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Original Article

Whole Exome Sequencing of Non-Syndromic Hearing Loss Patients

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Abstract

Background: Hearing loss is the second most common disease after mental retardation in Iran. Autosomal recessive non-syndromic hearing loss (ARNSHL) is an extreme and highly heterogeneous disease, for which more than 70 genes have been identified. Considering the frequency of family marriage as well as the importance of ARNSHL in Iran, we evaluated the genetic factors involved in this type of deafness.

Methods: We performed the whole exome sequencing (WES) of eight Iranian subjects with severe non-syndromic hearing loss selected from 110 well-characterized subjects with non-syndromic hearing loss from 2017-2019. The patients with mutated *GJB2* and *GJB6* genes were excluded from the study.

Results: The use of the whole exome sequencing method revealed 10 different mutations in 7 genes, including *SLC26A4 (*c.1234G>T), *FGF3* (c.45DelC, c.466T>C), *ADGRV1* (c.12528-2A>C, c.16226-16227insAGTC), *OTOG* (c.7454delG), *OTOF* (c.3570+2T>C), *ESPN* (c.992G>A), *OTOA* (c.2359G>T, c.2353A>C). Seven new variants were observed in seven families including *SLC26A4* (c.1234G>T), *FGF3* (c.45DelC), *ADGRV1* (c.12528-2A>C), *OTOG* (c.7454delG), *ADGRV1* (c.16226-16227insAGTC), *OTOF* (c.3570+2T>C).

Conclusion: The causal mutation of ARNSHL was found in all patients using the WES. Meta-analysis studies can help to identify common mutations causing deafness in any population to facilitate identification of carriers and subjects with deafness.

Keywords: Heterogenous disease; Inherited non-syndromic hearing loss; Whole exome sequencing

Introduction

The most common sensory impairment in humans is hearing loss and on average 1 in every 1000 newborn infants is affected by pre-lingual deafness (1). The prevalence of deafness in Iran is 2 to 3 times higher than that in other parts of the world (1). It is also the second most common disability after mental retardation in Iran (2). Hearing loss is very heterogeneous etiologically, and it can occur due to genetic mutations, environmental factors, or both. The type and distribution of mutations in deafness vary in different groups and populations of the world. Despite several studies, there are no exact statistics on the prevalence of deafness in Iran. Statistics indicate that over 60% of hearing loss cases are inherited, and recent studies suggest that approximately 1% of all human genes (approximately 200 to 250 genes) is involved in hearing loss (3). More than 1200 mutations and 140 loci in more than 90 genes have been identified as the involved genes in hereditary hearing loss (4).

Approximately 75% to 85% of hereditary hearing loss cases are non-syndromic, and the main pattern of inheritance; is autosomal recessive (DFNB). The other inheritance patterns of deafness are including autosomal dominant (20%-25% and most often post-lingual), X-linked (1%-2%) and mitochondrial which is less than 1% (5). All mendelian hereditary patterns of pre-lingual deafness have been observed in Iran, but autosomal recessive non-syndromic hearing loss (ARNSHL) is the most common one. Mutation in 76 different genes has been so far reported for ARNSHL in the world (6) and mutation in the Gap Junction Protein Beta 2 (*GJB2*) gene encoding connexin 26 is the most common cause of ARNSHL in many populations. Interestingly, a mutation in the *GJB2* gene is not the leading cause of ARNSHL in the Iranian population, and this gene is responsible for 16% of ARNSHL cases (7) (Fig. 1).

Several methods have been used to investigate the genetic architecture responsible for ARNSHL, and most mutations in ARNSHL genes are rare and may be seen only in one or a limited number of families (8). Therefore, due to high heterogeneity in ARNSHL, performing the next-generation-sequencing (NGS) technique with unprecedented ability can overcome these limitations, particularly, the whole-exome- sequencing (WES) method is useful to detect variants and rare genes causing ARNSHL.

Considering the high rate of family marriage in Iran, whole exome sequencing (WES) can help to identify the genetic causes of deafness in each region, according to the founder effects. This study aims to find the causal mutations in eight families with at least four subjects with ARNSHL without mutation in *GJB2* and Gap Junction Protein Beta 2 (*GJB6*) genes using WES.

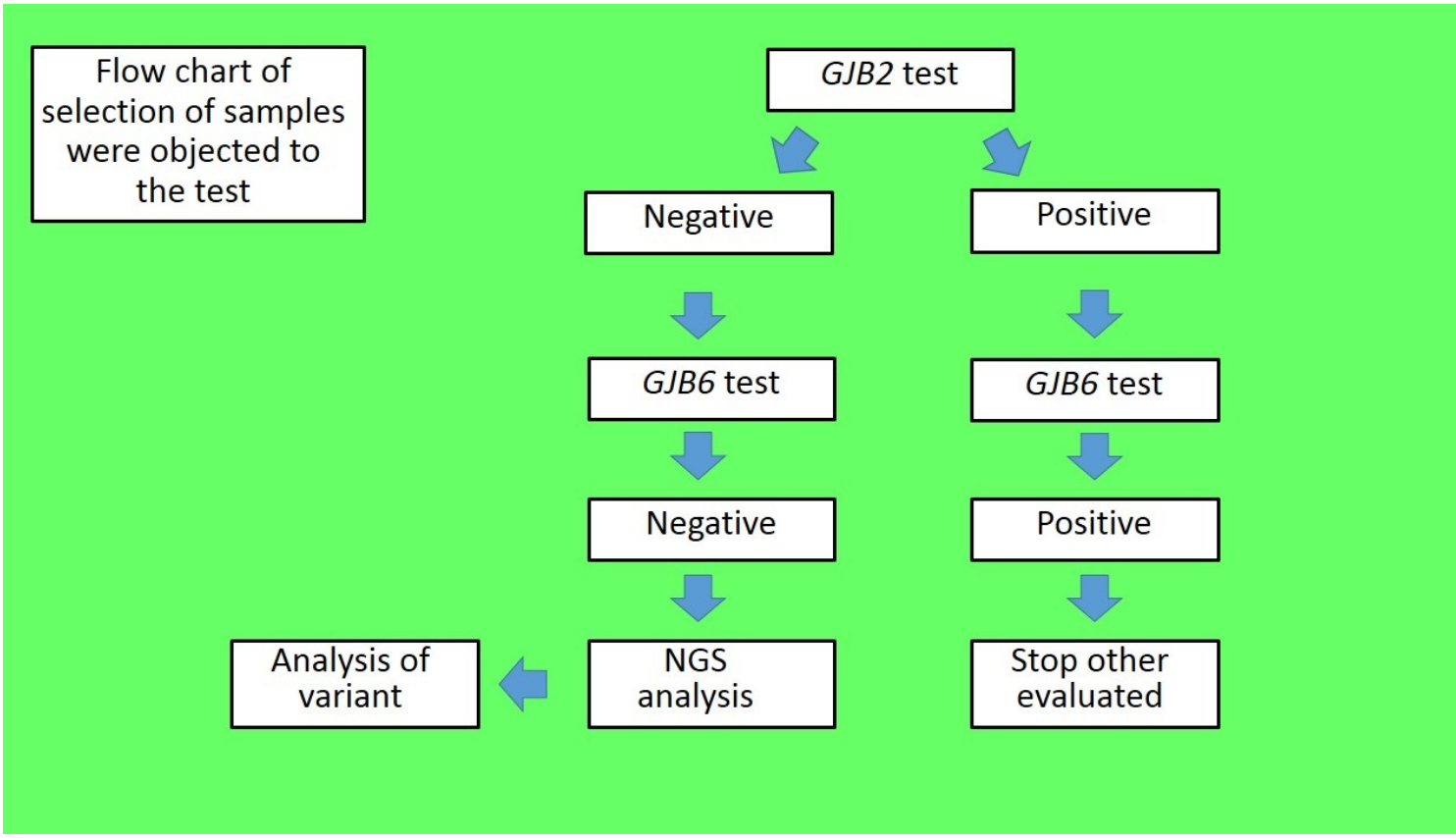

Fig. 1: Flow chart of sample selection that were objected to the tests

Materials and Methods

Patients and screening

At first, from a large group of patients recruited from Welfare Center of Qum Province, Iran, from 2017-2019. One hundred and thirty-one patients with pre-lingual hearing loss including 25 patients with sporadic inheritance and one hundred and six patients with autosomal recessive inheritance were selected based on family history of hearing loss and pedigree analysis. Non-genetic and syndromic causes of hearing loss based on medical history and ear, nose, and throat (ENT) were rejected. Medical history was evaluated based on our previous study (9).

Informed consent was obtained from all families and ethical approval for this study was received from the ethics committee in biomedical research of Islamic Azad University committee (reference IR.IAU.SRB.REC.1396.75).

All samples were screened for the mutations in the exons and UTRs (Untranslated region) of the *GJB2* gene using the conventional Sanger sequencing. The detailed method has been explained in our previous publish paper (9). We excluded 21 samples with a homozygous mutation in the *GJB2* gene. None of the samples showed mutation in the *GJB6* gene.

Exome Sequencing

Genomic DNA was extracted from the peripheral blood using the QIAamp DNA Blood Mini Kit QIA (Cat. No. 51104, Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Following library preparation, exome trapping on 50 ng of DNA was performed using the Nextera Rapid Capture Exome kit and DNA Sequencing was carried out on Illumina HiSeq 4000 platform as paired-end 150-200 bp reads according to the manufacturer's protocol.

The raw data were filtered on Minor Allele Frequency (MAF) and Insilico analysis, allele frequency, databases such as PubMed, inheritance pattern and tools such polyphen-2 (available at : http://genetics.bwh.harvard.edu/pph2), SIFT (available at: https://sift.bii.a-star.edu.sg) and SNP nexus(available at: https://www.snp-nexus.org/v4).

Generally, the investigation of the candidate genes of deafness was the primary goal of this study, conducted in several steps. In the first step, the variants with an allelic frequency higher than 0.01 in a database of 1000 genomes or exome variant server were deleted (Fig. 2).

The rest variants were novel, not reported so far. In the second step, the relationship between the genes associated with ARNSHL was investigated by examining the databases such as OMIM, Gene and PubMed. Unrelated genes were excluded from list of studied genes and the remaining variants were classified based on the autosomal recessive inheritance pattern, assuming that parents were obligated carriers and patients must be the homozygous mutated for that trait. Regarding the type of variants, the effect of the variants on the protein products was investigated using the polyphen-2, SIFT and online SNP nexus software and the benign or tolerated variants were excluded from the list of candidate variants. The candidate variants were screened in the ExAC (Exome Aggregation Consortium) database and confirmed as a candidate in the families using Sanger sequencing.

The results of the Q in this subject indicated that more than 98% of identified variants had the probability of an error per 100.

All found variants have been validated and co-segregation with the disease tested and approved in each family including all deaf people and parents by Sanger-base sequencing (PCR conditions and primer sequences and are available).

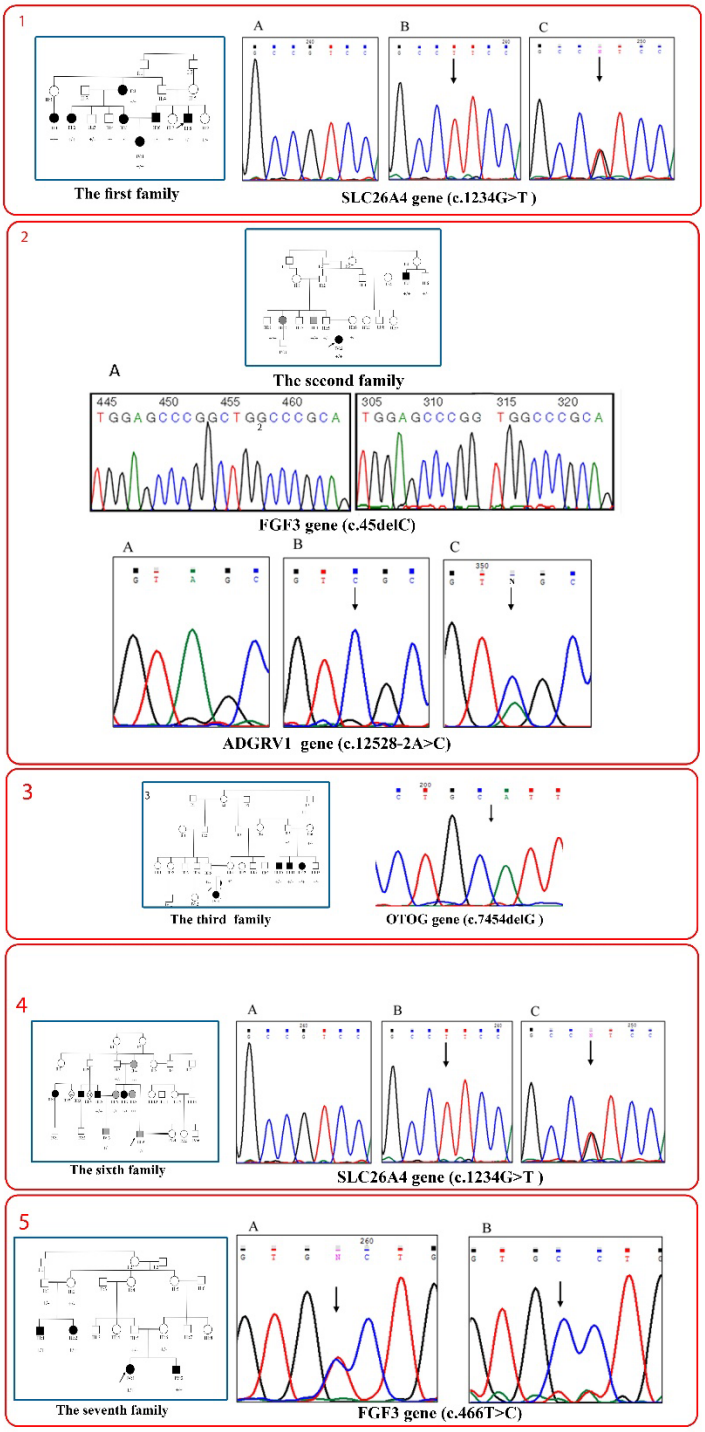

Fig. 2: Chromatogram of whole exome sequencing of samples selected for mutation analysis. The pedigrees of families 1, 2, 3, 6 and 7 and chromatogram of new variants related to any family. Individuals with severe and moderate deafness are indicated by black and gray filled symbols, respectively. In addition, and minus signs indicate the presence and absence of the mutation, respectively. The arrow indicates the changed base

Results

We focused on eight families with at least four deaf members. In all eight families, according to the parents' information and available documents, deaf people did not have any complications during childbirth, including embryonic anomalies, preterm labor, low birth weight, birth injuries and viral infection. The pedigrees of the families 1, 2, 3, 6 and 7 are depicted in Fig. 2. The most severe deaf person in each family was investigated for the causative variants using WES that were negative for general causative mutations like GJB2 or GJB6 genes. Segregation analysis was conducted to determine the causative mutation in each family.

All the patients had different degrees of hearing loss. All patients in the third, fifth, seventh and eighth pedigrees showed severe deafness (according to audiogram analysis, data not shown). Some patients in the second, fourth and sixth pedigrees had moderate to severe clinical deafness signs (Fig. 2). The variants filtering results for eight families have separately listed in Table 1. We found seven novel variants in seven out of eight families. The chromatogram of some new variants shows in Fig. 2.

The severity of hearing loss in patients was correlated to these variants: *OTOG* (c.7454delG), *OTOF* (c.3570+2T>C) and *FGF3* (c.466T>C) Respectively.

The homozygote c.1234G>T variant (p.Val412Phe) was found in the *SLC26A4* (Solute carrier family 26 member 4) gene in the first and sixth family. The homozygous frame shift mutation c.45DelC (p.Trp16Glyfs61) in the *FGF3* (Fibroblast growth factor 3) gene was found in the second family. The c.12528-2A>C variant in the *ADGRV1* (Adhesion G protein-coupled receptor V1) gene found in the in the second family is a splice acceptor site mutation. In the third family, the frame shift c.7454delG variant (p.Arg2458Hisfs77) in the *OTOG* (Otogelin) gene was detected as the causal mutation. The c.16226-16227insAGTC variant in the *ADGRV1* (Adhesion G protein-coupled receptor V1) gene was detected in the fourth family. The c.3570+2T>C variant in the *OTOF* (Otoferlin) gene was detected in homozygote state (3 subjects) as well as healthy or heterozygote state in control subject in the fifth family. Finally, the homozygous missense mutation c.466T>C (p.Ser156Pro) in the *FGF3* (Fibroblast growth factor 3) gene was found in the seventh family.

Table 1: The variants filtering results for eight families (number 1 to 8, respectively) (*e.g.* c.1521_1523delCTT (p.Phe508del), pathogenic, cystic fibrosis, autosomal recessive)

Family No	*Gene (transcript)*	*Nucleotide (protein)*	*Zygosity Index*	*In silico analysis*	*MAF*	*Variant Classification*	*Disorder (#OMIM;inheritance)*
1	*SLC26A4 (NM_000441)*	c.1234G>T (p.Val412Phe)	Homo	Strong	Not Reported	Likely pathogenic	Deafness, autosomal recessive 4, with enlarged vestibular aqueduct (#600791;) Autosomal recessive, population data
2	*FGF3 (NM_005247)*	c.45DelC (p.Trp16GlyfsTer63)	Homo	Strong	Not Reported	Likely pathogenic	Deafness, congenital with inner ear agenesis, microtia and microdontia (#610706;) Autosomal recessive, population data

Table 1: Continued…

	ADGRV1 (NM_032119)	c.12528-2A>C (splicing)	Hetero	Strong	Not reported	Likely pathogenic	Usher syndrome, type 2C (#605472;) Autosomal recessive, population data
3	*OTOG* (NM_001277269)	c.7454delG (p.Arg2485Hisfs*77)	Homo	Strong	Not reported	Likely pathogenic	Deafness, autosomal recessive 18B (#694145;), population data
4	*ADGRV1* (NM_032119)	c.16226-16227insAGTC (p.Thr5411Serfs3)	Homo	Strong	Not reported	Likely pathogenic	Usher syndrome, type 2C (#605472;) Autosomal recessive, population data
5	*OTOF (NM_001287489.1)*	c.3570+2T>C splicing variant	Homo	Strong	Not Reported	Likely pathogenic	Deafness, autosomal recessive 9 (#601071;) Autosomal recessive, population data
6	*SLC26A4 (NM_000441)*	c.1234G>T (p.Val412Phe)	Homo	Strong	Not Reported	Likely pathogenic	Deafness, autosomal recessive 4, with enlarged vestibular aqueduct (#600791;) Autosomal recessive, population data
7	*FGF3* (NM_005247)	c.466T>C (p.Ser156Pro)	Homo	Strong	Not Reported	Likely pathogenic	Deafness, congenital with inner ear agenesis, microtia and microdontia (#610706;) Autosomal recessive, population data
	ESPN (NM_031475)	c.992G>A (p.Ser331Asn)	Hetero	Strong	Not Reported	Likely pathogenic	Neurosensory deafness without vestibular involvment (#605472;)Autosomal dominant, population data
8	*OTOA* (NM_144672)	c.2359G>T (p.Glu787X)	Hetero	Strong	Reported[1]	Likely pathogenic	Deafness, autosomal recessive 22 (#607039;) Autosomal recessive, population data
		c.2353A>C (p.Thr785Pro)	Hetero	Benign	Reported[2]	uncertain significance	Deafness, autosomal recessive 22 (#607039;) Autosomal recessive, population data

[1,2]: ExAC 0.0012, ExAC 0.0017, respectively

Discussion

In our previous study, the allele frequency of *GJB*2 c.35delG mutation in the central region of Iran (Qom province) is 9.5% (9) and in this study, following the exclusion of deaf people with mutations in the *GJB2* gene, we used the whole exome sequencing followed by Sanger validations and segregation analyses to identify exome variants of the likely causes of ARNSHL in eight families.

Homozygous or compound heterozygous mutation in the *SLC26A4* gene on chromosome 7q22 is the cause of autosomal recessive deafness.

Therefore, this gene can cause both syndromic and non-syndromic hearing loss. Thus far, more than 300 mutations in the *SLC26A4* gene were identified. It is the second cause of autosomal recessive non-syndromic deafness in many populations, particularly Iran, which ranges from 4.3% to 18.0% in different areas (10-12).

We found a novel homozygote variant c.1234G>T (p.Val412Phe) in the *SLC26A4* gene in all the deaf individuals in two out of eight families. These people had a range of mild to severe non-syndromic hearing loss. This variant is not presented in both ExAC and 1000 Genomes databases. The prediction of the evolutionary conservation of nucleotides is effective in the variant's prioritization, and the evolutionary conservation of pathogenic mutations is more than benign variants (13). It is as a damaging variant classified as a likely pathogenic variant; however, the result should be addressed cautiously. To confirm the pathogenicity of the variant c.1234G>T, a whole family co-segregation was performed using Sanger sequencing. Our findings showed that all the affected members (seven and nine affected members in the first and the sixth family, respectively) of pedigree were homozygote and obligate carriers were healthy heterozygotes (Fig. 2). None of the healthy individuals was homozygous for this mutation. Therefore, our findings imply that this variant is the major genetic cause of non-syndromic hearing loss in these individuals.

Mutations in the *FGF3* gene causes syndromic deafness. There are no reports of mutation in the *FGF3* gene in Iran. The homozygous frame shift mutation c.45DelC (p.Trp16Glyfs61), and the homozygous missense mutation c.466T>C (p.Ser156Pro) were found in the second and seventh families, respectively. Both variants could be damaging and affect the protein function. In the affected individuals in both families of this study, the symptoms were not clear and showed very mild clinical symptoms. Two subjects showed homozygote in the variant c.12528-2A>C of the *ADGRV1* gene. This gene encodes a member of the G-protein coupled receptor superfamily to bind to calcium ion. It is expressed in the central nervous system (14).

Recent studies have reported considerable deletion in the *ADGRV1* gene in Iranian population (15). The variant c.12528-2A>C is the splice acceptor site mutation, and such variants usually have damaging effects on the protein structure. All four deaf members of the seventh family had the homozygous missense mutation c.466T>C (p.Ser156Pro) in the *FGF3* gene. None of the healthy and control subjects showed this mutation in homozygote state. Therefore, this variation causes deafness in this family.

The various mutations in *OTOG* (Otogelin) gene have been identified as the cause of ARNSHL (16, 17). The mutation in the *OTOG* gene has not been reported in the Iranian population. We detected the c.7454delG (p.Arg2458Hisfs77) mutation in the *OTOG* gene as the cause of deafness in the third family. The c.7454delG (p.Arg2458Hisfs77) mutation was predicted to be a frameshift one. Until now, no report of this variant has been found in the Clinvar database. Following identification of these variants in the proband, other profound deaf people (3 subjects) were examined in the third pedigree family for this variant by the Sanger sequencing. The homozygosity for this variant in all the three subjects. However, the result of sequencing of individuals in this pedigree showed healthy or obligated heterozygote.

In the fifth family, we detected the homozygote c.3570+2T>C mutation in the *OTOF* gene in the proband and other affected individuals (3 subjects) as well as in the healthy or heterozygote control subjects in this family. The various mutations in the gene have been introduced as a form of autosomal recessive non-syndromic hearing loss (DFNB9). The variant c.3570 + 2T> C is a deleterious variation modifying the splicing acceptor site in IVS 28. Other splicing variants in this site lead to non-syndromic hearing loss (17).

The prevalence of the mutation in the *OTOA* gene in the previous study in Iran was reported to be 2% among the affected individuals with deafness (5). The result of the WES in the proband from the eighth family showed two variants, including the c.2359G>T (p.Glu787X) and

the c.2353A>C (p.Thr785Pro) in heterozygote state in the *OTOA* gene.
As the earlier reports (11) described, about the variants related to hearing loss after excluding the *GJB2* mutations, we showed the relation of *SLC26A4* (c.1234G>T), *FGF3* (c.45DelC, c.466T>C), *ADGRV1* (c.12528-2A>C, c.16226-16227insAGTC), *OTOG* (c.7454delG), *OTOF* (c.3570+2T>C), *ESPN (c.992G>A), OTOA* (c.2359G>T, c.2353A>C) variants with hearing loss. The finding of exact meaningful dependence has to be evaluated with more samples and analysis.

Conclusion

Despite initial ENT assessment of non-syndromic hearing loss, in some proband, we identified the genes associated with syndromic deafness such as the *FGF*3 gene in the seventh family. Therefore, the use of the NGS panel alone is not enough to identify genes responsible for non-syndromic hearing loss in deaf subjects. Despite numerous examinations such as ENT and CT scan, in many cases, it is not easy to distinguish between syndromic and non-syndromic deafness. It is recommended that the common genes associated with hearing loss among deaf people be identified before applying NGS. Owing to the high cost of NGS, a targeted genes panel can be used to orientate molecular diagnostic testing.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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