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Clarification of glycosylphosphatidylinositol anchorage of OTOANCORIN and human *OTOA* variants associated with deafness

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Abstract

Otoancorin (OTOA), encoded by *OTOA,* is required for the development of the tectorial membrane (TM) in the inner ear. Mutations in this gene cause non-syndromic hearing loss (DFNB22). The molecular mechanisms underlying most of DFNB22 remains poorly understood. Disruption of glycosylphosphatidylinositol (GPI) anchorage has been assumed to be the pathophysiology, mandating experimental validation. From a Korean deaf family, we identified two *trans OTOA* variants (c.1320+5G>C and p.Gln589ArgfsX55 (NM_144672.3)). Pathogenic potential of c.1320+5G>C was confirmed by a minigene splicing assay. To experimentally determine the GPI anchorage, wildtype and the mutant OTOA harboring p.Gln589ArgfsX55 was

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Author contributions

B.J.K., D.-K.K., S.P., and B.Y.C. designed the study. H.-R.P. contributed to sample preparation. D.-K.K., J.H.H., J.O, and M.Y.K. performed the experiments. B.J.K., J.H.H., J.O., A.R.K., C.L., N.K.D.K., D.Y.O., S.P., and B.Y.C. analyzed the data including bioinformatics analysis. S.L. designed the figures. B.J.K., D.-K.K., S.P., and B.Y.C. wrote the first draft of the manuscript. W.-Y.P provided critical feedback. All authors discussed the results and contributed to the final manuscript.

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Conflict of Interest

The authors declare that no competing interests exist.

expressed in the HEK293T cells. The mutant *OTOA* with p.Gln589ArgfsX55 resulted in an uncontrolled release of *OTOA* into the medium in contrast with phosphatidylinositol-specific phospholipase C-induced controlled release of wildtype OTOA from the cell surface. Together, this reverse translational study confirmed GPI-anchorage of OTOA and showed that downstream sequences from the 589th aa is critical for GPI-anchorage.

Keywords

OTOA; Deafness; DFNB22; Tectorial membrane; Glycosylphosphatidylinositol (GPI)-anchorage

OTOA encodes otoancorin (OTOA), a protein required for limbal attachment of the tectorial membrane (TM), which is necessary for conditioning the proper stimulation of the inner hair cells (Lukashkin et al., 2012). Association of *OTOA* and hearing loss (DFNB22) was first reported in 2002 (Zwaenepoel et al., 2002). Until recently, most alterations of *OTOA* associated with deafness have been detected in North African or Middle Eastern nationals, including Palestinians, Pakistanis, Qataris, Turkish, and Algerians (Alkowari et al., 2017; Bademci et al., 2014; K. Lee et al., 2013; Shahin et al., 2010; Walsh et al., 2006; Zwaenepoel et al., 2002).

Copy number variations (CNVs) including large deletions or segmental duplications of *OTOA,* which could have evaded conventional NGS-based sequencing, have been reported in DFNB22. Comprehensive analysis of the CNVs of *OTOA* (Shearer et al., 2014) first reported DFNB 22 in patients of non-Middle Eastern ethnicity. DFNB22 was later reported in other ethnicities including Han Chinese and European populations etc. (He et al., 2018; Sloan-Heggen et al., 2016; Sommen et al., 2016). Currently, eight missense variants, two frameshift variants, one nonsense and one splice site variant of *OTOA* have been known to be associated with DFNB22 (Supp. Table S1), in addition to large/ whole deletions or duplications of this gene (Ammar-Khodja et al., 2015; He et al., 2018; K. Lee et al., 2013; Sloan-Heggen et al., 2016; Sommen et al., 2016; Walsh et al., 2006; Zwaenepoel et al., 2002). However, no point mutation or CNV of *OTOA* has yet been reported in the Korean deaf population.

OTOA, which contains an N-terminal secretory signal peptide (SP) and a C-terminal hydrophobic patch, was previously predicted as a glycosylphosphatidylinositol (GPI)-anchored protein (AP) using a computational GPI-anchorage prediction tool (Zwaenepoel et al., 2002). Since this report, disruption of GPI-anchorage has been assumed to play a role in the pathophysiology of *OTOA*-related deafness. However, it is critical to experimentally validate the GPI-anchorage of predicted proteins, since the efficiency of a GPI-anchorage varies depending on GPI-attachment sequences. Indeed, some proteins that pass the prediction algorism are not GPI-anchored (Galian, Bjorkholm, Bulleid, & von Heijne, 2012). Conversely, other proteins, which were regarded as a type I transmembrane protein, were proven to be GPI-anchored, contrary to prediction (Davies et al., 2010). GPI-anchorage of OTOA itself has not yet been experimentally verified, and the mechanism involving alterations in the *OTOA* gene leading to hearing loss is still not known.

Here, we report a novel causative variant of *OTOA* in the Korean deaf population. Further, through reverse translational research, we experimentally confirmed that OTOA is indeed GPI anchored and showed at least some downstream sequences from the 589th amino acid is critical for its function, suggesting that disruption of GPI-anchorage could be involved in the pathophysiology of DFNB22.

We identified two potential *OTOA* pathogenic variants, c.1320+5G>C and c.1765delC (p.Gln589ArgfsX55) in a Korean hearing-impaired pedigree (SB285) through the whole-exome sequencing (WES) and subsequent bioinformatics analysis (Chang et al., 2018; Han et al., 2017; Kim et al., 2018) (see the Supplementary Materials and Methods). We obtained a written informed consent from SB285–618 and SB285–586. For SB285–576, the written informed consent was obtained from his parents (Supp. Figure S1).

Because the patient (a 21-month-old Korean male, designated here as SB285–576) failed to pass universal newborn screening and displayed moderate to severe sensorineural hearing loss (Supp. Figure S1A and S1B), we analyzed the SB285, to identify potentially pathogenic genes. A stepwise filtering process including *in silico* analysis and a segregation study revealed two variants possibly responsible for the hearing loss: c.1320+5G>C in the splice region site just downstream of exon 13 and c.1765delC (p.Gln589ArgfsX55) in exon 17 of *OTOA* (NM_144672.3) (Supp. Figure S1C and Supp. Table S2). The c.1320+5G>C variant was identified as a novel splice region variant while c.1765delC (p.Gln589ArgfsX55) was previously detected as a single heterozygous state in a profoundly deaf patient, where it was assumed to be fortuitously detected irrespective of its pathogenic potential and causality were doubted (Park et al., 2014). In contrast, c.1765delC (p.Gln589ArgfsX55) was confirmed to be in a *trans* configuration with another potentially pathogenic variant in our present study, increasing the likelihood of the pathogenic contribution of this variant to deafness of SB285.

Disruption of the splicing region by c.1320+5G>C was initially suggested by ESEfinder, NNSplice, MaxEntScan::score5ss, and NetGene2. The c.1320+5G>C and p.Gln589ArgfsX55 alleles were rarely or never detected in the ExAC database, gnomAD and among 1,722 ethnicity-matched Korean control subjects (KRGDB database), suggesting its potential pathogenicity (Supp. Table S2). It was also classified as likely pathogenic in Deafness Variation Database (http://deafnessvariationdatabase.org/) (Accessed: December 31, 2018) (Azaiez et al., 2018). In addition, the c.1320+5G and p.Gln589 residues were well conserved across several different species in vertebrates, as supported by high GERP++ scores of 5.21 and 5.22, respectively, and showed deleterious effects according to the Mutation taster and CADD score (Supp. Table S2). Taken together, the two variants of *OTOA* found in SB285 were predicted to be likely pathogenic (c.1320+5G>C) or pathogenic (c.1765delC), according to the guidelines for the interpretation of variants (Oza et al., 2018; Richards et al., 2015).

The altered splice region site by c.1320+5G>C may have generated alternative transcripts, resulting in production of pathogenic OTOA mutants with variable C-terminal domains. To test this hypothesis, we carried out a minigene splicing assay using plasmids harboring downstream of intron 12 and exon 13, and upstream of intron 13, of either the wild-type

(WT) *OTOA* or c.1320+5G>C (Figure 1A). We transfected the pSPL3 vector only, the WT, and c.1320+5G>C into Cos-7 cells independently, and collected cells to extract transcripts. Using the reverse transcription-polymerase chain reaction (RT-PCR), the quantity and size of transcripts from each construct were visualized on an agarose gel (Figure 1B). While the WT-transfected cells expressed two major transcripts (479 bp and 519 bp), the mutant-transfected cells expressed shorter (200 bp) or longer (643 bp) transcripts, with a smeared banding pattern. The splicing patterns of the transgenes were further confirmed by Sanger sequencing (Figure 1C). The WT construct generated two forms of transcripts: 1) [(Exon A-Exon 13-Exon B) (6/9 colony pick, 66.7%)] and 2) [(Exon A-Exon 13–40 bp of upstream sequences of Intron 13) (3/9 colony pick, 33.3%)], indicating (Exon A-Exon 13-Exon B) was a dominant form, consistent with the RT-PCR data shown in Figure 1B. However, the mutant construct generated 12 distinctive types of transcripts [mutant (MT) type 1, 61/113 colony pick, 54.0%; MT type 6, 16/113 colony pick, 14.2%], each featuring different splicing patterns (**Figure 1D and** Supp. Table S3). Notably, no normal splicing pattern was detected in the c.1320+5G>C mutant. Statistical analysis showed a significantly higher portion of normal splicing patterns in the WT than in the mutant ($P < 0.00001$), indicating that c.1320+5G>C resulted in the production of multiple mutant forms of OTOA (Supp. Table S3). A minigene splicing assay performed on the previously proven pathogenic variant: c.1320+2T>C (Zwaenepoel et al., 2002) for doublechecking the integrity of this system showed a similar pattern on an agarose gel with that of c.1320+5G>C and the most prominent band turned out to be identical with the MT type 7 from c.1320+5G>C (Supp. Figure S2).

Regarding a GPI-anchorage of OTOA, we performed an *in vitro* assay to test whether WT OTOA contains a GPI-anchor for the surface-tethering of the protein. In detail, we transfected HEK293T cells with a full-length OTOA construct, which was myc-tagged just downstream to the N-terminal SP (myc-OTOA), and the levels of OTOA in the cell lysate and the medium were then determined by western blotting (Figure 2A). While OTOA was not initially detected in the medium, treatment of transfected cells with a bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) induced the release of OTOA into the medium. Migration of the released OTOA by PI-PLC was slower than that in the cell lysate, and additional peptide:N-glycosidase (PNGase) F treatment produced the same size band from the cell lysate and the medium, indicating that hyper-glycosylated OTOA was expressed on the cell surface, while hypo-glycosylated proteins were retained inside the cells (Figure 2A). Given that PI-PLC is expected to cleave the GPI-anchor on the cell surface and thus release OTOA into the medium, our results strongly indicate that mature OTOA protein was tethered on the plasma membrane via a GPI-anchor. To determine the degree of the GPI-anchorage of OTOA on the cell surface, we performed surface staining of OTOA in live cells, followed by total staining in permeabilized cells. OTOA was normally expressed on the cell surface and was completely removed from the cell surface by the PI-PLC treatment, showing that surface OTOA was predominantly GPI-anchored on the cell surface (Figure 2B). Schematic depiction of the anchoring of WT OTOA to the cell membrane by GPI anchor is illustrated (Figure 2C and 2D).

Since a substantial portion of the human DFNB22 phenotypes resulted from point mutations of *OTOA* rather than from total absence of the protein (He et al., 2018; K. Lee et al., 2013; Sloan-Heggen et al., 2016; Sommen et al., 2016; Walsh et al., 2006; Zwaenepoel et al., 2002), *Otoa* knockout mice could not faithfully model DFNB22. Noting that cell surface WT OTOA is exclusively GPI-anchored (Figure 2B), we characterized the pathogenic potential of our variants, focusing on the GPI-anchorage and surface expression. p.Gln589ArgfsX55 was chosen for this experiment because it was predicted to generate the longer translation product than c.1320+5G>C, if it was not subject to nonsense medicated decay (NMD). Using this second variant with the longer translation product, we expected to be able to better reduce the portion of the C-terminal part with unknown functions than using c.1320+5G>C.

First, we monitored the cellular localization of OTOA (p.Gln589ArgfsX55), which lacked the C-terminal domain, presumably with the GPI-anchorage signal. OTOA (p.Gln589ArgfsX55) was constitutively secreted into the medium and PI-PLC treatment did not facilitate its release (Figure 2E). Consistent with the western blotting data, we could not detect the surface expression of the mutant by surface staining (Figure 2F). Schematic depiction of the uncontrolled secretion of the OTOA (p.Gln589ArgfsX55) is illustrated (Figure 2G and 2H).

TECTA is another GPI-anchored protein that plays critical roles in the formation of the TM via crosslinking TM components (Andrade, Salles, Grati, Manor, & Kachar, 2016). Both *Otoa* and *Tecta* are expressed in the spiral limbus during TM development (Lukashkin et al., 2012; Zwaenepoel et al., 2002). Thus, we tested whether TECTA sequestered OTOA (p.Gln589ArgfsX55) on the cell surface. Expression of TECTA did not block the constitutive release of OTOA (p.Gln589ArgfsX55) (Supp. Figure S3).

Overall, the data suggested that OTOA is predominantly GPI-anchored on the cell surface and also that the loss of surface tethering followed by uncontrolled release of OTOA (p.Gln589ArgfsX55) may have been the cause of hearing deficits observed in the proband. However, we could not exclude the possibility that another mechanism yet to be identified from the C-terminal domain downstream to the 589th amino acid (Gln) may have exerted more deleterious pathogenic effects alone or in combination with disruption of the GPI-anchorage. Nonetheless, our present study of the frameshift variant provides the first reported evidence that OTOA is a GPI-anchored protein. The pathogenic potential of the splice region variant, c.1320+5G>C was further supported by the similar minigene splicing assay results with those obtained from the previously reported pathogenic variant, c. 1320+2T>C, which is only 3 bp ahead of our variant.

To exclude the possibility that our two variants (c.1320+5G>C and p.Gln589ArgfsX55 located between exon 13 and exon17) reside in the pseudogene, *OTOAP1*, we checked the sequence similarity between *OTOA* (NM_144672.3) and *OTOAP1* (NR_003676) using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and MAFFT (https://mafft.cbrc.jp/alignment/software/), which showed 28.29% sequence homology on the region between the exon 1 and exon 20, while 99.57% homology was observed on the remaining region

(between exon 21 and C-terminal), further supporting the presence of our two variants in the real *OTOA* gene.

The contribution of *OTOA* variants, including CNVs encompassing this gene, to deafness in Koreans was indirectly verified through the analysis of normal control whole-exome sequencing (WES) data. CNVs were analyzed in 944 cases of normal control data from the Korean Variant Archive (KOVA) (S. Lee et al., 2017) with the detection of only two CNVs of *OTOA* in contrast to the significantly higher detection rate (18/686) of *OTOA* CNVs in the non-syndromic hearing loss (NSHL) population (Shearer et al., 2014), indicating significantly more CNVs in the NSHL group than in the normal control population ($p < 0.001$), although the difference in ethnicity might have biased the interpretation. Indels were also investigated in 1,944 cases of combined normal control data from KOVA and Samsung Genome Institute (SGI), with only three indels in the intron detected (unpublished data), supporting the pathogenic potential of variants of *OTOA* in the deaf population.

Although *OTOA* was reported to be associated with DFNB22 in 2002, the molecular mechanism underlying the *OTOA*-related hearing loss (DFNB22) in humans has not been clearly elucidated. Initially, the association of TM and hearing loss was proposed for *OTOA*-related DFNB22 (Zwaenepoel et al., 2002). The TM of the mammalian cochlea is a complex structure composed of collagen fibrils embedded in a non-collagenous matrix, where a number of different glycoproteins, including Tecta, Tectb, otogelin, otolin, and Ceacam16 are located (Cohen-Salmon, El-Amraoui, Leibovici, & Petit, 1997; Deans, Peterson, & Wong, 2010; Hasko & Richardson, 1988; Legan, Rau, Keen, & Richardson, 1997; Zheng et al., 2011). Alterations in these glycoproteins have been reported to be associated with both multiple autosomal dominant and recessive hereditary deafness (Alasti et al., 2008; Mustapha et al., 1999; Verhoeven et al., 1998). A recent study using a null function mouse model of *Otoa* revealed that detachment of TM from the spiral limbus and subsequent disruption of inner hair cell stimulation might play an important role in the pathogenesis of DFNB22 (Lukashkin et al., 2012). However, mice with complete loss of *Otoa* function did not always faithfully model the human phenotype (DFNB22), with a varying range of OTOA functional losses. A delicate and sophisticated subtle pathogenic mechanism would be masked with an overwhelming null function, and may not have been elucidated with this null mutant model.

In the study, we could not properly evaluate the possibility of NMD for two mutant truncating transcripts from c.1320+5G>C and p.Gln589ArgfsX55, because *OTOA* transcript was not detected even from wild-type lymphoblastoid cell line (unpublished data). Therefore, there still remains the possibility of significant reduction of transcripts and proteins due to NMD. However, failure in GPI-anchorage of OTOA can still hold true even for null alleles of OTOA

In the present study, we experimentally proved GPI-anchorage of OTOA and also suggest the failure of GPI-anchorage as an important mechanism underlying the *OTOA*-associated, TM-related hearing loss, which was confirmed by an actual human *OTOA* variant from hearing-impaired patients and by subsequent, reverse translational molecular analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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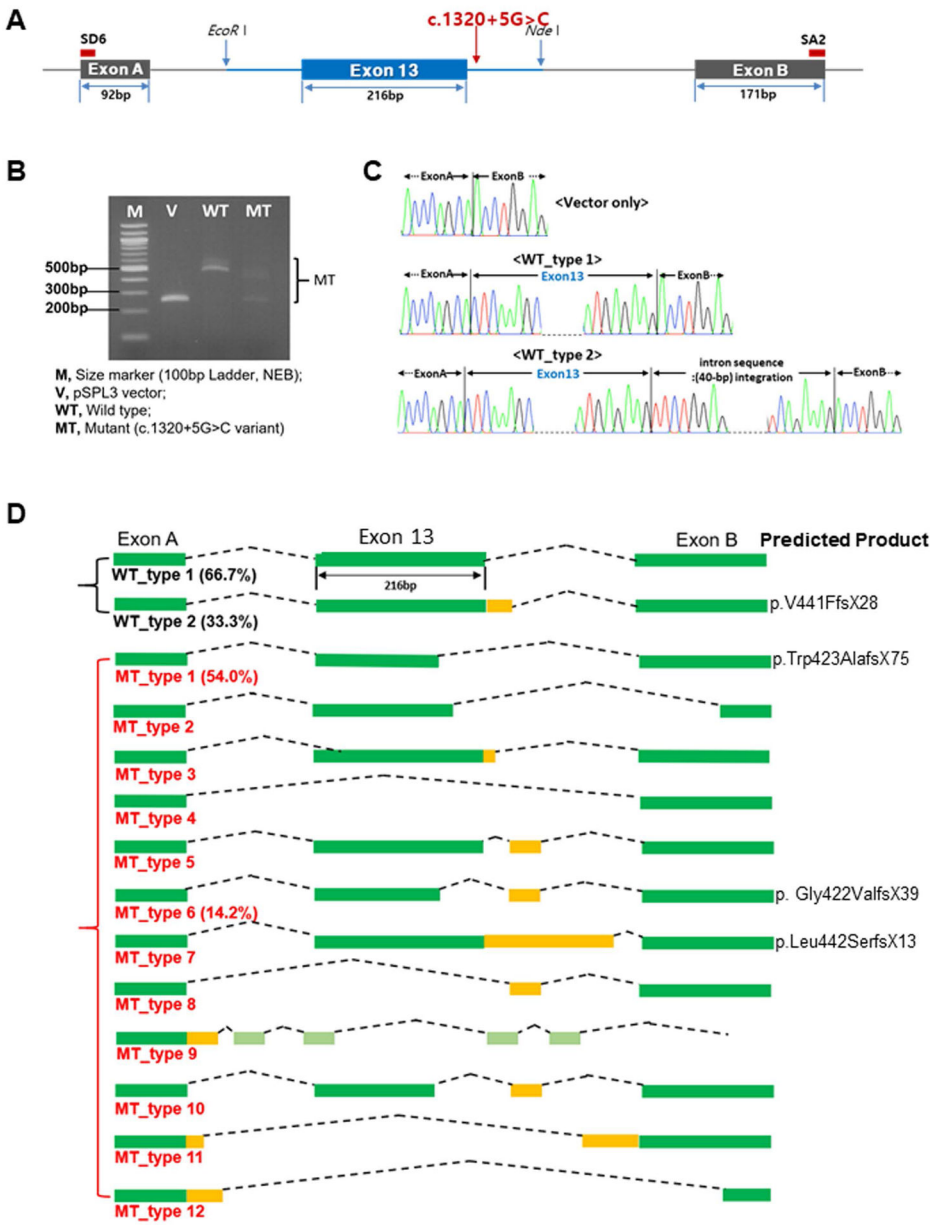

Figure 1. Mutation in the *OTOA* splicing region site by c.1320+5G>C generated multiple splicing isoforms and affected transcript stability.

(A) Generation of pSPL3 plasmid incorporating the c.1320+5G>C splicing variant.

(B) Reverse transcription-polymerase chain reaction products shown on an agarose gel with multiple bands for the wild-type and mutant samples.

(C) Sanger sequencing chromatograms of two types of spliced transcripts from the wild-type and one type of transcript from the vector only, directly quantitated using the polymerase chain reaction, TA cloning, and Sanger sequencing results.

(D) Schematic illustration of the two types of splicing products from the wild-type and 12 types of those from the mutants, with the two most prevalent ones showing percentages, directly quantitated using the polymerase chain reaction, TA cloning, and Sanger sequencing.

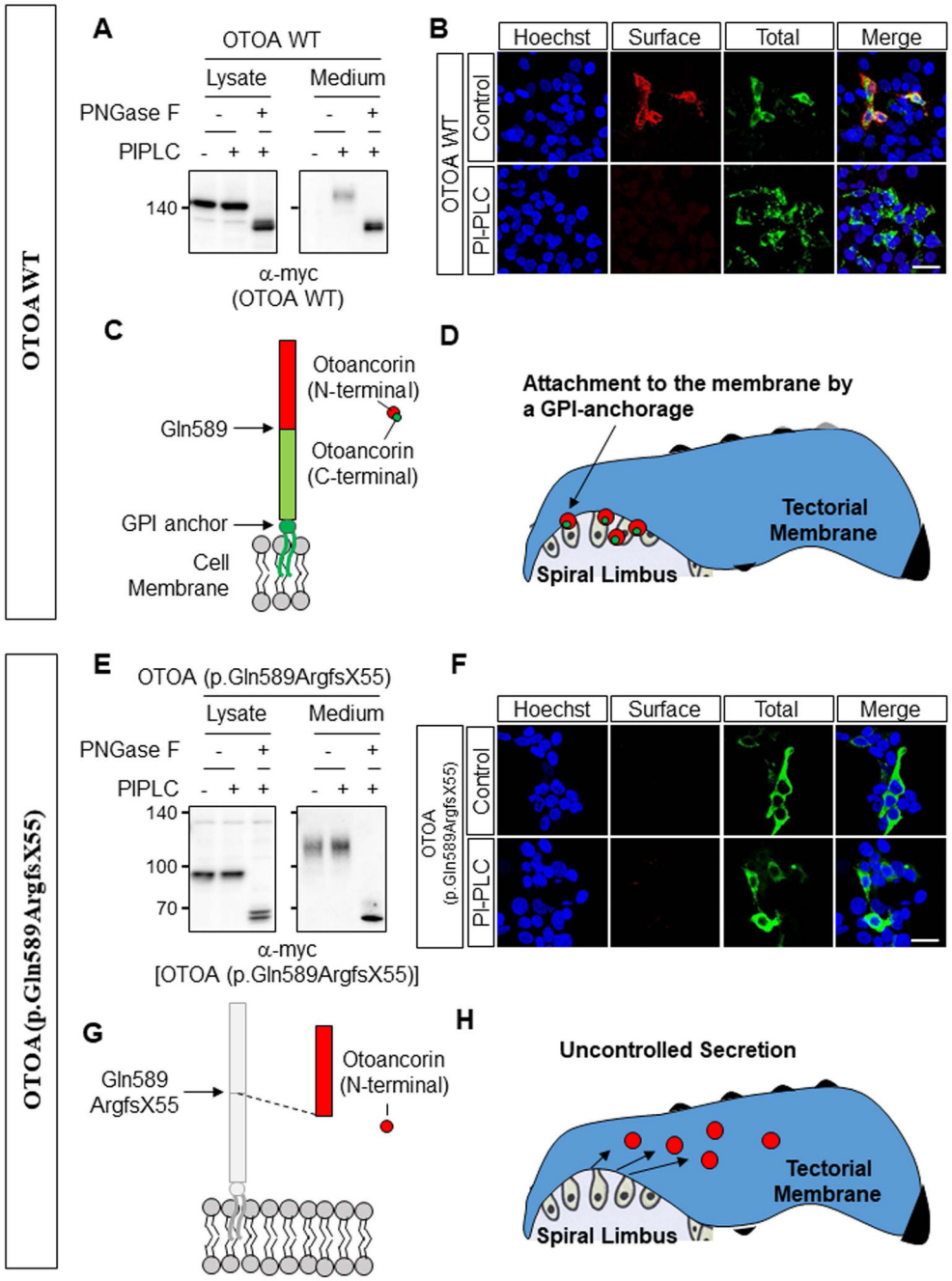

Figure 2. Wild-type (WT) OTOA is localized on the cell surface via a GPI-anchor, while truncated OTOA (p.Gln589ArgfsX55) is constitutively secreted from cells.

(A) HEK293T cells were transfected with myc-tagged WT OTOA. Western blot of cell lysate and medium samples with α-myc antibody showed that OTOA is not released into the medium. Treatment of cells with bacterial phosphatidylinositol- phospholipase C (PI-PLC), which cleaves a GPI-anchor on the cell surface, facilitates the release of OTOA into the medium. The released OTOA by PI-PLC migrated slower than that in the cell lysate. Deglycosylation by PNGase F treatment produced the same smaller size bands in the cell

lysate and the medium, indicating hyper-glycosylated OTOA is localized on the cell surface via a GPI-anchorage.

(B) Live cell surface labeling. HEK293T cells were transfected with WT OTOA and subsequently immunostained with an α-myc antibody (raised in rabbit) in the absence of a detergent for surface labeling. Cells were then treated with blocking solution containing 1% Triton X-100, and incubated with an α-myc antibody (raised in mouse) for total staining. Surface and total Otoa is shown as red and green, respectively. OTOA is observed on the cell surface, which is completely removed by PI-PLC treatment.

(C and D) Schematic cartoons illustrating the shape and function of WT OTOA. OTOA is tethered to the plasma membrane via a GPI-anchorage, which is attached to the C-terminus of the mature protein. Red and green color of the mature protein indicated the preserved and truncated domain of Gln589ArgfsX55 mutant, respectively.

(E) HEK293T cells were transfected with myc-tagged truncated OTOA (p.Gln589ArgfsX55). The truncated OTOA was constitutively secreted into the medium without PI-PLC treatment. The released protein migrated slower than that in the cell lysate. Deglycosylation by PNGase F treatment produced the same smaller size bands in the cell lysate and the medium, indicating hyper-glycosylated is secreted into the medium.

(F) Live cell staining of HEK293T cells transfected with myc-tagged truncated OTOA (p.Gln589ArgfsX55) showed that the truncated OTOA is not tethered to the cell surface.

(G and H) The truncated OTOA lacks a C-terminal GPI-anchorage and is constitutively secreted from the producing cells.