

Congenital Deafness: High Prevalence of a V371 Mutation in the GJB2 Gene Among Deaf School Children in Alor Setar

B H I Ruszymah, MD, PhD*, **I Farah Wahida, MMedSc***, **Y Zakinah, MS(ORL)****, **Z Zahari, BSurv(Land)*****, **M D Norazlinda, BSc***, **I Saim, FRCS****, **B S Aminuddin, MS (ORL)******

*Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, **Department of Otorhinolaryngology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Hospital Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, 56000 Cheras, Kuala Lumpur, ***Department of Survey and Mapping Malaysia, Bangunan Ukur, Jalan Semarak, 50578 Kuala Lumpur, ****Ear, Nose & Throat Consultant Clinic, Suite 1-1, First Floor Ampang Puteri Specialist Hospital, Jalan Mamanda 9, Taman Dato' Ahmad Razali, 68000 Ampang, Selangor

Summary

Twenty percent of all childhood deafness is due to mutations in the GJB2 gene (Connexin 26). The aim of our study was to determine the prevalence and spectrum of GJB2 mutations in childhood deafness in Malaysia. We analyzed the GJB2 gene in 51 deaf students from Sekolah Pendidikan Khas Alor Setar, Kedah. Bidirectional sequencing indicates that 25% of our childhood deafness has mutation in their GJB2 gene. Sixty two percent of these children demonstrate V371 missense mutation. Interestingly, V371 mutation in the GJB2 gene have been reported as polymorphism in Western countries, however in our country it behaved as a potentially disease-causing missense mutation, causing childhood deafness as it was not found in the normal control.

Key Words: Congenital childhood deafness, Mutation, GJB2, Connexin 26, V371

Introduction

Identification of genes involve in non-syndromic deafness has significantly improve the molecular knowledge on the auditory and vestibular organs and on the pathological mechanism, leading to hearing loss. As the hearing organ is a complex system that requires thousands of genes for it to function normally, it is not surprising that to date, at least 82 loci for non-syndromic hearing impairment have been identified through linkage analysis: 35 autosomal dominant (DFNA1-41), 33 autosomal recessive (DFNB 1-33) and 5 X-linked (DFN1-8). As for August 2003, only 36 auditory genes have been cloned which includes 17 genes for autosomal recessive, 16 genes for autosomal

dominant, 2 genes for mitochondrial and 1 gene for X-linked. With more than 300 different syndromes that have hearing impairment as one of the clinical feature, only 24 genes have been successfully identified so far.

Gap junctions are thought to play an important role for maintaining hearing function by the local circulation of potassium between the fluids of the inner ear¹. Among the non-syndromic hearing loss genes playing a role in the K⁺ recycling pathway are a potassium channel (KCNQ4), an anion transporter (SLC26A4), and 4 gap junction genes (GJB2, GJB3, GJB6 and GJA1). Recently, mutations in the GJB2 gene encoding connexin 26 protein (Cx26) located on human chromosome 13q11 (DFNB1)², have been shown to be

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Corresponding Author: Ruszymah Bt Hj Idrus, Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur

responsible for a majority of hearing impairment in children with approximately up to 50% of them have autosomal recessive non-syndromic hearing loss^{3,4}. Cx26 is expressed in the epithelial cells surrounding the hair cells and also in the adjacent connective tissue system of fibrocytes positioned under and around the marginal cells of the stria. Thus, mutations in Cx26 can result in severe hearing loss⁵. More than 100 mutations have been reported so far. The most frequent mutation of GJB2 associated with deafness is 35delG, which is common in white and European populations^{6,7}. Other populations may have additional or different specific mutations, such as 167delT among Ashkenazi Jews⁸, R143W in Africans⁹ and 235delC in Japanese¹⁰ and Koreans¹¹.

Connexins are the protein subunit of gap junctions; hexameric connexin oligomers are arranged in the plasma membrane as connexon hemichannels that dock with partners in adjacent cells to generate intercellular communication pathway¹²⁻¹³. Cx26 is one of 14 isoforms located on chromosome 13q11-q12 and has a molecular weight of 26.5kD¹⁴.

Since there were no reports regarding GJB2 gene mutations in Malaysian population, it is interesting to identify whether GJB2 mutations are also an important cause of non-syndromic hearing loss here. To realize further our objective, we report here the results of a sequence-based mutation analysis of the GJB2 gene from 51 children of Sekolah Pendidikan Khas Alor Setar, Kedah.

Materials and Methods

This study was approved by Medical Faculty Universiti Kebangsaan Malaysia Ethical Committee. It was conducted on 51 non-syndromic, unrelated patients, aged 8 to 14 years old from Sekolah Pendidikan Khas, Alor Setar, Kedah, Malaysia. Consent was obtained from all the participants' parents. Details of the consented subjects are as shown in Table I. The subjects were further examined by an extensive questionnaire regarding medical and family history. Later, they were clinically well characterized by a series of auditory examinations (pure tone audiometry). Ten ml of venous blood was collected from each patient and DNA extracted from peripheral blood lymphocytes using a standard extraction procedure. DNA was also analyzed from 100 normal hearing controls aged from 19-23 years old. The details of the control subjects are as tabulated in Table II.

Mutation analysis

DNA fragments containing the entire coding region were amplified from genomic DNA samples using two set of primers: 5'-TCTTTTCCAGAGCAAACCGC-3' with 5'-GACACGAAGATCAGCTGCAG-3' and 5'-CCAGGCTGCAAGAACGTGTG-3' with 5'-GGGC AATGCGTTAAACTGGC-3'. The same primers were used as the template for sequencing. Amplification conditions were as follows: 94°C for three minutes, 35 cycles of 94°C for 30 seconds, annealing temperature at 63°C for 30 seconds and 72°C for 10 seconds, followed by 72°C for two minutes. PCR products were separated on a 1.5% (w/v) agarose gel and bands size 285 base pairs for first set primer and 519 base pairs for second set primer were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). For sequencing, approximately 400ng PCR product was used as template with the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia). The products were visualized on an ABI377 DNA sequencer (PE Applied Biosystems).

Results

In this study, 13 out of 51 (25%) deaf subjects have mutation in their GJB2 gene. All V37I, R117H and E114G were the mutations detected. None of the mutations mentioned were found in the control population (0/100). In V37I mutation, a G to A transition at residue 109 was involved. This missense mutation which converts valine residue to isoleucine at codon 37 was found in eight subjects. V37I was the commonest mutation found in this study. For R117H mutation, G to A transition at residue 349 was involved. This missense mutation which converts arginine to histidine was found in one subject. Table III summarizes the mutations observed in this study. Analysis of GJB2 showed three mutations, all of which were missense mutations. Interestingly, one mutation, E114G (involving A to G transition at residue 341) which occurs as heterozygous mutation always presents with a particular polymorphism; V27I (G→A transition at residue 79). This combination of mutation-polymorphism was found in four subjects. In this study, all the subjects identified with mutations in their GJB2 gene have negative family history of deafness suggesting that their hearing loss might be due to sporadic genetic cause. The degree of hearing loss in these subjects shows variability from mild to profound hearing loss.

Table I : Race and gender composition of participating subjects

Gender	Race	
	Malay	Chinese
Male	24	7
Female	19	1
Total	43	8

Table II : Race and gender composition of the control population

Gender	Race		
	Malay	Chinese	Indians
Male	34	6	7
Female	39	8	6
Total	73	14	13

Table III: Mutations in the GJB2 gene detected in the 13 samples with various degree of hearing loss

Sample	Age	Gender	Race	Level of Hearing Loss		Mutation Type	GJB2 Genotype		Sporadic/ Familial
				Right	Left		Allele 1	Allele 2	
A	8	M	Malay	Profound	Moderate	Missense	V371	-	Sporadic
B	10	F	Malay	Profound	Profound	Missense	V371	-	Sporadic
C	13	M	Malay	Mild	Mild	Missense	V371	-	Sporadic
D	8	F	Malay	Moderate	Severe	Missense	V371	-	Sporadic
E	11	M	Malay	Profound	Profound	Missense	V371	-	Sporadic
F	14	F	Malay	Moderate→ severe	Moderate→ severe	Missense	V371	-	Sporadic
G	14	M	Malay	Profound	Profound	Missense	V371	-	Sporadic
H	14	F	Malay	Severe→ profound	Severe	Missense	V371	-	Sporadic
I	14	F	Malay	Severe→ profound	Severe→ profound	Missense	R117H	-	Sporadic
J	13	M	Chinese	Profound	Profound	Polymorphism/ missense	V271	E114G	Sporadic
K	10	M	Malay	Severe→ profound	Severe→ profound	Polymorphism/ missense	V271	E114G	Sporadic
L	14	F	Chinese	Moderate	Severe→ profound	Polymorphism/ missense	V271	E114G	Sporadic
M	11	M	Malay	Severe	Severe	Polymorphism/ missense	V271	E114G	Sporadic

* M=Male, F=Female

Discussion

Connexins are gap junction proteins which constitute a major system of intercellular communication important in the exchange of electrolytes, second messengers and metabolites. In the inner ear, connexin-26 expression was demonstrated in two groups of cells. The first group, non-sensory epithelial cells, includes interdental cells of the spiral limbus, inner and outer sulcus cells, sensory supporting cells, and within the root of spiral ligament. The second group, the connective tissue cell gap junction system, includes fibrocytes within the spiral ligament and spiral limbus, basal and intermediate cells of the stria vascularis, and mesenchymal cells which line the scala vestibule and interconnect the two populations of cell-type¹⁵. The loss of connexin-26 in the gap junction complex would expect to disrupt the recycling of potassium from the synapses at the base of hair cells through the supporting cells and fibroblasts of potassium ions back to the high potassium containing endolymph of the cochlea duct and therefore would result in a local intoxication of the Organ of Corti's by potassium, leading to the hearing loss¹⁶.

Mutations in GJB2 encoding for gap junction protein connexin-26 have been established as the basis of autosomal recessive non-syndromic hearing loss and proposed in some rare cases of autosomal dominant form of deafness². The 35delG mutations accounts for the most common type of non-syndromic recessive deafness among white populations⁴.

There is evidence that different combinations of GJB2 mutation exist in different ethnic, as in the Ashkenazi Jews where 167delT mutation is the most common mutation⁸ and the R143W in the Africans^{2,17}. However, in the Japanese and Korean populations, 235delC is the most frequent mutation^{10-11,18}. The same mutation exists in Thailand population¹⁹.

As Malaysians are part of the Asian community, we realize that the chances of finding either 35delG or 167delT, which was once thought as mutational hotspot, is rare. We are hoping that our data might be similar with those of our counterpart in : Japan and Thailand. However, some of the data observed in this study has proven otherwise. For instance, 235delC which was a common mutation among Japanese¹⁰ and other Asian country¹⁹ was not found in this study. Instead, V37I which was reported as polymorphism in the US population¹⁶ was found to contribute 62% (8/13)

of our mutated deaf cases. It is thought that the ethnic backgrounds suggest the possibility that this mutation may be more frequent among eastern-Asian population²⁰ and also Southeast Asia (this study).

Though it is debatable whether this particular mutation is a polymorphism, functional studies on V37I have shown that it is indeed pathologically significant²¹. Furthermore, few studies done in Israel⁸, Ghana² and a few other countries have not detected V37I mutation in their control populations. Interestingly, all of the eight cases of V37I in this study are heterozygous mutation. Bidirectional sequencing strategy failed to detect second mutation in the same gene suggesting that there might be a second unidentified mutations in the non-coding portion of the GJB2 gene; or mutations in another closely linked hearing loss gene²²; namely GJB6 gene encoding connexin 30 (digenic mode of inheritance). It is not impossible, given its similarity in sharing the same locus, namely DFNB1. Recently, there are reports demonstrating that two thirds of deafness patients who are heterozygous for GJB2 mutation have deletion in their GJB6 gene²³. Therefore, it might be possible that a proportion of our eight cases of V37I heterozygous mutation will harbor the GJB6 mutation (not examined).

Only one mutations identified in this study is novel; R117H. Since this mutation represented here was not found in any of the controls, it is likely that this is potentially pathological mutation rather than non-pathological polymorphic changes. V153I was thought to be a mutation as it was not found in our normal hearing controls (0/100). However, studies in India²⁴ and Turkey²² have reported it as polymorphism. As we do not have enough proof to support this particular amino acid change as disease-causing mutations, V153I identified in this study will remained as polymorphism. As with V153I, E114G was also not detected in our control subjects (0/100). But both E114G and V27I were reported as polymorphism and each occurred as separate case in Japan, Korea and Taiwan^{10-11, 25}. Intriguingly, in Thais¹⁹ and North Americans²⁶ have reported three and four deaf subjects respectively which were V27I heterozygotes were also heterozygous for E114G. And in this study, four subjects with deafness were reported to have E114G/V27I, all of which have severe to profound hearing loss. It is interesting to identify whether this particular combination of alleles is deaf-causing mutation as the pathogenic nature of E114G /V27I compound heterozygosity still remain unclear.

Prelingual deafness hampers speech acquisition, normal communication, and social integration and the first year of life is known to be a critical time in the development of language skills. Children diagnose with hearing loss after this time may face a lifetime of developmental delay. Therefore, the search for mutations in the GJB2 gene is important for genetic counseling as well as in providing clinical practitioner essential information regarding genetic basis of hearing loss. Early identification and intervention of hearing loss in children is critical for their intellectual, social and emotional development. Thus, knowledge of a gene involved in congenital deafness is useful for the study of the pathophysiology and development of strategies meant for therapeutic intervention.

Conclusion

The present results from Sekolah Pendidikan Khas Alor Setar suggested that the mutations of the GJB2 gene are

an important factor in the etiology of non-syndromic hearing loss in Kedah population. It should be used as a tool to detect genetic origin of deafness in Kedah and Malaysian population. Our study indicates that 25% of our deaf cases are caused by the mutations in the Cx26 gene. V37I is the most frequent mutation found in this study which account for 62% (8/13) of our mutated cases.

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References

1. Salt AN, Melichar I, Thalmann R. Mechanisms of endocochlear potential generation by stria vascularis. *Laryngoscope* 1987; 97: 984-91.
2. Hamelmann C, Amedofu K, Albrecht K et al. Pattern of connexin 26 (GJB2) mutations causing sensorineural hearing impairment in Ghana. *Hum Mutat* 2001; 18(1): 84-85.
3. Green GE, Scott DA, McDonald JM, Woodworth GG, Sheffield VC, Smith RJ. Carrier rates in the Midwestern United States for GJB2 mutations causing inherited deafness. *JAMA* 1999; 281(3): 2211-16.
4. Bruzzone R, White TW, Paul DL. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem* 1996; 238: 1-27.
5. Kikuchi T, Kimura RS, Paul D, Adams JC. Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. *Anat Embryol* 1995; 191: 101-18.
6. Zelante L, Gasparini P, Estivill X. Connexin 26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. *Hum Mol Genet* 1997; 6: 1605-609.
7. Denoyelle F, Weil D, Maw MA. Prelingual deafness; high prevalence of a 30delG mutation in the connexin 26 gene. *Hum Mol Genet* 1997; 6: 2173-77.
8. Morell RJ, Kim HJ, Hood LJ et al. Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *N Engl J Med* 1998; 339: 1500-505.
9. Martin PEM, Coleman SL, Casalotti SO, Forge A, Evans WH. Properties of connexin 26 gap junctional proteins derived from mutations associated with non-syndromal hereditary deafness. *Hum Mol Genet* 1999; 8: 2369-76.
10. Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ. Prevalent connexin 26 gene (GJB2) mutations in Japanese. *J Med Genet* 2000; 37: 41-43.
11. Park HJ, Hahn SH, Chun YM, Park K, Kim HN. Connexin 26 mutations associated with nonsyndromic hearing loss. *Laryngoscope* 2000; 110(9): 1535-38.

12. Goodenough DA, Goliger JA, Paul D. Connexins, connexons, and intercellular communication. *Annu Rev Biochem* 1996; 65: 475-502.
13. Kumar NM, Gilula NB. The gap junction communication channel. *Cell* 1996; 84: 381-88.
14. Forge A, Becker D, Evans WH. Gap junction connexin isoforms in the inner ear of gerbil and guinea pigs. *Br J Audiol* 1997 ; 31: 76-77.
15. Chang, EU, Van Camp, G, Smith, RJH. The role of connexons in human diseases. *Ear Hear* 2003; 24(4): 314-23.
16. Kelley PM, Harris DJ, Comer BC, et al. Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *Am J Hum Genet* 1998; 62: 792-99.
17. Brobby GW, Muller-Myhsok B, Horstmann RD. Connexin 26 R143W mutation associated with recessive non-syndromic sensorineural deafness in Africa. *N Engl J Med* 1998 ; 338: 548-50.
18. Fuse Y, Doi K, Hasegawa T, Sugil A, Hibino H, Kubo T. Three novel connexin 26 gene mutations in autosomal recessive non-syndromic deafness. *Neuroreport* 1999; 10: 1853-57.
19. Kudo T, Ikeda K, Oshima T et al. GJB2 (connexin 26) mutations and childhood deafness in Thailand. *Otol Neurotol* 2001; 22(6): 858-61.
20. Bason L, Dudley T, Lewis K et al. Homozygosity for the V37I Connexin 26 mutation in three unrelated children with sensorineural hearing loss. *Clin Genet* 2001; 61: 459-64.
21. Bruzzone R, Veronesi V, Gomès D et al. Loss-of- function and residual channel activity of connexin-26 mutations associated with non-syndromic deafness. *FEBS Lett* 2003; 533 (1-3): 79-88.
22. Bayazit YA, Cable BB, Cataloluk O et al. GJB2 gene mutations causing familial hereditary deafness in Turkey. *Int J Pediatr Otorhinolaryngol* 2003; 67(12): 1331-35.
23. Pallares-Ruiz N, Blanchet P, Mondain M, Claustres M, Roux AF. A large deletion including most of GJB6 in recessive non syndromic deafness: a digenic effect? *Eur J Hum Genet* 2002; 10(1): 72-6.
24. Ramshankar M, Girirajan S et al. 2003. Contribution of Connexin 26 (GJB2) mutations and founder effect to non-syndromic hearing loss in India. *J Med Genet* 2003; 40(5): e68.
25. Hwa HL, Ko TM, Hsu CJ et al. Mutation spectrum of the connexin 26 (GJB2) gene in Taiwanese patients with prelingual deafness. *Genet Med* 2003; 5(3): 161-65.
26. Pandya A, Arnos KS, Xia XJ et al. Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands. *Genet Med* 2003; 5(4): 295-303.