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Research Article

A *De Novo* from Mutational Analysis in PAX3 in Hearing Loss Children Caused Waardenburg Syndrome Type 1 at Children Hospital-1 in Ho Chi Minh City, Vietnam

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Abstract

Purpose: To detect a de novo paired box gene 3 (PAX3) mutations, mutational analysis, and associated phenotypes in the Vietnamese Children patients with Waardenburg syndrome type I (WS1). We conduct this study to provide initial information about types, rates, and influences of this particular PAX3 mutations gene in the Vietnamese patients.

Patients and Methods: Three unrelated patients with suspected WS1 were selected from our Genomic extraction DNA, probe and sequence the primers, PCR technique. PAX3 were amplified by polymerase chain reaction (PCR), and then amplicons PCR were analyzed by cycle sequencing. Variations were detected, documented and recognized as "*de novo*" mutation. We also compared the severity of hearing impairment, phenotypically variations among these individual affected patients.

Results: Number variations of mutations in PAX3 were detected in three patients, respectively: c.955delC (Gln319fsX380), c.667C>% (Arg223Stop). One mutation proved to be de novo as their parents did not carry the mutations, and had never been found in any previous study in Vietnam. All three patients with PAX3 mutations had different iris color, fundi between their two eyes, dystopia canthorum and profound hearing loss. We report the phenotypic expression of WS1 in these three patients and explore the implications for possible genotype-phenotype correlations. Sensorineural hearing loss was present in 80% of affected individuals, and spectrum of hearing loss with very high frequency.

Conclusion: *De novo* mutation in PAX3 has not been reported in Vietnam before. Finally, our finding has detected deletion mutation c.667>T (p.Arg223X) in PAX3 identified in three children individual with WS1. Our analyses indicated that these mutations might constitute a pathogenic-genetic-hearing loss associated with WS1. It is very clinical importance as such patients may be misdiagnosed as congenital hearing loss since it is uncommon in Southern Vietnamese population.

Introduction

Waardenburg Syndrome (WS) is named after Dutch ophthalmologist Petrus Johannes Waardenburg (1886-1979), who described the syndrome in detail in 1951. It is the most common type of autosomal dominant syndromic hearing loss. It consists of variable degrees of hearing loss and pigmentary abnormalities of skin, hair (white forelock), and eye (*Heterochromia iridis*). Ten subtypes and four types are recognized: WS1, WS2, WS3, and WS4. Phenotypic among these types are difference: WS1 is characterized by presence of dystopia canthorum while WS2 is characterized by its present. In WS3, upper-limb abnormalities are present, and finally in WS4, Hirschsprung disease is present. Overall, the syndrome affects approximately 1 in 45000 people [1].

WS1 is a pigmentary-auditory disorder comprising congenital sensorineural hearing loss and other pigmentary disturbances. The hearing loss in WS1, observed in approximately 70% of affected individuals. It is congenital, typically non-progressive, either unilateral or bilateral, and sensorineural [2]. Mutations in the paired box gene 3 (PAX3) have been identified to be responsible for WS1. Identification of a heterozygous PAX3 pathogenic variant by molecular genetic testing establishes the diagnosis if clinical features are inconclusive [3]. PAX3 is a gene that belongs to the paired box (PAX) family of

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transcription factors. This gene was formerly known as splotch. PAX3 has been identified with ear, eye and facial development. It is expressed in early embryonic phases in pigment system, and in dermatomyotome of paraxial mesoderm which it helps to demarcate. PAX3 encodes a member of the mammalian PAX family of transcription factors, which contains two highly conserved domains for DNA binding, paired box domain and paired-type homeodomain [4]. PAX3 is active in neural crest cells. In conjunction with MSX1, PAX3 guides the expression of the molecule; this allows neural crest cell plays very important role on organ development. Thus, mutating of PAX3 results neural crest cell can't complete their function as formation development role, secondary to impair hearing loss [5]. This mutation results in replacing the amino acid asparagines with lysine, inhibiting the PAX3 protein from binding to the necessary DNA. Alternative splicing of PAX3 results in several different length transcripts, of which the longest transcript contains 10 exons, and consequent proteins with distinct carboxyl termini. Heterozygous mutations in PAX3 have been reported in familial and sporadic WS1, while heterozygous or homozygous mutations have been detected in patients with WS3. Numerous mutations have been identified in Caucasians, minimal cases have been determined in Asian population. However, WS1 patient with PAX3 mutations in Vietnam have not been studied or reported.

In this study, we conducted detailed, thorough clinical and genetic analysis of several Vietnamese children individuals with WS1-afflicted. Several mutations in PAX3 were identified in three individual children from unrelated families with WS1. A de novo heterozygous deletion mutation c.667C>T (p.Arg223X) was also detected in the patient. All patients with mutations presented dystopia cathorum, different colors of irises, and profound hearing loss. Since pathogenic variant has been identified in an affected children, prenatal testing option from a clinical laboratory that offers either testing for this gene or custom prenatal testing. Molecular genetic testing of relatives at risk allows for early screening of those at risk for hearing loss.

Subjective and Methodology

Patients

Three unrelated patients were referred from our Pediatric and Genetic Ophthalmology Department at Nhi Dong I Children Hospital, HCM City Vietnam. There are several highlighted clinical features, but they are very inconclusive. In addition to profound hearing loss, one patient has hypoplastic blue irides, other had delayed speech pathology. No other clinical symptoms have met major, minor criteria to establish the diagnosis of WS1, thus, diagnosis of WS1 aren't confirmed to two major criteria or one major plus two minor criteria aren't met. Therefore, we tried to identify of a heterozygous pathogenic variant in PAX3 by molecular genetic testing, and result is positive. Informed consent conforming to Center for Molecular Biomedicine at The University of Medicine and Pharmacy at HCM City, HCM City, Vietnam; all participants received detailed ophthalmological and otolaryngological examinations performed by ophthalmologist, and otolaryngologist. All patients signed the consents and followed the IRB Review of Vietnam guideline/protocols.

Research methodology

We are reporting 3 cases in each three individual patients with congenital sensorineural hearing loss with features characteristic for WS1, and PAX3 gene have been extracted, analyzed for further evaluation of de novo gene definition.

Case presentation 1: The 12 years old boy was referred from Ophthalmology Department for genetic counseling and further evaluation due to congenital hearing loss. The proband is the only child of non-consanguineous parents. He was born at term after an uneventful pregnancy. His birth weight was 3200 gram; length 53 cm. Apgar score was 10. His early psychomotor development was normal. Due to his profound hearing loss, he attended a school for children with hearing impairment. At the age of 10, he was hospitalized due to minor car accident has been hit by motorcycle in Ho Chi Minh City. Phenotype examination at genetic counseling center revealed the presence of wide set eyes of hypoplastic blue irides, and dystopia canthorum. He did not present white forelock characteristic for WS. Intellectual development was normal. Both parents were phenotypically normal and neither of them presented any features suggestive of WS.

Case presentation 2: The 8 years old boy was also referred for genetic counseling due to hypoplastic irides in addition to congenital hearing loss. The proband is the first child of non-consanguineous parents. His younger sister-5 years old, who has the same problem. They were born at term after an uneventful pregnancy. Their early psychomotor development was normal, without any notable delay up to 2 years of age. Later both presented speech impairment due to hearing problems, noted at the age of 3. Both attended a school for children with hearing impairment. Both parents were congenital hearing impairment, but lacking hypoplastic irides. Interesting, their first auntie was also congenital hearing impairment, and noted features of hypoplastic irides suggestive of WS. Due to economic unstablility, living with social inequality; both parents and their first auntie have relocated to different regions of the country, thus we can't obtain their DNA sample for gene mutations analysis.

Methodology

DNA genomic extraction

For molecular testing, DNA was extracted from peripheral blood (venous leukocytes) samples using standard nonorganic protocols. Bloods were stored with EDTA agent, and transported to Molecular Genetic Institute at HCMU School of Medicine and Pharmacy. Genomic DNA was extracted from 200 µL peripheral blood by using QIAamp DNA kit (Quiagen, USA), followed by manufacturing instruction.

Probe and sequencing primers

To detect mutations in exon 3 of the PAX3 gene, we used the strategy of directly sequencing PCR-amplified DNA of an affected individual from each patient. Oligonucleotide primers CH2-1 and Ch2-3 were used to amplify human PAX3 exon3. The amplification product from 50 ml reaction was purified by membrane. To amplify human PAX3, the first strand cDNA was used as a template for a first round of amplification to amplify from exon 2 to the last 18 bases of coding sequence with oligonucleotide primers HUP2-4 and PAX3-1. Three additional nested amplifications were performed by using the product of each preceding reaction as a template. The primer pairs used were HUP2-5 (5' end of exon 3) with PAX3-1 (last 18 bases of coding sequence), HUP2-5 (5' end of exon 3) with PAX3-13 (64 bases upstream from stop codon). PAX3 is code as "NG_011632" on GenBank. Pairs of primer were designed based on primer3 http://bioinfo.ut.ee/primer3-0.4.0/). The primers for

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Table 1: Reaction of PCR and temperature/interval on gen PAX3 gene.

Reactions		Temperature and Interval of probe PCR			
PCR conducted	**Volume (µl)**	**Periods**	**Temp**	**Times**	**Interval**
10X PCR buffer	1.5		98°C	3 min	
2.5 mM dNTP	1.5	Denatured	98°C	10 sec	40
10 µM primer (F_2 and R_2)	1.5	Binding	60°C	20 sec	
H_2O	8.4	Prolonged	72°C	40 sec	
Takara HS Taq	0.1		72°C	3 min	
gDNA	2.0				
Total Volume	25				

exon F2 were as follows: (5'-GTCCTAGCTAGTGATTCCTG-3') and exon R2 (5'-GTTGCCTCATCCCTCTCATG-3'), a 50-bp product was obtained after amplification. The amplicons from individual exon were purified and analyzed by cycle sequencing with Integrated DNA Technologies (USA). Sequencing results from patients were imported into NCBI Human Genome Database for comparing and filtering any de novo mutation gene. Each variation was confirmed by bidirectional sequencing.

PCR technique

PCR of the PAX3 exons was performed in a total volume of 25 µL containing 50-100ng of genomic DNA, 500 nM each of the forward and reverse primers, 500 µM dNTPs, (250 µM for each and 1.25 U LA Taq DNA polymerase with GC buffer I from TAKARA (Tokyo, Japan). The amplification consisted of an initial denaturation stage at 98°C for 3 min, followed by 40 cycles consisting of denaturation at 98°C for 30 s, annealing for 15 s at 60°C, and extension at 72°C for 1 minute, with an extension step performed at 72°C for 5 min (Table 1).

PCR product were dyed in agarose gel 1.5% with ethidium bromide and observed by camera photographer Geldoc-It (UVP, USA). Finally, PCR was purified, buffered by illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, England) based on the manufacturing instruction.

Perform DNA sequencing

PCR fragments were sequenced using the forward and/or reverse primers and ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) and ABI 3130 Genetic Analyzer. The product is then precipitated with ethanol, dissolved in Hi-Di formamide, denatured at 95°C in 2 minutes before the sudden cooling. DNA sequence later read by ABI 3130 Genetic Analyzer, With POP-7 polymer and 80 cm capillary (Applied Biosystems, USA). Results are plugged in and analyzed by CLC Main Workbench software.

Statistical analysis

All experiment results were analyzed by Excel and IBM SPSS v.20.0 with 95% Confident Interval (CI)

Results and Discussion

General characteristic of experiments

Inclusion criteria were: 1) congenital; 2) clinical presentation: have one sign or symptoms belongs to major or minor criteria 3) type of hearing loss: sensorineural; 4) prognosis: stable/progressive HL; 5) any ear affected; 6) sporadic or familial case in his/her family

Exclusion criteria were: 1) non-syndromic hearing loss; and 2) congenital hearing loss caused by infections. The period of study took place between April, 2015 to September, 2016.

Variation detection

In the 3 patients, numerous heterozygous mutations in PAX3 were detected, including c.955delC (Gln319fsX380), c.567_586+17del (p.Asp189_Gln505delinsGluGlyGlyAlaLeuAlaGly), c.456_459dupTTCC (p.Ile154PhefsX162), c.795_800 delCTGGTT(p.Trp266_Phe267del), c.799T>A (p.Phe267Ile), and c.667C>T (p.Arg223X). The c.567_586+17del mutation was identified in a baby from Family A. Direct sequencing revealed a heterozygous variation involving multiple nucleotides in exon 4 region. Cloning sequencing revealed a 37bp deletion exon 4 and intron 4. A new splice site is predicted to be created downstream. The encoded protein would be truncated. The c.456_459dupTTCC and c.795_800delCTGGTT mutations were only present in the probands. Mutation c.667C>T (p.Arg223X) is proved to be de novo as their parents did not carry the mutations, suggesting that natural occurring new mutations in PAX3 of the Vietnamese population is not uncommon. Based on available information, *de novo* mutations in PAX3 have rarely been mentioned before. C.667C>T(p.Arg223X), are predicted to encode premature truncated proteins affecting the paired-type homeodomain.

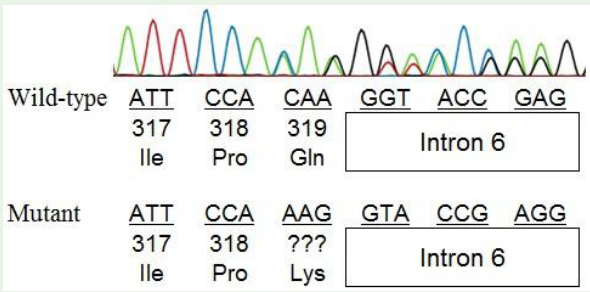

Figure 1: Premature truncated proteins affecting the paired-type homeodomain.

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Figure 2: The encoded truncated protein.

Conclusion

In summary, we described a de novo heterozygous deletion mutation in 2 Vietnamese families with WS1. Our analyses indicated that these mutations are highly associated with congenital hearing loss. This will be the root solution to confirm diagnosis with better treatment.

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