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The Spectrum of *GJB2* Mutations in Korean Patients with Genetic Hearing Loss: a Functional Study and Study of Cell Growth Control by Dominant Type of *GJB2* Mutants

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The *GJB2* mutation is mostly recessive in non-syndromic hearing loss, but specific mutations display a dominant type and syndromic hearing impairment. Both N54K and R75Q mutations present a dominant type in pedigrees with associated skin disorders. The purpose of this study was to investigate whether two *GJB2* mutations can exhibit a dominant-negative effect on the growth abrogation and the gap junctional intercellular communication capacity exerted by wild-type connexin 26. A specific mutant region of *GJB2* showed a loss of gap junction activity and a dominant negative effect on wild-type *GJB2*. The two mutants exerted a dominant-negative effect on the GJIC capacity and have independently effected *GJB2* regulated growth of Hela cells; however, they have no dominant-negative growth effect on wild-type *GJB2*. It is proposed that the different mechanisms of the dominant-negative effect on wild-type *GJB2* involve cell growth and GJIC function. This study describes mutations found in Korean deaf patients and that are typical of other east Asian regions.

Key Words: GJB2, Gap junction, Connexin 26, Deafness, Mutations, Genetic disease

INTRODUCTION

Among connexins, connexin 26 is also referred to as Gap Junction Protein Beta-2 (GJB2). The number 26 refers to the molecular weight of the protein (226 amino acids, ~26 kDa). There are two isotypes of the protein, though similar in amino acid sequence that are designated as alpha and beta. The *GJB2* gene is located on chromosome 13q11-q12 (Hsieh et al., 1991; Mignon et al., 1996).

The frequency of congenital hearing impairment is about 1 in 1000 births, half of which are due to genetic predisposition (Marazita et al., 1993). Mutations of the *GJB2* gene constitute approximately 50% of the autosomal recessive non-syndromic sensorineural hearing loss of the western population (Zelante et al., 1997; Estivill et al., 1998). However, many reports have shown that there is a decreased incidence of *GJB2* mutations in the Asian population than in the Western population (Park et al., 2000; Abe et al., 2000; Xiao and Xie, 2004). Out of approximately 70 genes responsible for hearing loss, mutation of *GJB2* was frequently found in the Asian population (Xiao and Xie, 2004; Oguchi et al., 2005). Therefore mutation screening of the *GJB2* gene is essential in the Asian population (Choung et al., 2002; Shi et al., 2004). As shown in several reports, the most prevalent *GJB2* mutation in East Asia was the 235delC mutation.

Over 90 kinds of *GJB2* mutations have been reported at an established database the connexins-deafness homepage (http://www.crg.es/deafness). In general *GJB2* mutations are of the non-syndromic recessive type, but a small portion of the *GJB2* mutations are dominant or of the syndromic type

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(Gerido and White, 2004). These recessive mutations invariably result in hearing impairment; the dominant non-conservative missense mutations of *GJB2*, *GJB3* and *GJB6* manifest with a spectrum of clinical phenotypes affecting hearing and the skin (Rouan et al., 2001). Specific region mutations of the *GJB2* gene showed syndromic hearing impairment with skin disorders (Heathcote et al., 2000; Maestrini et al., 1999). It has also been reported that the *GJB2* mutation exerted a trans-dominant negative effect on other co-expressed wild type connexins (Thomas et al., 2004). The mutated connexins can prevent the growth inhibition exerted by wild-type connexins. Connexins regulate growth control by an independent mechanism separate from the formation and the function of gap junctions (Duflot-Dancer et al., 1997).

To review in the turn-over process of gap junction formation, following translation of the connexon, it first migrates to a Golgi complex, and the monomer connexin is then combined as a hexamer and then migrates to the cell membrane along microbutubles in the trans-Golgi, and on the cell membrane, and connexons in the combined form produce plaques, and a complete gap junction channel is formed. Although there can be a situation where connexin 26 can not be detected on the cell membrane, the possibility of it remaining in the Golgi complex exists.

By using a dye transfer method that uses fluorescent substances smaller than 1,000 daltons that are able to penetrate connexon, the presence or absence of the gap junction exerting a normal function between adjacent cells could be assessed, and the functional gap junctions were found to be present in normal mammalian skin cells expressing connexin 26 and connexin 43, however, in cancer cells, the presence of gap junctions was not detected (Lee et al., 1992). By applying the dye transfer method, not only the presence or absence of a gap junction could be also examined but also whether it mediates normal functioning. In addition, based on cell cyle analysis using cell sorting (FACS), effects on cell proliferation can be determined.

In this study, cell based systems and molecular biological techniques were used. To screen the *GJB2* gene in deaf Korean patients and to investigate the properties of the gap junctions with two mutants (N54K and R75Q). Both N54K and R75Q were reported as dominant types of mutations. However, there are no reports about the functional studies of these mutations, and on the growth effect of wild-type connexin 26.

MATERIALS AND METHODS

1. Patients

One hundred unrelated Korean patients with congenital hearing loss showing *GJB2* mutations were screened. All of the patients included in this study were from the Soree Ear Clinic. Patients who had an abnormal tympanic membrane or middle ear cavity, who had taken toxic drugs, or who had a history of meningitis, trauma, or other known causes of hearing loss were not included in this study. The studies were approved by the institutional review board and performed with informed consent of all participants.

2. Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes using the Puregene® DNA Purification Kit (Gentra, Minneapolis, MN). To identify the *GJB2* mutations, the translational region of the *GJB2* gene was amplified from genomic DNA using the polymerase chain reaction (PCR) using a forward primer TCTTTTCCAGAGCAAACCGC and a reverse primer CCTCATCCCTCTCATGCTGT. The PCR mixture (20 μl) contained 100 ng of genomic DNA, 1 × PCR buffer, 2.5 mM dNTPs, 25 pmol of each PCR primer, and 1 U of *Taq* DNA polymerase. These primers yield a product of 764 bp. The PCR reaction was carried out bay an initial denaturing step at 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 1 min, with a final extension at 72℃ for 5 min. The PCR products were resolved on a 2% agarose gel. The corresponding bands on the 2% agarose gel were cut out, and the DNA was purified by the use of a QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). Primers for DNA sequencing were then added: the forward primer CTGCAGCTGATCTTCGTGTCC and the reverse primer TTGAGTAGGTGGACCACAGG. A 3100 Capillary DNA sequencer (Applied Biosystems, Foster, CA) was used for DNA sequencing. The patients with N54K or R75Q mutations in the *GJB2* gene were also screened for mutations in the *GJB3* and *GJB6* genes as well. The primer set for *GJB3* DNA sequence analysis was forward primer TCTCTCAGGTAGGCACGGC and reverse primer CACCTATTCATTCATACGATGG, The primer set for GJB6 DNA sequence analysis was forward primer TGTCTGTTTAGGGATAA-

ACC and reverse primer GACGCAGCTACATTTTACC. Both *GJB3* and *GJB6* primer sets covered the entire coding region, respectively. The PCR conditions were identical to that described for *GJB2*.

3. Cloning and transfection of *GJB2*

The primers for cloning were prepared, including the coding region of *GJB2* and the recognized restriction sites are as follows; Cx-*Eco*RI: TTAG*GAATTC*CCTCATCCC-TCTCATGCTGT and Cx-*Hin*dIII: GCAT*AAGCTT*TCTT-TTCCAGAGCAAACCGC. Three samples including a wild type, a N54K mutant and a R75 mutant were amplified by PCR using the same process described above. Each PCR product and the two types of vectors were digested with *Eco*RI and *Hin*dIII (New England Biolabs, Beverly, MA) The wild type *GJB2* gene was inserted into pcDNA3.1(+) (Invitrogen, Carlsbad, CA), and mutant types (N54K and R75Q) of the *GJB2* gene were inserted into pcDNA3.1(+)/ Hygro (Invitrogen, Carlsbad, CA). Prepared PCR products were mixed with pcDNA3.1(+) or pcDNA3.1(+)/Hygro vectors, T4 DNA ligase (Promega, Madison, WI), 1 × ligase buffer and were then incubated at 16℃ for 8 hours. Ligation products were transformed into *E. coli* DH5α. The transformed *E. coli* was incubated at 37℃ for 16 hours. Ampicillin resistant colonies were incubated in LB broth containing ampicillin. The QIAprep® spin miniprep kit (Qiagen, Valencia, CA) was used for purification of plasmid DNA according to the manufacturer's protocol.

One day before transfection, $0.5\sim2\times10^6$ cells were inoculated 5 ml of growth medium without antibiotics so that cells would be 90~95% confluent at the time of transfection. Transfection was performed using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

4. Immunocytochemistry

Fluorescent immunocytochemistry was used to verify expression and subcellular localization of Cx26. The Lab-TekII® Chamber Slide System (Nalge Nunc International, Naperville, IL) was used according to the manufacturer's protocol. The slides were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The slides were washed 3 times for 5 minutes with PBS containing 100 mM glycine. Cells were mixed with 0.1% Triton X-100 in PBS for and incubated for 4 minutes. The cells were washed 3 times with PBS, and then incubated with 1% BSA in PBS for 30 minutes. Primary rabbit anti-connexin 26 (20 μl, 10 μg/ml) (Zymed, South San Francisco, CA) was dropped in 1% BSA on the chamber. After incubation for 1 hour, the slides were washed 3 times for 10 minutes with PBS. A fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (0.4 mg/ml; Santa Cruz, Santa Cruz, CA) was diluted 1 : 100 and added to the chamber (final volume, 20 μl). The slides were then incubated for 1 hour in a dark room, washed with PBS, and mounted with 10 μl glycerol. Slides were examined with an Axivert 200 inverted High-end Microscope (Carl Zeiss, Jena, Germany) and images were stored digitally.

5. Functional study

The functionality of the mutated Cx26 was determined by the method of scrape-loaded dye transfer described by El-Fouly et al. (El Fouly et al., 1987) with some modifications. Transfected Hela cells were incubated in 60 mm plates for 24 hours. The cells were washed twice with PBS, flooded with PBS containing 0.5% Lucifer yellow (Sigma, St.Louis, MO), then scrape-loaded with a surgical scalpel. The dye solution was left on the plates for 10 minutes for spreading, and the plates were then rinsed with PBS to remove unloaded dye and background fluorescence. 10% formalin was added to the plates for fixation. Cell communication was observed under a Olympus IX70 (Olympus, Melville, NY). Images were stored digitally using scientific imaging software IPLab (scanalytics, Fairfax, VA). Images were processed using Jasc Paint Shop Pro (Corel, Eden Prairie, MN).

6. *In vitro* proliferation assay

For analysis of cell proliferation, Hela cells and each Hela transfectant were plated at a density of 5×10^3 cells/ well in 12-well plates in triplicate and were counted at day 3, 5, 8, and 10.

7. Statistical analyses

The results were analyzed using the JMP software package (version 4.0; SAS Insitutute, Cary, NC). Data was compared with the Dunnett *t* Test after ANOVA analysis on an IBM computer. For all comparisons, a *P* value less than 1% ($P<0.01$) was considered to be statistically significant.

Table 1. *GJB2* mutations and polymorphism detected in 100 Korean deaf patients

Codon location	Nucleotide change	Patients number		Frequency of mutation	Type of mutation
		Hetero-zygous	Homo-zygous		
V37I	109G → A	3	•	15.8%	Missense
N54K	162C → A	1	•	5.3%	Missense
R75Q	224G → A	1	•	5.3%	Missense
79	235delC	4	4	63.2%	Deletion/frameshift
100	299-300del (AT)	1	•	5.3%	Deletion/frameshift
R143W	426C → T	1	•	5.3%	Missense
V27I	79G → A	31	9	•	Polymorphism
E114G	341A → G	19	7	•	Polymorphism
I203T	608T → C	51	1	•	Polymorphism

RESULTS

1. Mutations in the *GJB2* gene

Among one hundred congenital unrelated deaf patients, 13 patients had mutations in *GJB2*. Six kinds of mutations and three kinds of polymorphisms were discovered in this study (Table 1). V37I, 235delC, 299-300delAT and R143W show autosomal recessive inheritance, but both N54K and R75Q show autosomal dominant patterns. The three polymorphisms were V27I, E114G and I203T.

It was found that the frequency of mutations varies. The frequency of 235delC was 63.2% (12 alleles/19 alleles) and that of V37I was 15.8% (3 alleles/19 alleles). Others were found once, respectively. The other allele was not found to be a mutation in five patients. Because the N54K and R75Q mutations were inherited in an autosomal dominant pattern, the other allele had no mutation.

2. Immunocytochemistry

The GJB2 protein was analyzed in Hela cells transfected with the *GJB2* gene using flurescent immunocytochemistry. Hela cells transfected with a wild-type gene gave patterns of reaction in the cell membrane. However, cells expressing N54K or R75Q did show a relatively rare staining around the cell membrane.

In Fig. 1(B), in examining the area indicated by the arrow, it could be found that in cell lines transfected with the *GJB2* wild-type, the distribution of the GJB2 protein along the cell membrane could be detected; however, in cells transfected with the two types of mutation, such a pattern could not be detected. The un-transfected Hela cells did not show any staining on the cell membrane or on the cytoplasm.

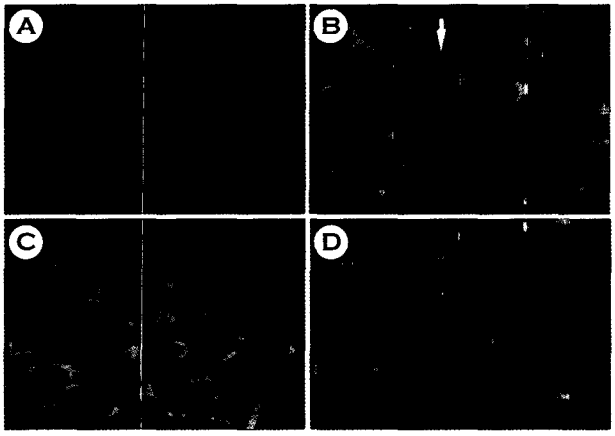

Fig. 1. Immunocytochemistry of Hela cells using a primary antibody against the N-terminal portion of Cx26. **(A)** untransfected Hela cell, **(B)** Wild-type (WT), **(C)** N54K, and **(D)** R75Q transfected cells. Untransfected Hela cells showed no reaction.

3. Functional study of GJB2

To investigate whether the mutant GJB2 could form functional gap junctions, the intercellular transfer of Lucifer yellow was observed in Hela cells expressing GJB2 using the scrape-loaded dye transfer technique (Fig. 2). Hela cells expressing N54K or R75Q showed no dye transfer. Furthermore, to examine the dominant negative effect on the wild-type *GJB2*, each N54K and R75Q transfected Hela cells also expressed wild type Cx26 by co-transfection. No dye transfer was observed in both co-transfected Hela cells.

The fluorescence images were converted into the average

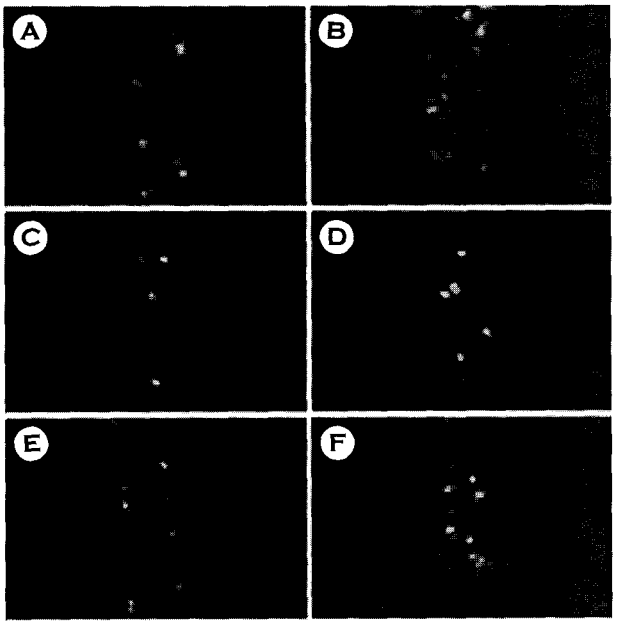

Fig. 2. Functional analysis of GJB2 using the scrape-loaded dye (Lucifer yellow) technique. **(A)** Hela Cell. **(B)** Wild-type (WT), **(C)** N54K, **(D)** WT-N54K, **(E)** R75Q, **(F)** WT-R75Q transfected cells (fluorescent microscopic view). Cell communication was examined by scrape-loaded lucifer yellow dye transfer into adjacent cells. The transfer of lucifer yellow into contiguous cells was detected in WT, but not in the others transfected Hela cells.

number of neighboring cell receiving dye out of five fields (Fig. 3). Further dominant negative effects were displayed in the wt/N54K-Cx26 and wt/R75Q-Cx26. The wt/wtCx26 was in a transient cell state and exhibited a higher cell number than wtCx26.

4. *In vitro* proliferation assay

The N54K and R75Q mutations of the *GJB2* gene accompany deafness and dermatolotical diseases. In cases of dermatological diseases, due to these mutations, the cell growth rate is changed. The level of the shedding of cells from the skin by apoptosis does not occur synchronously, and such phenomenon occurs in some areas at different times. This can be detected macroscopically, and the effect of each mutation induces the acceleration of cell growth, and the skin in some areas peels more readily. Therefore, shedding was analyzed by enumerating the cell number of 7 types of cells: Hela cells, Hela cells transfected with vector(s) only, cells expressing the wild-type *GJB2* gene, cells transfected with N54K mutation, cells transfected with R75Q mutation, cells co-transfected with the wild type and N54K, and cells co-transfected with the wild-type and R75Q.

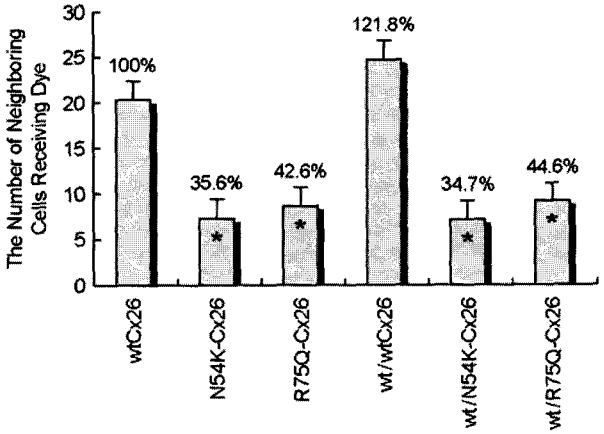

Fig. 3. Functional analysis of wild-type, mutant Cx26, and mutant Cx26 co-expressed with wild-type. The bars represent the number of cells that received dye. The relative functional activity of gap junctional channels is given in percent. wt/wtCx26 is a transient cell state but the others are stable cell lines. The cell number is reported as the mean ± S.E. using five independent experiments, $P<0.01$.

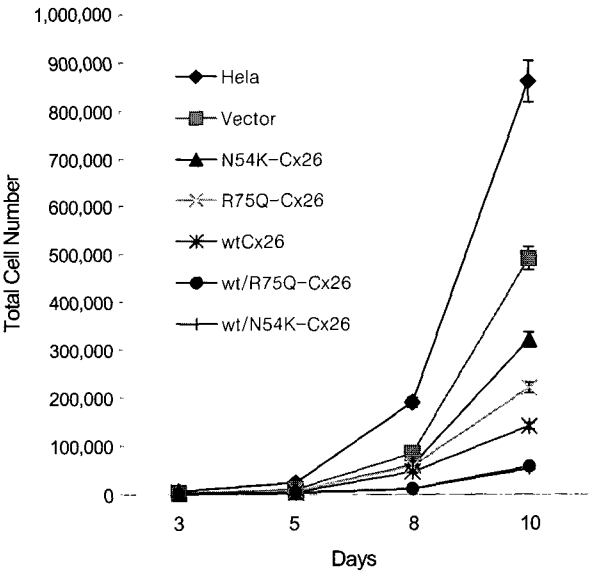

Fig. 4. Cell growth analysis of wild-type, mutant Cx26, and mutant Cx26 co-expressed with wild-type. For the analysis of cell proliferation, the cells were plated in 6-well plates in triplicate and were counted at day 3, 5, 8 and 10 after plating. The experiments were done in triplicate.

The growth rate of Hela cells was fastest, followed by Hela cells transfected with the empty vectors. The growth rate of N54K and R75Q mutation followed, and the wild-type was next. Therefore, based on such results, it was found that due to these two types of mutations, cell growth rate was increased. However, cells co-transfected with wild-type and either a N54K or R75Q mutation showed a cell growth

rate slower than the wild-type; this indicates that based on the effect of the cell growth due to the mutation, the dominant negative effect was not present (Fig. 4).

DISCUSSION

Thirteen patients with *GJB2* gene mutations were found in 100 Korean patients with a hearing impairment. This is similar to the frequency of the *GJB2* mutation found in the Chinese population--12.2% (16/131) as reported previously (Xiao and Xie, 2004). The allele frequency of 235delC and V37I in the patients were 42% and 16% respectively, and these results were in accordance with previous reports of the *GJB2* mutation frequency in east Asian populations (Park et al., 2000; Liu et al., 2002; Shi et al., 2004; Xiao and Xie, 2004; Oguchi et al., 2005). Two patients with 235delC did not have a second mutation, and neither did the 3 patients with the V37I mutation. The missing mutation was probably located outside of the coding region. Both N54K and R75Q mutations were reported to be dominant and one case of each was discovered.

This study and recent reports support that the 235delC mutation in the Mongoloid ethic group is a hot spot (Xiao and Xie, 2004). Therefore, it should be considered as the primary mutation to screen for patients with congenital deafness (Oguchi et al., 2005).

Most of Cx26 mutants are found in patients with non-syndromic deafness, but some patients with a Cx26 mutant showed syndromic deafness. Hearing loss due to the dominant inheritance type of mutations is frequently accompanied with the dermal disorder (Rouan et al., 2001; Marziano et al., 2003).

Each type of connexin shows a different tissue distribution, and in many types of cells, more than one type of connexin may be expressed. This allows the combination of different connexin isoforms and thus not only is a homo-oligomeric connexon formed by the combination of the identical type of connexin, but also a hetero-olgomeric connexon may be generated. Therefore, the patterns of connexon formed in such tissues appear to be more diverse (Brink et al., 1997).

The phenomenon accompanying dermatological diseases could be explained in the cases of different types of gap junctions forming hetero-oligomeric type connexons, a specific gap junction with a mutation could mediate an effect on the hetero-connexon function. Therefore, the phenomenon of deafness with concomitant dermatological diseases should be considered to be the effect of a special mutation of connexin 26 on a different gap junction expressed on the skin (Thomas et al., 2004).

By examining whether the combined connexon mediates its function properly through the analysis of the connexon assembly formed by a wild-type connexin and abnormal connexin, the dominant negative effect of identical connexins and connexins different from each other can be assessed (Lagree et al., 2003).

The severity of Cx26-linked skin diseases is probably related to a selective trans-dominant interaction with other connexins expressed with the skin. The connexin protein has N-terminal and C-terminal regions, a single intercellular loop and two extraecllular loops. The first extracellular loop is critical for Cx26 transport to the cell surface as well as the function of the gap junction channels (Thomas et al., 2004). The asparagine at position 54 is located in the first extracellular loop. The N54K and R75Q mutant patient in this study had skin diseases in the hands. Both the *GJB3* and *GJB6* genes expressed in the skin were sequenced, including the entire coding region, however, no mutations were discovered (data not shown). Therefore this finding alludes to the trans dominant interaction of N54K muations and R75Q.

The arginine at position 75 is critical for the formation of a functional gap junction channel. Arg-75 is especially responsible for intersubunit interactions in the connexon. Other amino acid substitutions of Arg-75 have been reported to have reduced channel activities (Oshima et al., 2003). Uyguner and colleagues (Uyguner et al., 2002) reported a R75Q mutation in the *GJB2* gene, and their case was accompanied with palmoplantar skin. *GJB2* mutations are related to hearing impairment alone, or in association with the thickening of the skin of palms and soles, diagnosed as palmoplantar keratoderma. Involvement of the skin remains unexplained, because the complete loss of Cx26 function in individuals with recessive *GJB2* mutations has never been associated with any skin disorder.

Many reports have found *GJB2* mutations as being responsible for 50% of autosomal recessive non-syndromic sensorineural hearing loss in western populations, but the east Asian populations showed a lower level.

In Korea, mutations of the *GJB2* gene were reported in

2000 for the first time (Park et al., 2000). In Koreans, the frequency of the carriers of a GJB2 gene defect is one in 100 normal individuals; therefore, even if a marriage were between normal individuals, if the couple were both carriers, they have a 1/4 probability of the birth of deaf progeny. Therefore, in Asians including Koreans, the analysis of the *GJB2* gene is of great interest (Park et al., 2000; Shi et al., 2004).

In this study, *GJB2* mutations of Korean patients with congenital hearing loss are estimated to be 13%. It is probably a major gene responsible for genetic hearing loss in Korea; however, over 80% of the cause of hearing loss is due to other genes. Therefore, it is necessary to find other causative genes in the Korean patients with genetic hearing impairment. Two kinds of mutations (N54K and R75Q) with dominant negative effect were investigated. According to recent reports, patients with N54K and R75Q mutations showed syndromic deafness with skin disease. These two mutants have an expressed dominant negative effect on wild-type GJIC function, but not on cell differentiation. Therefore to evaluate the exact mechanism of the trans-dominant negative effect in skin, additional functional studies are needed.

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