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Functional analysis on mutations in POU3F4 in DFNX2 patients

DFNX2 형 난청 환자의 POU3F4 유전자 돌연변이에 의한 단백질 기능 변화에 대한 연구

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Functional analysis on mutations in POU3F4 in DFNX2 patients

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Studies on effect of mutations in POU3F4 found in DFNX2 patients

by
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ABSTRACT

Introduction: Most X-linked nonsyndromic hearing loss is caused by various types of mutations of the POU domain class 3 transcription factor 4 (POU3F4) gene. Therefore, how various mutations of POU3F4 lead to hearing loss needs to be investigated.

Methods: For POU3F4 mutations found in DFNX2 patients, required constructs for further assays were cloned. RT-PCR and polysome fraction analysis were carried out for testing the expression of POU3F4 at RNA level. Western blot and immunofluorescence staining were performed for confirming protein level and localization, and finally luciferase assay was carried out for testing activity of protein.

Results: Five unique missense or frameshift truncation and extension mutations were found in Korean patients and the information was given. Two missense mutations (p.Thr211Met and p.Gln229Arg) disturbed transcriptional activity. Two frameshift extension mutations (p.Thr354GlnfsX115 and p.X362ArgextX113) were located outside of POU domain and nuclear localization signal at the C-terminus. POU3F4 protein

levels were low and could be restored by MG132, a proteasome inhibitor in vitro. These mutant POU3F4 proteins were exclusively localized to the cytoplasm and did not have transcriptional activity. Frameshift mutation (p.Leu317PhefsX12) in POU3F4 leads to the truncation of the C-terminal 44 amino acids spanning the POU domain and nuclear localization signal. This frameshift truncation mutant protein was located in both the nucleus and cytoplasm and was present at a low protein levels. This mutant was also transcriptionally inactive, even in presence of MG132.

Conclusions: From these results, I conclude that frameshift truncation and extension mutations in the C-terminus of POU3F4 lead to cytoplasmic localization and subsequent proteosomal degradation due to structural aberrations, which cause transcriptional inactivity and thus nonsyndromic hearing loss.

Keywords: Nonsyndromic hearing loss, POU3F4, frameshift mutation, proteasome, subcellular localization

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CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

X-linked nonsyndromic hearing loss is known to be responsible for about 2% of congenital deafness. DFNX2 (OMIM# 304400), also known as DFN3, is one of them whose causative gene is identified. Clinical feature of DFNX2 is characterized by conductive hearing loss, sensorineural hearing loss, bone deformity near meatus and cochlea, and etc (de Kok et al., 1995 and Song et al., 2010). In 1988 Brunner and his colleagues pointed out the gene underlying DFNX2, which was Xq21, by linkage analysis.(Brunner, et al., 1988). Later, it was identified that POU domain class 3 transcription factor 4 (POU3F4) transcription factor (OMIM# 300039) was the causative gene (de Kok, et al. 1995). Although nonsense and missense mutations of POU3F4 were the first to be identified (de Kok, et al. 1995)., not only mutation inside amino acid coding sequence of POU3F4, but also association of microdeletion upstream of POU3F4 with DFNX2 was reported (Hildebrand, et al., 2007). As a transcription factor, DNA sequence CAATATGCTAAT, to which POU3F4 binds is well known. This sequence locates in promoter region of POU3F4 and is related to autoregulation (Malik, et al., 1996).

Expression of Pou3f4 is detected from 10.5 days post coitus (dpc) in the periotic mesenchyme ventral to the otic vesicle (Phippard, et al., 1998). Expression of Pou3f4 continues to 18.5dpc and this stage is critical for morphogenesis of inner ear. Therefore, this expression pattern of Pou3f4 implies that Pou3f4 is important for development of ear and also its function (Trowe, et al., 2008). Brn4(Pou3f4)-deficient mice had no gross morphological change, but exhibit profound deafness with a dramatic reduction in endocochlear potential, alteration of cochlear spiral ligament fibrocytes (Minowa, et al., 1999), and a loss of Kir4.1 in the stria vascularis (Song, et al., 2011).

There are various kinds of disease-causing mutation for mendelian disorder. Missense mutation of coding sequence that results in nonsynonymous amino acid change and nonsense mutation that results in premature termination of translation are two common scenarios. However, even a simple protein can be comprised of several functional domains, specific position of mutations can be related to different mechanism of diseases. For example, missense mutation can cause structural change, which disrupt original role such as transcriptional activity during development (Bitner-Glindzicz, et al., 1995; de Kok, et

al., 1995; Lee, et al., 2009b). In some cases, endoplasmic reticulum stress is induced which might be the cause of retinal degeneration (Datta, et al., 2009). Nonsense-mediated decay(NMD) is a well-known mechanism which removes mRNA with nonsense mutation in the N-terminus to keep cell from producing truncated or errorneous proteins (Hentze and Kulozik, 1999). However, currently it is largely unknown how C-terminal frameshift extension mutation affects function of a gene or through which mechanism it causes the cause of a Mendelian disorder. Comprehensive understanding of C-terminal frameshift extension mutation may lead us to novel translational and post-translational control mechanism.

To date, most pathogenic mutations of POU3F4 are located in the POU-specific domain or POU homeodomain (Reardon, et al., 1991; de Kok, et al., 1995). Here, I report functional analysis on five novel mutations of POU3F4, including C-terminal frameshift extension mutations. Patient recruitment, mutation analysis and description of clinical features including radiologic features were done by Choi et al. Based on the genetic findings I revealed that although frameshift extension mutations are transcribed like wildtype, the mutant protein is degraded by the

proteasome. Moreover they were not properly translocated into the nucleus albeit its intact nuclear localization signal. From this result, I identified the molecular mechanism of frameshift extension mutations involved in the initiation of deafness.

MATERIALS AND METHODS

1. Study participants

A total of six Korean families segregating an incomplete partition type III anomaly (Fig. 1A) were ascertained and evaluated at the SNUBH and SNUH. Clinical phenotype evaluations included medical and developmental history interviews, physical examinations, pure tone audiometry, and imaging studies (temporal bone computed tomography and/or magnetic resonance images).

2. Mutation analysis

Bidirectional nucleotide sequence analysis of one exon of POU3F4 (GenBank Accession NM_000307) was performed by Choi et al. POU3F4 exon was amplified and sequenced as previously described (Lee, et al., 2009b). All participating affected probands and their mothers were included for sequence analyses. Mutations were numbered according to NM_000307.3 (c.DNA) and NP_000298.2 (protein). cDNA number +1 corresponds to the A of the ATG translation

initiation codon. Names of all variants were checked using Mutalyzer (http://www.LOVD.nl/mutalyzer/).

Novel splice-site variants, intronic variants, and synonymous changes were considered pathogenic when they were not detected in the normal Korean population. Names of all variants were checked as described above. BDGP (http://www.fruitfly.org/seq_tools/splice.html) and ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) software predicted changes that create or eliminate a splice-acceptor or -donor site. Novel variants that were found were submitted to the LSDB (http://grenada.lumc.nl/LOVD2/MR/submitters_variants.php?order=&hide_col=&show_col=&genes%5B%5D=POU3F4&submitterid=00045&submitter_select=00045).

3. Plasmids and transfection

pGL3-basic and pRL-SV40 plasmids for luciferase assay were purchased from Promega (Madison, WI), and pGL3-basic-3x-copy plasmid (pGL3-basic vector containing three copies of POU3F4-binding sites, CAATATGCTAAT) was kindly

provided by Dr. Jinwoong Bok (Lee, et al., 2009b). Sequences for wild-type POU3F4 as well as five mutant types of POU3F4 were HA-tagged and inserted into the pcDNA3 vector (Invitrogen, Grand Island, NY). Transient transfection was performed with various plasmids using X-tremeGENE HP (Roche, Manheim, Germany) according to the manufacturer's protocol.

4. Cell culture

293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 1% penicillin-streptomycin under 5% CO2 at 37°C.

5. RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) or TRIzol reagent (Invitrogen). After digesting possibly contaminated genomic DNA by recombinant DNase I (Roche), 0.5 to 1 ug RNA was reverse transcribed using a ReverseTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The forward and reverse primers for POU3F4 were 5'-

ATCATCCATCACCGCTCGCCACA-3' and 5'-GGTTGGCCGCTTGACGTGATAGAC -3', respectively.

6. Western blot

Cells were washed twice with phosphate-buffered saline (PBS) and solubilized in RIPA buffer [15 mM Tris-Cl (pH 7.4), 15 mM MgCl2, 300 mM NaCl, 1% Triton X-100] containing 1 mM phenylmethylsulfonyl fluoride. Whole-cell lysates were centrifuged for 20 min at 12,000 × g at 4°C, and protein concentrations of the supernatants were determined using the BCA protein assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and immunoblotted with anti-HA (Roche) and anti-actin (Santa Cruz Biotechnology Inc, Santa Cruz, CA) antibodies. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (BioRad, Hercules, CA) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) were used for visualization.

7. Luciferase assay

Firefly and Renilla luciferase activity were measured using a Dual Luciferase Assay Kit (Promega) and a Tecan Infinite M200 plate reader. To assess capability as a transcription factor, ratios of luciferase activity between pGL3-basic transfected cells and pGL3-basic-3x-copy were calculated. Renilla luciferase was used as an internal control of transfection efficiency.

8. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After blocking with PBS containing 2% bovine serum albumin for 30 min, cells were incubated first with anti-HA antibody (Roche) and then with Alexa Fluor® 488-labeled anti-mouse antibody (Invitrogen) at room temperature for 1 h and 30 min, respectively. All antibodies were diluted before incubation according to manufacturer's recommendations. Nuclei were counterstained with 4,6-diamidino-2-phenylindole.

9. Polysome fraction isolation

Cells were treated with 100 μg/ml cycloheximide for 5 min 24 h post-transfection. After briefly rinsing with ice-cold PBS containing 100 μg/ml cycloheximide three times, cells were lysed in lysis buffer [15 mM Tris-Cl (pH 7.4), 15 mM MgCl2, 300 mM NaCl, 1% Triton X-100, 100 μg/ml cycloheximide, 1 mg/ml heparin, and 200 U RNasin] and incubated on ice for 10 min. Lysates were transferred to 1.5-ml tubes and centrifuged for 10 min at 12,000 × g to remove nuclei and debris. Supernatants were layered onto a 10%-50% sucrose gradient and fractionated by ultracentrifugation in a Beckman ultracentrifuge using an MLA-130 rotor. Each fraction was collected from the top for further RT-PCR analysis.

RESULTS

Detection of novel mutations in patients

All six Korean families segregating an incomplete partition type III inner ear anomaly (Fig. 1A) in an X-linked recessive manner (Fig. 1B) had a mutation in a coding region of POU3F4. Previously reported p.Leu208X mutation of POU3F4 was found in one family (SB13), whereas the other five probable pathogenic variants were novel: these comprised two frameshift mutations (p.Thr354GlnfsX115 [SB7], p.Leu317PhefsX12 [SB8]), two missensemutations (p.Gln229Arg [SB2], p.Thr211Met [SB9]), and one nonstop extension mutation (p.X362ArgextX113 [SB11]) (Fig. 2A, Table 1). SB7 and SB11mutants result in a partial loss of the canonical 3′untranslated region (UTR) in POU3F4 (Fig. 2A). All of the novel variants were not identified in 100 ethnically matched control subjects (Table 1). I also checked 1000 genome and National Heart, Lung, and Blood Institute exome sequencing data to further confirm that these alleles are not detected in normal controls. Most of the probands manifested profound hearing loss and have had cochlear implantation, with the exception of SB8 and SB9, who showed severe and moderate

degrees of hearing loss, respectively. There did not seem to be any genotype-phenotype correlation given that themilder degree of hearing loss observed for SB8 and SB9 was not limited to a particular mutation of POU3F4 (Table 1).

Expression of missense and frameshift mutants

To reveal whether the mutations identified in POU3F4 alter its expression, I first checked the expression of POU3F4 at the RNA level. When they were transfected into HEK-293T cells, all five mutants showed comparable expression levels to wild type (Fig. 2B). Despite their similar degrees of expression at the RNA level, expression measured at the protein level was variable. I transfected cells with equal amounts of plasmids and performed Western blotting after 36 hr (Fig. 2B). In the absence of MG132, only the expression of SB2 and SB9 was similar to that of wild-type POU3F4. The amount of SB8 mutant protein increased to a similar level of wild type after treatment with MG132 (Fig. 2B), whereas SB7 and SB11 mutants remained at a lower level despite a substantial increase of proteins. Because the 3′UTR is highly important for regulation of translation, polysomal fraction analysis was

carried out to evaluate the effects of extended translation of the 3UTR, the consequence of a stop codon mutation identified in SB7 and SB11. I hypothesized that the two stop codon mutations might affect the initiation of translation. Their mRNAs might exist predominantly in a nonpolysomal fraction. However, for both mutant alleles, the majority of the mRNA was found in the polysomal fraction, which is similar to the patterns of SB2, SB8, SB9, and wild-type mRNA (Fig. 2C). These results suggest that the three C-terminal frameshift truncation and extension mutations (SB7, SB8, and SB11) identified in POU3F4 do not significantly alter either transcription of mRNA or protein translation initiation, but do alter the stability of the synthesized protein.

Intracellular localization of POU3F4

To determine whether any of the pathogenic effects of mutations in this study are due to abnormal subcellular localization, I examined the subcellular localization of each mutant protein. Immunohistochemical staining revealed that the five mutants could be categorized into three groups with respect to subcellular localization (Fig. 3A). First, two

missense mutants of POU3F4 (SB2 and SB9) properly localized to the nucleus as did wild-type POU3F4 protein. In contrast, SB7 and SB11 failed to localize to the nucleus. The majority of the POU3F4 protein was located in the cytoplasm. Finally, the SB8 allele, which has a C-terminal 34-amino acid truncation, was present in both the nucleus and cytoplasm, although the proportion of nuclear localization of this mutant allele was still significantly lower than that of wild type ($P = 0.0004$ according to Student's t-test; Fig. 3B)

Transcriptional activity of mutants

Because two missense POU3F4 mutants (SB2 and SB9) were localized in the nucleus, I determined whether SB2 and SB9 retained their ability as transcription factors. Using pGL3-Basic-3× copy, a pGL3-basic vector containing three consecutive copies of the POU3F4-binding site as a promoter for the luciferase gene, luciferase assays were performed. Unlike wild-type POU3F4, all five mutants showed significantly reduced luciferase activity similar to that of empty vector-transfected HEK-293T cells (Fig. 4). The transcriptional activities of these mutants did not improve even after MG132

treatment except in SB8, which showed significant improvement after MG132 treatment (P = 0.03, Student's t-test), albeit not to the level of the wild-type POU3F4 transcript (Fig. 4). Together, these data suggest that these mutations interrupt the normal function of POU3F4, primarily by damaging DNA-binding ability and have additional mislocalization effects, coupled not only with damaged nuclear localization but also with instability of the translation products according to mutation type.

Table1. Five novel *POU3F4* sequence variants in Korean families with IP−III anomaly (Choi, et al., 2012)

	SB7	SB8	SB13	SB2	SB9	SB11[d]
Sex/age	M/9	M/12	M/8	M/2	M/16	M/17
Type of mutation	Frameshift extension	Frameshift truncation	Nonsense	Missense	Missense	Extension
Nucleotide variation[a]	c.1069delA	c.950dupT	c.623T>A	c.686A>G	c.632C>T	c.1084T>C
Predicted effect	**p.Thr354Gln*fs*X115**	**p.Leu317Phe*fs*X12**	p.Leu208X	**p.Gln229Arg**	**p.Thr211Met**	**p.X362Arg*ext*X113**
Mw (kDa)	54.0	36.9	23.7	41.3	41.3	54.1
Reference	Novel	Novel	Lee et al.(Lee, et al., 2009a)	Novel	Novel	Novel
Allele frequency[b]	0/120	0/120	Known mutation	0/120	0/120	0/120
Degree of hearing loss	Profound	Severe	Profound	Profound	Moderate	Profound
Rehabilitation	CI[c]	Hearing aid	CI	CI	Hearing aid	CI

[a]Numbering from exon according to GenBank Accession NM_000307.3
Mutations were numbered according to NM_000307.3 (c.DNA) and NP_000298.2 (protein). cDNA number +1 corresponds to the A of the ATG translation initiation codon.
[b]Allele frequency in Korean control population. ; [c]CI: Cochlear implantation; [d]Sporadic case
Novel mutations in bold

(A) (B)

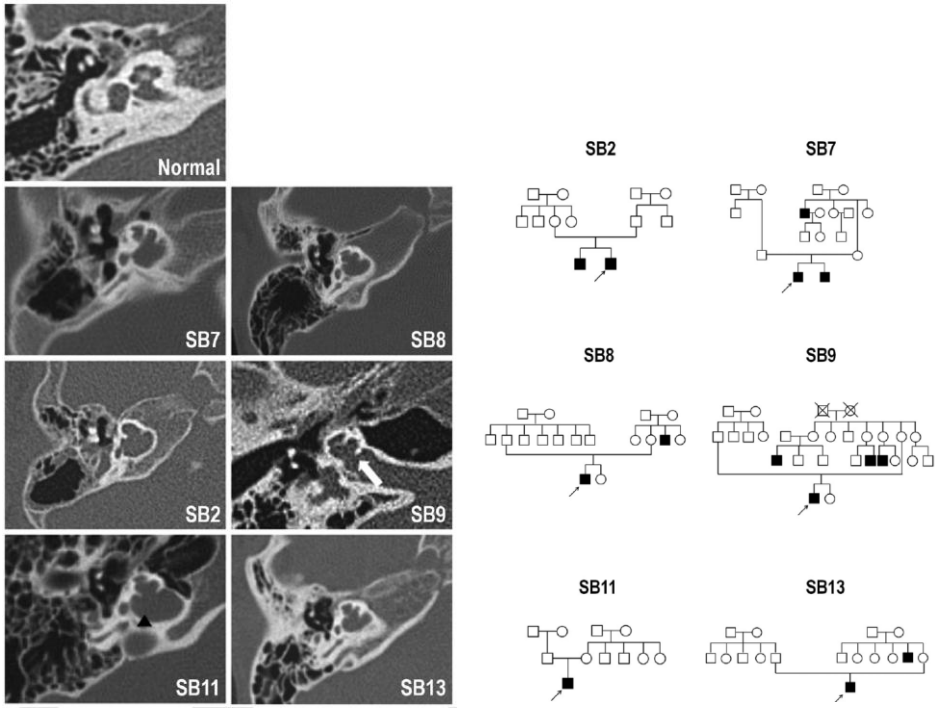
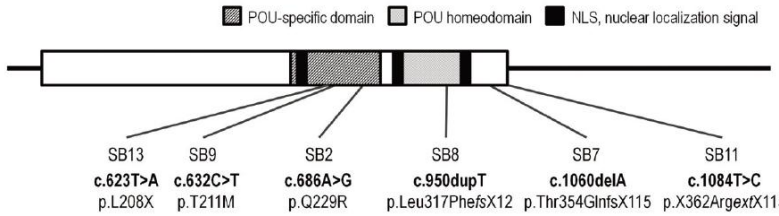


Figure 1. Characteristic inner ear radiological features of POU3F4 mutations and pedigrees. (Choi, et al., 2012)

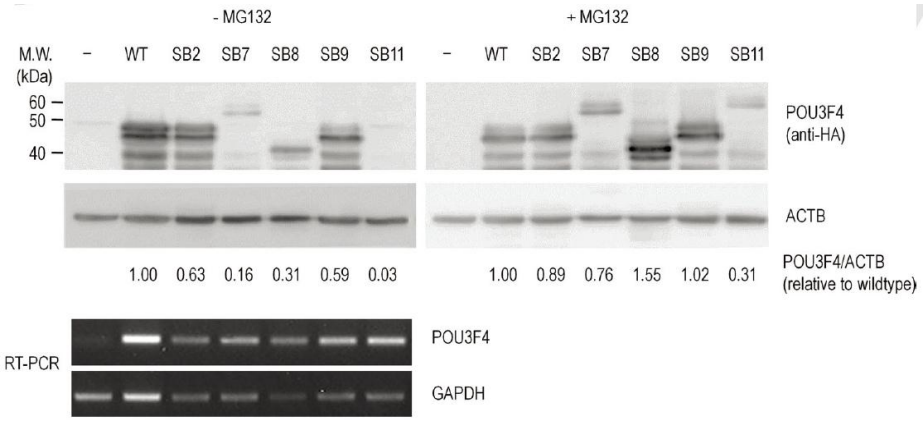
(A) All of the probands from six families showed characteristic inner ear radiologic features compatible with incomplete partition type III. The top figure shows a normal inner ear from controls. Anomalous fusion of the internal auditory canal (IAC) and the basal turn of the cochlea (white arrow) as well as the bulbous dilatation of the fundus of the IAC (black arrow head) are noted. (B) Segregation of deafness and an incomplete partition type III inner ear anomaly in six families is compatible with an X-linked recessive inheritance pattern.

(A)



Mutation	Protein Sequences (201-231....315-362)
Wild type, 39.4kDa	201 231 315 362 FKQRRIKLGFTQADVGLALGTLYGNVFSQTT.....LQLEKEVVRVWFCNRRQKEKRMTPPGDQQPHEVYSHTVKTDTSCHDL
SB2, 39.5kDa p.Q229R	FKQRRIKLGFTQADVGLALGTLYGNVFSRTT.....LQLEKEVVRVWFCNRRQKEKRMTPPGDQQPHEVYSHTVKTDTSCHDL
SB7, 52.1kDa p.Thr354GlnfsX115	FKQRRIKLGFTQADVGLALGTLYGNVFSQTT.....LQLEKEVVRVWFCNRRQKEKRMTPPGDQQPHEVYSHTVKQTHLAMISDWRKRGGGRPH WEQRGFLFLSHSLPFILVFFIIFLSLSFARSLVLSLFSLLSFFFPFPFFFPFFFPFLSFHFLSFPLPFPSLPSLPFLSFLFFCFPFLFFPFLSFS
SB8, 35.1kDa p.Leu317PhefsX12	FKQRRIKLGFTQADVGLALGTLYGNVFSQTT.....LQFGEGSGACLVL
SB9, 39.5kDa p.T211M	FKQRRIKLGFMQADVGLALGTLYGNVFSQTT.....LQLEKEVVRVWFCNRRQKEKRMTPPGDQQPHEVYSHTVKTDTSCHDL
SB11, 52.3kDa p.X362ArgextX113	FKQRRIKLGFTQADVGLALGTLYGNVFSQTT.....LQLEKEVVRVWFCNRRQKEKRMTPPGDQQPHEVYSHTVKTDTSCHDLRLEEARRRPAALG AARISLSLSLSSFHSSILYYFSLSLVRSLSRTLSLFPPFLFLSFPLFLSLLFPFPFLSFSFLSPSLPFPSISSFPFLSFLLLSFPFFPFSFLFIRGSNFC
SB13, 22.3kDa p.L208X	FKQRRIKLX

(B)



(C)

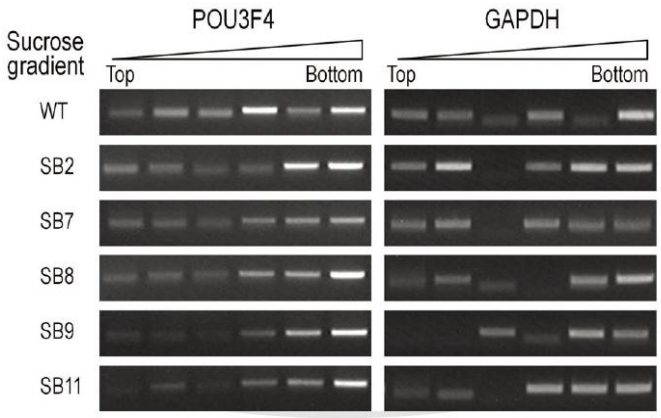


Figure 2. Novel mutations in POU3F4 and in vitro expression of mutants from Korean families with IP-III anomaly.

(A) Two novel pathogenic missense mutations of POU3F4 are located in the POU-specific domain, and one frameshift truncation mutation is in POU homeodomain. Two frameshift extension mutations are located at the C-terminus. Detailed amino acids changes are shown in for each mutant allele. (B) HEK-293T cells were transiently transfected with 1 μg pcDNA3-HA-POU3F4 plasmid. mRNA expression was analyzed by RT-PCR. POU3F4 proteins were detected with anti-HA antibody. Proteins of truncated or extended form are shown as larger or smaller size than wild type (41 kDa), respectively. To inhibit proteosome-mediated protein degradation, cells were treated for 12 hr with 25 μM MG132 24 hr after transfection. (C) POU3F4-transfected HEK-293T cell extracts were fractionated by sucrose density gradient. Each fraction was analyzed by RT-PCR

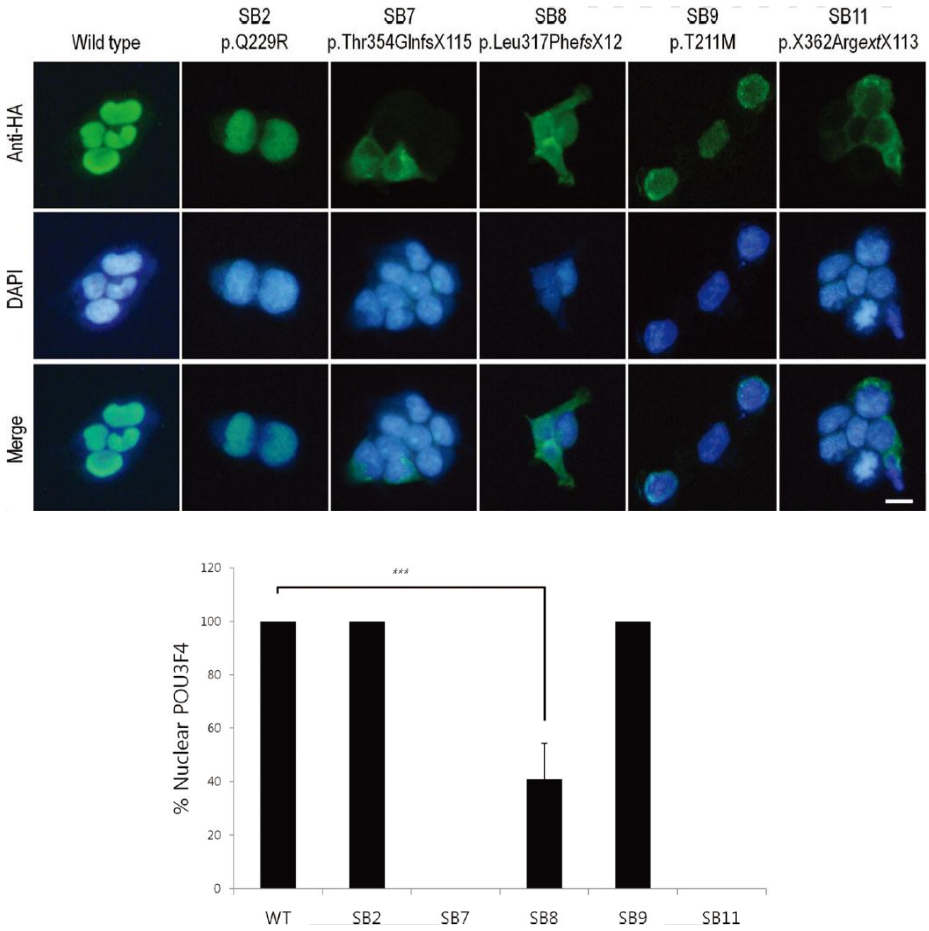


Figure 3. Subcellular localization of five novel POU3F4 mutants. HEK-293T cells were transiently transfected with each mutant expression vector, fixed, stained for POU3F4 and nuclear DNA, and visualized by indirect immunofluorescence microscopy. Top: While the SB2 and SB9 mutants were primarily localized in the nucleus similar to wild-type POU3F4, SB7 and SB11 mutants were found mostly outside of the nuclei. SB8 mutant protein was localized in both cytoplasm and nucleus. Bottom: Percentage of nuclear POU3F4 for each mutant. Scale bar: 10μm (***P = 0.0004 according to Student's t-test)

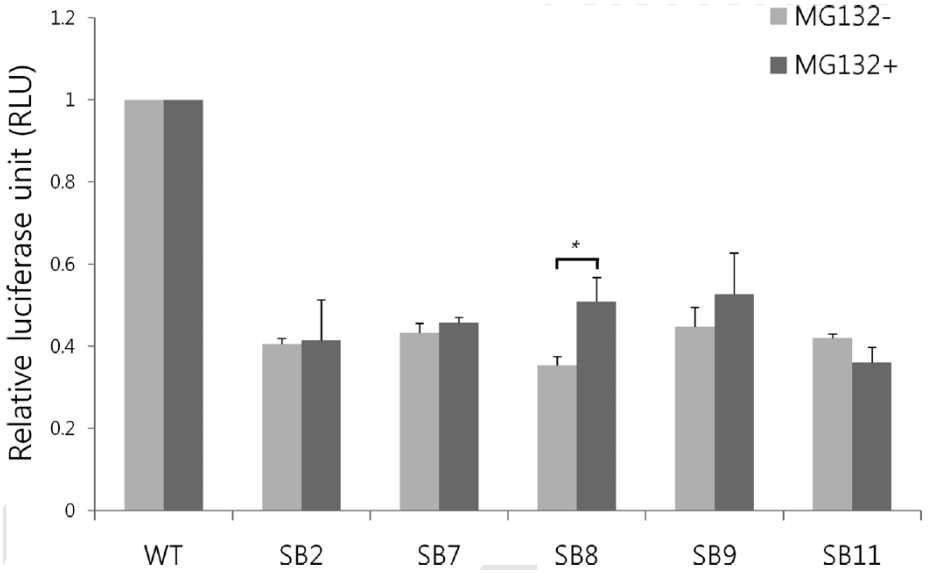


Figure 4. Transcriptional activity of the five POU3F4 mutants

Each construct was cotransfected with either pGL3-basic-3×, which has three consecutive POU3F4-binding sites or control pGL3-basic empty vector. Unlike wild-type POU3F4, upregulation of the reporter gene was not detected when mutant POU3F4 was transfected. In addition, luciferase activity was analyzed after the treatment of transfected cells with MG132. (*P =0.03 according to Student's t-test)

DISCUSSION

Until now, five types of X-linked nonsyndromic hearing loss were identified. POU3F4, which is responsible for 2% of congenital deafness, is the causative gene of X-linked deafness 2 (DFNX2). There are a number of studies that concern with role of POU-III class proteins during developmental processes. They include studies about the regulation of striatal neuron precursor differentiation (Shimazaki, et al., 1999), formation of pancreatic glucagon cell identity (Heller, et al., 2004), mesenchymal differentiation in the superior canal(Sobol, et al., 2005), and etc. However, molecular targets of the POU3F4 transcription factor have not been identified. Also, even if more than 30 mutations of POU3F4 have identified, most of the mutations are missense alleles or small deletions in the coding region, multiple deletions up to 1.0 Mb upstream of POU3F4 have been found in DFNX2 patients (Naranjo, et al., 2010).

Analysis of the consequences of C-terminal frameshift mutation was confronted with a couple of difficulties. First of all, due to the low frequency of C-terminal frameshift mutation previous studies on pathogenesis almost doesn't exist. In silico simulation of the protein structure was not possible either,

because prior structural information on the new, extended amino acid sequences could not be provided from anywhere. In vitro analysis for protein expression level using Western blot showed that plasmid construct transfection was not efficiently produce protein especially for two C-terminal frameshift mutation, SB7 and SB11 (Fig2B). It was noticeable that those two mutations eventually eliminates 3'UTR, which includes poly A sequences, which suggests the possibility that protein translation initiation complex formation could be inhibited by lack of polyA-binding complex formation (Gray and Wickens, 1998). However, none of the mutation of POU3F4 causes NMD in reverse transcription(RT)-PCR (Fig. 2B), and result of polysomal fraction analysis was almost similar regardless of mutation type (Fig. 2C). Presumably, a 113~115 amino acid-long extension of the POU3F4 protein may be thermodynamically unstable and fail to maintain structural integrity. This hypothesis was supported by Western blot with adding MG132, a proteasome inhibitor. Albeit increased level of protein, it did not followed by increased activity as a transcription activity shown by luciferase assay (Fig. 4). It was not surprising, because two frameshift extension mutations of

POU3F4 in SB7 and SB11 patients were mislocalized to the cytoplasm instead of nucleus where wild-type protein was localized (Fig. 3). In this sense, frameshift extension mutations without any discrete function may affect the function of the parent protein by destabilization and mislocalization due to extended amino acids. It is possible that mislocalization and destabilization due to the extended protein cannot be effectively rescued by MG132.

It is noteworthy that transcriptional activity of frameshift truncation mutation in patient SB8 (p.Leu317PhefsX12) has been partially restored by MG132 treatment. This mutant protein was localized to both nucleus and cytoplasm, whereas two C-terminal frameshift extension mutation proteins were localized exclusively at cytoplasm. The fact that two of three original NLSs are intact might possibly explain this phenomenon. Interestingly, MG132 increased transcriptional activity ($P<0.05$, Student's t test) as well as protein expression levels as shown in Figure 4, even though the activity still remained at far lower level than wild-type protein. The incomplete restoration of transcriptional activity of the SB8 mutant with MG132

treatment is likely due to the absence of one NLS in the C-terminus.

To date, most of the coding mutations in POU3F4 reported in the literature are confined to the POU-specific domain and POU homeodomain (Bitner-Glindzicz, et al., 1995; de Kok, et al., 1995; de Kok, et al., 1996; Lee, et al., 2009a; Lee, et al., 2009b; Song, et al., 2012). However, for the first time, two non-synonymous mutations are reported here that are localized to the non-functional domain of POU3F4 but affect the function of both the NLS and POU-specific domain. I elucidated that frameshift truncation and extension mutations are not confined to DNA-binding domains and are pathogenic, as they cause functional deterioration by reducing protein stability and mislocalization within the cell. Furthermore, I showed that MG132 treatment led to slightly increased transcriptional activity in a frameshift truncation mutation, which may shed light upon the importance of this pathogenic mechanism as a potential target for future therapies.

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국문 초록

서론: 대부분의 X 염색체 연관 비증후군성 난청은 POU 도메인 클래스 3 전사인자 4 (POU3F4) 유전자의 돌연변이에 의해서 유발된다. 따라서 이 POU3F4 유전자의 다양한 돌연변이가 어떻게 난청을 일으키는지에 관한 연구가 필요하다.

방법: 양방향 염기서열 분석을 통하여 발견된 POU3F4 유전자 내의 돌연변이 정보를 기반으로 하여 후속 실험에 필요한 발현벡터 클론을 제작하였다. 리보핵산 수준의 발현을 확인하기 위하여 역전사-중합효소연쇄반응 및 폴리리보솜 구획 분석을 하였으며, 단백질 수준의 발현 분석을 위해서는 웨스턴블롯 및 세포면역염색법을 사용하였다. 이후 루시페라아제 발현 분석을 통하여 야생형과 돌연변이형 단백질이 갖는 활성의 차이를 보았다.

결과: 한국인 환자들로부터 다섯가지의 미스센스 돌연변이 및 프레임시프트 절단 혹은 연장 돌연변이가 발견되었고 이들 중 두 개의 프레임시프트 연장 돌연변이(p.Thr354GlnfsX115 and p.X362ArgextX113)는 POU 도메인 및 핵 위치 신호의 바깥에 위치하였고, 감소되어 있던 단백질 양은 MG132 를 처리함으로서 증가하였다. 또한 이들 프레임시프트 연장 돌연변이 POU3F4 단백질은 거의 세포질에 위치하고 전사활성 능력을 가지고 있지 않았다. 또 다른 프레임시프트 돌연변이(p.Leu317PhefsX12)

에서는 POU3F4 단백 카르복시 말단의 44 개의 아미노산이 손실되는데, 이 돌연변이 단백은 핵과 세포질 모두에 분포하나 그 양이 적고 MG132 처리 시 단백의 양이 올라가더라도 전사활성에는 영향을 주지 못하였다. 마지막으로 루시페라아제 발현 실험을 통하여 두 개의 미스센스 돌연변이(p.Thr211Met and p.Gln229Arg)를 포함한 모든 POU3F4 돌연변이에서 전사인자 활성이 감소함을 확인하였다.

결론: 결론적으로 POU3F4 의 카르복시 말단 쪽 프레임시프트 절단 및 연장 돌연변이는 단백질의 3 차원 구조에 영향을 미치고 그 결과로 세포 내 위치 변화와 단백질의 분해를 유도한다고 할 수 있다. 또한 이러한 차이는 본래의 전사 활성 능력을 저하시켜 결과적으로 비증후군성 난청을 유발할 것이다.

주요어 : 비증후군성 난청, POU3F4, 프레임시프트 돌연변이, 프로테오솜, 세포 내 위치

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