Ion transporting activities of pendrin mutants identified in Korean patients with genetic hearing impairment

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Ion transporting activities of pendrin mutants identified in Korean patients with genetic hearing impairment

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ABSTRACT

The *SLC26A4* gene encodes pendrin, which is a transmembrane protein that functions as an anion exchanger of formate, iodide, chloride, and bicarbonate. Pendrin is expressed in the thyroid, kidney, mammary gland, uterus, testes, placenta, and inner ear. Mutation of Pendrin is responsible for Pendred syndrome and autosomal recessive non-syndromic hearing loss at the DFNB4 locus on chromosome 7q31.

More than 90 mutations of pendrin, including 16 mutations identified in Koreans, have been reported.

In regard to hearing process, Cl^-/HCO_3^- exchange by pendrin at the stria vascularis of the inner ear is important to maintain the endocochlear potential (EP), which provides the main driving force for the hearing mechanism.

To identify the underlying mechanism of defective pendrin function, this study evaluated the Cl^-/HCO_3^- exchange activities and membrane expression patterns of eight frequent pendrin mutants (S28R, P142R, M147V, S166N, G497S, E625X,

L676Q and H723R) in Korean patients with genetic hearing impairment and four relatively common pendrin mutants (L117F, L236P, E384G and T416P) in Caucasian patients.

Pendrin-mediated Cl^-/HCO_3^- exchange activities in HEK293 cells were markedly decreased in eight mutants identified in Koreans regardless of their location (extracellular, plasma membrane, intracellar).

Three common mutants reported in Caucasian also showed decreased activity.

The L117F mutant, which was reported in Caucasian, demonstrated about a 20% decrease in activity compared to WT, but was statistically insignificant.

Appropriate trafficking to the plasma membrane of pendrin was observed in S166N and L117F mutants when the Myc-specific antibody-tagged pendrin mutant constructs transient expressed in HeLa cells.

In conclusion, this study observed that the pendrin mutants identified in Korean patients with genetic hearing impairment did not move to the cell membrane but rather were retained in the cytoplasm. These results can cause decreased Cl^-/HCO_3^- anion exchange activity

Key words: *SLC26A4*, Pendrin , mutation, genetic hearing loss, functional study

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I. INTRODUCTION

Hearing loss is a common disorder that has been estimated to occur in about 10% of the population.[1] Half of these cases were caused by genetic disorders in newborns.[1] *SLC26A4* mutation produces hearing loss, accounting for up to 10% of all hereditary hearing loss, and is considered the most common cause of congenital deafness.[2]

The gene that codes for pendrin, is located at chromosome 7q and is a 73kDa glycoprotein that contains 12 transmembrane domains.[2] Pendrin is an isoform of ion-transporter that is expressed in the inner ear non-sensory epithelia, thyroid folliculocyte, renal cortical collecting ducts, placental trophoblast, and uterine epithelium.[3]

There are two clinical manifestations of pendrin mutants, Pendred syndrome and Non-syndromic hearing loss. Pendred syndrome is an autosomal recessive disorder first described in 1896 as the combination of congenital deafness and goiter.[4]

Non-syndromic recessive hearing loss (DFNB4) has been reported as an allelic disorder of Pendred syndrome.[5]

Over 90 different pendrin mutations have been reported, and each ethnic population has its own distinctive mutant allele series with a few prevalent founder mutations.[6] The variable thyroid phenotype of affected patients, even within the same family, can be explained by endogenous and environmental factors.[7]

Park et al. identified 16 different mutations in sensory neural hearing loss patients, which were associated with an enlarged vestibular aqueduct in Koreans. Among them, five mutations were recurrent and account for 80% of all mutant alleles; H723R (2169A>G), Splice acceptor (IVS7-2A>G), Splice donor (IVS9+3A>G), M147V (439A>G), and Frameshift (365insT).[8,9] (Table1.)

Coyle et al, studied samples from Caucasian patients, and found four of mutants which represented 74 percents of all mutants. This research suggested that a causal mechanism of pendrin mutation is the common founder effect rather than the hot spot.[10]

Pendrin which is located in the apical pole of thyroid folliculicytes, has an efficient iodide-trapping mechanism that leads to oxidation and organification of iodide by a sodium iodide symporter (NIS).[11,12,13,14,15]

As a result of this mutation, Pendred syndrome patients have a defect in iodide organification, which is shown by an abnormal perchlorate discharge test.[7]

Most of the hearing loss caused by pendrin mutation is prelingual, to a severe degree and symmetrical. It can be manifested as asymmetrical, mild degree hearing loss, which may get worse or fluctuate by head trauma. Most affected subjects had an enlarged vestibular aqueduct observed by a temporal bone CT scan and mondini malformation of the cochlear sometimes.[7]

Endolymph has a positive voltage (+80 mV) compared with perilymph or plasma, which is called the endocochlear potential (EP). The mechanisms required for the maintenance of this voltage were poorly understood, but Wangermann et al. postulated that the anion transport performed by pendrin was critical for maintaining

the appropriate ionic balance within the inner ear fluid, which is known to serve a critical role in the hearing process.[16,17]

The physiological importance of pendrin in the inner ear auditory function was indicated by the evaluation of Cl^-/HCO_3^- exchanger activity. The mechanism of hearing loss caused by pendrin was the functional loss of Cl^-/HCO_3^- exchange activity leading to the functional loss of the potassium channel, KCNJ10 by the elevated intracellular pH in the stria vasculalis. This ultimately results in a loss of EP and hearing loss.[16,17]

To identify the underlying mechanism of defective pendrin function, this study evaluated the effect of mutant allele on pendrin-mediated Cl^-/HCO_3 transport in transfected HEK293 cells. Immunochemistry was used to evaluate expression patterns of the cell membrane in transfected Hela cells. Eight mutants were identified in Korean patients with genetic hearing impairment was targeted to make mutants construct.

Because of the challenges in evaluating the Pendred syndrome phenotype clinically, most studies haxe not reliably distinguished non-syndromic deafness from Pendred syndrome.

Pendrin phenotypes of 16 patients with hearing loss were compared to the pendrin genotype in the cellular level.

Table 1. Pendrin mutants identified in Koreans

Location	Nucleotide change	Mutation	Frequency(%)*	Reference
2	82A>C	S28R	1(1.5)	(9)
4	365insT	Frameshift	2(3.0)	(9)
IVS4	IVS4+4A>G	Splice donor	1(1.5)	(9)
5	425C>G	P142R	1(1.5)	(9)
5	439A>G	M147V	3(4.5)	(18)
5	497G>A	S166N	1(1.5)	(9)
IVS7	IVS7-2A>G	Splice acceptor	9(22.4)	(19)
IVS9	IVS9+3A>G	Splice donor	4(6.0)	(9)
10	1229C>T	T410M	1(1.5)	(10)
13	1489G>A	G497S	1(1.5)	(5)
IVS14	IVS14-1G>A	Splice acceptor	1(1.5)	(9)
IVS15	IVS15+5G>A	Splice donor	1(1.5)	(9)
17	1873G>T	E625X	1(1.5)	(9)
17	2027T>A	L676Q	2(3.0)	(9)
19	2162C>T	T721M	1(1.5)	(20)
19	2168A>G	H723R	31(46.3)	(20)

*****Reported frequency was extracted from reference (9).

II. MATERIALS and METHODS

1. Materials, Solution, and Cells

2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was purchased from Molecular Probes (Eugene, OR). Anti-Myc monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated fluorescence antibody was purchased from Zymed (Carksbad, CA). Lipofectamine reagent was purchased from Invitrogen (Carksbad, CA). Restriction enzymes and peptide-*N*-glycosidase F (PNG) were purchased from New England Biolabs (Beverly, MA). The QuikChange II site-directed mutagenesis Kit was purchased from Stratagene (La Jolla, CA). All other chemicals were purchased from Sigma (St Louis, MO). The standard HEPES-buffered solution A contained (mmol/L) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). The HCO_3^--buffered solution B contained (mmol/L) 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4 with NaOH). Cl^--free solutions were prepared by replacing Cl^- with gluconate. HEK293 cells were maintained in DMEM-HG supplemented with 10% fetal bovine serum, and HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

2. Cloning of pendrin cDNA and site-directed mutagenesis.

The human wild-type Pendrin cDNA (Genbank NP000432) was generated by RT-PCR using total RNA from normal human thyroid tissue and pfu polymerase (Stratagene, LaJolla,CA).

The sequences of the sense and antisense primers were 5`-GCGAGCAGAGACAGGTCAT-3` and 5`- TCATAGAGACCTCCCGAACC-3`, containing *Xho I* and *Not I* restriction enzyme cleavage sites, respectively. Amplification was performed with a ABI 9700 (Applied Biosystems, Foster City, CA). Samples were denatured for 30 seconds at 98°C and then subjected to 30 cycles

of 10 seconds at 98°C, 45seconds at 72°C. The last extension was performed for 10 minutes at 72°C.

The Pendrin cDNA fragment was purified, digested with the *Xho I* and *Not I* restriction enzymes over 2 hours at 37°C, and then subcloned into the pCMV-Myc vector (Clontech, Palo Alto, CA).

Oligonucleotide-directed mutagenesis using the QuikChange II site-directed mutagenesis Kit was performed to generate the pendrin mutants in the expression vector pCMV-Myc according to the manufactures' protocol.

The primer sequences used are shown in Table 3.

PCR was performed in a final volume of 50 μl, which contained 50ng of dsDNA template, 125ng of each primer, 1μl dNTP and 1μl of Phusion DNA polymerase.

Amplification was performed with a ABI 9700 (Applied Biosystems, Foster City, CA). Samples were denatured for 30 seconds at 95°C and then subjected to 12 cycles of 30 seconds at 95°C, 1 minute at 55°C, 8 minutes at 68°C. The last extension was performed for 10 minutes at 72°C. PCR products were electrophoresed in 1.5% agarose gel and visualized directly by ethidium bromide staining. Add 1 μl of the Dpn I restriction enzyme (10U/μl) directly to each amplification reaction. Spin down the reaction mixtures in a microcentrifuge for 1 minute an immediately. Then incubate each reaction at 37°C for 1 hour to digest the parental supercoiled dsDNA.

Then transformation of XL1-Blue supercompetent cells was performed:

Gently thaw the XL1-Blue supercompetent cell on ice. Transfer 50 μl XL1-Blue supercompetent cells by adding 1 μl of the Dpn I-treated DNA. Swirl the transformation reactions for 45 seconds at 42°C and the place the reactions an ice for 10 minutes. Add 1ml of LB broth shaking incubation for 1 hour. Spread cells on LB-ampicillin agar plates the incubation at 18 hours at 37°C.

Then LA broth shaking incubation for 16 hours at 37°C.

Sequencing :

Cycle sequencing reactions were performed using 10ng plasmid and the Big dye Terminator (version 3.1) Ready Reaction kit (Applied Biosystems, Foster City, CA) Primer(1.6 pmol) was used in a total reacton volume of 10 μl. Cycling parameters were: 30cycles of 30 seconds at 95°C, 15 seconds at 50°C, 4 minute at 60°C. After the sequencing PCR, each reaction was ethanol-precipitated to remove excess dye terminators. Prior to loading on the automated DNA sequencer, pellets were dissolved in distilled water and heated at 95°C for 5min (to denture) and then quenched on ice for 5 minutes. The samples were then mixed and centrifuged briefly. Finally, samples were loaded into the autosampler tray of an ABI 3100 (Applied Biosystems, Foster City, CA)

Table 2. Pendrin mutants analyzed in this study and their location

	Pendrin mutants	Location
1	S28R	N terminal (intracellular)
2	L117F	Plasma membrane
3	P142R	Plasma membrane
4	M147V	Plasma membrane
5	S166N	Extracellular
6	L236P	Intracellular
7	E384G	Extracellular
8	T416P	Intracellular
9	G497S	Plasma membrane
10	E625X	Intracellular
11	L676Q	C terminal, * STAS (intracellular) domain
12	H723R	C terminal, STAS (intracellular) domain

*STAS domain **:** sulfate transporter and anti-sigma antagonist domain

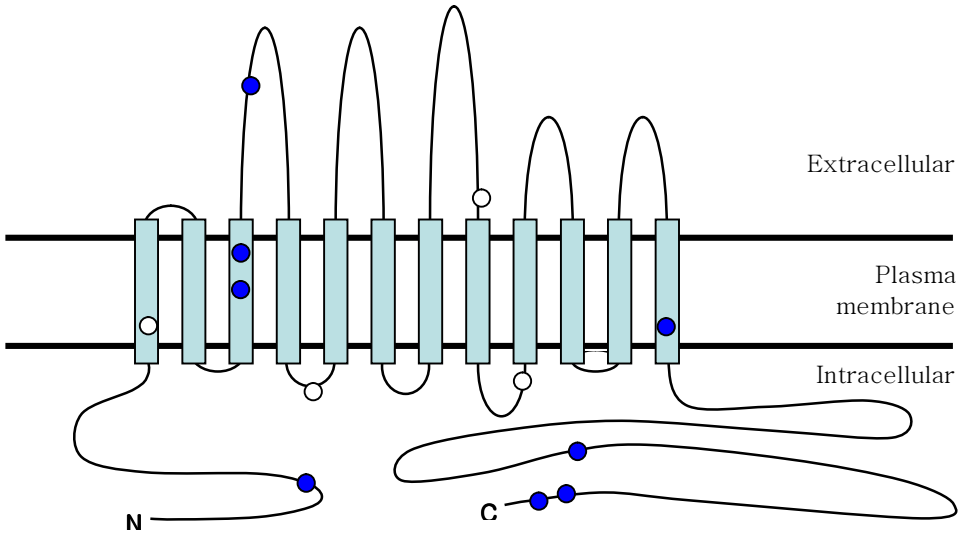


Fig. 1. Structure of pendrin and the locations of its mutants studied in this cohort.

Among the 16 pendrin mutants identified in Koreans, eight mutants were selected for functional study that can be made artificially. Eight mutants identified in Korean patients with genetic hearing impairment and four relatively common mutations reported in Caucasian patients were investigated. Five different programs (PHD, TMHMM, HMMTOP, TOPPRED, and TMPRED) were used in predicting protein topological structures. They were used to classify extracellular, plasma membrane, and intracellular proteins. The black circle represents the locations of the mutants identified in Korean patients and the white circle represents the mutation locations reported in Caucasian patients.

Table 3. Primers used for site directed mutagenesis

		Primer	Melting temperature(°C)
1	S28R(82A>C)	GGCCGGTCTACCGCGAGCTCGCTTT	78.4
2	L117F(349C>T)	GCAGTTCCTGTCGGATATGGTTTCTACTCTGC	79.1
3	P142R(425C>G)	GACATATCTCAGTTGGACCTTTTCGAGTGGTGAG	78
4	M147V(439A>G)	GACCTTTTCCAGTGGTGAGTTTAGTGGTGGGATC	79.2
5	S166N(497G>A)	CGACGAACACTTTCTCGTATCCAGCAACAATGGAAC	79.3
6	L236P(707T>C)	CAAGTGCTGGTCTCACAGCCAAAGATTGTCCTC	79.1
7	E384G(1151A>G)	GATGGGAACCAGGGATTCATTGCCTTTGGGATC	79.1
8	T416P(1246A>C)	GTCCAGGAGAGCCCTGGAGGAAAGACAC	78.7
9	G497S(1489G>A)	CCATCATTCTGGGGCTGGATCTCAGTTTACTAGC	79.2
10	E625X(1873G>T)	AGGATATTGAAGATCTGTAG	59.2
11	L676Q(2027T>A)	CGTTGTTGGAGTGAGATCACAGCGGGTGATTG	79.1
12	H723R(2169A>G)	GGACACATTCTTTTTGACGGTCCGTGATGCTATAC	78.1

3. Measurement of Cl^-/HCO_3^- exchange activitiy

HEK 293 cells were plated on a sterile 22 X 40-mm coverslip at a density of 2.5 X 10^5 cells/cm^2 for intracellular pH (pH_i) measurements. On the following day, 1 μg of WT or each mutant construct was transfected using Lipofectamine. The cells were used for Cl^-/HCO_3^- exchange activity measurements 48-72 h after transfection. Cl^-/HCO_3^- exchange activity was measured by recording the intracellular pH (pH_i) in response to $(Cl^-)_0$ changes of the perfusate. Cells attached to glass coverslips were washed once with HEPES-buffered solution A, and then assembled to form the bottom of a perfusion chamber. Glass coverslip-attached cells were loaded with a fluorescent pH probe BCECF by incubating for 10 min at room temperature in solution A containing 2 uM BCECF-AM. After dye loading, the cells were perfused

with a HCO_3^- buffered solution B and BCECF fluorescence was recorded at the excitation wavelengths of 488 and 442 nm at a resolution of 2/s using a recording setup (Delta Ram; PTI Inc.) under a continuously gassed condition of 95% O_2 and 5% CO_2.

The results of multiple experiments were analyzed using non-paired Student's *t*-test.

4. Immunoblotting of wt-pendrin and pendrin mutants

Cell lysates (50 μg of protein) from HEK293 cells transfected with WT or each mutant Myc-Pendrin were suspended in SDS sample buffer and separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred to polyvinylidene difluoride membranes, which were blocked by 1 h incubation at room temperature in a solution containing 5% nonfat dry milk, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20. The membranes were then probed with a Myc monoclonal Antibody.

5 . Immunocytochemistry of wt-pendrin and pendrin mutants

HeLa cells were transfected with WT or each Myc-pendrin mutant using Lipofectamine. After 48-72 h, the cells were washed with phosphate-buffered saline (PBS), and fixed with cold methanol for 10 min at -20°C. The cells were blocked with PBS containing 5% goat serum, 1% bovine serum albumin, and 0.1% gelatin for 1 h at room temperature, and then stained with primary antibody, anti-Myc polyclonal antibody, overnight in a cold room. After three washes, FITC secondary antibody was applied for 1 h at room temperature. Coverslips were mounted for confocal image analysis. Images were obtained with a Zeiss LSM 510 confocal microscope**.**

Myc-tagged WT and pendrin mutants expressing HeLa cells were fixed and permeabilized by incubation in cold methanol for 10 minutes at -20°C. Nonspecific binding sites were blocked by incubation for 1 h at room temperature with 0.1 ml of phosphate-buffered saline containing 5% goat serum, 1% bovine serum albumin, and

0.1% gelatin (blocking medium). After blocking, The cells were stained by incubation with anti-Myc antibodies and then treated with fluorophore-tagged secondary antibodies. Images were obtained with a Zeiss LSM 510 confocal microscope.

III. RESULTS

1. Cl^-/HCO_3^- exchange activity according to pendrin mutants

To identify the underlying mechanism of defective pendrin function, the inner ear transporter for Cl^-/HCO_3^- exchanger was evaluated at the cellular level.

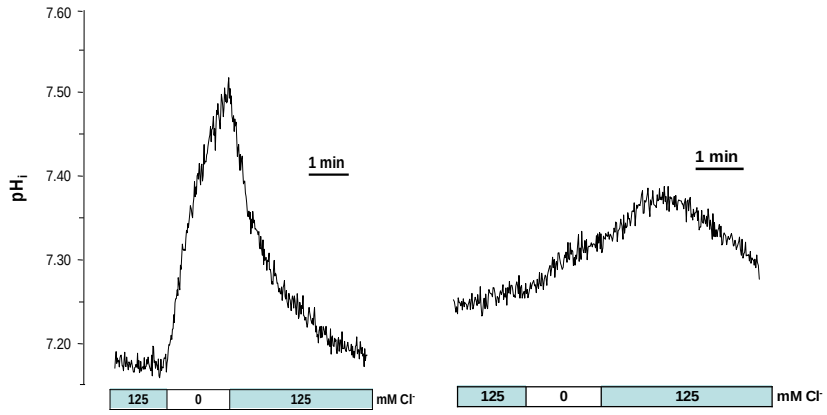
Cl^-/HCO_3^- exchange activities according to wt-pendrin and pendrin mutants were investigated using HEK 293 cells transfected with pendrin mutants. Intracellular pH was measured in response to $(Cl^-)_0$changes of the perfusate. The Cl^-/HCO_3^- exchange activities according to wt-pendrin and pendrin mutants were shown in Fig. 2.

Pendrin-mediated Cl^-/HCO_3^- exchange activities were markedly decreased in eight mutants identified in Koreans regardless of their location (extracellular, plasma membrane, intracellar).

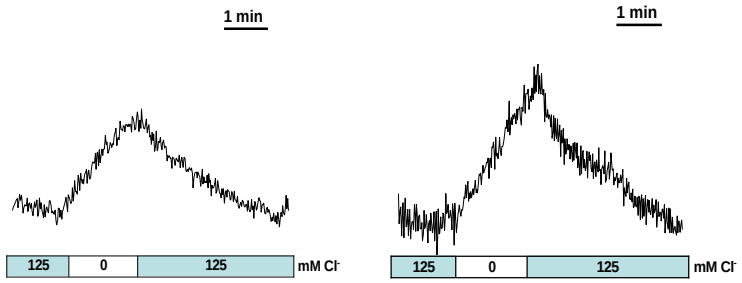
Three common mutants reported in Caucasian also showed decreased activity.

The L117F mutant, which was reported in Caucasians, demonstrated about a 20% decrease in activity compared to WT, but was statistically insignificant.

a. Cl^-/HCO_3^- exchange activity in extracellular portion

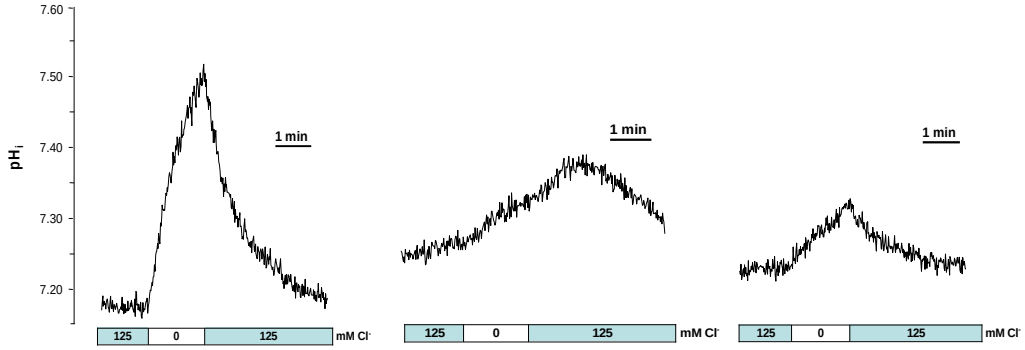


WT **Mock**

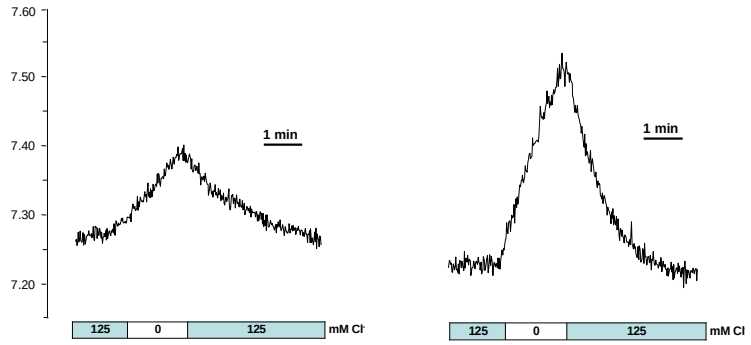


S166N **E384G**

b. Cl^-/HCO_3^- exchange activity in plasma membrane

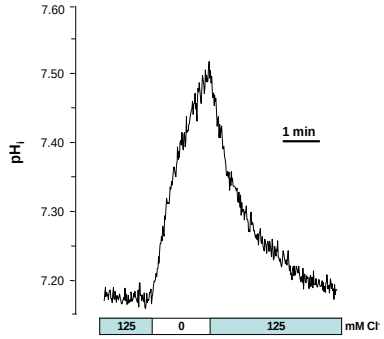


WT **Mock** **P142R**

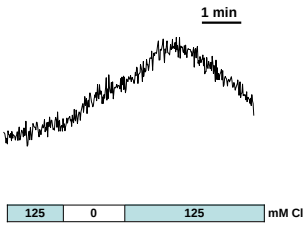


M147V **L117F**

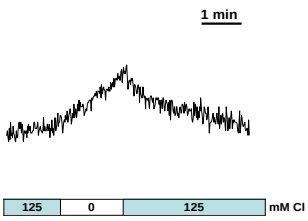
c. Cl^-/HCO_3^- exchange activity in intracellular portion



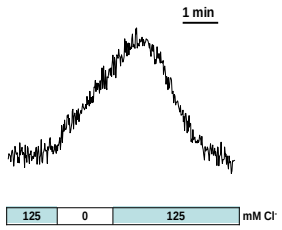
WT



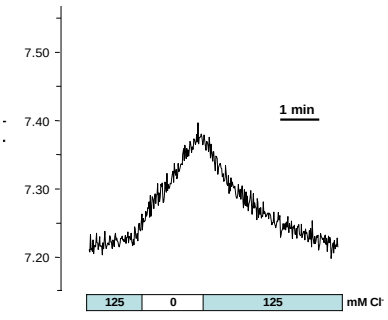
Mock



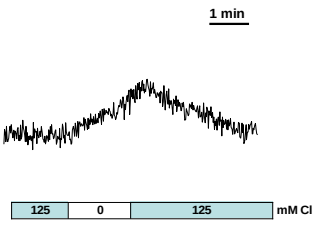
S28R



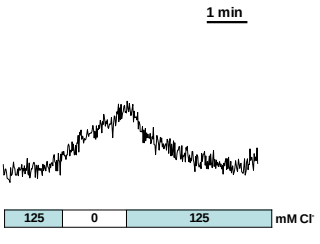
L676Q



H723R



L236P



T416P

d. Average and standard error of wt-pendrin and pendrin mutants.

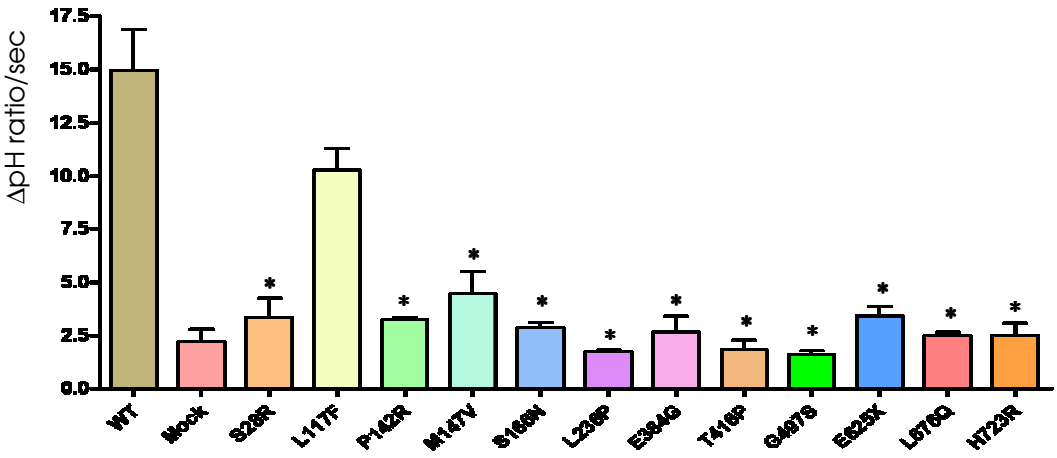


Fig. 2. Cl^-/HCO_3^- exchange activity according to pendrin mutants in HEK293 cells.

a~c. After expressing wt-pendrin and pendrin mutants in HEK293 cells, intracellular pH, which changes by Cl^-/HCO_3^- exchange activity, was measured using BCECF fluorescent material. d, ΔpH ratio/sec average and standard error of wt-pendrin and pendrin mutants. (*$p<0.05$) Pendrin - mediated Cl^-/HCO_3^- exchange activities showed a marked decreased in eight mutants identified in Korean patients regardless of their location (extracellular, plasma membrane, intracellar) Three common mutants reported in Caucasian patients also demonstrated decreased activities. The L117F mutant which was reported in Caucasians, demonstrated about a 20% decreased in activity compared to WT, but was statistically insignificant.

2. Immunoblotting of wt-pendrin and pendrin mutants

Cell lysates from HEK293 cells transfected with WT or each mutant Myc-Pendrin were suspended in SDS sample buffer and separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membranes and probed with the monoclonal myc- monoclonal antibody.

After being treated with anti-mouse goat IgG secondary antibody, protein bands were visualized using an enhanced chemiluminescence kit.

WT showed two bands in immunoblotting. The S166N and L117F mutants showed two bands in immunoblotting similar to that of the WT.

The E625X mutant was not detected via immunoblotting.

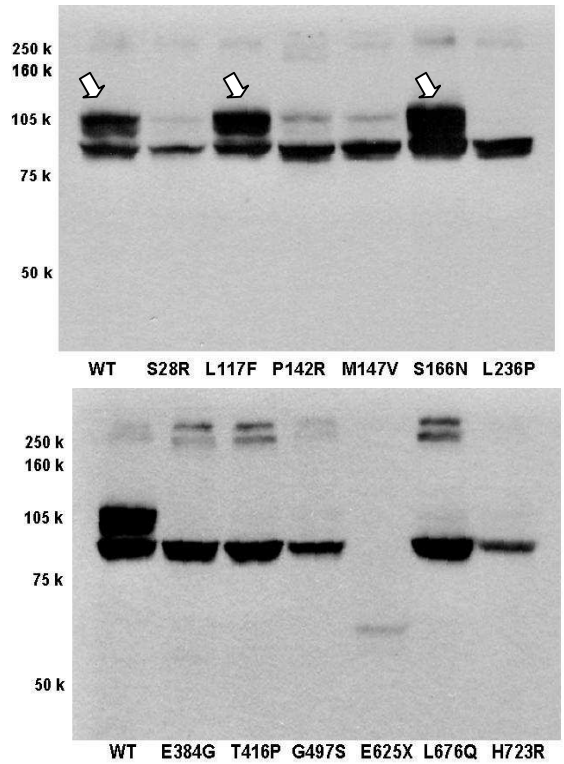


Fig. 3. WT-pendrin and pendrin mutants expression in HEK293 cells.
After wt-pendrin and pendrin mutants attached to a Myc tag were expressed in HEK293 cells, pendrin expression was observed using a Myc-specific antibody. The E625X mutant was not detected via immunoblotting. The S166N and L117F mutants showed two bands in immunoblotting similar to that of the wt-pendrin (indicated by arrow).

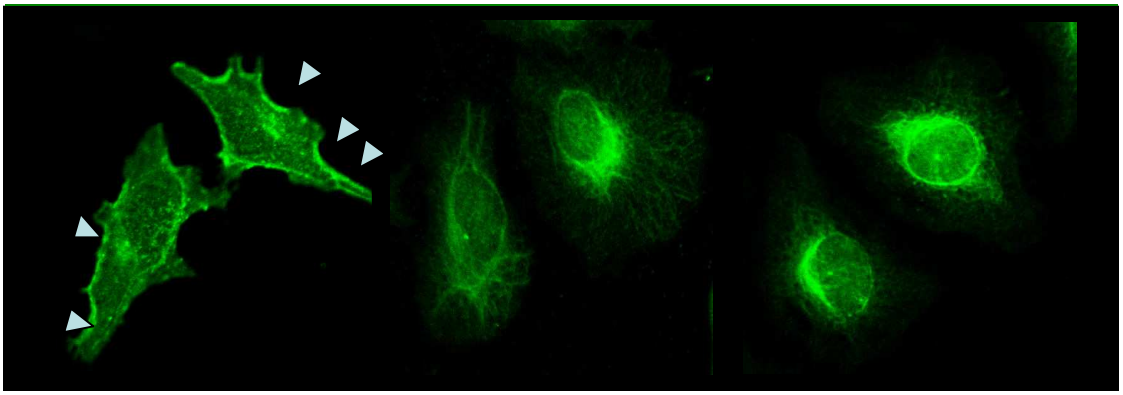
3. Cellular localization of wt-pendrin and pendrin mutants

To assess the effect of mutations on membrane targeting, HeLa cells were transfected with WT or each Myc-pendrin mutant. Myc specific antibody-tagged WT and pendrin mutants were applied and observed for localization using immnofluorescence.

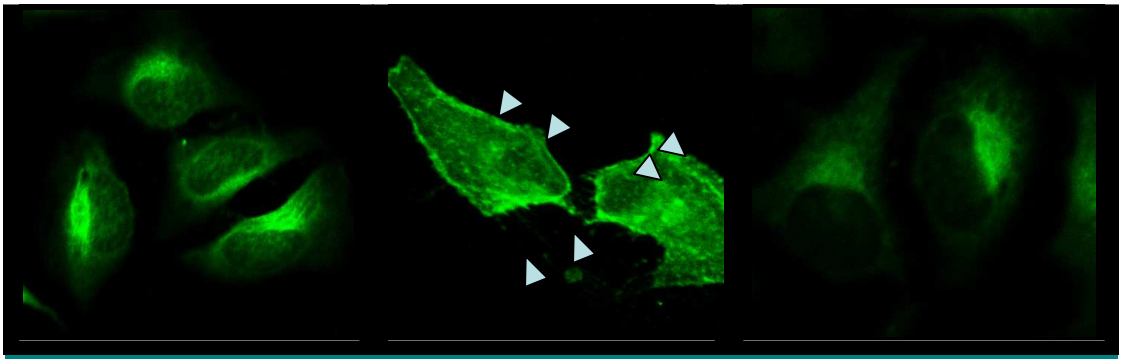
WT was clearly present at the cell membrane. The L117F and S166N mutants showed a cell membrane protein distribution similar to that of WT.

All mutants except L117F and S166N failed to express at the cell membrane and appeared to be localized at the cytoplasm.

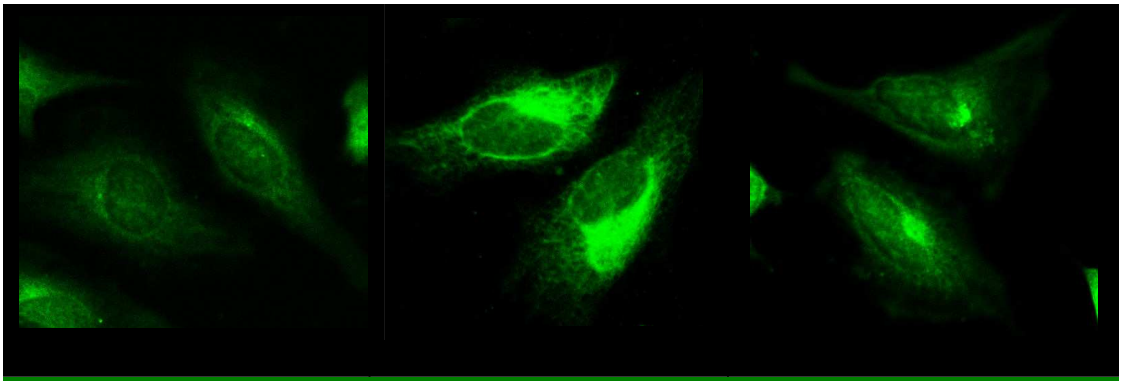
a. Wild type b. S28R c. P142R



d. M147V e. S166N f. G497S



g. E625X h. L676Q i. H723R



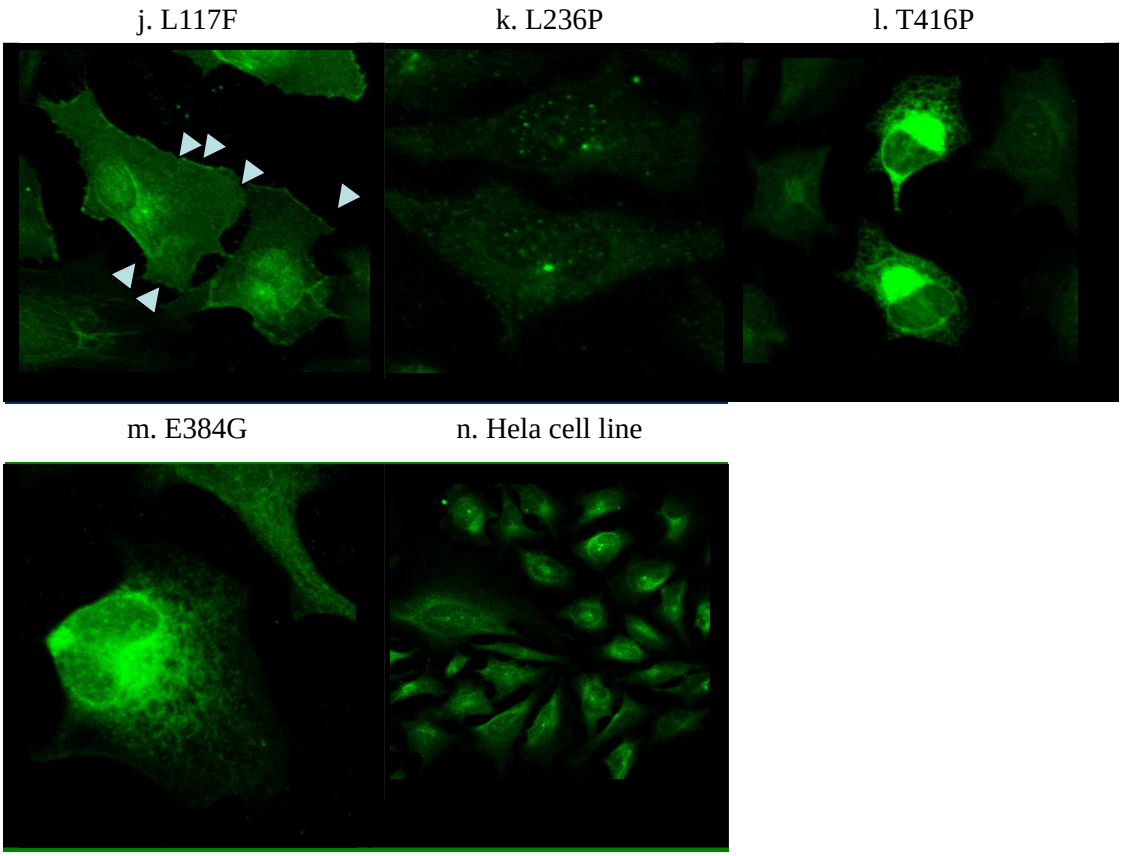


Fig. 4. Intracellular expression of wt-pendrin and pendrin mutants in HeLa cells.
After HeLa cell transfection, 1st Ab and 2nd Ab reactions were done and confocal microscope imaging showed localization differences between wt-pendrin and pendrin mutants.
n.: control , Non-transfected HeLa cells
WT was clearly present at the cell membrane (arrow denotes plasma membrane expression).The L117F and S166N mutants showed a cell membrane protein distribution similar to that of WT.The remaining mutants failed to express at the cell membrane and appeared to be localized at the cytoplasm.

4. Genotype – phenotype correlation

To investigate the association between the different pendrin mutants and clinical manifestations, this study included sixteen patients with the conditions as detailed in Table 4.

The hearing level described in the table below is at the time of detection.

This investigation focused on hearing level and found variations especially at the onset of severe degree hearing loss (Data are not shown).

Sixteen patients had a severe to profound hearing impairment, which indicated that pendrin mutation is related to hearing impairment.

Table 4. Phenotype associated with each of the eight mutants

Pat No.	Sex	Age	Genotype	Hearing level(dB)*		Remark
				Rt.	Lt.	
1	F	24	H723R/WT	90	60	Goiter
2	F	6	H723R/H723R	75	90	
3	M	5	H723R/WT	65	65	
4	F	5	IVS7-2A>G/H723R	70	70	
5	M	6	E625X/H723R	80	80	
6	F	4	H723R/H723R	90	90	
7	F	12	H723R/H723R	80	80	
8	F	10	H723R/T410M	80	70	
9	F	1 1/12	H723R/WT	90	90	
10	M	1 5/12	H723R/H723R	90	90	
11	F	3	H723R/H723R	90	90	
12	F	3	IVS7-2A>G/H723R	50	55	
13	M	3	H723R/H723R	90	90	
14	F	34	L676Q/L676Q	90	90	
15	M	41	IVS7-2A>G/H723R	90	90	Bilateral vestibular dysfunction
16	M	18	S166N/IVS4+4A>G	90	90	

*Pure-tone averages (dB HL) at 500 Hz, 1kHz, and 2kHz.

IV. DISCUSSION

SLC26A4 is a member of the SLC26 gene family, Over the past decade, 11 human genes belonging to the solute linked carrier 26(SLC26) family of transporters, have been identified. Special interest has been focused on four members of the SLC26 family that are associated with the distinct recessive diseases which are Chondrodysplasias (*SLC26A2*), congenital chloride diarrhea (*SLC26A3*), Pendred syndrome and non-syndromic deafness (*SLC26A4*) and non-syndromic hearing impairment (*SLC26A5*).[13,14,21]

The SLC26 proteins, which include SAT-1, DRA/CLD, pendrin, prestin, PAT-1/CFEX and Tat-1, are structurally related and have been shown to transport one or more of the following substrates: sulfate, chloride, bicarbonate, iodide, oxalate, formate, hydroxyl or fructose. Because *SLC26A4* has a similar base sequence to a sulfate transporter (sulfate transporter and anti-sigma antagonist domain : STAS domain), *SLC26A4* was thought to have some relations to a sulfate transporter, but several electrophysiologic studies showed that it is not associated with sulfate transportation and does not transport SO_4^{2-} or oxalate compared to other SLC26 members. *SLC26A4* was proved to have some important functions in the development of the musculoskeletal system, synthesis of thyroid hormone and secretion of bicarbonate in digital glomerulus or pancreas secreting cells.[13,14,21]

This study, the first of its kind in Korea, evaluated that the Cl^-/HCO_3^- exchange activities and membrane expression patterns of eight frequent mutants (S28R, P142R, M147V, S166N, G497S, E625X, L676Q and H723R) in Korean patients with genetic hearing impairment, and four relatively common mutants (L117F, L236P, E384G & T416P) in Caucasian patients.

In this study, eight artificially mutated genes with substitutions had exonal mutations that were reported to be present in sixteen mutated genes.

Among these mutants, H723R is the most commonly found mutant in Koreans, and it is also frequently found in Japanese patients. The functional study of gene mutations including H723R is important. Mutants in this cohort accounted for 60 percents of the all *SLC26A4* mutations found in Korean patients with genetic hearing impairment.[9]

All of the mutants reported in Koreans except S166N were conserved in studies comparing Rat (Genbank NP035997) and Mouse (Genbank NP062087), which indicate that those regions have important functions.[22]

Based on topological structures, H723R (2469A>G) and L676Q (2027T>A) as shown in Fig.1 are located on the sulfate transporter and anti-sigma antagonist (STAS) domain. These domains perform an important function in the SLC family and different results were expected from these two mutants compared to other mutants in this cohort.

However, Cl^-/HCO_3^- exchange function of the mutants reported in Koreans were markedly decreased compared to WT. Therefore, it was impossible to detect functional differences of mutants which were located at extracellular, plasma membrane and intracellular portions by measuring Cl^-/HCO_3^- exchange functions.

This study revealed that Cl^-/HCO_3^- ion transport function was almost lost in all mutants in this cohort except L117F. All mutants were determined to be related to deafness. Therefore, this study suggests that Cl^-/HCO_3^- ion transport function is very important in the hearing mechanism. After all, change in ion transport due to pendrin mutation makes it impossible to maintain homeostasis in the inner ear, which results in profound hearing impairment.

The L117F mutant, which had been reported by Reaedon et al., performed similarly to wt- pendrin in this Cl^-/HCO_3^- exchange function assay.[23] The Cl^-/HCO_3^- ion transport function of L117F was decreased 20 percent compared to WT; however, the decrease in activity was statistically insignificant.

The L117F mutant was selected in this study to compare other mutants reported in Koreans. According to other earlier studies that measured iodide transport functions, L117F, which was different from L236P, E384G and T416P, commonly reported in Caucasian patients, was reported to have nearly the same function as WT, and did localize to the cellular membrane.[24]

It was shown that homozygous PDS knockout mice have a marked loss of hearing without thyroid manifestation, suggesting that the ear may be more sensitive to pendrin loss, and raising the possibility of an alternative iodine transporter in the murine thyroid gland.[25]

Further investigation is necessary to understand the correlation of L117F between normal ion transport function and hearing impairment.

It may be allowed that the designation of mutations in the same allele with additional nucleotide substitutions.

In order to study whether defective membrane trafficking was involved in the mechanism of Cl^-/HCO_3^- ion transport function, we used Myc–specific antibody-tagged HeLa cells to investigate the intracellular distribution and trafficking of WT and twelve mutants.

As demonstrated by transient expression in HeLa cells , appropriate trafficking to the plasma membrane was observed for only two mutants (L117F and S166N).

These data suggest that the main cause of the defective Cl^-/HCO_3^- anion exchange activity of pendrin is the defective pendrin. Defective pendrin is targeted to the plasma membrane.

Prior studies showed the expression of pendrin mutants in cellular membrane by immunofluorescent staining WT is targeted to the cellular membrane while L236P was retained in the endoplasmic reticulum (ER).[26]

The possible mechanism of this result could be due to improper protein folding or elimination of an essential targeting signal causing immobilization and aggregation of the mutant protein in the ER membranes.[22] Furthermore, previous studies have been shown that L236P could not prevent the movement of normal pendrin when L236P and WT are co-expressed, which supports the mechanism of hereditary pattern of Pendrin is recessive.[21]

In this confocal study, L236 was compared with other mutants identified in Korean patients and observed proteins around the nucleus, which is similar to that of other reports.[26,27]

Proteins that remain in the ER and cannot be expressed are also found in cystic fibrosis transmembrane conductance protein (CFTR),[27] Nehls used the chemical chaperone like dimethylsulfoxide, trimethylamine-N-oxide to express proteins in the cellular membranes that remain in the ER. It was experimentally proven that pendrin function improved partially, suggesting that this approach to medical treatment was worth while.[28]

The S166N, an amino acid substitution of asparagines to serine in the same amino group, was the mutant initially identified in Korean patients.[9]

This is the only form that was not conserved, when compared to Rat and Mouse among the eight mutants identified in Korean patients. S166N is the only mutant out of the eight that can be expressed in the cellular membrane. S166N also decreased Cl^- /HCO_3^- exchange function.

With these results, there may be other mechanisms that can be used to determine ion transportation. The expression of the gene on the cellular membrane, and the location in pendrin may have a specific effect on Cl^-/HCO_3^- ion transport.

Within this cohort, only one individual was associated with the S166N mutant. The patient was 18 year old male with S166N and IVS4+4A>G, a compound heterozygote, who had an average auditory capacity of 90dB HL at diagnosis, but low frequency capacities of 250Hz and 500Hz were conserved at around 60dB HL. This patient was rehabilitating through the use of hearing aids. His auditory capacity was favorable compared to the other genetic mutations, and did not exhibit any thyroid pathology ; this patient was a non-syndromic type.

The G209V mutant had similar results with S166N in other previous studies and the function of iodine transportation was decreased compared to normal persons, but was still functioning.[24] G209V was also expressed on the cellular membrane,.

Furthermore, an individual with G209V was a compound heterozygote and had profound hearing impairment but no goiter; this patient was a non-syndromic type.[24]

Therefore, further investigation is necessary to understand the clinical features based on genotype by measuring iodine transportation function in S166N and ion transportation function in G209V mutants.

There have been many studies about mutual interactions between genotype and phenotype and it was reported that the goiter which can be found in the patients with the *SLC26A4* mutation, is rarely found in Koreans.[9]

Diverse thyroid phenotypes have been studied and are considered to be caused by environmental factors such as thyroid hormone intake, difference in iodide intake, and genetic factors.[7]

Scott et al. suggested that the degree of genetic functional loss and phenotype are correlated. Based our results, the ion transportation function is preserved in non-

syndromic deafness compared to Pendred syndrome, in which ion transportation ability is lost. It was measured using Xenopus oocyte.[29]

On the other hand, Lopez studied the genotypes of family members diagnosed with Pendred syndrome in which members have different hearing levels. However, he cannot explain the phenotype differences within the family.[30]

A study carried out by Wu. found that the pendrin mutation was present in 78% of 25 families who had a mondini deformity or an enlarged vestibular aqueduct. Furthermore, he did not find the pendrin mutation in ten other families with different inner ear deformities.[31]

Considering these studies, there may be other existing mechanisms in the development of inner ear deformity other than the traditional idea of the developmental arrest.

After korean pendrin mutants were reported in 2004 by Park et al. [9], the clinical features of those patients were recorded. This study tried to determine the correlations between the phenotypes of sixteen patients who carried eight different mutants in this cohort and genotypes at the cellular level.

Most of the sixteen patients had severe to profound hearing impairments, which indicates that the pendrin mutation is related to hearing impairment.

However, there were some limitations to this study, which suggest the need for more research.

First, all mutants with decreased Cl^-/HCO_3^- ion transport function did not show any differences from each other.

Second, among the different mutants, only H723R and L676Q have homozygotes that can be compared using allele-specific assays.

Third, the perchlorate discharge test, the most important test known to distinguish syndromic type from non-syndromic type, was not done for most patients. Therefore, abnormal function of the thyroid cannot be fully evaluated.

Fourth, the degree of hearing impairment was described at the time of detection, and the hearing levels continuously decreases with time. Therefore, it is irrational to associate the degree of deafness to cellular transportation function.

For these reasons, it is necessary to detect, diagnosis, and follow up on patients earlier, so that the features of phenotypes can be thoroughly elucidated and compared to the variable genotypes.

However, the H723R mutant, which makes up 40 percent of pendrin mutations, has a great number in comparison to the phenotypes and genotypes of six homozygote patients. The times of origination and discovery of deafness were analyzed. The originating time of a profound hearing impairment showed different patterns resulting in diverse phenotypes. Although it has the same genotype, it was impossible to analyze phenotype differences using ion transport function and intracellular expression.

The majority cause non-functional products which confirms that the same primary genetic defect may be associated with different phenotypes and supports the fact that other genetic and environmental factors play a fundamental role in determining the phenotype of pendrin mutants.

V. CONCLUSION

This study examined the mechanism of pendrin-mediated ion transport function with a spectrum of genetic hearing impaired patients.

To identify the underlying mechanism of defective pendrin function, Cl^-/HCO_3^- exchange activities according to wt-pendrin and pendrin mutants using HEK293 cells were investigated for the first time in Korea.

In order to study whether defective membrane trafficking was involved in the mechanism of Cl^-/HCO_3^- ion transport function, we used Myc–specific antibody-tagged HeLa cells to investigate the intracellular distribution and trafficking of WT and twelve pendrin mutants.

This study concluded the following:

1. Decreased Cl^-/HCO_3^- exchange activity caused by pendrin mutation has an important role in hearing mechanism.

2. Most of the mutants, which were not seen on the cell membrane, showed a markedly decreased their anion exchange activity.

3. Considering the confocal microscopy imaging results, the main cause of the defective Cl^-/HCO_3^- anion exchange activity of pendrin is the defective pendrin. Defective pendrin is targeted to the plasma membrane.

4. The S166N showed decreased Cl^-/HCO_3^- exchange activity even with normal expression on the cell membrane. This results suggested another mechanism related to anion activity function of pendrin. The mechanism for S166N should be investigated further.

In this study, it was observed that the pendrin mutants identified in Korean patients with genetic hearing impairment did not move to the cell membrane but rather were

retained in the cytoplasm. These results can cause decreased ion transporting activities.

Furthermore, the correcting methods of pendrin mutants expression in the cell membrane can be used to restore ion transport functionality. Thus, further investigations are needed to explore the clinical significance of this study.

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국문요약

한국인 유전성 난청환자에서 발견된 *SLC26A4* 유전자 변이의 세포 내 이온 수송기능

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유 신 영

*SLC26A4*의 유전자 변이는 유전성 난청을 일으키는 것으로 알려져 있으며 이 유전자는 상염색체 7번에 위치하고 있다. *SLC26A4* 에 의해 생성 된 단백질인 pendrin은 세포막 단백질로서 내이와 갑상선, 신장, 태반등의 장기에 분포하고 있다.

Pendrin은 이온 수송기능을 담당하고 있으며 주로 chrolide, bicarbonate, formate 그리고 iodide의 운반을 담당하고 있다.

Pendrin은 내이에서 stria vascularis내에 위치하며, pendrin에 의한 Cl^- /HCO_3^-이온 수송기능 청각에 중요한 역할을 수행하는 endocochlear potential을 유지하는 데 있어 매우 중요한 것으로 알려져 있다.

지금까지 전 세계적으로 90여가지의 pendrin 유전자 변이가 보고되었으며 한국에서도 9가지의 novel 유전자 변이를 포함한 16가지의 유전자 변이가 보고되었다.

본 연구는 한국인에게서 발견 된 8가지의 *SLC26A4* 유전자 변이(S28R, P142R, M147V, S166N, G497S, E625X, L676Q, H723R) 를 대상으로

이들의 Cl^-/HCO_3^- 이온 수송기능을 측정하고 세포막에서의 발현 여부를 알아보았다.

또한 서양에서 흔하게 보고 된 *SLC26A4* 유전자 변이 (L117F, L236P, E384G, T416P) 들도 같은 방법으로 측정하여 국내에서 발견 된 유전자 변이와 비교해 보았다.

HEK 293 cell을 이용하여 Cl^-/HCO_3^- 교환에 따른 세포 내로의 이온 수송기능은 L117F을 제외하고는 측정한 모든 유전자 변이에서 정상에 비해 현격하게 저하되어 있었다.

L117F은 정상 유전자에 비해 약간 감소된 이온 수송기능을 보였으나 통계적으로 유의하지 않았다.

또한, 정상 pendrin은 Immunoblotting상에서 두 개의 Band를 나타내었으며 이와 유사한 결과를 보인 것은 대상 유전자 변이 중 S166N과 L117F였다.

Hela cell에서 myc 특이 항체를 이용하여 시행한 면역 형광 염색에서 Immunoblotting상에서 두 개의 Band를 나타내었던 S166N과 L117F만이 정상 유전자와 같이 세포막에 발현하였으며, 그 외의 대상 유전자 변이는 세포막에 발현이 미미하며 세포질에 머무르고 있는 것으로 나타났다.

이것으로 pendrin 에 의한 Cl^-/HCO_3^- 이온 수송은 난청에 있어 중요한 역할을 담당하고 있음을 알 수 있었으며, 유전자 변이들의 Cl^-/HCO_3^- 이온 수송기능의 저하는 세포막으로 이동되지 못하는 것이 주된 원인임을 알 수 있었다.

핵심되는말 ; *SLC26A4*, 유전자변이 , 유전성 난청, 기능 연구