTTAA in intron 11 of the *BRCA2* gene was detected both in heterozygosity and homozygosity. The pathogenic variants were all detected in heterozygosity (Table 2) except for the c.8946delA in the *BRCA2* gene which was detected in homozygosity in the forward reads. This is due to the location of the c.8946delA in a homopolymer stretch. In total, all 62 variants were detected resulting in a sensitivity of 100%.

We detected 37 different false positives with the AVA software 2.7 (Supplementary Table 2). All of them correspond to deletions located in homopolymeric stretches and are generated as a result of the use of the pyrosequencing technology as has been described previously [21]. 35 out from 37 correspond to deletions in homopolymeric stretches of 6 bp or longer. The remaining 2 false positives correspond to two deletions at homopolymers of 4 nucleotides. In the total of three runs 71 false positives were called resulting in a specificity of 97.35%. The analysis of the homopolymers stretches of 6 bp or longer using the BRCA HP v2.0 kit (Multiplicom) allows the exclusion of all variants detected in homopolymers $\geq$ 6 bp from the variant list reported by the AVA 2.7 software, increasing the specificity of the detection of *BRCA1* and *BRCA2* mutations to 99.99%.

3.2. Detection of BRCA1 and BRCA2 Mutations in a Cohort of 101 Patients with Inherited Breast and Ovarian Cancer. We next decided to implement our parallel pyrosequencing protocol and sequence analysis approach to screen for mutations in the *BRCA1* and *BRCA2* genes in a series of 101 patients with breast and ovarian cancer. Our objective was to further analyze the performance of massive parallel pyrosequencing in terms of number of sequences obtained per run, coverage uniformity, and number of variants detected as well as in the identification of pathogenic mutations responsible for the disease.

Table 2: Pathogenic mutations in the validation set tested for the evaluation of the AVA 2.7 software.

Variant HGVS	Gene	Variant freq. % (number of reads)	
		forward	reverse
c.70_71insTGTC	*BRCA1*	55.88 (68)	59.32 (59)
c.1121-1123delCACinsT	*BRCA1*	35 (60)	49.25 (67)
c.1961delA	*BRCA1*	38.68 (106)	31.07 (103)
c.2921T>A (p.L974X)	*BRCA1*	51.16 (43)	36.21 (58)
c.3767_3768delCA	*BRCA1*	26 (50)	54.69 (64)
c.3770-3771delAG	*BRCA1*	50 (50)	47.46 (59)
c.4107-4110dupATCT	*BRCA1*	54.24 (59)	51.85 (54)
c.5123C>A	*BRCA1*	52.38 (42)	56.25 (48)
c.1842dupT	*BRCA2*	56.00 (25)	55.17 (29)
c.5350-5351delAAinsT	*BRCA2*	48.92 (139)	54.08 (98)
c.6275-6276delTT	*BRCA2*	47.41 (116)	38.13 (139)
c.7617+1G>A	*BRCA2*	34.78 (23)	48.28 (29)
c.8946delA[a]	*BRCA2*	100 (52)	44.44 (36)
c.9026_9030delATCAT	*BRCA2*	50.94 (53)	40.54 (37)

[a]Mutation located in a homopolymeric region.

All samples were first analysed for mutations in the homopolymer stretches of >6 bp using the BRCA HP v2.0 kit. Three frameshift mutations were detected. Although they are not strictly located in the homopolymer stretch, they are within the fragments amplified by the BRCA HP v2.0 kit (Table 4). Sanger sequencing identified one deletion, one insertion, and a combined InDel (c.4030del6insC, c.5189dupA, and c.5722_5723delCT in the *BRCA2* gene).

The remaining 98 samples were distributed in 14 Junior runs in groups of seven samples. We decided to sequence seven samples per run instead of eight in order to increase the coverage per amplicon and to decrease the number of amplicons with low coverage (<38 reads).

The number of reads obtained per run was very variable. The reads that passed the quality filters ranged between 69455 reads and 150722 reads with an average of 99864 reads (±28215) (Table 3). As a result of the differences between the reads obtained per run, the average coverage per amplicon and most importantly the number of amplicons with less than 38 reads were also variable (Table 3). Depending on the run the number of amplicons with less than 38 reads ranged between 1 and 26.

In the total 14 GS Junior runs, we identified 14 patients with deleterious mutations of which 7 are frameshift mutations (one mutation was found in three different patients), 4 are nonsense, 2 are missense, and 1 is an in frame deletion that affects splicing (Table 4). All mutations were confirmed by Sanger sequencing discarding the presence of false positives. One *BRCA1* mutation, c.68_69delAG, was found in three different patients. This mutation accounts for the 30.4% *BRCA1* mutations in the Mediterranean area [22]. Although only found once in our series, mutations c.211A>G, c.5123C>A in *BRCA1*, and c.3264dupT in *BRCA2* are also considered recurrent in the Spanish population [22]. We have identified 5 novel mutations in our cohort. Mutation c.2900_2901dupCT in the *BRCA1* gene and mutations c.4030del6insC, c.5189dupA, c.8009delC, and c.9274delT in the *BRCA2* gene are mutations not described previously. In addition we detected 9 variants with unknown clinical significance (VUS). All of them are missense mutations except one located in an intronic sequence (c.68-7T>A in the *BRCA2* gene). Finally, large genomic deletions and duplications were screened using the MAQ assay, which consists in a semiquantitative PCR that amplifies all exons in the *BRCA1* and *BRCA2* genes together with control regions. We detected an exonic deletion that comprises exons 16 and 17 of the *BRCA1* gene. This mutation is predicted to produce an inframe deletion of 132 amino acids that disrupts the BRCT-N domain (p.Glu1559_Thr1691del) and it has been described to be deleterious by functional analysis [23].

4. Discussion

Molecular genetic testing of mutations in the *BRCA1* and *BRCA2* genes is currently performed using highly sensitive but labour-intensive direct Sanger sequencing of individual exons. The advances in sequencing technologies have increased the speed and efficiency of DNA testing and next generation platforms are becoming the standard in molecular genetic diagnosis.

Here, we have tested and implemented a method for the molecular analysis of the *BRCA1* and *BRCA2* genes based on massive parallel pyrosequencing of pooled *BRCA1* and *BRCA2* gene enriched samples. *BRCA1* and *BRCA2* exon enrichment has been performed by PCR amplification using the Multiplicom BRCA MASTR kit, which amplifies all *BRCA1* and *BRCA2* coding exons in 97 amplicons. PCR enrichment was chosen over a hybridisation based method because PCR enrichment has been shown to cover all the amplicons of interest and to provide less variation in coverage between regions [10]. In addition, PCR enrichment has also

Table 3: Summary of reads obtained and coverage results in 14 GS Junior runs.

Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Passed filter reads	113809	69455	82287	73127	88687	138625	72728	110271	90416	136237	69811	121332	80589	138238
Mapped reads	113445	69184	81904	72835	87529	137794	72522	109691	89982	135982	69613	12107	77080	138190
Coverage (number of reads/amplicon)														
Mean	213,28	106,88	127,26	113,81	134,03	202,36	110,44	167,54	137,11	207,24	106,07	184,66	117	196.9
Minimum	5	29	22	34	19	24	26	27	31	7	23	21	11	8
Maximum	750	377	394	417	1560	1455	823	556	439	663	383	728	509	734
Amplicons <38 reads (%)	7	13	10	1	20	2	20	2	1	10	8	8	26	18

Table 4: Summary of BRCA1 and BRCA2 pathogenic mutations and variants of unknown significance (VUS) detected using our proposed workflow.

Variant HGVS	Gene	Detected with assay	Clinical significance	
c.68_69delAG (p.Glu23Valfs*16)	*BRCA1*	NGS	Pathogenic	Spanish recurrent mutation
c.211A>G (p.Arg71Gly)	*BRCA1*	NGS	Pathogenic	Spanish recurrent mutation
c.2410C>T (p.Gln804*)	*BRCA1*	NGS	Pathogenic	Reported
c.2900_2901dupCT (p.Pro968Leufs*32)	*BRCA1*	NGS	Pathogenic	Novel
c.3406C>A p.(Pro1136Thr)	*BRCA1*	NGS	VUS	Novel
c.3708T>G (p.Asn1236Lys)	*BRCA1*	NGS	VUS	Reported
c.4935G>C (p.Arg1645Ser)	*BRCA1*	NGS	VUS	Reported
c.5078_5080delCTG (p.1692del26)	*BRCA1*	NGS	Pathogenic	Reported
c.5123C>A (p.Ala1708Glu)	*BRCA1*	NGS	Pathogenic	Spanish recurrent mutation
Δ Exons 16/17	*BRCA1*	MAQ	Pathogenic	Reported
c.68-7T>A	*BRCA2*	NGS	VUS	Reported
c.754G>A (p.Asp252Asn)	*BRCA2*	NGS	VUS	Novel
c.3264dupT (p.Gln1089Serfs*8)	*BRCA2*	NGS	Pathogenic	Spanish recurrent mutation
c.4030del6insC (p.Asn1344Hisfs*5)	*BRCA2*	HP	Pathogenic	Novel
c.4316C>A (p.Ala1439Asp)	*BRCA2*	NGS	VUS	Reported
c.4681C>A (p.His1561Asn)	*BRCA2*	NGS	VUS	Reported
c.4965C>A (p.Tyr1655*)	*BRCA2*	NGS	Pathogenic	Reported
c.5189dupA (p.Asn1730Lysfs*12)	*BRCA2*	HP	Pathogenic	Novel
c.5722_5723delCT (p.Leu1908Argfs*1)	*BRCA2*	HP	Pathogenic	Reported
c.6215C>G (p.Ser2072Cys)	*BRCA2*	NGS	VUS	Reported
c.6613G>A (p.Val2205Met)	*BRCA2*	NGS	VUS	Reported
c.7180A>T (p.Arg2394*)	*BRCA2*	NGS	Pathogenic	Reported
c.7480C>T (p.Arg2494*)	*BRCA2*	NGS	Pathogenic	Reported
c.8009delC (p.Ser2670Trpfs*2)	*BRCA2*	NGS	Pathogenic	Novel
c.9274delT (p.Tyr3092Ilefs*11)	*BRCA2*	NGS	Pathogenic	Novel

NGS: next generation sequencing; HP: homopolymer; MAQ: multiple amplicon quantification.

a lower cost and requires less DNA compared to hybridisation based methods. Currently, PCR based enrichment is chosen for molecular diagnosis when analysing few genes simultaneously.

We validated our approach in a cohort of 23 patients with previously characterised *BRCA1* and *BRCA2* mutations and polymorphisms. We detected all mutations and polymorphisms in both heterozygosity and homozygosity achieving 100% sensitivity and 97.35% specificity. To increase the specificity of the method the variants called in homopolymeric regions should be excluded. Because both *BRCA1* and *BRCA2* genes comprise homopolymeric stretches in their coding regions a complementary assay is then needed to screen for changes in homopolymers. We used the BRCA HP assay developed by Multiplicom which screens for deletions and insertions in all exonic homopolymers of 6 bp or longer. This allowed the exclusion from our final variant list all changes detected in homopolymeric regions of ≥6 nucleotides resulting in a specificity of 99.99%.

Other works have analysed the performance of pyrosequencing in the detection of mutations in the *BRCA1* and *BRCA2* genes using different approaches to obtain the *BRCA1* and *BRCA2* DNA library and using the 454 GS FLX and GS Junior platforms [15–18]. Here, we used a multiplex amplicon based assay which amplifies all *BRCA1* and *BRCA2* coding regions and exon-intron boundaries and attaches the MIDs and sequencing adaptors in a second PCR (BRCA MASTR, Multiplicom). Multiplex PCR has been demonstrated to result in higher coverage per amplicon compared to singleplex [15] or long PCR fragments [17] and allows the joint sequencing of seven samples in each run. Recently, Feliubadaló et al. [18] have developed and validated a workflow using the BRCA MASTR kit amplicon followed by 454 GS Junior pyrosequencing. Data analysis combines the use of the three types of software VIP, R, and AVA and numerous filters followed by visual inspection of fragments. Their workflow achieves a specificity of 99.99% and a sensitivity of 100% when adding the BRCA HP assay to detect insertions and deletions in homopolymeric regions. In contrast to Feliubadaló et al. [18] our data analysis is based exclusively on the AVA 2.7 software making it simpler and completely automated. The AVA2.7 software in contrast to previous versions is able to call small InDels and achieve a sensitivity of 100% in variant calling ([16] and this report). Using our filtering parameters in the AVA 2.7 software together with BRCA HP assay we achieve a specificity of 99.99% and a sensitivity of 100%.

It is recommended that mutations detected by NGS technologies be validated by Sanger sequencing in the context of molecular diagnostics. Here, all deleterious mutations and VUS detected in our cohort of 101 patients have been

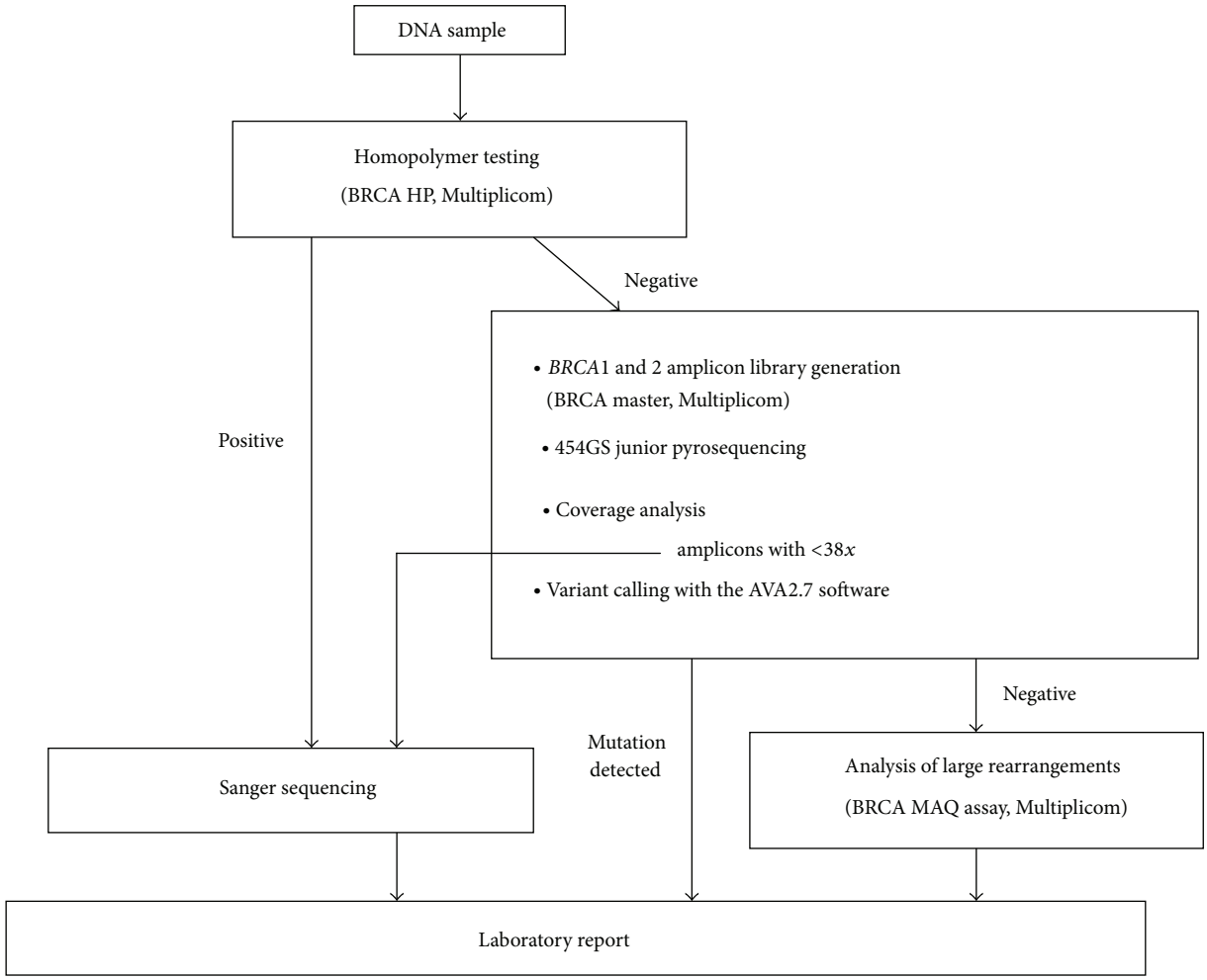

Figure 1: Proposed workflow using massive parallel pyrosequencing for analysing *BRCA1* and *BRCA2* genes.

confirmed by Sanger sequencing. These results together with the ones obtained in our validation set show that when using massive parallel pyrosequencing only deleterious mutations detected in homopolymeric tracts should be confirmed by Sanger sequencing [16].

Analysis of the coverage in our series of 14 runs showed that the number of amplicons with less than 38x ranged from 1 to 26 (0.14–3.8%) of a total number of 679 amplicons sequenced per run. This means that seven samples can be screened in a single GS Junior run with more than 95% of sequences covered sufficiently to provide a minimum power of 99.9% to detect heterozygous mutations in at least 25% of the reads. We detected that the number of reads obtained per run was very variable. After carefully reviewing our whole procedure, we realised that the addition of a lower number of molecules of DNA library per bead in the emulsion PCR resulted in the higher number of reads that passed quality filtering. Taking into account this observation we are now increasing the number of samples per run, which will result in a lower cost per sample analysed. We have checked that the cost and time consuming per sample of our sequencing approach improves the overall cost (approximately 50% less) and makes the process faster compared to direct Sanger sequencing alone.

Our proposed workflow to screen for mutations in the *BRCA1* and *BRCA2* genes consists first in the use of the *BRCA1* and *BRCA2* homopolymer assay (BRCA HP) followed by massive parallel sequencing with the 454 GS Junior sequencer and using the BRCA MASTR amplicon kit to generate the patient libraries. Coverage and variant calling is done using the AVA 2.7 software. Amplicons with low coverage should be Sanger sequenced. Finally, large rearrangements in the *BRCA1* and *BRCA2* genes are detected using the BRCA MAQ kit (Figure 1). Using our validated workflow, we have identified 18 deleterious mutations in 101 patients (17,8%) which is in accordance with the prevalence of *BRCA1* and *BRCA2* mutations reported in the Spanish hereditary breast and ovarian cancer population. In addition, we have detected 10 VUS, nine of which are unique and two of them have not been previously reported.

5. Conclusions

We show here that massive parallel pyrosequencing can be used as a diagnostic strategy to test for *BRCA1* and *BRCA2* mutations meeting very stringent sensitivity and specificity parameters and could be used in diagnostic laboratories replacing traditional Sanger sequencing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Genetic Diagnosis of Charcot-Marie-Tooth Disease in a Population by Next-Generation Sequencing

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Charcot-Marie-Tooth (CMT) disease is the most prevalent inherited neuropathy. Today more than 40 CMT genes have been identified. Diagnosing heterogeneous diseases by conventional Sanger sequencing is time consuming and expensive. Thus, more efficient and less costly methods are needed in clinical diagnostics. We included a population based sample of 81 CMT families. Gene mutations had previously been identified in 22 families; the remaining 59 families were analysed by next-generation sequencing. Thirty-two CMT genes and 19 genes causing other inherited neuropathies were included in a custom panel. Variants were classified into five pathogenicity classes by genotype-phenotype correlations and bioinformatics tools. Gene mutations, classified certainly or likely pathogenic, were identified in 37 (46%) of the 81 families. Point mutations in known CMT genes were identified in 21 families (26%), whereas four families (5%) had point mutations in other neuropathy genes, *ARHGEF10, POLG, SETX,* and *SOD1*. Eleven families (14%) carried the *PMP22* duplication and one family carried a *MPZ* duplication (1%). Most mutations were identified not only in known CMT genes but also in other neuropathy genes, emphasising that genetic analysis should not be restricted to CMT genes only. Next-generation sequencing is a cost-effective tool in diagnosis of CMT improving diagnostic precision and time efficiency.

1. Introduction

Charcot-Marie-Tooth (CMT) is the most common inherited neuropathy, affecting 40 to 81 cases per 100,000 in the Norwegian general population [1, 2]. CMT is clinically, neurophysiologically, and genetically heterogeneous. The clinical classification is based on age at onset, distribution of muscle weakness, sensory loss, walking difficulties, and foot deformities [3]. CMT is neurophysiologically subdivided into a demyelinating (CMT1) and axonal (CMT2) form depending on whether the median motor nerve conduction velocity (NCV) is below or above 38 m/s, respectively. A third form, intermediate CMT, has both demyelinating and axonal features and NCV between 25 and 45 m/s [2, 3].

The mode of inheritance is autosomal dominant, autosomal recessive, or X-linked [3]. At present more than 40 CMT genes have been identified and there are several genes associated with related conditions [4–8]. Genetic heterogeneity and pleiotropic genes, that is, mutations in different genes, cause a similar phenotype and mutations in a single gene cause different phenotypes, which adds to the complexity of CMT [3, 6, 7]. Furthermore, sporadic cases of CMT are not uncommon due to autosomal recessive inheritance, reduced penetrance, late onset, small family size, and *de novo* mutations [2, 8, 9].

The duplication of *PMP22* is the most common cause of CMT. The prevalence was ~15% in two Norwegian studies and up to 40% in other selected populations [2, 9–13]. Otherwise, CMT is caused by point-mutations, with rare exception of non-*PMP22* copy-number variations (CNVs) [14, 15]. Establishing a genetic CMT diagnosis provides patients and

families with information about prognosis and recurrence risk, as well as future options for specific treatment [16, 17].

Current strategy for diagnosing CMT is based on the clinical and neurophysiological phenotype. It is favourable to initially test CMT1 patients for the *PMP22* duplication due to its high prevalence. Genes are thereafter traditionally tested sequentially by Sanger sequencing, but the low prevalence of specific CMT point-mutations renders sequential testing unfavourable due to time and cost. Furthermore, most diagnostic laboratories only have capacity for sequencing a few genes [3, 7, 12, 17]. Hence, it is important to develop a more comprehensive approach for clinical diagnosis of heterogeneous disorders such as CMT, dystonia, hereditary spastic paraplegia (HSP), and Parkinson's disease [6]. Next-generation sequencing (NGS) makes it possible to sequence several genes in parallel and at a low cost compared to traditional methods.

We applied NGS on 59 CMT families from the Norwegian general population and sequenced 32 CMT genes along with 19 genes causing other inherited neuropathies, since the phenotypes of CMT, distal hereditary motor neuropathy (dHMN), and other inherited neuropathies overlap [3, 4, 6].

2. Materials and Methods

2.1. Study Population. People with CMT residing in eastern Akershus County, Norway, January 1, 1995, were included in the study [2]. Akershus County has rural and urban areas and was inhabited by 297, 539 persons [18]. A total of 245 affected persons from 116 CMT families were identified. DNA was available in 81 CMT families, 189 affected individuals. The neurophysiology among the families was 38 CMT1, 33 CMT2, two intermediate CMT, and 8 families with an unknown neurophysiological phenotype. The families were previously tested for the *PMP22* duplication by real-time quantitative PCR and point mutations in *PMP22*, *GJB1*, *MPZ*, *LITAF*, *MFN2*, and *EGR2* by conventional Sanger sequencing [2]. Later, a duplication of *MPZ* was identified in one CMT family, and another CMT family had a point mutation in the *SOD1* gene [14, 19]. A mutation was identified in 22 CMT families. A more comprehensive description of the study population has been published previously [2].

This study applied NGS on 70 affected individuals from 59 CMT families without a genetic diagnosis; these were 22 CMT1 families, 29 CMT2 families, one intermediate CMT family, and seven families with unknown neurophysiological phenotype. A control group of 180 healthy individuals were included in order to detect polymorphisms present in ≥1% of the population [20]. The Norwegian Regional Ethical Committee for Medical and Health Research approved the project, and the participants gave written informed consent.

2.2. Targeted Capture and DNA Sequencing. Table 1 shows the 51 neuropathy genes included in the panel [3, 4, 6]. Illumina's DesignStudio (Illumina Inc., San Diego, USA) for TruSeq Custom Enrichment was used to target all exons and flanking 5′ and 3′UTR (untranslated region) sequences (default settings). In total, 909 oligonucleotide probes covering 256,248 bp (base pairs) were included. Genomic DNA was extracted from whole blood using standard techniques; DNA samples were prepared in multiplex according to standard TruSeq Sample Prep and Custom Enrichment protocols (Illumina), and 75 base pairs were sequenced in each direction (paired-end). Sequencing was performed on the Illumina HiScan SQ. Samples from affected and controls were run in two separate runs.

2.3. Sequence Analysis. Bioinformatic analysis consisted of a standard protocol including image analysis and base calling by Illumina RTA 1.12.4.2, demultiplexing by CASAVA 1.8 (Illumina), and alignment of sequence reads to the reference genome GRCh37/hg19 by BWA [23]. Picard (http://picard.sourceforge.net/) was used for removing PCR duplicates. The GATK (Genome Analysis Toolkit) was applied for base quality score recalibration, INDEL (insertion and deletion) realignment, and SNP (single nucleotide polymorphism) and INDEL discovery [24, 25]. Annotation of sequence variants was performed by Annovar [26]. Variants present in exons ±10 bp intron sequences and 3′- and 5′UTR (untranslated region) were included in further analysis.

2.4. Classification of Variants. Variants were classified into five pathogenicity classes (Table 2). Variants were classified based on frequency data from 1000 gen-omes (http://www.1000genomes.org/), dbSNP 135 (http://www.ncbi.nlm.nih.gov/projects/SNP/), 180 in-house normal controls, pathogenicity predictions through the Alamut interface v2.2-0 (Interactive Biosoftware, Rouen, France), and reports in HGMD, IPNMDB, and the literature [4, 6, 27]. Variants with prevalence ≥ 0.1% in dbSNP 135 or 1000 genomes and presence in ≥ 2 in-house normal controls were removed unless homozygous or compound heterozygous. Variants with frequency < 0.1% were considered possible pathogenic as the SNP databases may contain information from individuals with disease, especially traits with debut during life such as CMT. Data from the ESP (the exome sequencing project) (http://evs.gs.washington.edu/EVS/) was also used in classification but only as a guidance as this database contains data from both the selected affected and controls for specific traits. Synonymous, intronic, and UTR variants not predicted to have an effect on splice site were also removed. The remaining variants were defined as the candidate variants. Variants classified likely or certainly pathogenic in recessive genes had to be present in a homozygous or compound heterozygous state. Classification into these classes also required phenotype-genotype correlation with previously published literature, and/or segregation of the variant(s) within the affected in the families, and/or the possible dual (digenic) effect of two variants in different genes. Identified variants classified certain, likely, and uncertain pathogenic were submitted to the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/).

Table 1: Sequence capture performance results for the 51 neuropathy genes included in the panel.

Gene	GenBank accession and version number	Neuropathy phenotype	Average coverage	% Bases ≥ 2x coverage	% Bases ≥ 30x coverage
AARS	NM_001605.2	CMT2	642	100.0	100.0
ARHGEF10	NM_014629.2	Slow NCV	620	100.0	97.7
ATP7A	NM_000052.6	dHMN	295	100.0	94.4
BSCL2	NM_001122955.3	dHMN	845	100.0	100.0
CTDP1	NM_004715.4	CCFDN	488	100.0	87.7
DCTN1	NM_001135041.2	dHMN	972	100.0	99.8
DNM2	NM_001005361.2	CMT2 and ICMT	449	100.0	96.6
DYNC1H1	NM_001376.4	CMT2	662	100.0	99.3
EGR2	NM_000399.3	CMT1, DSN, and CMT4	1328	100.0	100.0
FAM134B	NM_001034850.2	HSAN	296	100.0	88.5
FGD4	NM_139241.2	CMT4	410	100.0	100.0
FIG4	NM_014845.5	CMT4	480	100.0	100.0
GAN	NM_022041.3	GAN	472	100.0	93.1
GARS	NM_002047.2	CMT2, dHMN	427	100.0	92.9
GDAP1	NM_001040875.2	CMT2, CMT4, and ICMT	591	100.0	100.0
GJB1	NM_000166.5	CMTX	513	100.0	98.0
HK1	NM_033500.2	CMT4	654	100.0	95.9
HSPB1	NM_001540.3	CMT2 and dHMN	429	100.0	80.4
HSPB3	NM_006308.2	dHMN	535	100.0	100.0
HSPB8	NM_014365.2	CMT2 and dHMN	536	100.0	96.9
IGHMBP2	NM_002180.2	dHMN	540	100.0	99.2
IKBKAP	NM_003640.3	HSAN	488	100.0	99.8
KIF1B	NM_015074.3	CMT2	579	99.9	98.9
LITAF	NM_001136472.1	CMT1	454	100.0	90.0
LMNA	NM_170708.3	CMT2	533	100.0	93.8
MED25	NM_030973.3	CMT2	476	100.0	84.4
MFN2	NM_001127660.1	CMT2	602	100.0	99.3
MPZ	NM_000530.6	CMT1, CMT2, ICMT, and DSN	417	100.0	82.2
MTMR2	NM_016156.5	CMT4	338	100.0	97.5
NDRG1	NM_001135242.1	CMT4	501	100.0	97.2
NEFL	NM_006158.4	CMT1 and CMT2	439	100.0	98.5
NGF	NM_002506.2	HSAN	475	100.0	87.8
NTRK1	NM_001012331.1	HSAN	528	100.0	85.8
PLEKHG5	NM_001042664.1	ICMT and dHMN	463	99.5	95.2
PMP22	NM_153322.2	CMT1, DSN, and HNPP	597	100.0	100.0
POLG	NM_001126131.1	CMT associated [21, 22]	485	100.0	95.5
PRPS1	NM_002764.3	CMTX	373	100.0	99.0
PRX	NM_181882.2	CMT4 and DSN	966	100.0	100.0
RAB7	NM_004637.5	CMT2	486	100.0	98.4
REEP1	NM_001164731.1	dHMN	476	100.0	95.5
SBF2	NM_030962.3	CMT4	443	100.0	97.4
SEPT9	NM_001113493.1	HNA	449	100.0	86.3
SETX	NM_015046.5	dHMN	566	100.0	99.4
SH3TC2	NM_024577.3	CMT4	506	100.0	100.0

Table 1: Continued.

Gene	GenBank accession and version number	Neuropathy phenotype	Average coverage	% Bases ≥ 2x coverage	% Bases ≥ 30x coverage
SLC12A6	NM_001042497.1	ACCPN	760	100.0	99.3
SOD1	NM_000454.4	CMT associated [19]	485	100.0	100.0
SOX10	NM_006941.3	PCWH	311	98.8	77.5
SPTLC1	NM_001281303.1	HSAN	449	100.0	95.7
TRPV4	NM_001177428.1	CMT2 and dHMN	454	100.0	100.0
WNK1	NM_014823.2	HSAN	883	100.0	99.1
YARS	NM_003680.3	ICMT	475	100.0	95.0

ACCPN = agenesis of the corpus callosum with peripheral neuropathy; CCFDN = cataract, congenital, with facial dysmorphism and neuropathy; CMT1 = demyelinating Charcot-Marie-Tooth disease with autosomal dominant inheritance; CMT2 = axonal Charcot-Marie-Tooth disease; CMT4 = demyelinating Charcot-Marie-Tooth disease with autosomal recessive inheritance; CMTX = Charcot-Marie-Tooth disease, X-linked inheritance; dHMN = distal hereditary motor neuronopathy; DSN = Dejerine-Sottas neuropathy; *GAN* = giant axonal neuropathy; HNPP = hereditary neuropathy with liability to pressure palsies; HSAN = hereditary sensory and autonomic neuropathies; ICMT = intermediate Charcot-Marie-Tooth disease; NCV = nerve conduction velocity; PCWH = peripheral demyelinating neuropathy, central dysmyelination.

Table 2: Classification of variants into five pathogenicity classes.

Pathogenicity class	Conclusion	Criteria
5	certainly pathogenic	(1) Reported pathogenic in at least two unrelated cases (2) and/or functional studies reveal effect on protein structure/function (3) and zygosity/inheritance of phenotype fits the variant (4) and phenotype-genotype correlation with previously published literature
4	likely pathogenic	(1) Reported pathogenic in one case (2) and/or predicted pathogenic in at least 2 of 4 variant prediction tools: SIFT [28], Polyphen [29], Align GVGD [30], and Mutation Taster [31] through the Alamut interface (3) and/or predicted loss or gain of splice site predicted in at least 4 of 5 splice site predictors: SpliceSiteFinder [32], MaxEntScan [33], NNSPLICE [34], GeneSplicer [35], and Human Splicing Finder [36] through the Alamut interface (4) and/or close proximity to known pathogenic mutations with similar or lower variant prediction score (5) and zygosity/inheritance of phenotype fits the variant (6) and phenotype-genotype correlation with previously published literature
3	uncertain pathogenic	(1) Present in ≤0.1% of dbSNP135 or 1000 genomes (2) and/or present in ≤1 in-house control (3) and zygosity/inheritance of phenotype in family fits the variant (4) Variants in class 2 may be lifted to this class if present in several affected patients with similar phenotype
2	unlikely pathogenic	(1) Present in 0.1–1% of dbSNP135 or 1000 genomes (2) and/or present in 2-3 in-house controls (3) and/or predicted no loss or gain of splice site predicted by 5/5 splice site predictors (applies only to synonymous variants and variants in introns and UTRs) (4) and/or reported benign in the literature
1	certainly not pathogenic	(1) Present in ≥1% of dbSNP135 or 1000 genomes (2) and/or present in ≥4 in-house controls

dbSNP = the single nucleotide polymorphism database.

2.5. Verification by Sanger Sequencing. Candidate variants were verified by Sanger sequencing in all available family members to establish genotype-phenotype correlation. Primer design and sequence analysis were performed in CLC Main Workbench (CLC bio, Aarhus, Denmark); the sequencing was carried out using standard procedures and sequenced on the ABI3130XL (Life Technologies Ltd., Paisley, UK) as previously described [2].

3. Results

3.1. Sequencing Performance Results, Variant Identification, and Verification. Analysis of sequence data revealed uniform coverage and high read depths in all samples. On average among the affected patients, the percentage of nucleotides with at least 30x and 2x coverage was 97.73% and 98.73%, respectively, and the mean coverage depth was

516. On average, 202 variants were detected among the 51 investigated genes per patient. Table 1 shows sequence capture performance results per gene and Table 3 shows sequence capture performance and variant identification results among the 70 affected analyzed by NGS. In the group defined as candidate variants, 63 nonsynonymous exonic variants, zero synonymous variants, and four nonexonic variants remained among the 70 patients. The candidate variants were sorted in the five classes: (5) certainly pathogenic—seven variants, (4) likely pathogenic—ten variants, (3) uncertain pathogenic—15 variants, (2) unlikely pathogenic—15 variants, and (1) certainly not pathogenic—20 variants. All candidate variants were verified by Sanger sequencing.

3.2. Prevalence of CMT Variants. The distribution of variants among the CMT families is illustrated in Figure 1. Table 4 shows phenotype-genotype correlations for certain and likely pathogenic variants and Supplemental Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/210401) shows genotype-phenotype correlations for all candidate variants in the 59 CMT families analysed by NGS.

NGS identified seven certain, 10 likely, and 15 uncertain pathogenic variants in 24 CMT families. One family was compound heterozygote for likely pathogenic variants in *POLG* (family 62) and six CMT families had possible dual pathology, that is, mutations in two different genes. Family 252 had one certain variant and one uncertain variant in *SH3TC2* and *AARS*, respectively. Family 95 had two likely pathogenic variants in *REEP1* and *SETX*. Three families had one likely and one uncertain pathogenic variant, *LMNA* and *DCTN1* in family 27, *LM NA* and *ARHGEF10* in family 54, and *DYNC1H1* and *GAN* in family 231. Family 11 had two uncertain pathogenic variants in *SEPT9* and *SETX*. Eleven of the certain, likely, and uncertain pathogenic variants were novel. Two families had mutations in previously sequenced genes, *GJB1* in family 5 and *MFN2* in family 90. These were not detected in the previous study due to mix-up of DNA of an affected and unaffected and due to an unknown laboratory mistake.

Of the total 81 CMT families, 37 CMT families had certain or likely pathogenic variants in 16 different genes (Table 4). Twelve CMT families had a CNV (11 families had the *PMP22* duplication and one family had a *MPZ* duplication) and 25 CMT families carried a point mutation. Figure 2 illustrates the gene frequencies among the CMT1 and CMT2 subgroups. Of the 38 CMT1 families, 55% (21/38) of the families had certain or likely identified genotypes; that is, 29% (11/38) had a CNV and 26% (10/38) had a point mutation. Thirty-six percent (12/33) of the CMT2 families had a certain or likely identified genotype. One of the two families with intermediate CMT had an identified genotype. Among the eight families with unknown neurophysiological phenotype, one family had *PMP22* duplication and two families had point mutations. Four families had likely pathogenic variants in non-CMT genes, *ARHGEF10*, *POLG*, *REEP1*, *SETX*, and *SOD1* [4, 6]. Forty-one percent (11/27) of the sporadic case families had certain or likely identified genotypes; that is, three families had *PMP22* duplication and eight families had point mutations.

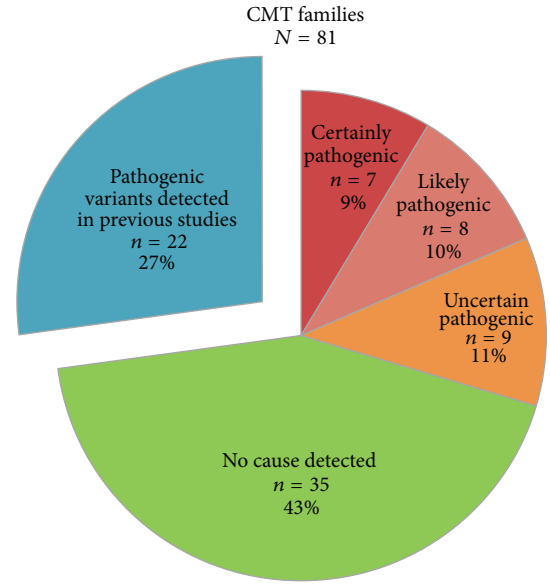

Figure 1: Identified variants in 81 Norwegian CMT families from the general population. Our previous studies identified copy-number variations in 12 CMT families and pathogenic point mutations in 10 CMT families [2, 14, 19]. The remaining 59 CMT families were investigated by next-generation sequencing.

4. Discussion

4.1. Main Findings. This is to our knowledge the first study to provide prevalence data for most of the currently known CMT genes in a population based sample by targeted NGS. The main result of our study is as follows. After extracting CMT families with the *PMP22* duplication, sequencing identifies certain and likely pathogenic point mutations in 36% (25/70) of the CMT families. The duplication of *PMP22* is the most common cause of CMT, found in 14% (11/81) of our families, whereas one family had a duplication of the *MPZ* [2, 14]. Large CNVs are not detected by our NGS-pipeline but require other methodologies, such as MLPA (multiplex ligation-dependent probe amplification). Thus, before NGS is applied, patients with CMT1 should be tested for the *PMP22* duplication. Other CNVs are considered rare [48]. The known CMT genes accounted for the majority of our identified mutations supporting a correct clinical diagnosis. However, phenotypically certain CMT families had certain and likely pathogenic variants in the non-CMT neuropathy genes, that is, *ARHGEF10*, *POLG*, *SETX*, and *SOD1*, thus expanding the number of known CMT genes. This highlights the importance of including all neuropathy genes in the NGS panel due to the genetic heterogeneity and pleiotropic genes of inherited neuropathies.

4.2. Study Population. Our material included 27 CMT families with only one affected; that is, the diagnostic certainty

Table 3: Sequence capture performance results and variant identification among 70 affected patients.

		Average	Standard deviation	Min	Max
Coverage	Coverage, all regions[1]	515.5	105.1	77.1	828.7
	% Base ≥ 2x coverage, all regions[1]	99.0	0.001	98.6	100.0
	% Base ≥ 30x coverage, all regions[1]	97.7	0.007	93.5	99.2
Variant identification	Variants in all regions[1]	202	18.2	163	241
	Variants in all regions[1] after filtering[2]	11	3.2	6	21
	Nonsynonymous variants in exons[2]	3	1.4	1	7
	Synonymous variants in exons[2]	3	1.0	2	6
	Variants in ± 10 bp intron, 3′UTR and 5′UTR variants[2]	5	2.7	2	14

[1] All regions = exons ± 10 bp intron sequence, 3′UTR and 5′UTR.
[2] Filtering against presence in ≥1% of dbSNP135 or 1000 genomes and presence in ≥4 in-house unrelated controls.
bp = base pair; UTR = untranslated region.

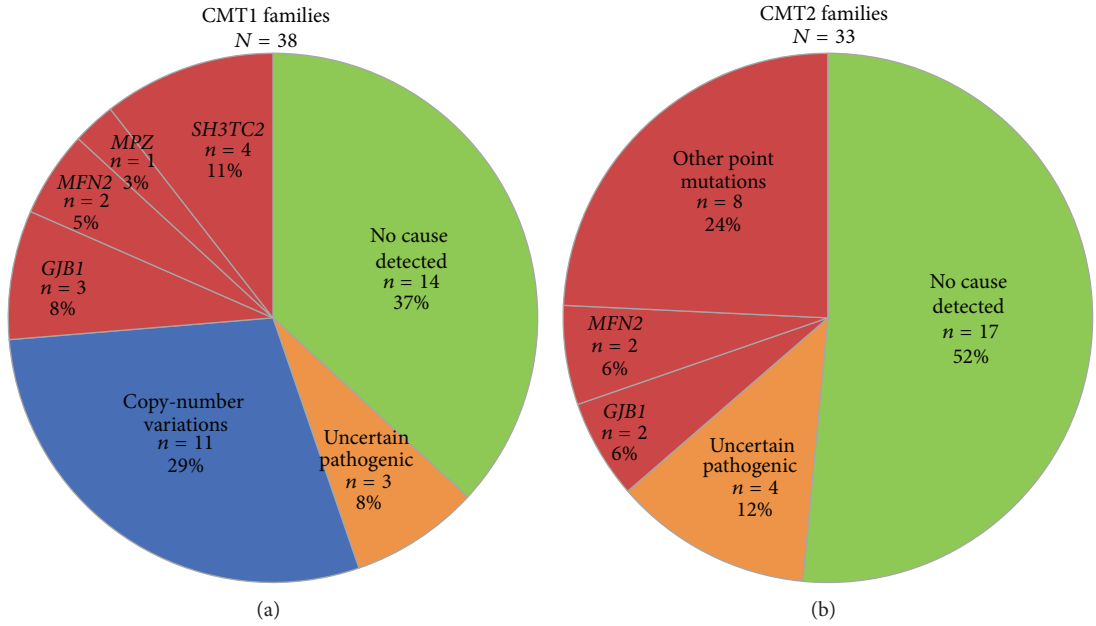

Figure 2: Frequencies of certain and likely pathogenic variants in CMT1 and CMT2 families from the Norwegian general population.

of the phenotype might be less than in CMT families with several affected. However, it would be incorrect to exclude sporadic cases, as CMT may be caused by autosomal recessive inheritance, reduced penetrance, and *de novo* mutation as well as nonpaternity. Autosomal recessive CMT accounts for about 4% of all cases in Europe, while it is considerably more frequent in countries with a high rate of consanguinity [7]. *De novo* duplication of *PMP22* may occur in about 10% of the patients [8]. We identified the *PMP22* duplication in three and a point mutation in eight of the total 27 sporadic CMT families. Thus, CMT variants were identified almost equally frequent in the sporadic and nonsporadic CMT families, that is, *PMP22* duplication 11% (3/27) versus 15% (8/54) and point mutations 30% (8/27) versus 35% (19/54), justifying the inclusion of the 27 sporadic CMT families in our material.

4.3. Methodological Considerations. Technically, the NGS panel demonstrated excellent results for coverage, read depth, and robustness for all genes in all 250 patients and controls, except one control with poor DNA quality. Lowering the possibility of technical errors is important in a clinical setting. NGS has several diagnostic advantages in heterogeneous diseases; that is, all known genes can be effectively sequenced and interpreted simultaneously. Furthermore, Sanger sequencing does not detect dual pathology, as sequencing is usually finalized when the first pathogenic variant is identified. This may be the reason why the literature rarely reports dual pathology, except from an American study which identified dual pathology in 1.4% of the CMT patients [9]. We identified possible dual pathology in 10% (6/59) of our CMT families. Thus, CMT dual pathology may not be

Table 4: The genotype-phenotype correlation in 81 Norwegian CMT families carrying certain or likely pathogenic variants.

Gene[1]	Nucleotide change	Protein change	Family ID	CMT type	Genotype-phenotype correlation
Certainly pathogenic					
GJB1	c.688C>T	p.Arg230Cys	5	CMT2	Family with axonal CMT and X-linked inheritance. Previously reported in [37].
HSPB1	c.380G>T	p.Arg127Leu	102	CMT2	Novel variant, highly conserved, predicted pathogenic. Classified certainly pathogenic as previously reported CMT families had a pathogenic variant in the same codon, causing p.Arg127Trp [6]. Present in an affected patient and his affected daughter, not in his unaffected daughter.
MFN2	c.310C>T	p.Arg104Trp	90	CMT1	Severely affected CMT1 patient with slightly decreased motor NCV, 36 m/s. Previously reported to cause early onset severe CMT2 by several [38].
SH3TC2	c.2860C>T	p.Arg954*[2]	142, 252, 285, 295	CMT1	Present as homozygous in four patients from four different families with demyelinating CMT. Reported to cause CMT1 in several populations [6]. Scoliosis at variable degree was found in all cases, which is often associated with mutations in this gene. Found as heterozygous in ten unaffected family members and in five in-house controls.
Likely pathogenic					
ARHGEF10	c.1013G>C	p.Arg338Thr	257	CMT2	Novel variant, highly conserved, predicted benign but extensive change in amino acid physiochemical properties. Sporadic case with CMT2 and decreased NCV. Close proximity to another heterozygous variant (Thr332Ile) associated with decreased NCV and thin myelination [39]. Functional studies show that the Thr332Ile mutant stimulates increased actomyosin contraction, regulating cell morphology in Schwann cells [40]. Classified likely pathogenic due to similar phenotype, NCV in the same range, and close proximity to the previous reported variant.
DNM2	c.1241A>G	p.Lys414Arg	9	CMT	Totally conserved, predicted pathogenic, situated in the dynamitin central domain. Sporadic case with unknown CMT. Not present in the unaffected daughter but in one in-house control and in one control in the ESP database; but considering the relatively high age of onset (85 years), it is uncertain whether these controls could develop neuropathy at higher age or whether the variant display reduced penetrance. Variants in *DNM2* cause both axonal CMT and intermediate CMT. Situated in the same domain as another variant associated with CMT [41].
DYNC1H1	c.1700G>A	p.Arg567His	231	CMT2	Novel variant, highly conserved situated in the dynein heavy chain, domain-1. Recently discovered as a CMT causing gene, reported to cause autosomal dominant CMT [42]. The previously reported variant (His306Arg) is situated in the same highly conserved domain as our variant and apart from some higher age of onset in our family the phenotypes correlate well. DNA was only available from one case in this family.

Table 4: Continued.

Gene[1]	Nucleotide change	Protein change	Family ID	CMT type	Genotype-phenotype correlation
KIF1B	c.881A>G	p.Lys294Arg	123	CMT2	Totally conserved, predicted pathogenic, situated in the kinesin motor domain, found among one individual in the ESP database (0.008%). Situated in the same highly conserved domain as a heterozygous variant (Gln98Leu) reported in another CMT2 family [43]. Researchers have been cautious about classifying *KIF1B* a CMT causing gene since only one family has been reported. As functional studies of the previously reported variant have confirmed loss of motor activity and variants in other motor proteins (*KIF1A*, *DYNC1HI*, and *DCTN1)* also are involved in neuropathy, we consider *KIF1B* a possible CMT causing gene and classify our variant likely pathogenic. DNA was only available from one case in this family.
LMNA	c.1930C>T	p.Arg644Cys	27 54	CMT CMT2	Totally conserved, predicted pathogenic. The pathogenicity of this variant has been questioned due to extreme phenotypic diversity including neuropathy and also low penetrance in affected families. But in support of its pathogenicity, found in 19 patients and not in 1000 controls (including our results), totally conserved, and studies of fibroblast carrying this variant show abnormalities of nuclear shape [44]. Found in the ESP database among 14 individuals (0.1%), but included in this database are also the affected carrying traits associated with *LMNA* mutations. Digenic inheritance with another variant, which is observed in three reported cases, may explain the phenotypic diversity and nonpenetrance [44]. Intriguingly, the CMT family with unknown neurophysiology carried a heterozygous variant in *DCTN1*, p.Arg651Trp and the sporadic CMT2 case carried a heterozygous variant in *ARHGEF10*, p.His733Tyr, both classified uncertain pathogenic.
POLG	c.1491G>C c.2243G>C	p.Gln497His p.Trp748Ser	62	CMT2	p.Gln49His: highly conserved, predicted pathogenic. p.Trp748Ser: highly conserved, predicted pathogenic in one tool. Situated in the same domain. Sporadic case with CMT2. These two variants are reported to cause severe ataxic neuropathy with additional features in two Norwegian patients as compound homozygous [45]. Additionally the p.Trp748Ser variant is reported to cause neurodegenerative disorders with ataxia in three patients and dHMN in five patients as compound heterozygote [22, 46]. A patient in our clinic with simular phenotype presented with the same two variants, not seen in in-house controls or in SNP databases.
REEP1 *SETX*	c.524A>G c.3075_3076insTGA	p.*175Trpext*55 p.Arg1026*	95	CMT2	REEP1: novel stop loss variant lengthening the protein by 55 residues. SETX: novel nonsense (stop codon) insertion at position 1026, shortening the protein by 1652 amino acids. Sporadic case with CMT2 and spasticity. Variants in *SETX* are associated with dHMN, a phenotype that overlaps with CMT2 and variants in REEP1 are associated with hereditary spastic paraplegia and in some cases dHMN [6, 47]. Thus, we assume digenic pathogenicity: the *SETX* variant causing CMT2 and the *REEP1* variant causing spasticity.

Table 4: Continued.

Gene[1]	Nucleotide change	Protein change	Family ID	CMT type	Genotype-phenotype correlation
Pathogenic variants identified among the same epidemiological population, prior to the NGS study [2, 14, 19]					
GJB1	c.187G>A	Val63Ile	118, 256	CMT1	
GJB1	225delG	Leu76Cysfs*8	83	ICMT	
GJB1	c.491G>A	Arg164Gln	44	CMT1	
GJB1	c.658C>T	Arg220*	398	CMT2	
MFN2	c.280C>T	Arg94Trp	8	CMT1	
MFN2	c.281G>A	Arg94Gln	38	CMT2	
MFN2	c.2119C>T	Arg707Trp	258	CMT2	
MPZ	c.161C>G	Ser54Cys	39	CMT1	
MPZ	Duplication		124	CMT1	
PMP22	Duplication		17, 51 56, 82, 136, 148, 155, 225, 309, 367	CMT1	
PMP22	Duplication		371	CMT	
SOD1	c.140A>G	His46Arg	1	CMT2	

[1] All relevant GenBank accession and version numbers are given in Table 1.
[2] Present as homozygous.
CMT = unspecified Charcot-Marie-Tooth; CMT1 = demyelinating Charcot-Marie-Tooth; CMT2 = axonal Charcot-Marie-Tooth; dHMN = distal hereditary neuronopathy; ESP = the exome sequencing project; ICMT = intermediate Charcot-Marie-Tooth; NCV = nerve conduction velocity.

as rare as the earlier literature implies. The digenic effect of two variants in different genes may modulate the phenotype, depending on whether the gene products work in the same pathways or not.

Another shortcoming with selective gene testing of a specific CMT phenotype is that unknown genotype-phenotype correlations are missed. An example is *MFN2,* usually tested only in CMT2 families; thus *MFN2* mutations in CMT1 or intermediate CMT families are missed [49, 50].

The diagnostic benefit of NGS targeted sequencing has been highlighted in other heterogeneous diseases such as cardiomyopathies and epilepsy [51, 52]. The technical quality of NGS targeted sequencing has previously been questioned in relation to clinical diagnostics, but increasing quality is now obtained of which two examples are the study on cardiomyopathies and ours indicating that NGS targeted sequencing is ready for clinical diagnosis [52].

Exome sequencing is another NGS approach, where every exon in the genome is sequenced. It has been frequently applied on rare Mendelian disorders as well as on some CMT patients [42, 53]. Targeted sequencing as compared to exome sequencing shows higher technical performance, increased capacity per run (192 versus 12 samples in our laboratory), easier data analysis, lower cost of data storage, fewer problems with incidental findings, and lower cost (approximately € 175 (500x coverage) versus € 1165 (70x coverage) in our laboratory). Furthermore, it is easier to adopt in small laboratories. However, exome sequencing can discover new disease genes, while targeted sequencing only can if the gene panel is expanded. In families with unknown CMT genotype exome sequencing could be beneficial as a next step towards a genetic diagnose.

Precise classification of variants with exclusion of non-pathogenic and inclusion of pathogenic variants is extremely important in a clinical setting. Stringent criteria were applied, in order to avoid misclassification. The analysis of the 179 controls secured that ethnically specific normal variants were excluded. Detailed clinical data and family history were necessary for matching genetic data with the phenotype. A limitation with the interpretation of novel variants detected in this study is that no functional tests have been performed, but currently this is rarely available as part of routine genetic testing and beyond the scope of our present study.

4.4. Genotype-Phenotype Correlations. Among the seven certain pathogenic variants, four families were homozygous for the *SH3TC2* Arg954* mutation, previously reported in several populations [6]. The prevalence of 5% (4/81) shows that *SH3TC2* should not be considered an unusual CMT gene in Northern Europe.

Eight CMT families had variants classified likely pathogenic. Phenotypically certain CMT families had pathogenic variants in the non-CMT neuropathy genes. *ARHGEF10* has been associated with slow NCV [39]. CMT2 and dHMN phenotypes have previously been reported for patients with *POLG* variants [21, 22]. *SETX* variants are associated with dHMN among other phenotypes, but this patient had a neurophysiological CMT2 phenotype. In a previous study on the same material, the affected in a large CMT2 family carried a certain pathogenic variant in SOD1, a gene usually associated with ALS [19]. The identification of certain and likely pathogenic variants in non-CMT genes and in CMT genes, regarded unusual, is especially important in the CMT2 families, where an accurate molecular diagnosis often has been lacking. It could also be speculated whether these genes might be more common than first thought but has been considered unusual due to lack of routine analysis.

Among the 15 uncertain pathogenic variants, 9 families had heterozygous variants in *GAN*, *MTMR2*, and *SH3TC2* genes usually associated with autosomal recessive inheritance but dominant inheritance has also been reported in a few cases often related to lighter phenotypes [6]. In our cases the variants were predicted pathogenic and the phenotype matched previous reports for these genes, except for one variant in *MTMR2*. Further analysis is required in order to establish whether the heterozygous state can cause a mild phenotype.

The identification of dual pathology is important to increase our knowledge of interplay between different gene variants. Dual pathology was observed in six of our CMT families. One sporadic case with CMT2 and spasticity had likely pathogenic variants in *SETX* and *REEP1*; we assume the *SETX* variant causes CMT2 and the *REEP1* variant causes spasticity. Thus, in this case we do not consider *REEP1* a CMT causing gene. The pathology of the *LMNA* variant observed in two CMT families has been questioned due to extreme phenotypic diversity and low penetrance in affected families [44]. It is speculated that the pathogenic effect of this variant might be due to the digenic inheritance with another variant [44]; intriguingly this was observed in both our cases with variants in *ARHGEF10* and *DCTN1*. *LMNA* and *ARHGEF10* are both involved in myelination and cell morphology [39, 54]. *LMNA* and *DCTN1* are situated in the same pathway, activation of the transcription factor *XBP1,* which has been associated with neuron differentiation [55].

4.5. Research in Context. A comparison of our results with the prevalence of identified CMT point mutations in four large clinic populations of affected individuals from Japan, Spain, United Kingdom, and USA is shown in Figure 3 [9, 10, 12, 13].

After exclusion of the *PMP22* duplication, point mutations were detected in 36% of our, Japanese, and British CMT1 patients, 66% of the American CMT1 patients, and 79% of the Spanish CMT1 patients [9, 10, 12, 13]. Point mutations in *GJB1* or *MPZ*, the two genes most frequently mutated in CMT1,were identified in 44% of the American patients, 60% of the Spanish patients, and only 14–22% of the patients in the other three studies. We identified a higher percentage of pathogenic CMT2 variants than the British and Japanese studies but lower than the American and Spanish studies [9, 10, 12, 13]. *GJB1*, *MPZ,* and *MFN2* variants, the most common causes of CMT2, accounted for 12% in our study and 18, 20, 28, and 34% in the British, Japanese, Spanish, and American studies. Apart from the *GJB1*, *MPZ,* and *MFN2* genes, variants were identified in 24% of our CMT2 families and in 35% of the Spanish patients but only in 1, 6, and

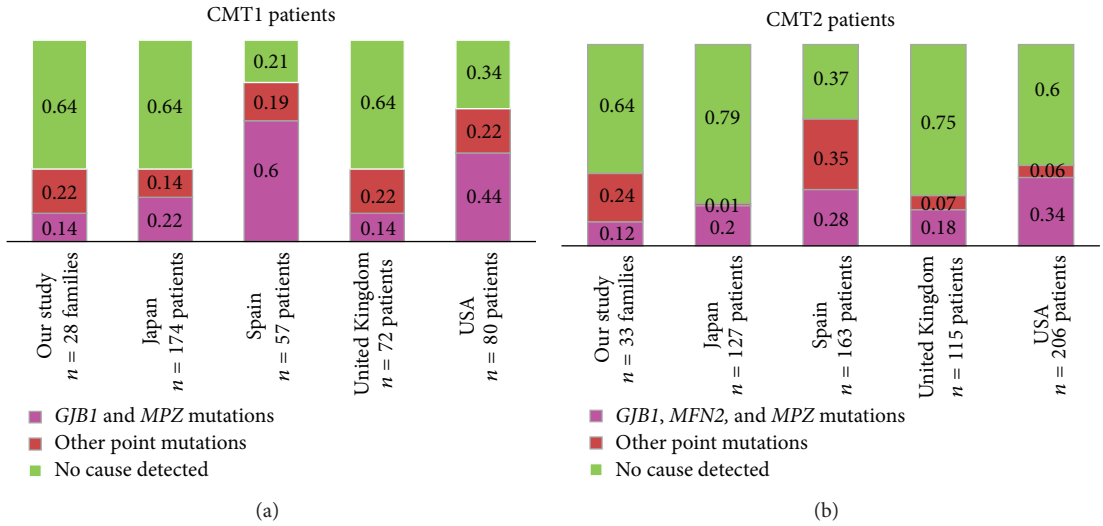

Figure 3: Frequencies of CMT1 and CMT2 point mutation in five studies [9, 10, 12, 13]. Numbers from our study include certainly and likely pathogenic variants, whereas the families with variants of uncertain pathogenicity have been included in the no cause detected group. Patients with intermediate forms of CMT were excluded in our study and the British and American studies but not in the Spanish and Japanese studies as intermediate forms were not differentiated; this may lead to a screw comparison. Numbers from the British study include only patients seen in their inherited neuropathy clinic and numbers from the American study include those with reported neurophysiology.

7% of the Japanese, American, and British CMT2 patients. High yield of identified "uncommon" CMT2 variants in our study and the Spanish study is most likely due to analysis of almost all CMT2 genes and the additional 19 neuropathy genes in our study. In the Spanish CMT2 patients, 26% had variants in *GDAP1,* also accounting for the high yield. In another study from northern Norway, CMT patients were analysed for the *PMP22* duplication and point mutations in seven genes (*EGR2*, *GJB1*, *LITAF*, *MPZ*, *MFN2*, *NEFL*, and *PMP22*), a genetic diagnose was established in only 17% of the patients [11]. These results together with ours indicate that other genotypes might be more common in Norway. At least part of the gene frequency differences is likely due to geographical differences, while different ascertainment might also affect the results. This emphasizes the difficulties of having a common sequential testing scheme for a rationale diagnosis of CMT but rather highlights the benefits of NGS targeted analysis.

Why do half of the CMT families lack a molecular diagnosis in our study? Several CMT genes are still to be identified, and there might be unidentified founder variants in the Norwegian population. After the analysis of these families, more than ten new genes have rapidly been identified mainly due to exome sequencing; these may count for a few unidentified cases [5, 6, 8]. Small tandem repeats, copy-number variations, mutations in regulatory elements distant from the gene, or cellular changes other than mutations in genomic DNA might be relevant in some cases. Dual pathology is easy to overlook. We also applied stringent criteria for the classification of variants; all heterozygous variants in autosomal recessive genes were classified as uncertain or unlikely pathogenic and variants with prevalence ≥ 0.1% in 1000 genomes or dbSNP135 were removed; thus some of these might be pathogenic. Clinical misclassification cannot be ruled out but it probably explains only a minority of the cases, since pathogenic variants were identified equally frequent in familial and sporadic cases.

5. Conclusion

Sequential testing scheme is useful for the *PMP22* duplication as an initial first step in CMT1; otherwise it is advantageous to start with NGS targeted sequencing.

The insight of pathological mechanisms caused by mutations in CMT genes has prompted promising reports of specific targeted treatments. Examples are treatment with HDAC6 inhibitors in *HSPB1* mutant mice, restoring axonal transport defects [16], and treatment with curcumin improving outcome of neuropathy in *MPZ* mutant mice [56]. Specific treatments require a precise genetic diagnose. The NGS technology has now become a robust and powerful tool with high technical quality, delivering increased diagnostic precision at a low cost. The NGS technology is likely to change clinical practice in complex diseases over the next years.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Helle Høyer, Geir J. Braathen, Camilla F. Skjelbred, and Michael B. Russell conceived and designed the study. Geir J. Braathen, Camilla F. Skjelbred, and Michael B. Russell supervised the study. Geir J. Braathen acquired the data. Geir

J. Braathen constructed the patient database and Marit Svendsen constructed the result database. Helle Høyer, Øystein L. Holla, and Linda Strand did the NGS sequencing. Øyvind L. Busk did the bioinformatic and statistical analysis. Helle Høyer and Øyvind L. Busk interpreted the NGS data. Helle Høyer and Hilde T. Hilmarsen did the Sanger sequencing and interpreted the data. Helle Høyer, Øyvind L. Busk, Geir J. Braathen, Camilla F. Skjelbred, and Michael B. Russell did the genotype-phenotype correlations. Helle Høyer and Michael B. Russell drafted the paper. Helle Høyer and Øyvind L. Busk prepared the figures and tables. All authors critically revised the paper and approved the final version.

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Research Article

Molecular Testing for Fragile X: Analysis of 5062 Tests from 1105 Fragile X Families—Performed in 12 Clinical Laboratories in Spain

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Fragile X syndrome is the most common inherited form of intellectual disability. Here we report on a study based on a collaborative registry, involving 12 Spanish centres, of molecular diagnostic tests in 1105 fragile X families comprising 5062 individuals, of whom, 1655 carried a full mutation or were mosaic, three cases had deletions, 1840 had a premutation, and 102 had intermediate alleles. Two patients with the full mutation also had Klinefelter syndrome. We have used this registry to assess the risk of expansion from parents to children. From mothers with premutation, the overall rate of allele expansion to full mutation is 52.5%, and we found that this rate is higher for male than female offspring (63.6% versus 45.6%; $P < 0.001$). Furthermore, in mothers with intermediate alleles (45–54 repeats), there were 10 cases of expansion to a premutation allele, and for the smallest premutation alleles (55–59 repeats), there was a 6.4% risk of expansion to a full mutation, with 56 repeats being the smallest allele that expanded to a full mutation allele in a single meiosis. Hence, in our series the risk for alleles of <59 repeats is somewhat higher than in other published series. These findings are important for genetic counselling.

1. Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability (ID) [1]. It is an X-linked dominant disease with incomplete penetrance, affecting approximately 1/3717 to 1/8918 Caucasian males [2]. Although rare, it is one of the most prevalent genetic disorders; this is the reason for its medical and social importance.

In affected males, the syndrome is characterised by moderate-to-severe mental retardation with behavioural disturbances such as hyperactivity and stereotypic hand flapping in addition to variable dysmorphic features such as large everted ears, elongated face, and postpubertal macroorchidism [1]. Affected females tend to have milder symptoms of FXS than males and they rarely show physical features.

The *FMR1* gene was identified in 1991, and FXS is now known to be caused by the anomalous expansion of a trinucleotide CGG repeat located in the 5′ end of this gene at the FRAXA locus in Xq27.3 [3]. The number of CGG repeats in the population has been classified into four groups depending on repeat size: normal (N), with 6 to 44 repeats; intermediate (IA)—also called "grey alleles"—with between 45 and 54 CGG repeats; premutation (PM) with between 55 and 200 repeats; and the so-called full mutation (FM) with over 200 repeats [4]. In the last group, a second mechanism is triggered, the hypermethylation of the adjacent CpG island, resulting in a shutdown of transcription and therefore lack of production of the FMRP protein, which is the underlying cause of the syndrome [5].

The term "premutation" was coined to reflect the fact that PM carriers do not generally have ID but that their alleles are usually unstable, resulting in an expansion of the CGG repeats when transmitted by a female and, hence, offsprings of female PM carriers are at risk of having FXS [3]. The *FMR1* PM affects both males and females and it seems that as many as 1/130–260 females and 1/250–810 males are carriers of a PM [6]. In recent years, it has been seen that IAs may or may not be unstable [7]. It has also been demonstrated that the risk of expansion is related to the number of CGG repeats, with smaller alleles being less likely to expand to a full mutation than larger ones [5, 8]. The smallest premutation that has been reported to expand to a full mutation allele in one generation had 59 CGG repeats [7].

It was initially thought that females with a PM were completely asymptomatic, but it was soon realized that this is not the case: in 1996, a family was described in which the women with a PM presented with clinical symptoms seemingly unrelated to ID: a premature ovarian failure leading to premature menopause [9]. Over the years these findings have been confirmed in numerous studies, all pointing to fragile X-associated primary ovarian insufficiency (FXPOI), as a phenotypic characteristic of PM carriers although only about 13–26% of them present with this trait [10]. Interestingly, full mutation carriers do not seem to present FXPOI. Furthermore, PM alleles are associated with a significant elevation of *FMR1* mRNA levels [11, 12] and it has been shown that carriers of the *FMR1* premutation are at risk of developing fragile X-associated tremor/ataxia syndrome (FXTAS), a late-onset neurodegenerative disorder usually affecting males over 50 years of age [13]. In short, fragile X (FX) is now understood to be a family of disorders including FXS, FXPOI, and FXTAS [1].

In Spain, the frequency of the FM alleles has been estimated to be approximately 1 in 2633 [14] and 1 in 2466 [15] in two different studies in male newborns, and the disease prevalence has been estimated to be 1 : 5000–1 : 6800 in males [16]. However, the real number of individuals with FM or PM remains unknown, not only in Spain, but also in other countries. Furthermore, clinical features are neither specific nor constant in carriers of the FM or the PM and, hence, the exact frequencies of all of these types of clinical involvement remain unknown in most of the populations studied.

With the overall aim of adding our knowledge to what is already known about this syndrome, we created a Fragile X Registry so that we could use it to improve diagnosis, prevention, and genetic counselling in these families. Thanks to the collaboration of 12 clinical laboratories with members of GIRMOGEN (a Spanish Network for the study of intellectual disabilities of genetic origin) that have received samples from patients all over Spain, we have collected clinical and molecular information from 19 years of diagnostic work on a large number of members of FX families. In this work, we present the first part of the statistical analysis of this data to provide new information to guide clinical practice, specifically concerning the clinical indications, molecular results, and transmission of the expansions for the purposes of genetic counselling. Despite the large series already published [7, 8, 14, 15, 17–19], this work is one of the few including individuals within FX families; this is the reason for the importance of this report.

2. Materials and Methods

2.1. Patients. Data from FX patients and their direct relatives were retrospectively collected from clinical laboratories at 12 diagnostic reference centres of several Spanish regions. Table 1 lists these centres and numbers of cases and families recruited. The total number of individuals studied includes all index cases (ICs) plus all their relatives, including prenatal cases. We considered an IC as the first individual in a family seeking genetic testing, the result of which indicated that he/she was a carrier of a FM (in general, probands with ID) or PM (patients with normal intelligence) (Table 2). A custom-designed program (see Section 2.3) perfectly identified the cases recorded in more than one laboratory, so that they could be taken into account to ascertain the exact number of different ICs. Each new positive PM or FM case not related to others was considered a new family meaning that the number of ICs is the same as the number of families. In total, we have registered 1105 ICs or families and a total of 5062 cases, ICs included, that is, a total of 3957 relatives. It is important to note that we have only included direct relatives at risk by pedigree, excluding all that were not directly related (spouses/partners of individuals with PM or FM, etc.). Some of these families have been included in previous studies [20,

Table 1: Participating laboratories/centres and their total number of individuals registered.

Laboratories/centres	Number of cases in each centre	Shared cases	Number of IC or families in each centre
Hospital Clínic (Barcelona)	1.623	116	405
Hospital V. Arrixaca (Murcia)	1.001	9	104
Hospital Universitario Cruces (Basque Country and Navarra)	744	24	125
Hospital La Fe (Valencia)	608	33	108
Hospital Ramón y Cajal (Madrid)	403	1	133
C. Sanitaria Parc Taulí (Sabadell-Catalonia)	225	117	97
Hospital Virgen Macarena (Sevilla)	184	29	78
Hospital Son Espases (Illes Balears)	149	12	35
Hospitales Miguel Servet and Lozano Blesa (Zaragoza)	101	3	32
Hospital regional de Málaga (Málaga and Sevilla)	82	27	21
IBGM, Universidad de Valladolid (Castilla-León)	76	4	35
F. Jiménez Díaz (Madrid)	54	1	27
Total number of individuals studied	5062[a]	378	1105[b]

[a]The total sum is 5250 cases, but subtracting the 188 shared cases yields a total of 5062 different cases.
[b]The total sum of families is 1200 families, of which 95 were shared, giving a total of 1105 different families.

21]. The registry was completed with individuals who underwent molecular genetic testing between 1991 and 2009 who provided informed consent for diagnostic testing.

2.2. Molecular Analysis. Molecular analysis of the *FMR1* CGG repeat region was performed in different laboratories following the same method, with an initial screen using PCR analysis of the CGG repeat to exclude males with a normal repeat or females showing two normal alleles, based on the protocol proposed by Fu et al. [22]. In recent years, some of the participating laboratories have used PCR amplification using fluorescent-labelled primers, analysing the size of the amplified fragments on a sequencer ABI310 (Applied Biosystems, Foster City, CA) [14] or PCR amplification followed by detection with nonradioactive methods [23]. Finally, to confirm suspected PMs, mosaic alterations, or FMs, all laboratories used analysis by Southern blot with the StB12.3 probe in DNA double digested with EcoRI + EagI [24].

2.3. Data Collection. We created a standardised registry program called ProGGen (an application developed on Lotus Notes) for which Excel files were sent to each collaborating laboratory along with detailed instructions on how to complete each file. The design was developed according to criteria established by clinicians, geneticists, and molecular geneticists, members of the GIRMOGEN network and involved in the genetic diagnosis of FX. Clinical and molecular information comprised the date of birth, year of sampling and study, cellular origin of DNA, sex, number and methylation status of CGG repeats, CGG repeats of the carrier mother/father, mental status, facial dysmorphic features, presence of macroorchidism (in males), and diagnoses of FXPOI or FXTAS. Once received, the completed files were imported into the aforementioned program, which detects and cleans duplicate cases and then carries out the data extraction, processing, and the statistical analysis with its own software.

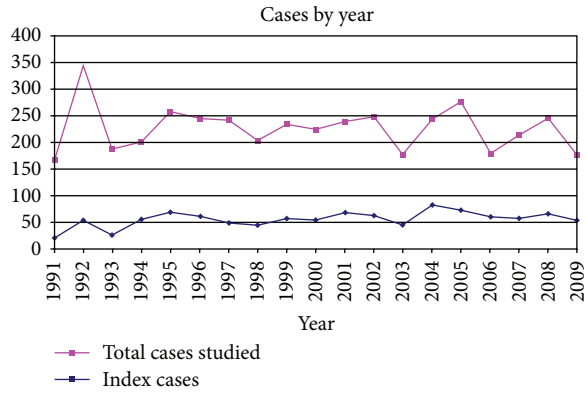

Figure 1: Distribution of cases studied by the year of diagnosis (1991–2009).

3. Results

In total, 1200 index cases or families were registered but 95 were shared by two or more laboratories, and hence there were in fact 1105 different families. The total number of individuals entered on the database was 5250, but subtracting 378 shared cases, the total number of different cases—ICs included—was 5062 (Table 1). This yields a total of 3957 females and males tested for each registered IC; that is, the average pedigree size was 3.58. In 268 families we were only able to study. or record, the IC and, at the other extreme, the largest family registered comprises 55 individuals, including the IC.

Figure 1 shows the distribution of the number of diagnoses per year. The mean number of ICs studied per year was

Table 2: Medical indication for referral.

	Number of males	Number of females	Total number
Patients with ID			
With family history of ID			
GDD, ID, and autism (all ages)	97	8	105
Studies in ID institutions	22	0	22
With no family history of ID			
Children with GDD and or autism	461	55	516
ID in young patients and adults	113	13	126
Patients with normal intelligence			
With family history of ID			
Relative of a patient with FXS diagnosed in another centre	30	112	142
Individual with history of ID with unknown aetiology in his/her family	2	16	18
With no family history of ID			
POI and/or menopause	0	17	17
FXTAS	10	3	13
Unknown reason to be studied	106	40	146
Total	841	264	1105

Table 3: Distribution of the 769 ICs with ID, by the age of the diagnosis of FXS.

Age of diagnosis	0–9	10–19	20–29	30–39	40–49	50–59	60+	Age unknown	Total
Number of males	333	125	43	26	7	5	4	150	693
Number of females	37	16	5	5	2	0	1	10	76
Total	370	141	48	31	9	5	5	160	769

54.89 and this figure appears to be stable over the years. A peak is observed in the total of individuals studied in 1992 because many of the FX families diagnosed before 1991 by cytogenetic analysis were restudied that year with molecular techniques. There were other smaller peaks in 2004-2005 when families began to be referred for testing because of FXTAS or FXPOI.

Table 2 lists the ICs as a function of the reasons for referral, separating those with ID, developmental problems, and/or autism from those with normal intelligence. As can be seen, 17 ICs were detected among women tested because they had ovarian failure and 13 (10 males and 3 females) were detected among patients with suspected FXTAS. The average age of the IC at diagnosis was 15.8 years (mean calculated with the 840 ICs in which the age at diagnosis was known), being this mean so high due to individuals seeking genetic testing with normal intelligence (Table 2). As for the subgroup of patients with ID, Table 3 classifies them by the age of diagnosis, in 10 year bands, showing that 60.75% of ICs (370/609) were less than 10 years old at diagnosis.

Table 4 shows the results of all the molecular tests performed in the 5062 cases (postnatal and prenatal). Of the total, 969 were FM males and 541 were FM females; 145 were mosaic (96 males and 49 females); 351 were PM males and 1487 PM females, with the remaining 102 cases having IAs. In addition, we have found three deletions, two in prenatal diagnosis (1 female and 1 male). Interestingly, three FM patients also carried a chromosomal abnormality: two males had Klinefelter syndrome and one female (previously reported [25]) had a mosaic Turner syndrome. It is also notable that two FM males were classified as normal from the point of view of intelligence; that is, they were high-functioning men. Finally, one male with a PM also carried a mutation in the FBN1 gene, causing Marfan syndrome.

Among the 271 prenatal diagnoses (Table 5) performed in carrier pregnancies (PM or FM), the mutated allele had been passed to the foetus in 147 cases (147/271 = 54.24%) and the normal allele in 124 (124/271 = 45.75%). The difference is not significant and thus there was no evidence of segregation distortion of the alleles. Similarly, we found an excess of males carrying the chromosome with the FX mutation but the difference was not significant (78/147 = 53.06% versus 68/147 = 46.25%), so there does not appear to be any sex ratio distortion among FX offsprings.

With all these molecular data, we wanted to analyse the instability of the CGG repeat alleles inside the families, and in order not to bias the analysis, we excluded ICs. Table 6 shows the risk of expansion for females: the results are expressed as the total number of sons and daughters with FMs and PMs in pedigrees, having a mother with PM or IA whose number of CGG repeats is known. These maternal repeat sizes are distributed from ten to ten repeats. In general, there is a growing likelihood of unstable transmissions with increasing repeat size but four points are important to emphasize in interpreting this table: (1) although the great majority of the alleles with more than 110 repeats expanded to a full mutation, the expansion risk was only 100% in two repeat ranges (140–149 and 160–169); (2) in all ranges, except in one, the risk

Table 4: Results of the 5,062 molecular diagnostic tests performed in the 1105 different families (ICs and prenatal cases included).

	FM	Mosaics	PM	IA	Deletions	Normal	Total
Number of males	969	96	351	42	2	692	2152
Number of females	541	49	1487	60	1	757	2895
Total	1510	145	1839[a]	102	3	1458[a]	5057[b]

[a]The sex was unknown in 1 premutation and in 9 normal cases. All were prenatal cases (see Table 5).

[b]In 5 cases, the molecular status was not recorded in the database.

Table 5: Molecular results of the prenatal studies performed in pregnant women carriers of an FM or PM.

	FM	Mosaics	PM	Deletions	Total fragile-X	Normal-X	Total
Number of males	61	1	15	1	78	64	142
Number of females	51	2	14	1	68	51	119
Total	112	3	30[a]	2	147[a]	124[a]	271[a]

[a]The sex was unknown in 1 premutation and in 9 normal cases.

of allelic expansion to FM is higher for male than female offspring (63.6% versus 45.6%; $P < 0.001$); (3) in the IA group, there were 10 cases of expansion to a PM allele; and (4) in the range of the smallest PM alleles (55–59 repeats), there was a 6.4% risk of expansion to a full mutation, with 56 repeats being the smallest allele that expanded to a FM.

Finally, Table 7 shows the instability in the paternal transmissions. It is interesting that there were more expansions than regressions (99 versus 40; $P < 0.001$) but for men with more than 140 repeats all daughters showed regressions of the paternal repeat size.

4. Discussion

In clinical practice, the established technique for FX diagnosis is testing for the CGG expansion in the *FM1* gene, what we call molecular genetic diagnosis. In line with this, in Spain all patients with suspected FX are referred for diagnosis to reference centres which use molecular techniques and the results are recorded in local databases that have been operating within these centres since the identification of the *FMR1* gene. In 2006, the 12 largest molecular laboratories (Table 1) decided to bring together the information contained in the individual databases creating a single national registry. The present study summarizes the compilation of molecular data of 5062 individuals from 1105 different FX families from 19 years of diagnostic work. Although FXS is one of the most prevalent genetic disorders, few analyses have been published on large series of individuals belonging to fragile X families, and hence the importance of our report.

From the point of view of clinical practice, we first want to take note of the reasons for referral that resulted in the diagnosis of a new FX family (Table 2). As we can see, 190 ICs (190/1105 = 17.2%) had normal intelligence and they were referred because they had a family history of ID, or because they had an ovarian failure or there was suspicion of FXTAS, and all were found to be carriers of a PM. The diagnosis of new FX families with these selection criteria confirms what has been recommended by the FX American Expert Working Group [26] in the sense that all these reasons for referrals are associated with a high rate of identification of affected individuals and carriers.

Considering the distribution by the age at diagnosis of the 609 ICs with ID for which this age was recorded (Table 3), we observe that only 370/609 (60.75%) were less than 10 years old and as many as 98 ICs were adults (98/609 = 16%). These data can be explained by the fact that in the early years, most studies were performed in adults from institutions for individuals with intellectual disabilities. Table 3 also shows that the IC was a female in 10% of the detected FMs (76 out of 769 cases), a figure similar to others published [17]. Figure 1 shows the distribution of the patients according to the year of diagnosis. Looking at the curve of the ICs, we can see that the number of diagnoses per year has been more or less stable with three small peaks: the first one in 1995, which corresponds to studies in institutions; the second one in 2001, which may correspond to a more widespread awareness of these tests among paediatricians; finally, a third peak in 2004, when tests were introduced for FXPOI and FXTAS. Overall, despite the fact that the number of tests carried out in our centres has increased considerably in recent years (data not shown), it seems that we have reached a ceiling in the detection of new families per year with an incidence of about 50 families. Returning to the cases of ID, we have found no changes in the age at diagnosis of FXS during the last 10 years of records and hence our data indicate that, despite all the information paediatricians and teachers have about FXS, the identification of new cases at younger ages continues to be a challenge [27].

In the event of a positive diagnosis (a new IC), an extension of the molecular study to relatives has always been proposed in Spain, following standard recommendations [4, 17, 26, 28] on cascade testing in the extended family. In total, 3957 females and males were tested (3.58/IC), including 271 prenatal diagnoses (Tables 4 and 5). Table 4 shows that there were more males identified with the full mutation and more females with the premutation as has been reported previously in families [5, 17, 20, 21] and this is attributable to the fact that the great majority of males tested had intellectual disability (Table 2), and their mothers were frequently carriers of

Table 6: Mutation expansion risks for carrier females of a PM or IA (including PN cases but not ICs).

Maternal repeat size	Male offspring			Female offspring			Total		
	Number of premutation	Number of full mutation	% full mutation	Number of PM	Number of. full mutation	% full mutation	Number of PM	Number of full mutation	% full mutation
44–49	0	0	0.0	0	0	0.0	0	0	0.0
50–54	4	0	0.0	6	0	0.0	10	0	0.0
55–59	11	0	0.0	33	3	8.3	44	3	6.4
60–69	22	7	24.1	60	11	15.5	82	18	18.0
70–79	26	24	48.0	53	30	36.1	79	54	40.6
80–89	18	31	63.3	36	30	45.5	54	61	53.0
90–99	2	21	91.3	12	28	70.0	14	49	77.8
100–109	2	22	91.7	5	22	81.5	7	44	86.3
110–119	1	7	87.5	0	15	100.0	1	22	95.7
120–129	0	10	100.0	2	7	77.8	2	17	89.5
130–139	0	13	100.0	1	12	92.3	1	25	96.2
140–149	0	3	100.0	0	5	100.0	0	8	100.0
150-159	0	3	100.0	1	2	66.7	1	5	83.3
160–169	0	5	100.0	0	7	100.0	0	12	100.0
170–199	1	6	85.7	0	3	100.0	1	9	90.0
Total	87	152[a]	63.6[a]	209	175[a]	45.6[a]	296	327	52.5

[a]Differences in the risk of expansion from PM to FM between males and females offsprings has statistical significance ($P < 0.001$).

the premutation. Furthermore, there is an excess of total females studied (2895 women versus 2152 males) indicating the importance given to the knowledge of the carrier status in females for reproductive purposes. In relation to mosaic cases, although some other research in families [5] indicated that male carriers with a full mutation have mosaic patterns more frequently than females, our results do not corroborate these data (96/1065 = 9% in males versus 49/590 = 8.3% in females).

Regarding the finding of cases of Klinefelter and Turner syndromes, it is interesting to recall that one of the advantages of the FX test is the ability to detect some sex chromosome abnormalities [8], in particular, with the observation either in PCR or Southern blot analysis of two X chromosomes in a male patient. In the large series published by Strom et al. [8] and by Youings et al. [29] not a single case was found of a male patient with both a sex chromosome aneuploidy and a PM or FM allele. They reported a frequency of Klinefelter syndrome of 1 : 702 [8] and 1 : 249 [29] among males studied for FXS. In our registry, 2 of the 969 male patients with FM also had Klinefelter syndrome; a rate intermediate between those found in the aforementioned studies but in accordance with the fact that Klinefelter Syndrome is, by far, the most common sex chromosome aneuploidy. Since the FXS is the most frequent genetic cause of ID, it should not be expected to be rare for the two syndromes to cooccur in a patient.

In relation to prenatal diagnosis, in general, our results corroborate those of previous large series [7, 18, 19] in the sense that there was no evidence of segregation distortion of the allele transmitted nor in the sex segregation. Although it seems there is an excess of male foetuses compared to female foetuses this difference was not statistically significant and is consistent with other reports [8]. We underline that it is important to bear the results in Table 5 in mind for genetic counselling, because the overall cases recorded (Table 4) correspond to a retrospective analysis of fragile X families that may suffer from ascertainment bias, while prenatal data are always considered prospective and not subject to this source of error [19].

Concerning the mutation expansion risks for females with IA or PM alleles (Table 6), our work also confirms previous studies [7, 8, 18, 19] in the sense that the instability of PM alleles increases with the size of alleles. It has been suggested that the lower expansion rate of the smaller PMs is due to the presence of an AGG sequence in the middle of the CGG repeats that creates an anchor protecting against expansion [7]. Since our molecular data were recorded with the size alone, we have no way of knowing which cases have AGG sequences. In any case, there are some differences with the previous studies that we want to highlight. First, in mothers with 50 to 54 repeats (IAs), they expanded to a PM allele in 10 cases and in the range of the smallest PM alleles, 6.4% of alleles of 55 to 59 repeats expanded in a single meiosis to a FM allele [30, 31]. Furthermore, for ICs (not included in Table 6), the smallest maternal allele observed to expand to a FM allele contained 56 repeats. Hence, in our series the risk for alleles of <59 repeats is somewhat higher than in other published series [8, 18, 19] and recommendations for prenatal testing must be established in that range. Furthermore, in our study, the alleles of up to 79 repeats expanded more frequently to PM than to FM alleles and the highest rate of full mutation expansion appeared in mothers with 90 repeats

Table 7: Paternal transmissions to their daughters.

Paternal repeat size	Number with the same number of repeats	Number with regression	Median of the repeats average difference	% regression	Number with expansion	Median of the repeats average expansion	% expansion
50–59	11	1	5.0	3.4	17	11.6	58.6
60–69	8	6	2.3	14.3	28	13.1	66.7
70–79	3	4	7.8	15.4	19	17.4	73.1
80–89	12	3	7.0	9.1	18	32.1	54.5
90–99	5	6	22.0	33.3	7	32.4	38.9
100–109	0	3	19.3	37.5	5	35.8	62.5
110–119	1	3	15.3	37.5	4	26.8	50.0
120–129	1	3	39.0	60.0	1	17.0	20.0
130–139	1	0	0	0.0	0	0	0.0
140–149	0	3	26.7	100.0	0	0	0.0
150-159	0	0	0	0.0	0	0	0.0
160–169	0	0	0	0.0	0	0	0.0
170–179	0	0	0	0.0	0	0	0.0
180–198	0	4	73.5	100.0	0	0	0.0
190–199	0	0	0	0.0	0	0	0.0
>200	0	4[a]	471.0	100.0	0	0	00.0
Total	42	40		22.1	99		54.7

[a]These 4 daughters had PMs, with their fathers being 2 high functioning males.

or more, whereas Nolin et al. [19] observed this higher rate for mothers with 80 repeats or more. We also observed that, in all ranges except one (110–119 repeats), the risk of expansion to a FM is higher for male than female offspring (63.6% versus 45.6%; $P < 0.001$), and that provides strong evidence that the transition from PM to FM is a postzygotic event; that is, it occurs after fertilization of the carrier oocyte [5]. These patterns are of vital importance for genetic counselling.

Evidence from other triplet-repeat disorders also points to postzygotic events that contribute to these differences in the repeat instability. They have also been observed in maternal transmissions of CAG repeats in Huntington disease, with a tendency for expansion in male offspring and contractions in female offspring [32]. In myotonic dystrophy although contractions of the CTG repeats are much less frequent than expansions, they are more frequently transmitted by males [33]. In our study, contractions also occur in maternal transmissions but only in a 2.6% of them, with no differences between male and female offsprings. By the contrary, it is interesting that, for carrier men, there were more transmissions with expansions than contractions (99 versus 40; $P < 0.001$) but, for men with more than 140 repeats, all daughters showed regressions in size—including four daughters with a PM whose fathers had >200 repeats—indicating once more that expansions are a postzygotic event. Thus, events occurring after fertilisation may play a role in determining repeat size in FX as it does in other expansion disorders [32], and these events may be heritable as we suggested in a previous work the possible existence of an intrafamilial effect [12].

5. Conclusions

In conclusion, our data fully validate the use of molecular genetic tests for fragile X in clinical practice. It also supports and completes previous studies, adding more evidence and additional data that may be useful for the purposes of genetic and reproductive counselling.

Conflict of Interests

None of the authors have reported any conflict of interests.

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Clinical Study

The Analysis of Genetic Aberrations in Children with Inherited Neurometabolic and Neurodevelopmental Disorders

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Inherited encephalopathies include a broad spectrum of heterogeneous disorders. To provide a correct diagnosis, an integrated approach including genetic testing is warranted. We report seven patients with difficult to diagnose inborn paediatric encephalopathies. The diagnosis could not be attained only by means of clinical and laboratory investigations and MRI. Additional genetic testing was required. Cytogenetics, PCR based tests, and array-based comparative genome hybridization were performed. In 4 patients with impaired language abilities we found the presence of microduplication in the region 16q23.1 affecting two dose-sensitive genes: *WWOX* (OMIM 605131) and *MAF* (OMIM 177075) (1 case), an interstitial deletion of the 17p11.2 region (2 patients further diagnosed as Smith-Magenis syndrome), and deletion encompassing first three exons of Myocyte Enhancer Factor gene *2MEF2C* (1 case). The two other cases represented progressing dystonia. Characteristic GAG deletion in *DYT1* consistently with the diagnosis of torsion dystonia was confirmed in 1 case. Last enrolled patient presented with clinical picture consistent with Krabbe disease confirmed by finding of two pathogenic variants of *GALC* gene and the absence of mutations in *PSAP*. The integrated diagnostic approach including genetic testing in selected examples of complicated hereditary diseases of the brain is largely discussed in this paper.

1. Introduction

Neurometabolic and neurodevelopmental disorders have complex behavioural and cognitive phenotypes and in some cases may cause diagnostic dilemmas. To provide a diagnosis of these challenging medical conditions the patient has to be subjected to an integrated set of clinical, imaging, and laboratory analyses [1–3]. Combining extensive clinical workup with neuroimaging and sophisticated biochemical testing is essential to reach an accurate diagnosis, but some cases still remain unresolved. The analysis of data from broad patient phenotyping, together with genetic testing results, is warranted to attain a diagnosis in difficult cases. Genetic testing provides the final confirmation of clinical suspicions or constitutes an essential addition to prior examinations performed to reach a clinical diagnosis [4, 5]. In the latter case, both normal and abnormal results of such analyses can be of potential value to the clinician. Sometimes, genetic testing

may provide completely new, important information about the complex human cognitive functions, including language development. A range of molecular genetic techniques, such as array comparative genomic hybridization, the identification of copy number variations, and in the future sequencing of genes, can support the clinical diagnosis and serve as a rich resource of the knowledge about disease mechanisms [4–6].

The aim of the current study was to analyse the genetic etiologies of congenital disorders of the central nervous system in children.

2. Patients and Methods

Seven patients from Polish families with inborn paediatric encephalopathies were retrospectively enrolled. The patients were categorized into 2 subgroups: first group (4 children) with nonprogressive neurodevelopmental disorders and the second group of 3 children with progressive neurological process. The depiction of specific entities only by means of clinical and biochemical analysis was difficult in all patients. Additional genetic testing was required in all cases either to fulfil the criteria of a defined genetic syndrome or to associate known phenotype with a given variant.

All patients underwent detailed examination using an assessment protocol based on a multidisciplinary approach and administered by a child neurologist experienced in developmental disorders. Clinical investigations included family history and medical records of the child's pre- and postnatal period. All children were subjected to an in-depth analysis of the developmental phenotype—motor patterns in terms of gross and fine motor skills and motor functions, social relationships, receptive and expressive language development, behavioural and emotional regulation (mood regulation, attention, sleep/arousal, and feeding behaviour), and cognitive development evaluation. All subjects have undergone detailed neurological examination and have been assessed by psychologists. The clinical characteristics of all the enrolled patients are presented in Table 1.

The developmental and intellectual characteristics of all the enrolled patients are presented in Table 2.

Routine haematology and biochemistry including glucose, ammonia, lactate, and thyroid function tests in plasma were performed. Neuroimaging tests including brain MRI and awake and asleep electroencephalogram were performed in every case. If the patient history and clinical signs, as well as MRI abnormalities, indicated a neurometabolic or neurodegenerative disease, a battery of specific neurometabolic and genetic tests was performed. Depending on the clinical picture, biochemical diagnostics included serum, cerebrospinal fluid and urine amino acids, acylcarnitines analyses in dried blood spot by tandem mass spectrometry (MS/MS), urine organic acids analysis by gas chromatography-mass spectroscopy (GC/MS), lysosomal enzymes activities, urinary oligosaccharides and glycosaminoglycans, plasma ceruloplasmin, biogenic amines metabolites in cerebrospinal fluid (CSF), and pterins in urine. The results of magnetic resonance imaging (MRI), electroencephalography (EEG), and metabolic testing are shown in Table 3.

Genomic DNA was extracted from patients' fresh blood samples. Molecular cytogenetic, PCR, and/or array-based comparative genome hybridization were performed in examined individuals. Array CGH was performed using 180 K microarray: 4 × 180 K from Agilent (Agilent 180 K, 021924). DNA digestion, labeling, and hybridization were performed according to the manufacturer's instructions. Scanned images were quantified using Agilent Feature Extraction software (v10.0). The customized web2py software was used for genomic copy-number analysis. All genomic coordinates are based on the NCBI36/hg18 reference genome.

For DYT1 mutation, DNA was extracted from whole blood following standard protocols. We used published primers, 6418 and 6419 [7], for PCR amplification across the critical region of the DYT1 gene. PCR products were resolved in a denaturing 6% polyacrylamide gel and visualized by silver staining.

Karyotype analysis was performed using peripheral blood lymphocyte cultures and a standard GTG banding method [8].

FISH analysis was performed in phytohemagglutinin-stimulated peripheral blood lymphocytes using standard procedures with LSI SMS (Vysis) probes specific for 17p11.2 Smith-Magenis syndrome critical region.

GCH1, GALC, and PSAP sequencing and electrophoresis experiments were done according to standard protocols and manufacturer's instructions on ABI Prism Genetic Analyzer 310 (Applied Biosystems).

The results of genetic analysis are presented in Table 4.

3. Discussion

Genetic testing is the optimal strategy in patients with complex, multisystem disorders in whom a disease cannot be identified despite exhaustive diagnostic efforts. Inherited alterations driving neurodevelopmental disorders are complex, including not only de novo mutations and common, low-risk polymorphisms but also high or moderate risk variants including copy number variation. In the current study we report 7 difficult clinical cases of congenital disorders of the brain diagnosed on the basis of integrative approach including extensive clinical and radiological examination, biochemical analysis, and genetic testing. Enrolled patient belonged to the two groups. First group included children with intellectual disability and the other group consisted of children with neurometabolic disorders. The clinical symptoms of those conditions most frequently appeared in the first years of life. Depending on the affected structure or function, various clinical pictures were present, being the starting point for further diagnostics. Basically routine clinical and laboratory investigations, MRI imaging of the central nervous system, and specialized metabolic testing did not yield a correct diagnosis.

Four patients (numbers 1–4) displayed speech and language difficulties of varying degrees. In patient number 1 both expressive and receptive language abilities were impaired to a greater extent than could be expected on the basis of child IQ. Interestingly, no pragmatic disorder has been observed

Table 1: The clinical characteristics of enrolled subjects.

Patient number, sex/age, family history (FH)	Course of pregnancy, perinatal period (PP)	First symptoms	Age at the time of diagnosis	Dysmorphic features	Clinical symptoms
Patient 1, female 18 yrs, FH-negative	Premature uterine contractions, PP-uneventful	2 yr develop mental delay	16 yr	yes	Dysmorphic features; convergent strabismus of the left eye, hirsutism; scoliosis; epilepsy—valproic acid resulted in a complete EEG normalization; simple stereotypic movements of the upper limbs
Patient 2, male 7 yrs, FH-negative	Premature uterine contractions, PP-uneventful	1 mo dysmorphic features	1.8 yr	yes	Dysmorphic features; neonatal oedema of the legs, cryptorchidism, brachydactyly; short stature; pronounced hypotonia; gastroesophageal reflux; severe sleep disturbances; severe autoagression, aggression, inadequate long temper tantrums, and stereotypic movements
Patient 3, female 6 yrs, FH-negative	Gestational diabetes, PP-uneventful	1 mo dysmorphic features	2 yr	yes	Dysmorphic features; poor suck, hypoacusia-cochlear implant, significant hypotonia, no sleep disturbances, cheerful, without aggression or autoagression
Patient 4, male 7.7 yrs, FH-negative	Mother's hyperthyroidism and toxoplasmosis, birth at 35 w., 2480 g 10 p. Apgar	2 mo abnormal movements	2.10 yr	yes	Dysmorphic features, vertical and horizontal nystagmus up to 5 mo, global hypotonia, ataxia, decomposition of the movement. Obligatory mirror movement of upper limbs up to 3–5 yrs, exaggerated startle for unexpected stimuli with head retraction and trunk retropulsion and tremor of stiff limbs; decreased pain sensation. Epilepsy improvement after lamotrigine therapy.
Patient 5, male 21 yrs, FH-negative	No foetal or perinatal problems	5 yr dystonia	8 yr	no	Motor deterioration, dystonia; 8 yr—only slight voluntary movements of the left upper limb, forced posture, increased muscle tone (rigidity), deep tendon reflexes symmetrical, bilateral dorsal hallux sign; treatment with l-dopa led to deterioration; improvement after bilateral deep brain stimulation of the internal pallidum
Patient 6, female 26 yrs, FH-negative	No foetal or perinatal problems	4 yr dystonia	13 yr	no	Since 4-5 years of age she began falling and her gait was compromised. Dystonia; remission after l-dopa therapy
Patient 7, male, died at 15 mo, FH-negative	No foetal or perinatal problems	Before 6 mo tetraparesis	13 mo	no	Motor deterioration; tetraparesis spastica

and child successfully communicated using gestures and onomatopoeia. Array CGH revealed presence of microduplication in the region 16q23.1 with the size ranging from 0.744 Mb to 0.827 Mb. The variant has never been described in the literature before. Its parental origin could not be fully traced either; however, copy-number variations in the 16q22-q24 region have been linked to autism and developmental delay phenotypes [9, 10]. The duplication found in Patient 1 affects two dose-sensitive genes: *WWOX* (OMIM 605131) and *MAF* (OMIM 177075). Human WW domain-containing oxidoreductase (WWOX, FOR, or WOX1) induces apoptosis, probably via the mitochondrial pathway and is a proapoptotic protein and a tumour suppressor [11]. In rats, it was found that WOX1 plays an essential role in the 1-methyl-4-phenyl-pyridinium induced neuronal death and is present in the condensed nuclei and damaged mitochondria of degenerative neurons in the striatum and cortex, ipsilaterally to intoxication [12]. Protein encoded by the *MAF* gene (v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (c-MAF)) is a DNA-binding leucine zipper-containing transcription factor [13, 14]. The expression of transcription factor c-Maf plays an important role in the development of

Table 2: The developmental and intellectual characteristics of enrolled patients.

Patient number	Development of gross motor skills	Development of fine motor skills	Speech and language development	Intellectual functioning	Autistic features
Patient 1	Abnormal motor coordination	Significant deficits in praxis and visual-motor coordination	Speech, especially active, not developed (single words or phrases, often the first syllable instead of the full word). Oral dyspraxia.	6 yr—nonverbal Leiter scale IQ-75; 11 yr and 13 yr—nonverbal scale of the WISC-R test IQ 52; 17 yr—nonverbal scale of the WISC-R test IQ 42	no
Patient 2	Gross motor function-clumsy, probably partly due to significant hypotonia	Fine motor skills-retarded: at age of 3 developed the pincer grasp	Active and passive speech delayed. At age of 3—a few sounds (not naming objects, not imitating any sounds or words), communication using gestures.	Mental retardation with autistic features; symbolic play absent.	yes
Patient 3	Walked alone at the age of 26 mo.	Abnormal	Deficits of speech connected with hypoacusia and defective articulation	Cognitive development at 2 yr corresponding with the chronological age; symbolic play was present. 5 yr—nonverbal Leiter scale IQ-99	no
Patient 4	Began to walk alone in the third year of life-shaky, wide-based stance and gait	"Jerky movements" with reduced complexity, variability, and fluency. Up to 5 yr of age no pincer grasp.	7 yr—total lack of spoken language, reacted to very simple commands.	ASD with profound mental retardation	Profound
Patient 5	Dystonia at 5 yr	Dystonia at 5 yr	Normal	Cognitive functions normal	no
Patient 6	Dystonia at 4 yr	Dystonia at 4 yr	Normal	Cognitive functions normal	no
Patient 7	Abnormal due to tetraparesis spastica	Abnormal due to tetraparesis spastica	Lack of development	Lack of development	no

interneurons of laminae III/IV in the dorsal horn of mouse spinal cord, which receive inputs from mechanoreceptive dorsal root ganglion neurons [14].

C-MAF-inducing protein (CMIP) is involved in the c-maf signalling pathway. CMIP interacts with filamin A (plays an important role in the formation of the dendritic spine) and the NF-kappaB subunit RelA (in a mouse model it plays a role in memory formation, cognition, and behaviour; NF-κB signalling pathway is altered in many chronic neurodegenerative diseases in humans). CMIP regulates nonword repetition performance and modulates phonological short-term memory commonly impaired in specific language impairment (SLI) [15]. *CMIP* was recently recognized as one of the important genes involved in the aetiology of specific language impairment [16]. It is difficult to attribute deep speech and language impairment, which dominates in the clinical picture of this patient, to the duplication of a specific gene, but one should consider the eventuality of the interaction of the c-maf signalling pathway and CMIP. In the course of clinical observation these patients decelerated overall mental development with age (especially deductive reasoning, generalization, anticipation, planning, and construction abilities). Initially, development dynamics were constant but then slowed down or maybe even deteriorated. This could be due to the dysfunction of WWOX, that is, its impact on apoptosis. The significance of this change in our patient is limited by the lack of paternal DNA and detailed clinical data about speech development in the father.

The language problems of patient number 2 were further blunted by impaired social development and joint attention. Speech comprehension in this child was relatively good. In patient number 3 speech difficulties were secondary to hearing impairment. Following the implantation of a cochlear implant the patient quickly improved verbal communication skills. In both patients genetic testing allowed to diagnose the Smith-Magenis syndrome (SMS), usually a sporadic disorder characterized by dysmorphic features, hypotonia, developmental delay, speech difficulties, sleep disturbance, and behavioural problems (hyperactivity, aggression, and autoagression) [17]. In both cases a deletion affecting the same genes: the human homologue of the Drosophila flightless-I gene (*FLII*); cytosolic serine hydroxymethyltransferase

Table 3: The results of MRI imaging, EEG, and metabolic testing in all enrolled subjects.

Patient number	Brain ultrasound	Brain MRI	EEG	Biochemical diagnostics tests abnormalities
Patient 1	Not performed	At the age of 5 yr T2-hyperintense changes of the periventricular white matter around the centre of the body of the lateral ventricles. 18 yr—normal	Generalized spikes and spike-and-wave discharges (2.5–5 Hz) as well as focal spikes	Not found
Patient 2	12 mo.—dilatation of the lateral ventricles (11 and 14 mm)	Not performed	Not performed	Hypogammaglobulinemia; mild elevation of alpha-fetoprotein (13 IU, reference value up to 5.5 IU).
Patient 3	Dilatation of the lateral ventricles	Dilatation of the lateral ventricles	Normal	Not found
Patient 4	Normal	10 mo abnormal-mild thinning of the corpus callosum and delayed white matter myelination	Ictal activity in the form of slow waves with sharp waves and spikes	Strong signal of 2-ketoglutaric acid in urine; elevation of alpha-fetoprotein (13 IU/normal up to 5.5 IU/).
Patient 5	Not performed	Normal	Normal	Not found
Patient 6	Not performed	Normal	Normal	Decreased concentrations of HVA and 5-HIAA in CSF. Pterine profile in urine—very low concentration of neopterin and biopterin, below the reference range: 0.16 mmol/mol creat (ref. range: 0.3–4.0) and 0.27 mmol/mol creat (ref. range: 0.5–3.0), respectively. Phenylalanine loading test in blood—Phe concentration and Phe to Tyr ratio significantly increased after 1 h, 2h, and 4 h.
Patient 7	Not performed	T2-hyperintense changes of the periventricular white matter and cerebellar white matter	Not performed	β-galactocerebrosidase—in two different samples of blood leukocytes revealed 2.1 and 2.0 nmol/mg protein/18 hr, respectively (reference values: 4–9.4 nmol/mg protein/18 hr; in patients with Krabbe disease below 2.0 nmol/mg protein/18 hr). In cultured skin fibroblasts: 3.7 nmol/mg protein/18 hr (reference values 3.9–15.2 nmol/mg protein/18 hr).

HVA: homovanillic acid (dopamine metabolite); 5-HIAA: 5-hydroxyindoleacetic acid (a serotonin metabolite); Phe: phenylalanine; Tyr: tyrosine.

Table 4: Results of genetic testing in all enrolled patients. All genomic coordinates are based on the NCBI36/hg18 reference genome.

Patient number	Cytogenetic/molecular tests	Detected variant (start–end hg18)	Size (Mb)	Inheritance
1	Array CGH	arr 16q23.1 (77,445,915–78,190,209) dup	0.744–0.827	Unknown
2	GTG karyotype	del(17)(p11.2)	—	De novo
3	Fluorescence in situ hybridization	ish del(17) (p11.2p11.2)	—	De novo
4	Array CGH	arr 5q14.3 (88,121,748–88,232,276) del	0.111–0.148	De novo
5	*DYT1* PCR	c.907_909delGAG	—	De novo
6	*GCH1* sequencing	Not found	—	—
7	*GALC* and *PSAP* sequencing	30 kb deletion encompassing exons 11–17 within the *GALC* gene; mutation in exon 11 (c.1186C>T, p.R396W)	—	—

(*SHMT1*); 21,23 the human homologue of Drosophila lethal 2 giant larva (*LLGL1*); and 18 topoisomerase IIIa (*TOP3A*), was found confirming the clinical diagnosis of SMS. Nevertheless, the phenotypic differences between both patients cannot be explained only on the basis of the performed genetic tests.

Patient number 4 previously described by Nowakowska et al. [18] suffered from very deep speech impairment. The lack of speech and language development was accompanied by deep mental retardation, autistic symptoms, and tremor. Moreover, this patient displayed an atypical movement pattern called mirror movement of the upper limbs, which persisted even in his second/third year of life. Mirror movements (m. m.) are involuntary movements executed by one side of the body during voluntary movements of the contralateral homologous body parts. This is a physiological phenomenon, which normally occurs at the early stage of development [19]. It is clearly visible in the first months of life, but in children older than 6 months it is no longer the dominant pattern and completely disappears by the 10th month. The persistence of this symptom up to 3–5 years can be explained by delayed/abnormal maturation of inhibitory processes in the brain. The overall movement decomposition and wide-based stance and gait were also noted pointing to developmental cerebellar dysfunction. Furthermore, an impact of retarded myelination on the boy's clinical picture cannot be excluded. Genetic testing of this patient revealed a deletion encompassing the first three exons of *MEF2C*. MEF-2 (Myocyte Enhancer Factor 2) proteins are transcription factors that belong to the MADS (MCM1, Agamous, Deficiens, and serum-response factor) box family of transcription factors. In mammals there are four isoforms MEF-2 A, B, C, and D encoded in four genes; the expression of which overlaps in developing muscle and neural cells during embryogenesis [20]. MEF2C is expressed preferentially in certain neuronal layers of the cortex and that expression declines during postnatal development [21]. MEF2C plays a crucial role in the homeostatic control of activity-dependent synaptogenesis [12]. It is an important process in the establishment of functional neuronal circuits during development and memory storage [22]. Barbosa and coworkers proved that the deletion of the MEF2C transcription factor in mouse brain impairs hippocampal-dependent learning and memory [22]. MEF2C plays an essential role in neurodevelopment by suppressing the number of excitatory synapses during activity-dependent refinement of synaptic connectivity and thus regulating basal and evoked synaptic transmission without affecting synapse structure [22]. A strong dominance of excitation over inhibition observed in the described patient could be related to this mechanism.

It is generally recognized that speech and language disorders tend to cluster in families; therefore, a genetic testing is always warranted. The first description of a family with severe speech and language impairments dates back to 1990 [23]. It was related to a mutation within the *FOXP2*, the first known gene associated with communication disorders. *FOXP2* is a member of the forkhead family of transcription factor genes and plays a key role in brain development [24]. A systematic genetic analysis of developmental speech disorders could contribute to defining specific phenotypes of language impairments and facilitate the diagnosis and treatment of such conditions. Genetic studies in this group of children may reveal molecular etiology of speech impairment in a single patient; moreover, the identification of genes linked to language phenotypes and further characterization of normal and aberrant functions of these genes can provide valuable insight into the biological foundations of complex symbolic form of communication that speech and language represent. On the other hand, genetic testing of a subset of genes associated with speech and language development can be screened in children with speech and language disorders in order to guide early and effective therapy.

The other examined group consisted of three patients with neurometabolic and movement disorders presenting as various neurological syndromes. The detailed analysis of those patients clearly shows that it is not possible to reach a correct diagnosis of neurometabolic or neurodegenerative disorder in children only on the basis of clinical features, neuroimaging, and electrophysiological examinations. Both detailed biochemical analyses of different body fluids, including CSF, accompanied with genetic testing are crucial to recognize a specific disease entity and to implement appropriate therapy.

Patient number 5 presented with a fast progression of dystonia beginning from the trunk muscles while cognitive functions remaining unimpaired. The normal results of urine organic acid analysis by GC/MS and a normal brain MRI allowed excluding organic acidurias (e.g., glutaric aciduria type I) and pantothenate kinase-associated neurodegeneration. The diagnosis of autosomal dominant GTP-cyclohydrolase I (GTPCH I) deficiency was excluded as the early impairment of trunk muscles and a serious deterioration following L-dopa treatment is unusual for those disorders. In this case, PCR-RFLP analysis revealed the presence of characteristic GAG deletion in the *DYT1* consistently with the diagnosis of torsion dystonia. The analysis of this case clearly shows that analysis of *DYT1* is necessary to confirm or exclude the diagnosis of torsion dystonia.

The clinical picture of patient number 6 pointed to l-dopa responsive dystonia without hyperphenylalaninemia, which may occur in autosomal dominant GTPCH I deficiency, sepiapterin reductase (SR) deficiency, or in tyrosine hydroxylase (TH) deficiency. The diagnosis of inherited biogenic amine metabolism disorders (BAD) is almost exclusively based on the quantitative determination of the metabolites in CSF [25].

The characteristic clinical symptoms like diurnal variation and dopa-responsive dystonia, analysis of biogenic amine metabolites in CSF, pterin profile in CSF/urine, and phenylalanine loading test are usually sufficient for diagnosis. Genetic tests are primarily required to distinguish between the two forms of GTPCH (autosomal dominant versus autosomal recessive form). This information is crucial for genetic counselling of the family.

One limitation of AD GTPCH I deficiency testing is that molecular analyses in the coding region cannot identify the mutations in this gene in approximately 40% of patients [26]. In some patients a large gene deletion or an intragenic duplication or inversion of the *GCH1* gene or abnormalities in noncoding regions of the gene can cause an enzyme

deficit. Such cases require the use of other methods, such in next generation sequencing order to recognize intragenic copy-number variation. As in patient number 6 molecular investigation did not detect a pathogenic mutation in the *GCH1* gene and genetic examination; without a prior analysis of biogenic amine and pterin metabolites in CSF, it would have failed to identify the cause of the defect.

In patient number 7 [27] clinical picture was consistent with Krabbe disease and borderline beta-galactocerebrosidase activity pointed to the impairment in a pathway of galactocerebroside degradation. The high residual activity of beta-galactocerebrosidase was not typical of infantile-onset Krabbe disease and suggested the deficiency of saposin A [28]. The clinical picture and prognosis in both defects does not differ much. The sequencing of the *GALC* gene revealed two pathogenic variants and the absence of mutations in the *PSAP* gene, thus confirming the diagnosis of globoid leukodystrophy [27]. Proper molecular diagnostics has enabled genetic counselling and family planning for this family.

4. Closing Remarks

The vast majority of neurometabolic and neurodegenerative diseases belongs to the group of rare diseases (frequency less than 1 : 2000 live births). These sporadic diseases are diagnosed with delay. The availability of new modalities of genetic testing like microarray high-resolution CGH have greatly contributed to the assessment of copy number variation and their role in diverse phenotypes. Moreover, whole genome association studies and exon sequencing in orphan diseases have revealed new candidate genes. In this work, we have taken advantage of array CGH to analyze genomic DNA in patients with the aim to identify potential molecular variants that could be associated with differential clinical outcomes. The proper paediatric and neurological examination together with neuroimaging and biochemistry supplemented with genetic testing was necessary to confirm or rule out the diagnosis. This study also highlights the advantage of an integrated approach to a patient with complicated hereditary disease to establish a proper and prompt diagnosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Experience of Preimplantation Genetic Diagnosis with HLA Matching at the University Hospital Virgen del Rocío in Spain: Technical and Clinical Overview

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Preimplantation genetic diagnosis (PGD) of genetic diseases, combined with HLA matching (PGD-HLA), is an option for couples at risk of transmitting a genetic disease to select unaffected embryos of an HLA tissue type compatible with that of an existing affected child. Here we present the results of our PGD-HLA program at the Department of Genetics, Reproduction and Fetal Medicine of the University Hospital Virgen del Rocío in Seville. Seven couples have participated in our program because of different indications. Overall, 26 cycles were performed, providing a total of 202 embryos. A conclusive molecular diagnosis and HLA-typing could be assured in 96% of the embryos. The percentage of transfers per cycle was 26.9% and the birth rate per cycle was 7.7% per transfer. Our PGD-HLA program resulted in the birth of 2 healthy babies, HLA-identical to their affected siblings, with successful subsequent haematopoietic stem cell (HSC) transplantations. Both HSC-transplanted children are currently doing well 48 and 21 months following transplantation, respectively. All the procedures, including HSCs umbilical cord transplantation, were performed in our hospital.

1. Introduction

The human leukocyte antigen (HLA) system is the name of the major histocompatibility complex (MHC) in humans. The superlocus resides on chromosome 6 and contains a large number of genes that encode cell-surface antigen-presenting proteins that, among several functions, play a major role in the immune system function in humans. Diversity of HLAs in the human population is one aspect of disease defense, and, as a result, the chance of two unrelated individuals with identical HLA molecules on all loci is very low. HLA genes have historically been identified as a result of the ability to successfully transplant organs between HLA-similar individuals. In other words, HLA complex is responsible for rejection following organ/tissue transplantation. Haematopoietic stem cell transplantation (HSCT) from an HLA-identical donor is the best therapeutic option for genetic diseases affecting the haematopoietic and/or immune system in children (e.g., β-thalassemia, Fanconi anaemia, etc.) and may be a therapeutic option for acquired diseases (e.g., leukaemia, acquired medullary aplasia, etc.) as well [1, 2]. The frequent unavailability of HLA-identical donors for affected children within the corresponding families or in HSC banks has made the combination of in vitro fertilization (IVF) with HLA-typing for the selection of HLA-identical embryos, a therapeutic approach for these affected children. Moreover, in case of the genetic cause of the disease affecting the children, preimplantation genetic diagnosis (PGD) combined with HLA-typing has emerged as a tool for couples to select unaffected embryos of an HLA tissue type identical to that of an existing affected child [3]. At delivery, HSC from the newborn umbilical cord blood can be used to treat the affected sibling. This approach, firstly applied for Fanconi anaemia by Verlinsky et al. in 2001, is valuable for life-threatening disorders that require

an HLA-compatible HSC donor, where HLA identity seems to provide the best chance of avoiding graft rejection and other serious complications of bone marrow transplantation. HLA-typing on one cell is complex because the HLA locus is highly polymorphic and large (4 Mb) and recombination within the locus has been observed [4, 5]. Worldwide, current HLA testing on preimplantation embryos is usually performed using STRs, since multiple STRs throughout the HLA region allow 100% accuracy HLA-typing and detect possible recombination events, as well as the copy number of chromosome 6 [6, 7]. The Centre for Medical Genetics UZ Brussel was the first to report a novel approach for HLA-typing using four evenly distributed informative STRs in multiplex PCR on single cells [6]. Since then, several methodological approaches have been reported with the aim of developing flexible and reliable methodologies for PGD-HLA molecular analyses. Nevertheless, to date a limited number of cases with successful pregnancies and births of healthy HLA compatible donors for patients have been reported [8–18]. Here we present the results of our program of preimplantation HLA-typing, alone or in combination with PGD.

2. Materials and Methods

2.1. Protocol for the Inclusion of Couples in Our PGD-HLA Program and Ethical Approval. Since 2007, a total of 12 couples have been attended in our department whose reason for consultation was the inclusion in our PGD-HLA-typing program. All of these couples had children affected by either a genetic or an acquired disease affecting their haematopoietic and/or immune system. During the first consultation, the couples provide a detailed medical report from the specialist (generally a hematologist) with an evaluation of the clinical status of the disease in their child, indicating if HSCT is either a consolidated or an experimental therapeutic option for such specific case. In addition, the application of the inclusion in our program must be accompanied by the justification of the lack of matched family donors and, if indicated, the unavailability of other nonrelated matched donors in our National Marrow Donor Registry (Registro de Donantes de Médula Ósea, REDMO). Express agreement of a Service specialized in HSCT must also be included in the documentation, warranting the transplantation of cells from the newborn umbilical cord blood to his affected sibling, in case of success of the PGD-HLA procedure. In addition, in case of a genetic disease, a clear and accurate genetic test report of the corresponding disease for the affected child must be also provided. Extensive genetic counselling and information about the PGD procedures, success rate and possibility of misdiagnosis inherent to techniques, are then given by our multidisciplinary team of geneticists, embryologists, and gynaecologists to the couple.

Informed consent concerning PGD and related procedures as well as the fate of the nontransferred embryos must be signed by the couples. Then, a basic test is prescribed to evaluate the reproductive state of the couples, which includes a hormone analysis and transvaginal ultrasound in the female, seminogram in the male, and serology for hepatitis B and C, HIV, and syphilis in both of them. The results of this test, together with all the documentation previously mentioned, is then sent to our Health Authority (Government of Andalucia) with the aim to obtain its authorization to conduct the PGD-HLA. A prior favorable ruling from our National Commission of Human Assisted Reproduction (Comisión Nacional de Reproducción Humana Asistida, CNRHA), which evaluates the social, therapeutic, and clinical characteristics of each case, is a requisite to obtain the final authorization.

2.2. Couples Treated for HLA-Typing Alone or in Combination with PGD. To date a total of seven out of the twelve couples have been authorized to be treated in our PGD-HLA program because of different indications (Table 1). One couple was dismissed; two couples are still pending of the final decision of our health authority, and for the other two couples all the documentation required for evaluation is being currently compiled.

Two out of the seven couples required exclusively HLA-typing to select HLA-matched embryos for their children, who were affected by acquired severe bone marrow aplasia and by a *de novo* mutation related to diamond-blackfan anemia (OMIM#105650), respectively. Regarding the remaining five couples, HLA-typing in combination with PGD was required, with β-thalassemia (OMIM#613985) as the indication for 4 of them and adenosine deaminase immunodeficiency (ADA, OMIM#102700) in another one.

2.3. Selection of Markers for the Genetic Analyses. Informativity testing for segregation analyses is always developed on the DNA samples from the corresponding family members (father, mother, and affected child) using standard PCR protocols, to identify the "disease haplotypes" and the specific HLA combinations carried by the affected children in the context of their corresponding families.

A first selection of up to 10 STRs was initially made according to their localization along the HLA locus. The policy is to select, for the subsequent PGD, the maximum number of informative STR markers evenly spaced throughout the HLA complex to obtain an accurate haplotyping, allowing identification of double recombination events, which if not detected may lead to misdiagnosis in HLA-typing. Using this panel, we achieved the first successful PGD with HLA-typing performed in Spain [16]. Subsequently, and following the ESHRE PGD guidelines [19], the method has been updated with the inclusion of a selection of another 10 markers along the HLA locus [16].

A panel of six polymorphic short tandem repeats (STRs) located in the neighbouring regions to the *β-globin* gene was selected to test the status for β-thalassemia [16]. Regarding the ADA, we selected 2 STRs surrounding the *ADA* gene (*D20S55* and *D20S16*) and other 2 intragenic STRs located in intron 3 and intron 8, respectively. In each case, the selection was based in the heterozygosity values (>30%) detected for each marker in a group of 30 normal controls, and in their specific location with respect to the genes responsible for

Table 1: Results of the HLA or PGD/HLA cycles performed in HUVR.

Couple	Mode of transmission	Theoretical probability of suitable embryos	Cycle	Oocytes retrieved	Mature oocytes injected	Fertilized oocytes	Analyzed embryos	Diagnosed embryos	Genetically suitable embryos*	Transferred embryos	Pregnancy/Clinical pregnancy
1 (β-THALASSEMIA)	Autosomal Recessive	3/16 (18.75%)	*1*	20	17	14	11	11	1	1	No
			2	25	22	18	17	17	2	1	Yes/Yes
			Overall	**45**	**39 (86.6%)**	**32 (82%)**	**28 (87.5%)**	**28 (100%)**	**3 (10.7%)**	**2**	
2 (β-THALASSEMIA)	Autosomal Recessive	3/16 (18.75%)	1	9	5	5	0	0	0	0	—
			2	9	2	2	2	2	0	0	—
			3	10	6	5	5	5	0	0	—
			Overall	**28**	**13 (46.4%)**	**12 (92.3%)**	**7 (58.33%)**	**7 (100%)**	**0 (0%)**	**0**	**—**
3 (ADA)	Autosomal Recessive	3/16 (18.75%)	*1*	29	23	12	9	9	0	0	—
			2	19	17	12	12	11	0	0	—
			3	23	20	9	9	9	0	0	—
			4	18	18	12	8	8	2	0	—
			5	17	14	11	10	9	0	0	—
			Overall	**106**	**92 (86.8%)**	**56 (60.87%)**	**48 (85.7%)**	**46 (95.83%)**	**2 (4.4%)**	**0**	
4 (β-THALASSEMIA)	Autosomal Recessive	3/16 (18.75%)	*1*	11	11	4	4	4	0	0	—
			2	19	13	7	7	7	0	0	—
			3	20	16	8	8	5	0	0	—
			4	29	22	12	11	11	1	1	No
			5	18	16	9	8	8	0	0	—
			6	14	11	7	7	6	1	1	Yes/No
			7	18	14	9	7	7	1	0	—
			8	29	15	7	6	6	0	0	—
			9	30	27	10	10	10	4	2	No
			Overall	**188**	**143 (76.0%)**	**73 (51.1%)**	**68 (93.2%)**	**64 (94.1%)**	**7 (10.9%)**	**4**	
5 (β-THALASSEMIA)	Autosomal Recessive	3/16 (18.75%)	1	8	4	3	3	3	0	0	—
			2	16	14	6	6	6	0	0	—
			Overall	**24**	**18 (75%)**	**9 (50%)**	**9 (100%)**	**9 (100%)**	**0 (0%)**	**0**	**—**
6 (BONE MARROW APLASIA)	Acquired	1/4 (25%)	1	29	28	14	14	13	3	2	No
			2	34	27	19	19	18	1	1	Yes/Yes
			Overall	**63**	**45 (71.4%)**	**33 (73.3%)**	**33 (100%)**	**31 (93.9%)**	**4 (12.9%)**	**3**	
7 (DIAMOND-BLACKFAN ANEMIA)	De novo	1/4 (25%)	1	12	9	4	4	4	0	0	—
			2	14	11	6	3	3	0	0	—
			3	15	12	4	2	2	0	0	—
			Overall	**41**	**32 (78.0%)**	**14 (43.8%)**	**9 (62.3%)**	**9 (100%)**	**0 (0%)**	**0**	

Cycles in which molecular diagnosis and HLA-typing were performed on 2 cells biopsied from each embryo, are in italic. For the remaining cycles, just 1 blastomere was biopsied per embryo.
*"Genetically suitable embryos" were those non-affected embryos that were HLA-identical to the affected patient within the context of each family.

the disease, warranting the possibility to detect any recombination event.

2.4. Assisted Reproductive Techniques and Embryo Biopsy. Controlled ovarian stimulation is performed through a long protocol as previously described [20]. Oocytes are carefully denuded from cumulus cells and intracytoplasmic sperm injection (ICSI) is used to prevent contamination with residual sperm adhered to the zona pellucida [19, 21]. Blastomere biopsy is performed on the morning of day three after fertilization. Laser technology (Octax Laser) is used to create an opening in the zona pellucida and one blastomere is gently aspirated for each embryo. Cells are transferred into thin-walled 0.2 mL PCR tubes containing 2.5 μL of lysis buffer and frozen at −80°C before cell lysis.

2.5. Multiple Displacement Amplification (MDA) on Single Cells. We adapted the protocol described by Kumar et al. in 2008 [22] to obtain whole genome amplification (WGA) of the blastomeres biopsied from the embryos resulting from the PGD-HLA cycles of the couple with the child affected by ADA. Optimal cell lysis protocol and MDA conditions were set up on single cells biopsied from supernumerary IVF embryos not suitable for transfer or cryopreservation. Efficiency of the MDA protocol on single cells was tested by measurement of absorbance, at $\lambda = 260$ nm, and the performance of different multiplex PCR protocols on the MDA products.

After blastomeres biopsy, cells are transferred into thin-walled 0.2 mL PCR tubes containing 2.5 μL of lysis buffer [600 mM NaOH, 10 mM EDTA, and 100 mM dithiothreitol (DTT)] and frozen during at least 30 minutes at −80°C before cell lysis. Cell lysis is carried out for 10 min at 65°C, followed by the addition of 1.5 μL neutralizing buffer (Tricine 200 mM, pH = 4.93). In addition, 4 μL sample buffer, 9 μL reaction buffer, and 1 μL enzyme mixture supplied with the Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare Life Sciences) are added to complete the reaction. The amplification is then carried out at 30°C for 4 h followed by heat inactivation at 65°C for 10 min. Subsequently absorbance of the MDA products at $\lambda = 260$ nm is measured, and proper dilutions are prepared to obtain aliquots at a final DNA concentration of 25 ng/μL.

2.6. Multiplex PCR Protocol on Either Single Cells Or MDA Products. A one-step multiplex single-cell fluorescent PCR is used for the simultaneous amplification of several combinations of markers at the HLA locus alone or in combination with the β-globin locus, using the QIAGEN Multiplex PCR kit (QIAGEN, GmbH; Hilden, Germany) and a protocol previously described [16, 23]. Primer sequences and PCR conditions for HLA-typing in combination with PGD for β-thalassemia have been previously described [16].

In the case of ADA, a multiplex fluorescent PCR is used for the amplification of a combination of markers linked to the *ADA* locus, in a separate reaction of that used for the HLA typing, using 25 ng of the MDA product in each case. Primers for specific amplification of the ADA-linked markers (available on request) were designed to have a melting temperature of around 55°C, so that the corresponding fragments could be successfully amplified with the same PCR program that the one used for HLA-typing [16].

The different multiplex PCR products are analyzed on an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA).

Prior to the analyses it was established that embryos showing monosomy, trisomy, or uniparental disomy of the chromosomes analyzed would be considered to be abnormal. The embryos with a recombination pattern at the HLA locus are considered to be HLA-nonidentical and therefore not suitable for transfer.

3. Results

3.1. Diagnosis and HLA-Typing of the Embryos. After analysis of the STR markers for β-globin/ADA and/or HLA haplotypes in the context of each family, a specific panel of markers was selected to be further used in the diagnosis/HLA-typing of the embryos resulting from the cycles. The selection of such STRs was made according to their amplification efficiency at the single-cell level, the informativity in the family, and their localization along the tested loci.

At the start of the PGD-HLA program, two cells were taken from each embryo in order to verify the results, but once we experienced that a conclusive and reliable diagnosis for the embryos could be obtained on the basis of one cell, we limited to one cell per embryo.

The percentage of cells with no amplification was 6.0%, which leads to a 3.9% of embryos being undiagnosed, based on the result of at least one cell with PCR amplification. Allele drop-out occurs when a sample is typed and one or more alleles (but not all) are not present, in contrast with what one could expect in the case of monosomy. By the previous segregation analysis in the context of each family, we know the specific combinations of markers associated or not to the disease, as well as the specific HLA-identical haplotypes. Moreover, we specifically select the informative markers to have the warranty to appropriately select embryos. In our experience reported here, we have not detected any ADO for the markers selected in each case. Contaminations were not detected either. Abnormal embryos with monosomies, trisomies, or uniparental disomy comprised 4.6% of cases. Taking into account exclusively the embryos with a conclusive diagnosis for HLA, the global percentage of HLA-identical embryos was 8.8%, and the percentage of HLA-identical unaffected embryos in case of PGD was 8.4% (Tables 1 and 2, and see supplementary table in Supplementary Material available online at http://dx.doi.org/10.1155/2014/560160). A total of 6 embryos showed recombination within the HLA locus (3.0%).

The remaining unaffected embryos resulting from all the cycles that did not achieve enough quality to be cryopreserved (46 embryos), as well as the 50 affected embryos, were retested for the corresponding markers in each case, and the initial results were confirmed in all of them. A

Table 2: Clinical data for preimplantational HLA typing at HUVR.

	HLA + PGD	HLA-only	Total
No of couples treated	5	2	7
Maternal age	31.0 ± 2.4	28.0 ± 2.8	30.1 ± 2.7
No of cycles performed	21	5	26
No of cycles performed per couple	4.2 ± 2.9	2.5 ± 0.7	3.7 ± 2.6
No of oocytes retrieved	391	104	495
No of oocytes retrieved per cycle	18.6 ± 7.0	16.3 ± 9.0	17.9 ± 7.6
No of mature oocytes submitted to ICSI	305	77	382
% of oocytes injected	78.0%	74.0%	77.2%
No of mature oocytes submitted to ICSI per cycle	14.6 ± 6.5	13.1 ± 9.3	14.2 ± 7.3
No of oocytes fertilized	182	47	229
% of oocytes fertilized	59.7%	61.0%	59.9%
No of oocytes fertilized per cycle	8.7 ± 3.9	7.3 ± 5.5	8.3 ± 4.4
No of embryos analyzed	160	42	202
% of embryos analyzed	87.9%	89.4%	88.2%
No of embryos analyzed per cycle	7.6 ± 3.8	6.2 ± 6.4	7.2 ± 4.6
No of transfers	5	2	7
% of transfers	23.8%	40%	26.9%
No of embryos transferred	6	3	9
No of pregnancies	2	1	3
No of clinical pregnancies	1	1	2
No of clinical pregnancies per cycle	4.8%	20%	7.7%
No of clinical pregnancies per transfer	20%	50%	28.6%
No of embryos implanted	2	1	3
Implantation rate	33.3%	33.3%	33.3%
No of pregnancies went to term	1	1	2
No of babies born	1	1	2
Live birth rate per cycle	4.8%	20%	7.7%

total of 42 unaffected and/or non HLA-identical embryos suitable to be cryopreserved were vitrified using the VitKit Freeze kit (Irvine Scientific) and the protocol provided by manufacturers.

3.2. Clinical Results. The results of the clinical HLA or PGD-HLA cycles for the seven couples are shown in Table 2. Comparison with the clinical results of other Centers is shown in Table 3. As indicated, a total of 26 cycles were performed, accounting for 5 cycles of exclusively HLA-typing (19.2%) and 21 cycles of PGD-HLA (80.8%). The percentage of mature oocytes suitable to be submitted to ICSI procedures was 77.2%. The fertilization rate, considering the correctly fertilized oocytes out of the total number of mature injected oocytes, was 59.9%. Finally, the overall number of embryos analysed per cycle (88.2% of the embryos) was very variable ranging from 8 to 30 and generally depending exclusively on the couple treated.

Overall 9 embryos were transferred in 7 out of the 26 cycles, which corresponds to a transfer rate of 26.9% (Table 2 and supplementary table).

Of note, in two of the seven couples, efforts resulted in respective pregnancies, with the birth at term of healthy children whose cord blood hematopoietic stem cells were obtained and frozen for a subsequent successful HSCT to their affected siblings. All the procedures, including HSCT, were performed at the University Hospital Virgen del Rocío (HUVR) in Seville. Taking into account these 2 cases, both the clinical pregnancy and live birth rates were 7.7% per cycle and 28.6% per transfer. One of those cases was the first successful case of PGD-HLA in Spain, previously published [16]. The second case corresponds to the couple with a son affected by acquired severe bone marrow aplasia. In this case two HLA-typing cycles were necessary to obtain a successful pregnancy, with the birth at term of a healthy girl. Cord blood HSC was obtained and frozen for later use. The stem cells number in the cord blood was high and HSCT was performed 3 months later. The child is currently doing well and off all treatments 21 months following transplantation.

In summary, 7 couples were treated in 26 cycles and 2 healthy HLA-matched babies were born, leading to a live birth rate of 28.6% per transfer and of 7.7% per initiated cycle.

4. Discussion

Verlinsky and collaborators described the first case of PGD-HLA-typing in 2001 [3]. A PGD for Fanconi anaemia in combination with HLA testing was performed to give birth

Table 3: Comparison of the clinical data for preimplantational HLA typing at different Centres.

	Reproductive Genetics Institute Chicago (Rechitsky et al., 2004) [7]	UZ Brussel (Van de Velde et al., 2009) [12]	Genoma (Van de Velde et al., 2009) [12]	Istanbul Memorial Hospital's (Kahraman et al., 2011) [9]	HUVR, Spain (this work)
No of couples treated	26	32	107	171	7
No of cycles performed	46	85	199	327	26
% of oocytes injected	NA	82.7%	76.8%	NA	77.2%
% of oocytes fertilized	NA	68.0%	88.5%	NA	59.9%
% of embryos analyzed	NA	40.3%	76.5%	NA	88.2%
% of embryos diagnosed	93.0%	99.1%	94.2%	92.0%	96.0%
No of transfers	33	27	138	NA	7
% of transfers	71.7%	31.8%	69.3%	64.8%	26.9%
No of embryos transferred	50	34	216	NA	9
No of clinical pregnancies	6	9	48	NA	2
% of clinical pregnancies per cycle	13.0%	10.6%	24.1%	NA	7.7%
% of clinical pregnancies per transfer	18.2%	33.3%	34.8%	34.9%	28.6%
Implantation rate	12.0%	32.4%	28.7%	26.3%	33.3%
No of pregnancies went to term	5	8	37	52	2
Live birth rate per cycle	10.9%	9.4%	18.6%	15.9%	7.7%

to an unaffected HLA matching sibling. The successful haematopoietic reconstitution in the affected child by HSCT from the HLA-matched offspring was described later [24]. After that, several successful HSC transplantations for genetic and acquired diseases have been reported [8–12, 16–18], representing one of the most relevant achieved challenges in reproductive medicine.

HLA-typing in combination with PGD is a practice allowed only in a few European countries and since 2006 also in Spain. The first Spanish law regulating assisted reproduction in Spain dates back to 1988 (Law 35/1988, of November 22nd). Given the experimental stage of PGD in such date, the regulation of the technique was left to future legislative interventions, but the absence of a specific regulation characterized the Spanish IVF regime until the 2006 Assisted Reproduction Act (Law 14/2006, of May 26th). This law did not establish a closed list of genetic conditions but framed PGD in broader terms in order to introduce a more flexible regulatory regime and to accommodate future technological advances and new genetic conditions without the need to modify the normative framework. The 2006 Act regulated PGD in very permissive terms, supporting the use of this technique not only to avoid the transmission of diseases for which no treatment existed, but also for the selection of embryos for HLA matching. In this specific regard (PGD-HLA matching), the law stipulates a number of conditions, including that cases have to be approved by the National Committee for Assisted Human Reproduction on a case-by-case basis after evaluating the clinical and therapeutic characteristics and weighing carefully the potential risks and benefits to all those involved. As a general rule, preimplantation genetic testing techniques are not paid by public healthcare, although IVF techniques are subsidized in the majority of the regional healthcare systems. In 2005, the Andalusia Regional Government authorized PGD to avoid the implantation of an embryo presenting a genetic profile related to a closed list of specific monogenic diseases (156/2005 decree, of June 28th). For these conditions, IVF and PGD became accessible through the public healthcare system through the HUVR in Seville, one of the leading centers for genetic-based research in Spain. Moreover, since 2006, PGD-HLA was also accessible through our hospital, making it the only public healthcare institution in Spain providing this service.

In our study, the percentage of mature oocytes submitted to ICSI was 77.2%, quite similar to those reported by other centers (Table 3). However, the rate of fertilization was some lower than in other institutions, although it was the same that the general fertilization rate obtained in our hospital for ICSI procedures with exclusively reproductive aims.

In general, the success rate in accurate genetic analyses is quite good, since 96% of the embryos got a correct HLA or PGD/HLA diagnosis (96.3% and 95.2% of the analyzed embryos for PGD/HLA and for HLA, resp.). Initially, 2 cells were biopsied from each embryo to perform the analyses (9 cycles accounting for a total of 88 embryos, Table 1). However, 2 important technical innovations allowed us to reduce the number of biopsied cells to 1 per embryo. The first one was the optimization of a one-step multiplex PCR-based method for HLA-typing and preimplantational genetic diagnosis of β-thalassemia. The advantage of such method is that it involves only a round of single PCR for multiple markers amplification (up to 10 markers within the HLA and 6 markers at the β-globin loci), leading to a current genotyping success rate of 100% [16]. The second one was the use of MDA as a tool for WGA of the cell, which let us obtain enough DNA quantity to perform a wide spectrum of independent genetic analyses and achieve an

accurate molecular diagnosis and HLA-typing, without ADO events among other advantages. Comparative studies suggest that MDA-based WGA procedures produce amplified DNA which is more suitable for a wide range of genetic analysis than DNA from PCR-based WGA methods [22]. This is due to the relatively unbiased amplification by φ29 DNA polymerase and the high molecular weight of the amplified DNA compared with PCR-based methods. In fact, MDA had been previously applied to either PGD or PGD-HLA-typing [25] giving satisfactory results, as in our case.

Also worth of note is that the percentage of transfers in our institution (26.9%) is lower than in other centers [7, 9, 12]. This rate is mainly due to the low number of HLA identical embryos obtained per cycle. As shown in Table 1, only 4 out of the 42 embryos for exclusively HLA-typing (9.5%) and 13 out of the 160 embryos for PGD/HLA (8.1%) were HLA-identical, in contrast with the theoretically expected rate of 25%. Moreover, 3 of the 7 couples (2 for PGD/HLA and 1 for HLA only) have not had any transfer yet, although a detailed inspection shows that in those particular cases the response to ovarian stimulation was not good, leading to low figures of oocytes retrieved, mature oocytes injected, fertilized oocytes, and analyzed embryos. This fact obviously affects the percentage of clinical pregnancies per initiated cycle, also relatively lower than in other institutions [7, 9, 12], although the clinical pregnancies per transfer is similar to those previously reported elsewhere.

Finally, although the live birth rate per cycle is slightly lower than other previously reported (7.7%), it is important to note that 2 out of the 7 couples (28.6%) resulted with successful pregnancies and deliveries of HLA-matched embryos. Moreover, HSCT, also performed in our hospital, was successful in both cases (100%).

5. Conclusions

The balance of our PGD/HLA program during this period is therefore quite satisfactory, and our results have constituted a relevant advance in the Spanish Public Health system, converting our institution into a referral centre for this therapeutic intervention in our country.

Conflict of Interests

The authors declare no financial relation with the trademarks mentioned in this paper, as well as no conflict of interests.

Authors' Contribution

Raquel María Fernández and Ana Peciña contributed equally.

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Research Article

A 3'UTR Polymorphism of IL-6R Is Associated with Chinese Pediatric Tuberculosis

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Background. IL-6 is a proinflammatory cytokine that plays a critical role in host defense against tuberculosis (TB). Genetic polymorphisms of IL-6 and its receptor IL-6R had been discussed in adult TB recently. However, their role in pediatric TB is still unclear. Due to the obvious differences in TB pathophysiology in children, which may also reflect differences in genetic background, further association studies in pediatric populations are needed. *Methods*. A case-control study was carried out in a Chinese pediatric population including 353 TB patients and 400 healthy controls. Tag-SNPs of IL-6 and IL-6R genes were selected by Haploview software, genotyped using MassArray, and analyzed statistically. *Results*. One polymorphism, rs2229238, in the 3'UTR region of IL-6R was observed to be associated with increased resistance to TB (adjusted $P = 0.03$). The rs2229238 T allele contributed to a reduced risk to TB in recessive heritable model (OR, 0.53; 95% CI, 0.35–0.78). *Conclusions*. By tag-SNP genotyping based case-control study, we identified a genetic polymorphism in the IL-6R 3'UTR that regulates host resistance to pediatric TB in a Chinese population.

1. Introduction

Caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) infection, tuberculosis (TB) remains to be a major global public health concern. In China, the prevalence of TB is 1.08‰ [1] in adults and 0.918‰ [2] in children. Host genetic factors play an essential role in determining TB susceptibility or resistance [3]. Compared with adults, children present a special risk group for TB due to rapid progression, significant morbidity, and mortality [4–6]; thus, the genetic background of pediatric TB might be quite different from adult TB. While most of association studies have been focused on TB in adults, childhood TB has been relatively neglected. Studies of TB genetics in well-defined pediatric populations are therefore needed.

As a major proinflammatory cytokine, Interleukin-6 (IL-6) takes part in the protection from pathogens infection. By binding to Interleukin-6 receptor (IL-6R), IL-6 triggers the intracellular signaling cascade that leads to inflammatory responses [7]. Elevated IL-6 from bronchoalveolar lavage cells seemed to be biomarkers of noncavitary TB [8]. An association study of genetic polymorphisms of IL-6 and its receptor IL-6R had recently been conducted in a group of Chinese population, suggesting a promoter polymorphism in IL-6 associated with adult TB [9]. However, the potential associations and molecular roles of IL-6 and IL-6R in regulating susceptibility or resistance to pediatric TB are still undiscovered. As childhood TB seems to have more genetic predisposition [5], we thus tried to discuss the associations of IL-6 and IL-6R with Chinese pediatric TB by single polymorphism genotyping (SNP) based case-control study.

2. Materials and Methods

2.1. Ethics Statement. Clinical investigation had been conducted according to the principles expressed in the Declaration of Helsinki. This research has been approved by the Ethics Committee of Beijing Children's Hospital. Written

informed consent was obtained from all the participants or their guardians in this research.

2.2. Study Sample. All the participants involved in this research were Han ethnicity. The pediatric TB patients (n = 353) were newly diagnosed to be pulmonary TB (PTB, pathological changes limited to lung) or extrapulmonary TB (EPTB, pathological changes involving other tissues) by at least two experienced pediatricians in Beijing Children's Hospital according to the pediatric TB clinical diagnosis standard [10–13]. The diagnostic criteria of pediatric TB had been described in our previous paper [14].

Participants of the control group (n = 400) were recruited among those admitted to Beijing Children's Hospital for physical examination. All of them had negative tuberculin PPD skin-test results (<5 mm) and no history of TB or HIV infection and were matched with TB cases for age, sex, and ethnicity.

2.3. DNA Extraction and Genotyping. Tag-SNPs of IL-6 and IL-6R were selected following data release from Phase II of the International HapMap project [15]. Sample based genotypes were downloaded for all variants in genomic regions including from 5,000 bp 5-prime upstream to 5000 bp 3-prime downstream of IL-6 and IL-6R independently.

Since the study populations under investigation were from the Chinese population, downloaded genotypes were restricted to those for the Han Chinese in Beijing, China (CHB) population (http://hapmap.ncbi.nlm.nih.gov). Tag-SNPs were selected using a pairwise tagging algorithm by Haploview software (available at http://www.broadinstitute.org/haploview), with a correlation coefficient (r2) exceeding 0.8 for all downloaded SNPs with minor allele frequency (MAF) >5% [16]. Because the tag-SNP probabilities were discrete, accordingly, functional ranking of tag-SNPs with the same probability was used.

Blood samples from all participants were collected and stored at −20°C. Genomic DNA was extracted from peripheral leukocytes by using a Genomic DNA Extraction kit (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). MassArray (Sequenom, USA) was used for genotyping selected tag-SNPs (Gabriel et al., 2009), and this assay was accomplished by Bio Miao Biological Technology (Beijing, China). The primers were designed using iPLEX GOLD (Sequenom, USA) [17].

2.4. Statistical Analysis. Statistical analysis was carried out using the Statistical Package for SNP Stats software (http://bioinfo.iconcologia.net/snpstats/start.htm) and PLINK software (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/). The Hardy-Weinberg equilibrium (HWE) was performed using the SHEsis program (http://analysis.bio-x.cn/myAnalysis.php). Chi-square tests were used for unordered categorical variable. Significant differences were indicated by a P value <0.05. Bonferroni correction for multiple testing was used. Adjusted odds ratio (AOR) and 95% confidence interval (CI) were calculated by logistic regression analysis.

3. Results

3.1. Patients and Controls. The mean age was SD, 4.7; range, 2 months–16.5 years for TB patients and 6.1 years (SD, 3.8; range, 3 months–17 years) for the non-TB control subjects. TB cases in our research include 156 (44.2%) PTB patients and 197 (55.8%) EPTB patients. Here, we also defined severe TB (SeTB) to be patients with disseminated TB (DTB) and tuberculosis meningitis (TBM), which both belonged to EPTB but presented severe clinical manifestations and usually poor outcomes. SeTB was identified in 81.2% of EPTB cases (160/197). Detailed characteristics of study population are shown in Table 1.

3.2. Selected Tag-SNPs. To conduct the association study, gene polymorphisms were selected using the criteria mentioned above in Section 2. Thus, two IL-6 tag-SNPs and ten IL-6R tag-SNPs were finally selected for genotyping. Of the 2 selected tag-SNPs of IL-6, one existed in the near 5′ of IL-6 (rs17147230) and another in the IL-6 promoter region (rs1800796). The rs1800796 SNP is located in the IL-6 promoter and has been thought to be associated with adult TB in Chinese population recently [9]. The tag-SNP rs17147230 was thought to be functionally associated with plasma adrenomedullin levels by one research group [18]. Meanwhile, 10 tag-SNPs of IL-6R were included in our genotyping. Of the 10 tag selected SNPs of IL-6R, one (rs3887104) is located in the promoter region, one (rs4845617) in the 5-prime untranslated region (5′UTR), three (rs7411976, rs4845618, rs4845626) in the intron region, two (rs2228145, rs8192284) present to be missense SNPs in coding region and other three (rs2229238, rs4072391, rs3828078) in 3-prime untranslated region (3′UTR).

3.3. Genotyping and Genetic Analysis. Total selected 12 single-nucleotide polymorphisms (SNPs) were genotyped. One SNP (rs8192284) data was wiped off for nonspecific amplification, according to the clustering performance. The rs4072391 SNP was not in Hardy-Weinberg equilibrium (HWE, P = 0.013) in the control group and thus was ruled out for further analysis. The rest 10 tag-SNPs were in Hardy-Weinberg equilibrium (HWE, $P > 0.05$) in the control group, which were sent to further analysis. The genotyping results of detected SNPs are summarized in Table 2.

Genetic association of rs2229238, an IL-6R 3′UTR SNP, with TB disease was observed after an application of the Bonferroni correction for multiple testing. The frequency of T allele in TB group was significantly lower than that in control group (OR: 0.57, 95% CI: 0.39–0.83, Bonferroni P = 0.033). Genotypic distribution of rs2229238 also revealed significant difference between TB group and control group (Bonferroni P = 0.05). Further, a $2 \times 2\chi^2$ test was used by combining different genotype combinations to test dominant (TC + TT versus CC) and recessive (TT versus CC + TC) models of inheritance. The OR for the T allele of rs2229238 as a possible risk factor was 0.53 (95% CI: 0.35–0.78, P = 0.001) under a dominant model and 1.13 (95% CI: 0.16–8.07, P = 0.9) under a recessive model (Table 3(a)). Thus, a Mendelian dominant trait of T allele was accepted for the inheritance pattern.

Table 1: Demographic characteristics of study population.

Characteristic	TB (n = 353)	Control (n = 400)	P
Gender			
Male, n (%)	223 (63.2)	236 (59.0)	0.385[a]
Female, n (%)	130 (36.8)	164 (41.0)	
Age			
Mean year (SD)	5.7 (4.7)	6.1 (3.8)	0.144[a]
TB type			
PTB (%)	156 (44.2)		
EPTB (%)	197 (55.8)		
DTB	71		
TBM	90		
Abdominal TB	21		
TBL	12		
Bone or joint TB	2		
Other EPTB	1		

TB: tuberculosis; PTB: pulmonary tuberculosis; EPTB: extrapulmonary TB; DTB: disseminated TB; TBM: tuberculous meningitis; TBL: tuberculous lymphadenitis. [a]P value was calculated by t test.

To further examine associations of the rs2229238 polymorphism genotypes with different clinical forms of TB, we compared PTB and EPTB subgroups with control group independently (Table 3(b)). Both the frequencies of rs2229238 T allele and rs2229238 TC + TT combined genotypes decrease progressively from controls to PTBs, then to EPTBs. Significant differences of rs2229238 allele were found between EPTB and controls ($P = 0.003$), but not between PTB and controls ($P = 0.109$). The frequency of TC + TT genotypes (T allele carrying) was significantly lower in EPTB patients than that in controls (OR: 0.44, 95% CI: 0.26–0.74, $P = 0.0035$), while the frequency of TC + TT genotypes was not significantly lower in the PTB group than that in controls (OR: 0.64, 95% CI: 0.39–1.06, $P = 0.073$).

4. Discussion

Unlike adults, children present rapid progression from a recent infection towards disease and are vulnerable to severe disease and death [4–6]. Certain pediatric TB reflects Mendelian predispositions, while adult TB seems to be more complex for genetic predisposition [5]. Studying the effects of the candidate susceptibility genes on pediatric TB may aid in the establishment of more efficient prevention of TB spread.

Interleukin-6 (IL-6) is a pleiotropic cytokine with important roles in immunoregulation [7]. But the role of IL-6 in limiting *M. tuberculosis* infection is still under discussion. Recently, Zhang et al. [9] proved in a Chinese Han population that an IL-6 promoter variation, which functionally downregulated IL-6 producing, was protective against TB. Elevated IL-6 from bronchoalveolar lavage cells seemed to be a biomarker of noncavitary TB [8]. In mice, increased IL-6 level was found to be correlated with TB progression [19]. IL-6 could downregulate the microbicidal activity of macrophage [20, 21]. But Ladel et al. [22] believed that IL-6 could play critical role in host resistance to *M. tuberculosis* infection that IL-6 deficient mice had increased bacterial loads when infected by *M. tuberculosis*. Some other researchers demonstrated that although IL-6 could induce early interferon-gamma production in the infected lung and the absence of IL-6 led to a delay in the induction of protective immunity with a subsequent early increase in bacterial load, however, the absence did not affect the induction of normal protective memory responses, which means IL-6 might not be required for generation of specific immunity to *M. tuberculosis* infection [23].

As the receptor of IL-6, IL-6R plays an important role in IL-6 signaling cascade [7]. IL-6 acts through binding specifically to the IL-6R to form a complex, and then this complex binds to the ubiquitousgp130 subunit to trigger intracellular signaling. IL-6R could be expressed both in membrane-bound form and a cleaved soluble form of IL-6R (sIL-6R). Increased level of sIL-6R has been reported in immune-related diseases, such as diabetes and allergic asthma [24, 25]. IL-6R controls lung $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) development, with sIL-6R regulating Th2 cell functions in $CD4^+CD25^-$ effectors T cells lacking mIL-6R and mIL-6R controlling cell fate at the beginning of T-cell differentiation by directing $CD4^+$ naive cells toward Th2 pathways and inhibiting Treg differentiation [26]. The imbalance between effectors T cells and Tregs was also thought to play an important role in TB etiology [27].

In this study, we try to illustrate the association of IL-6 and its receptor IL-6R with pediatric TB by tag-SNP genotyping. A tag single-nucleotide polymorphism (SNP) is representative SNP in a region of the genome with high linkage disequilibrium (the nonrandom association of alleles at two or more loci). It is possible to identify genetic variation without genotyping each SNP in a chromosomal region. Tag-SNPs are useful in gene association studies. Previously, rs1800796 (−572 C/G) of IL-6 was detected to be associated with adult TB and the regulatory effects of this SNP on IL-6 production in plasma and $CD14^+$ monocyte cultures stimulated with a *M. tuberculosis* product were also conformed [9]. $CD14^+$ monocytes with rs1800796 GG genotype produced

Table 2: The genotyping results of SNPs in IL-6 and IL-6R.

Gene	Position	Rs No.	Allele/genotype	TB (*n*, %)	Control (*n*, %)	OR	*P*	Bonf *P*
IL6	5 near	rsl7147230	A	334, 47.3	388, 48.7	0.94 [0.77–1.56]	0.58	5.79
			T	372, 52.7	408, 51.3			
			AA	83, 23.5	94, 23.6			
			TA	168, 47.6	200, 50.2		0.67	6.70
			TT	102, 28.9	104, 26.1			
	Promoter	rsl1800796	G	243, 33.7	269, 34.4	1.03 [0.83–1.28]	0.77	7.72
			C	463, 66.3	529, 65.6			
			GG	43, 12.2	49, 12.3			
			GC	157, 44.5	171, 42.9		0.90	9.00
			CC	153, 43.3	179, 44.9			
IL-6R	Promoter	rs3887104	A	65, 9.4	56, 7.2	1.33 [0.92–1.93]	0.13	1.34
			G	629, 90.6	720, 92.8			
			AA	3, 0.9	0, 0			
			AG	59, 17.0	56, 4.4		0.06	0.63
			GG	285, 82.1	332, 85.6			
	5 UTR	rs4845617	A	370, 47.4	322, 48.6	0.95 [0.77–1.17]	0.65	6.48
			G	358, 52.6	392, 51.4			
			AA	78, 22.9	90, 23.6			
			AG	166, 48.8	190, 49.9		0.87	8.70
			GG	96, 28.2	101, 26.5			
	Intron	rs7411976	C	39, 5.6	34, 4.4	1.27 [0.79–2.04]	0.32	3.19
			A	661, 94.4	732, 95.6			
			CC	2, 0.6	0, 0			
			CA	35, 10	34, 8.9		0.20	2.00
			AA	313, 89.4	349, 91.1			
	Intron	rs4845618	G	320, 46.1	342, 44.0	1.09 [0.89–1.34]	0.41	4.08
			T	374, 53.9	436, 56.0			
			GG	77, 22.2	73, 18.8			
			GT	166, 47.8	196, 50.4		0.51	5.10
			TT	104, 30	120, 30.9			
	Intron	rs4845626	T	57, 8.1	71, 8.9	0.90 [0.63–1.30]	0.58	5.78
			G	649, 92.9	729, 91.1			
			TT	4, 1.1	1, 0.2			
			TG	49, 13.9	69, 17.2		0.15	1.50
			GG	300, 85.0	330, 82.5			
	Missense	rs2228145	C	290, 41.7	350, 46.5	0.82 [0.67–1.01]	0.06	0.62
			A	406, 58.3	402, 53.5			
			CC	60, 17.2	81, 21.5			
			CA	170, 48.9	188, 50.0		0.17	1.70
			AA	118, 33.9	107, 28.5			

Table 2: Continued.

Gene	Position	Rs No.	Allele/genotype	TB (*n*, %)	Control (*n*, %)	OR	*P*	Bonf *P*
			T	46, 6.5	87, 10.9	0.57 [0.39–0.83]	0.003	0.03
			C	660, 93.5	711, 89.1			
	3 UTR	rs2229238	TT	2, 0.6	2, 0.5			
			TC	42, 11.9	83, 20.8		0.004	0.04
			CC	309, 87.5	314, 78.7			
			A	43, 6.1	34, 4.3	1.46 [0.92–2.31]	0.11	1.08
			G	663, 93.9	764, 95.7			
	3 UTR	rs3828078	AA	0, 0	2, 0.5			
			AG	43, 12.2	30, 7.5		0.03	0.29
			GG	310, 87.8	367, 92.0			

Logistic regression analyses were used for calculating OR (95% CI: confidence interval). Value was determined by Fisher's exact test.

Table 3: Comparing rs2229238 polymorphisms in controls with those in TB cases.

(a) Analysis of the inheritance models of IL-6R rs2229238 polymorphism in the combined samples

Model	Genotype	TB (*n*, %)	Control (*n*, %)	*P* value	OR (95% CI)
Dominant	CC	309, 87.5	314, 78.7	0.001	0.53 (0.35–0.78)
	TC + TT	85, 21.3	44, 12.5		
Recessive	CC + TC	351, 99.4	397, 99.5	0.9	1.13 (0.16–8.07)
	TT	2, 0.6	2, 0.5		

(b) Comparison of the genotypes and alleles distribution in different patient subgroups stratified by diagnosis

Subjects (*n*)*	Genotype (*n*, %)		P^1	OR[1] (95% CI)	Allele (*n*, %)		P^2	OR[2] (95% CI)
	TC + TT	CC			T	C		
Control (400)	85, 21.3	314, 78.7	—		87, 10.9	711, 89.1	—	
TB (273)	44, 12.5	309, 87.5	0.004	0.53 (0.35–0.78)	46, 6.5	660, 93.5	0.003	0.57 (0.39–0.83)
PTB (156)	23, 14.7	133, 85.3	0.073	0.64 (0.39–1.06)	24, 7.7	288, 92.3	0.109	0.68 (0.42–1.09)
EPTB (197)	21, 10.7	176, 89.3	0.004	0.44 (0.26–0.74)	22, 5.6	372, 94.4	0.003	0.48 (0.30–0.78)

*PTB: pulmonary tuberculosis; EPTB: extrapulmonary. [1]*P* value and OR (95% CI) of 2 × 2 χ^2 test for dominant inheritance of T allele; [2]*P* value and OR (95% CI) of 2 × 2 χ^2 tests for allele. [1]OR (95% CI) for dominant inheritance of T allele. [2]OR (95% CI).

less IL-6 in response to *M. tuberculosis* 19 kDa lipoprotein than those with CC or CG genotype. To our surprise, we did not find this potential association of rs1800796 with TB in our pediatric group. This might depend on the genetic and clinical differences between TB on set childhood and adulthood as we have described above. Another possibility of this inconformity might be due to different genetic backgrounds between two populations: our samples are mainly from North China, while Zhang's samples are from South China. We noticed that the G allele frequencies between these two researches are obviously different. In Zhang's research, the allele frequencies of rs1800796 G are 26.1% in controls and 21.3% in cases, while in our research, these frequencies are 34.4% and 33.7% independently.

Our results indicate that a 3′UTR polymorphism variation within IL-6R, rs2229238, contributes to pediatric TB resistance. The data showed that both the frequencies of rs2229238T allele and rs2229238 TC + TT combined genotypes decrease progressively from control to PTB, then to EPTB. As we know, to children, most of EPTB are developed from PTB, which means the rs2229238 SNP might contribute to TB disease progression. Unlike adults, pediatric EPTB usually presents more serious and complicated clinical symptoms and also poor outcomes. In our research, SeTB, which presents severe clinical manifestations, was identified in 81.2% of EPTB cases. Thus, rs2229238 T allele might protect children from both TB on set and disease progression.

Previously, in analyzing the potential genetic associations between four polymorphisms of IL-6R and atherosclerotic lipid profiles among young adolescents in Taiwan, Chu et al. [28] found the IL-6R rs2229238 C/T variants being associated with dyslipidemia in girls. By screening the association of eleven IL-6R gene variants with type 2 diabetes in Northern European Caucasian and African American ethnic groups, Wang et al. [24] identified the rs2229238 polymorphism in the 3′UTR showing a trend to an association with type 2 diabetes in a Caucasian population ($P = 0.055$). But no association study of rs2229238 C/T variants with pediatric TB has been confirmed before.

One weakness of this study is that we did not investigate how rs2229238 C/T was functionally involved in the susceptibility and development of TB. In the future, we could try to do some functional studies, for instance, to discover whether different alleles of this SNP differ in IL-6R expression.

5. Conclusion

In conclusion, we discussed the associations of IL-6 and IL-6R SNPs with TB in a Chinese pediatric population and identified rs2229238 T allele presenting a protective role in both pediatric TB on set and disease progression. Additional studies are warranted to test out result in other pediatric populations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Chen Shen and Hui Qi contributed to this work equally.

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