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Population-Based Newborn Hearing Impairment Screening Test Using *GJB2* Mutation Analysis

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Hearing loss is a common congenital disorder that is frequently associated with mutations in the Cx26 gene (*GJB2*). Recently, the mutation analysis of *GJB2* has been used in a newborn screening test for the detection of hearing impairment. Population-based studies should be performed before the application of genetic testing for the identification of deaf newborns. In this study, 8 positions of *GJB2* mutations-including 35delG, 167delT, 235delC, V27I, V37I, M34T, E114G, and I203T-were analyzed using PCR-direct sequencing in a total of 437 healthy Korean neonates. DNAs from dried blood spots were extracted using a commercial DNA extraction kit. The PCR-amplified products (783 bps) of the *GJB2* gene were detected using 2% agarose gel electrophoresis and subjected to direct sequencing. The sequences were compared with those in the GenBank database by using the BLAST program. In this study, 5 *GJB2* mutations -including V27I (79G>A), V37I (109G>A), E114G (341A>G), I203T (608T>C), and 235delC- were found. Of the 437 neonate samples, 301 subjects showed *GJB2* mutations (68.9%, 301/437). The V27I mutation was found in 271 subjects and was the most frequent (62.0%, 271/437). The E114G, I203T and V37I mutations were shown in 146, 17 and 14 subjects, respectively. The 235delC mutation was found in 1 subject. The E114G mutation was frequently accompanied by the V27I mutation. V27I/E114G (97.2%, 143/147) was the most common double mutation and 3 subjects had the double mutation V27I/I203T. A triple mutation, V27I/E114G/I203T, was found in 1 subject. In conclusion, PCR-direct sequencing is a convenient tool for the rapid detection of *GJB2* mutations and this data might provide information for the genetic counseling of the *GJB2* gene.

Key Words : hearing loss, newborn hearing screening, *GJB2* mutation

I. INTRODUCTION

Congenital hearing loss occurs in approximately 1 in every 1,000 live births and 50% of these cases are hereditary (Morton et al, 1991). Genes causally related to nonsyndromic hearing loss (NSHL) have been reported at more than 100 localizations across the genome sites, and 37 different genes encoding proteins with a wide variety of functions have been identified (Denoyelle et al, 1997). Recently, mutations in the *GJB2* (Gap junction protein, Beta-2; *GJB2*; gene map locus 13q11-q12; MIM121011) gene have been reported in a great number of familial and sporadic cases of congenital deafness in Caucasians. Mutations of the *GJB2* gene of Cx26 were initially reported by Kelsell et al (1997). These mutations have recently become of particular interest because they account for up to 50% of the cases of autosomal recessive

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NSHL in many of the worlds populations, which makes *GJB2* the most frequently associated gene with this condition (Estivell et al, 1998; Kelly et al, 1998; Kenneson et al, 2002). To date, more than 80 *GJB2* mutations have been reported: a few of them are very common, while the others are extremely rare (Yan et al, 2003). The combined frequency of the *GJB2* mutations is sufficiently high in most populations to accomplish the mutation analysis of this gene (Cryns et al, 2004). Extensive screening studies of the *GJB2* mutations have been undertaken within the deaf population in many different ethnic backgrounds (Lench et al, 1998). Recently, *GJB2* mutation analysis is used more frequently in the newborn screening program for the detection of hearing impairment. Generally, a population-based screening study in healthy subjects should be performed before the application of genetic testing. In this study, *GJB2* mutation analysis was performed using the PCR-direct sequencing technology for establishing a database in Koreans.

II. MATERIALS AND METHODS

DNAs were extracted using G-DEX DNA extraction kit (Intron Co, Korea) using hill punched blood spots of 437 neonatal subjects with normal hearing. Dried blood spots were incubated with 1.5 mL of blood lysis buffer for 30 min and centrifuged at 1,5000 rpm for 10 min. After removing the supernatant, the pellet was incubated at 60 ℃ overnight with 300 μL of cell lysis buffer. After that, 100 μL of G-DEX PPT buffer was added and spun down at 15,000 rpm for 5 min and 400 μL of 2-propanol was added. After removing the supernatant, the pellet was washed with 70% (v/v) ethanol and dried at room temperature. The pellet was resuspended with 20 μL of G-DEX rehydration buffer. For amplification of the *GJB2* gene, PCR was performed with the *GJB2* gene specific primers described by Scott et al (1988). PCR amplifications were performed using 4.5 μL of template DNA, 0.5 μM of primer set, 2 mM of $MgCl_2$, 25 mM of KCl, 30 mM of Tris-HCl (pH 9.0), 0.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (Intron Co., Korea) in a final volume of 20 μL. The amplification conditions included an initial denaturation for 3 min at 94℃, 40 cycles of amplification with denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 30 sec and followed by final extension at 72℃ for 7 min. The PCR-amplified products (783 bps) of the *GJB2* gene were detected using 2% agarose gelelectrophoresis. The PCR products were purified and sequencing was accomplished in both directions by use of the ABI PRISM BigDye terminator cycle sequencing kit and DNA automatic sequencer (ABI 3730 Genetic analyzer, Perkin Elmer, USA). The sequences were compared with those in the GenBank database by using the BLAST program.

III. RESULT

The mutation analysis of the *GJB2* gene was performed on a total of 437 Korean newborns with normal hearing. Eight clinically significant mutation sites of the *GJB2* gene in oriental people -including 35delG, 167delT, 235delC, V27I, V37I, M34T, E114G, and I203T- were analyzed. Among the 8 *GJB2* mutations analyzed, 5 mutation sites -including V27I (79G>A), V37I (109G>A), E114G (341A>G), I203T (608T>C), and 235delC -were identified in the subjects. *GJB2* mutations were found in 301 Korean healthy neonates (68.9%, 301/437), including 154 subjects with a single mutation and 147 subjects with multiple mutations (Table 1). In this study, 2 polymorphic mutations, V27I and V37I, were detected. The V27I mutation, substitution of valine for isoleucine in codon 27 (79G>A), was found in 271 subjects and consisted of 204 and 67 subjects with heterozygous and homozygous mutations, respectively; this was the most frequently observed mutation. The V37I mutation, substitution of valine for isoleucine in codon 37 (109G>A), was found in

Table 1. Mutation frequencies of the *GJB2* gene in healthy Korean neonates.

Genetic changes		Mutation	No. of subjects	Total No. of subject(N=437)
Mutation	single mutation	V271	124	154
		V371	14	
		E114G	2	
		I203T	13	
		235delC	1	
	Double mutation	V27I/E114G	143	146
		V27I/I203T	3	
	Triple mutation	V27I/E114G/I203T	1	1
Negative			136	136

V27I : substitution of valine for isoleucine in codon 27, V37I : substitution of valine for isoleucine in codon 37
E114G : substitution of glycine for glutamate in codon 114, I203T : substitution of isoleucine for threonine in codon 203
235delC : deletion at codon 79

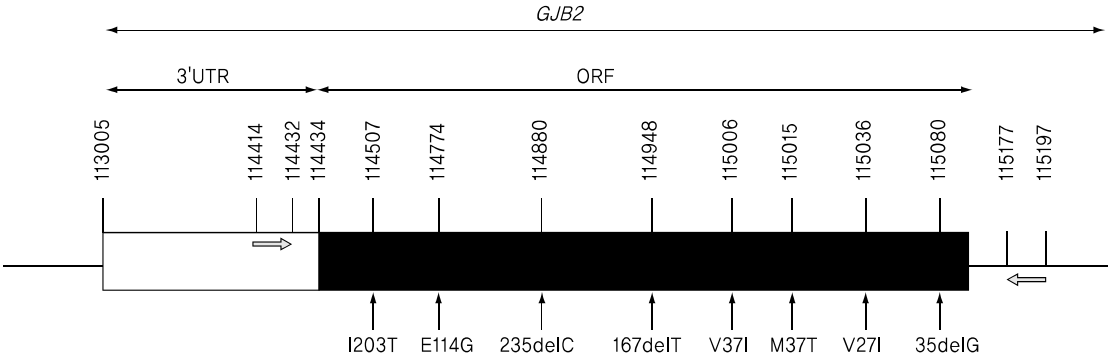

Fig. 1. The genetic positions of mutations of *GJB2* genes derived from the genomic sequence of clone RP11264J4; GeneBank accession no. AL138688 (Yan et al, 2003)

14 subjects. Two missense mutations, E114G and I203T were observed. The E114G mutation, substitution of glycine for glutamate in codon 114 (341A>G), was found in 146 subjects-including 121 and 25 subjects with heterozygous and homozygous mutations, respectively. The I203T (608T>C) mutation was observed in 17 heterozygote subjects. The deletion at codon 79 (235delC) was found in 1 case (Table 2). Most of the E114G mutations were accompanied by the V27I (V27I/E114G) mutation. A triple mutation, V27I/E114G/I203T, was found in 1 case (Table 1).

IV. DISCUSSION

NSHL comprises a majority of hereditary hearing loss with autosomal recessive forms accounting for 85% of these cases (Fuse et al, 1999; Mehl and Thomson, 2002). Recently, *GJB2* mutations have been reported to cause deafness with a significant difference in the frequency and distribution among different populations (Houseman et al, 1998; White et al, 1998). *GJB2* encodes the connexin 26 (Cx26) protein, a member of a large family of transmembrane gap-junction proteins that mediate electrical and metabolic coupling between adjacent cells (Xiao et al,

Table 2. Number of homozygous and heterozygous subjects according to each mutation site of the *GJB2* gene

Codon location	Nucleotide change	Types of mutation	No. of subjects		
			Heterozygote	Homozygote	total
V27I	79G>A	polymorphism	204	67	271
V37I	109G>A	polymorphism	13	1	14
E114G	341A>G	missense	121	25	146
I203T	608T>C	missense	17	0	17
79	235delC	deletion		1	1

Table 3. Comparison of mutations of the *GJB2* gene among other ethnic groups

Codon location	Nucleotide change	This study (n=437)	Korean[23] (Park et al) (n=100)	Japanese[1] (Ave et al) (n=192)	Chinese[30] (Xio et al) (n=200)	Caucacians[26] (Roux et al) (n=7032)
V27I	79G>A	271 (271/437)	68 (68/100)	75 (75/192)	8 (8/200)	10 (10/7032)
M34T	101T>C	0	0	0	0	81 (81/7032)
V37I	109G>A	14 (14/437)	0	2 (2/192)	15 (15/200)	30 (30/7032)
79	235delC	1 (1/437)	1 (1/100)	2 (2/192)	1 (1/200)	0
E114G	341A>G	146 (146/437)	38 (38/100)	25 (25/192)	0	1 (1/7032)
I203T	608T>C	17 (17/437)	3 (3/100)	16 (16/192)	1 (1/200)	0

2004). *GJB2* mutations and the disruption of Cx26 gap junctions presumably impair the recycling of potassium ion thereby causing hearing impairment (Lautermann et al, 1998). The Transient Evoked Otoacoustic Emissions (TEOAE) test or Auditory Brainstem Response (ABR) test have been used for detecting neonatal hearing impairment. To date, *GJB2* mutation analysis has been applied to the hearing impairment screening test and it has greatly improved the management of deafness. However, a population study in healthy subjects might be preliminary performed before its application in clinical samples, because of the genetic heterogeneity of deafness. In this study, we analyzed *GJB2* mutations in a population-based database study among Korean newborns with normal hearing. A total of 437 subjects were studied; 301 subjects showed mutations in the *GJB2* gene (68.9%) (Table 1). The positions of mutations with their respective nucleotide numbers are shown in Fig. 1 (Yan et al, 2003). Five *GJB2* mutation types-including V27I, V37I, E114G, I203T, and 235delC-were identified. The mutation patterns of the *GJB2* gene are presented in Fig. 2. V27I was the most frequently found mutation (271 subjects). The E114G (146 subjects), I203T (17 subjects), V27I (14 subjects), and 235delC (1 subject) were observed in this study (Table 2). The results correlated well with those from the previous study in Koreans (Park et al, 2000).

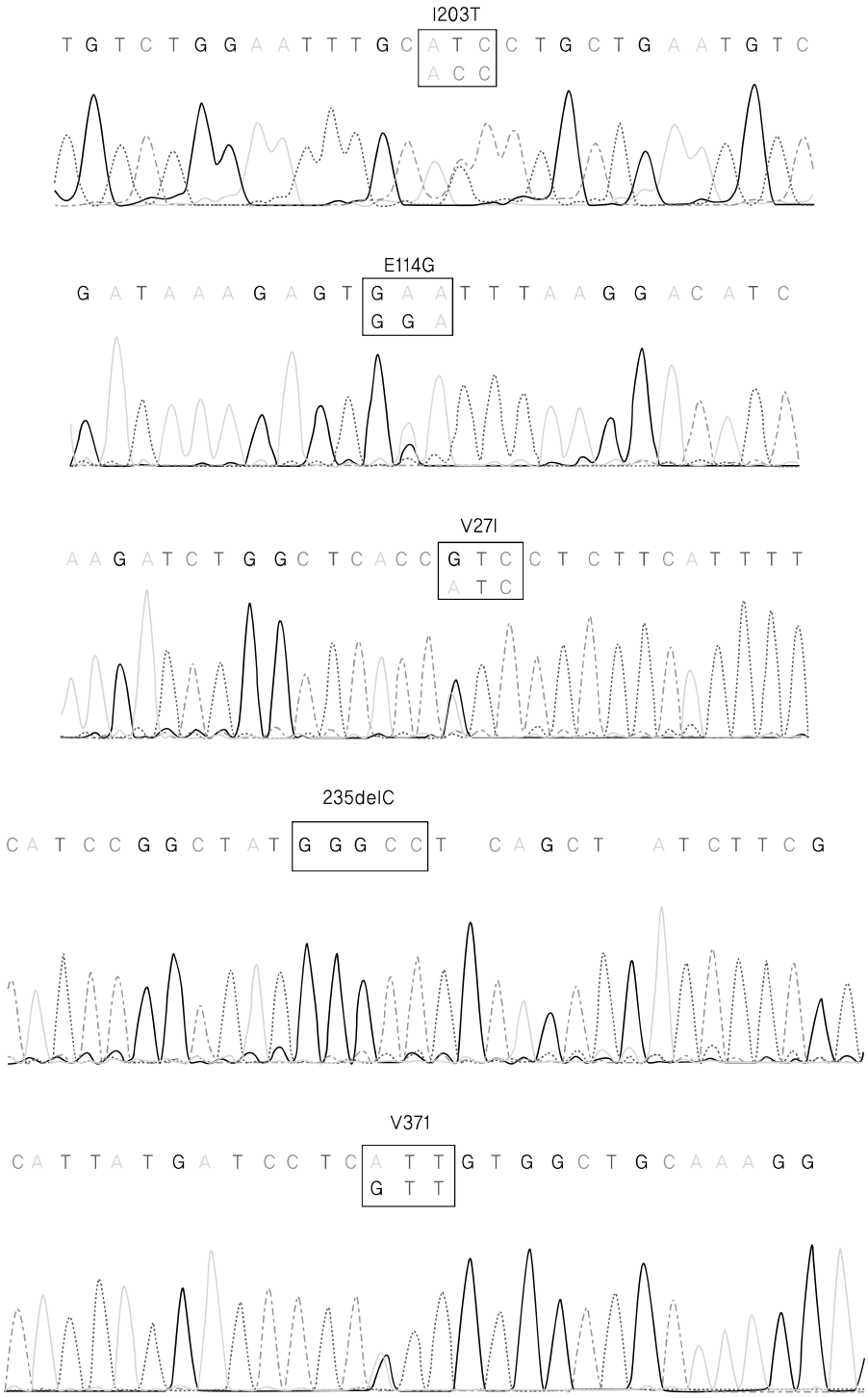

Fig 2. Mutation patterns of the *GJB2* gene determined by PCR-direct sequencing.

In genetic counseling, it is clinically significant to identify whether certain molecular changes in the *GJB2* gene are real mutations or genetic polymorphism. Mutations could be more clinically significant in patient care than genetic polymorphisms. Some reports have described that polymorphisms have no correlation with hearing loss and appear to be non-pathological changes (Kelly et al, 1998). The V27I and V37I mutations have been reported as polymorphisms. The mutation frequencies of the *GJB2* gene among healthy subjects from several countries are shown in Table 3. The frequency of the V27I mutation is extremely high in Korean and Japanese populations (Abe et al, 2000; Park et al, 2000). Park et al (2000) reported that 6 *GJB2* mutation types-including 35delG, V27I, S72C, 235delC, E114G, and I203T-were observed in healthy Koreans. Among these mutations, V27I and E114G were the most frequent. This correlated well with our data. However, 35delG and S72C were not found in our study, even though the number of subjects tested was 4 times higher than the previous study (Table 3). According to Fuse et al (1999) and Ave et al (2000), the V27I, E114G, and I203T mutatoins were also frequently found in healthy Japanese. However no correlation between these mutations and hearing loss was found among the Japanese. In the Chinese population, Xiao et al (2004) reported the frequency of the *GJB2* mutation to be 26% in healthy subjects. The mutation frequency observed was low compared with our study on Koreans (26.0% vs 68.9%). One of the reasons for the difference may be due to the method of detection. V37I and V27I were frequent mutations in the Chinese (Liu et al, 2001). None of the mutations they found were associated with deafness (Xiao et al, 2004). In healthy Caucasians, the most frequent mutation of the *GJB2* gene was M43T. The 35delG and V37I mutations were also frequently found (Roux et al, 2004). Two mutations, V27I and E114G that are frequently found in Koreans were very rare in the white population (Table 3). The *GJB2* mutations in healthy Korean newborns were similar to those in the Japanese, but some differences were observed with respect to those among the Chinese and the Caucasians. In several studies of patients with hearing loss, the GJB mutation spectrum diverges substantially among populations, as reflected by the specific ethnic biases toward common mutations like 35delG among the Caucasians (Zelante et al, 1997; Estivill et al, 1998; Green et al, 1999; Janecke et al, 2002), 235delC among the Japanese (Abe et al, 2000; Kudo et al, 2000), 167delT in the Ashkenazi Jewish population (Morell et al, 1998), and V37I in the Taiwanese population (Hwa et al, 2003). Therefore a population screening study of healthy subjects might be performed before using a genetic screening marker in each ethnic group.

For mutation analysis, various methods-including sequence specific primer (SSP), single strand conformation polymorphism (SSCP) (Angeli et al, 2000), restriction fragment length polymorphism (RFLP) (Scott et al, 1998), denaturating gradient gel electrophoresis (Antoniadi et al, 2000), denaturating high-performance liquid chromatography (Azaiez et al, 2004; Rikket et al, 2005), sequencing (Wu et al, 2002), and DNA chip technology (Simering et al, 2006)-have been used. Among these methods, the PCR-direct sequencing technique used in this study, was a simple and rapid method of mutation analysis and might be useful for a central reference laboratory with large volumes of samples. In conclusion, this data could be useful for inclusion in the population database of the *GJB2* gene mutation in Koreans. Further study in the deaf patient population is required in order to determine the association between *GJB2* gene mutation and NSHL.

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국문 초록

선천성난청은 비교적 흔한(1/1,000) 유전성질환으로 여러 유전자와 관련이 있으며, 최근에는 connexin 26 단백질내의 *GJB2* 유전자 돌연변이와의 관련성이 보고되고 있다. 유전질환을 예측하기 위한 유전자선별검사를 임상에 적용하기 위해서는 각 해당 국가별로 정상인에서 유전자돌연변이의 빈도를 구하고, 환자의 결과를 비교하여 활용성을 검토한 후 사용하여야 한다. 본 연구에서는 청력검사(TEOAE)가 정상인 신생아에서 *GJB2* 유전자 돌연변이 빈도를 구하여 screening test를 위한 한국인의 database를 수립하고자 하였다. 검체로는 437 명의 건강한 신생아의 발꿈치를 천자하여 얻은 혈액을 이용하였고, DNA는 Intron 사의 킷트를 사용하여 추출하였으며, *GJB2* PCR을 실시한 후 증폭산물(783 bps)은 2% agarose gel로 전기영동을 실시하였고, DNA 자동염기서열분석기를 이용하여 분석을 실시하였다. 총 437명의 한국인 신생아에서 *GJB2* 유전자 중 8곳의 돌연변이(35delG, 167delT, 235delC, V27I, V37I, M34T, E114G, I203T)를 분석하였으며, 이 중 5곳에서 돌연변이가 발견되었다. 총 437명 중 301명(68.9%)에서 *GJB2* 유전자돌연변이가 발견되었는데, 그 중 154명이 단일돌연변이였다. V27I 변이가 271명으로 가장 많이 발견되었으며, 대부분의 V27I 변이는 E114G 변이와 함께 나타났다. E114G 변이는 총 146명, I203T 변이는 17명, V37I 변이는 14명, 235delC 변이는 1명의 순으로 나타났다. 이중돌연변이의 대부분은 V27I/E114G였으며, V27I/I203T는 3명에서 나타났고, 삼중돌연변이 V27I/E114G/I203T는 1명에서 발견되었다. 본 연구결과, PCR을 이용한 자동염기분석검사는 *GJB2* 유전자의 돌연변이 검출에 매우 유용하며, 본 결과는 향후 신생아 난청선별검사를 위한 한국인의 database로 활용될 수 있을 것으로 사료된다.