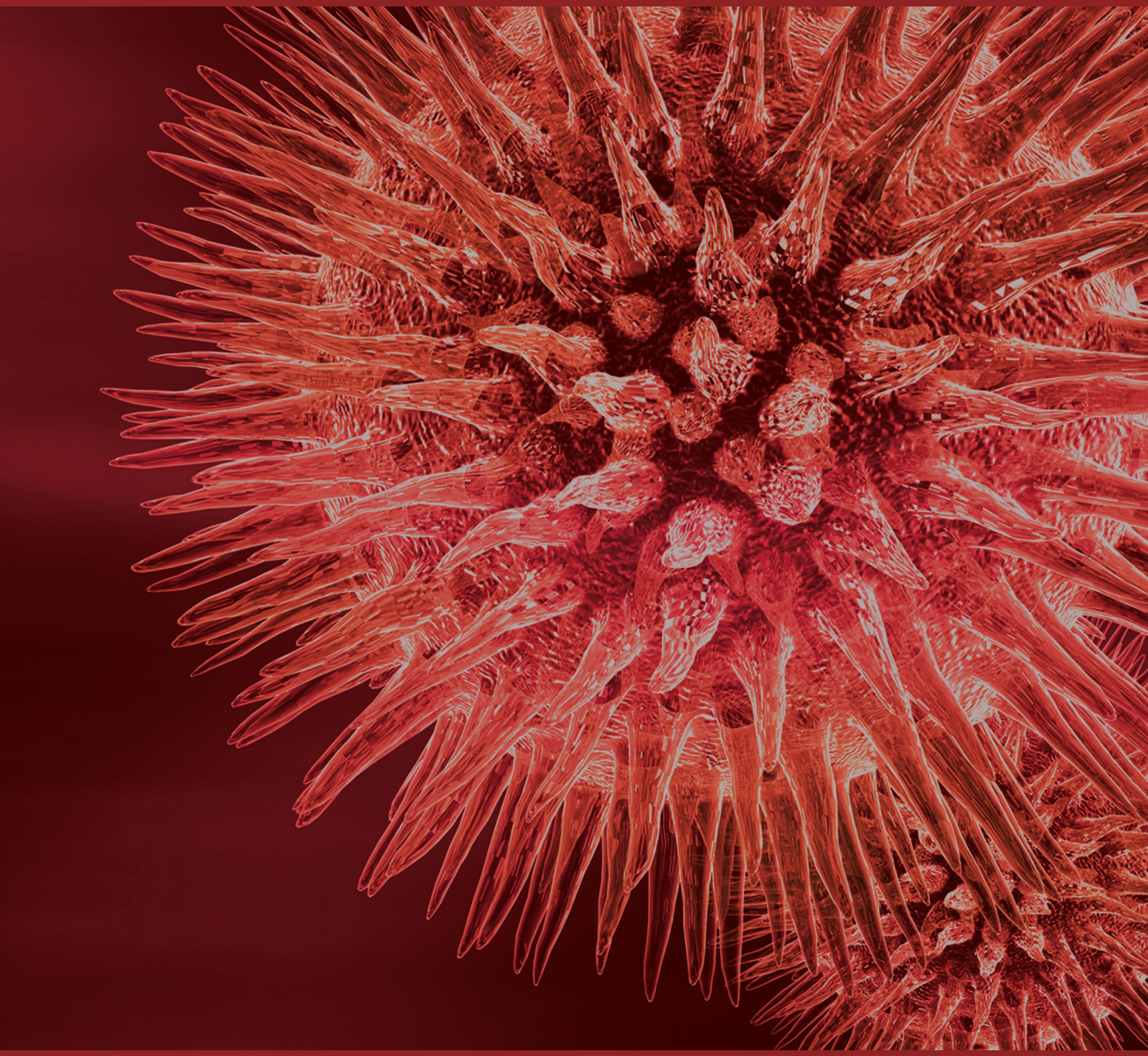
BioMed Research International

Human Genetic Diseases

Guest Editors: Hao Deng, Peter Riederer, Han-Xiang Deng, Weidong Le, Wei Xiong, and Yi Guo



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Editorial
Human Genetic Diseases

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There is no question that the rapid advance in genetic technology is changing our viewpoint on medical practice, which is dramatically improving the diagnosis, prognosis, and therapy of human genetic disease. In particular, the next-generation sequencing (NGS) technologies, such as exome sequencing and whole-genome sequencing, and gene editing technology have been applied to several areas, such as genomes, transcriptomes, and epigenomes, and have transformed the genetic research of human diseases. As a powerful and cost-effective discovery and diagnostic tool, exome sequencing was widely used in detecting disease-associated variants underlying genetic disease and developed genetic research such as personalized medicine and personal genomics.

Through rigorous peer review, this special issue includes high-quality papers. We provide a general description as follows.

In the paper, "A Novel *COL4A5* Mutation Identified in a Chinese Han Family Using Exome Sequencing," X. Xiu et al. have explored the disease-related gene in a four-generation Chinese Han pedigree of Alport syndrome. Their results showed that a novel deletion *COL4A5* mutation, c.499delC (p.Pro167Gln*fs**36), may be responsible for AS in this family. Also, their works indicate that exome sequencing is a fast, sensitive, and relatively low-cost method to identify disease-associated mutation(s).

By using NGS, Q. Zhou et al. intended to study the possible association of certain genes with X-linked retinitis pigmentosa (RP) in a Chinese family. They discovered a novel c.C1555T (p.R519T) mutation in the *CACNA1F* gene on X chromosome, which showed perfect cosegregation with the disease in the family. The identification may have significant contribution for the RP diagnosis, genetic counseling, and clinical management.

C. Wang and Q. Tian determined the long-term quality of life (QOL) in Chinese patients with disorders of sex development (DSD). Their works suggest that, compared with the Chinese urban population, the QOL score of DSD patients in China was not significantly lower. With proper treatment, including the follow-up and psychological support, the QOL of DSD patients cannot be significantly reduced, and more attention should be paid to the potential psychological and sexual problems.

A. P. Grillo et al. investigated the association of nine single nucleotide polymorphisms (SNPs) located within the *DFNB1* locus with the occurrence of autosomal recessive nonsyndromic hearing loss (ARNSHL). Their works showed that there were statistically significant differences between patients and controls, and the SNPs presented in the *GJB2* and *GJB6* genes may have an influence on ARNSHL in humans.

R. S. Honjo et al. intended to report the clinical findings of 55 Brazilian Williams-Beuren syndrome (WBS) patients confirmed by Multiplex Ligation-Dependent Probe Amplification (MLPA). Their results indicate that MLPA was a promising method in the diagnostic investigation of WBS and was effective in detecting the microdeletion.

A. Gordon-Shaag et al. summarized the current research development in keratoconus (KC) epidemiology and genetic etiology. Risk factors, including environmental, socioeconomic, and familial factors, were also discussed. The detailed molecular mechanism will significantly advance our understanding of KC and promote the development of potential therapies.

G. N. Cerbino et al. reviewed the molecular heterogeneity of Acute Intermittent Porphyria (AIP) in Argentinean patients. Thirty-five different mutations were identified in the *HMBS* gene, and a founder effect (p.G111R) was found. Their works also indicated the importance of molecular techniques as the most appropriate tools for detecting and identifying specific mutations in carriers of affected families.

R. Farhat et al. intended to explain the variable phenotypes of *CFTR* c.3909C>G mutation in Cystic Fibrosis patients in the Lebanese. They identified the association between the *CFTR* c.3909C>G and complex allele c.[744-33GATT(6); 869+11C>T]. Splicing studies revealed no impact of the c.3909C>G mutation on splicing, whereas the associated complex allele induces minor exon 7 skipping.

With the development of NGS, it has been used for uncovering the genes underlying unsolved Mendelian disorders, explaining the heritability of complex and health-related trains, and even setting the stage for applying to facilitate clinical diagnosis and personalized disease-risk profiling. New mutations, if validated, contribute to find specific and selective biomarkers for certain diseases, which are important prerequisites for early diagnosis and treatment. Few diseases could be due to a single factor, and most common diseases are multifactorial. Exploring the interaction of multiple factors, including modificator genes and environmental and geographical factors, may help us to better understand the genetics mechanism of human disease.

This special issue is intended to develop and expand the association between human disease and genetics. By soliciting paper, we hope this special issue will help stimulate the understanding of molecular pathology underlying human genetics diseases and provide new insight in diagnosis, therapy, and genetic counseling of human genetic disease.

Acknowledgments

We would like to express appreciation to the authors for their contribution in assisting us. We also thank the anonymous reviewers who helped improve the quality of the papers.

Hao Deng
Peter Riederer
Han-Xiang Deng
Weidong Le
Wei Xiong
Yi Guo

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Research Article

Williams-Beuren Syndrome: A Clinical Study of 55 Brazilian Patients and the Diagnostic Use of MLPA

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Williams-Beuren syndrome (WBS) is a genetic disease caused by a microdeletion in the 7q11.23 region. It is characterized by congenital heart disease, mainly supravalvular aortic stenosis, mental retardation, mild short stature, facial dysmorphisms, and variable abnormalities in different systems. *Objectives*. To report the clinical findings of 55 Brazilian patients confirmed by multiplex ligation-dependent probe amplification (MLPA). *Methods*. Patients were followed up for 4 years at the Genetics Unit of the Instituto da Criança of the Hospital das Clínicas, FMUSP, Brazil. A kit specific for WBS was used to detect the 7q11.23 microdeletion. *Results*. Two patients with negative FISH results had positive MLPA results for WBS. The characteristics of the patients with the deletion were as follows: typical WBS facies (98.2%), neuropsychomotor delay (98.2%), hypersocial behavior (94.5%), hyperacusis (94.5%), and congenital heart disease (81.8%). *Conclusions*. MLPA was effective in detecting the microdeletion in the 7q11.23 region to confirm the diagnosis of WBS. MLPA was also able to confirm the diagnosis of WBS in two patients with typical clinical characteristics but negative FISH results. Thus, MLPA is a promising method in the diagnostic investigation of WBS. WBS is a multisystemic disorder and therefore requires multidisciplinary care and specific follow-up to prevent complications.

1. Introduction

Williams-Beuren syndrome (WBS) is a genetic multisystemic disease characterized by congenital heart disease, mainly supravalvular aortic stenosis (SVAS), mental retardation, mild short stature, facial dysmorphisms, and variable abnormalities in the genitourinary, endocrinological, ophthalmological, and skeletal systems [1, 2]. The incidence is estimated to be 1 in 20,000 live births [3], but some authors report a prevalence of approximately 1 in 7,500 [4].

The typical facial dysmorphisms found in WBS are as follows: high forehead, medial broadening of the eyebrows, periorbital fullness, depressed nasal bridge, malar hypoplasia, thick lips, and long nasolabial philtrum [1, 2, 5]. Short stature is common [6] but not severe.

Several studies report that patients with WBS have unique cognitive and behavioral profiles, with characteristic dissociations among different domains, such as better skills in language and deficits in motor and visuospatial activities [7–9]. Patients also have characteristic hypersocial behavior, even with strangers [10, 11].

WBS is caused by a 1-2 Mb microdeletion in 7q11.23, a region that contains 28 genes [12]. Approximately 90% of WBS patients have a 1.55 Mb microdeletion and 8% have a 1.84 Mb microdeletion. These are considered "typical" WBS microdeletions. Microdeletions larger than 1.84 Mb or

smaller than 1.55 Mb are termed "atypical," are often associated with atypical clinical manifestations, and occur in only 2% of cases [13]. The recognition and description of these cases have been very helpful for genotype-phenotype correlation studies. Whether the parental origin of the microdeletion has any impact on the phenotype of the patient remains under debate [14–16].

WBS is generally sporadic [16–18], is caused by de novo deletions, and has a recurrence risk lower than 5% [19–21]. A few cases of vertical transmission have been reported [12, 22–24]. People with microinversions of 1.5–1.9 Mb in the WBS critical region are predisposed to having children with WBS [25–27].

The chromosomal region 7q11.23 comprises a region of approximately 1.2 Mb of single copy genes and three blocks of low copy repeat sequences. Due to the high similarity of those blocks, nonallelic homologous recombination is possible and can result in microdeletion or microduplication within the region [12, 16, 28].

Although individuals with WBS present with a highly characteristic phenotypic profile, the diagnosis of WBS is often confirmed by molecular testing. Currently, the microdeletion in the 7q11.23 region can be detected by several methods, including fluorescence in situ hybridization (FISH), polymorphic microsatellite markers, chromosomal microarray analysis (CMA), and *multiplex ligation-dependent probe amplification* (MLPA).

Some studies have shown that MLPA is an alternative to FISH, which is the current gold-standard method for diagnosing WBS. Cho et al. [29] found concordant results in four patients using both techniques.

Here, we report the clinical findings of 55 Brazilian patients with WBS confirmed by MLPA.

2. Methods

The patients were evaluated at the Genetics Unit of the Instituto da Criança of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (ICr, HCFMUSP), São Paulo, Brazil. The study was approved by the local Ethics Board, and informed consent form was obtained from all families. All patients were evaluated and followed up by a single examiner over a period of 4 years (2008–2011). Clinical and laboratory data were collected following a protocol that included the following: anamnesis, physical examination, cardiovascular assessment (arterial pressure and echocardiogram), urinary tract evaluation (renal ultrasonogram, BUN, and creatinine), plasmatic and urinary calcium, thyroid function tests, and referral to specialists for baseline and/or follow-up evaluations (ophthalmology, cardiology, nephrology, psychiatry, and endocrinology, among others).

DNA was extracted from peripheral blood by the salting-out method [30]. MLPA analyses were performed using kit P029 from MRC Holland (Amsterdam, Netherlands), following the manufacturer's instructions. This kit contained probes of genes mapped to the WBS critical region (*ELN*, *CLIP2*, *LIMK1*, *TBL2*, *STX1A*, *RFC2*, *FZD9*, and *FKBP6*) and controls. Data were analyzed using the GeneMarker software.

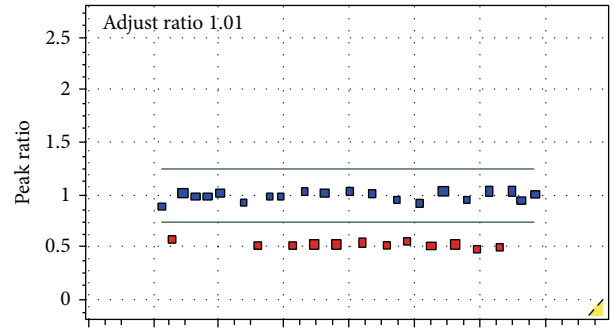


Figure 1: MLPA showing deletion of the probes in the 7q11.23 region (red squares).

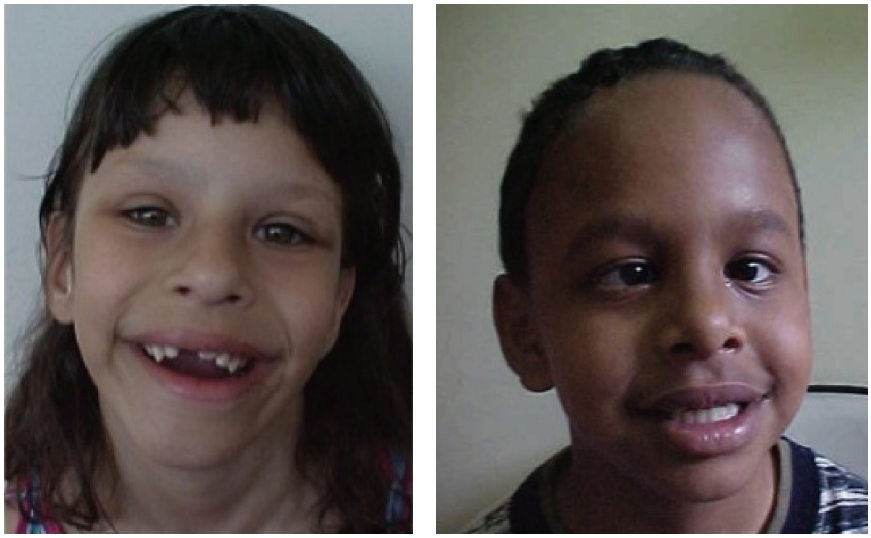
3. Results

Fifty-five patients (34 males and 21 females) with clinical diagnoses of WBS were evaluated. The age at diagnosis ranged from 2 to 30 years old (median = 14 years). MLPA confirmed the microdeletion in 7q11.23 (Figure 1).

FISH results were available for 18 of the 55 patients; the results were positive for all but two. MLPA confirmed the diagnosis in these two patients. The first patient was a girl (Figure 2(a)), born at term, after an uneventful pregnancy except for the use of penicillin by the mother in the 2nd month. The mother had had one previous spontaneous abortion and had another child with autism. The patient was born by cesarean section due to fetal bradycardia, with 2620 g and 47 cm. She had meningitis within 21 days and presented with neuromotor development delay (sat after 8 months, walked at 2 years of age, and spoke first words at 5 years). The patient has typical WBS behavior and facies, as seen in Figure 2, besides constipation, scoliosis, enuresis, precocious puberty, and mental retardation. Pituitary microadenoma was diagnosed at 11 years of age. The second patient (Figure 2(b)) was a boy, born at term, cyanotic, with no available information regarding weight and height at birth. He had neuromotor development delay (sat at 2 years, walked at 3 years, and spoke at 2 years of age) and showed typical WBS facies and behaviour, hypothyroidism, unilateral radioulnar synostosis, and bladder diverticulum.

The most prevalent clinical characteristics of the 55 patients are shown in Table 1. These included typical WBS facies (98.2%), developmental delay (98.2%), hypersocial behavior (94.5%), hyperacusis (94.5%), and congenital heart disease (81.2%).

Congenital heart disease was present in 45/55 patients, and SVAS was the most prevalent type (19/45 or 42.2% of the cases). Isolated SVAS was present in 12 patients; SVAS was associated with other cardiac anomalies in 7 patients. Three patients had echocardiogram reports of aortic stenosis, but it was not supravalvular. Pulmonary stenosis was the second most frequent abnormality, detected in 12/45 (26.7%) of the patients. Other cardiac anomalies were found in the other patients (14/45), either in isolation or in combination, as follows: mitral valve prolapse, aortic coarctation, pulmonary artery stenosis, interatrial septal defect, ventricular septal



(a) (b)

Figure 2: Patients with positive MLPA but negative FISH results for WBS.

Table 1: Clinical characteristics of WBS patients.

Clinical characteristic	*n*	%
Typical WBS facies*	54/55	98.2
Developmental delay	54/55	98.2
Hypersocial behavior	52/55	94.5
Hyperacusis	52/55	94.5
Congenital heart disease	45/55	81.2
Genitourinary symptoms	47/55	85.5
Short stature	24/55	43.6
Hypertension	20/55	36.4
Microcephaly	17/55	31.0

*Typical WBS facies based on the score proposed by the Genetics Committee of the American Academy of Pediatrics (2001).

defect, tricuspid insufficiency, pulmonary valve insufficiency, mitral valve insufficiency, and bicuspid aortic valve.

One patient with SVAS that was surgically repaired in childhood presented with congestive heart failure at 19 years of age and needed a heart transplant. However, she died due to CMV infection on the 30th day after transplant.

Two other patients, a 13-year-old girl and a 19-year-old boy, died due to cardiovascular complications.

Ten out of 55 patients (18.2%) did not have congenital heart disease.

Short stature was present in 24 patients (12 females and 12 males). Microcephaly was present in 17 patients, 13 of whom were females.

Hypercalcemia was detected in only one patient, at 1.6 years of age. Another patient had serum calcium in the upper limit of normal. Two other patients presented with nephrocalcinosis, and one presented with hypercalciuria but normal serum calcium.

Noncongenital hypothyroidism was diagnosed in 8 patients (14.5%). Seven patients (7.3%) had subclinical hypothyroidism. None of the patients had abnormal fasting blood glucose levels.

Strabismus was present in 19 patients (34.5%), and hernias, either umbilical or inguinal, were present in 20 patients (36.4%). Three patients (5.4%) presented with lacrimal duct obstruction.

Genitourinary symptoms, including mainly urinary urgency and nocturnal enuresis, were reported in 45 patients (85.4%).

Twenty patients (36.4%) presented with arterial pressures above the normal levels for their age, gender, and height percentiles. The ages of these patients ranged from 4 to 23 years old. Four of them (20% of the patients with hypertension) had renal artery stenosis. Three (aged 7–13 years old) underwent corrective surgery, and one is awaiting the intervention. One patient required 2 procedures at 8 and 11 years of age.

Scoliosis was present in 31 of the 55 patients (56.4%), and radioulnar synostosis was present in 6 (10.9%).

All patients had delays in at least one motor milestone and/or had mental retardation. One patient was diagnosed with panic disorder, and one had visual hallucinations.

Some clinical manifestations rarely described in WBS were found in our patients as follows: vertebral fusion (2 patients with cervical vertebral fusion and another with lumbosacral vertebral fusion), accessory spleen ($n = 1$), scrotal nodule ($n = 1$), labia majora hypertrophy ($n = 1$), sagittal craniosynostosis ($n = 1$), neonatal tooth ($n = 1$), and muscular hernia in the leg ($n = 1$).

4. Discussion

For geneticists, WBS is a well-known syndrome. It is usually promptly recognizable by the characteristic facial dysmorphisms and typical hypersocial behavior. Severe cases of

neonatal hypercalcemia can result in death before a diagnosis of WBS has been considered. For this reason, neonatologists should consider WBS diagnosis in neonates with hypercalcemia and/or intrauterine restriction and SVAS.

Regarding the frequency of congenital heart disease in this cohort (81.8%) and the most prevalent type, SVAS (42.2%), the data are concordant with the literature [2]. A significant number of the patients were referred to our service from the WBS National Patient Association (ABSW), not from the cardiology department of the hospital. This could have contributed to the SVAS frequency being below 50%. Although it is an important sign of the syndrome, SVAS is not pathognomonic. It is important to reinforce that the absence of congenital heart disease does not rule out WBS diagnosis. Because cardiovascular disease in WBS has been credited to the *ELN* gene deletion, it is intriguing that at least 15% of the patients with this deletion do not have cardiac abnormalities. Epigenetic factors such as copy number variation in other regions of the genome might play a role.

Sudden death is one of the complications in WBS [31–35]. Some necropsy cases revealed stenoses of the coronary arteries and severe biventricular obstruction with myocardial ischemia, decreased cardiac output, and arrhythmias as causes of death [31]. Other patients died after anesthetic procedures [36]. A phenomenon called Kounis syndrome can occur when inflammatory mediators, possibly due to massive mast cells degranulation, induce coronary spasm or obstruction in patients with preexisting coronary disease [37, 38]. We had 3 patients that died during adolescence due to cardiovascular complications. One underwent heart transplantation; the indications and outcome of this procedure in WBS are scarce in the literature.

The prevalence of other findings in WBS in this cohort is the same as reported by other groups in regard to facial dysmorphisms, hypersocial behavior, neuromotor delay, hyperacusis, short stature, and microcephaly [39]. However, only one patient presented with hypercalcemia, a feature that is usually linked to WBS because of its description. Hypercalcemia often manifests in the first years of life [2], and some of our patients might have presented this abnormality before the diagnosis of WBS was considered. Because serum calcium is not routinely measured in the neonate unit or the pediatric ER and most of our patients have not been diagnosed with WBS by this time, this may be a bias in our cohort. In addition, hypercalcemia can present at any time in a WBS patient's life; thus, although our patients did not have hypercalcemia during the assessment period, they are still at risk and should be periodically monitored for calcium disturbances [39, 40].

The prevalence of thyroid abnormalities in our cohort (14.3% of hypothyroidism and 7.3% of subclinical hypothyroidism) is similar to that of other studies, even though there is a wide range of the reported prevalences (2–38%) [39, 41]. Because hypothyroidism can aggravate some of the clinical manifestations of WBS and is a treatable condition, periodic monitoring of thyroid hormone levels in WBS patients is recommended. Currently, there is a recommendation of assessment every two years (American Academy of Pediatrics, 2001); however, in our protocol, we tested yearly, and several cases were diagnosed; thus, the interval of testing should be shorter.

Diabetes mellitus is a well-described late manifestation of WBS [40]. Although none of our patients had abnormal fasting glucose levels, most of them were children and adolescents; diabetes in WBS is more common in the adult population [40, 42].

The frequencies of other manifestations in this cohort, such as strabismus, hernias, scoliosis, and radioulnar synostosis, were concordant to the prevalence and variations described in literature [16, 39, 43, 44].

On the other hand, urinary problems were somewhat more frequent in this cohort (85.4% in our study versus 68% in the literature) [16, 39]. This could be due to the fact that a urologist evaluated all of our patients.

Arterial hypertension was detected in 20/55 patients (36.4%), one of whom was diagnosed at 4 years old; this can be an early complication in WBS. In this syndrome, there is a lifetime risk of developing arterial hypertension of 50% [45], and this has been reported even in 1-month-old patients [46]. Four out of 20 patients in our group (20%) had renovascular disease. Arterial hypertension due to renal artery stenosis is described in 44% of WBS patients [47]. Thus, every patient with WBS, regardless of age, should be monitored for blood pressure (American Academy of Pediatrics, 2001), and, in the case of hypertension (using appropriate curves for age and height percentiles), evaluation of the renal arteries is mandatory.

The occurrence of other rare findings in our patients (e.g., accessory spleens, neonatal tooth, and muscle hernias) and their relation to WBS could not be determined because the prevalence of each finding separately was low. Lacrimal duct stenosis and craniosynostosis, although uncommon, were already described in WBS [48, 49]. One patient presented with recurrent patellar dislocation, which has also already been described in WBS [50].

FISH has been the gold-standard method for the diagnosis of WBS. Among the 55 patients studied by MLPA in this study, 16 also had positive FISH results. However, two patients with typical physical and behavioral characteristics of WBS had negative FISH results but positive polymorphic marker analysis and MLPA results, which detected the typical deletion. After the positive results in both cases using MLPA method (deletion of all the probes, not an atypical deletion), we contacted the laboratory that had performed FISH. The FISH tests were repeated and the results were positive for the microdeletion. This emphasizes the importance of testing with another method or repeating the test when clinical and laboratory analyses diverge. A group from Netherlands studied 63 patients by FISH and MLPA. In 53/63 patients, the microdeletion was detected by both methods. In 10 patients, the results were negative with MLPA and FISH. However, one patient with a small, atypical microdeletion could only be diagnosed with MLPA; FISH using commercial probes was negative [51]. Thus, the gold-standard test to the diagnosis of WBS should be revised.

MLPA is also used to diagnose many other syndromes of microdeletion and microduplication, such as Smith-Magenis, DiGeorge, Alagille, Prader-Willi, and Angelman syndrome.

There are specific kits for each syndrome or kits with a few probes of multiple syndromes (e.g., kits to diagnose some mental retardation syndromes). MLPA has also been proven useful for prenatal diagnosis using amniotic fluid for microdeletion and microduplication syndromes and for the diagnosis of trisomies [52–55].

5. Conclusions

The assessment and long follow-up of WBS patients by several medical specialties is of great relevance due to the relatively high prevalence of multisystem manifestations and complications.

MLPA was effective in confirming the diagnosis of WBS and can be used as the first exam in developing countries due to its lower cost compared with FISH. In addition, MLPA has the advantage of detecting atypical deletions and can be useful when FISH is negative in patients with clinical characteristics that are highly suggestive of WBS.

Conflict of Interests

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

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Review Article

The Genetic and Environmental Factors for Keratoconus

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Keratoconus (KC) is the most common cornea ectatic disorder. It is characterized by a cone-shaped thin cornea leading to myopia, irregular astigmatism, and vision impairment. It affects all ethnic groups and both genders. Both environmental and genetic factors may contribute to its pathogenesis. This review is to summarize the current research development in KC epidemiology and genetic etiology. Environmental factors include but are not limited to eye rubbing, atopy, sun exposure, and geography. Genetic discoveries have been reviewed with evidence from family-based linkage analysis and fine mapping in linkage region, genome-wide association studies, and candidate genes analyses. A number of genes have been discovered at a relatively rapid pace. The detailed molecular mechanism underlying KC pathogenesis will significantly advance our understanding of KC and promote the development of potential therapies.

1. Introduction

Keratoconus (KC), a term which comes from the Greek words *keras* (cornea) and *konos* (cone), was first described in the literature in 1854 (Nottingham). Yet its etiology, which is multifactorial with genetic and environmental influences, remains elusive [1]. It is a corneal disorder in which the central portion of the cornea becomes thinner and bulges forward in a cone-shaped fashion resulting in myopia, irregular astigmatism, and eventually visual impairment. Until some years ago, the definition of KC included the notion of a noninflammatory process [2, 3]. However, recent evidence of overexpression of inflammatory mediators such as cytokines and interleukin 6 (IL-6) in tears of KC patients and in subclinical KC may refute this concept [4, 5] and inflammation is currently considered by some researchers to play a role in the pathogenesis of KC [1, 6] (reviewed in [7]). Further evidence comes from the reduced levels of superoxide dismutase [8] in KC whose function is to remove reactive oxygen species known to be associated with inflammatory reactions.

1.1. Signs and Symptoms. The onset of the disease usually occurs in the second decade of life, although some cases may develop in early adulthood [3]. It is a progressive condition which usually stabilizes by the fourth decade of life [2, 9, 10]. Early in the disease, the patient is typically asymptomatic. As the disease progresses, visual acuity decreases and eventually the patient notices visual distortion with significant vision loss. These changes are due to the development of irregular astigmatism, myopia, and in many cases corneal scarring. In addition, the cornea becomes thinner [11, 12] and less touch-sensitive [13, 14]. The disease is bilateral, although asymmetrical [3]. Initially it is often unilateral, the prevalence of which ranges from 14.3% to 41% [2, 15, 16] when detected by keratometry alone. With computerized topography the prevalence of unilaterality is greatly diminished from 0.5% to 4% [17–22]. However, the majority of patients eventually develop bilateral KC. In one study it was shown that 50% of the nonaffected fellow eyes developed the disease within 16 years [23].

KC affects both men and women. However, it remains unclear whether men or women have higher prevalence of KC. The majority of recent papers published after 1970s [13, 24–32] indicate a preponderance of men over women with KC while other studies published prior to 1970s and two recent studies reported the opposite [16, 33, 34]. In

a retrospective study conducted in Netherlands [35], using data relating to over 100,000 contact lens wearers obtained from four university clinics and five noncontact lens centers between the years 1950 and 1986, the ratio of men to women was 0.5. In cases diagnosed in the period from 1950 to 1954, it remained less than 1.0 until 1970s when the number of male patients significantly increased while the number of female patients remained virtually unchanged. The ratio of KC affected men compared to women reached 1.58 for patients diagnosed in 1985 and 1986 and this difference was made more manifest with the advent of corneal topography. Several reasons may account for this observation. First, this study is based on clinics instead of population or community. Second, possible sample errors could affect the study result. Third, different technologies to diagnose KC may be used throughout the study period. Finally, hormonal differences have been invoked and it has been noted that keratoconus develops earlier and progresses more rapidly in men than women [36], which could account for its higher prevalence.

Early *biomicroscopic* signs include Fleischer's ring, which is a partial or complete circle of iron deposition in the epithelium surrounding the base of the cornea and Vogt's striae, which are fine vertical lines produced by compression of Descemet's membrane [37]. As the disease progresses, a Munson's sign, a V-shaped deformation of the lower lid, becomes noticeable as the eye looks in the downward position, as well as a bright reflection of the nasal area of the limbus called Rizzuti's sign [37]. Less common are breaks in Descemet's membrane known as hydrops, which cause stromal edema, vision loss, and associated pain [38, 39]. For patients who wear contact lenses, corneal scarring is a very common feature [40].

1.2. Diagnosis. Since KC is typically characterized by the progression of irregular astigmatism, thinner cornea, and increased steepening of corneal curvature, KC is often first detected in the course of an eye examination and patients may be unaware of it, even though they complain of poor vision and have sought ocular care [27, 41]. The practitioner may note a suspicious reduction in visual acuity, scissors movements in retinoscopy, distortion of keratometric images, smaller values of pachymetric corneal thickness, which often precedes ectasia, or some of the known signs of the disease during the slit-lamp examination.

The most sensitive method of detecting and confirming a diagnosis of KC is unequivocally corneal topography based on the principles of Placido disc and Scheimpflug imaging, the latter being the most sensitive method of assessing corneal shape. Topography has become the gold standard method to diagnose and monitor KC [3, 42]. It allows the early detection of subclinical cases, also called *forme fruste* or KC suspect, as well as grading the severity of the disease by producing a color-coded topographic map of the corneal surface and various indices. Several quantitative methods based on these indices have been developed. The most common are the KC prediction index (KPI), which is derived from eight quantitative indices and the KC Index (KCI %) itself derived from the KPI and four other indices [42], and the KISA % which is based on keratometric value, inferior-superior asymmetry (I-S), asymmetric bow-tie astigmatism (AST), and skewed radial axis (SRAX) values [43]. Instruments that are based on Scheimpflug imaging [44, 45] are especially important in light of recent studies that suggest that KC starts from the posterior cornea and that posterior curvature may be the best way of identifying early KC [46–49]. Pellucid marginal degeneration is easily distinguished from KC by slit-lamp examination and by a distinct videokeratographic pattern [3].

The measurement of corneal thickness made by optical coherence tomography (OCT) has been shown to be as sensitive and as specific as the topographic KISA index [12]. Other attempts at detecting KC have been made with corneal aberrometers [50], since the keratoconic corneas display a large amount of higher order aberrations, especially vertical coma. However, in a study comparing the aberrations to the inferior-superior topographic values, the latter was proved to be just as good as a detector of KC [51].

1.3. Treatment. A number of different treatments are used to correct the vision caused by KC. In the early stages, the condition is usually well managed by spectacles. As the condition progresses to a mild or moderate stage with irregular astigmatism, the treatment of choice is contact lenses, especially rigid gas permeable lenses. However, about 20% of patients with advanced or severe KC cannot tolerate or improve their vision sufficiently with contact lenses and will eventually need surgery. The traditional surgical intervention has been penetrating keratoplasty in which the entire thickness of the cornea is excised and replaced by a donor cornea. This operation has yielded better vision than the partial removal of a superficial corneal layer (called lamellar keratoplasty) [52] but it caused more graft rejection [53]. Recently, a technique called collagen cross-linking (CXL) has been introduced and it has been proven to be successful not only at improving visual acuity but also at stiffening thus arresting and, even in many cases, regressing the progression of KC by preventing enzymatic degradation of stromal collagen [54–57]. Further research with KC will significantly improve our understanding and therefore potential therapy for KC.

2. Prevalence of KC

The burden of a disease in a community is evaluated by the knowledge of how widespread is that disease. This is demonstrated by its *prevalence*, which is a proportion (or percentage) of the total number of cases at a period in time divided by the size of the population from which the cases have been determined. Another measure of burden of disease is *incidence*, which is the number of new cases presenting during a defined period of time divided by the population size from which the cases have been determined and existing during that same period of time. Moreover, if the disease is chronic, then prevalence = incidence × duration. However, these measures of disease occurrence are used to characterize the KC population at risk of the disease. In particular, it is aimed at identifying the KC population at

Table 1: Hospital/clinic based epidemiological studies of KC.

Author	Location	Age in years	Sample size	Incidence/100,000	Prevalence/100,000	Method
Tanabe et al. (1985) [58]	Muroran, Japan	10–60	2601-P		9	Keratometry
Kennedy et al. (1986) [15]	Minnesota, USA	12–77	64-P	2	54.5	Keratometry + retinoscopy
Ihalainen (1986) [59]	Finland	15–70	294-P	1.5	30	Keratometry + retinoscopy
Gorskova and Sevost'ianov (1998) [60]	Urals, Russia				0.2–0.4	Keratometry
Pearson et al. (2000) [30]	Midlands, UK	10–44	382-P	4.5-W 19.6-A	57 229	Keratometry + retinoscopy
Ota et al. (2002) [28]	Tokyo, Japan		325-P	9		Keratometry?
Georgiou et al. (2004) [25]	Yorkshire, UK		74-P	3.3-W 25-A		Clinical examination
Assiri et al. (2005) [61]	Asir, Saudi Arabia	8–28	125-P	20		Keratometry
Nielsen et al. (2007) [62]	Denmark		NA	1.3	86	Clinical indices + topography
Ljubic (2009) [63]	Skope, Macedonia		2254		6.8	Keratometry
Ziaei et al. (2012) [64]	Yazd, Iran	25.7 ± 9	536	22.3 (221)		Topography

A, Asian (Indian, Pakistani, and Bangladeshi); W, white; P, patient; NA, not available.

risk (e.g., gender, age, parental consanguinity, and associated factors), the geographic location of greater occurrence, and the time when disease occurs most frequently (e.g., exposure to a risk factor and introduction of computer topography).

2.1. Hospital/Clinic Based Reports. The majority of prevalence studies have been conducted in a hospital clinic because of the ease of collecting data. Although these findings offer an estimate of prevalence, they are likely to underestimate the true prevalence of the disease, as patients presenting in hospitals are usually symptomatic and early forms of the condition are thus missed. In addition, these studies neglect the number of patients treated by independent optometrists and ophthalmologists. They do not take into consideration an ascertainment bias in access to health care. Although these studies are commonly cited, they must be interpreted with caution.

Until a few years ago most publications on KC referred almost exclusively to one prevalence value obtained in Minnesota, USA, in 1986 which had been found to be 0.054% (54 persons out of 100,000 people) [15]. The diagnosis was based on a mixture of scissors movements in retinoscopy and keratometry, as were the majority of prevalence studies published prior to 2011. Nevertheless, this figure was similar to those reported in Finland [59] or Denmark [62] but much higher than those reported in the Urals, Russia, at 0.0004% [60] or 0.0068% in Skope, Macedonia [63]. Still, it must be noted that the more precise videokeratography is likely to yield higher prevalence than the older methodology. Indeed, recent studies using this method report higher prevalence or incidence [27, 41, 64, 67–71], but other factors may confound a possible correlation with the method used, since they come principally from the Middle East and India with different climates and ethnic groups than Europe or North America, even if merely diagnosed with a keratometer [33]. Table 1 presents the epidemiological studies conducted in a hospital/clinic. Comments on the ethnic differences will be discussed in Section 3.2.4.

2.2. Population-Based Studies. Cross-sectional studies typically enroll people who volunteer to participate in the investigation, even though the population selected may represent a broad socioeconomic spectrum. Nevertheless, a selection bias may occur, since individuals with the disease may refrain from participating. On the other hand, others with visual problems may be keen to volunteer. However, the majority of volunteers are likely to have felt no particular bias. Selection bias is unlikely to cause a significant error because in some studies it was observed that a certain proportion of the volunteers who had been totally unaware of their condition were discovered to have the disease during the survey [27, 41]. Therefore, population-based screening studies are the best methodology to assess the true prevalence of the disease.

Modern videokeratography is the best method to screen subjects in a population-based study. However, for the purpose of completeness we will also mention studies using less reliable methodology. The first cross-sectional survey was carried out in 1957 at the Indiana State Fair in Indianapolis over a period of 10 days by 25 different optometrists, using a Placido disc [65]. 13,345 people were thus examined and

Table 2: Population-based epidemiological studies of KC.

Author	Location	Age in years (mean)	Sample size	Prevalence/100,000 (cases)	Method	Sampling method
Hofstetter (1959) [65]	Indianapolis, USA	1–79	13345	120 (16)	Placido disc$^{\Psi}$	Rural volunteers
Santiago et al. (1995) [66]	France	18–22	670	1190	Topography	Army recruits
Jonas et al. (2009) [33]	Maharashtra, India	>30 (49.4 ± 13.4)	4667	2300 (128)	Keratometry$^{\Psi}$	Rural volunteers (8 villages)
Millodot et al. (2011) [27]	Jerusalem, Israel	18–54 (24.4 ± 5.7)	981	2340 (23)	Topography	Urban volunteers (1 college)
Waked et al. (2012) [67]	Beirut, Lebanon	22–26	92	3300 (3)	Topography	Urban volunteers (1 college)
Xu et al. (2012) [68]	Beijing, China	50–93 (64.2 ± 9.8)	3166	900 (27)	Optical low coherence reflectometry$^{\Psi}$	Rural + urban volunteers
Hashemi et al. (2013) [69]	Shahrud, Iran	50.83 ± 0.12	4592	760 (35)	Topography	Urban volunteers from random cluster
Hashemi et al. (2013) [70]	Tehran, Iran	14–81 (40.8 ± 17.1)	426	3300 (14)	Topography	Urban volunteers (stratified cluster)
Shneor et al. (2014) [41]	Haifa, Israel	18–60 (25.05 ± 8.83)	314	3180 (10)	Topography	Urban volunteers (1 college)
Hashemi et al. (2014) [71]	Mashhad, Iran	20–34 (26.1 ± 2.3)	1073	2500 (26)	Topography	Urban volunteers (stratified cluster in 1 university)

$^{\Psi}$The methods for detecting KC used in these studies are now considered inadequate and the results should be interpreted with caution.

50 individuals exhibited a doubtful or definite keratoconic pattern, thereby indicating a prevalence of 0.37% for doubtful and definite types and only 0.12% for definite keratoconic patterns. The possible discrepancy in subjective assessment of the corneal pattern through a Placido disc, an inadequate method, by the large number of examiners rendered this study unreliable. The Central India Eye and Medical Study is a population-based study that included 4,667 subjects in rural India [33] and found a prevalence of 2.3%. KC was defined as an anterior corneal refractive power exceeding 48 D, as measured by keratometry. Since keratometry measures the central corneal power, it is likely to miss some inferior cones. In addition, not all subjects with refractive power exceeding 48 D will have KC. Therefore, this estimation must be viewed with caution. The population-based Beijing Eye Study included 3468 individuals [68]. Steep cornea/KC was found to be 0.960 ± 2%, defined as an anterior corneal refractive power exceeding 48 D measured using optical low coherence reflectometry biometry of the right eyes only. These results must be interpreted with the same caution as the previous study. Another investigation of French army recruits using videokeratography arrived at a prevalence of 1.2%, but the results of the various indices were more compatible with suspect than definite cases [66].

More definite prevalence studies have been conducted since 2009 in the Middle East and Asia, using in most instances videokeratography, which afford better detection. For example, Millodot et al. [27] described how they diagnosed KC with a combination of topographic pattern, dioptric power of the corneal apex, and inferior-superior asymmetry to determine normal KC suspect and definite KC. Table 2 illustrates the population-based studies published thus far. It can be seen from the table that in the last few years almost all prevalence studies have relied on the use of videokeratography. As shown in Table 2, these modern studies result in a higher prevalence of KC than previously thought, ranging from 0.9% to 3.3%. Comments on the ethnic and geographical differences will be discussed in Section 3.2.4.

3. Risk Factors for KC

3.1. Environmental Factors. It is commonly accepted that the etiology of KC is multifactorial combining environmental and genetic factors [1, 101–103]. Moreover, it seems that an environmental factor may be essential to act as a trigger of the condition in genetically predisposed individuals. Environmental factors, which have been recognized, are eye rubbing, atopy, and UV exposure, although the relative contribution of all these factors is currently unknown [6]. An excess of any of these environmental factors cause oxidative damage to KC corneas because of the inability of KC corneas to process reactive oxygen species (ROS), which leads to a degradation process leading ultimately to corneal thinning and loss of vision [104] due to a lack of corneal enzymes such as aldehyde dehydrogenase class 3 (ALDH3), catalase, or superoxide dismutase to remove or neutralize the ROS [105].

3.1.1. Eye Rubbing. An association between eye rubbing and KC has long been described [24, 78, 81, 102, 106, 107] and accepted as a risk factor. Most authors report that about half of KC patients rub their eyes, although the percentage varies according to the study (see review in [82]). Obviously, there are some variations in this association whether the eye rubbing is gentle or vigorous [79, 108] and the usual length of rubbing in KC patients is much longer (from 10 to 180 seconds) than the typically less than 15-second duration of rubbing in allergic or infective ocular disorders [109] and less than 5 seconds in people without any eye condition [78]. Noteworthy are cases of asymmetric KC in which the most affected eye was the one which was rubbed most vigorously [78, 110, 111]. Coyle [112] reported the case of an 11-year-old boy who, at the age of 5, discovered he could stop his paroxysmal atrial tachycardia by vigorously massaging his left eye (up to 20 minutes a day). At the age of 7, his ocular examination was normal. By the age of 11, the child had developed unilateral KC in his left eye. Another case reported a patient with a history of vigorous daily ritual massaging of the left eye which had led to unilateral KC in that eye [113]. A series of cases confirm the asymmetric expression of the disease in patients who habitually rub the more affected eye [110, 111, 114].

Case-control studies provide the most convincing evidence of an association between KC and eye rubbing. The first was by Bawazeer et al. [24], who conducted a logistic regression analysis that included atopy and family history of KC and found that only eye rubbing was significantly associated with the disease, with an odd ratio (OR) of 3.98. This was confirmed in other logistic analyses [115, 116]. Nevertheless, this strong association has not been reported by all authors. Although they usually find a large percentage of KC patients who rub their eyes, the control group does as well [27, 41, 79]. The discrepancy may stem from the amount of dust in dry climates inducing frequent eye rubbing in both patients and controls, thus concealing a possible association.

Still, most authors who reviewed the pathogenesis of KC consider eye rubbing to be strongly associated with the disease [6, 104, 117]. There is mechanical trauma which could be caused by chronic eye rubbing, as well as a result of poorly fitted rigid contact lenses [104, 118, 119]. Nevertheless, this association is not necessarily causative. Indeed a fair percentage of individuals develop KC without any history of eye rubbing. It could be that abnormal rubbing habits start as KC develops and vision is impaired. However, there are a large number of patients with a history of habitual eye rubbing before the development of KC [15, 120, 121] and one is compelled to accept eye rubbing as a risk factor at least in some forms of KC in genetically susceptible people [122].

The microtrauma caused to the epithelium by rubbing KC corneas generates elevated levels of matrix metalloproteinases MMP-1 and MMP-13 [123, 124], which are secreted by epithelial and stromal cells, and inflammatory mediators including IL-6 and TNF-α [5, 125]. The release of these factors form part of the process that leads to KC and its progression. The processes include apoptosis of keratocytes as a result of increased levels of interleukin IL-1 with subsequent loss of stromal volume [126]. Direct experimental evidence of an association between KC and eye rubbing has been demonstrated in a group of volunteers without the disease and not wearing contact lenses who were instructed to rub their eyes in a controlled fashion for 60 seconds. Basal tears were collected before and after eye rubbing and it was found that levels of MMP-13, IL-6 and, TNF-α were significantly increased after rubbing. The authors concluded that persistent eye rubbing, common in KC patients, may contribute to the progression of the disease by continuous elevated levels of these protease, inflammatory mediators and protease activity [127, 128].

3.1.2. Atopy. Atopy is a hypersensitivity reaction, which comprises allergy, asthma, and eczema. There are some conflicting reports of an association between KC and atopy. A positive association has been noted by many authors [80, 82, 129, 130], but others did not find a statistically significant association when compared to a control group [24, 75, 131, 132]. It should be noted that in the nonsignificant findings [75] the control group came from the general population rather than an age- and sex-matched group. The discrepancy may stem not only from different severity of the condition or methods of assessment, which is based on patients' self-report, but also from the fact that some authors did not differentiate between the effects of the hypersensitivity reaction [24, 27, 75, 131, 132], whereas others only assessed one symptom of atopy, such as allergy, but did not include asthma or eczema [78, 102] and others assessed only allergy and asthma and not eczema [80]. Using a multivariate logistic regression analysis, Bawazeer et al. [24] concluded that atopy was not significantly associated with KC but with eye rubbing. These authors suggested that atopy was only associated indirectly because the itch that it induced led to eye rubbing. Still, Kaya et al. [130] showed that people with KC and atopy had a steeper and thinner ectatic cornea than age- and sex-matched people with KC but without atopy.

Allergy, induced by pollen, dust, antibiotics, or animal fur, is often associated with KC compared to controls or the general population [11, 29, 39, 59, 78–80, 82, 115]. It is found in about a third of KC patients, but the percentage varies according to the study (see Table 3). It should be noted that most of these studies were dependent on self-reported allergies. In some of these studies the control group came from the general population [29, 39, 79, 82], but a significant association was shown in several studies, which included an age- and sex-matched group [74, 78, 80, 115, 132]. Although allergy may cause eye rubbing, it is not the only provocative factor, since a much higher percentage of patients rubbed their eyes than the percentage of patients with allergy. Asthma and particularly eczema are reported less commonly than allergy (see Table 3) and it would appear that these reactions are less frequently reported in some of the studies conducted in the Middle East, India, and Singapore [34, 61, 67, 81, 115, 133]. This may be due to the hot and sunny climate of these countries, although Georgiou et al. [25] reported small percentages among Asian living in the UK

Table 3: Percentage of allergy, asthma, and eczema in KC patients from several studies.

Study	Year	Allergy	Asthma	Eczema
Copeman [72]	1965	27		32
Karseras and Ruben [73]	1976	34.6	34.6	18.6
Rahi et al. [74]	1977	15	3	2
Gasset et al. [75]	1978	35.7	17.9	8.2
Swann and Waldron [76]	1986	42.2	15.8	12.3
Ihalainen [59]	1986	35	8	24
Harrison et al. [77]	1989	37.3	28.4	31.3
Tuft et al. [10]	1994	35.2	25.2	19.9
Zadnik et al. [39]	1998	53	14.9	8.4
Owens and Gamble [29]	2003	57	34	30
Mcmonnies and Boneham [78]	2003	39		
Georgiou et al. [25]	2004	20 W, 9 A	38 W, 18 A	14 W, 7 A
Assiri et al. [61]	2005	39.2	5.6	8
Weed et al. [79]	2008	30	23	14
Nemet et al. [80]	2010	17.6	8.2	
Jordan et al. [11]	2011	25.5	26.2	22.4
Khor et al. [81]	2011	1.8	26	18.4
Shneor et al. [82]	2013	34.4	13.2	6.6

A, Asian; W, white.

compared to white, suggesting an ethnic difference. Table 3 presents the percentage of patients with atopic reaction in several studies.

3.1.3. Sun Exposure. Ultraviolet light (UV) is a source of reactive oxygen species (ROS) and excessive exposure to sunlight leads to oxidative damage to KC corneas, in which there is a reduced amount of the enzymes including aldehyde dehydrogenase class 3 (ALDH3) and superoxide dismutase necessary to remove the ROS [104, 105]. Hence, the higher prevalence of KC in hot, sunny countries compared to Europe and North America has led to the belief that the high sun exposure in these countries accounts for the high prevalence (see Tables 1 and 2). For example, in Jerusalem where the prevalence was found to be 2.34% [27], the mean annual number of hours of sunshine is 3397 according to the "Climatological information for Jerusalem, Israel" (http://www.hko.gov.hk/wxinfo/climat/world/eng/europe/gr_tu/jerusalem_e.htm). Such weather conditions are not unlike those prevailing in Saudi Arabia [61], Lebanon [67], India [33], and Iran [64, 69–71] in contrast to Finland [59], Minnesota [15], Urals [60], Japan [28, 58], or Denmark [62]. Additional evidence comes from animal experiments in which mice exposed to UV light demonstrated a degeneration of stromal collagen and stromal thinning with a marked loss of keratocytes [134]. This last study confirmed an earlier report of UV exposure of an anaesthetized rabbit cornea, which resulted in apoptosis of cells in all layers of the cornea as well as keratocytes [135].

However, it must be noted that UV radiations might provide a beneficial effect by inducing cross-linking of corneal collagen, thus mitigating either the development or the progression of the disease [136]. Moreover, sun exposure cannot explain the discrepancy found in the English Midlands where Indians, Bangladeshi, and Pakistani have 4.4 and 7.5 times [25, 30] higher KC prevalence than whites living in the same ambient environment. And neither can it account for the 7.9% KC prevalence reported in Tehran among non-Persians (Arabs, Turks, and Kurds) compared to 2.5% prevalence of Persians [70] or the significantly steeper corneas of Indians compared to Chinese or Malays, all living in Singapore [137]. Nevertheless, it is likely that the oxidative damage caused by UV radiations combined with a genetic factor such as consanguinity precipitates or accelerates the disease process. Research is needed to elucidate the role of sun exposure in KC, possibly in the form of a case-control investigation using a validated questionnaire.

3.1.4. Miscellaneous. Exposure to environmental neurotoxins such as nicotine in the form of cigarette smoking has not been found to be associated with KC, neither in a case-control study [115] nor in observational studies [33]. In fact, there may be a negative correlation between cigarette smoking and KC possibly because the by-products of smoke may lead to cross-linking of collagen in the cornea [138]. On the other hand one report from the Urals indicated more cases of KC in the urban centers with polluting industries than in the rural areas [60].

3.2. Socioeconomic Factors

3.2.1. Age. KC onset varies between the early teenage years and young adulthood and it seldom appears after the age of 35 years [2]. In a cohort of 196 patients, 18 years was the most frequent age of onset [59] and it was 15.39 (±3.95) in another study [139]. However, most reports give the age of diagnosis, which is some years after onset because the disease is usually

asymptomatic at first. The mean diagnostic age ranged from 20.0 years (±6.4) [140] to 24.05 (±8.97) [31] in most studies [29, 141–143]. Interestingly, the age of first presentation was found to be significantly younger in Asians than in white patients by 4 to 5 years in three different studies carried out in the English Midlands (22.3 ± 6.5 versus 26.5 ± 8.5 [30]; 21.5 versus 26.4 [25]; and 23.0 ± 7.0 versus 27.8 ± 8.1 [144]). Recent reports on pediatric CXL demonstrate onset at the end of the first decade of life or early in the teen years [145–148]. This leads to the notion that either the age of onset has decreased or the medical community is being more diligent in early diagnosis.

Since the disease is chronic one would expect to find at least a similar proportion of patients in older compared to in younger patients. That is not the case, especially after the age of 50 years and this has intrigued many authors [9, 149–154], although in one study the number of old KC patients was found to be substantial [155]. Most of these studies report low percentage of KC patients beyond 50 years, ranging from 7.4% [152] to 15% [39], with one exception 40% [155]. The reason may rest in the more efficient methods of diagnosis of the disease in recent years, such as videokeratography, or it may be because there are now more people with an allergy in the general population [156]. Another possibility is that KC patients have reduced longevity compared to the general population, as has been suggested by some authors [150, 152, 154] because of an associated fatal condition, for example, mitral valve prolapse [157, 158], obesity [159, 160], or obstructive sleep apnea [160, 161], although the mortality rate of a population of KC patients was not found to be significantly different than that of the general population [151]. Nevertheless, the question as to what happens to KC patients beyond the age of 50 years remains to be elucidated, possibly by comparing the corneas of older KC patients with an age-matched control group.

About 20% of KC patients will eventually require surgery, although there are wide variations in percentages among the studies, with a seemingly lower percentage in the Far East (India, China, Singapore, and Japan) than in the rest of the world (see review in Kok et al. [162]). Nevertheless, the deleterious effects of this chronic disease, in which a substantial percentage of patients will require invasive surgery and for the other patients a lifelong need for specialized contact lens fitting, represent a serious burden not only for the individual but also for the national health services of a country.

3.2.2. Geographic Location. It was thought that KC affected all countries equally [3]. However, it has become obvious, especially in the past decades, that KC prevalence is not the same throughout the world, as the presently available studies can reveal (see Tables 1 and 2). Northern Europe and the Urals have low prevalence [25, 30, 59, 60, 163], as well as northern USA [15, 65]. Prevalence is also low in Japan [28, 58]. On the other hand it is relatively high in countries of the Middle East [27, 41, 61, 64, 69–71], India [33], and China [68]. The Middle East countries in particular, as well as parts of India, are characterized by hot and sunny climates with very little rain as distinct from the other countries. Could the climate influence the development of KC, especially the oxidative damage caused by excessive sun exposure to ultraviolet light [104]? Is there an inherent difference in the people, such as ethnic backgrounds, or could the very different styles of life with nutrition play a role? There is also the possibility that in these countries the disease affects more the poor people, a factor known to increase the proportion of chronic diseases [164]. These are puzzling questions that need elucidation to better understand the pathogenesis of KC.

3.2.3. Parental Education. It has been suggested that there exists an association between low parental education and KC, because parental education is associated with socioeconomic status [165]. Children living in poverty are brought up in environments with air, water, and waste contamination problems [166], which are hazardous to their health. As a consequence, these children are at risk or suffer from a host of disorders, such as asthma, cancer, hyperactivity, and obesity [167]. Several investigators have reported an association between obesity and KC [159–161, 168]. Therefore, it could be inferred that there exists an association between low parental education and KC since low parental education is linked to low socioeconomic status. To the best of our knowledge there is not as yet a report of such an association.

3.2.4. Ethnic Differences. Until some years ago it was assumed that KC affected all races equally [3]. However, it has now been demonstrated unequivocally that there are differences in KC prevalence among ethnic groups. It was first noted by Pearson et al. [30] who found that Asians (Indians, Bangladeshi, and Pakistani) living in the English Midlands had an incidence of the disease 4.4 times higher than in whites. This was confirmed in two other investigations also conducted in the Midlands where the difference in incidence was 7.5/1 [25] and 9.2/1 [144]. Other studies have demonstrated a difference among ethnic groups of the same country. In Iran, KC prevalence was found to be three times less in the Persian ethnic population than in the non-Persians (Arabs, Turks, and Kurds) [70]. In Singapore, steep cornea possibly reflecting KC was found to be significantly steeper in Indians than in Malays or Chinese [137]. In addition, the age of onset of the disease has been found to be generally younger in Asians than in Caucasians [25, 30, 79, 144]. The age of onset, or more specifically diagnosis, of most Asians is in the early 20s whereas it was much older in the CLEK study (n = 1209 patients) [39]. Differences in KC prevalence and age of onset among ethnic populations strongly suggest that genetic influences play an important role in the pathogenesis of the disease. This is discussed below.

3.3. Familial Factors. A large positive family history of the disease may stem from either environmental or genetic causes. It is not always clear which of the two is most influential in the pathogenesis of the disease without establishing a family pedigree. The recent data on the strong association of parental consanguinity/endogamy with KC suggests a strong genetic component to the development of KC in many studies [115].

3.3.1. KC in the Family. Although the most common type of KC is sporadic [102], many studies have reported the presence of large number of familial KC. The rate ranges from 5% to 27.9% [15, 41, 59, 79, 82, 102]. In the study in which a rate of 27.9% of KC was found in at least one person in the family, it was further noted that affected first-degree relatives represented 20.5% [82]. It was much lower (3.34%) in first-degree relatives when the family history was not self-reported by the cases but determined by videokeratography [169]. This was still 15–67 times higher in those who had developed the disease than in those who did not have relatives with KC. In another study in which relatives (first-degree and others) were evaluated topographically, 14% of family members were found to have KC [170]. The discrepancy between the latter two studies may reflect a greater prevalence of KC in the general population of the second, which was conducted in Turkey whereas the other was in America. Most percentages of general family history are usually lower than 20%. Typical results of family history from large sample population of KC patients are 12.4% [11], 13.5% in the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) [39], and 17.8% in another large cohort [171]. Interestingly, in the Dundee University Scottish Keratoconus Study (DUSKS) [79] the rate for Caucasians was 5% but it was 25% for the small Asian subgroup (Indian subcontinent) who participated in the study. This last result is not surprising as one would expect a higher level of positive family history in communities with a greater prevalence of KC. This was the case in several studies in which KC prevalence was high and so was family history, 23% [27], 22.9% [115], and 27.9% [82], as well as in a study involving KC patients in families with a lot of children as found in northern Finland 19% versus 9% in southern Finland, where families had few children [59]. The large variation in the percentage of family members with the disease (3.34%–27.9%) may indicate different expression of KC with different modes of inheritance [59, 101, 115, 169].

3.3.2. Consanguinity. Consanguinity, the marriage between relatives, has been shown to be associated with a host of disorders: childhood mortality [172], deafness [173], sickle-cell anemia [174], hydrocephalus, postaxial polydactyly and facial clefts [175], heart disease [176], multiple sclerosis [177], tuberculosis and hepatitis B [178], preterm birth [179], and physical and mental handicap [180–182].

Over the years several authors have alluded to a possible association between KC and consanguinity [25, 101, 144, 183]. Evidence was provided by a report by van der Hoeve in 1924 [184] who presented a family pedigree in which three of the six children of a consanguineous couple had KC. In another report with suggestive evidence one in 400-Pakistani family, who came from a tradition of consanguinity and living in England, was found to have KC compared to one in 30,000 whites [185]. However, the first study to establish a significant association was performed in a hospital in east Jerusalem in which KC Arab patients and controls, age- and sex-matched, were examined and all subjects completed a questionnaire asking about their parents' relationship. It was found that children of consanguineous parents had a fourfold risk of KC compared with children of unrelated parents after adjusting for other factors, using multivariate logistic regression analysis [115], and this association was much stronger with parents married to first cousins than second cousins. This result was further confirmed in a similar study conducted with students from an Arab College in Haifa in which a fivefold (or 5,1, 95% 1.41–18.33) risk of KC in offspring of consanguineous marriages [41] was found.

As already suggested by Georgiou et al. [25] and Cozma et al. [144] the large discrepancy in the prevalence of KC between Asian, mostly of Pakistani origin, and white patients could be attributed to the tradition of consanguineous, especially first-cousin marriages. In fact, practically all countries with a high KC prevalence as noted in Tables 1 and 2 are from the Middle East and India which have a tradition of consanguinity, especially in their Muslim ethnic communities [186–189]. In Pakistan, approximately 60% of marriages are consanguineous, over 80% of which are between first cousins [190]. In Israel, population surveys have found that Israeli Arabs have a high rate of consanguinity, 42–45%, with 28% being first-cousin marriages [191]. For Israeli Jews, consanguinity is much lower ranging from 1.5 to 7.1% depending on the community, with 0.4 to 1.2% being first cousins [192]. However, endogamy is relatively common among Israeli Jews and it may play a role contributing to the high prevalence of KC in Israel [27]. The high corneal steepness found in Indians compared to Chinese or Malays all living in Singapore was also suggested to have been caused by consanguinity among the former [137].

If both parents are first cousins, they could both be carriers of a mutant allele at the same locus leading to corneal ectasia. The result of these studies points not only to a genetic component of the disease, but more specifically to an autosomal recessive inheritance. This is in contrast to other forms of KC in which many patients with a positive family history described in the literature, but mainly from western countries, present a family pedigree suggesting an autosomal dominant inheritance [101, 102]. Strong support for a genetic basis for KC comes from segregation analysis of genetic models based on 95 keratoconic families evaluated by videokeratography [169]. It appears undeniable that the genetic effect of consanguinity plays an important role in the pathogenesis of KC and is the principal factor that accounts for the differences in prevalence among ethnic groups and possibly geographic locations. It may, however, require to be combined with an environmental factor to be activated and lead to KC.

Twin studies in which there is a concordance in the topographic pattern of a monozygotic pair add evidence to a genetic contribution to KC. To date, 21 pairs have been reported, although many of these were described before the advent of videokeratography. Nevertheless, more than half of these pairs were found to be concordant [31, 59, 193–195], the others being discordant [196–198]. A study comparing dizygotic (DZ) and monozygotic (MZ) twins has been reported [142] in which significantly more concordance was found in MZ than in DZ providing further evidence of a genetic contribution to the disease.

Table 4: List of the identified genomic loci through linkage studies.

Population	Location	Mode of inheritance	Gene	Reference
Australian	1p36.23-36.21	Autosomal dominant		[83]
Ecuadorian	2q13-q14.3	Autosomal dominant		[84]
European, Arabic, Caribbean African	2p24			[85]
Italian	3p14-q13	Autosomal dominant		[86]
Caucasian, Southern Italian	5q14.3-q.21.1	Autosomal dominant		[87, 88]
Caucasian, Hispanic	5q23.2			[89]
Southern Italian	5q32-q33			[87]
Australian	8q13.1-q21.11	Autosomal dominant		[83]
Caucasian, Hispanic	9q34			[89]
Ecuadorian	13q32	Autosomal dominant	DOCK9	[90–92]
Southern Italian	14q11.2			[87]
Caucasian, Hispanic	14q11.2			[89]
Multiethnic	14q24.3			[93]
Southern Italian	15q2.32			[87]
Northern Irish	15q22.33-24.2	Autosomal dominant	miR-184	[94–97]
Finnish	16q22.3-q23.1	Autosomal dominant		[98]
Pakistani	17p13	Autosomal recessive		[99]
Ecuadorian	20p13-p12.2			[84]
Australian, Tasmania	20q12	Autosomal dominant		[100]

4. Genetic Studies of KC

4.1. Traditional Linkage Studies. As discussed above, genetics plays an important role in the pathogenesis of KC. Relatives of KC patients have an elevated risk compared to those with unaffected relatives. Most of the familial KC is autosomal dominant while autosomal recessive pattern has also been suggested. Family-based linkage studies have identified at least 19 candidate genetic loci that may harbour genetic mutations for KC (Table 4) [199]. This clearly indicates the genetic heterogeneity of KC pathogenesis. Although most of these genomic loci have not been independently replicated, the chr5q21.2 region has been independently replicated in three separate studies [87–89]. Recently this region has been further confirmed with high density single nucleotide polymorphisms (SNPs) based linkage [200]. The overlapping region from these three studies strongly suggests the possibility of a common locus for KC pathogenesis. Another linkage locus chr5q32-33 reported by Bisceglia et al. was identified as suggestive linkage with KC by Li et al. [87, 89]. A suggestive linkage locus in chr14q11.2 was reported by these two studies. A linkage locus chr16q22.3-q23.1 identified by Tyynismaa et al. is very close to a suggestive linkage region identified by Bisceglia et al. [87, 98]. It should be noted that Burdon et al. reported two genomic regions chr1p36.23-36.21 and chr8q13.1-q21.11 with equal evidence of linkage (LOD score of 1.9 each) [83]. Analysis of both loci concurrently, meaning digenic inheritance of two loci, suggests a two-locus LOD score of 3.4. However, no mutations were identified in six candidate genes that were expressed in the cornea [83].

A number of efforts have been performed to identify the genetic mutations in these linkage regions. A 5 Mb genomic region on chr15q22-q25 was originally mapped in a large three-generation Northern Irish family with 18 affected individuals [94, 95]. All the affected family members had severe anterior KC and early-onset anterior polar cataract [95]. The inheritance was autosomal dominant. All genes in this 5 Mb genomic region were enriched using a custom sequence capture array from NimbleGen followed by second generation sequencing (a Genome Analyzer II from Illumina). A mutation (r. 57c>u) was identified within the seed region of *miR-184*. *miR-184* is a microRNA (miRNA), which is small regulatory strands or RNA with 19–25 nucleotides in size [94]. miRNA mostly binds to complementary sequences in the 3′ untranslated region (UTR) of mRNA of target genes, leading to mRNA degradation or translational repression. *miR-184* is abundantly expressed in cornea and lens. It was considered that miR-184 with this specific mutation fails to compete with another miRNA—*miR-205* for overlapping target sites on the 3′-UTR of two target genes, *INPPL1* (inositol polyphosphate phosphatase-like 1) and *ITGB4* (integrin beta 4). These two genes are involved in corneal healing after injury as the principal component of corneal basal epithelial hemidesmosomes [94]. The same mutation in *miR-184* has been replicated in other KC patients with congenital cataracts [96, 201]. Two additional mutations (r.8c>a and r.3a>g) were reported in sporadic KC patients with very low frequency (2 in 780 patients) [97]. These two sporadic KC patients did not have congenital cataracts. These two mutations may have incomplete or reduced penetrance in the studied families. However, we did not find any mutations in over 140 KC patients from Saudi Arabia (unpublished data). All these indicate that mutations in *miR-184* only account for a relative small number of KC patients or that miR-184 contributes to the causal of congenital cataract instead of KC. The identification of *miR-184* in KC patients suggests that regulatory

variants may directly impact transcriptional activity of key target genes in cornea development and maintenance. More research will be necessary to study whether miR-184 may regulate the expression of other KC candidate genes.

Chr13q32 was originally identified to be linked with familial KC in Ecuadorian families, under an autosomal dominant model [90]. Mutation screening of 8 candidate genes in this region identified a potential mutation c.2262A>C (p. Gln754His) in *DOCK9* (dedicator of cytokinesis 9) in a large Ecuadorian KC family [91]. *DOCK9* (OMIM 607325) encodes a member of the DOCK protein family with GTP/GDP exchange factor activity that specifically activates G-protein CDC42 [202]. *DOCK9* is expressed in human cornea [91]. However, it still requires to be replicated in other KC families and patients [92] as well as functional work of the reported mutation in cornea.

4.2. Genome-Wide Association Studies. Genome-wide association studies (GWAS) examine several hundred thousand to over a million SNPs in hundreds to thousands of individuals using high throughput DNA genotyping technology [203]. GWAS has been shown to be very powerful to identify the genetic factors of many complex traits and diseases, including central corneal thickness (CCT) and KC. A number of GWAS reported the association of CCT with sequence variants near or within many genes, including *ZNF469*, *COL5A1*, *RXRA-COL5A1*, *COL8A2*, *AKAP13*, *AVGR8*, *FOXO1*, *FNDC3B*, *TJP1*, *NR3C2*, *LRRK1*, *FDF9-SGCG*, *LCN12-PTGDS*, *ADAMTS6*, *CHSY1*, *HS3ST3B1-PMP22*, *GLT8D2*, *SMAD3*, *VKORC1L1*, *COL4A3*, *FAM46A-IBTK*, *LPAR1*, *ARID5B*, *TBL1XR1-KCNMB2*, *ARHGAP20-POU2AF1*, *C7ORF42*, *MPDZ-NF1B*, *USP37*, *GPR15*, and *TIPARP* [204–208]. Two CCT-associated genomic regions *FOXO1* and *FNDC3B* have been associated with KC risk [207]. These genetic discoveries implicate the role of the collagen and extracellular matrix pathways in the regulation of CCT [207] and potentially KC. Recently, two studies identified that missense variants in *ZNF469* have been identified in 12.5% and 23.3% of sporadic KC patients in UK/Switzerland and New Zealand, respectively [209, 210], indicating the potential role of *ZNF469* in the development of KC. However, more replicative sequencing and further functional studies will need to determine the relative role of *ZNF469* in the pathogenesis of KC. Recently, our group has identified several genomic deletions in familial KC patients in several CCT-associated regions, including *RXRA-COL5A1* and *HS3ST3B1-PMP22*, as well as a refractive error-associated region of *GRIA4* [211]. The genetic variants in *ZNF469* and genomic deletions in these genes indicate the potential contributions of these CCT-associated genes in the pathogenesis of KC.

The first GWAS with KC was reported by Li et al. in 2011 in a Caucasian population of 222 patients and 3324 controls [212]. Although no genome-wide significant associations (P value $< 5 \times 10^{-8}$) were identified, a suggestive association (P value 1.6×10^{-7}) was reported with a genomic region located near the *RAB3GAP1* (RAB3 GTPase activating protein subunit 1 (catalytic)) gene on chromosome 2q21.3. This association has been replicated in a separate study by Bae et al. [213], suggesting the genetic contribution of this region to KC susceptibility. RAB3GAP1 is involved in regulation of RAB3 activity by forming a heterodimer with RAB3GAP2 to convert active RAB3-GTP to the inactive form RAB3-GDP [214]. Interestingly, mutations in *RAB3GAP1* are associated with Warburg Micro Syndrome, a rare autosomal recessive syndrome with ocular and neurodevelopmental defects, such as microphthalmos, microcornea, congenital cataracts, and optic atrophy [214–217].

The second GWAS with KC was followed by Burdon et al. in a population of patients from Australia using pooled DNA from 97 KC patients and 216 controls [218]. While no variants reached genome-wide significance, the most significant association (9.9×10^{-7}) was located upstream of the *HGF* (hepatocyte growth factor) gene. The specific variant was also associated with serum HGF level in normal individuals [218]. This association has been independently replicated by Sahebjada et al. [219]. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine signalling cascade [220]. The genomic region of *HGF* has been associated with refractive error in several populations including Han Chinese and Caucasians [221–223]. The association of *HGF* with KC suggests the potential involvement of HGF-related inflammatory pathways.

4.3. Candidate Genes. A large number of candidate genes have been studied in relation to KC pathogenesis. We will focus on two main candidate genes, visual system homeobox 1 (*VSX1*) and superoxide dismutase 1 (*SOD1*). *VSX1* is located within a linkage locus for a corneal dystrophy called posterior polymorphous dystrophy (PPCD) [224–226], which has been associated with KC [227–233]. Since PPCD and KC have similar corneal curvature and the involvement of posterior surface of cornea, specifically Descemet's membrane, PPCD and KC might be linked due to poor case definition. In 2002 *VSX1* mutations were first reported in PPCD and KC patients [234], in which two mutations (R166W and L159 M) were originally identified in KC patients. *VSX1* encodes a pair-like homeodomain protein which binds to the core of the locus control region of the red and green visual pigment gene cluster and may regulate expression of the cone opsin genes during embryonic development [235, 236]. It is expressed in several ocular tissues including the retina [224, 226, 234]. The expression of *VSX1* in human or mouse cornea remains unclear since many studies did not confirm the expression in cornea [234, 236, 237]. Mouse models with the loss of *VSX1* function did not show cornea-related phenotypes [235]. Since the original report in 2002, many studies have examined the potential mutations of *VSX1* in KC patients [90, 238–255]. Most of the identified variants are polymorphic [199]. It remains unclear whether *VSX1* mutations contribute to the pathogenesis of KC [37, 162, 256]. It is possible that mutations in *VSX1* only affect a very small percentage of KC patients, which is consistent with the concept of genetic heterogeneity of KC. It is also more possible that *VSX1* may not play a significant role in the pathogenesis of KC. We recommend future research efforts focus in the identification of novel genetic factors in KC.

SOD1 encodes a major cytoplasmic antioxidant enzyme that metabolizes superoxide radicals and provides a defence against oxygen toxicity [257]. Mutations in *SOD1* have been implicated in familial amyotrophic lateral sclerosis (ALS) [257, 258]. However, no corneal phenotypes have been reported in ALS patients. To date, it is widely accepted that oxidative stress plays a critical role in the progression of KC [37, 240]. An accumulation of cytotoxic by-products, mitochondrial DNA damage, and high levels of oxidative stress in KC-affected corneas [259–262] have been reported. *SOD1* has been selected as a candidate gene and examined in many KC-related studies [239, 249, 255, 263–265]. However, no mutations in *SOD1* have been identified in KC patients. It remains undetermined whether *SOD1* plays a role in the pathogenesis of KC.

4.4. Future Direction. Recent development in genome technology has enabled the application of novel and high throughput genetic approaches in ocular genetics research. Among these technologies, whole exome or genome sequencing will be very powerful in the identification of causal mutations in multiplex families with KC [266–268]. Many research laboratories around the world, including our group, have applied the whole exome sequencing to identify causal mutations in multiplex KC families. Previously identified linkage region will be tremendously helpful to assist the interpretation of exome or genome sequencing data. As discussed earlier, the genetic heterozygosity of KC may prevent a single research group from identifying and replicating novel genetic mutations. It will be necessary for different KC research groups to collaborate with each other, by sharing DNA samples and phenotype data. A genetics research consortium may be one of the approaches. The integration of next generation sequencing has recently led to the identification of *miR-184* mutations in KC patients. We expect to see more peer-reviewed reports using next generation sequencing in the near future. At the same time, in comparison to GWAS studies with small sample size, GWAS approach with large number of cases and controls in different ethnic groups will greatly improve the chances of avoiding type I errors and will continue to identify novel genomic variants that are associated with KC and cornea-relative phenotypes. In addition, gene expression profile in normal and diseased human cornea will provide further information to help narrow down the list of potential causal genes.

5. Conclusion

In summary, KC is the most common ectatic disorder of cornea with the onset of puberty. It affects both genders and all ethnic groups worldwide. Both environmental and genetic factors contribute to the pathogenesis of KC. Significant achievements have been made in the understanding of its epidemiology and etiology. Newly developed genetic technologies including whole exome or genome sequencing and genome-wide association technologies have promoted and will continue to improve our knowledge on the pathogenesis of KC. This knowledge will eventually lead to future development of improved early diagnostics, targeted therapeutics, and potential prognosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Acute Intermittent Porphyria in Argentina: An Update

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Porphyrias are a group of metabolic diseases that arise from deficiencies in the heme biosynthetic pathway. A partial deficiency in hydroxymethylbilane synthase (HMBS) produces a hepatic disorder named Acute Intermittent Porphyria (AIP); the acute porphyria is more frequent in Argentina. In this paper we review the results obtained for 101 Argentinean AIP families and 6 AIP families from foreign neighbour countries studied at molecular level at Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP). Thirty-five different mutations were found, of which 14 were described for the first time in our population. The most prevalent type of mutations was the missense mutations (43%) followed by splice defects (26%) and small deletions (20%). An odd case of a double heterozygous presentation of AIP in a foreign family from Paraguay is discussed. Moreover, it can be noted that 38 new families were found carrying the most frequent mutation in Argentina (p.G111R), increasing to 55.66% the prevalence of this genetic change in our population and adding further support to our previous hypothesis of a founder effect for this mutation in Argentina. Identification of patients with an overt AIP is important because treatment depends on an accurate diagnosis, but more critical is the identification of asymptomatic relatives to avoid acute attacks which may progress to death.

1. Introduction

The porphyrias are a heterogeneous group of metabolic disorders that result from the decreased activity of a specific enzyme of the heme pathway and are characterized by the overproduction and excretion of heme intermediates in urine and/or stool and their accumulation in certain tissues [1–3].

Acute Intermittent Porphyria (AIP, OMIM 176000) is the most common of the acute hepatic porphyrias. It is an autosomal dominant disorder caused by a deficient activity of hydroxymethylbilane synthase (HMBS, EC 4.3.1.8), also referred to as porphobilinogen deaminase, producing a markedly increase in the urinary excretion of ALA and PBG. The symptoms may frequently appear at any time after puberty and are characterized by acute neurovisceral signs which include intermittent attacks of abdominal pain, constipation, vomiting, hypertension, tachycardia, fever, and various peripheral and central nervous system manifestations. Acute attacks may frequently result from exposure to diverse porphyrinogenic drugs, alcohol ingestion, reduced calories intake due to fasting or dieting, infections, and hormones which stimulate heme synthesis by ALA-synthase induction, thereby increasing the production of the porphyrin precursors ALA and PBG [4, 5].

HMBS is the third enzyme involved in heme pathway and catalyzes the head to tail condensation of four molecules of PBG to form the lineal tetrapyrrole HMB. It is encoded by a single gene localized at the chromosomal region 11q23.3. The cDNA and the entire 10 kb gene have been sequenced including the 5′ regulatory, 3′ regulatory, and intronic regions. The gene contains 15 exons and 2 distinct promoters that generate housekeeping and erythroid transcripts by alternative

splicing and cDNAs encoding the 44-kD housekeeping and the 42-kD erythroid-specific isoenzymes, which have been isolated and characterized [6].

AIP is the most common acute porphyria in our country [7]. It is an autosomal dominant disorder with incomplete penetrance although some cases of homozygosity or double heterozygosity have been described, in most cases associated with childhood and more severe manifestations [8–21]. The identification of asymptomatic heterozygotes in families with affected individuals is essential for their counselling to avoid specific precipitating factors, but as the enzyme assay is only about 80% accurate [1], the use of molecular techniques to identify specific mutations in the *HMBS* gene is essential for accurate diagnosis of affected members in AIP families [7].

To date about 390 different mutations have been identified in the *HMBS* gene causing AIP (Human Gene Mutation Database HGMD, http://www.hgmd.cf.ac.uk/ac/index.php); most of them were either private or found in a few unrelated families, showing the molecular heterogeneity of AIP.

We review here all the mutations found in 101 Argentinean and 6 foreign AIP families and studied during the last 20 years at CIPYP. Four new mutations and 31 already described genetic changes were found; some of them were detected for the first time in our population. It must be highlighted that 59 unrelated families carry the same mutation, p.G111R (55.66%), increasing its number with respect to that previously found [22], suggesting a founder effect for this genetic change as has been described for different mutations in other populations [23–25].

2. Materials and Methods

2.1. Patients. Informed consent was obtained from all patients following the standards of UNESCO Declarations-DD.HH Genome and Genetic Data (http://www.unesco.org/shs/ethics), Declaration of Helsinki was taken into consideration, and the study was approved by the Institutional Research Ethics Committee of the CIPYP, National Scientific and Technical Research Council (CONICET), University of Buenos Aires (UBA).

From March 1994 to July 2014, 106 unrelated Argentinean families were studied at biochemical and molecular level. All patients had current symptoms of AIP and the diagnosis was made on the basis of their clinical history of at least one acute attack associated with increased excretion of ALA and PBG in urine and reduced HMBS activity in red blood cells [1]. The final diagnosis of the patients was established by genetic studies. Unrelatedness was determined by family inquiries.

2.2. Identification of Mutations

2.2.1. DNA Isolation and HMBS Amplification. Genomic DNA was extracted from peripheral blood collected in EDTA using the commercial kit illustra^{TM} blood genomicPrep Mini Spin Kit (GE Healthcare). Mutational analysis was performed amplifying the promoters, all exons, and the intron/exon boundaries of the *HMBS* gene by PCR using the specific primers shown in Table 1. Promoter regions and genomic sequence from exon 3 to noncoding exon 15 were amplified in only two fragments using Platinum Taq DNA Polymerase High Fidelity enzyme (Invitrogen by Life Technologies). Alternatively, exons 3 to 15 and their flanking intron regions were amplified in 5 fragments as indicated (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/946387), employing recombinant Taq DNA Polymerase (Invitrogen by Life Technologies).

2.2.2. RT PCR. RNA was isolated from the leukocytes using the commercial kit Ribo Pure–Blood (Ambion) and reverse transcribed with M-MLV Reverse Transcriptase and Oligo $(dT)_{12-18}$ primers (Invitrogen), according to manufacturer's instructions. The HMBS cDNA was amplified with the primers Fc (5′ aaagcctgtttaccaaggagc 3′)–Rc (5′ caccaccagctccaagatgt 3′).

All PCR products were checked in 1.5% agarose gel.

2.2.3. Sequencing Analysis. The amplified products were purified with the Bioneer Accuprep PCR Purification Kit (Bioneer) or QIAquick PCR/Gel Purification Kit (QIAGEN) and were automatically sequenced by Macrogen (Macrogen Inc., Gangseo-gu, Seoul, Korea, ABI3730XL, Macrogen). The sequencing primers are listed in Supplementary Material. All mutations were confirmed by sequencing both DNA strands of at least two different PCR products. To validate the new mutations, their absence in 50 control individuals has been performed. Nucleotides were numbered according to the cDNA sequence for the housekeeping isoform of HMBS transcript variant 1 (GenBank Accession NM_000190.3) in which the A of the ATG initiation codon was numbered as 1.

2.2.4. Databases. The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/) was used for information about reported mutations in the *HMBS* gene.

3. Results

At present 177 AIP families (299 affected individuals) were diagnosed at CIPYP. Of them, 107 were also studied at molecular level and results for 48 families were already described [7, 28, 29, 34]. In the last 10 years 58 new families were also biochemically diagnosed as AIP and molecular analysis revealed 19 mutations, 4 new and 15 already reported of which 7 were described for the first time in our population (Table 1).

From the novel mutations, two were splice site mutations at acceptor splice sites. One was an A to G transition in the penultimate base of intron 8 leading to the in-frame deletion of 15 bp with the loss of the first 5 amino acids of exon 9 (c.423-2A>G) by the use of a cryptic site (Figure 1). The other was also an A to G transition in the last base of intron 14 (c.913-1G>A) predicting the skipping of exon 15.

In another family, an out of frame new duplication of 7 bp in exon 7 which generates a stop codon 17 bp upstream (c.301_307dupCCCACTG) was found (Figure 2).

Table 1: All 106 families studied at biochemical and molecular level in the last 20 years at CIPYP.

Exon/intron	Mutation	Nucleotide change	Effect	Number of families (affected individuals)	First reported
E3	p.R26C	c.76C>T	26 Arg > Cys	2 (9)	[26]
I3	IVS3ds+1G>A	c.87+1G>A	5′splice site mutation, exon 3 deletion	1 (4)	[27]
E4	p.Q34P	c.101A>C	34 Gln > Pro	6 (12)	[28]
	p.Q34X	c.100C>T	34 Gln > Stop	1 (2)	[26]
	p.T35M	c.104C>T	35 Thr > Met	1 (1)	[29]
	p.Y46X	c.138C>A	46 Tyr > Stop	1 (1)	[30]
E5	p.L68fsX69	c.202_203delCT	Out of frame deletion of 2 bp	1 (1)	[31]
E6	p.L81P	c.242T>C	81 Leu > Pro	1 (3)	[14]
E7	p.G111R	c.331G>A	111 Gly > Arg	59 (171)	[32]
	p.V103fsX120	c.298_304dupCCCACTG	Out of frame duplication of 7 bp with a stop codon at +17	1 (3)	This report
I7	IVS7+1G>C	c.344+1G>C	5′splice site mutation, deletion exon 7	1 (3)	[33]
E8	p.R116W	c.346C>T	116 Arg > Trp	1 (1)	[32]
I8	IVS8as-2A>G	c.423-2A>G	3′splice site mutation, deletion 15 bp	1 (5)	This report
	IVS8as-1G>T	c.423-1G>T	3′splice site mutation, deletion of 15 bp	2 (8)	[34]
E9	p.R149Q	c.446G>A	149 Arg > Gln	1 (1)	[35]
	p.A152del	c.453_455delAGC	Del Ala 152	1 (1)	[29]
E10	p.R173Q	c.518G>A	173 Arg > Gln	1 (4)	[36]
	p.R173W	c.517C>T	173 Arg > Trp	3 (4)	[37]
	p.R201W	c.601C>T	201 Arg > Trp	1 (1)	[38]
	p.Q204X	c.610C>T	204 Gln > Stop	1 (3)	[37]
I10	IVS10ds-1G>T	c.612G>T	Deletion of 3 aa in exon 10	3 (8)	[35]
E12	p.V221fsX242	c.665insA	Out of frame insertion of A at 665	1 (3)	[28]
	p.T243fsX249	c.728_729delCT	Out of frame deletion of 2 bp at 728-729	1 (5)	[28]
I12	IVS12ds+1G>A	c.771+1G>A	5′splice site mutation, deletion of exon 12	1 (1)	[28]
	IVS12as-1G>A	c.772-1G>A	3′splice site mutation, deletion of exon 13	1 (3)	[39]
E13	p.K272fsX287	c.815_818delAGGA	Out of frame deletion of 4 bp at 815	1 (3)	[28]
E14	p.G281del	c.841_843delGGA	In-frame deletion of GGA at 841	2 (7)	[28]
I14	IVS14-2A>G	c.913-2A>G	3′splice site mutation, deletion exon 15	1 (1)	[40]
	IVS14as-1G>A	c.913-1G>A	3′splice site mutation, deletion of exon 15	1 (1)	This report
E15	p.H301fsX306	c.913insC	Out of frame insertion of C at 913	2 (5)	[41]
	p.V315fsX328	c.948delA	Out of frame deletion of A at 948	1 (2)	[28]
	p.L329fsX341	c.985delTTGGCTGCCCAG	In-frame deletion of 329 LAAQ	1 (5)	[28]
	p.R321H	c.962G>A	321 Arg > His	1 (4)	[22]
	p.G335S	c.1003G>A	335 Gly > Ser	1 (4)	[28]
	g.3078_8306del5228bp		Deletion of 5228 bp from intron 2 to intron 15	2 (7)	This report

Table 2: Biochemical data and mutation status of the family from Paraguay.

Patient	Age	ALA mg/24 h	PBG mg/24 h	Porph. μg/24 h	PPIλ: 619 nm	HMBS activity	Mutation status
Proband	38	2.7	8.7	188	1.80	44.72	c.772-1 G>A
Mother	67	—	—	—	1.85	46.24	c.772-1 G>A
Husband	62	—	—	—	1.00	61.20	p.R321H
Daughter	23	6.6	32.5	589	1.23	58.06	c.772-1 G>A/p.R321H
Daughter	26	6.1	24.7	782	1.29	31.75	c.772-1 G>A/p.R321H
Daughter	30	1.0	1.2	37	1.00	44.77	p.R321H

Age in years at diagnosis. Porph.: porphyrins.
Normal values: ALA: ≤4 mg/24 h: PBG: ≤2 mg/24 h; porphyrins: 2–250 μg/24 h; Plasma Porphyrin Index (PPI) ≤1.30 (λ: 619); HMBS activity: 84.51 ± 11.96 U/ml GR (F); 73.13 ± 13.62 U/ml GR (M).

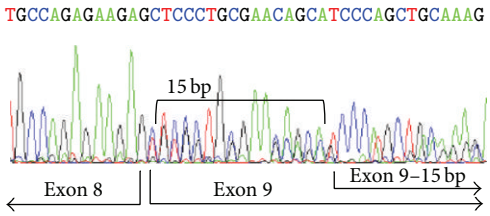

Figure 1: Electropherogram showing c.423-2A>G mutation RT-PCR product.

In another two families, 2 bands were found when *HMBS* gene was amplified, one of the expected size and another of 300 bp (Figure 3(a)). The sequencing of the small purified band revealed a large deletion of 5228 bp spanning from intron 2 to noncoding exon 15 (Figures 3(c) and 3(d)).

The other 15 mutations were previously described pointing that 7 of them were identified for the first time in our population (Table 1).

Of note are the results obtained for a foreign family from Paraguay. Two female symptomatic members carried two already described mutations. One was a splice site mutation in the last base of intron 12 inherited from the mother (c.772-1G>A) which leads to exon 13 skipping [39]. The other was a point mutation (c.962G>A) in exon 15 which produces an amino acid change (p.R321H) and was inherited from the father [22]. Another asymptomatic sister carried only this last mutation. The biochemical values and molecular results for this family are shown in Table 2.

In addition, 38 new families carry the p.G111R mutation previously described for another 21 unrelated families [7] ascending the number of unrelated Argentinean families that carry this mutation to 59 (55.66%) adding further support to our previous hypothesis of a founder effect [28].

4. Discussion

During the last 20 years, 35 different mutations were found: 14 described in Argentina for the first time and 21 already reported for other populations. These 35 genetic changes include 15 missense mutations, 9 splice defects, 7 small deletions, 2 small insertions, one gross deletion, and 1duplication.

One of the splice site mutations is located in the −2 position of the acceptor splice site of intron 8 (c.423-2A>G, GENBANK HM856802) leading to the in-frame deletion of 15 bp (Figure 1). The same result has been found for a point mutation in the last base of intron 8 already described for another Argentinean family [34].

The other novel splice site mutation was an A to G transition in the last base of intron 14 (c.913-1G>A). Although no sample was available to carry out RT-PCR studies, as this base is 100% conserved in the consensus splice site, it is very likely that this substitution leads to exon 15 skipping as it has been described by Puy et al. for a different mutation affecting the same acceptor splice site [39].

The novel frameshift mutation (Figure 2), c.301_307dupCCCACTG (GENBANK HQ7315521), introduces a premature stop codon at exon 8 so the transcript codified by this allele is most likely to be degraded by the nonsense-mediated mRNA decay (NMD) [42]. This mutation has been found in two unrelated Argentinean families (Table 1).

In two families, two bands were found when PCR product was run in an agarose gel, one of the expected size and another of 300 bp (Figure 3(a)). When these were sequenced the large one did not show any genetic change but it showed an apparent homozygosis of the 6 variable SNPs in the studied population (g.3119T>G, g.3581A>G, g.3982T>C, g.6479T>G, g.7064C>A, g.7539C>T) Cerbino [43]. However, as it is shown in Figure 3(b), for two of these SNPs, (g.6479T>G and g.7539C>T), the proband and her sister carry the same allelic variant (6479 G and 7539 C), but the symptomatic daughter of one of them carries another allelic variant (6479 T and 7539 T) inherited from her father. The analysis of the smaller band indicated that this corresponded to the other allele with a large deletion of 5228 bp spanning from intron 2 to noncoding exon 15. As indicated in Figures 3(c) and 3(d), positions g.3078_g.3081 and g.8306_g.8309 shared the same region (CCCC) so it was impossible to determine the breakpoint of the deletion. Only another gross deletion of 4620 bp but including promoter and exon 1 has been described by Di Pierro et al. [44].

In the family from Paraguay two double heterozygotes relatives were found. The proband of this family has been

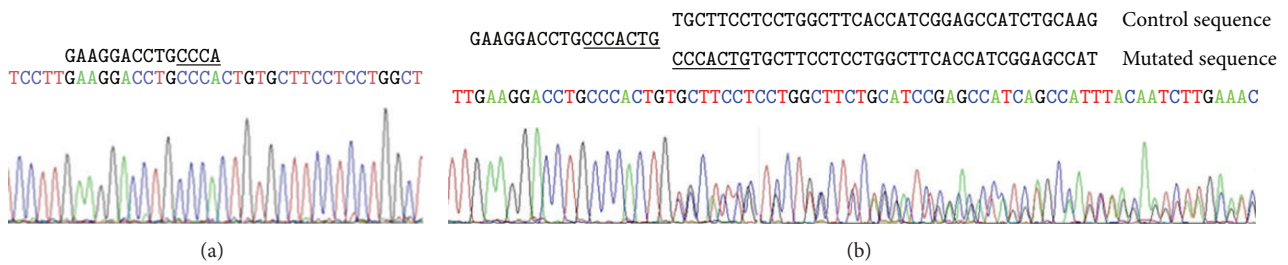

Figure 2: Electropherograms showing (a) control sequence; (b) c.301_307dupCCCACTG mutation; the duplicated sequence is underlined.

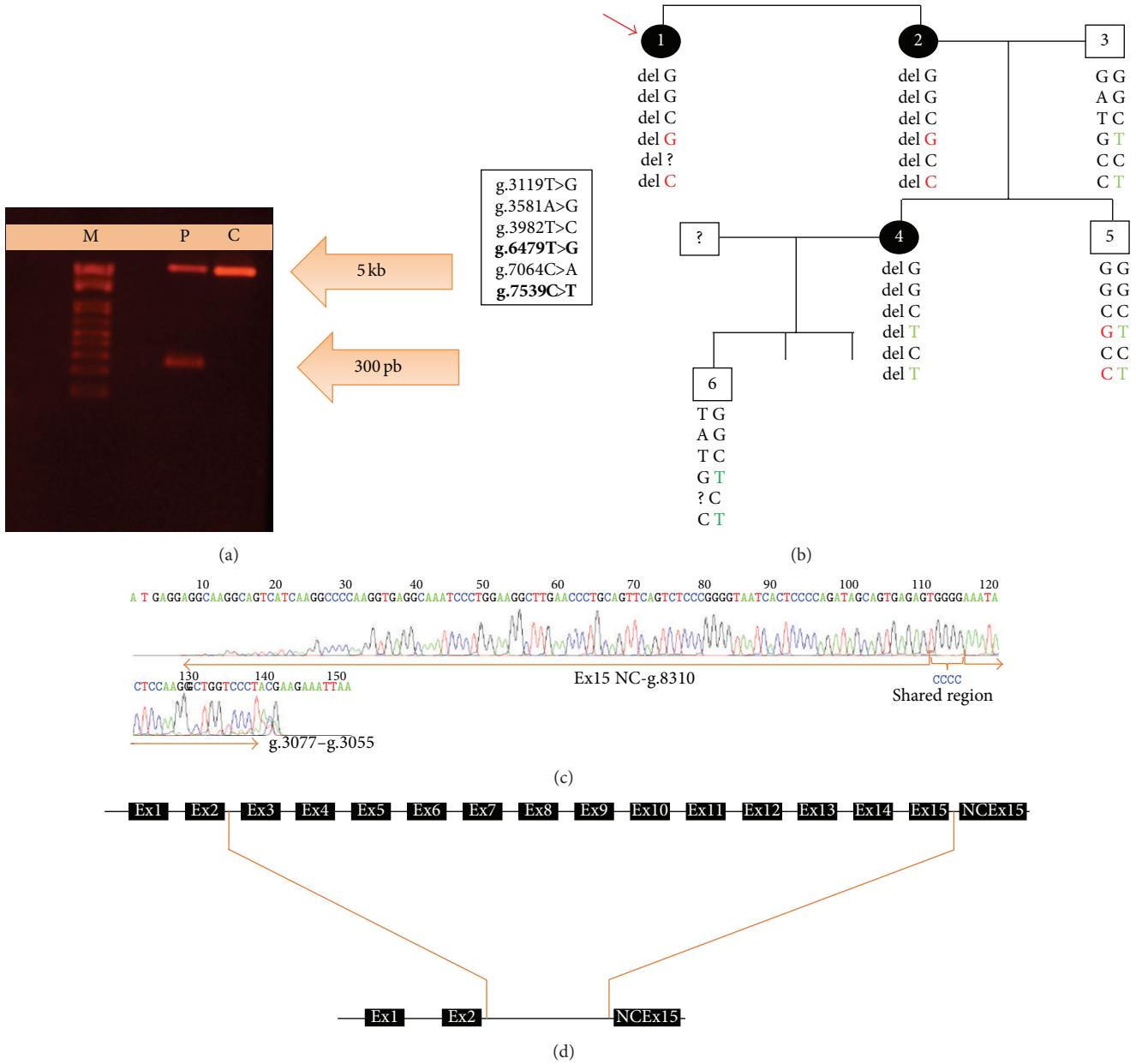

Figure 3: Gross deletion of 5228 bp. (a) PCR product of patient (P), control individual (C), M: 1 kb marker; (b) family SNPs analysis; (c) electropherogram of the 300 bp band showing the shared region between intron 2 and noncodifying exon 15; (d) scheme showing the deleted region.

diagnosed as AIP 15 years ago carrying one reported mutation, c.772-1G>A, a splice site mutation which leads to exon 13 skipping [39]. When three of her daughters came for diagnosis, it was found that one of them, asymptomatic but with a HMBS activity reduced to 50% of control value, did not carry the family mutation. A reexamination of mother DNA confirmed only the previous mutation but a more extensive study of her daughters indicated that two of them carry two mutations, the maternal mutation and another previously described missense mutation, p.R321H [22], inherited from their father. The asymptomatic daughter carries this last genetic change. It is interesting to note that these patients developed the symptomatology recently (23 and 26 years old) and only one of them occasionally suffered from some abdominal pain. In most of homozygous or double heterozygous AIP reported cases the enzyme activity was severely reduced and the symptomatology was developed at an early age with severe neurological manifestations [13, 15, 16, 18–21]. In several of these cases essential arginine residues for enzyme activity are affected [45–47]. In these new cases the mutations found do not seem to be essential for enzyme expression or activity. This is likely true for the missense mutation which affects a nonconserved arginine residue in exon 15 located in the 29-residue insert between strands β3 and α2 in the domain 3 only present in the human enzyme sequence [47]. However, the splice site mutation leads to exon 13 skipping [39], an exon where the residue Cys^{261} is located to which the essential DPM (dipyrromethane) cofactor is bound [47]. Nevertheless this combined heterozygous genotype does not seem to have a more serious impact on HMBS activity than in the heterozygous form, since the activity of the three sisters is approximately the same. These results highlighted the importance of carrying out a complete genotype investigation of family members of a known carrier.

Finally 38 new families carrying the frequent p.G111R mutation have been characterized leading to 59 (55.66%) the number of Argentinean families carrying this genetic change. The other mutations were found in one or in a few families. Efforts were made to know if the AIP patients showing this mutation have a common ancestral origin. Detailed pedigrees were unavailable, because either relatives of many of the patients were dead or the relatives themselves had limited knowledge of their families ancestry. Since the p.G111R mutation occurs at a hot spot CpG dinucleotide, it can be possible that the mutation had been originated several times independently. However, a preliminary analysis of four intragenic and four flanking DNA polymorphic markers indicated that all tested patients with p.G111R mutation (7/21) had at least one common allele for all intragenic and flanking markers. Argentinean AIP patients with other mutations had different alleles for the markers [28]. These previous results had suggested that individuals carrying this mutation were most likely related. Extended haplotype analysis on a large group of families with the p.G111R mutation and their relatives add further evidence to our previous hypothesis about a founder effect for this mutation in the Argentinean population [43]. Microsatellite studies in these families are being carried out.

5. Conclusions

This study emphasizes the molecular heterogeneity of AIP and the importance of molecular techniques as the most appropriate tools for detecting and identifying specific mutations in carriers of affected families to avoid the contact with precipitating agents.

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

N1303K (c.3909C>G) Mutation and Splicing: Implication of Its c.[744-33GATT(6); 869+11C>T] Complex Allele in *CFTR* Exon 7 Aberrant Splicing

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Cystic Fibrosis is the most common recessive autosomal rare disease found in Caucasians. It is caused by mutations on the *Cystic Fibrosis Transmembrane Conductance Regulator* gene (*CFTR*) that encodes a protein located on the apical membrane of epithelial cells. c.3909C>G (p.Asn1303Lys, old nomenclature: N1303K) is one of the most common worldwide mutations. This mutation has been found at high frequencies in the Mediterranean countries with the highest frequency in the Lebanese population. Therefore, on the genetic level, we conducted a complete *CFTR* gene screening on c.3909C>G Lebanese patients. The complex allele c.[744-33GATT(6); 869+11C>T] was always associated with the c.3909C>G mutation in cis in the Lebanese population. *In cellulo* splicing studies, realized by hybrid minigene constructs, revealed no impact of the c.3909C>G mutation on the splicing process, whereas the associated complex allele induces minor exon skipping.

1. Introduction

Cystic Fibrosis (CF) is the most common autosomal recessive genetic disease in Caucasians caused by mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene. The prevalence of CF varies and depends on the geographical location [1, 2]. The disease is less frequent in the Arab population in the Middle East region than in Europe [3, 4].

In the Lebanese population, the incidence of both common and rare genetic diseases is relatively high compared to neighbour countries [3] due to the existence of several communities and consanguineous marriage. Around 17% of the patients admitted to the Pediatric Service of American University in Beirut during 1961, 1966, and 1971 were found to suffer from a genetically caused or predisposed disorder [5]. Even though the first Arab CF child was detected in Lebanon in 1958 [6], few accurate pieces of data were presented during the following years to study this disease in the Lebanese population. However, the mutational *CFTR* spectrum of the Lebanese population was roughly elucidated in two previous studies [3, 7]. The major characteristic of this spectrum concerns the c.3909C>G mutation. This widespread mutation appears to have the highest worldwide frequency in Lebanon.

Soon after its identification, the c.3909C>G mutation was characterized by its severity on the pancreas and the variability of the pulmonary status [8]. Despite the low effect in the previous studies, almost all c.3909C>G Lebanese CF patients showed severe pancreatic and pulmonary phenotypes [3, 7]. The presence of a complex allele may aggravate its clinical

outcome [9, 10] and can explain the variability of the CF phenotype in CF c.3909C>G patients.

Therefore, to explore the presence of a complex allele, we sequenced *CFTR* genes existing in Lebanon, by recruiting Lebanese c.3909C>G heterozygous and homozygous families. We have detected two variations c.744-33GATT(6) in intron 6 and c.869+11C>T in intron 7 always associated in cis with the c.3909C>G mutation. Therefore, we performed *in cellulo* studies using hybrid minigene constructions to determine firstly the impact of the c.3909C>G mutation on splicing and secondly that of its associated complex allele c.[744-33GATT(6); 869+11C>T].

2. Material and Methods

2.1. DNA Extraction from Blood Samples. The blood samples were collected in EDTA (ethylenediaminetetraacetic acid) from 7 Lebanese families carrying at least on one allele the c.3909C>G mutation. Genomic DNA was extracted from peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer protocol. The DNA samples were quantified using the Nanodrop 2000 (Thermo).

2.2. DNA Amplification. Each of the 27 exons of the *CFTR* gene and their flanking introns were amplified by specific primers. The Pre-PCR reaction (25 μL) consisted of 5 μL of DNA, 2.5 μL of 10x buffer, 2 mmol/L of $MgCl_2$, 250 μmol/L of each dNTP, 10 pM of specific primers, and 3 U Taq polymerase. Pre-PCR was performed using a 9700 GeneAmp Thermo Cycler (Perkin Elmer) with the following cycling conditions: initial denaturation (94°C, 2 min), followed by 30 cycles (94°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec), and a final extension step (72°C, 5 min). To eliminate the excess of primers, a step with the ExoSAP (Affymetrix) was realized (15 min at 37°C). The enzyme was directly degraded at 80°C during 15 min.

2.3. Direct Sequencing of the CFTR Gene. To perform the direct sequencing, PCR reactions were realized on the Pre-PCR product. Sequencing is realized on the purified products using the ABI PRISM Big Dye Terminator TM cycle sequencing Reading Reaction Kit (Applied Biosystem). PCR was performed with the 25 cycles: initial denaturation (96°C, 10 sec), primers hybridization (44°C, 30 sec), and an extension step (60°C, 4 min). Then, purifications of the product were realized by filtration on DyeEx colon (Qiagen) according to the manufacturer protocol. Reactions were run on an ABI PRISM 3100 automatic sequencer (Applied Biosystems). The obtained sequences are aligned and compared to the *CFTR* data base sequences (http://www.genet.sickkids.on.ca/cftr).

2.4. Construction of Minigene for Splicing Study. The pTB*Nde*I plasmid (generously provided by F. Pagani) is a strong support to examine the impact of intronic or/and exonic variations on aberrant splicing in transfected mammalian cells. The genomic DNA region of interest, containing a putative splicing mutation, is introduced into the minigene *via* a unique restriction site (*Nde*I) located in a fibronectin intron. The construction and validation of the hybrid minigene used in this study has been described elsewhere [11].

To evaluate the impact of the c.3909C>G mutation on splicing, a PCR fragment, encompassing the 90 bp of exon 24 and 100 pb of each surrounding intron, was amplified from human genomic DNA (Figure 1(a)). Another PCR fragment was amplified to study the impact of the c.[744-33GATT(6); 869+11C>T] complex allele. This fragment contains the 126 pb of exon 7 and nearly 300 pb of each flanking intron (Figure 1(a)). PCR amplifications of these fragments were realized by specific primers described in Table 1.

After plasmid digestion with the *Nde*I restriction enzyme, PCR products were inserted with the DNA ligase (Figures 1(b) and 1(c)). Directed mutagenesis using specific primers was performed to obtain the different minigenes (Figure 1(c)) using the gene tailor site-directed mutagenesis kit (Invitrogen) and specific primers (Table 1). All hybrid minigene constructs were sequenced to verify the correct insertion of WT and mutated DNA fragments (Table 1).

2.5. Cell Culture and Transient Transfections. HeLa, HT29, and HEK293 cells were grown in DMEM medium with Glutamax-I (Life Technologies) supplemented with 10% foetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL of streptomycin in a humidified incubator at 37°C in the presence of 5% CO_2. Cells were transiently transfected by WT and mutant *CFTR* plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At least three independent transfections for each cell line were performed for RNA extraction experiments.

2.6. RT-PCR Analyses. Total mRNA was extracted from cell lysates using the RNeasy Mini Kit (Qiagen, Germany) and dissolved in 30 μL of sterile water. cDNA synthesis was carried out at 37°C for 1 h after adjustment of the mixture to contain 5 μL of 5x buffer (Gibco-BRL, France; 250 mmol/L of Tris-HCl pH 8.3, 375 mmol/L of KCl, 15 mmol/L of $MgCl_2$), 10 mmol/L of dithiothreitol (Gibco-BRL, France), 1 mmol/L of dNTPs (Roche Diagnostics, France), 2.4 μg of random hexamer primers, 10 μL of RNA, 40 U RNAguard (Amersham Biosciences, Orsay, France), and 400 U Moloney murine leukemia virus (MMLV) reverse transcriptase. The reaction medium was made up to 25 μL with sterile water and the reaction was stopped by incubation at 100°C for 2 min. The PCR reaction (25 μL) consisted of 5 μL of cDNA, 2.5 μL of 10x buffer, 2 mmol/L of $MgCl_2$, 250 μmol/L of each dNTP, 10 pM of specific primers for the cDNA (Table 1), and 3 U Taq polymerase. PCRs were performed using a 9700 GeneAmp Thermo Cycler (Perkin Elmer) with the following cycling conditions: initial denaturation (94°C, 2 min), followed by 30 cycles (94°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec), and a final extension step (72°C, 5 min). Amplification products were analyzed by 1.5% agarose gel electrophoresis.

For cDNA obtained from cultured cells, each fragment was purified from a nondenaturing 10% polyacrylamide gel and sequenced with specific primers (Table 1).

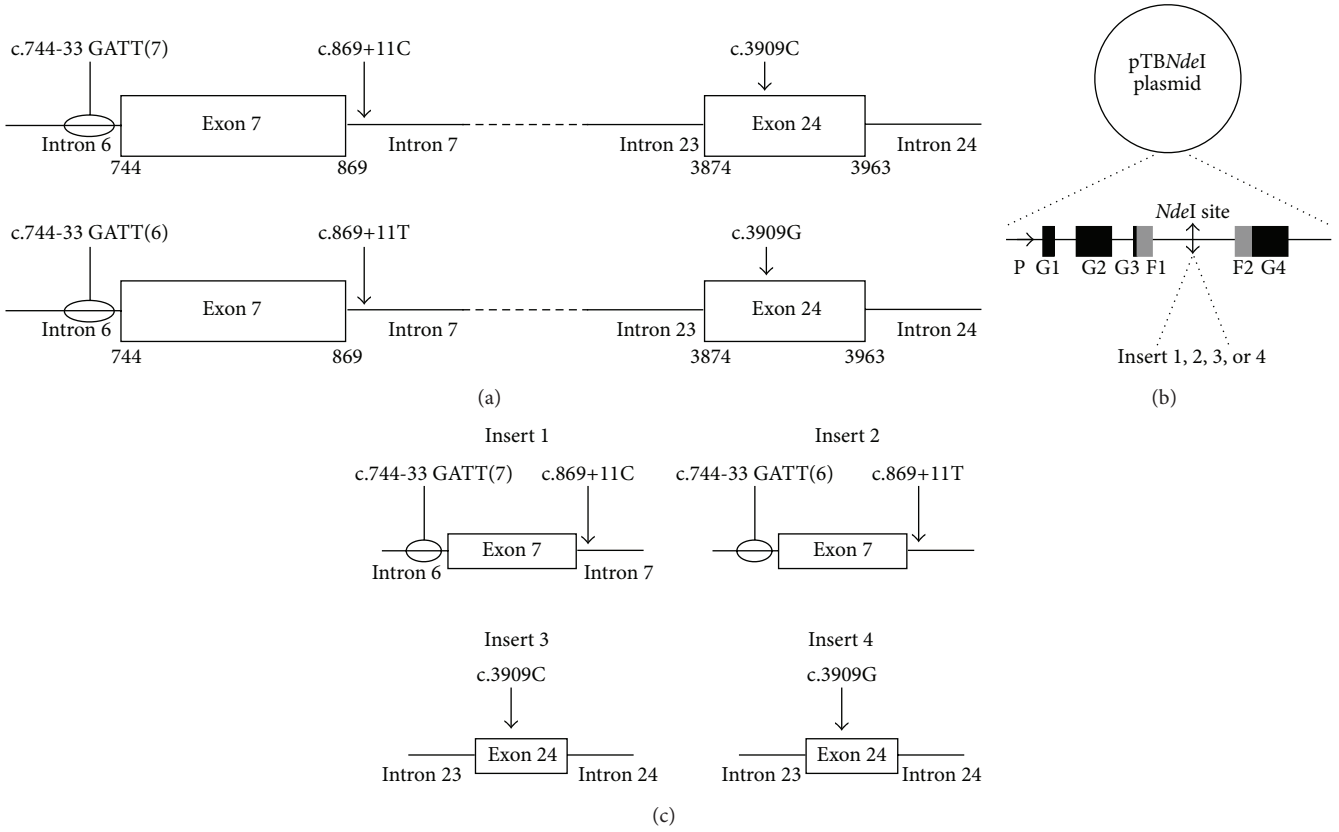

Figure 1: Identified complex allele in the Lebanese population and constructions of four hybrid minigenes. (a) The identified *CFTR* complex allele combining the 744-33GATT(6) polymorphism (intron 6), c.869+11C>T polymorphism (intron 7), and the c.3909C>G mutation (exon 24). (b) The pTB*Nde*I plasmid used in the hybrid minigene approach. This plasmid contains a reporter gene used to study the mRNA splicing. The reporter gene contains, at the 5′ end, a promoter/enhancer sequence indicated by the arrow. This is followed by α-globin (G1, G2, G3, and G4) and fibronectin (F1 and F2) exons separated by intronic sequences. The fibronectin intronic region, located between F1 and F2, contains a unique *Nde*I restriction site. Fragments of interest can be inserted in this site. (c) The four inserts used in this study. The impact of the c.[744-33GATT(6); 869+11C>T] complex allele on splicing was evaluated by the use of insert 1 (c.[744-33GATT(7); 869+11C]) and insert 2 (c.[744-33GATT(6); 869+11C>T]). Inserts 1 and 2 contain a part of intron 6 (335 bp), exon 7 (126 bp), and a part of intron 7 (326 bp). The impact of the c.3909C>G mutation on splicing was assessed using inserts 3 (WT) and 4 (c.3909C>G). Inserts 3 and 4 contain intron 23 (100 bp), exon 24 (96 bp), and intron 24 (100 bp). Inserts 1, 2, and 3 are obtained from patients and were inserted in the pTB*Nde*I plasmid. Plasmid containing insert 4 was obtained by directed mutagenesis realized on the plasmids containing insert 1.

3. Results

3.1. CFTR Gene Sequencing in c.3909C>G Patients. The sequencing of *CFTR* 27 exons and their surrounding introns, in the 7 families, confirmed the presence of the c.3909C>G mutation and another mutation in trans, previously determined by the CF30 kit (Elucigene). Furthermore, the sequencing revealed the presence of other different polymorphisms and mutations presented in Table 2. The polymorphism GATT of intron 6 had 6 repeats and the polymorphism c.869+11C>T in intron 7 was present in at least one allele in all the studied patients. The sequencing of exon 7 and part of its surrounding introns of the parental DNA indicated that the GATT(6) and c.869+11C>T polymorphisms are always associated in cis with the c.3909C>G mutation in all the studied patients. Moreover, the allele that does not carry the c.3909C>G mutation has the GATT(7) and no c.869+11C>T polymorphism. Therefore, all the Lebanese patients of this study possess the c.[744-33GATT(6); 869+11C>T; 3909C>G] complex allele.

3.2. No Impact of the c.3909C>G Mutation on CFTR mRNA Normal Splicing. The sequencing revealed no length difference between the WT and c.3909C>G cDNA, meaning that the c.3909C>G mutation has no effect on splicing regarding the *in cellulo* analyses (Figure 2). Results were identical in all of the three independent transfections in the three tested cell lines.

3.3. Splicing Study of the Associated Polymorphisms. Following transient transfections of HeLa, HT29, and HEK293 cells, with the WT (c.[744-33GATT(7); 869+11C]) and mutated (c.[744-33GATT(6); 869+11C>T]) plasmids, mRNA was analysed by RT-PCR and directly sequenced using β-globin-specific primers (Table 1). A polyacrylamide gel was

Table 1: Primers used in amplification and sequencing of studied regions.

Use	Hybridization	Primers
(a) Insert preparation containing the WT exon 24	Intron 23/intron 24	5′ACTTGATGGTAAGTACATGG3′ 5′AGGTATGTTAGGGTACTCCA3′
(b) Insert preparation containing c.[744-33GATT(7);869+11C] or c.[744-33GATT(6);869+11C>T]	Intron 6/intron 7	5′CCAGATTGCATGCTTACTA3′ 5′AGTTACCAATCAGCCTTCA3′
(c) Directed mutagenesis to introduce the c.3909C>G mutation on the pTB*Nde*I plasmid containing the WT exon 24 insert	Exon 24	5′TCTGGAACATTTAGAAAAAAGTTGGATCCCT3′ 5′TTTTTTCTAAATGTTCCAGAAAAAATAAATACTTT3′
(d) Verifying the correct introduction of the inserts in pTB*Nde*I and the correct realization of the direct mutagenesis	Intron fibronectin 1/intron fibronectin 2	5′ACTTCAGATATTATGTCTAGG3′ 5′CCCCATGTGAGATATCTAG3′
(e) Sequencing cDNA of cultured cells	Exon globin 3/fibronectin 2	5′CAACTTCAAGCTCCTAAGCCACTGC3′ 5′AGGGTCACCAGGAAGTTGGTTAAATCA3′

Table 2: *CFTR* mutations and polymorphisms identified on each allele of c.3909C>G in Lebanese patients. *No DNA was obtained for the patient number 5 parents; thus the association in cis for the TG(m)T(n) was not determined. As the patient is CF the two detected mutations are in trans.

Individual	Allele	Intron 6	Intron 7	Intron 9		Exon 11	Exon 12	Exon 15	Exon 23	Exon 24	Exon 27
1	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
2	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(7)	c.869+11C	TG(10)	T(7)			c.2562G	**c.3846G>A**		c.4521A
3	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(6)	c.869+11T	TG(10)	T(9)	**c.1521_1523delCTT**					
4	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(6)	c.869+11T	TG(10)	T(9)	**c.1521_1523delCTT**					
5	1	GATT(6)	c.869+11T	10/11TG						**c.3909C>G**	
	2	GATT(6)	c.869+11T	9/7T*			**c.1647G**				
6	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(6)	c.869+11T	TG(10)	T(9)	**c.1521_1523delCTT**					
7	1	GATT(6)	c.869+11T	TG(10)	T(9)					**c.3909C>G**	
	2	GATT(7)	c.869+11C	TG(11)	T(7)	c.1408G					

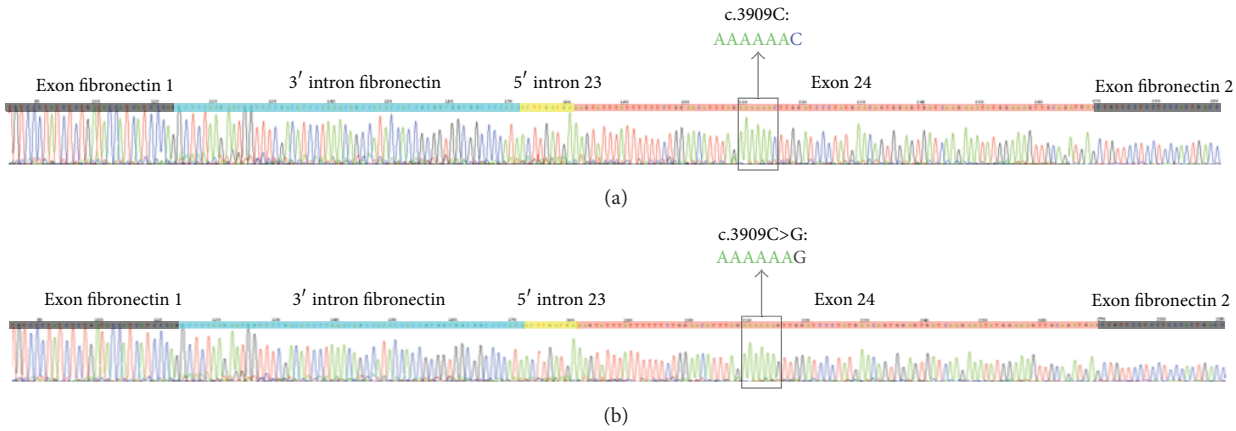

Figure 2: Impact of the c.3909C>G mutation on exon 24 splicing. Sequences of the cDNA were obtained from transfected cells with the pTB*Nde*I minigene plasmid carrying (a) WT or (b) c.3909C>G exon 24 *CFTR* minigenes. In both plasmids the sequencing reveals the same transcript, excluding an impact of the c.3909C>G mutation on splicing (the presence of intronic parts in WT and mutated results from plasmid construction and has been detected in the three cell lines).

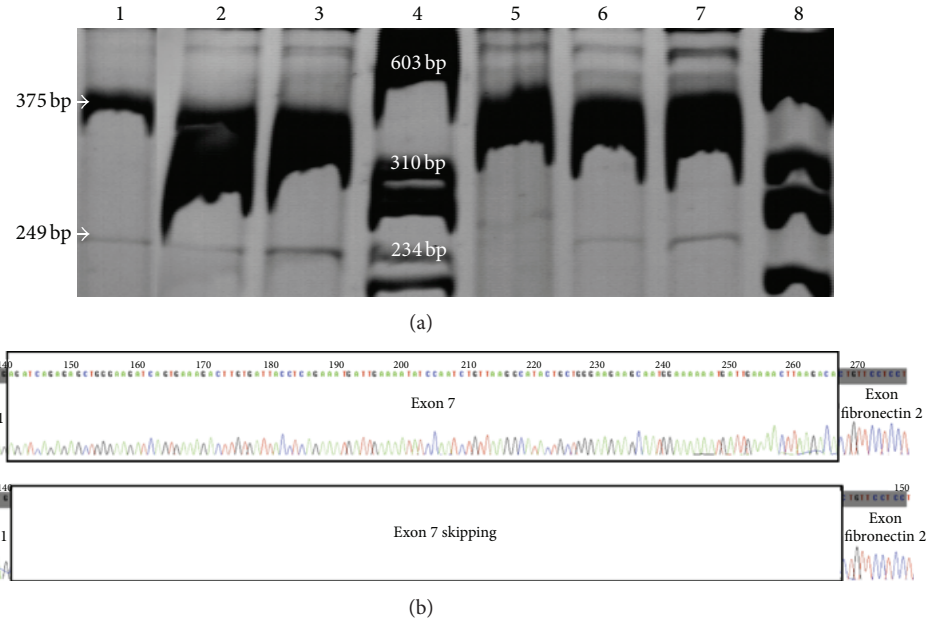

Figure 3: Impact of the c.[744-33GATT(6); 869+11C>T] complex allele on splicing patterns. (a) Polyacrylamide gel showing, for each plasmid construction, the different fragments of cDNA using 2,3α and Bra2rev primers on the total cDNA. This polyacrylamide gel shows three independent transfections of the c.[744-33GATT(7); 869+11C] (Lines 1, 2, and 3) and c.[744-33GATT(6); 869+11C>T] (Lines 5, 6, and 7). The results were confirmed in three different cell lines. Two fragments are visible: normal splicing with exon 7 (375 pb) and exon 7 skipping (249 pb). Ladder: ϕX174 DNA/*Bsu*RI (*Hae*III) Marker (Fermentas) was used (Lines 4 and 8). (b) Sequencing of the 375 bp fragment revealing a normal splicing and that of the 249 bp fragment revealing an exon 7 skipping.

used for more precise separation of the resulting fragments (Figure 3(a)). Each fragment was isolated and sequenced with specific primers (Table 1) to determine the different mRNA products.

Polyacrylamide gel shows, for each plasmid construction, two fragments: normal splicing with exon 7 (375 bp) and exon 7 skipping (249 bp). The exon 7 skipping is present in both WT and mutated plasmids. The sequencing, of the different fragments obtained after their purification from the gel, confirmed that the first fragment represents the normal splicing with the complete exon 7 and the second fragment represents that of the exon 7 skipping (Figure 3(b)). This last mRNA is in frame and so induces a shorter fragment than WT-CFTR (−126 bp). Surprisingly, in HEK293 cells, another detected transcript is deleted of one nucleotide (data not shown).

4. Discussion

Since its initial identification, the c.3909C>G mutation presented an unclear phenotype-genotype correlation. While the first collaborative study has provided conclusive evidences of the c.3909C>G grave consequences on the pancreas, the severity on pulmonary level remained unpredictable in both homozygous and heterozygous states [8]. In fact, 100% (61 patients) of c.3909C>G/c.1521_1523delCTT and c.3909C>G/c.3909C>G have pancreatic insufficiency, while 72% (23/32 patients) of them present sputum colonization of *P. aeruginosa*. In the Lebanese population, also 100% (8/8) of the same category showed pancreatic insufficiency or growth retardation, and 88% (7/8 patients) revealed pulmonary manifestation [3, 7]. The variable severity regarding the lung disease in c.3909C>G homozygous and heterozygous patients has been previously related to ethnic variation [8]. Environmental factors and/or the presence of a complex allele could modulate the initial consequence of the c.3909C>G mutation. This has led us to recruit all the patients where the c.3909C>G mutation has been detected between 2005 and 2011 in the University of Saint-Joseph genetics laboratory. The complete sequencing of *CFTR* 27 exons and their flanking intronic parts, in all the studied patients, revealed the existence of both c.744-33GATT(6) and c.869+11C>T polymorphisms always associated with c.3909C>G mutation (Table 2). Thus, in this study, we have identified in the Lebanese population the c.[744-33GATT(6); 869+11C>T; 3909C>G] complex allele. This complex allele was already detected in different populations [12, 13].

The examined effect on the c.3909C>G mutation denied the predicted aberrant exon 24 splicing (Figure 2). Despite the early identifications of two associated intronic variations, their impacts on splicing have never been assessed yet neither independently nor in association. The GATT polymorphic region, described in 1990 by Horn et al. in CFTR data base (http://www.genet.sickkids.on.ca), is located in the 5′ flanking region of exon 7 and presents 5 to 7 GATT repeats. The GATT(7) is considered WT since it is the most frequent allele [14]. The c.869+11C>T polymorphism, identified in 1991 by Cuppens et al. in CFTR data base, occurs in the 3′ flanking region of exon 7. In our study, we assessed the influence on splicing, using the minigene assay, in HeLa, HT29, and HEK293 cells, since the severity of the splicing defect may be varied among the cultured cell lines using the same pTB*Nde*I hybrid minigene construct [15]. The WT complex allele (c.[744-33GATT(7); 869+11C]) and the mutated one (c.[744-33GATT(6); 869+11C>T]) uncovered a minor alternative exon 7 splicing in both genotypes. Therefore, the c.[744-33GATT(6); 869+11C>T; 3909C>G] complex allele seems to have no notable influence on the CF phenotype and it is extremely low to explain the variable clinical phenotypes in c.3909C>G patients. However, *in vivo* assessment can validate the splicing outcome and provide justification for further experimental examination of patients samples when available [16].

In conclusion, on the gene level, we identified in the Lebanese population the complex allele associating the c.3909C>G mutation with the c.869+11C>T polymorphism. On the mRNA level, no aberrant splicing was detected with the c.3909C>G. However, we reported minor exon 7 skipping in both WT (c.[744-33GATT(7); 869+11C]) and mutated complex allele (c.[744-33GATT(6); 869+11C>T]). This is unlikely to explain the observed variable phenotype in c.3909C>G patients. However, it is important to note that splicing results differ in function of the cells types. In order to detect the impact of the complex allele on splicing *in vivo*, it is essential to have nasal epithelial cell of homozygote patients with this genotype.

Conflict of Interests

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

Acknowledgments

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Research Article

Single Nucleotide Polymorphisms of the *GJB2* and *GJB6* Genes Are Associated with Autosomal Recessive Nonsyndromic Hearing Loss

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Single nucleotide polymorphisms (SNPs) are important markers in many studies that link DNA sequence variations to phenotypic changes; such studies are expected to advance the understanding of human physiology and elucidate the molecular basis of diseases. The *DFNB1* locus, which contains the *GJB2* and *GJB6* genes, plays a key role in nonsyndromic hearing loss. Previous studies have identified important mutations in this locus, but the contribution of SNPs in the genes has not yet been much investigated. The aim of this study was to investigate the association of nine polymorphisms located within the *DFNB1* locus with the occurrence of autosomal recessive nonsyndromic hearing loss (ARNSHL). The SNPs rs3751385 (C/T), rs7994748 (C/T), rs7329857 (C/T), rs7987302 (G/A), rs7322538 (G/A), rs9315400 (C/T), rs877098 (C/T), rs945369 (A/C), and rs7333214 (T/G) were genotyped in 122 deaf patients and 132 healthy controls using allele-specific PCR. There were statistically significant differences between patients and controls, in terms of allelic frequencies in the SNPs rs3751385, rs7994748, rs7329857, rs7987302, rs945369, and rs7333214 ($P < 0.05$). No significant differences between the two groups were observed for rs7322538, rs9315400, and rs877098. Our results suggest that SNPs present in the *GJB2* and *GJB6* genes may have an influence on ARNSHL in humans.

1. Introduction

Hearing loss (HL) is a common congenital sensory disorder worldwide, affecting almost 600 million people. Approximately 2 to 6 children in 1,000 are affected by severe hearing loss at birth [1] or during early childhood. This is defined as prelingual deafness, with about half of cases attributable to genetic causes. Furthermore, many cases of late onset progressive HL also have a genetic origin, in addition to progressive HL associated with ageing [2]. According to the Hereditary Hearing Loss (HHL) homepage (http://hereditaryhearingloss.org/), over 140 loci for nonsyndromic HHL have been mapped, together with approximately 80 genes, and more than 1000 mutations have been identified in humans, making it one of the most genetically heterogeneous traits. However, in most cases, genetic hearing loss is a monogenic disorder. Biallelic mutations in 47 different genes have been reported for autosomal recessive nonsyndromic hearing loss (ARNSHL), which in many populations accounts for 80% of families with this type of deafness [3].

The occurrence of ARNSHL has been related to the *DFNB1* locus (deafness, autosomal recessive 1) (OMIM 220290) at chromosome 13q11-q12, which contains two genes associated with hearing loss: *GJB2* (gap junction protein,

beta-2) (OMIM 121011) and *GJB6* (gap junction protein, beta-6) (OMIM 604418), which codify the proteins encoding connexins 26 (Cx26) [4] and 30 (Cx30) [5], respectively. Mutations in these genes are the most frequent causes of ARNSHL in most populations worldwide, sometimes accounting for up to 50% of cases [6].

Direct intercellular communication is mainly mediated by gap junction channels, which in vertebrates are formed by members of the connexin protein family. Connexins are transmembrane proteins that regulate electrical signals and the passage between neighboring cells of ions, small biological molecules (<1000 Da) including sugars, nucleotides, and amino acids, secondary messengers such as Ca^{2+}, cyclic AMP, and inositol triphosphate, and metabolic precursors [7]. Cochlear gap junctions, especially connexins Cx26 and Cx30, have been implicated in the maintenance of K^+ homeostasis in the inner ear [8]. Cx26 and Cx30, the two most abundantly expressed gap junction proteins in the cochlea, are coexpressed as heteromeric connexons in nonsensory cells of the organ of Corti as well as in cells of the spiral ligament and stria vascularis [9]. Extensive genetic studies have been conducted to identify mutations in the *DFNB1* locus; however, information is lacking concerning the potential association between nonsyndromic hearing loss and SNPs of the *GJB2* and *GJB6* genes [10].

Single nucleotide polymorphisms (SNPs) are the most abundant genetic markers at a specific location in the genome, occurring at a frequency of more than 1% in the human population [11]. The International HapMap Project has characterized over 3.1 million human SNPs, indicating a SNP density of approximately one per kilobase [12]. SNPs are of great interest in medical and pharmacological studies of disease susceptibility and drug response. They also provide powerful tools for a variety of medical genetic studies [13].

In view of all these observations, in an attempt to identify polymorphisms related to autosomal recessive nonsyndromic hearing loss, this study investigated the frequency of SNPs in the *GJB2* and *GJB6* genes by means of a case-control association study.

2. Materials and Methods

2.1. Ethics Statement. Written informed consent forms were obtained from all the participating subjects or from their parents. The study was approved by the Ethics Committee at Centro Universitário Hermínio Ometto de Araras (UNIARARAS), under protocol number 744/2010.

2.2. Study Subjects. The study involved 122 unrelated newborn patients of both genders (69 males and 53 females) with moderate to profound ARNSHL, together with 132 normal controls (CTL). The patients were from the Hearing and Language Stimulation Therapy Association, in Jundiaí (SP, Brazil). This institution is concerned with auditory and communication disorders, and since 2001 it has developed a universal hearing screening program. The majority of subjects studied were classified as Caucasian; however, many Brazilians have unique mixtures of Amerindian, European, and African ancestries in their genomic mosaic [14].

Table 1: Summary of selected SNPs.

Gene	rs number	Position	SNP type	MAF
GJB2	rs3751385	3′ UTR	C/T	T = 0.360
	rs7994748	Intron	C/T	C = 0.481
	rs7329857	3′ UTR	C/T	T = 0.079
	rs7987302	Downstream	G/A	A = 0.098
GJB6	rs7322538	Downstream	G/A	A = 0.214
	rs9315400	Intron	C/T	T = 0.362
	rs877098	Intron	C/T	T = 0.429
	rs945369	Intron	A/C	C = 0.352
	rs7333214	3′ UTR	T/G	T = 0.302

rs number, NCBI reference SNP (rs) number, is an identification tag assigned by NCBI to SNPs [36]. MAF (ref): minor allele frequency information from public database, NCBI dbSNP Build 132; MAF ≥ 0.05.

All patients underwent molecular analysis of the coding region and part of exon 1 and the flanking donor splicing site of *GJB2* and the two deletions affecting the *GJB6* gene (del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854)). Also the 1555A>G, 827A>G, and 1494C>T mitochondrial mutations in the *MTRNR1* gene and the 74555A>G mutation in the *MTTS1* gene were analyzed. Following this screening, all individuals with an identified molecular cause for ARNSHL were excluded from the study. These previous analyses were performed at the Center of Molecular Biology and Genetic Engineering (CBMEG), Human Molecular Genetics Laboratory, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

The normal controls were recruited from amongst the employees of UNIARARAS. In order to exclude the influence of inherited susceptibility factors, information about the control individuals was collected using questionnaires containing items concerning general health, medical conditions, and hereditary factors. Subjects with histories of head injury, otological disease, other diseases that could affect hearing, and previous or present treatment with ototoxic substances were excluded.

2.3. SNP Selection. Several SNPs covering the *DFNB1* locus were analyzed, seven of these (rs3751385, rs7994748, rs7329857, rs7987302, rs877098, rs945369, and rs7333214) were selected from the HapMap database (available at http://www.hapmap.org/), and SNP genotyping data from the YRI population were downloaded into Haploview [15]. Only SNPs that passed quality control criteria (call rate ≥ 95%, minor allele frequency (MAF) ≥ 0.05, and Hardy-Weinberg disequilibrium $P > 0.01$) were included in the genetic analysis. Two other SNPs (rs7322538 and rs9315400) were selected based on previous reports of association with deafness phenotypes [10]. Nine SNPs were analyzed, four in the *GJB2* gene and five in the *GJB6* gene. A summary of the selected SNPs is provided in Table 1.

2.4. Genotyping. Genomic DNA was extracted from whole blood leukocytes using the standard phenol–chloroform method, as described previously [16]. For genotyping, all

Table 2: Validation methods for each SNP analyzed.

SNP	Gene	Method (enzyme)
rs3751385	*GJB2*	RFLP-PCR (*Nhe* I)
rs7994748		RFLP-PCR (*Bstx* I)
rs7329857		RFLP-PCR (*Dpn* II)
rs7987302		Direct sequencing
rs7322538	*GJB6*	Direct sequencing
rs9315400		RFLP-PCR (*Acu* I)
rs877098		RFLP-PCR (*Eco* RI)
rs945369		RFLP-PCR (*Mva* I)
rs7333214		RFLP-PCR (*Mae* II)

the DNA samples were normalized to a concentration of 50 ng/μL.

The SNP genotypes were determined by allele-specific PCR (AS-PCR) amplification. For analysis of allelic variants, two forward primers were designed, with the 3′ base of each primer matching only one of the biallelic SNP bases to be evaluated. Incorporation of a primer mismatch at the second or third base from the 3′ end of the primer has been shown to enhance the specificity of the PCR by further destabilizing the extension of the doubly mismatched primer [17–19]. A common reverse primer (COM) was designed downstream of the polymorphic site. Control primers were also used for SNP coamplification of a portion of the human amelogenin (*AMELX*) gene [20]. These primers were therefore used as internal amplification controls. The sequences of the primers used in this study are listed in Table 2.

Approximately 10% of samples, randomly selected, were regenotyped for cross-validation by restriction fragment length polymorphism PCR (RFLP-PCR) using restriction enzyme (New England Biolabs Inc., USA) (see Table 2) or by direct sequencing of PCR products using ABI BigDye Terminator, with analysis using an ABI PRISM 3700 DNA sequencer (Applied Biosystems, Foster City, USA). No inconsistencies were observed.

2.5. AS-PCR Amplification and Electrophoresis. The PCR procedures were performed using a 30 μL reaction volume containing 50 ng of template DNA, 0.5–0.7 pmol of each forward and reverse primer, 0.2 pmol of each control primer, 170 μM dNTP, an appropriate concentration of $MgCl_2$ (Table 3), 0.01% (v/v) BSA, 1X reaction buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), and 1 U *Taq* polymerase (Invitrogen, Itapevi, SP, Brazil). The samples were incubated at 95°C for 5 min, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing (the times and annealing temperatures for different PCRs are described in Table 3), 1 min extension at 72°C, and a final extension at 72°C for 10 min. The amplicons (Table 3) were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and were visualized and photographed using the Syngene G:BOX gel documentation system.

2.6. Statistical Analysis. The Pearson goodness of fit χ^2 test was used to assess deviation from Hardy-Weinberg equilibrium (HWE). Differences in genotype and allele frequencies between the study groups were compared by the chi-square test and/or Fisher's exact test. Association between the SNPs and the risk of autosomal recessive nonsyndromic hearing loss was analyzed using the binary logistic regression test. Risk was expressed as odds ratio (OR) with 95% confidence intervals (CI). An OR > 1.0 was used as the cutoff for the baseline of risk-associated SNPs, and the baseline for risk-lowering SNPs was OR < 1.0. The Bonferroni post hoc test was used to compare multiple groups, using the definition P value (single tests) × number of tests. The minor allele frequency (MAF) for the study was calculated, and a χ^2 test was performed to determine whether there was a significant difference between the MAF value obtained here and that in the public MAF database (NCBI dbSNP Build 132). All the statistical analyses were conducted using GraphPad Prism 5.0 software. A P value < 0.05 was taken as statistically significant.

3. Results

A total of 254 individuals (132 in the control group and 122 in the patient group) were genotyped for 9 SNPs in the *DFNB1* locus, using AS-PCR. Figure 1 shows a representative AS-PCR gel corresponding to the SNPs rs945369 and rs7333214. Significant deviations from Hardy-Weinberg equilibrium were detected in the genotype frequencies of both groups. HWE P values for all the studied SNPs are summarized in Table 4. Analysis of the results indicated that the minor allele frequencies of the significant SNPs were in accordance with the information in the public database, although different frequencies were found for two of the SNPs, rs7994748 (MAF = 0.173) and rs7987302 (MAF = 0.026) (Table 5).

Out of the nine SNPs included in the study, six (66.7%) were found to be significantly associated with ARNSHL. Four of these (rs3751385, rs7994748, rs7329857, and rs7987302) were found in the *GJB2* gene and two (rs945369 and rs7333214) in the *GJB6* gene (Table 6). No significant associations were found for the other three SNPs (33.3%) genotyped. Three of the four significant SNPs of the *GJB2* gene (rs3751385, rs7994748, and rs7329857) and one SNP of the *GJB6* gene (rs7333214) remained statistically significant after application of the Bonferroni correction for multiple testing (×9, P values of 1.011×10^{-12}, 0.036, 3.478×10^{-4}, and 0.0027, resp.). These four SNPs were therefore of sufficient interest to warrant further investigation. As revealed by the odds ratio (OR), four of the six SNPs were associated with a high risk of ARNSHL, with OR > 1, while the remaining two were associated with a decreased susceptibility to ARNSHL (OR < 1). The rs7329857 SNP of the *GJB2* gene was associated with the highest risk of ARNSHL, with OR of 11.70.

Considering the risk-increasing SNPs, the rs3751385 (C/T) T allele (*GJB2* gene) was identified in 90 out of 122 ARNSHL cases (74%), with 40 in heterozygous and 50 in homozygous genotypes. Likewise, the T allele of rs7994748 (C/T) was present in 92% of the cases (10 in heterozygous and 102 in homozygous genotypes). Other risk-associated SNPs in the *GJB2* gene were only observed to occur in heterozygous genotypes, and these included 20 cases (16%) for rs7329857

Table 3: AS-PCR primers and conditions.

Gene	SNP ID (nucleotide change)	Sequence (5′-3′)*	Annealing temperature	Mg^{2+}	Amplicon size
GJB2	rs3751385 (C/T)	C allele: GCTCAGCTGTCAAGGCTCAGTCTCC T allele: GCTCAGCTGTCAAGGCTCAGTCTCT COM: TTGTCCTCAGAGAAAGAAACAAATGCC	60°C (1 min)	2.5 mM	284 bp
	rs7994748 (C/T)	C allele: GCTGAGAGCTGGGTTCCGTGTC T allele: GCTGAGAGCTGGGTTCCGTGTT COM: AGGGGCTCAGAAGCAGGACG	60°C (1 min)	2.5 mM	435 bp
	rs7329857 (C/T)	C allele: TTTCCCAACACAAAGATTCTGCC T allele: TTTCCCAACACAAAGATTCTGCT COM: CTTACACCAATAACCCCTAACAGCC	58°C (1 min)	2.5 mM	199 bp
	rs7987302 (G/A)	G allele: GGCATATCAGTCTATGGACAATGGGG A allele: GGCATATCAGTCTATGGACAATGGGA COM: AGAGGTTGCAGTGAGCCAAGG	58°C (1 min)	1.7 mM	157 bp
GJB6	rs7322538 (G/A)	G allele: CTAATGCAACTAGGGAAATTCG A allele: CTAATGCAACTAGGGAAATTCA COM: GCAATCTAGTTTTTCCTCATCC	56°C (45 s)	2.2 mM	106 bp
	rs9315400 (C/T)	C allele: GCAGCCTAGCATTTTACATC T allele: GCAGCCTAGCATTTTACATT COM: GTCTCTTTTTCGCAACCTTG	55°C (45 s)	2.5 mM	100 bp
	rs877098 (C/T)	C allele: AAGGGAGCTTGGAAATGAAGTC T allele: AAGGGAGCTTGGAAATGAAGTT COM: GAGGTGGAGCTTGCAGTGAG	56°C (1 min)	2.5 mM	227 bp
	rs945369 (A/C)	A allele: GTCCCTGTTTTTAGAAAAAAAGAA C allele: GTCCCTGTTTTTAGAAAAAAAGAC COM: GGAAGTAAACAGATCAGGGAG	59°C (1 min)	2.5 mM	187 bp
	rs7333214 (T/G)	T allele: AACATTTATCCAGGAATTGATATT G allele: AACATTTATCCAGGAATTGATATG COM: CAAATTTGCCAACAGACAATGC	57°C (1 min)	2.5 mM	230 bp
Controls primers					
AMELX		CTLA: CCCACCTTCCCCTCTCTCCAGGCAAATGGG CTLB: GGGCCTCAGTCCCAACATGGCTAAGAGGTG			360 bp

COM: common primer (reverse). *The mismatches of the allele-specific primers are underlined. SNP ID: identification of the SNP. AMELX: human amelogenin gene used as an internal amplification control.

Table 4: Hardy-Weinberg proportions in the groups studied.

Gene	SNP	HWE *P* value (CTL)	HWE *P* value (patients)
GJB2	rs3751385	0.143	**0.001**
	rs7994748	0.711	$\mathbf{6.692 \times 10^{-11}}$
	rs7329857	0.994	0.615
	rs7987302	0.917	0.895
GJB6	rs7322538	0.740	0.435
	rs9315400	0.653	0.557
	rs877098	$\mathbf{4.312 \times 10^{-18}}$	$\mathbf{3.221 \times 10^{-27}}$
	rs945369	**0.0082**	**0.003**
	rs7333214	0.995	0.156

HWE: Hardy-Weinberg equilibrium test was done using Pearson's goodness of fit χ^2 test and *P* value <0.05 was considered to show significant deviation of the observed genotypes from Hardy-Weinberg proportions. Significant deviations values from Hardy-Weinberg equilibrium are shown in boldface.

(C/T) and 10 cases (8%) for rs7987302 (G/A). The presence of rs945369 (A/C) in the *GJB6* gene was observed to be associated with decreased risk of ARNSHL, with 38 of the patients heterozygous (31%) and 23 of the cases (19%) CC homozygous. A genetic association of SNP rs7333214 (T/G) with ARNSHL was identified in 94% of the patients, with 61 heterozygous and 54 GG homozygous genotypes. The results of the association analysis are summarized in Table 6.

4. Discussion

Genome-wide association studies (GWAS) have successfully identified numerous loci that influence disease risk. Such techniques have been proved to offer powerful approaches for the screening of genes involved in complex diseases [21], including hearing loss, which occurs in around one per 1,000 newborns on average [22].

The current study evaluated the relationships between polymorphisms in the *GJB2* and *GJB6* genes and autosomal recessive nonsyndromic hearing loss in a sample of the Brazilian population. Evidence was found that SNPs in both genes were significantly associated with ARNSHL. Data for 122 patients and 132 controls indicated that *GJB2*

Table 5: Comparative analysis between minor allele frequency described in the database and minor allele frequency in the whole study group.

Gene	SNP ID (nucleotide change)	MAF ref	MAF study	*P* value (ref × study)
GJB2	rs3751385 (C/T)	T = 0.360	0.405	0.513
	rs7994748 C/T	C = 0.481	0.173	$\mathbf{3.442 \times 10^{-6}}$
	rs7329857 C/T	T = 0.079	0.043	0.287
	rs7987302 G/A	A = 0.098	0.026	**0.035**
GJB6	rs7322538 G/A	A = 0.214	0.114	0.056
	rs9315400 C/T	T = 0.362	0.451	0.200
	rs877098 C/T	T = 0.429	0.486	0.418
	rs945369 A/C	C = 0.352	0.400	0.472
	rs7333214 T/G	T = 0.302	0.236	0.293

MAF (ref) ≥0.05: minor allele frequency information from public database, NCBI dbSNP Build 132; MAF (study): minor allele frequency in the whole study group. Significant values are shown in boldface.

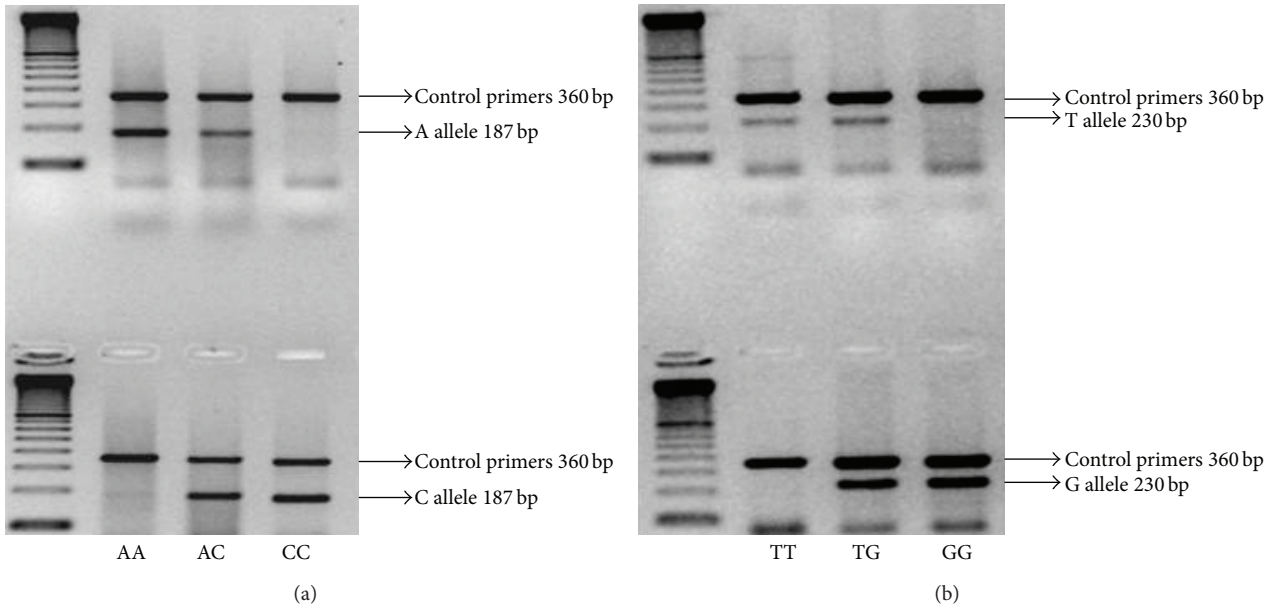

Figure 1: Electrophoretic patterns observed on AS-PCR analysis of the SNPs rs945369 (a) and rs7333214 (b). DNA ladder molecular weight marker (100 bp) was run in the first lane as labeled. The photo shows all three genotypes obtained from study individuals seen on the ethidium bromide-stained 1.5% agarose gel. AA: homozygote for the allele A; AC: heterozygote for the alleles A and C; CC: homozygote for the allele C (a). TT: homozygote for the allele T; TG: heterozygote for the alleles T and G; GG: homozygote for the allele G (b).

rs3751385 (C/T) was associated with hearing loss susceptibility, in agreement with previously published research [21, 23, 24].

The T allele of this SNP (rs3751385) has also been shown to be a risk factor associated with chronic plaque psoriasis in Chinese Han population patients [25]. A similar finding was reported for Chinese patients with psoriatic arthritis [26], possibly because connexin 26 (Cx26) has been found to be highly expressed in psoriasis plaques. Cx26 is widely expressed in most human tissues, including the ectoderm-derived epithelia of the cochlea, cornea, and skin [27].

Wilch et al. [28] previously showed that the expression of *GJB2* and *GJB6* diminished in the presence of the rs7333214 G allele. Conversely, in this study, the presence of the T allele was found to be positively associated with ARNSHL.

Here, to the best of our knowledge, we describe for the first time the association between the rs7994748, rs7329857, rs7987302, and rs945369 SNPs and ARNSHL. The results indicate that these genetic changes may be important determinants of hearing impairment risk in the studied population. The rs7994748 and rs945369 SNPs are present in the intronic regions of the *GJB2* and *GJB6* genes, respectively. It has been shown previously that SNPs present in the noncoding region can modulate gene expression [29]. The rs3751385 and rs7329857 SNPs are located in the 3′UTR region of the *GJB2* gene, while rs7333214 is found in the *GJB6* gene. The 3′UTR region of a gene is usually important for regulation of processes such as transcript cleavage, alternative polyadenylation, and mRNA nuclear export [30]. Mutations in 3′UTR of certain genes have been reported to be associated with several genetic diseases [31–33]. Recently,

Table 6: Association analysis of selected SNPs in *GJB2* and *GJB6* genes with the ARNSHL.

Gene	SNP	Samples	Allele distribution		P/P^* corrected	OR (95% CI)	Genotype distribution			P value
			1	2			1 1	1 2	2 2	
GJB2	rs3751385	CTL	198 (0.75)	66 (0.25)	$\mathbf{1.12 \times 10^{-13}/1.011 \times 10^{-12}}$	4.04 (2.77–5.89)	70 (0.53)	58 (0.44)	4 (0.03)	$\mathbf{5.83 \times 10^{-13}}$
	1 = C 2 = *T*	Patients	104 (0.43)	*140 (0.57)*			32 (0.26)	40 (0.33)	50 (0.41)	
	rs7994748	CTL	58 (0.22)	206 (0.78)	**0.004/0.036**	2.01 (1.25–3.25)	8 (0.06)	42 (0.32)	82 (0.62)	$\mathbf{1.91 \times 10^{-5}}$
	1 = C 2 = *T*	Patients	30 (0.12)	*214 (0.88)*			10 (0.08)	10 (0.08)	102 (0.84)	
	rs7329857	CTL	262 (0.99)	2 (0.01)	$\mathbf{3.86 \times 10^{-5}/3.48 \times 10^{-4}}$	11.70 (2.70–50.61)	130 (0.98)	2 (0.015)	0 (0.00)	$\mathbf{2.53 \times 10^{-5}}$
	1 = C 2 = *T*	Patients	224 (0.92)	*20 (0.08)*			102 (0.84)	20 (0.16)	0 (0.00)	
	rs7987302	CTL	261 (0.99)	3 (0.01)	**0.035**/0.315	3.72 (1.01–13.68)	129 (0.98)	3 (0.02)	0 (0.00)	**0.0323**
	1 = G 2 = *A*	Patients	234 (0.96)	*10 (0.04)*			112 (0.92)	10 (0.08)	0 (0.00)	
GJB6	rs7322538	CTL	232 (0.88)	32 (0.12)	0.704	0.87 (0.50–1.50)	101 (0.76)	30 (0.23)	1 (0.01)	0.764
	1 = G 2 = A	Patients	218 (0.89)	26 (0.11)			96 (0.79)	26 (0.21)	0 (0.00)	
	rs9315400	CTL	154 (0.58)	110 (0.42)	0.108	1.33 (0.94–1.89)	43 (0.33)	68 (0.51)	21 (0.16)	0.129
	1 = C 2 = T	Patients	125 (0.51)	119 (0.49)			35 (0.29)	55 (0.45)	32 (0.26)	
	rs877098	CTL	139 (0.53)	125 (0.47)	0.336	1.19 (0.84–1.69)	11 (0.08)	117 (0.89)	4 (0.03)	**0.0006**
	1 = C 2 = T	Patients	122 (0.50)	122 (0.50)			0 (0.00)	122 (1.0)	0 (0.00)	
	rs945369	CTL	145 (0.55)	119 (0.45)	**0.0144**/0.126	0.64 (0.45–0.92)	31 (0.23)	83 (0.63)	18 (0.14)	$\mathbf{1.5334 \times 10^{-6}}$
	1 = *A* 2 = C	Patients	*160 (0.65)*	84 (0.35)			61 (0.50)	38 (0.31)	23 (0.19)	
	rs7333214	CTL	45 (0.17)	219 (0.83)	**0.0003/0.0027**	0.46 (0.30–0.70)	4 (0.03)	37 (0.28)	91 (0.69)	**0.0004**
	1 = *T* 2 = G	Patients	*75 (0.31)*	169 (0.69)			7 (0.06)	61 (0.50)	54 (0.44)	

CI: confidence interval; odds ratio (OR) between groups was determined by logistic regression. *P*: *P* value calculated by chi-squared test or Fisher's exact test for difference in allele and genotype frequency between cases and controls. * *P* value after Bonferroni's correction. The allele frequency statistically significant is shown in italic. Significant values are shown in boldface (*P* value <0.05).

Ramsebner et al. [34] showed that the rs117685390 C allele in the regulatory region of the human *GJB2* gene could contribute to autosomal recessive nonsyndromic hearing loss.

Our study showed no significant differences between control and hearing loss subjects in terms of the distribution of alleles of the rs7322538, rs9315400, and rs877098 SNPs in the *GJB6* gene. Similarly, no significant differences were detected between noise-induced hearing loss (NIHL) and normal groups when Abreu-Silva and colleagues [35] compared allele and genotype frequencies for the rs877098 SNP. Elsewhere, negative associations of rs7322538 with sporadic hearing impairment were found in the Chinese population [10]. The same study showed a significant difference in allele frequency for rs9315400, most likely due to the different ethnicity.

5. Conclusions

The present findings indicate that the rs7322538, rs9315400, and rs877098 SNPs in the *GJB6* gene are not a significant risk factor for the development of ARNSHL in the Brazilian population. However, in contrast to these SNPs, it was found that carriers of the T allele of the SNPs rs3751385 (C/T), rs7994748 (C/T), rs7329857 (C/T), and rs7333214 (T/G) are at increased risk of ARNSHL.

Further studies are required to confirm these findings and to explore the hypothesis that the rs7994748 T, rs7329857 T, rs7987302 A, and rs945369 A alleles could be used as biomarkers for the development of autosomal recessive nonsyndromic hearing loss in Brazilian populations and could be included in assessments of the risk of developing ARNSHL. Finally, we provide important evidence of the association of SNPs in the *GJB2* and *GJB6* genes with hearing loss. The findings of this study contribute to an understanding of the intricate associations and gene interactions involved in hereditary hearing loss.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Camila Andréa de Oliveira conceived and designed the experiments. Ana Paula Grillo, Flávia Marcorin de Oliveira, Gabriela Queila de Carvalho, Ruan Felipe Vieira Medrano, and Sueli Matilde da Silva-Costa performed the experiments. Camila Andréa de Oliveira analyzed the data. Ana Paula Grillo, Flávia Marcorin de Oliveira, Gabriela Queila de Carvalho, and Edi Lúcia Sartorato contributed reagents, materials, and analysis tools. Camila Andréa de Oliveira wrote the paper.

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Research Article

A Novel *COL4A5* Mutation Identified in a Chinese Han Family Using Exome Sequencing

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Alport syndrome (AS) is a monogenic disease of the basement membrane (BM), resulting in progressive renal failure due to glomerulonephropathy, variable sensorineural hearing loss, and ocular anomalies. It is caused by mutations in the collagen type IV alpha-3 gene (*COL4A3*), the collagen type IV alpha-4 gene (*COL4A4*), and the collagen type IV alpha-5 gene (*COL4A5*), which encodes type IV collagen α3, α4, and α5 chains, respectively. To explore the disease-related gene in a four-generation Chinese Han pedigree of AS, exome sequencing was conducted on the proband, and a novel deletion mutation c.499delC (p.Pro167Gln*fs**36) in the *COL4A5* gene was identified. This mutation, absent in 1,000 genomes project, HapMap, dbSNP132, YH1 databases, and 100 normal controls, cosegregated with patients in the family. Neither sensorineural hearing loss nor typical *COL4A5*-related ocular abnormalities (dot-and-fleck retinopathy, anterior lenticonus, and the rare posterior polymorphous corneal dystrophy) were present in patients of this family. The phenotypes of patients in this AS family were characterized by early onset-age and rapidly developing into end-stage renal disease (ESRD). Our discovery broadens the mutation spectrum in the *COL4A5* gene associated with AS, which may also shed new light on genetic counseling for AS.

1. Introduction

Alport syndrome (AS) is a monogenic disease of the basement membrane (BM), resulting in progressive renal failure due to glomerulonephropathy, variable sensorineural hearing loss, and ocular anomalies. It is caused by defects of type IV collagen, which is the major structural component of BM and necessary for BM maintenance [1]. Type IV collagen comprises six α chains (α1–α6) encoded by the collagen type IV alpha-1 gene (*COL4A1*) to the collagen type IV alpha-6 gene (*COL4A6*), respectively. These six α chains share a common primary structure: an approximately 25-residue "7S" domain at the amino terminus, a collagenous domain of approximately 1,400 Gly-X-Y repeats, and an approximately 230-residue noncollagenous (NC1) domain at the carboxyl terminus [2]. AS is caused by mutations in the collagen type IV alpha-3 gene (*COL4A3*), the collagen type IV alpha-4 gene (*COL4A4*), and the collagen type IV alpha-5 gene (*COL4A5*), encoding type IV collagen α3, α4, and α5 chains, respectively [3]. The estimated gene mutation frequency is 1/5,000–1/10,000 [4]. Three inheritance patterns of AS have been reported: the most common X-linked inheritance (mutations in the *COL4A5* gene; ~85%), the less common autosomal recessive inheritance (mutations in the *COL4A3* gene and the *COL4A4* gene; ~15%), and the rare autosomal dominant inheritance [5]. Genotype-phenotype correlations of AS have been extensively described. Patients may present with a wide spectrum of phenotypes, ranging from benign familial

hematuria (BFH) or thin basement membrane nephropathy (TBMN) to end-stage renal disease (ESRD) resulting from various mutations, though the *COL4A5*-related BFH and TBMN were considered to be the milder subtypes of AS [5–7].

The purpose of our study is to explore the disease-related gene in a four-generation Chinese Han pedigree of AS. Exome sequencing is a powerful and cost-effective tool for uncovering the genetic basis of diseases [8, 9]. Conventional mutation screening by Sanger sequencing is time consuming and expensive due to genetic heterogeneity of AS and large size of those three genes (*COL4A3*, *COL4A4*, and *COL4A5*). Therefore, we detected the proband of the family using exome sequencing to identify the gene responsible for this disease. A novel deletion mutation c.499delC (p.Pro167Gln*fs**36) in the *COL4A5* gene was identified, and it cosegregated with the disease in the family. Our data broaden the genotypic spectrum of *COL4A5* mutations associated with AS.

2. Materials and Methods

2.1. Subjects. A pedigree consisting of 10 individuals across 4 generations of Chinese Han family was enrolled in this study (Figure 1). Peripheral blood samples were collected from 6 members of this family, including 4 patients. Peripheral blood samples were also collected from 100 unrelated ethnically matched normal controls (male/female: 50/50, age 40.6 ± 8.4 years). All participants underwent clinical evaluation, auditory and typical *COL4A5*-related ophthalmological examinations (dot-and-fleck retinopathy, anterior lenticonus, and the rare posterior polymorphous corneal dystrophy). The protocol of this study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, and all participants signed informed consent.

2.2. Clinical Data. All family members underwent urinalysis and renal function evaluation. Members with no more than trace amount of hematuria or proteinuria and normal renal ultrasound examination were considered normal [10]. Kidney biopsy was performed for the proband. Global and segmental sclerosis and mesangial expansion were identified by light microscopy. Electron microscopy revealed irregular thickening and splitting of the glomerular basement membranes (GBMs). Immunofluorescence and electron microscopy detected no immunoglobulin A (Ig A) deposition. None of the family members showed any evidence of auditory, typical *COL4A5*-related ophthalmological (dot-and-fleck retinopathy, anterior lenticonus, and posterior polymorphous corneal dystrophy), or platelet abnormalities or leiomyomatosis.

2.3. Exome Capture. Genomic DNA was isolated from peripheral blood leukocytes by standard phenol-chloroform extraction method [11]. Three micrograms (μg) of genomic DNA was used to construct the exome library. Genomic DNA of the proband was sheared by sonication and hybridized to the Nimblegen SeqCap EZ Library for enrichment, according to the manufacturer's protocol. Enriched exome fragments were sequenced on the HiSeq 2000 platform (Illumina, San Diego, CA, USA) to get paired-end reads with read length of 90 bp. A mean exome coverage of 81.65× was obtained to accurately call variants at 99.41% of the targeted exome [12, 13].

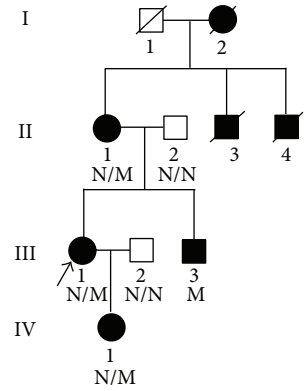

Figure 1: Pedigree of the family with X-linked Alport syndrome. N: normal, M: *COL4A5* c.499delC (p.Pro167Gln*fs**36) mutation.

2.4. Read Mapping and Variant Analysis. The sequence reads were aligned to human genome reference obtained from UCSC database (http://genome.ucsc.edu/), version hg19 (build 37.1), using the program SOAP aligner. Single nucleotide polymorphisms (SNPs) were called using SOAPsnp set with the default parameters after the duplicated reads (produced mainly in the PCR step) were deleted [14]. Short insertions or deletions (indels) altering coding sequence or splicing sites were also identified by GATK. We filtered candidate SNPs with the following criterion: SNP quality ≥20, sequencing depth ≥4, the estimated copy number ≤2, and the distance between two SNPs >5 (the quality score is a Phred score, generated by the program SOAPsnp1.03, and quality score 20 represents 99% accuracy of a base call) [6]. Candidate mutations were filtered against databases including the single nucleotide polymorphism database (dbSNP132, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi/), 1,000 genomes data (1,000 genomes release_20100804), HapMap (2010-08_phase II + III) and YanHuang1 (YH1) project, and synonymous substitutions. Potential disease-causing variants were evaluated by SIFT prediction (http://sift.jcvi.org/). Sanger sequencing was employed to verify the identified potential disease-causing variants with ABI3500 sequencer (Applied Biosystems, Foster City, CA, USA). Sequences of the primers were as follows: 5′-TGA-ATCTTCAGATCATTTTTCTGG-3′ and 5′-GAGGGA-TTGTTGTAATCTTCTGG-3′.

3. Results

We performed exome sequencing of the proband (III: 1, Figure 1) in a Chinese Han family with AS. We generated 8.14 billion bases of 90-bp paired-end read sequence for the patient. Among the 8.14 billion bases, 7.88 billion (96.81%) passed the quality assessment, 7.37 billion (93.53%) aligned

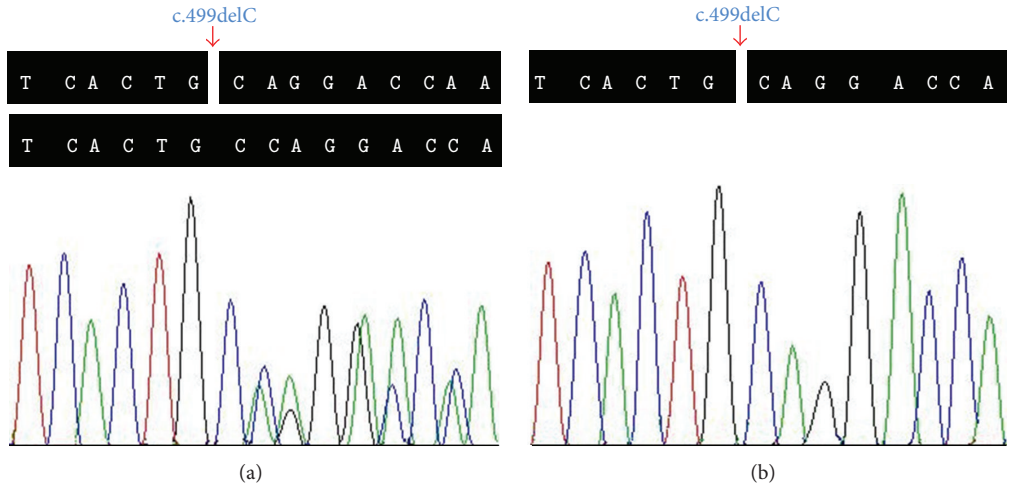

Figure 2: Sequencing analysis of *COL4A5* c.499delC (p.Pro167Gln*fs**36) mutation. The arrow shows site of the novel c.499delC (p.Pro167Gln*fs**36) deletion mutation in the *COL4A5* gene. (a) Heterozygous mutation carrier (III: 1). (b) Hemizygous mutation carrier (III: 3).

to the human reference sequence, and 3.60 billion bases (48.85%) mapped to the targeted bases with a mean coverage of 81.65-fold. 105,963 genetic variants, including 14,723 nonsynonymous variants, were identified in either the coding regions or the splice sites. A prioritization scheme was applied to identify the pathogenic mutation in the patient, similar to recent studies [6, 15]. We excluded known variants identified in 1,000 genomes project, HapMap, dbSNP132, and YH1. Applying the above strategy, we reduced the number of candidate genes by more than 90.33%.

A novel deletion mutation, c.499delC (p.Pro167Gln*fs**36), was identified in exon 9 of the *COL4A5* gene in the proband. This mutation results in premature stop codon and a truncated protein. The same mutation was subsequently verified in all four affected family members (II: 1, III: 1, III: 3, and IV: 1; Figure 1), while being absent in unaffected members and 100 ethnically matched normal controls by Sanger sequencing (Figure 2). It is also absent in 1,000 genomes project, HapMap, YanHuang1 (YH1) project, and dbSNP. The mutation is located in the Gly-X-Y repeats. The p.Pro167 is a highly conserved amino acid residue among different species from chicken to human, suggesting its structural and functional importance (Figure 3). This mutation was predicted to affect the protein features and be disease causing (predicted by http://www.mutationtaster.org/). SIFT prediction also showed a damaging effect with a confidence score of 0.858 (http://sift.bii.a-star.edu.sg/www/SIFT_indels2.html).

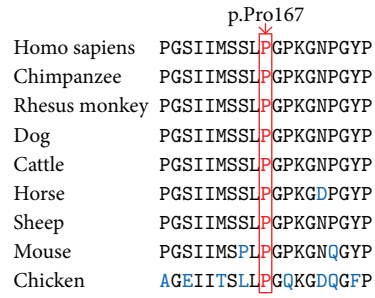

Figure 3: Conservation analysis of *COL4A5* p.Pro167 amino acid residue.

4. Discussion

AS is a clinically and genetically heterogeneous disease, and severity of this disease is usually equal between males and females in the autosomal recessive form (autosomal recessive AS, ARAS), while greater in males with X-linked form (X-linked AS, XLAS). XLAS is caused by mutations in the *COL4A5* gene with an approximately prevalence of 1/10,000 [5], and it accounts for 40%–45% of female patients with AS [16]. Female patients with XLAS have a variable and generally mild clinical course with 12% reaching ERSD by the age of 40 years and about 30% by the age of 60 years in European cohorts [17]. While male patients are more severe than females with 70% of affected males developing into ESRD before the age of 30 years (juvenile form), the remaining 30% are progressing toward ESRD after the age of 30 years (rare adult form) [18]. Furthermore, hearing loss and ocular abnormality happened in 90% and 35% of male patients, respectively [10].

In our family, four patients presented with heterogeneous clinical phenotypes of glomerulopathy, while none of them showed any clinical features of either sensorineural hearing loss or typical *COL4A5*-related ocular abnormalities. A *COL4A5* c.499delC (p.Pro167Gln*fs**36) mutation in exon 9, cosegregating with the disease, was identified. The deletion mutation leads to a truncated protein and is absent in 1,000 genomes project, HapMap, YanHuang1 (YH1) project, dbSNP, and 100 normal controls. Our clinical and genetic data also support an X-linked inheritance form of AS in this family.

Table 1: Clinical and genetic data of 4 *COL4A5* c.499delC (p.Pro167Gln*fs**36) mutation carriers.

Subject	II: 1	III: 1	III: 3	IV: 1
Gender	F	F	M	F
Age (year)	42	24	22	6
Onset-age (year)	13	20	3	3
Genotype	Heterozygote	Heterozygote	Hemizygote	Heterozygote
Renal function	ESRD at 36 years	Normal	Normal	Normal
Microscopic hematuria	Yes	Yes	Yes	Yes
Gross hematuria	Yes	No	Yes	No
Proteinuria	Yes	No	Yes	No
Uremia	Yes	No	No	No
Audiological examination	Normal	Normal	Normal	Normal
Ophthalmic examination*	No	No	No	No

*Dot-and-fleck retinopathy, anterior lenticonus and posterior polymorphous corneal dystrophy; *COL4A5*, the collagen type IV alpha-5 gene; F, female; M, male; ESRD, end-stage renal disease.

The *COL4A5* gene is located at Xq22 and contains 51 exons, encoding type IV collagen α5 chain [6]. Type IV collagen α5 chain contains 1,685 amino acid residues, which consist of a 26-residue signal peptide, a 1,430-residue collagenous domain starting with a 14-residue noncollagenous sequence, a Gly-X-Y-repeat sequence interrupted at 22 locations, and a 229-residue carboxyl-terminal NC1 domain [19]. To date, 688 *COL4A5* mutations have been identified according to the Human Gene Mutation Database (http://www.hgmd.org/), including missense, nonsense, deletion, splicing mutation, and complex rearrangements [17, 20], without identification of any mutation hot spot. Genotype-phenotype correlations between *COL4A5* mutations and XLAS have been extensively described. For genotype-phenotype correlation purposes, typical XLAS is classified into three types: (1) severe type with ERSD at ~20 years (juvenile-onset ESRD), 80% of hearing loss, and 40% of ocular lesions, caused by large rearrangements, premature stop, frameshift, donor splice, and mutations in the NC1 domain; (2) moderate-severe type with ESRD at ~26 years, caused by non-Gly-X-Y-missense, Gly-X-Y mutations in 21–47 exons; (3) moderate type with ESRD at ~30 years (late-onset ESRD), 70% of hearing loss and <30% ocular lesions, caused by Gly-X-Y mutations in 1–20 exons [21, 22]. Four patients of our family showed no clinical features of either sensorineural hearing loss or typical *COL4A5*-related ocular abnormalities. Though our family is not large, a moderate type of XLAS is considered due to the mutation located in *COL4A5* exon 9 and the late-onset ESRD (ESRD at 36 years, II: 1; Table 1). More severe clinical phenotypes and earlier onset-age were observed in male patient of this family (III: 3), consistent with previous reports [6].

Mutations in genes encoding α chain of type IV collagen could lead to dysfunction of BM and then lead to the development of human disease in the eye, kidney, ear, and so forth [1]. Once the α5 chain is missing, the formation of the normal α3α4α5 (IV) protomer is disrupted in BM of glomerulus, ear, eye, and lung, which could lead to structural and functional defects [23]. This is supported by the immunohistochemical finding of frequent loss of α3, α4, and α5 signals in the GBM of XLAS patients [24, 25]. The cause of clinical heterogeneity of XLAS, such as difference in age of disease onset, disease severity, and disease progression, may be multifactorial, including random X chromosome inactivation, ethnic background, and environment factors.

Animal models with genetic deficiency may provide probabilities to reveal the pathogenesis and treatment of AS [26]. Two *Col4a5* truncation mutations have been identified in dogs (Samoyed and Navasota dogs) with clinical features of proteinuria and progressive kidney disease leading to terminal failure [27]. Intriguingly, a deletion in *Col4a5* resulting in disruption of the Gly-X-Y repeats, similar to human p.Pro167Gln*fs**36 mutation, was observed in a family of mixed-breed dogs with an inherited nephropathy that exhibits the clinical, immunohistochemical, pathological, and ultrastructural features of human XLAS, and the truncated peptide chain may probably prevent extracellular assembly in type IV collagen networks [28]. Further studies on the *Col4a5* genetic-deficient AS animal models will provide new insight into mechanism research, diagnosis, and target therapy of AS in human.

5. Conclusions

In our study, we identified a novel deletion mutation c.499delC (p.Pro167Gln*fs**36) in the *COL4A5* gene, which may be responsible for AS in this family. Our study showed that exome sequencing is a fast, sensitive, and relatively low-cost method to identify gene(s) responsible for AS. The discovery broadens the genotypic spectrum of *COL4A5* mutations associated with AS and has implications for genetic diagnosis, therapy, and genetic counseling of this family.

Conflict of Interests

The authors declare that there is no conflict of interests in this paper.

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