科技部補助專題研究計畫成果報告
期末報告

利用細胞模式探討 gap junction 通道蛋白造成聽障之分子機制

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1.公開資訊：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢
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中 文 摘 要 ： 遺傳性聽障一種常見的感音神經性疾病，在開發中國家大約60%由於基因突變所造成的。目前已知有59個基因的突變會導致聽障且每個基因的致病機制皆不盡相同。gap junction在聽力功能上扮演的角色已被許多研究所確認，其在鉀離子再循環、耳蝸內離子平衡及聽力形成過程皆扮演重要的角色。本研究計畫目的主要是著重於探討gap junction通道蛋白基因突變對其功能的影響，藉此瞭解gap junction通道蛋白基因於聽覺形成過程中所扮演的角色並瞭解其致病機制。在本年度研究計畫我們主要針對GJB3、GJB4和GJC3基因進行探討。在本研究中我們一共發表了兩篇國際期刊(Molecular Medicine Reports DOI: 10.3892/mmr.2014.2725 和Biochemistry and Cell Biology 92:251~257)和在103年「生醫年會」研討會中以壁報的方式發表。另有一篇相關的文章正在投稿中。在本研究計畫中我們已證實部分正常及突變gap junction通道蛋白基因在細胞內的表現情形及功能。在這一年所獲得的經驗和研究成果，提供了相關的資訊，可作為後續研究gap junction通道蛋白基因造成非症候群聽障致病機轉的參考。

中文關鍵詞： 基因、突變、聽障、功能研究

英 文 摘 要 ： Hearing loss is a common sensory disorder in the human population. In the developed countries, the incidence of congenital hearing loss is estimated at 1~3 in 1000 births, of which approximately 60% cases are attributed to genetic factors. Of the genes responsible for deafness, 59 genes have been identified. Previously, we have found many novel mutations in the gap junction (GJ) protein, connexin (CX) gene, from screening of 513 patients with nonsyndromic deafness. The prevalence of GJ gene mutations in this study was about10%. Therefore, the overall goal of this project will study the mechanisms of functional alterations in the deafness-gene (gap junction protein) mutant from patients with nonsyndromic hearing loss using cell model.
In this year, we will focus on study the mutation of GJB3, GJB4 and GJC3 gene. We have published two papers in the SCI journal (Molecular Medicine Reports DOI: 10.3892/mmr.2014.2725 and Biochemistry and Cell Biology 92: 251~ 257) and one paper has submitted to

SCI journal in this year. In additional, we have also published the results of GJB4 in the 29th Joint Annual Conference of Biomedical Sciences using poster form. These results of this research, we anticipate that the study will further our understanding of the functional role of GJ protein gene in nonsyndromic deafness.

英文關鍵詞： mutation, hearing loss, gap junction, functional study

中文摘要

遺傳性聽障一種常見的感音神經性疾病，在開發中國家大約60%由於基因突變所造成的。目前已知有59個基因的突變會導致聽障且每個基因的致病機制皆不盡相同。gap junction在聽力功能上扮演的角色已被許多研究所確認，其在鉀離子再循環、耳蝸內離子平衡及聽力形成過程皆扮演重要的角色。本研究計畫目的主要是著重於探討gap junction通道蛋白基因突變對其功能的影響，藉此瞭解gap junction通道蛋白基因於聽覺形成過程中所扮演的角色並瞭解其致病機制。

在本年度研究計畫我們主要針對*GJB3*、*GJB4*和*GJC3*基因進行探討。在本研究中我們一共發表了兩篇國際期刊(Molecular Medicine Reports DOI: 10.3892/mmr.2014.2725 和 Biochemistry and Cell Biology 92:251~257) 和在103年「生醫年會」研討會中以壁報的方式發表。另有一篇相關的文章正在投稿中。在本研究計畫中我們已證實部分正常及突變gap junction通道蛋白基因在細胞內的表現情形及功能。在這一年所獲得的經驗和研究成果，提供了相關的資訊，可作為後續研究gap junction通道蛋白基因造成非症候群聽障致病機轉的參考。

關鍵詞：基因、突變、聽障、功能研究

Abstract

Hearing loss is a common sensory disorder in the human population. In the developed countries, the incidence of congenital hearing loss is estimated at 1~3 in 1000 births, of which approximately 60% cases are attributed to genetic factors. Of the genes responsible for deafness, 59 genes have been identified. Previously, we have found many novel mutations in the gap junction (GJ) protein, connexin (CX) gene, from screening of 513 patients with nonsyndromic deafness. The prevalence of GJ gene mutations in this study was about10%. Therefore, the overall goal of this project will study the mechanisms of functional alterations in the deafness-gene (gap junction protein) mutant from patients with nonsyndromic hearing loss using cell model.

In this year, we will focus on study the mutation of *GJB3*, *GJB4* and *GJC3* gene. We have published two papers in the SCI journal (Molecular Medicine Reports DOI: 10.3892/mmr.2014.2725 and Biochemistry and Cell Biology 92: 251~ 257) and one paper has submitted to SCI journal in this year. In additional, we have also published the results of GJB4 in the 29th Joint Annual Conference of Biomedical Sciences using poster form. These results of this research, we anticipate that the study will further our understanding of the functional role of GJ protein gene in nonsyndromic deafness.

Keywords: mutation, hearing loss, gap junction, functional study

前言

目前約有1~3/1000比例的嬰兒於出生或幼年時期罹患中重度聽障，在已開發國家中發現60%的個案為遺傳因素所造成。先前針對台灣地區513位非症候群聽障患者的gap junction通道蛋白基因篩檢中，發現了許多的錯意(missense)突變(Yang et al., 2007, 2010)。在過去幾年我們一直在探討這些突變所造成的影響和治病機轉(Su et al., 2010; Hong et al., 2010a, 2010b; Wang et al., 2011; Su et al., 2013)。然而目前還是有些錯意突變所造成的功能影響及其致病機轉並不清楚，且同一基因在不同位置的突變常會產生蛋白功能影響上的差異。因此本計畫我們想藉由原本已建立好的細胞模式來加以探討gap junction通道蛋白這些錯意突變對其蛋白功能的影響是如何? 進一步的瞭解這些錯意突變所造成的聽障的機制為何?

研究目的

本研究的目的為進一步探討 gap junction 通道蛋白基因突變後對其功能的影響，藉由探討這些問題以瞭解 gap junction 通道蛋白基因在聽覺形成過程中所扮演的角色，並瞭解其致病機制。

文獻探討

聽障可因遺傳基因突變或環境因素，或兩者兼之引起的。約有 1/1000 嬰兒在出生時或在小孩早期罹患中重度聽障(severe or profound)，在已開發國家約有 60% 個案是遺傳因素(Marazita et al., 1993)。自 1996 年聽障（hearing loss）的基因陸續被發現，過去這段時間在研究聽力或聽障與基因之間的關係和其分子機制發展非常快速，聽障被認為是一種普遍的感音性和 heterogeneous 的遺傳性疾病。至目前為止超過 50 個基因和 80 個另外的基因座(loci)被發現牽涉造成不同程度的聽障(review in Dror and Avraham, 2010)。

耳蝸是一種非常精緻的器官，在聽覺的形成中佔相當重要的角色。內淋巴液(endolymph)、外淋巴液(perilymph)和 intrastrial 液體等三種細胞外液已經被發現充滿在耳蝸內。內淋巴液是具有高 K+低 Na+的細胞外液；而外淋巴液是低 K+ 高 Na+的細胞外液(Wangemann, 2006)。耳蝸內淋巴主要被 12 種不同上皮細胞所圍繞包括感音毛細胞(sensory hair cells)，同時內淋巴是一種獨特類似細胞內液的細胞外液，在內淋巴液中 K+濃度的改變主要造成感覺神經傳導的機制(Wangemann, 2006;)。當聲音由耳翼接收，經外耳道傳至鼓膜將它轉變成機械能量並傳送到聽小骨，最後經由聽小骨傳到內耳，刺表激內耳的 sprial organ。耳蝸將機械能轉成液能使外淋巴液形成波浪，引起耳蝸前庭腔(scala vestibule)和耳蝸鼓腔(scala tympani)的震動，接下來耳蝸前庭腔的能量會傳遞到耳蝸中膈(scala media)，造成耳蝸中膈內

淋巴的移動，再經由 organ of Corti 上的 stereocilia 來接收震動，當 stereocilia 受到震動時，位於纖毛頂端的機械訊號傳導通道(mechanotransduction channels)會被打開，此時內淋巴液中 K+會流經這些通道導致細胞膜去極化，並以神經衝動形式將此帶電訊息經由聽神經傳至中樞神經系統的大腦皮層聽覺中樞(Roberts et al., 1998; Pickles and Corey, 1992)。內淋巴液內的離子濃度不僅是高 K+低 Na+，此細胞外液也含有低 Ca2+、高 HCO3-和少量的蛋白質。高 HCO3-濃度可能牽涉到維持內淋巴液的 pH 值。低 Ca2+濃度在耳蝸內聽覺的傳導也具有關鍵性的角色，Ca2+會伴隨 K+離子進入纖毛頂端的機械訊號傳導通道參與神經傳導的機制(Ricci and Fettiplace, 1998; Holt and Corey, 2000)。故耳蝸中內淋巴液離子環境的維持對聽覺的產生相當的重要。

由於聽覺的產生與內淋巴液具有高 K+和 endocochlear potential (EP)的特性有極大的關係，因此 K+再循環(recycling)機制的進行和維持在整個聽覺中扮演重要的角色(Ortolano et al., 2008; review in Dror and Avraham, 2010; Mammano et al., 2007)。在整個 K+ 再循環(recycling)牽涉的基因很多，到目前為止主要包括(1). 形成 gap junction (GJ)的基因---connexin (CX) 基因族--- GJB2 (CX26)、GJB6 (CX30)、GJB3 (CX31)及 GJA1 (CX43)；(2).形成 tight junction (TJ)的基因族--- CLAUDIN 11 (CLDN 11)、TRICELLULIN (TRIC)和 CLAUDIN 14 (CLDN 14)；(3). KCNQ4、KCNN2、KCNMA1 基因。在內耳 gap junction 主要是細胞間離子通過的管道，而內耳內的外淋巴和內淋巴需要特殊的保護機制而將他們完全的區隔以維持高 K+和 endocochlear potential (EP)，為維持內淋巴高靜止電位，有很多研究已經發現在內淋巴的周圍細胞間須用各種 tight junction 將其緊密連接起來 (Anderson and van Itallie 1995; Schneeberger and Lynch 1992; Tsukita et al., 2001)。在國外很多研究報告指出這些基因如果發生突變將造成聽障(review in Dror and Avraham, 2010)。國內也有相關的研究，包括我們近幾年的研究(2007-2014)。

Gap junction (GJ)在耳蝸內主要分為兩大系統: (1) 上皮細胞(epithelial cell) GJ 系統，此系統包括所有 organ of Corti 的支持細胞、spiral limbus 的 interdental 細胞和 spiral ligament 的 root 細胞； (2)結締組織(connective tissue cell)細胞 GJ 系統，此系統包括 spiral ligament 和 suprastrial zone 的各種形式的纖維細胞(fibrocytes)、stria vascularis 的 intermediate 細胞、耳蝸前庭腔(scala vestibule)的 mesenchymal 細胞和 spiral limbus 的 mesenchymal dark 細胞。這上皮細胞 GJ 系統的組成細胞主要排列在基底膜上，且並無直接接觸結締組織(connective tissue cell)細胞 GJ 系統的組成細胞。這些 GJ 系統主要的功能是牽涉在耳蝸 K+再循環(recycling)機制(Kikuchi et al., 2000; Zhao et al., 2006)。另外也有報告指出 GJ 的功能並不只是牽涉 K+再循環(recycling)，他

們也建議細胞質內的訊息分子或代謝分子的通過 GJ 可能在聽力的形成過程中也是必須的(Beltramello et al., 2005; Zhang et al., 2005)。GJ 通道蛋白的突變已經牽涉在許多人類的遺傳疾病，包括遺傳性聽覺障礙 (Zoid1 and Dermietzel, 2010)。

Gap junction (GJ)是閘道式細胞內通道的群集，直接連接了兩個相鄰細胞的細胞質，因此允許小於 1k Da 或直徑小於 1.2nm 的分子在相連的兩細胞之間自由擴散，涉及細胞的發育及分化過程(Sáez et al., 2003)。構成 GJ 蛋白為 connexin (CX)，CX 在 Golgi-ER 聚合(oligomerize)成六聚體的(hexameric) connexons，再送到細胞膜，構成 hemichannels (gap junction 的其中一邊)，再與相鄰細胞的 connexons 對接(docking)，在細胞膜排列形成 gap junctions (Evans et al.,2006)。在人類已發現約 20 種 CX gene 家族成員(Willecke et al., 2002)，每個都由不同的基因編譯，並根據其分子量(molecular weight in kDa)給予命名，再基於核酸及胺基酸層級上的相似性分成 α 、β 、ε 子群體。(Söhl and Willecke, 2003)相同(homomeric)或不同的(heteromeric) connexins 可以組成多種不同的 connexon isoforms，由於所組成的蛋白大小及電荷不同，因此也會改變通道(channel)對分子的選擇性及調節的敏感度，如:正常 CX26 蛋白所形成的 GJ 可使 Leucifer yellow (457 Da)通過，但在 CX26 與 CX30 形成 heteromeric GJ 時，細胞只能使 neuroobiotin (287 Da)通過(Marziano, et al., 2003)。

在形成 GJ 的大部分細胞中常表現出不只一種 CX 蛋白，因此在細胞內可能會形成具有不同生理功能的 gap junction (Kumar and Gilula 1996)。另外在形成 connexon 時有可能是由相同的(homomeric)或不同的(heteromeric)CX 蛋白所組成，且在相鄰兩細胞的 connexon 的結合形成 GJ 也有可能是同質性(homotypic junction)或異質性(heterotypic junction)的 connexon 所組成(Falk 2000 a,b)。到目前為止大部分的研究發現正常只有單一群的 CX 會彼此互相結合形成 GJ，不同的群並不會互相結合，就是說α群只會和α群結合，β群只會和β群結合。在最近的研究有發現 *GJA1* (α 群)和 *GJB2* (β群)共同表現在 rat 的耳蝸內(Suzuki et al., 2003)，然而不同族群間的 CX 蛋白是否互相影響目前還不清楚。*GJC3*(CX30.2/CX31.3)是屬於ε 次群，在 2003 年 Ahmad 等人的研究利用 cDNA macroarray hybridizations 的方式發現 *GJC3* 基因在老鼠的耳蝸內有大量表現耳蝸內但並不清楚表現的位置。而我們實驗室在 2005 年利用組織免疫螢光染色法(Immuno- histochemistry；IHC)、細胞雷射擷取技術(Laser Capture Microdissection; LCM) 和 Reverse transcription-polymerase chain reaction (RT-PCR)方法發現 Cx29 (G*jc3*) [原先被稱為 Gje1；在人類相對應是 CX30.2/CX31.3(*GJC3*)]的表現在 mouse 和 rat 耳蝸內的 cochlea neurons、spiral limbus、spiral ligament、 organ of Corti 和 stria vascularis。Cx29 的表現位置有些和其他 *Cx* 基因族的表現位置是相類似。此結果已經發表在 *Biochemical and Biophysical Research Communications* 2005; 338: 723–728 (Yang et al., 2005)。另外也有報告利用基因剔除

(knock out)或基因轉殖(transgene)技術發現Cx29大量表現在耳蝸內，尤其是神經細胞(Schwann cells)(Tang et al., 2006; Eiberger et al., 2006)。所以 *Gjc3* 基因被我們認為在聽覺的形成過程中可能扮演重要的角色。最近我們的研究也指出 CX30.3(*GJB4*)表現在 rat 耳蝸的 spiral limbus、spiral ligament、 spiral ganglion、 和 stria vascularis 等區域(Wang et al., 2010)。根據先前的研究加上我們的研究，可以清楚的瞭解 CX26(*GJB2*) 、CX30(*GJB6*) 、CX30.3(*GJB4*) 、CX31(*GJB3*)、CX30.2/CX31.3(*GJC3*)和 CX43(*GJA1*)等基因皆有表現在內耳耳蝸內(Lautermann et al., 1998;Wang et al., 2010; Xia et al., 2000; Yang et al., 2005; Liu et al., 2001)。

在國內外的研究中，造成非症候群聽障的成因中以 GJB2(CX26)基因所佔的比例最高，對於 GJB2 基因的研究也最多(OMIM 121011)。在我們的基因突變篩檢中，也是以 GJB2 基因的突變所佔的比例最高(Yang et al., 2007; 2010)。另外由於 GJB2(CX26)與 GJB6(CX30)基因都位於相同的區域(13q11- q12)，且在內耳的表現 CX26 和 CX30 是共同表現在一起，因此 GJB2 和 GJB6 彼此之間的關係是最常被探討和研究(Ahmad et al.,2003; OMIM 121011; OMIM 604418)。在我們細胞模式的研究中也發現當 CX26 發生 R184Q 突變時會影響正常 CX30 的蛋白運送至細胞膜形成 GJ 通道(Su et al., 2010)。相反的，我們的研究發現當 CX30 發生 A40V 突變時會造成正常的CX26蛋白無法運送到細胞膜(Wang et al., 2011)。由此可見CX26和CX30在細胞中的功能是相互影響的。CX31 也被認為和 CX26(GJB2)、CX30(GJB6)、CX32(GJB1)和 CX45(GJA7)這 4 種 connexin 形成 heterotypic 的 gap junction 通道(OMIM, 603324)。有研究指出在老鼠的耳蝸中 CX31 和 CX26 可共同表現在一起，同時在 cotransfection CX31 和 CX26 到 HEK293 細胞中，可發現兩者共同形成 gap junction。另外，在同一個的研究中也指出 CX26 和 CX31 兩者的 heterozygous 突變的交互作用會造成非症候群聽障 (Liu et al., 2009)。Gjc 基因是 GJ 基因中較晚在老鼠中被選殖(clone)出來，人類的 GJC3 基因(NM_181538; hGJC3)座落於染色體 7q22.1，包含兩個 exons，840bp 開放式讀碼框(open reading frame)，其表現的蛋白為 CX30.2/CX31.3，含 279 個胺基酸，分子量為 31.29 kDa (Sohl et al., 2001; Altevogt et al., 2002)。Gjc3 mRNA 在中樞及周邊神經系統(central and peripheral nervous systems) 都有表現，而在周邊神經系統表現特別豐富(Sohl et al.,2001)。以免疫螢光標定發現多分布在坐骨神經(sciatic nerve)的雪旺細胞(Schwann cells)(Sohl et al.,2001)、寡突神經膠質細胞(oligodendrocyte)和髓鞘(myelinating)的膠質細胞(glial cell)（ Altevogt et al., 2002 ）。在我們的研究中CX30.2/CX30.3(GJC3)在 HeLa 細胞內的表現並不像其他GJ 蛋白一樣是以斑塊(plaque)的型式表現在細胞膜上，取而代之的是 CX30.2/CX31.3 蛋白是是圍繞在整個細胞膜上呈現連續性表現(Liang et al., 2011)。此結果反而和老鼠的水晶體 Cx23 蛋白的表現情形類似，由文獻中知道， 老鼠的水晶體 Cx23 無法形成有功能的 GJ 通道，但是可以藉由半通道(hemichannels)

來釋放 ATP 到細胞外 (Sonntag et al., 2009)。所以進一步我們探討 GJC3 功能時，利用染料轉移(dye transfer) 技術發現 CX30.2/CX31.3 蛋白在細胞膜上形成的 GJ 通道並無法通透染料，反而在低 Ca+的濃度時 CX30.2/CX31.3 蛋白形成的半通道會被打開，釋放出 ATP 到細胞外 (Liang et al., 2011)。所以 CX30.2/CX31.3 蛋白的表現情形和功能是類似與同樣可構成半通道及 GJ 通道的 Pannexin (Pax) 蛋白在細胞表現的特徵及功能相似 (Dahl and Locovei, 2006; Penuela et al., 2007)。因此 GJC3 的功能和角色可能和其他在耳蝸內表現的 CX 家族成員不一樣，值得我們進一步的探討。

研究方法

一、HeLa細胞模式

1. 建構正常及突變的gap junction通道蛋白基因於螢光表現載體
2. 基因轉殖(transfection)至HeLa細胞株表現
3. 利用細胞免疫螢光染色技術觀察正常或突變CX蛋白於細胞內的表現位置
4. 建立持續穩定表現正常或突變CX蛋白的HeLa細胞株
5. MTT assay
6. 利用染料轉移(dye transfer) 的方法研究GJ 通透性的功能差異

二、共同免疫沈澱法(co-immunoprecipitation)

1. 萃取細胞蛋白質
2. 磁珠與抗體作用
3. 攜帶抗體的磁珠與細胞蛋白質作用

三、Subcellular Fractionation

四、藥物作用

1. 5 μg/mL Brefeldin A (BFA)
2. 5 μg/mL Cytochalasin B (Cyto B)
3. 10 μg/mL Nocodazole (Noco)
4. MG132 (C26H41N3O5)
5. 氯奎寧(Chloroquine)

五、分析細胞外 ATP 的濃度

1. 製作標準曲線
2. 檢測細胞在不同鈣離子環境下 ATP 濃度
3. 檢測細胞在低鈣離子環境下藥物抑制半通道釋放的 ATP 濃度

3.1 加入非特異性 gap junction 半通道抑制劑 18α-glycyirrhetinic acid (18α-GA) 抑制半通道釋放 ATP

3.2 加入非特異性 gap junction 半通道抑制劑 Carbenoxolone (CBX) 抑制半通道釋放 ATP

六、shRNA 技術

結果與討論

一、 *GJB4*基因(CX30.3)突變之研究 (附件一和附件二)

先前我們的研究已經在國內 513 位非症候群聽障學童中發現到 5 個突變型的 GJB4 基因 (CX30.3)，分別為 64C→T/wt(R22C)、109G→A/wt(V37M)、220G→A/wt(V74M)、302G→A/wt(R101H)、507C→G/507C→G(C169W)。但這些突變型 GJB4 基因的功能性尚不清楚。為了探討 GJ 的功能性是否因為突變型 GJB4 基因的影響導致 GJ 發生異常，所以我們在本研究中以不含內生性 connexin 的 HeLa 細胞作為細胞模式來觀察野生型 CX30.3 (CX30.3WT) 與突變型 CX30.3 (mutant CX30.3) 在細胞內的表現型態。於是我們先將 CX30.3WT 與突變型 CX30.3 與綠螢光蛋白 (LEGFP) 融合之後，再把已融合蛋白轉染 (transfection) 到細胞中表現並使用螢光顯微鏡觀察 (CX30.3WT) 與突變型 CX30.3 在細胞內的表現型態。從先前實驗室的結果已知 CX30.3WT-TagRFP 融合螢光蛋白會表現在細胞質內，而在本研究的結果也可發現 CX30.3WT - LEGFP 融合螢光蛋白同樣會表現在細胞質內。且五種突變型的 CX30.3 - LEGFP 融合螢光蛋白與 CX30.3WT－LEGFP 同樣也會表現在細胞質內。而根據先前的文獻結果顯示野生型 CX31 (CX31WT) 會與 CX30.3WT 共同以 GJ 斑塊 (plaque) 的型態表現在細胞膜上。為了探討 CX31WT 是否會與先前文獻結果一致與 CX30.3WT 共同以 GJ plaque 的型態表現在細胞膜上，所以我們也同時將 CX31WT 與 CX30.3WT 共同轉染至 HeLa 細胞中觀察在細胞內的共同表現型態。從我們觀察的結果中得知野生型 CX31WT 會與 CX30.3WT 共同以 GJ plaque 的型態表現在細胞膜上，這個結果與先前文獻的結果是一致。接著我們繼續探討野生型 CX31WT 與突變型 CX30.3 在細胞內的共同表現型態。研究結果顯示突變型 CX30.3 無法與野生型 CX31WT 共同在細胞膜上表現。根據這些實驗結果，我們推測野生型 CX30.3WT 蛋白無法獨自形成 GJ，而需與野生型 CX31WT 共同表現才會以 GJ plaque 的型態表現在細胞膜上。但突變型 CX30.3 蛋白則無法與野生型 CX31WT 蛋白共同以 GJ plaque 的型態在細胞膜上表現。此部分結果已於 **103 年「生醫年會」研討會以壁報的方式發表(附件一)**。另外對於 GJB4 基因突變的 genotype 和

phenotype 之間的關係，我們也進行一系列的分析，其結果**也發表在 SCI 國際期刊 Molecular Medicine Reports DOI: 10.3892/mmr.2014.2725 (附件二)**。

二、GJB3 基因(CX31)突變之研究 (附件三)

先前針對台灣 513 位非症候群聽障患者所篩檢到的六個 *GJB3* 基因錯意突變點 p.L10R、p.P18S、p.V84I、p.V174M、p.E183K 和 p.A194T 以及正常的 *GJB3* 基因完成選殖，並分別將其建構於 pLEGFP 或 pTagRFP 螢光表現載體上。在本研究計畫即針對這些 *GJB3* 基因錯意突變點進行研究，利用免疫螢光法探討此錯意突變所造成的影響。研究的部分成果已經發表**在 SCI 國際期刊 Biochemistry and Cell Biology 92:251~257 (附件三)**。

三、 GJC3基因(CX30.2/CX31.3)突變之研究 (附件四)

先前針對台灣513位非症候群聽障患者所篩檢到的4個GJC3基因錯意突變點，實驗室已完成正常及突變GJC3基因的選殖，並分別將其建構於螢光表現載體上。在此計畫之前也已完成GJC3基因p.E269D突變點的研究(**此結果已發表在Human Genetics 2010;127:191-199**)和GJC3基因p.R15G和p.L23H兩個錯意突變的研究(**此結果已發表在Cell Biochemistry and Biophysics 66:277-286**) 。本研究持續針對GJC3 p.W77S突變進行研究，我們的結果發現這突變會堆積在endoplasmic reticulum (ER)，且在共同轉染實驗中發現此突變有dominant negative effect。另外，我們發現突變蛋白無法持續穩定表現於HeLa細胞中，進一步的分析發現這些突變蛋白會被lysosomes and proteosomes所降解，此結果已撰寫成論文，目前正在投稿中(附件四)。

計劃成果結論和建議:

我們已將本計畫的部分研究成果以壁報的方式在**103年「生醫年會」研討會發表(如附件一)**，同時我們也發表了兩篇論文在國際期刊(附件二和附件三)，另有一篇相關的文章正在投稿中(附件四)。在本研究計畫中我們已證實部分正常及突變gap junction通道蛋白基因在細胞內的表現情形及功能。在這一年所獲得的經驗和研究成果，提供了相關的資訊，可作為後續研究gap junction通道蛋白基因造成非症候群聽障致病機轉的參考。

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附件一

Functional Study of *GJB4* Gene Mutation in Nonsyndromic Deafness Using Cell Model

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ABSTRACT

Gap junctions (GJ) are groups of intercellular channels that allow transport of molecules with size less than 1KD. Each GJ is composed of two hemichannels, or connexons, which are themselves each constructed out of six connexin (CX) molecules. Mutations in the CX gene family, including *GJB2*, *GJB3*, *GJB4*, *GJB6*, *GJC3*, and *GJA1*, have been shown to underlie distinct genetic forms of hearing loss. Recently, we have identified five missense mutations in *GJB4* (CX30.3) gene, whose defect cause hearing loss. However, the functional change in these mutants remains unknown. In this study, we want to know whether the function of GJ will be interfered by these variations. To determine the functional phenotypes of these mutations, we transfected GJ-deficient HeLa cells with WT or mutants CX30.3 fused with TagRFP (TagRFP::CX30.3) or pLEGFP (pLEGFP::CX30.3). Wild-type or mutant CX30.3 protein expression in HeLa cells was analyzed by a direct fluorescent protein fusion method involving fusion of EGFP or RFP to the C-terminal ends. This membrane localization was confirmed by colocalization with pan-Cadherin antibody. Mounted slides were visualized and photographed using a fluorescence microscope. In fluorescent localization assay, we found that TagRFP::CX30.3WT were accumulates in the cytoplasm near the nucleus. The same results were also observed in the HeLa cells with WT pLEGFP::CX30.3. Mutants pLEGFP::CX30.3R22C, pLEGFP::CX30.3V37M, pLEGFP::CX30.3V74M, and pLEGFP::CX30.3R101H, and pLEGFP::CX30.3C169W were also accumulates in the cytoplasm near the nucleus, which is similar to the CX30.3 WT. In addition, we found that cells expressing both CX30.3WT and the CX31WT protein exhibited co-assembly expression in the cell membrane of HeLa cells. The expression pattern of HeLa cells was similar to previous study.

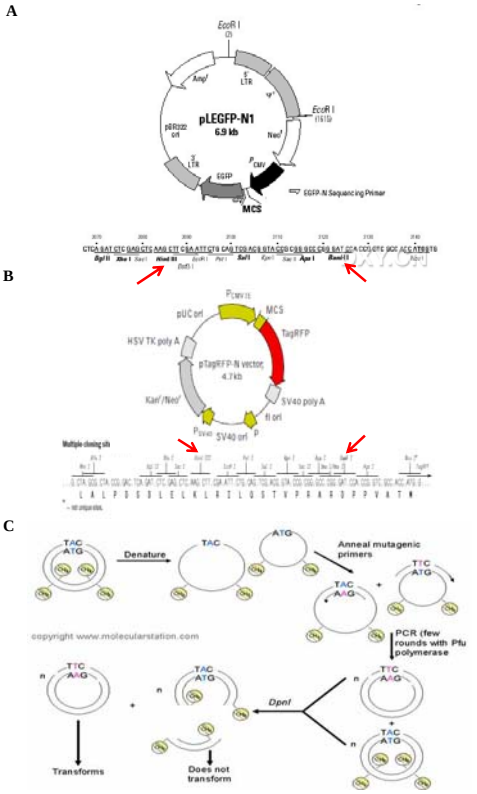

Fig 1. Construction of wild-type Cx30.3 or Cx31 gene in LEGFP or TagRFP vector and mutant Cx30.3 gene in LEGFP or TagRFP vector cDNA sequences of the autofluorescent reporter proteins LEGFP (pLEGFP-N1 vector; **A**) and TagRFP (pTagRFP-N vector; **B**) were fused in-frame to the sequences encoding wild-type (wt) Cx30.3 proteins at their C-terminus. Constructed mutant Cx30.3 gene in LEGFP using Site-Directed Mutagenesis (flow chart of Site-Directed Mutagenesis; **C**) encoding mutant CX30.3 protein at their C-terminus. All constructs were verified by sequencing.

Table 1. Completion of constructed plasmid used in the cell model

Fluorescent protein	Interested gene	Constructed plasmid
LEGFP	*Cx30.3*^{WT}	pLEGFP::*Cx30.3*^{WT}
	Cx30.3^{R22C}	pLEGFP::*Cx30.3*^{R22C}
	Cx30.3^{V37M}	pLEGFP::*Cx30.3*^{V37M}
	Cx30.3^{V74M}	pLEGFP::*Cx30.3*^{V74M}
	Cx30.3^{R101H}	pLEGFP::*Cx30.3*^{R101H}
	Cx30.3^{C169W}	pLEGFP::*Cx30.3*^{C169W}
TagRFP	*Cx30.3*^{WT}	pTagRFP::*Cx30.3*^{WT}
	Cx30.3^{R22C}	pTagRFP::*Cx30.3*^{R22C}
	Cx30.3^{V37M}	pTagRFP::*Cx30.3*^{V37M}
	Cx30.3^{V74M}	pTagRFP::*Cx30.3*^{V74M}
	Cx30.3^{R101H}	pTagRFP::*Cx30.3*^{R101H}
	Cx30.3^{C169W}	pTagRFP::*Cx30.3*^{C169W}

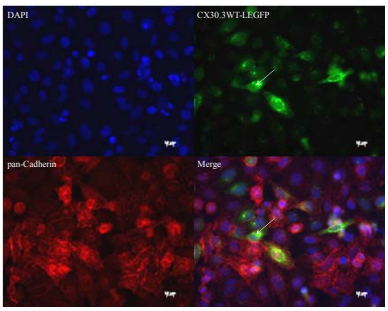

Fig.2 Expression analysis of CX30.3WT-LEGFP in transiently transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells transiently expressing **CX30.3WT-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3WT-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

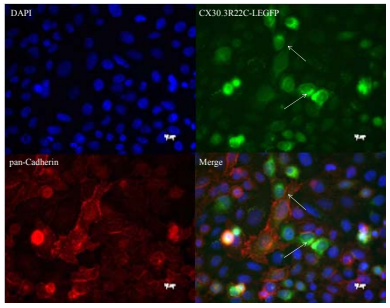

Fig.3 Expression analysis of CX30.3R22C-LEGFP in transiently transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells transiently expressing **CX30.3R22C-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3R22C-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

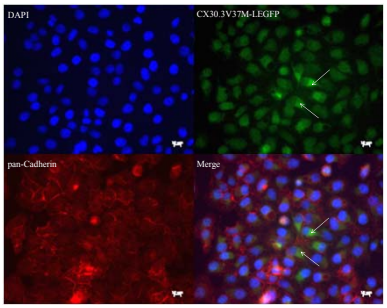

Fig.4 Expression analysis of CX30.3V37M-LEGFP in stable transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells stably expressing **CX30.3V37M-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3V37M-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

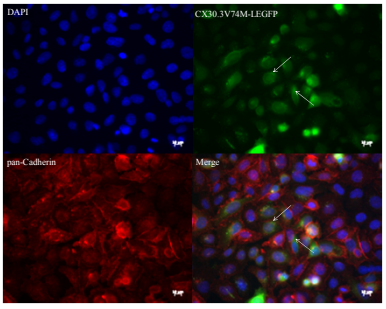

Fig.5 Expression analysis of CX30.3V74M-LEGFP in transiently transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells transiently expressing **CX30.3V74M-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3V74M-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

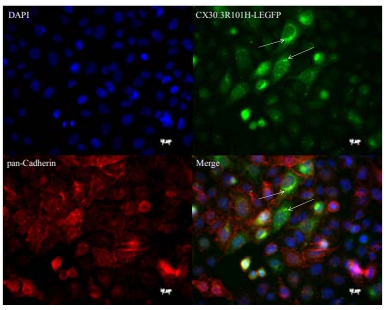

Fig.6 Expression analysis of CX30.3R101H-LEGFP in transiently transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells transiently expressing **CX30.3R101H-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3R101H-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

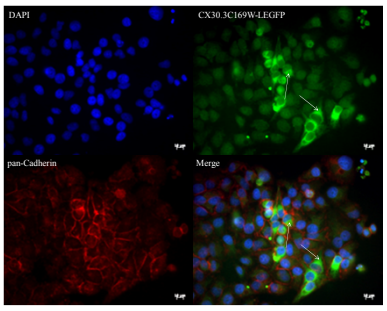

Fig.7 Expression analysis of CX30.3C169W-LEGFP in stable transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells stably expressing **CX30.3C169W-LEGFP** in the cytoplasm. White arrows indicate expression of CX30.3C169W-LEGFP. The cells were counterstained with DAPI to highlight the nuclei. Scale bars : 10 μm.

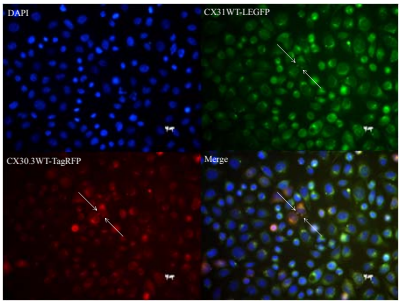

Fig.8 Expression analysis of co-transfection of CX31WT-LEGFP/CX30.3WT-TagRFP in transiently transfected HeLa cells counterstained with DAPI to highlight the nuclei. Fluorescence microscopy of HeLa cells expressing **CX31WT-LEGFP/CX30.3WT-TagRFP** in the cell membrane. White arrows indicate expression of CX31WT-LEGFP/CX30.3WT-TagRFP. Scale bars : 10 μm.

CONCLUSION

1. Only **CX30.3 WT protein** do not form gap junction.
2. **CX30.3WT** formed gap junction plaque between contiguous cells with **CX31WT** when they were co-expressed in HeLa cells.
3. Maintenance of the function of gap junction formed between mutant CX30.3 and CX31 WT protein requires further study.
4. The study of CX30.3 (*GJB4*) variants should help in defining the role of *Cx30.3* in the hearing loss.

ACKNOWLEDGEMENT

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附件二

Association between mutations in the gap junction β4 gene and nonsyndromic hearing loss: Genotype-phenotype correlation patterns

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Abstract. Numerous studies have confirmed that gap junctions, composed of connexin (Cx) protein, are essential for auditory function. However, few studies have investigated the correlation between variants in the gap junction β4 (*GJB4*) gene and phenotype in patients with nonsyndromic hearing loss. Our previous study identified 11 patients with *GJB4* gene variants in 253 unrelated patients with nonsyndromic hearing loss. In the present study, the phenotype-genotype correlation was examined in the 11 deaf patients with the different variants of *GJB4*. Analytical results revealed that the majority of probands had congenital hearing loss, which was bilateral, stable and without associated dermatological manifestations or morphological changes of the inner ear. An audiometric profile, including the observed consistency with severe-profound and flat shape dominance, may enable screening for variants of *GJB4*. On the basis of the above results, it was hypothesized that *GJB4* may be a genetic risk factor for the development of nonsyndromic hearing loss and the data from the present study can be used to direct the clinical evaluation and effectively manage the care of families of children with *GJB4*.

Introduction

Hearing impairment is the most common sensory disorder worldwide (1) and genetic inheritance presents a major source of the auditory system dysfunction resulting in hearing loss (2). Presently, 54 gene loci associated with an autosomal dominant mode of inheritance and 67 gene loci associated with an autosomal recessive mode of inheritance have been identified, of which seven are X chromosome-linked and four are mitochondrial (3). The cochlea is a complex organ in the ear, which is composed of several cell types and specialized regions that are involved in the normal process of hearing. A number of genes have been associated with hearing loss and several corresponding proteins have been identified as being expressed in the cochlea. Ionic homeostasis in the cochlear duct is associated with a several genes associated with deafness (4). In mice, endolymph (the fluid surrounding the upper surface of the hair cell) has a high concentration of potassium and a low concentration of sodium, and is maintained at a high positive resting potential of approximately +100 mV. This high resting potential is considered to be essential for the normal functioning of hair cells as, when its value is reduced to zero, deafness occurs (5).

Communication between the majority of cells in animal tissues is mediated by unique intercellular cytoplasmic channels, gap junctions, spanning across two cell membranes. These cell-to-cell channels consist of assemblies of proteins termed connexins (Cxs) or pannexins in vertebrates and innexins in invertebrates (6). Cxs belong to a protein family of >20 members, each of which is encoded by a different gene and they are assigned a number which is associated with their approximate molecular weights. Cxs share a common structure of four transmembrane segments, which extend into two extracellular and three cytoplasmic domains (7). Gap junction intercellular communication has a range of functions in order to meet the requirements of the organs, tissues and cell groups in which the Cx genes are expressed (8), and the importance of these gap junctions in auditory functions has been confirmed by numerous studies (9-13). In the sensory epithelia of the inner ear, gap junction channels are important in the recycling of potassium ions that enter the hair cells and are also involved in auditory signal transduction (14).

Immunolabeling analysis has identified several types of Cx product, including Cx26, Cx29, Cx30, Cx31 and Cx43, in the mature cochlea (10-12,15-17). Through immunohistochemical and reverse transcription-quantitative polymerase chain reaction analyses, our previous study indicated that Cx30.3 is present and localized in the rat cochlea (18). In addition, a study of 555 deaf patients revealed a common (4.1%; 23/555)

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Key words: connexin 30.3, hearing loss, phenotype, genotype, gap junction β4, gap junction

frameshift mutation (c.154del4) in gap junction β4 (*GJB4*, also termed Cx30.3) in deaf individuals (18). In the study, five amino acid variants (c.307 C>T, c.371 G>A, c.478 C>T, c.507 C>G and c.611 A>C) were detected in deaf individuals without skin disorders (19). In our previous genetic survey of 373 individuals, including 253 with nonsyndromic deafness and 120 with normal hearing, 11 mutations were detected in the patients with hearing loss (20). However, the correlation between the *GJB4* gene mutations and the audiology phenotype in deaf patients was not examined. Therefore, the present study investigated the phenotype-genotype correlation in deaf patients with mutations in *GJB4*, the results of which may provide assistance in the clinical evaluation and effective management of care for families of children with *GJB4*.

Materials and methods

Patient selection. A total of 253 individuals with hearing loss were screened for *GJB4* variants in the present study. For the patients with hearing loss, a total of 173 school children were selected from the National Tainan School for the Deaf (Tainan, China) and 80 individuals with hearing loss, who were managed at the Chang Gung Memorial Hospital (Chiayi, China), were selected. The frequency range of hearing loss was between 250 and 8,000 Hz, with a mean threshold (500, 1,000, 2,000 and 4,000 Hz) of >40 dB in the right and left ears. All probands were 17 years old or younger at the time of molecular diagnosis. In the present study, the 11 patients with *GJB4* missense and nonsense mutations had complete audiograms and were used for analysis.

Patients with syndromic hearing loss or environment-associated hearing loss were excluded from the present study, as determined by an otorhinolaryngologist. The complete medical history of each child was obtained to determine the age of onset of deafness and to exclude the possibility of environmental causes, including maternofetal infection, perinatal complications, meningitis, mumps, prenatal or postnatal drug ototoxicity and acoustic trauma. All procedures were approved by the Institutional Review Board of Chung Gung Memorial Hospital (96-1294B). Written informed consent was obtained from all patients.

Clinical evaluation. The genetic and audiological data were categorized according to recommendations on genetype-phenotype correlations by the Genetic Deafness Study Group (21). According to these guidelines, the groups were recognized as follows: Mild hearing loss (20-40 dB), moderate hearing loss (41-70 dB), severe hearing loss (71-95 dB) and profound hearing loss (>95 dB). The audiometric configurations were determined for each ear by differences in hearing level (HL) as follows: Ascending low frequency, >15 dB difference in HL between the poorer low frequency thresholds and the higher frequencies; U-shaped mid frequency, >15 dB difference in HL between thresholds at the poorest mid-frequencies and those at higher and lower frequencies; gently sloping high frequency, 5-29 dB difference in HL between the mean thresholds at 0.5 and 1 kHz and at 4 and 8 kHz; steeply sloping high frequency, >30 dB difference in HL between the above-mentioned frequencies; and flat, <15 dB difference in HL between the mean thresholds at 0.25 and 0.5 kHz, 1 and 2 kHz and 4 and 8 kHz. Asymmetric HL was defined as an interaural pure tone average (PTA) difference of >10 dB in at least two frequencies.

Computed tomography (CT) of the inner ear. CT images of the inner ears were examined in 11 probands in the cohort of the present study. All the images examined were high resolution 1-mm contiguous, axial and coronal images of the temporal bones. Digital or printed images were evaluated for abnormalities of the cochlea, vestibule, semicircular canals and endolymphatic aqueduct.

Results

Severity and configuration of hearing impairment and genotype. In our previous study, a total of nine different *GJB4* mutations were identified in 11 of the 253 probands (19). Of these mutations, eight were missense variants that led to amino acid substitution in the encoded proteins and one was a nonsense mutation. No vestibular symptoms or skin disorders were observed in any individual. Genetic assessment facilitates the determination of the cause of deafness and the prediction of the degree of hearing impairment and language development (22). Therefore, the present study investigated the phenotype-genotype correlation in the 11 deaf patients with mutations of *GJB4*. The severity of hearing impairment was assessed in the 11 patients with the *GJB4* mutations (Table I; Fig. 1), and the four-frequency PTA was calculated as the average of air-conduction thresholds at 500, 1,000, 2,000 and 4,000 Hz. The mean (± standard deviation) threshold of hearing for all *GJB4* mutations was 97.16 dB (± 13.52 dB). In the present study, 10 probands with the *GJB4* mutation were observed to have symmetrical HL. Asymmetric HL was observed in only one proband (TDF547), with an interaural PTA difference of 20 dB. This proband had a c.109G>A/WT heterozygous genotype. In addition, one proband (TDF521) was identified with a compound missense heterzogous mutation (c.376G>A/c.507C>G) of *GJB4* and had more severe hearing loss, compared with the proband exhibiting a heterozygous missense mutation (c.376G>A/wt), in the right and left ears (Fig. 2).

Cx30.3 protein structure and hearing loss. Similar to other Cx proteins, Cx30.3 consists of four transmembrane (TM) domains, TM1 (amino acid 21-40), TM2 (amino acid 76-98), TM3 (amino acid 127-149) and TM4 (amino acid 188-210). These are linked by one cytoplasmic and two extracellular loops with cytoplasmic C- and N-terminal ends. The p.R22C and p.V37M substitutions detected in the present study occurred in TM1 of Cx30.3, and the p.E67L and p.C169X substitutions occurred in the first extracellular loop (E1) and the second extracellular loop (E2) of Cx30.3, respectively. In addition, three variants, p.R98C, p.R124W and p.G126T, were located at the cytoplasmic domain and two variants, p.H221Y and p.T233L, were located at the C-terminal domain. The relative predictive values of PTA were then examined in the right and left ears of the patients with the *GJB4* mutations (Table I). The hearing threshold results revealed that cytoplasmic linking (CL) domain mutations of the Cx30.3 protein had a PTA of 68-72 dB, with the exception of the p.R124W missense mutation. However, the mean PTA was >96 dB in the other domains of the Cx30.3 protein (Table I), suggesting that the

Table I. Audibility thresholds for air conduction in pure tone audiometry of the 11 patients with gap junction β4 missense and nonsense variants at frequencies between 250 and 8,000 Hz.

Patient	Genotype variant (amino acid change)	Protein domain	Frequency (Hz)							Mean threshold[a]
			Ear	250	500	1,000	2,000	4,000	8,000	
KDF026	c.64 C>T/WT	M1	R	AR	95	100	100	90	AR	96.3±4.8
	(pR22C)		L	AR	105	95	110	105	AR	103.8±6.3
TDF547	c.109 G>A/WT	M1	R	70	90	100	100	110	100	100.0±8.2
	(p.V37M)		L	90	100	100	100	110	100	102.5±5.0
TDF553	c.109 G> A/WT	M1	R	95	100	100	105	115	100	105.0±7.1
	(p.V37M)		L	105	115	120	120	115	100	117.5±2.9
TDF067	c .199G >A/WT	El	R	110	110	110	110	110	110	110.0±0.0
	(p.E 67L)		L	110	110	110	110	110	110	110.0±0.0
CDF006	c.292 C>T/WT	CL	R	70	70	75	70	75	75	72.5±2.9
	(pR.98C)		L	60	65	75	70	70	70	70.0±4.1
LDF011	c.370 C>T/WT	CL	R	AR	95	105	105	100	AR	101.3±4.8
	(p.R124W)		L	AR	90	100	95	100	AR	96.3±4.8
TDF512	c376G>A/WT	CL	R	60	65	65	65	80	AR	68.8±7.5
	(p.G126T)		L	55	60	75	80	80	AR	73.8±9.5
LDF014	c.507 C>A/WT	E2	R	55	85	95	100	110	100	97.5±10.4
	(p.0 169X)		L	55	85	95	100	110	100	97.5±10.4
KDF012	c.661 C>T/WT	C	R	AR	100	100	100	110	AR	102.5±5.0
	(p.H221Y)		L	AR	90	100	110	110	AR	102.5±9.6
TD F035	c.698C>A/WT	C	R	90	100	100	110	110	100	105.0±5.8
	(p.T233L)		L	90	110	100	110	110	100	107.5±5.0
TDF521	c.376G>A/c.507 C>G	CL/E2	R	80	85	90	105	110	100	97.5±11.9
	(p.G126T)/(p.C169W)		L	80	95	100	105	100	95	100.0±4.1

[a]Mean, calculated from the frequencies of 500, 1,000, 2,000 and 4000 Hz. AR, absent response in the maximum intensity of the device; R, right ear; L, left ear; E1, first extracellular loop; E2, second extracellular loop; M1, transmembrane domain 1; CL, cytoplasmic linking domain.

Table II. Comparison of *GJB4* and *GJB2* on the basis of audiogram shapes.

Audiogram shape	Present study *GJB4*		Present study *GJB2*		Hişmi *et al* (21) *GJB2*		Liu *et al* (22) *GJB2*	
	Ears (n)	%	Ears (n)	%	Ears (n)	%	Probands (n)	%
Flat	16	72.7	12	30	75	59.5	48	24.7
Sloping	5	22.7	20	50	50	39.6	136	70.1
U-shaped	-	0.0	4	10	1	0.8	10	5.1
Ascending	1	4.6	4	10	-	0.0	-	0.0
Total	22	100.0	40	100	126	100.0	194	100.0

GJB4/2, gap junction β4/2.

degree of PTA was lower in CL domain mutations compared with mutations in others domains of the Cx30.3 protein. In addition, in the proband with the c.507C>A (p.C169X) mutation, the degree of hearing loss was more marked at high frequencies compared with low frequencies (Table I; Fig. 1).

Configuration of hearing loss. Furthermore, the relative frequencies of the configuration of hearing impairment in patients with *GJB4* and *GJB2* genotype variants in the present study were compared with those in previous studies by Hişmi *et al* (23) and Liu *et al* (24) (Table II; Fig. 3). The results indicated that the

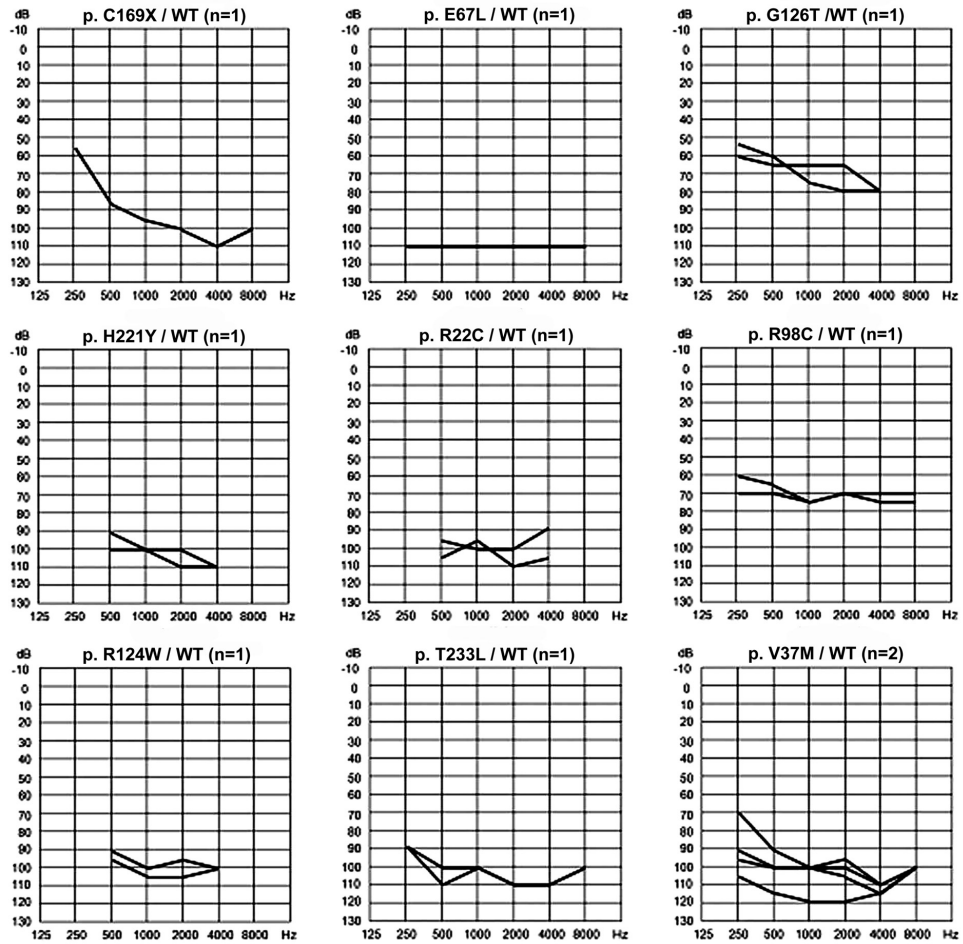

Figure 1. Audiogram of patients with heterozygous missense and nonsense variants of gap junction β4.

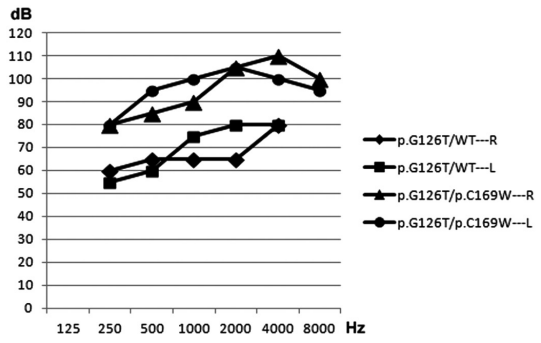

Figure 2. Audiogram of patients with a heterozygous gap junction β4 mutation (p.G126T) and a compound heterozygous mutation (p.G126T/p.C169W). R, right ear; L, left ear.

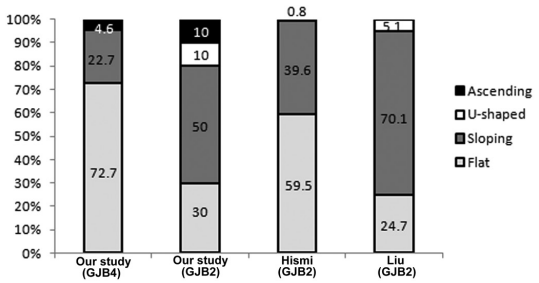

Figure 3. Relative frequencies of the configuration of hearing impairment in these genotypes. The actual number of patients is presented in Table II. The number of patients is detailed in each subgroup. P=0.016* between *GJB4* and *GJB2* in the present study. P=0.403 between *GJB4* in the present study and *GJB2* in the study by Hişmi *et al* (21). P<0.001* between *GJB4* in the present study and *GJB2* in the study by Liu *et al* (22). *P<0.05 indicates a statistically significant difference.

frequency of a flat audiometric configuration in patients with *GJB4* variants was significantly higher compared with that in patients with *GJB2* variants (P=0.016). Similarly, a significant difference was observed between the patients with *GJB4* variants in the present study and the patients with *GJB2* variants in the study by Liu *et al* (P<0.001). However, the difference in the frequency of this configuration between patients with *GJB4* in the present study and with *GJB2* in the study by Hişmi *et al* was small (P=0.403). This may be due to the difference in the point mutation site in the *GJB2* genotype, which was c.35delG in the Hişmi *et al* study and c.235delC in the present study, or due to different ethnicities resulting in different phenotypes. Therefore, in the present study, the flat shape was more predominant in patients with *GJB4* variants compared with *GJB2* variants, and this data may be applied to direct the clinical evaluation of children with *GJB2* or *GJB4*.

CT images of the 11 patients were also analyzed. A total of 10 probands (20 ears) exhibited normal CT images of the

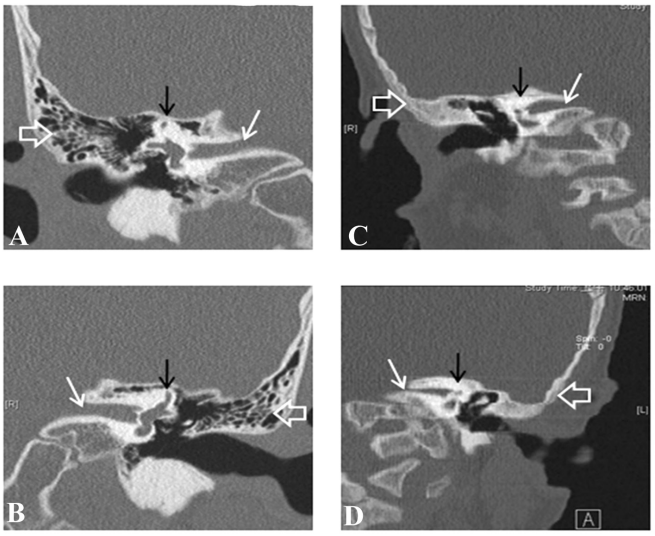

Figure 4. Computer tomograms of temporal bone in a normal subject and in a patient with inner ear and middle ear deformities. Normal IAC (white arrow), semicircular canals (black arrow) and mastoid air cells (open arrow) on the (A) right side and (B) left side in the normal subject. Bilateral IAC stenosis (white arrow), shortening of the superior and lateral semicircular canals (black arrow) and nonpneumatization of mastoid air cells (open arrow) on the (C) right side and (D) left side in a patient with the c.370 C>T heterozygous genotype. IAC, internal auditory canal.

temporal bones. In one patient (LDF011) with a c.370 C>T heterozygous genotype, inner ear and middle ear deformities were observed (Fig. 4). The CT findings included bilateral stenosis of the inner auditory canal, which was greater on the left side, bilateral shortening of the superior and lateral semicircular canals and bilateral non-pneumatization of the mastoid air cells. In conclusion, only one of the 11 patients (9%) with the *GJB4* variant in the present study had a morphological abnormality of the inner ear, as indicated on the CT images. Therefore, the number of patients with morphological abnormalities of the inner ear in the cohort was low.

Discussion

Several genetic studies have revealed the importance of Cxs in normal cochlear function (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/). Few studies have been conducted on the correlation of variants in the *GJB4* gene and its phenotype in patients with nonsyndromic hearing loss. These previous studies were compared and summarized in Table I (13,19,20). In these results, the proportion of patients with *GJB4* variants was determined to be 4.09% (21/513). The present study identified that variation in *GJB4* is the second most common genetic risk factor in the Cx gene family for the development of hearing loss in this population. In addition, the phenotype of patients with variants of Cx30.3 included prelingual, bilateral, severe-to-profound hearing loss. A flat audiometric configuration was also more frequently detected in patients with *GJB4* (Cx30.3) variants compared with patients with *GJB2* variants.

In total, >20 different Cx proteins have been identified in mammals. They all share a common structure, however, each has its own tissue distribution-specificity, electrophysiological characteristics and regulatory properties (25). Electrophysiological studies have indicated that gap junctions have multiple gating mechanisms. At least two regulation mechanisms respond to transjunctional voltage (Vj), including Vj gating (fast) and loop gating (slow) (26). In addition, membrane voltage (Vm) can also gate gap junctions, termed Vm-gating, and by chemical factors, including the phosphorylation, pH and Ca^{2+}, which is termed chemical gating (27). Therefore, patients may exhibit different phenotypes between mutations in different functional domains of the Cx protein.

A three-dimensional (3D) characterization of protein structures can be used to explain the functions of proteins and their disease formation associations (28,29). High-resolution characterization of proteins can be provided by either experimental methods, including X-ray crystallography, nuclear magnetic resonance or computational analysis (29). However, there is a significantly higher number of known protein sequences compared with experimentally solved protein structures. Use of a method comprising reliable models of proteins, which share ≥30% sequence identity between known structures and target proteins (28,30), may assist in understanding the function of target proteins in the absence of crystallographic structures. The crystalline structure of the gap junction channel, which is formed by human Cx26, has been previously described (31,32) and the N-terminal and TM13 domains have been identified as important in the permeation pathway of a gap junction channel with an intracellular channel entrance, pore funnel and extracellular cavity (31,32). In addition, analysis of the crystalline structure revealed that the TM2, TM4, E1 and E2 domains of Cx are associated with the structural organization of the hexameric connexon, and two neighbor connexons of the gap junction channel interact with the E1 and E2 domains (31). In classifying the Cx protein, human Cx26 and Cx30.3 are referred to as the same subgroup, termed group I or the β group, in phylogenetic tree analysis (33). Therefore, the Cx26 crystalline structure may assist in explaining why, in the present study, mutants in the CL domain of Cx30.3 affected the degree of hearing loss compared with the other functional domains of Cx30.3. However, in the present study, the functional effect of Cx30.3 was a prediction and the real functional effect remains to be elucidated. Therefore, in order to further investigate the effect of these variants at the protein level, the 3D structure of the Cx30.3 protein requires investigation.

The c.507C>G (p.C169W) missense mutation has been found in patients with nonsyndromic hearing loss (13,20). The results of the present study revealed that the heterozygous c.507C>G mutation was present in the normal hearing control group. In addition, the proband containing the homozygous c.507C>G mutation was inherited from parents with normal hearing, suggesting that the c.507C>G missense mutation had a recessive inheritance pattern (13). In the present study, a patient carrying the compound heterozygous mutation, c.376G>A/c.507 C>G (p.G126T)/p.C169W), had more serious hearing loss in the right and left ears compared with a patient carrying only a heterozygous mutation (c.376G>A/wt) (Fig. 2). This result demonstrated that the combination of two genetic mutations leads to a disease phenotype, however, this phenotype is not present or is present in a mild form when only one of these gene mutations is present. Analysis using the ConSeq server (34), a web server for the identification of

structurally and functionally important residues in protein sequences, determined that the location of position 169 in the Cx30.3 protein was at E2, which is exposed and highly conserved throughout evolution. The variants of p.C169 at E2 may result in incompatibility between the different species of connexin proteins to form heterotypic functional channels (35). Therefore, in the present study, it was hypothesized that the c.507C>G mutant of *GJB4* is a modifier and risk factor in the development of hearing loss.

In addition, no vestibular symptoms or skin disorders were found in patients with *GJB4* gene variants. Notably, one patient with the c.370 C>T heterozygous genotype had inner ear and middle ear deformities on CT analysis, whereas the other patients with Cx30.3 variants were normal. Therefore, it was suggested that c.370 C>T heterozygous variants of *GJB4* provide an important base for improving the clinical diagnosis of deaf patients with inner ear and middle ear deformities.

The present study demonstrated that *GJB4* may be genetic risk factor for the development of nonsyndromic hearing loss, and the data can be applied for the effective clinical evaluation and management of care for families of children with *GJB4*. Further investigation will be required to understand how interference of the mutation contributes to hearing loss. In addition, it may used in future prenatal genetic analysis.

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ARTICLE

Mechanism of a novel missense mutation, p.V174M, of the human connexin31 (*GJB3*) in causing nonsyndromic hearing loss

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Abstract: Hearing loss is the most common sensory disorder, worldwide. In a recent study, we have identified a missense mutation, p.V174M, in the connexin 31 encoded by the *GJB3* gene, in a patient with nonsyndromic hearing loss. However, the functional change in the CX31V174M mutant remains unknown. This study compared the intracellular distribution and assembly of the mutant CX31V174M with that of the wild-type (WT) CX31 in HeLa cells, and it examined the effect that the mutant protein had on those cells. A fluorescent localization assay of WT CX31 showed the typical punctuate pattern of a gap junction channel between the neighboring expression cells. Conversely, the p.V174M missense mutation resulted in the accumulation of the mutant protein in the lysosomes rather than in the cytoplasmic membrane. Moreover, dye transfer experiments have also demonstrated that the CX31V174M mutant did not form functional gap junction channels, probably due to the incorrect assembly or the altered properties of the CX31 channels. In addition, we found that CX31V174M-transfection can cause cell death by MTT assay. CX31V174M co-expressed with either CX31WT or CX26WT studies, suggested the impairment of the ability of CX26WT proteins to intracellular trafficking and targeting to the plasma membrane, but did not influence the trafficking of CX31WT. Based on these findings, we suggest that the CX31V174M mutant may have an effect on the formation and function of the gap junction, and CX31V174M has a *trans*-dominant negative effect on the function of wild types CX26. These results provide a novel molecular explanation for the role that *GJB3* plays in hearing loss.

Key words: CX31, *GJB3*, mutation, hearing loss.

Résumé : La perte auditive est le trouble sensoriel le plus fréquent à travers le monde. Dans une récente étude, les auteurs ont identifié une mutation non-sens dans le gène *GJB3* codant la connexine 31, p.V174M, chez un patient présentant une perte auditive non-syndromique. Cependant, le changement fonctionnel que produit la mutation CX31V174M demeure inconnu. Cette étude a comparé la distribution intracellulaire et l'assemblage du mutant CX31V174M et de la CX31 sauvage dans les cellules HeLa, et examiné l'effet que la protéine mutante exerçait dans ces cellules. Un test de localisation en fluorescence de la CX31 sauvage a révélé un patron ponctué typique des canaux des jonctions communicantes entre les cellules adjacentes qui les expriment. En revanche, la mutation non-sens p.V174M provoquait une accumulation de la protéine mutante dans les lysosomes plutôt que dans la membrane cytoplasmique. De plus, des expériences de transfert de colorant ont aussi démontré que le mutant CX31V174M ne formait pas de canaux fonctionnels dans les jonctions communicantes, probablement à cause d'un assemblage incorrect ou de propriétés déficientes des canaux de CX31. En outre, les auteurs ont trouvé par un dosage au MTT que la transfection de CX31V174M peut provoquer la mort cellulaire. Des études de co-expression de CX31V174M avec la CX31 ou la CX26 sauvages suggéraient que le trafic intracellulaire et le ciblage à la membrane plasmique de CX26 sauvage étaient diminués, contrairement au trafic de CX31 sauvage. Les auteurs suggèrent sur la base de ces résultats que le mutant CX31V174M puisse exercer un effet sur la formation et la fonction des jonctions communicantes, et que CX31V174M possède un effet négatif *trans*-dominant sur la fonction de CX26 sauvage. Ces résultats fournissent une explication moléculaire inédite du rôle de *GJB3* dans la perte auditive. [Traduit par la Rédaction]

Mots-clés : CX31, *GJB3*, mutation, perte auditive.

Introduction

Hearing loss, a common sensory disorder in the human population, is often caused by genetic inheritance of auditory system dysfunction (Apps et al. 2007). In developed countries, the estimated incidence of congenital hearing loss is 1 in 1000 births, of which approximately 60% are attributable to genetic factors (Morton 1991; Pallares-Ruiz et al. 2002). Of the genes responsible for deafness, 59 have been identified, some of which are the genes involved in ion recycling and maintenance (Morton 2002).

Connexins (CXs/Cxs) are the major constituents of the gap junction (GJ) channel, i.e., an important pathway for intercellular communication of ions and small biological molecules (<1 kDa) between adjacent cells. Each GJ consists of the docking of two independent hemichannels (or connexons) in the appositional plasma membranes of the two contacting cells. Six connexin mol-

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ecules assemble in an intracellular compartment (ER-Golgi) to form a hemichannel that is distributed to the plasma membrane by the secretory pathway (Saez et al. 2003). Hemichannels may also display functional roles at the plasma membrane, including the propagation of electrical signals, metabolic cooperation, growth control, the spatial buffering of ions, and cellular differentiation (Bruzzone et al. 1996). Mutations in connexin genes have been found in several disorders, such as X-linked Charcot-Marie-Tooth peripheral neuropathy, cataracts, and hearing loss (Bergoffen et al. 1993; Krutovskikh and Yamasaki (2000).

So far, six CX genes (CX26, CX29, CX30, CX30.3, CX31, and CX43) are known to be involved in human genetic deafness (Kelsell et al. 1997; Xia et al. 1998; Grifa et al. 1999; López-Bigas et al. 2002; Yang et al. 2007; Wang et al. 2010, Yang et al., 2010). The proteins they encode are located in the GJ-rich regions of the cochlear duct, suggesting that all six connexin proteins are essential components of GJs. The loss of connexin in the GJ complexes in the cochlea would be expected to disrupt the recycling of potassium from the synapses at the base of the hair cell through the supporting cells and the fibroblasts back to the high potassium-containing endolymph of the cochlear duct, thereby resulting in hearing loss due to local potassium intoxication of the organ of Corti (Kikuchi et al. 1995).

The *GJB3* gene (NM 024009), which contains two exons and an open reading frame of 813 base pairs, is localized on chromosome 1p34. The *GJB3* gene product, CX31 protein, contains 270 amino acid residues and has a molecular weight of 31 kDa (Xia et al. 1998; Wenzel et al. 1998). *GJB3* is highly expressed in the stratum granulosum in the upper differentiating layer of the epidermis (Common et al. 2005; Di et al. 2001), and it is also found in peripheral nerves and the cochlea (López-Bigas et al. 2001). Many studies have found a significant correlation between the *GJB3* gene mutation and erythrokeratodermia variabilis (EKV). Two amino acid variants (G12R and G12D) have been found in CX31 in the four EKV families (Richard et al. 1998). In addition, multiple autosomal dominant (R42P, C86S, and F137L) (Wilgoss et al. 1999; He et al. 2005) and recessive (L34P) (Gottfried et al. 2002) CX31 mutations have been found to be associated with EKV. *GJB3* (CX31) mutations have also been linked to nonsyndromic hearing loss. Xia et al. (1998) reported that the mutations (E183K and R180X) in CX31 may cause an autosomal dominant form of nonsyndromic hearing impairment. Lopez-Bigas et al. (2001) reported that a dominant mutation (D66del) in the *GJB3* gene affected peripheral neuropathy and hearing impairment. In contrast, the *GJB3* gene with a compound heterozygote (423-425delATT/I141V) mutation is a recessive mutation. The compound heterozygote mutation will damage the M3 domain of CX31 to affect the function of the CX31 protein and cause hearing loss (Liu et al. 2000).

At least five missense mutations [c.53C > T (P18S), c.250G > A (V84I), c.520G > A (V174M), c.547G > A (E183K), and c.580G > A (A194T)] of the *GJB3* gene have been detected in Taiwanese patients with nonsyndromic hearing loss in our previous studies (Yang et al. 2007; Yang et al. 2010). These findings demonstrate the requirement of CX31 for normal cochlear function and suggest that *GJB3* is a new candidate gene for studying auditory neuropathy. To better understand the pathogenic role that *GJB3* mutation plays in nonsyndromic hearing loss, it is necessary to investigate the functional properties of the mutant CX31 GJs. In the present study, we investigated the effect of the p.V174M (c.520G > A) mutation on the functional properties and the subcellular localization of the mutant CX31 protein in HeLa cells.

Materials and methods

Construction of plasmids capable of expressing WT and mutants CX31

For expression in HeLa cells, a full length cDNA of amplified human CX31WT cDNA was cloned into a pLEGFP vector (Invitrogen, Carisbad, Clif., USA) using a designed oligonucleotide primer pair, 5′-CAGAAGCTTATGGACTGGAAGACACTCCAG-3′ and 5′-ATAG TCGACAAGATGGGGGTCAGGTTGGGT-3′, to generate 5′Hin dIII (AAGCTT) and 3′SaII (GTCGAC) restriction sites for inframe ligation. Mutant CX31 GJ genes were obtained by performing oligonucleotide-directed mutagenesis using the Stratagene Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA). The following oligonucleotide primers (mutated nucleotide is underlined) were used to prepare the mutant *GJB3* gene: CX31V174M sense 5′-CCTGCCCCAACATCATGGACTGCTACATTGC-3′ and CX31V174M antisense 5′-GCAATGTAGCAGTCCATGATGTTGGGGCAGG-3′. The dideoxy DNA sequencing method, using a DNA Sequencing Kit (Applied Biosystems Corporation, Foster City, Calif., USA) with an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Corporation, Foster City, Calif., USA), was used to confirm the DNA sequence of all constructs.

Transfection and expression of CX31WT, CX31V174M, CX31WT/CX31V174M and CX26WT/CX31V174M chimerae protein in HeLa cell line

Human epitheloid cervix carcinoma cells (HeLa, ATCC CCL 2; American Type Culture Collection, Rockville, Md., USA) lacking the *GJIC* gene were used throughout this study. Cell lines were maintained in standard cell culture medium supplemented with 10% of fetal bovine serum, 2 mmol/L of L-glutamine, and 50 units/mL of penicillin–streptomycin. Cell cultures were maintained at 37 °C in a humidifed 5% CO_2 incubator. The vectors, pLEGFP and pTaqRFP, containing the DNA fragment encoding the wild-type or mutant CX31 and CX26WT protein were transfected or co-transfected to HeLa cells using jetPRIME (Polyplus transfection, NY, USA). To obtain HeLa cell colonies that stably expressed CX31 WT or CX31 mutants, 1 mg/mL of G418 (Geneticin, Gibco–BRL, Grand Island, NY, USA) was added to the growth medium. The growth medium was renewed at 2–3 day intervals. After 2–3 weeks, single cell colonies were obtained. Under a fluorescence microscope, cells displaying either green or red fluorescence were chosen for further culture.

Immunofluorescence staining of post-transfection HeLa cells

Wild-type or mutant CX protein expression in HeLa cells was analyzed by a direct fluorescent protein fusion method involving fusion of EGFP or RFP to the C-terminal ends of the CX proteins. Briefly, post-transfection HeLa cells grown on coverslips were fixed with 4% paraformaldehyde in 0.1 mol/L PBS for 20 min and then rinsed three times in PBS. Then, the coverslips were immersed in 10% normal goat serum and 0.1% Triton X-100 for 15 min. The primary antisera and dilutions were as follows: mouse anti-pan-cadherin antibody at 1:200 (anti-CH19; abcan) for cell membrane and mouse anti-Golgin-97 at 1:200 (Invitrogen) for Golgi apparatus. After incubation with primary antiserum at 4 °C overnight, the cells were rinsed in PBS three times before adding Alexa Fluor 488 and (or) Alexa Fluor 594 conjugated secondary antibodies (Invitrogen). Endoplasmic Reticulum (ER) was stained with ER-Tracker® Blue-white DPX Probes at 1:670 dilution (Invitrogen) for 10 min at room temperature. Lysosomes were stained with LysoTracker® Red-DNA-99 Probes (Invitrogen) at 1:1000 dilution for 20 min at room temperature. The nuclei of cells were counterstained with 49-6-diamidino-2-phenylindole (DAPI; 2 μg/mL) or propidium iodide (PI; 1 mg/mL; 1:400 dilution) for 5 min and rinsed with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany). All immunofluorescence cell experiments were performed more than five times and observed more than 200 pairs coupling cells per times with similar results in the study.

Dye transfer analysis

Dye transfer capabilities of the tagged GJs in HeLa cells were examined by exchanging a medium with pre-warmed 1 × PBS and

placing the dishes on a stage heated to 37 °C, mounted on an inverted epifluorescence microscope (Zeiss Axiovert100, Oberkochen, Germany). Clusters and pairs of cells expressing GJ plaques were selected. Next, single cells of the clusters with GJ plaques visible by fluorescence illumination in their plasma membranes were microinjected with 4% solutions of Lucifer yellow (Invitrogen). The transfected HeLa cells were injected ($n > 25$), along with injection into the non-transfected cells as the mock controls ($n > 25$).

Cell growth curve analysis

The MTT assay was used to detect the proliferation rate of HeLA cells. Briefly, 6×10^4 cells of WT or mutant CX31 per well were plated in 24-well plates and incubated for 1, 2, 3, 4, 5, and 6 d, respectively. Briefly, 50 μL of MTT reagent (1 mg/mL) was added and incubated for 4 h at 37 °C in a humidified incubator containing 5% CO_2. Supernatants were removed from the wells, and then 100 μL DMSO was added to solubilize the crystal products at room temperature for 10 min. The absorbance (OD) was measured with a microplate reader (Bio-Rad) at a wavelength of 570 nm.

Statistical analysis

All data are presented as the mean ± SD of the MTT assay. The statistical analyses between HeLa, WT, and mutant groups were done using two-way analysis of variance (ANOVA) by Statistix Analytical Software (Tallahassee, Fla., USA). A P value <0.05 was considered statistically significant. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

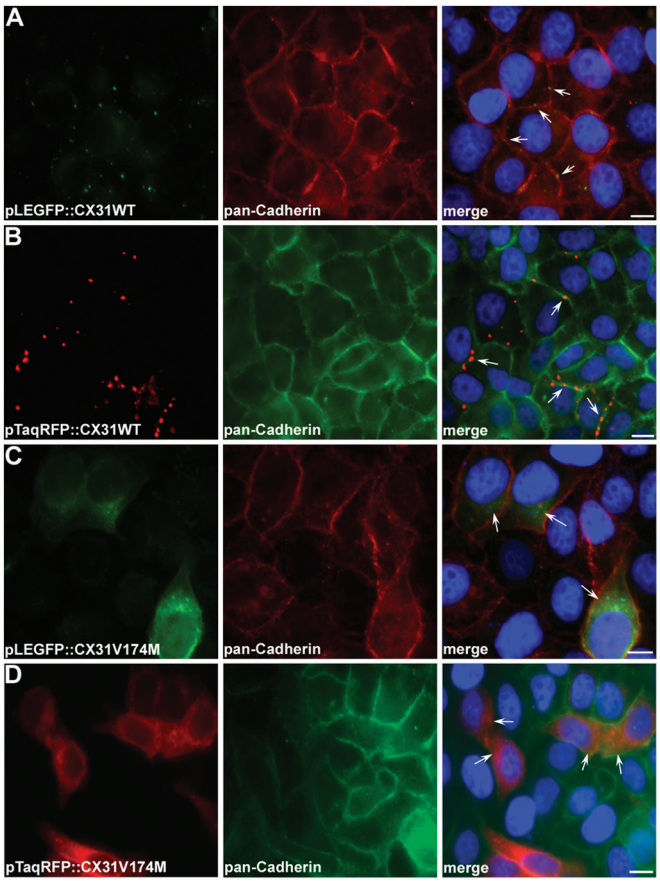
Results

The topological model based on the UniProtKB/Swiss-Prot O75712 (GJB3_HUMAN) database showed that the CX31 protein subunit, like other CX proteins, contains a short cytoplasmic amino-terminal domain (NT; amino acids 1–20) and four transmembrane domains TM, TM1 (amino acids 21–40), TM2 (amino acids 76–98), TM3 (amino acids 127–149), and TM4 (amino acids 188–210), which are separated by one cytoplasmic loop domain (CL; amino acids 99–126) and two extracellular loops (E1; amino acids 41–75 and E2; amino acids 150–187), and a carboxylterminal cytoplasmic domain (CT; amino acids 211–270). According to the predictive results mentioned above, we extrapolated that the p.V174M substitution was localized on the second extracellular loop (E2) domain (Supplementary data Fig. S1[1]). To clarify the roll of p.V174M, a basic ConSeqanalysis system (http://conseq.tau.ac.il/) was used to study the amino acid sequences of CX31 in the CX/Cx gene family of all species. After the protein sequence of CX31 was deposited, the system automatically detected homologous sequences of CX31/Cx31 and conducted multiple alignments. Of the 114 PSI-BLAST hits obtained by the system, 96 were unique sequences. In the next step, the system automatically calculated the 50 sequences with the lowest E-values. The calculation results revealed that p.V174M was highly conserved (Conseq score = 9) and buried in the CX/Cx protein (Supplementary data Fig. S2[1]). Therefore, mutation of the p.V174M residue may interfere with the normal function of the CX31 protein, and it may play an important role in GJ channel formation.

To identify the effects of p.V174M on the functional properties and subcellular localization of the CX31 protein, lipofection was used to transiently transfect the GJ-deficient HeLa cells with cDNA constructs of wild-type (CX31WT-pLEGFP) or mutant CX31 (CX31V174M-pTaqRFP) (Fig. 1). In cells transiently expressing CX31WT-pLEGFP, the GJ plaque formation was indicated by WT CX31 expression localized to the cell membranes at points of contact between the adjacent GFP-expressing cells. This membrane localization was confirmed by co-localization with pan-cadherin (Fig. 1A). Similarly, CX31WT-pTaqRFP was localized to the cell membrane (Fig. 1B). In contrast, the impaired trafficking of CX31V174M-pTaqRFP to the cell membrane resulted in cytoplasmic concentrations near the nucleus (Fig. 1C). To identify the organelles in which the cytoplasm of the mutant p.V174M of CX31 had localized, this study then analyzed HeLa cells transfected with CX31V174M cDNA using immunostaining with markers for endoplasmic reticulum (ER), lysosomes, and Golgi apparatus (Fig. 2). The results of the assay showed that the CX31V174M proteins were co-localized with ER-Tracker Blue-White DPX dye (E12353), a photostable probe that is selective for the endoplasmic reticulum in live cells (Fig. 2A). At the same time, we also observed that the CX31V174M proteins are accumulated in the lysosome using LysoTracker® Red-DNA-99 Probes (L12492), a high selectivity for acidic organelles in live cells (Fig. 2B). In contrast, we did not find the CX31V174M protein co-localized with Golgin 97 protein, which is probably involved in maintaining Golgi structure (Fig. 2C). Based on the above results, we suggest that most of the accumulation of p.V174M of the CX31 mutant proteins occurred in the ER and lysosomes.

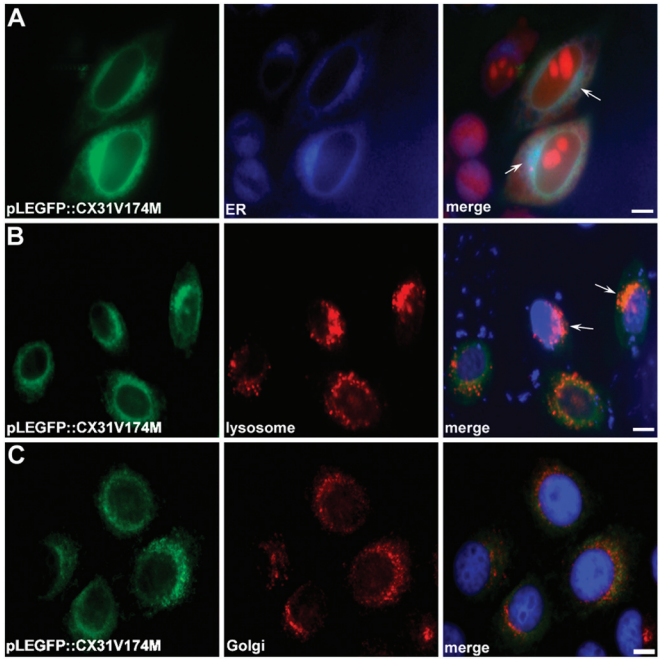
The distribution pattern of the mutant CX31 proteins appeared to be different from the distribution pattern of the WT CX31 in the transfected cells (Fig. 1), indicating that the mutant of CX31 impairs the ability for trafficking to the cell membrane. To confirm

Fig. 1. Expression analysis of CX31WT and CX31V174M in transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of CX31WT-pLEGFP (A) and CX31WT-pTaqRFP (B) HeLa cells showing expression of CX31 fusion protein in the plasma membranes. However, CX31V174M-pLEGFP (C) and CX31V174M-pTaqRFP (D) transfected HeLa cells show impaired trafficking of the CX31 protein with localization near the nucleus. The cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) to highlight the nuclei. Scale bars: 10 μm.



[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/bcb-2013-0126.

Fig. 2. Intercellular localization of mutant CX31 proteins. Photomicrographs of HeLa cells transfected with CX31V174M-pLEGFP cDNA after immunostaining for markers of the ER (ER-Tracker® Blue-white DPX Probes), lysosome (LysoTracker® Probes), and Golgi apparatus (red in (A)–(C), respectively). Arrows in the image overlays (right column) indicates co-localization of CX31V174M-pLEGFP and the organelle of interest. Mutant CX31 shows moderate co-localization with the ER and lysosomes marker. The cells were counterstained with Propidium iodide (PI) or 4′-6-diamidino-2-phenylindole (DAPI) to highlight the nuclei. Scale bars: 10 μm. Please see online version for colour reproduction.



whether or not the functional GJ channels were formed by the mutant CX31 proteins, a dye transfer method was used. That method entails that individual HeLa cells with WT or a mutant CX31 protein be microinjected with Lucifer yellow dye to transfer the dye across neighboring cells (Table 1 and Fig. 3). HeLa cells transfected with WT CX31 cDNA showed a significant amount of dye transfer (Fig. 3A). However, according to our results, the p.V174M CX31 mutants completely lost their dye transfer capabilities in the HeLa cells (Fig. 3B). Moreover, no dye transfer was observed between the untransfected HeLa cells (Fig. 3C).

In a previous study, we tried to acquire the CX31V174M stable expression cell line to facilitate follow-up experiments. Under a fluorescence microscope, however, we found that the positive cells displaying green fluorescence were noticeably decreased in the following days of culture in post-transfect mutant CX31V174M plasmids. Therefore, we detected the cell survival ratio by MTT assay (Fig. 4 and Supplementary data Fig. S3[1]). Our results found that the cell survival ratio of post-transfect mutant CX31V174M was 151 ± 9% after 6 days of transfection. In contrast, the post-transfect CX31WT cell was 230 ± 7%, and mock HeLa cell was 259 ± 6% in 6 days of culture. The results indicate that the cell survival ratio of post-transfect mutant CX31V174M was significantly lower than mock HeLa and post-transfect CX31WT. Therefore, we considered that the mutation point p.V174M caused cell death, and we were not able to obtain a stable performance of the cell lines.

To determine if the CX31V174M mutation was an autosomal dominant or recessive mutation, we examined the effects of the mutant proteins on CX31WT in HeLa cells using a co-expression study by co-transfecting both WT and mutant expression plasmids (Fig. 5). First, we found that cells expressing both CX31WT-

Table 1. Lucifer yellow transfer in expressed wit WT or mutant Cx31 HeLa cells.

Cell line	Dye-filled neighbor cell number (mean ± SE)	Number of injections (*n*)
HeLa-CX31WT	2.3±1.1	25
HeLa-CX31V174M	0	25
HeLa	0	25

Fig. 3. Imaging intercellular transfer of membrane-impermeant dye. Expression of (A) CX31WT and (B) CX31V174M HeLa cells was microinjected with Lucifer yellow. Intercellular diffusion of dye was assessed 5 min after injection. Additionally, (C) mock HeLa cell was negative control. Scale bars: 10 μm.

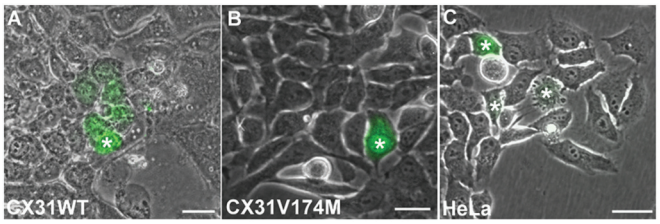
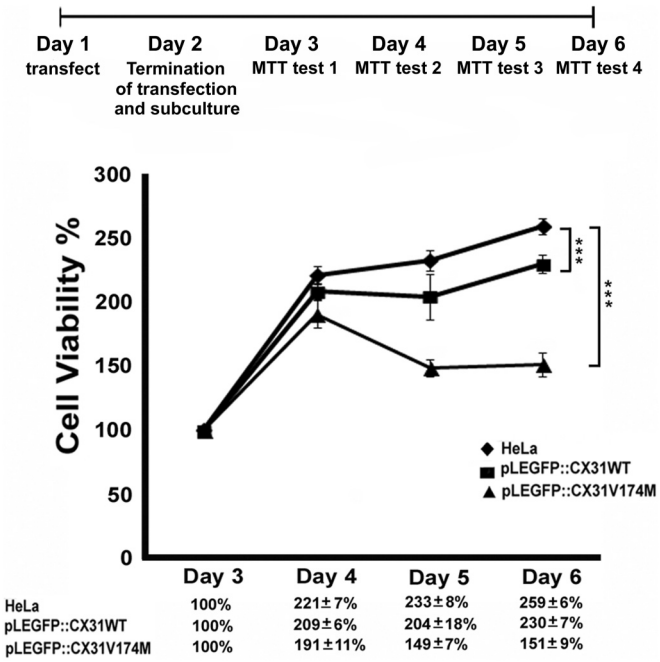
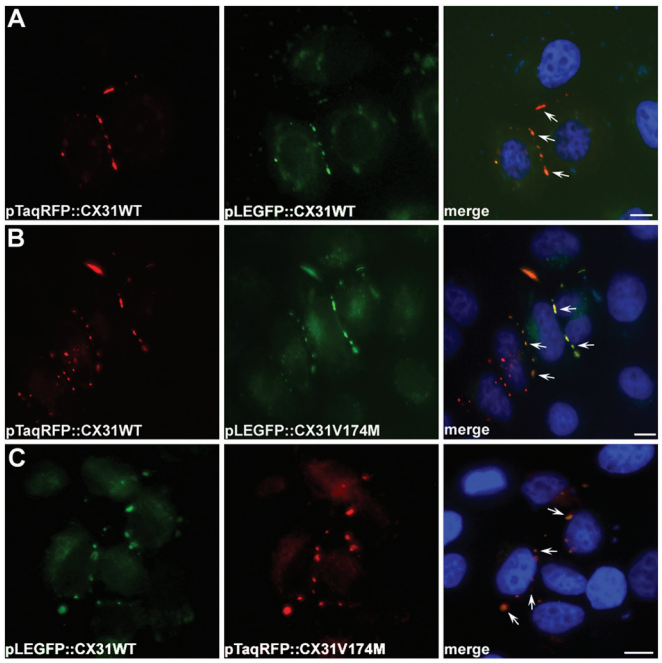


Fig. 4. Analysis of cell survival rate in transfected CX31WT and CX31V174M HeLa cells by MTT assay. The results showed that when the HeLa cells expressed p.V174M, the performance showed a slightly downward trend compared to the number of cells in CX31WT or HeLa cell after 6 days of transfection. The MTT analysis of each group were from three independent experiments. All data were presented as the mean ± SD. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.



TaqRFP and the CX31WT-pLEGFP protein exhibited co-assembly expression in the cell membrane of HeLa cells (Fig. 5A). Interestingly, both CX31WT-pTaqRFP and the CX31V174M-pLEGFP revealed expression patterns differing from those of p.V174M alone, which is expression at the cell membrane of adjacent HeLa cells (Fig. 5B). Similarly, both CX31WT-pLEGFP and the CX31V174M-pTaqRFP proteins were also observed to have co-expression at the cell membrane (Fig. 5C). Analytical upshots revealed co-localized CX31V174M mutant protein and CX31WT protein, resulting in no

Fig. 5. Co-expression of CX31V174M mutant proteins with CX31WT by co-transfection expression system. (A) HeLa cells co-expressing CX31WT-TaqRFP and CX31-pLEGFP, revealing co-localization of the two WT fluorescence proteins at the plasma membrane between cell–cell contact areas. In the HeLa cells co-expressing CX31WT-pTaqRFP and CX31V174M-pLEGFP (B) or CX31WT-pLEGFP and CX31V174M-pTaqRFP (C). Our results indicated that WT and mutant proteins are co-localized at the plasma membrane, resembling those of CX31WT alone, but is different only for CX31V174M expression around the nucleus regions. Arrows indicate co-expressed proteins. The cells were counterstained with DAPI to highlight the nuclei. Scale bars: 10 μm.



dominant negative effect of CX31WT protein trafficking to the cell membrane.

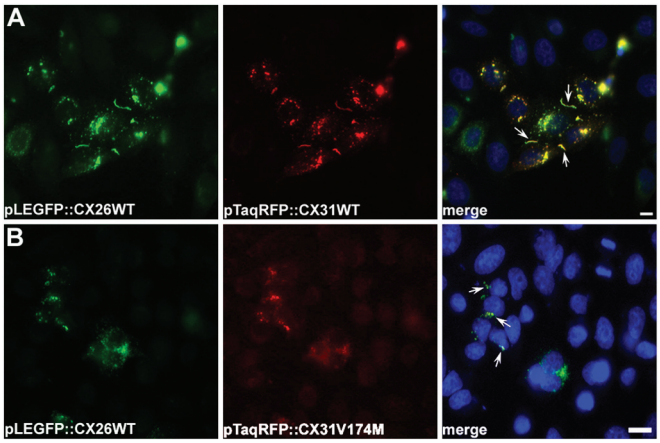
Mutations in the CX26 have been found to play a role in about half of the cases of inherited nonsyndromic hearing loss (Maw et al. 1995; Morell et al. 1998). Additionally, human genetic studies also revealed an interaction between CX26 and CX31 (Dinh et al. 2009; Liu et al. 2009). Thus, we were encouraged to conduct further tests of the effects of the mutant CX31V174M protein on CX26WT (Fig. 6). Both CX26WT-pLEGFP and CX31WT-pTaqRFP were co-localized at the cell membrane of contact between neighboring HeLa cells (Fig. 6A), which confirmed that both CX31 and CX26 can be trafficked to the same GJ plaque. Conversely, co-expression of Cx26WT-pLEGFP and CX31V174M-pTaqRFP changed the localization of these CX26WT proteins from the cell membrane to the cytoplasm near the nucleus (Fig. 6B). Investigative consequences revealed co-assembly of CX31V174M mutant protein and CX26WT protein and impaired CX26WT protein trafficking to the cell membrane. We therefore hypothesized that p.V174M of CX31 has a trans-dominant negative effect on CX26WT.

Discussion

For auditory function, the important role of intercellular communication of the GJs has been confirmed by findings that certain CX gene mutations cause hearing loss. Mutations in the CX26 (*GJB2*) alone have been found to make an important contribution to inherited nonsyndromic hearing loss (Maw et al. 1995; Morell et al. 1998). Less frequently reported are mutations in other CX genes, such as *GJB6*, *GJB3*, and *GJB1* (Grifa et al. 1999; Xia et al. 1998; Bergoffen et al. 1993; Liu et al. 2000). Similar to these earlier findings, our previous study found that CX gene mutations were a common factor in nonsyndromic deafness in Taiwan, and that mutations in the *GJB2* were the most prevalent of the CX genes surveyed for deafness (Yang et al. 2007). CX31 is highly expressed in the stratum granulosum in the upper differentiating layer of the epidermis (Common et al. 2005; Di et al. 2001), and it is also found in peripheral nerves and the cochlea (López-Bigas et al. 2001). Xia et al. (1998) identified heterozygous mutations in the *GJB3* gene (538C > T, 547G > T) from two Chinese families with autosomal dominant hearing loss. Subsequently, Liu et al. (2000) screened 25 Chinese families with recessive deafness to determine whether mutations in the *GJB3* can also cause recessive nonsyndromic deafness. They found compound heterozygotes (423A > G, 423-425delATT) of the *GJB3* mutations in the two families. Liu et al. (2009) found two other compound heterozygotes (497A > G; N166S, 580G > A; A194T) of the *GJB3* mutation with the 235delC and 299delAT of *GJB2* in three unrelated families with hearing loss. These findings suggest that *GJB3* may be associated with digenic inheritance. Nevertheless, we did not observe any of above-noted mutations in our previous study. Instead, we found a missense mutation, p.V174M, in three of our patients. Moreover, we were unable to identify any mutations in other members of the connexin gene family in patients with the CX31V174M mutation. However, we cannot rule out the possibility that an interaction of the CX31V174M mutant with a second gene, at the same or other locus, exerts a dominant effect.

In our previous study, we found a novel c.520C > A mutation in the second extracellular loop region of the *GJB3* gene in three patients with nonsyndromic deafness. This C > A transversion leads to a valine (V)→methionine (M) substitution at codon 174 (p.V174M) (Yang et al. 2007). ConSeq is a Web site server that can identify biologically important residues in protein sequences (Berezin et al. 2004). Using the ConSeq server, we found that p.V174 is highly conserved (Conseq score 9) in the second extracellular loop. Based on this finding, it is unlikely that the transversion of valine to methionine at codon 174 of the *CX31* gene plays a critical role in the function of the CX31 protein. However, our results showed that the p.V174M missense mutation resulted in the accumulation of the CX31 mutant protein in the ER and lysosomes instead of targeting the cytoplasmic membrane.

Fig. 6. Co-expression of CX31V174M mutant proteins with CX26WT by co-transfection protein expression system. (A) HeLa cells co-expressing CX26WT-pLEGFP and CX31WT-TaqRFP revealing co-localization of the two proteins at the plasma membrane between cell–cell contact areas. (B) HeLa cells co-expressing CX26WT-pLEGFP and CX31V174M-TaqRFP revealing co-localization of the two proteins in the cytoplasm concentrations near the nucleus regions. Arrows indicate co-expressed proteins. The cells were counterstained with DAPI to highlight the nuclei. Scale bars: 10 μm.



Further investigation into the functional roles of CX31 in the cell is needed. Functional studies of connexins have been carried out in expression systems by transfecting mammalian cells (e.g., HeLa cells) devoid of CXs with relevant cDNAs to reconstitute GJ communication (Beltramello et al. 2003). In this study, we studied the extracellular distribution and assembly of mutant CX31 (CX31V174M) in HeLa cells. The immunolabeling assay with EGFP revealed that the CX31-pLEGFP protein was expressed in plaques along the apposed cell membranes. This finding is consistent with the results reported by Oh et al. (2013). In contrast, our findings revealed that the p.V174M mutation in the *CX31* gene impaired trafficking of the protein to the plasma membrane and the concentration near the nucleus. In a previous study, we found that p.V174M of CX31 was a heterozygous mutation in two patients with hearing loss from the same family (Yang et al. 2007). In a family study, we also found that the mutant was inherited from his/her mother with normal hearing (data not shown). In this study, our results showed that CX31V174M did not interfere with CX31WT protein synthesis and CX31WT protein trafficking to the cell membrane. Based on the above results, we suggest that a CX31V174 residue may play an important role in the CX31 protein life cycle, and p.V174M mutation in the CX31 seems to be a recessive mutant form.

The mutation of CX26, 551G > A is a missense mutation that causes R to Q substitution at position 184 (Yang et al. 2007). Position 184 of CX26 is located at the second extracellular loop (E2) that is highly conserved among members of the human β group of the Cx family and throughout evolution, and it is the major determinant for compatibility between connexins. The R184Q of CX26 at E2 might result in incompatibility between different species of CX proteins to form heterotypic functional channels (Krutovskikh and Yamasaki 2000). Two other missense mutations (R184W and R184P) at the same position have been identified in patients with hearing loss (Wilcox et al. 2000; Denoyelle et al. 1997), confirming the importance of position 184 for the function of E2. In addition, two CX26 mutations (P175T and S199F) in the second extracellular loop (E2) have been demonstrated in patients with deafness (Denoyelle et al. 1997; Green et al. 1999). Previously reported findings have also demonstrated that two CX31 mutations, the missense (E183K) and the nonsense (R180X), located at the E2 are associated with deafness (Xia et al. 1998). Liu et al. (2009) found compound heterozygosity for a 497A-G transition in the *GJB3* gene, resulting in an asn166-to-ser (N166S) substitution in the second extracellular loop. Our previous study found one missense mutation, p.V174M of CX31, which was also located at the E2 (Yang et al. 2007). Furthermore, our results demonstrated that the CX31V174M mutation had a negative effect on the formation of the GJ channel. Therefore, we suggest that a 3D structure or X-ray crystallographic studies of the CX31 protein need to be performed to further understand the influence this mutation has at the protein level.

In this study, we found that these cells will continue to die after 3–6 days of transfection. The HeLa cells expressed p.V174M, and the performance compared to the number of cells in normal CX31 showed a slightly downward trend by MTT assay. The same feature of high incidence of cell death was observed in the dominant skin disease CX31 mutations (Di et al. 2002). Previous data demonstrated that the CX31 mutant (R42P) prevents the trafficking of the protein to the membrane (Di et al. 2002). In addition, defective trafficking was also observed in the dominant mutation of GJB3, 66delD segregating in a family with peripheral neuropathy and sensorineural deafness. This abnormal localization appears to be associated with cell death (Di et al. 2002). However, Tattersall et al. (2009) found that cell death is demonstrated by upregulation of components of the UPR, and they stressed that ER was the major mechanism in the CX31 mutants associated with EKV. Based on these results, we suggest that p.V174 amino acid in the E2 domain likely plays a critical role in CX31, and as a result a mutation in this residue will lead to loss of function of the protein and cause cell death. Therefore, further molecular mechanism studies are needed to clarify how this mutation affects the function of CX31 and cause celldeath.

Previous studies have shown a direct interaction between CX26 and CX31(Liu et al. 2009; Dinh et al. 2009). In addition, *GJB3* mutations occurring in compound heterozygosity with the *GJB2* mutations have been found in three unrelated Chinese families. Furthermore, they provide evidence that mutations at these two CX genes can interact to cause hearing loss in digenic heterozygotes in humans (Liu et al. 2009). In our co-expression CX31WT and CX26WT study, we found that CX26 and CX31 were able to co-localize in the same GJs plaque. The in vitro data are consistent with the previous study by Dr. Liu et al. (2009). Surprisingly, our co-expression study showed that CX31V174M mutant trafficking defects could not be rescued by co-expression with CX26WT; instead, the p.V174M of the *CX31* mutant causes loss of function in the CX26WT protein. Therefore, we hypothesize that the p.V174M missense mutation in *CX31* has a trans-dominant negative effect. So far, no studies have indicated that *CX31* mutations have trans-dominant inhibitory effects on the function of other connexins. Our study is the first evidence that p.V174M of the *CX31* mutation has a trans-dominant negative effect on *CX26*. However, the detailed mechanisms and components of the molecular machinery that mediate this unconventional process remain unclear. Thus, the patho-genetic nature of the p.V174M remains to be elucidated in the further.

Acknowledgements

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Figure S1. Schematic representation of the domain structure of the CX31protein with indication of known variants. The black arrows indicate the p.V174M (c.520A>T) variant in CX31. TM1-4: transmembrane domains; E1-2: extracellular domains; CL: cytoplasmic linking domain.

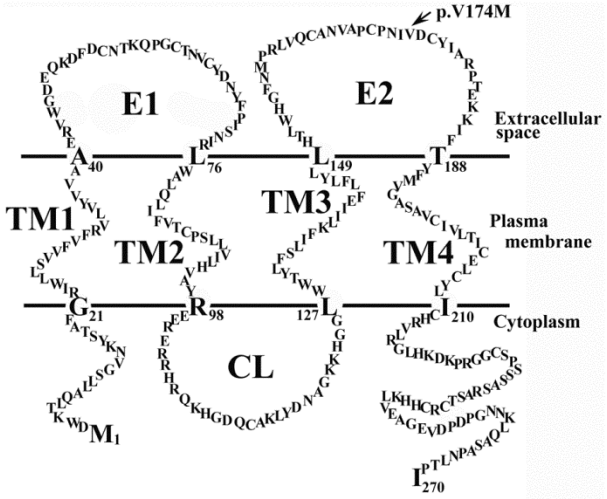


Figure S2. ConSeq predictions demonstrated on human CX31 (SWISS-PROT: O75712), using 50 homologues obtained from the Pfam database (family code: PF00029). The sequence of the CX31 protein is displayed with the evolutionary rates at each site colour-coded onto it (see legend). The residues of the CX31 sequence are numbered starting from 1. The first row below the sequence lists the predicted burial status of the site (i.e. “b”—buried versus “e”—exposed). The second row indicates residues predicted to be structurally and functionally important: “s” and “f”, respectively. Vertical arrows indicate amino acid codons (p.V174M).

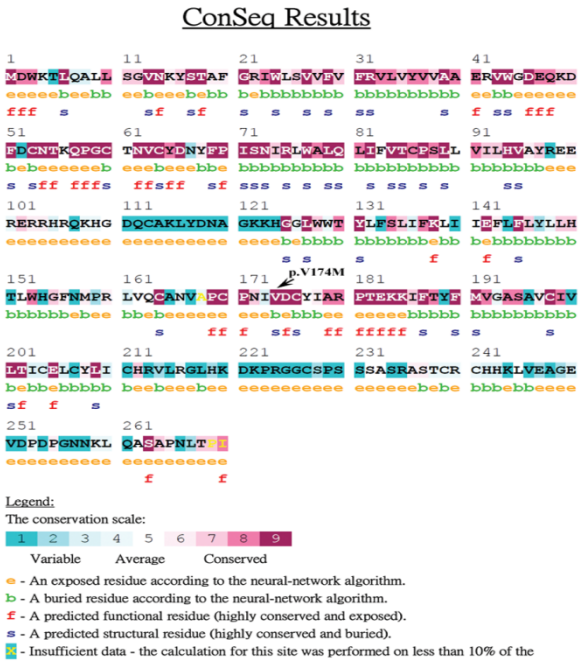
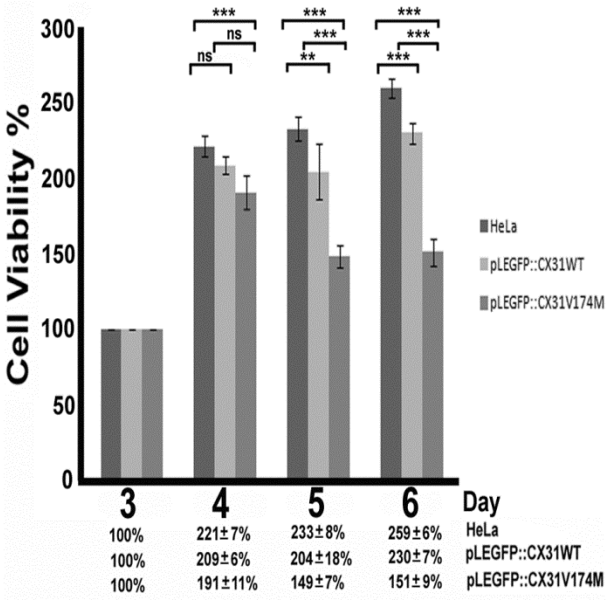


Figure S3. Analysis of cell survival rate in transfected CX31WT and CX31V174M HeLa cells by MTT assay. All data were presented as the mean±SD and were considered significant difference for $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ as compared with each other between mock HeLa, CX31WT and CX31V174M groups. The results showed that when the HeLa cells expressed p.V174M, the performance showed a slightly downward trend compared to the number of cells in HeLa from after 4 day of post-transfection CX31V174M ($p<0.001$). In addition, the number of cells in CX31V174M compared with CX31WT are slightly downward from after 5 day of post-transfection ($p<0.001$). This MTT analysis of each group were from three independent experiments, respectively.



附件四

Functional analysis of a nonsyndromic hearing loss-associated mutation in the transmembrane II domain of the GJC3 gene

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Keywords: **CX30.2/CX31.3, *GJC3*, mutation, hearing loss**

Abstract

In a previous study, we identified a novel missense mutation, p.W77S, in the *GJC3* gene encoding connexin30.2/connexin31.3 (CX30.2/CX31.3) from patients with hearing loss. The functional alteration of CX30.2/CX31.3 caused by the p.W77S mutant of *GJC3* gene, however, remains unclear. In the current study, our result indicated that the p.W77S missense mutation proteins in the intracellular distribution are different CX30.2/CX31.3WT, which showed continuous staining along apposed cell membranes, and an accumulation of the mutant protein in the endoplasmic reticulum (ER) of the HeLa cell. Furthermore, co-expression of WT and p.W77S mutant proteins by a bi-directional tet-on expression system showed that the heteromeric connexon accumulated in the cytoplasm, thereby impairing the WT proteins' expression in the cell membranes. In addition, we found that CX30.2/CX31.3W77S missense mutant proteins were degraded by lysosomes and proteosomes in the transfected HeLa cell. Based on these findings, we suggest that p.W77S mutant has a dominant negative effect on the formation and function of the gap junction. These results give a novel molecular elucidation for the mutation of *GJC3* in the development of hearing loss.

Introduction

The mammalin inner ear comprise the cochlea, which is the hearing organ. The functions of the organ is dependent on tightly controlled ionic environments, in particular for K^+ ions, for hearing transduduction (Oghalai, 2004). Gap junction system is highly probable pathway for cochlear K^+ ions recirculation in the cochlea (Kikuchi et al., 2000). CXs genes code for a large and highly homologous family of proteins that form intercellular gap junction chanels. More than 20 CXs have been described in the mammalian. There are twenty-one CXs genes within the human genome. The topological model of CX protein shows that the polypeptide comprise a short cytoplasmic amino-terminal domain (NT), four transmembrane domains (TM1 to TM4) linked by one cytoplasmic loop (CL) and two extracellular loops (E1 and E2), and a most variable carboxyl-terminal cytoplasmic domain (CT) (Willecke et al., 2002).

Mutations in the CXs have been identified as associated with a variety of human inherited disease, such as deafness, epidermal disease, neuropathies, oculoden todigital dysplsia and cataracts. The inheritance of this disease more likely to be autosomal dominant, autosomal recessive, or X-linked (Krutovskikh and Yamasaki, 2000). Disease-causing mutations can potentially take place anywhere in the CXs. These mutations may cause disease through a variety of mechanisms, most of which alter intercellular communication by affecting various processes of the CXs life cycle or channel function. The plurality of identified CXs mutations are located within the coding region of protein. These different mutations generate abnormalities at diverse steps in the CX life cycle, including synthesis, assembly, channel function, and degradation (Dinh et al., 2009).

Up to now, six CXs protein (CX26, CX30, CX31, CX30.3, CX30.2/CX31.3 and CX43) are reported expression in the gap junction-rich regions of the cochlear duct and association with human genetic hearing lose (Kelsell et al., 1997; Xia et al., 1998; Grifa et al., 1999; López-Bigas et al., 2002; Yang et al., 2007; Wang et al., 2010; Yang et al., 2010). The human *GJC3* gene,

coding for CX30.2/CX31.3, is located on chromosome 7q22.1 and the coding region is localized on both exon 1 and exon 2 and is interrupted by an intron. The CX30.2/CX31.3 contains 279 amino acid residues and has a molecular weight of 31.29 kDa. Human CX30.2/CX31.3, orthologs of the mouse Cx29, was first identified by database analysis in 2002 and has been shown to be highly expressed in the cochlea using cDNA macroarray hybridization (Sohl et al., 2001; Altevogt et al., 2002; Ahmad et al., 2003). Furthermore, previous animal studies also indicate that the Cx29 protein is expressed in the cochlear tissue of mice and rats (Yang et al., 2005; Tang et al., 2006). Previously, we have been identified four heterozygous missense mutations [c.807A>T (E269D), c.43C>G (R15G), c.68T>A (p.L23H) and c.230C>G (W77S)] of the *GJC3* gene in Taiwanese patients with nonsyndromic deafness (Yang et al., 2007; Wang et al., 2010; Yang et al., 2010). To understand the play role of *GJC3* mutation in nonsyndromic hearing loss, it is necessary to investigate the functional alteration of mutant Cx30.2/CX31.3 in intercellular communication. Previously, we have found that p.E269D mutation in the *GJC3* gene has a dominant negative effect on the formation and function of the gap junction (Hong et al., 2010). In addition, we found that p.R15G and p.L23H mutants do not decrease the trafficking of CX proteins, but the mutations in *GJC3* genes result in a loss of function of the CX30.2/CX31.3 protein (Su et al., 2013). However, the functional alternation of CX30.2/CX31.3 caused by the p.W77S mutant remains unclear. This study, therefore, investigates the affecting of the p.W77S mutations on the functional properties and subcellular localization of the mutant CX30.2/CX31.3 protein in tet-on HeLa cells.

Materials and Methods

Molecular cloning and construction of the plasmids expressing wild-type or mutants CX30.2/CX31.3

The wild-type CX30.2/CX31.3 expressing plasmids was constructed as previously describe (Hong et al., 2010). Mutant *GJC3* genes were generated by performing oligonucleotide-directed

mutagenesis using the Stratagene Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The following oligonucleotide primers (mutated nucleotide is underlined) were used to prepare the mutant *GJC* gene: CX30.2/31.3 W77S sense 5'-CCgCTgCgTTTCTCggTCTTCC AggTCATC-3' and CX30.2/31.3 W77S antisense 5'-gATgACCTggAAgACCgAgAAACgCAgC gg-3'. The cDNA sequences of the autofluorescent reporter proteins EGFP (pEGFPN1 vector; Clontech, Palo Alto, CA, USA) were fused in-frame to the C terminus of wild type and mutants for fusion protein generation. The coding region of CX30.2/31.3WT and that of mutant CX30.2/31.3W77S were amplified from plasmids containing the CX30.2/31.3 cDNA (CX30.2/31.3^{wt}-EGFP or CX30.2/31.3^{W77S}-DsRed) using two pair primers containing recognition sequences 5'- *Sal*I and 3'- *Not*I or 5'-*Nhe*I and 3'-*Eco*RV, respectively, and Platinum Pfx DNA polymerase (Invitrogen, Carisbad, CA). Purified products were subcloned into the corresponding site of the bi-directional expression vector pBI (Clontech, Palo Alto, CA). The dideoxy DNA sequencing method, using a DNA Sequencing kit (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM 3730 automated sequencer, were used to confirm the DNA sequence of all constructs.

***Transfection and expression of* *CX30.2/31.3*WT, *CX30.2/31.3*W77S, and *CX30.2/31.3*WT/ *CX30.2/31.3*W77S chimerae protein in tet-on HeLa cell line**

The tet-on HeLa cell line deficient in the *GJIC* gene was purchased from BD Biosciences Clontech (Palo Alto, CA, USA) and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS (Gibco BRL, Gaithersburg, USA), 100 μg/ml G418, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a moist atmosphere containing 5% CO2. Transfection was carried out using LipofectAMINE reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. A ratio of 1 μg DNA vs. 2 μl LipofectAMINE 2000 was used for the tet-on HeLa cells. Cells were harvested at 24 h post-transfection and grown on a coverslip for 24 h at 37℃ in a humidified 5% CO_2 incubator. Then, tet-on HeLa cells were

treated with 1 μg/ml doxycyclin (Dox) (Sigma-Aldrich Corporation, St. Louis, Mo) in cell culture medium to induce CX30.2/31.3WT or CX30.2/31.3W77S mutant protein expression. Cells were exposed to Dox for 5 h prior to immunofluorescence staining. Tet-on HeLa cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 20 min, rinsed three times in PBS, stained with DAPI for 5 min, and then washed three times with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from wild type or mutant CX30.2/CX31.3 expression cell lines using the Total RNA Extraction Miniprep System according to the manufacturer's directions (VIOGENE, Sunnyvale). cDNA was synthesized according to the manufacturer's directions in a reaction volume of 20 μl, containing 2-5 μg RNA, random hexamer primer, and 200 units Improm-II^{TM} Reverse Transcriptase (Promega, San Luis Obispo). With primers specific for the coding region of the *GJC3* gene (forward 5'- ATGTGCGGCAGGTTCCTGAG -3' and reverse 5'- CATGTTT GGGATCAGCGG-3'), PCR was performed (94 ^{o}C 30 sec, 58 ^{o}C 35 sec, 72 ^{o}C 1 min) for 35 cycles in a volume of 25μl containing 1 mM Tris-HCl (pH 9.0), 5 mM KCl, 150 μM $MgCl_2$, 200 μM dNTP, 1 units proTaq DNA polymerase (Promega, San Luis Obispo), 100 ng of cDNA, and 200 μM forward and reverse primers. A fragment of approximately 700 bp was amplified from cDNA of the *GJC3* gene. The PCR products were subjected to electrophoresis in an agarose gel (2 w/v %) stained with ethidium bromide. The signals were detected by an Alpha Image 2200 system (Alpha Image 2200 analysis software).

Immunofluorescence staining of post-transfection HeLa cells

Wild-type or mutant CX30.2/CX31.3 protein expression in tet-on HeLa cells was analyzed by a direct fluorescent protein fusion method involving fusion of EGFP or DsRed to the C-terminal ends of the CX30.2/CX31.3 proteins. Briefly, post-transfection tet-on HeLa cells grown on coverslips were fixed with 4% paraformaldehyde in 0.1 M PBS for 20 min and then

rinsed three times in PBS. Then, the coverslips were immersed in 10% normal goat serum and 0.1% Triton X-100 for 15 min. The primary antisera and dilutions were as follows: mouse anti-pan-cadherin antibody at 1:200 (anti-CH19; abcan) for cell membrane, mouse anti-Golgin-97 at 1:200 (Invitrogen, Carisbad, CA) for Golgi apparatus. After incubation with primary antiserum at 4℃ overnight, the cells were rinsed in PBS three times before adding Alexa Fluor 488 and/or Alexa Fluor 594 conjugated secondary antibodies (Invitrogen, Carisbad, CA). Endoplasmic Reticulum (ER) was stained with ER-Tracker® Blue-white DPX Probes at 1:670 dilution **(**Invitrogen, Carisbad, CA**)** for 10 min at room temperature. Lysosomes were stained with LysoTracker® Probes **(**Invitrogen, Carisbad, CA**)** for 20 min at room temperature. The nuclei of cells were counterstained with DAPI (2 μg/ml) for 5 min and rinsed with PBS. Mounted slides were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany).

Real-time Quantitative polymerase chain reaction (Q-PCR)

For quantitative real-time RT-PCR (q-PCR) analysis, total RNA was isolated from four positive stable cell lines using the Total RNA Extraction Miniprep System according to the manufacturer's directions (VIOGENE, Sunnyvale). Reverse transcription was performed using Improm-II^{TM} Reverse Transcriptase (Promega, San Luis Obispo) in the presence of oligo-dT18 primer. Quantitative PCR for mRNA was performed using the SYBR Green I Master Mix (Applied Biosystems, Foster city, CA) and detected in a ABI7000 thermocycler (Applied Biosystems, Foster City, CA). Real-time PCR primers for mRNA were designed using PrimerExpress software (Hong et al., 2010). The Primers, CX30.2/CX31.3 real-time F-5'CCTG GGATTCCGCCTTGT-3' and CX30.2/CX31.3 real-time R-5'- TGGGTGTGACACACGAAT TCA-3' were using for CX30.2/CX31.3 detection. Each measurement was performed in triplicate and the results were normalized by the expression of the GAPDH reference gene.

DNA fragmentation analysis

Both expressed CX30.2/CX31.3WT and CX30.2/CX31.3W77S HeLa cells (5x 10^6 cells) were cultured in DMEM medium for 4 days. After removing the nonadherent dead cells in the cultures by rinsing with PBS, the adherent cells were collected by centrifugation for 5 min (1000 rpm) at room temperature. DNAs were purified as previously describe (Su et al., 2013). Different DNA concentrations from 500μg to 3000μg were resolved in a 1 % (w/v) agarose gel in 1x TAE buffer. The DNA bands were stained with ethidium bromide (0.5 ug/ml) and photographed (Alpha Image 2200 analysis software).

Evaluation of cell viability

Cell viability was determined by MTT assay. Briefly, after MG63 cells were cultured on nanostructured alumina surface for 1, 2 and 4 days. 100 ml of MTT (5 mg/ml) (Wako, Japan) was added to each well and incubated at 37°C for another 4 h. Then, 0.5 ml dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance of each solutionwas measured at the wavelength of 490 nm with a microplate reader (Bio-Rad 680, Bio-Rad, USA).

Results

The predictable protein structure of CX30.2/CX31.3 was acquired from UniProtKB/Swiss-Prot database entry P17302. We found that the topological model of CX30.2/CX31.3 protein is similarily other members of CX family. The structure of CX30.2/CX31.3 protein was described previously (Su et al., 2013) and was displyed in supplemental Fig. 1. Our result indicated that p.W77S missense mutation is localized at the second membrane-spanning segments (TM2) and near border of the E1 domain of the CX30.2/CX31.3 protein (supplemental Fig. 1). Further, we examined and compared amino acid sequences of the CX30.2/CX31.3 domain among CX families in humans using Biology WorkBench Clustal W (1.81) Multiple Sequence Alignments (http://workbench.sdsc.edu/, San

Diego Supercomputer Center). According to that comparison, the p.W77S amino acid of the CX30.2/CX31.3 protein was highly conserved among the human CX family members (Fig. 1A). Additionally, we compared amino acid sequences of CX30.2/CX31.3 in CX families of all species using a basic ConSeq analysis system (http://conseq.tau.ac.il/; Berezin et al., 2004). The contrast results revealed that p.W77 is also highly conserved (Conseq score = 8~9) in all species (Fig.1B). These results indicated that p.W77 may be play an important role in the function of CX30.2/CX31.3 protein.

To confirm the contrast results and understand the effects of p.W77S missense mutation, we compared the functional properties and subcellular localization of the CX30.2/CX31.3 wild-type (WT) and mutant (p.W77S) protein in the gap junction-deficient HeLa cells. First, we transfected the cDNA constructs of WT (CX30.2/CX31.3WT-EGFP or CX30.2/CX31.3WT-DsRed) or mutant CX30.2/CX31.3 (CX30.2/CX31.3W77S-EGFP) into tet-on HeLa cells using lipofection. In the CX30.2/CX31.3WT-EGFP and CX30.2/CX31.3WT-DsRed expression cell line, the results indicated that the WT proteins were observed along apposed cell membranes between adjacent cell (Fig. 2A and 2B, right panel). Further, this membrane localization was confirmed by colocalization with pan-Cadherin (Fig. 2A, left panel). These results are consistent with our previous studies (Hong et al., 2010; Su et al., 2013). Contrary to CX30.2/CX31.3WT, as seen in the immunolabeling assay, CX30.2/CX31.3W77S mutant proteins were concentrated in the cytoplasm close to the nucleus (Fig. 2C). Following, we identify which organelles in the cytoplasm the mutant CX30.2/CX31.3 localized in HeLa cells. We analyzed the HeLa cell had been transfected with CX30.2/CX31.3W77S-EGFP cDNA by immunostaining with markers for lysosome, ER, and Golgi apparatus (Fig. 3). The results of the assay indicated that the most CX30.2/CX31.3W77S mutant protein was characteristically found in a reticular pattern co-localized with an ER marker (Fig. 3B). Base on above found, we consider that the p.W77S mutation interferes with normal CX30.2/CX31.3 trafficking.

In our previous investigating, we showed that the p.W77S mutation in CX30.2/CX31.3 is a heterozygous mutation in patients with nonsyndromal hearing loss (Yang et al., 2010). Therefore, co-expression studies were followed out to inspect the effects of the mutant protein on CX30.2/CX31.3WT using a bi-directional tet-on protein expression system with equal amounts of the two respective expression proteins. Our results showed that the CX30.2/CX31.3WT-DsRed and CX30.2/CX31.3W77S-EGFP co-expression pattern was similar to that in cells expressing only CX30.2/CX31.3W77S, which are concentrated in the cytoplasm close to the nucleus (Fig. 4). Based on this finding, we suggest that the p.W77S mutation seem to have a dominant negative effect on CX30.2/CX31.3WT.

In order to obtain HeLa cell colonies that stably expressed CX30.2/CX31.3W77S, a FACSAria™ cell sorter (BD Biosciences, USA) was used to sort positive cells (cell with green fluorescence) in the study. We did not, however, obtain the stably expressed CX30.2/CX31.3W77S cell lines. Under a fluorescence microscope, we found that the positive cells displaying green fluorescence had decreased noticeably from 8.96±0.91% to 0.63±0.49% in the days following the culture in post-transfect mutant CX30.2/CX31.3W77S plasmid (Fig. 5). In contrast, the post-transfect CX30.2/CX31.3WT cell with green fluorescence had decreased only slightly, from 14.1±4.28% to 10.03±0.58% in the days following the culture (Fig. 5). Therefore, one possibility reason is the accumulation of a great quantity of mutant proteins in the ER switches on unfolded protein response (UPR) within the ER that leads to the programmed cell death (apoptosis).

To explanation the possibility, we further analyzed cell death using two methods, DNA fragmentation and MTT assay. Both expressed CX30.2/CX31.3WT and CX30.2/CX31.3W77S HeLa cells were incubated for 4 days before being subjected to cell viability assays by DNA fragmentation. DNAs were purified from expressed HeLa cells, which are post-transfect CX30.2/CX31.3WT and CX30.2/CX31.3W77S plasmids and were then resolved by conventional

agarose gel electrophoresis to assay the potential apoptotic DNA fragmentation. The results obviously proved absence of the characteristic DNA laddering of those cells expressing CX30.2/CX31.3W77S (Fig. 6A). Simultaneously, the characteristics of cell viability were also determined by the MTT analysis in the days following the culture in post-transfect WT CX30.2/CX31.3 and mutant CX30.2/CX31.3W77S plasmids (Fig. 6B). This result of cell viability is consistent with DNA fragmentation analysis, which is not different between expressed WT and mutant HeLa cell. Thus, we suggest that the accumulation of CX30.2/CX31.3W77S mutant protein in the ER did not trigger cell death.

Further, we tried to find the factors contributing to green fluorescence decrease in the positive cells. To understand p.W77S of GJC3 mutant mRNA expression, real-time Quantitative–PCR was performed to assess the expression of transgenes in post-transfect HeLa cells (Figure. 7). Our results indicate that p.W77S of GJC3 mRNA expression is consistent with normal GJC3 mRNA expression in the post-transfect HeLa cells. Therefore, we suggest that the p.W77S mutant does not interfere with mRNA expression in the transcription process. As well, we used the lysosome inhibitor chloroquine (CQ) 20μg/ml and the proteasome inhibitor MG132 3μg/ml to understand whether the CX30.2/CX31.3W77S mutant proteins underwent degradation. These concentrations were decided according to cell viability using MTT assay (data not show). After treatment with both chloroquine (CQ) and MG132, we found that the positive cells were higher in number than in the untreated group 12, 24, and 48 hours after treatment (Figure. 8). The rate of positive cells was 20.42±8.3%, 39.28±3.42%, 19.88±3.57%, and 14.24±4.54% for CQ treatment at 0, 12, 24, and 48 hours respectively. At 0, 12, 24, and 48 hours after MG132 treatment, the rate of positive cells was 21.04±3.7%, 28.48±5.28%, 24.87±7.22%, and 9.38±2.06%. Based on these results, we suggest that a missense mutation of the CX30.2/CX31.3 p.W77S protein was degraded in the HeLa cell.

Discussion

At least seven heterozygous mutations and two heterozygous polymorphisms of the *GJC3* gene have been detected in Taiwanese patients with nonsyndromic deafness (Yang et al., 2007; Wang et al., 2010; Yang et al., 2010). Of the seven mutation, four heterozygous missense mutations [c.43C>G (p.R15G), c.68T>A(p.L23H), c.230C>G (p.W77S) and c.807A>T (p.E269D)] of the *GJC3* gene were identified. Previously, we had indicated that p.E269D missense mutation in the GJC3 gene resulted in accumulation of the CX30.3/CX31.2 mutant protein in the endoplasmic reticulum (ER), and had a dominant negative effect on the formation and function of the gap junction (Hong et al., 2010). In the p.R15G and p.L23H mutants, we have indicated that p.R15G and p.L23H mutants exhibited continuous staining along apposed cell membranes in the fluorescent localization assay, which is the same as the wild type. However, two mutations in *GJC3* genes resulted in a loss of ATP release (hemichannel function) function of the CX30.2/CX31.3 protein (Su et al., 2013). In this study, we found that the p.W77S mutation proteins in the intracellular distribution were different from those in CX30.2/CX31.3WT, which showed continuous staining along apposed cell membranes, and an accumulation of the mutant protein in the endoplasmic reticulum (ER). Simultaneously, we also found that the p.W77S heterozygous mutation has a dominant negative effect on the formation and function of the gap junction. These results of p.W77S mutation are consistent with p.E269D mutation, but there is no consistency with p.R15G and p.L23H mutations.

Similarly, the different site mutations in the *GJC* gene caused different effect mechanisms that were also found in the *GJB2* (CX26) gene, which is a major CX gene linked to hearing loss either alone or as part of a syndrome. For example, p.W44S and p.W44C of CX26 result in a protein that is trafficked to the plasma membrane. In contrast, the p.G59A and p.D66H mutations resulted in protein with impaired trafficking and were concentrated close to the nucleus (Martin et al., 1999; Marziano et al., 2003). Despite p.R127H mutant proteins of the Cx26 were mainly

localized in the cell membrane and prominent in the region of cell–cell contact, but this mutant proteins was a formation of defective junctional channels (Wang et al., 2003). In the CX30.2/CX31.3 protein, the p.E269D mutation occurred in the putative C-terminal cytoplasmic domain, and the p.W77S mutation was localized in the second membrane-spanning segments (TM2) and near border of the E1 domain protein. The p.L23H mutation was at the border of the N-terminal (NT) domain, and the first membrane-spanning segments (M1) and p.R15G occurred in the putative NT cytoplasmic domain (Supplemental Fig. 1). Based on these results, we believe that mutant sites within the protein are important in determining the functional effects of protein.

In a previous study, we found a novel p.W77S mutation in the *GJC3* gene from patients with nonsyndromic hearing loss (Yang et al., 2010). Tryptophan [W; Ph-NH-CH=C-CH2-CH (NH2)-COOH] and serine [S; HO-CH2-CH(NH2)-COOH] are similar, each having polar and unchanged side-chain amino acids (Baldwin and Lapointe, 2003). There are, however, some differences between tryptophan and serine. Tryptophan has heterocyclic aromatic amino side chains, weak basic, and is the largest of the amino acids (Nelson and Cox, 2000). Tryptophan is also a very hydrophobic amino acid and prefers to be buried in protein-hydrophobic cores. Tryptophan also can be involved in interactions with non-protein ligands that themselves contain aromatic groups via stacking interactions and in binding to polyproline-containing peptides, for example, in SH3 or WW domains. Serine is generally considered a slightly polar, weakly acidic, and small amino acid. Serine can reside both within the interior of a protein and on the protein surface. It is quite common in protein functional centers, and it is possible for the serine side-chain hydroxyl oxygen to form a hydrogen bond with the protein's backbone or with a variety of polar substrates. In addition, a common role for serine within intracellular proteins is phosphorylation (Baldwin and Lapointe, 2003; Nelson and Cox, 2000; Betts and Russell, 2003).

Position 77 of the CX30.2/CX31.3 protein is located at the second membrane-spanning segments (M2) and near the border of the first extracellular loop (E1), which is highly conserved

among CX/Cx family members and throughout evolution by Multiple Sequence Alignments and ConSeq analysis (Fig 1). The E1 domain of CX is involved in the interactions between the two adjoining connexons of the gap junction channel (Krutovskikh and Yamasaki, 2000; Maeda et al., 2009). Previous X-ray structure of the CX26 monomer study found that CX26 comprises a typical four-helical bundle in which any pair of neighboring helices is antiparallel. Moreover, this study also indicated that M1, E1, and M2 face the pore; meanwhile, TM3, E2, and TM4 are on the border of the hemichannel facing the lipid or extracellular environments. Additionally, the results disclosed that the cytoplasmic half of TM1 and TM2 are sheltered by the facing the lipid or extracellular environments (Nakagawa et al., 2010). In the CX26, the prominent intra-protomer interactions are in the extracellular part of the transmembrane region. Two hydrophobic cores around p.W44 (E1) and p.W77 (TM2) stabilize the protomer structure of CX26 (Maeda et al., 2009). In this study, our data confirmed that the p.W77S mutant protein of CX30.2/CX31.3 was retained in the ER of HeLa cells. Moreover, we observed significant inhibition of the functional activity of CX30.2/CX31.3-WT in HeLa cells when expressed in a manner mimicking a heterozygous genotype. Thus, p.W77S mutation has dominant negative effect on the function of WT CX30.2/CX31.3. Based on these findings, we suggested that the p.W77 amino acid likely plays a critical role in CX30.2/CX31.3, and as a result a mutation in this residue (W changed to S in position 77) will lead to loss of function of the protein. However, these are predictions, and these cell experiment results are restricted to this study. Therefore, we suggest that the X-ray and 3D structure of the CX30.2/CX31.3 protein needs to be studied to understand further the influence this mutation has at the protein level.

In eukaryotic cells, most secreted and transmembrane proteins fold and mature in the lumen of the endoplasmic reticulum (ER). Previously studies have indicated that CXs can assemble into functional hexameric connexons in the ER membrane (Falk et al., 1997). That CXs pass through the Golgi apparatus to reach the plasma membrane has been demonstrated by subcellular

fractionation studies and immunocolocalization analyses (Musil et al., 1991; Falk et al., 1994; Laird et al., 1995). In this study, we found that the mutant p.W77S proteins accumulate in the ER. The result is similar to the p.E269D mutation. Our previous study described that great quantities of mutant proteins accumulating in the ER might cause unfolded protein responses (UPR), which is a cellular stress response (ER stress) (Zhang and Kaufman, 2004). The ER responds to the accumulation of unfolded proteins in its lumen by activating intracellular signal transduction pathways, cumulatively called UPR (Ron and Walter, 2007). UPR increases the biosynthetic capacity of the secretory pathway through upregulation of ER chaperone and foldase expression. In addition, the UPR decreases the biosynthetic burden of the secretory pathway by downregulating the expression of genes encoding secreted proteins (Schröder and Kaufman, 2005). At least three such mechanisms were found in an imbalance (called ER stress) between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery, the first two of which are rectifying. The first mechanism is a transient adaptation, which reduces the protein load that enters the ER by lowering protein synthesis and translocation into the ER. The second mechanism is a longer-term adaptation that increases the capacity of the ER to handle unfolded proteins by transcriptional activation of UPR target genes, including those that function as part of the ER protein-folding mechanism. If homeostasis cannot be re-established, then a third mechanism, cell apoptosis, is triggered, presumably to protect the organism from rogue cells that display misfolded proteins (Ron and Walter, 2007). In our study, we discovered via cell-viability analysis that the accumulation of p.W77S mutant protein in the ER did not cause cell apoptosis. In conclusion, we found that CX30.2/CX31.3W77S missense mutant proteins were degraded by lysosomes and proteosomes in the transfected-HeLa cell. We have suggested that the accumulation of p.W77S mutant proteins in the ER triggered their degradation, which was insufficient to cause cell apoptosis.

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Figure 1. Multiple alignments of amino acid sequence in connexin proteins by bioinformatics. (A) Alignment of the amino acid sequences of the average domain of human CX30.2/CX31.3 and members of human CX family using Biology WorkBench Clustal W (1.81). The p.W77S are indicated by the frame. (B) ConSeq predictions demonstrated on human CX30.2/CX31.3 [NP_853516; SWISS-PROT: Q8NFK1 (CXG3_Human)], using 50 homologues obtained from the Pfam database (family code: PF00029). The sequence of the CX30.2/CX31.3 protein is displayed with the evolutionary rates at each site colour-coded onto it (see legend). The residues of the CX30.2/CX31.3 sequence are numbered starting from 1. The first row below the sequence lists the predicted burial status of the site (i.e. “b”—buried versus “e”—exposed). The second row indicates residues predicted to be structurally and functionally important: “s” and “f”, respectively. Vertical arrows indicate amino acid codons (p.W77).

Figure 2. Expression analysis of CX30.2/CX31.3WT and CX30.2/CX31.3W77S in transiently transfected HeLa cells by immunocytochemistry using pan-cadherin antibody. Fluorescence microscopy of HeLa cells expressing CX30.2/CX31.3^{WT}-EGFP (A) and CX30.2/CX31.3^{WT}-DsRed shows expression of the CX30.2/CX31.3 fusion protein in the plasma membranes. However, CX30.2/CX31.3^{W77S} -EGFP (C) transfected HeLa cells show impaired trafficking of the CX30.2/CX31.3 protein with localization near the nucleus. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

Figure 3. Intercellular localization of mutant CX30.2/CX31.3 proteins. Photomicrographs of HeLa cells transfected with CX30.2/CX31.3^{W77S}-EGFP cDNA after immunostaining for markers of the lysosome, Golgi apparatus, and ER (anti-PDI) (red in (A)–(C), respectively). Yellow signal in the image overlays (right column) indicates co-localization of CX30.2/CX31.3^{W77S}-EGFP and the organelle of interest. Mutant CX30.2/CX31.3 shows moderate co-localization with the ER marker. The cells were counterstained with 4'-6-Diamidino-2-phenylindole, DAPI, (blue) to highlight the nuclei. Scale bars: 10 μm.

Figure 4. Co-expression of mutant proteins with CX30.2/CX31.3WT revealed by tet-on protein expression system. (A) Tet-on HeLa cells co-expressing CX30.2/CX31.3WT-DsRed and CX30.2/CX31.3WT-EGFP. Co-localization of the two proteins is visible at the plasma membrane. (B) Tet-on HeLa cells co-expressing CX30.2/CX31.3WT-DsRed and CX30.2/CX31.3^{W77S}-EGFP. Co-localization of the two proteins is visible near the nucleus regions. Arrows indicate co-expressed proteins. Cells were counterstained with DAPI to highlight the nuclei. Scale bars: 10 μm.

Figure 5. Quantitative analysis of positive cells displaying after transiently transfected CX30.3/CX31.3WT and CX30.2/CX31.3W77s into HeLa cells. Fluorescence microscopy of HeLa cells expressing CX30.2/CX31.3WT and CX30.2/CX31.3W77S shows expression of the CX30.2/CX31.3 fusion protein in the transiently transfected HeLa cell. (B) Data represent

average ± SD of the percent, after transiently transfected, cells expressing flurencense (EGFP) versus the total cells. Total cell numbers are 1500. Results are representative of three separate experiments.

Figure 6. Cell viability analysis on expressed CX30.2/CX31.3WT and stably CX30.2/CX31.3W77S HeLa cells. Both cells were incubated in DMEM medium for 1-7 day and were then harvested for DNA fragmentation assay (A) and analysis of MTT (B). (A) DNA was prepared for agarose gel electrophoresis as described in the Materials and Methods. (B) The results showed that when the HeLa cells expressed p.W77S mutant proteins, the performance showed unobvious downward trend compared to the number of cells in WT CX30.2/CX31.3. Results are representative of three separate experiments.

Figure 7. Expression analysis of *GJC3* mRNA in the CX30.2/CX31.3WT and CX30.2/CX31.3 W77S transfected HeLa cells by RT-PCR (A) and Quantitative-PCR (B). Total RNA from HeLa cells expressing CX30.2/CX31.3WT and CX30.2/CX31.3W77S confirms expression of the corresponding mRNAs in transfected HeLa cell. β-actin served as reference of the amount of total RNA for each sample. Data represent average ± SD. Results are representative of three separate experiments..

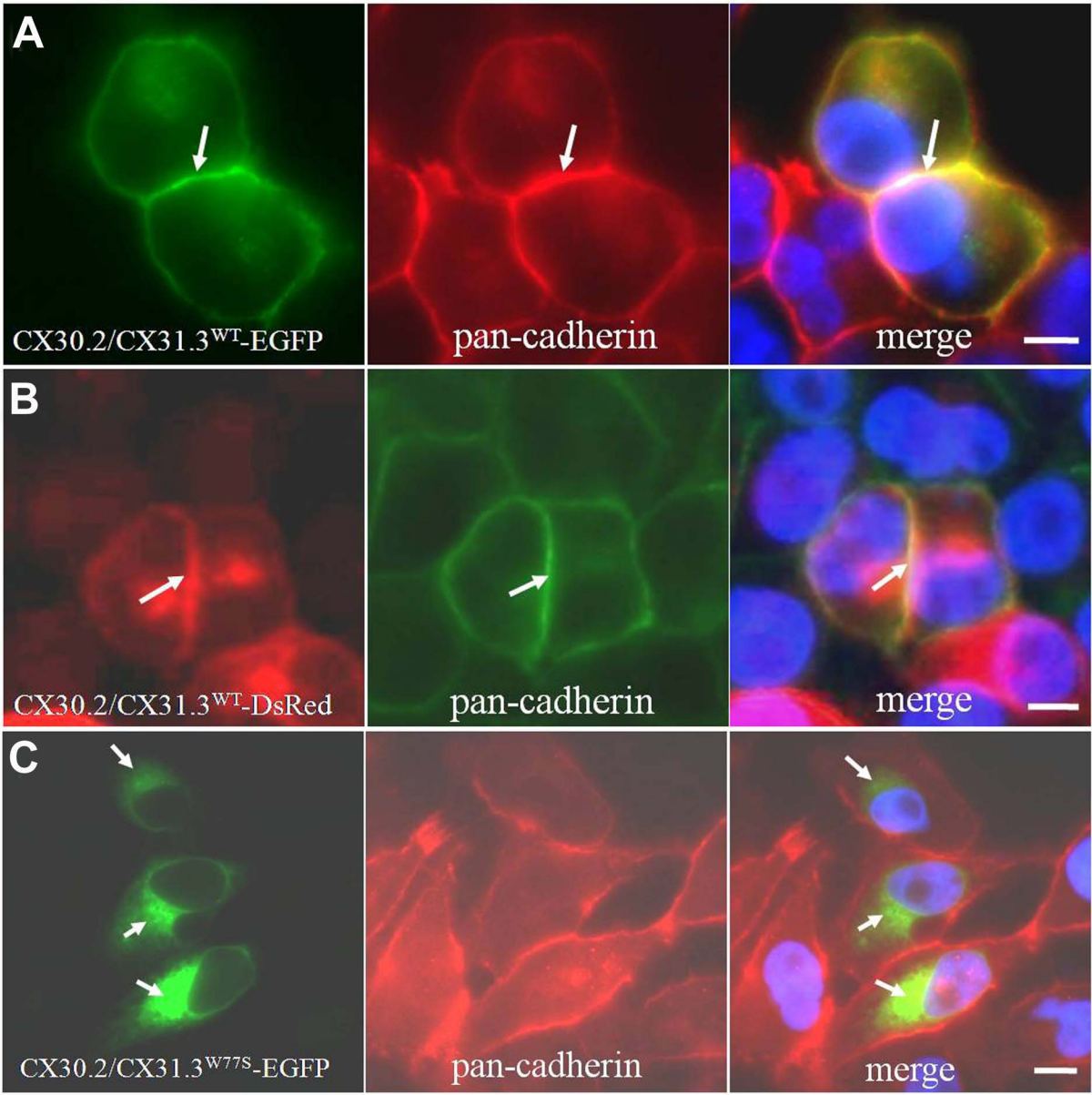
Figure 8. Quantitative analysis of positive cells displaying in the HeLa with transiently expressed CX30.2/CX31.3W77S after chloroquine (CQ) and MG132 treatment. HeLa cells that expressed mutant CX30.2/CX31.3W77S were cultured in the presence of 20μg/ml CQ or 3μg/ml MG132 medium, respectively. After 0, 12, 24, 48 hour, cells were washed and the culture medium was replaced using normal culture medium. Then, these cells were visualized and photographed using a fluorescence microscope (Zeiss Axioplam, Oberkochen, Germany). Data represent average ± SD of the percent, after transiently transfected, cells expressing flurencense (EGFP) versus the total cells. Total cell numbers are 1500. Results are

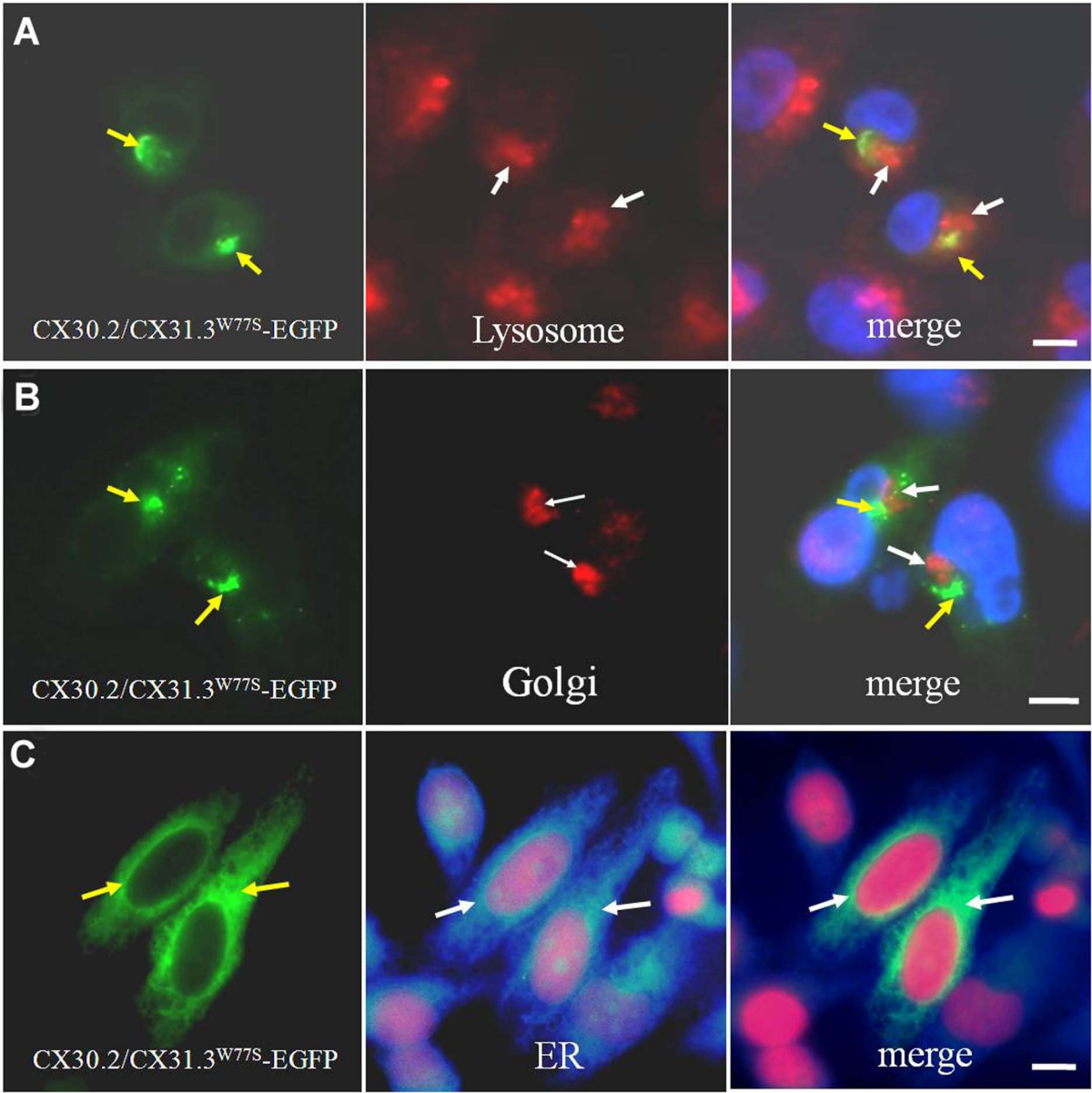
representative of three separate experiments.

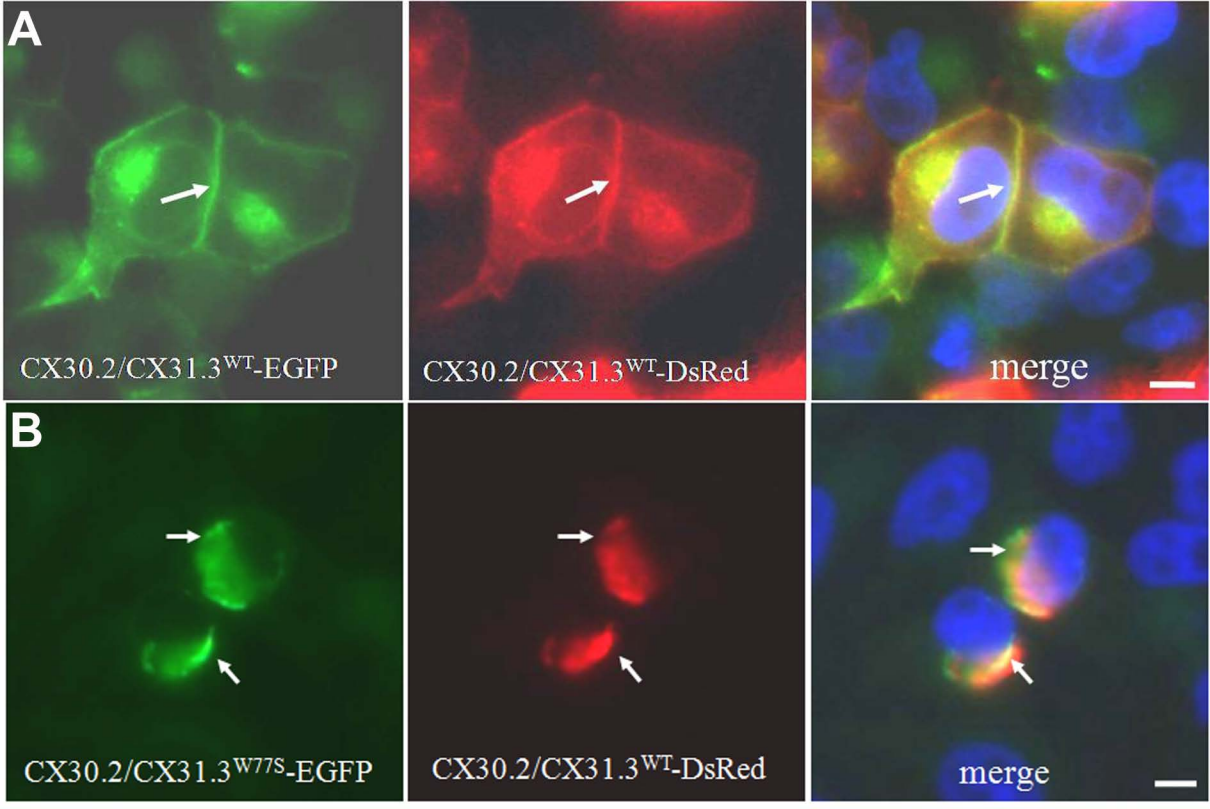
Supplemental Figure 1. Schematic representation of the domain structure of the CX30.2/CX31.3 protein with indication of known variants. The arrows indicate the mutations of CX30.2/CX31.3. TM1-4: transmembrane domains; E1-2: extracellular domains; CL: cytoplasmic linking domain

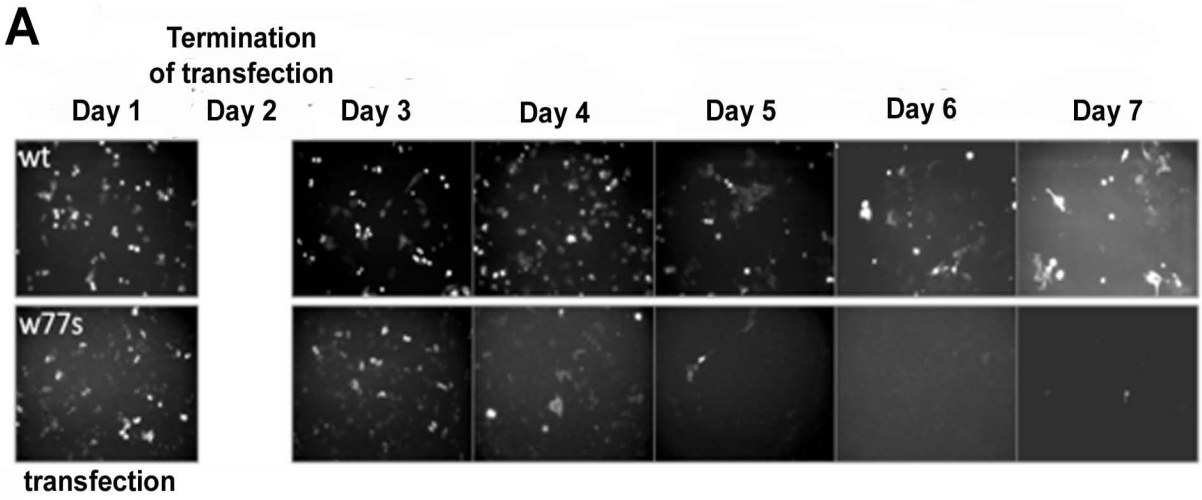

A
p.W77
CX26
CX30
CX32
CX30.3
CX31
CX43
CX30.2/CX31.3
mCx23
consensus

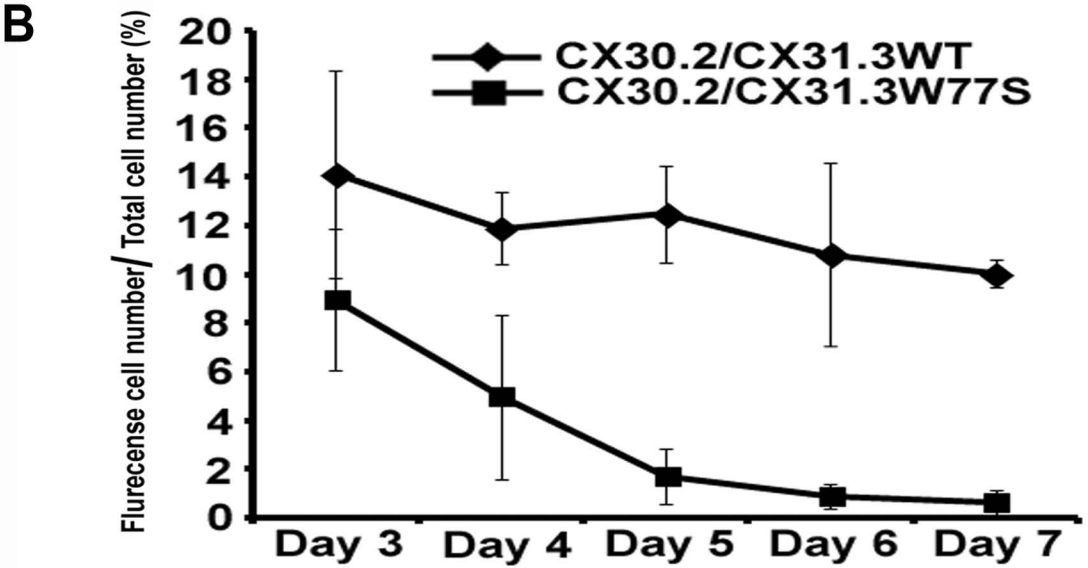
B
ConSeq results
p.W77S
Variable
Average
conserved
e - An exposed residue according to the neural-network algorithm.
b - A buried residue according to the neural-network algorithm.
f - A predicted functional residue (highly conserved and exposed).
s - A predicted structural residue (highly conserved and buried).
x - Insufficient data- the calculation for this site was performed on less than 10% of the sequences.


A
CX30.2/CX31.3WT-EGFP
pan-cadherin
merge
B
CX30.2/CX31.3WT-DsRed
pan-cadherin
merge
C
CX30.2/CX31.3W77S-EGFP
pan-cadherin
merge

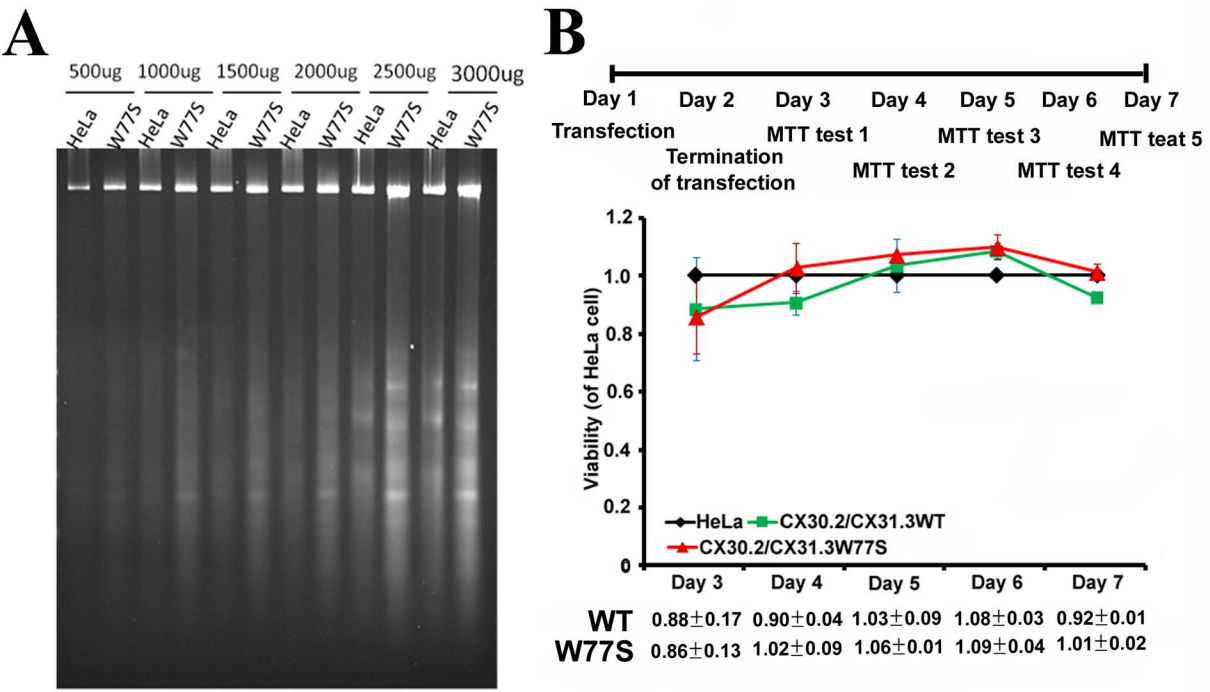

A
CX30.2/CX31.3W77S-EGFP
Lysosome
merge
B
CX30.2/CX31.3W77S-EGFP
Golgi
merge
C
CX30.2/CX31.3W77S-EGFP
ER
merge


A
CX30.2/CX31.3WT-EGFP
CX30.2/CX31.3WT-DsRed
merge
B
CX30.2/CX31.3W77S-EGFP
CX30.2/CX31.3WT-DsRed
merge

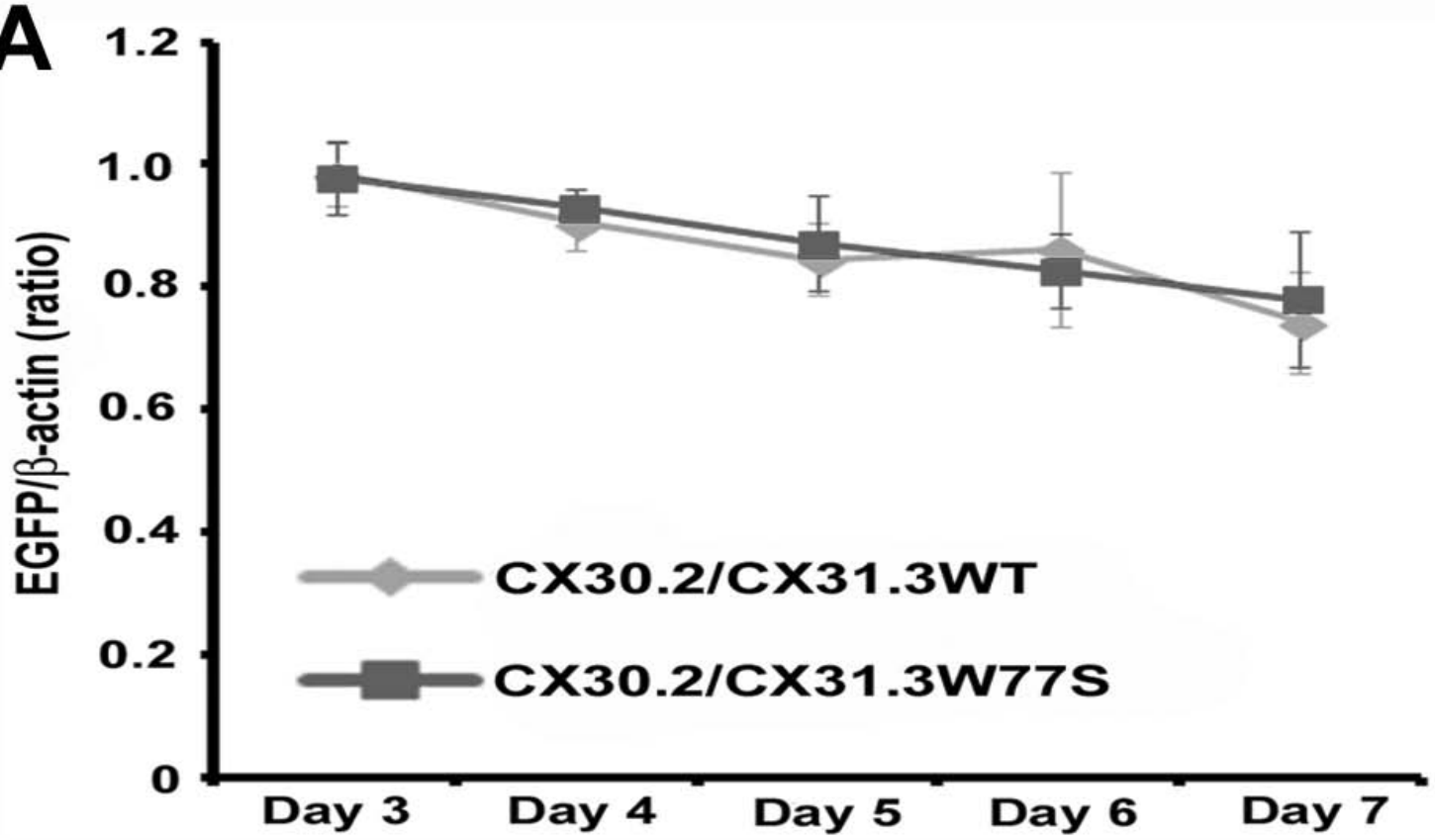




CX30.2/CX31.3WT	14.1±4.28%	11.87±1.47%	12.47± 2.0%	10.8 ±3.75%	10.03±0.58%
CX30.2/CX31.3W77S	8.96 ±2.91%	4.97±3.35%	1.7 ±1.13%	0.9 ±0.53%	0.63±0.49%

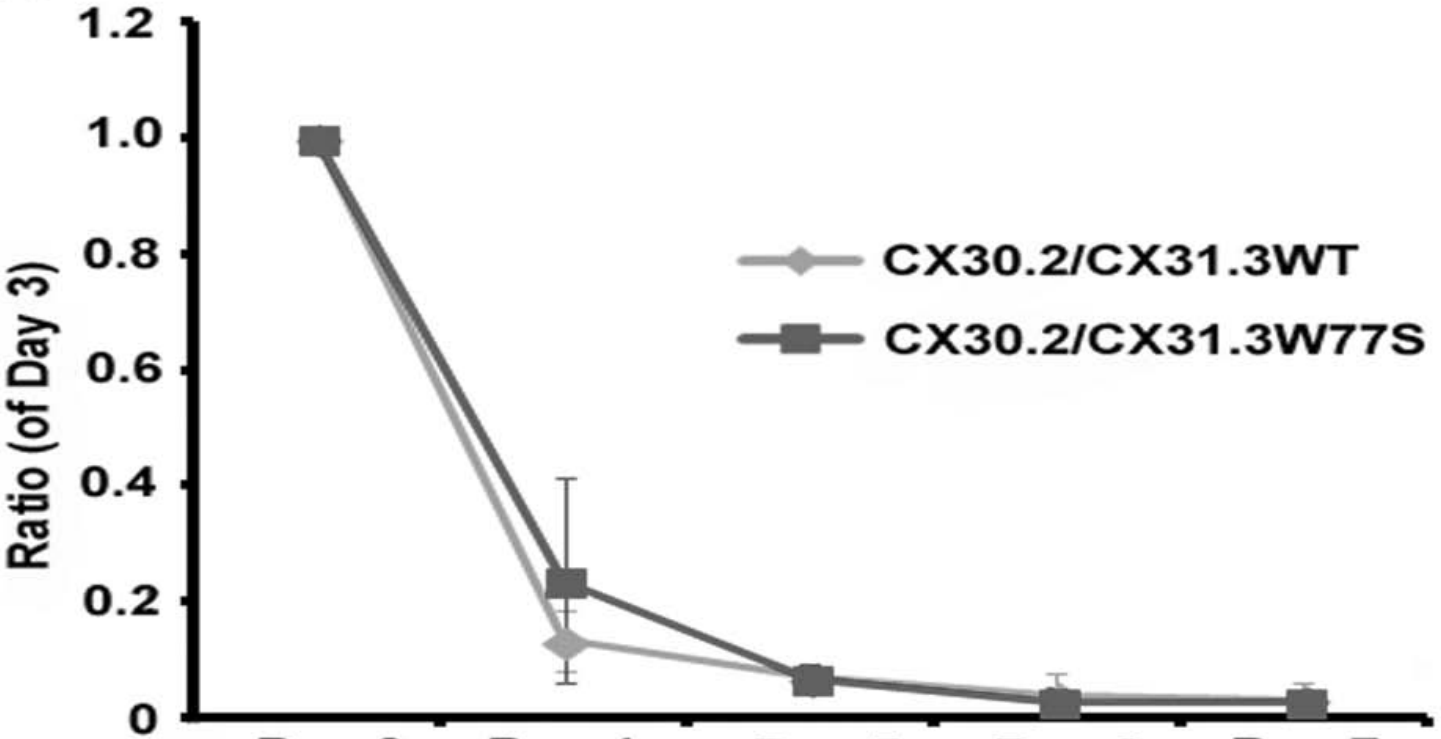

A
500ug
1000ug
1500ug
2000ug
2500ug
3000ug
HeLa
W77S
B
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Transfection
Termination of transfection
MTT test 1
MTT test 2
MTT test 3
MTT test 4
MTT teat 5
Viability (of HeLa cell)
1.2
1.0
0.8
0.6
0.4
0.2
0
HeLa
CX30.2/CX31.3WT
CX30.2/CX31.3W77S
Day 3
Day 4
Day 5
Day 6
Day 7
WT 0.88±0.17 0.90±0.04 1.03±0.09 1.08±0.03 0.92±0.01
W77S 0.86±0.13 1.02±0.09 1.06±0.01 1.09±0.04 1.01±0.02

A

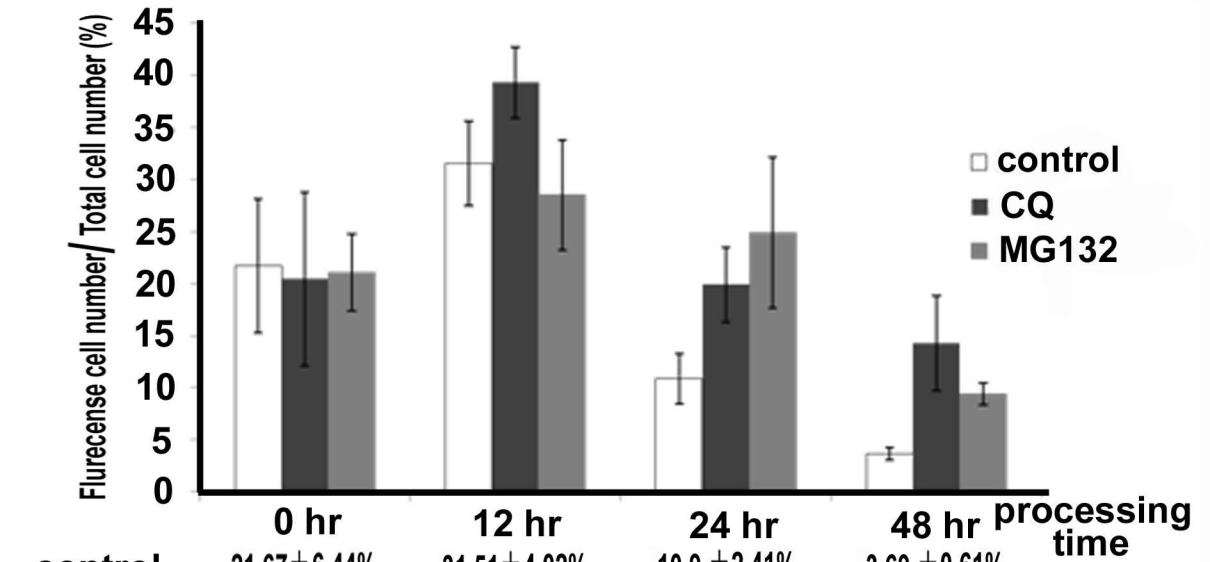


	Day 3	Day 4	Day 5	Day 6	Day 7
WT	0.98±0.05	0.91±0.05	0.85±0.06	0.86±0.13	0.74±0.08
W77S	0.98±0.06	0.93±0.03	0.87±0.08	0.83±0.06	0.78±0.11

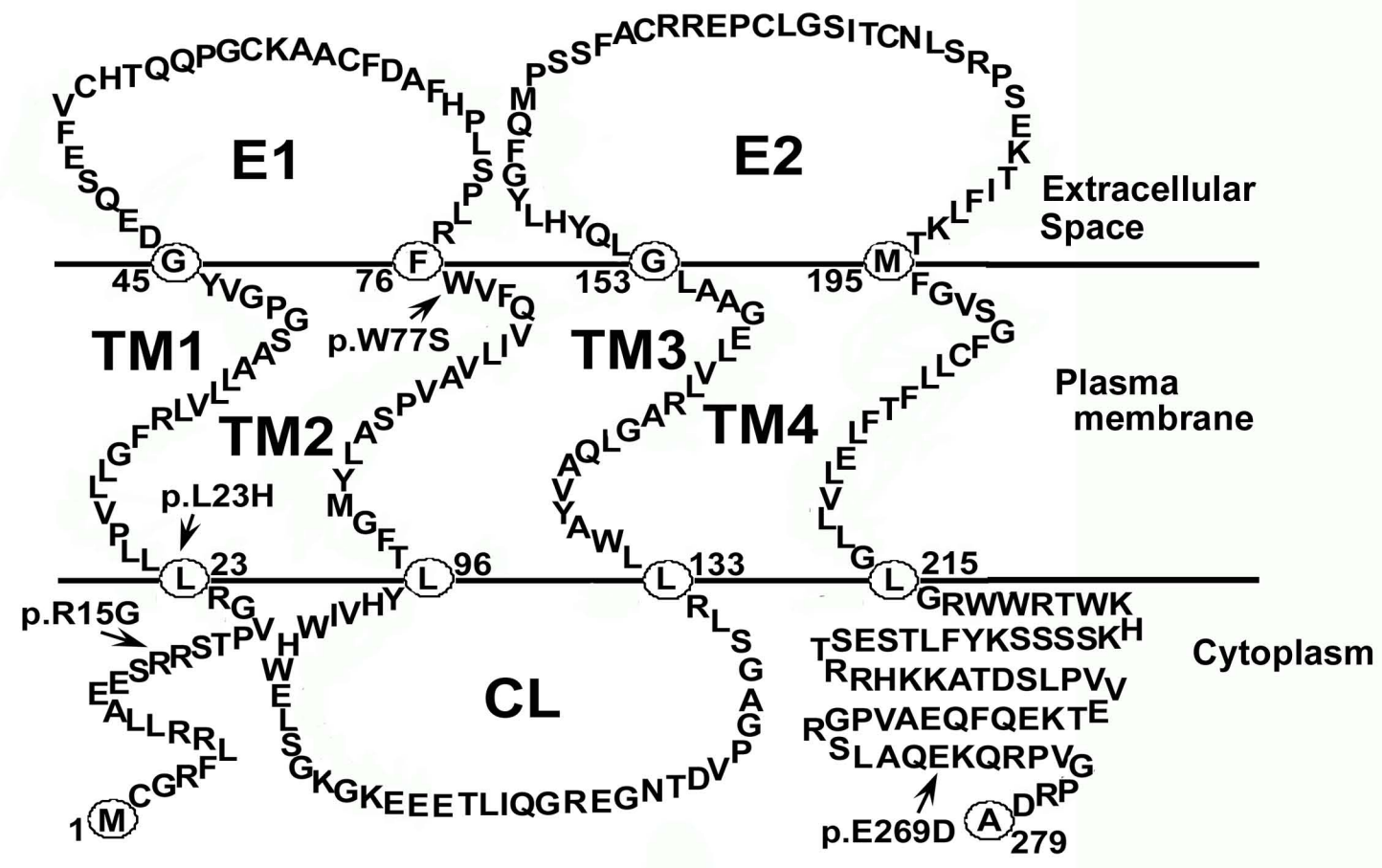
B



	Day 3	Day 4	Day 5	Day 6	Day 7
WT	1	0.33±0.05	0.07±0.02	0.04±0.04	0.03±0.03
W77S	1	0.24±0.18	0.07±0.02	0.03±0.02	0.02±0.01



	0 hr	12 hr	24 hr	48 hr
control	21.67±6.44%	31.51±4.02%	10.9 ±2.41%	3.69 ±0.61%
CQ	20.42± 8.3%	39.28±3.42%	19.88±3.57%	14.24±4.54%
MG132	21.04± 3.7%	28.48±5.28%	24.87±7.22%	9.38 ±2.06%


E1
E2
CL
TM1
TM2
TM3
TM4
Extracellular Space
Plasma membrane
Cytoplasm
45
76
153
195
23
96
133
215
1
279
p.W77S
p.L23H
p.R15G
p.E269D

科技部補助專題研究計畫出席國際學術會議心得報告

日期：103 年 07 月 31 日

計畫編號	MOST　102－2320－B－040－022－		
計畫名稱	利用細胞模式探討 gap junction 通道蛋白造成聽障之分子機制		
出國人員 姓名	楊建洲	服務機構 及職稱	中山醫學大學 生物醫學科學學系 副教授
會議時間	103 年 06 月 28 日至 103 年 07 月 01 日	會議地點	美國、威斯康辛州、麥迪遜
會議名稱	(中文) 2014 第七屆斑馬魚疾病模式國際會議 (英文) 2014 The 7th Zebrafish Disease Models Conference		
發表題目	(中文)研究人類非症候群聽障基因 TMPRSS3 在斑馬魚上的同源基因 (英文) The study of human TMPRSS3 homologous gene in zebrafish (Danio rerio) for non-syndromic hearing loss		

一、 參加會議經過

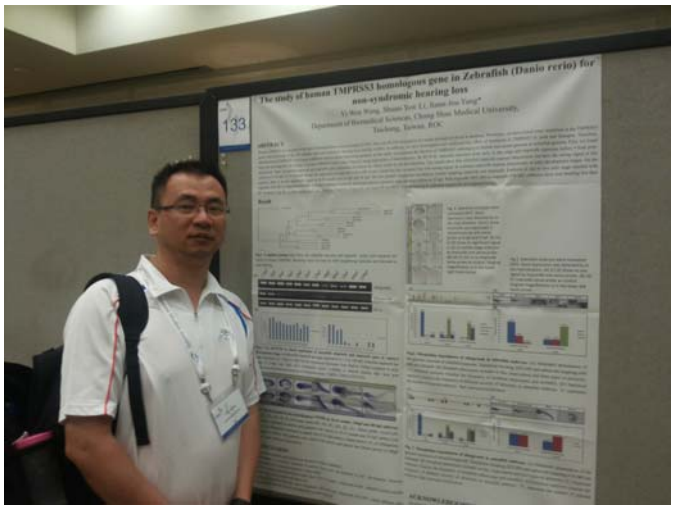
2014 第七屆斑馬魚疾病模式國際會議是 103 年 06 月 28 日至 103 年 07 月 01 日在美國威斯康辛州麥迪遜舉行，會議內容分為十一個部份(session)、兩個專題(Keynote lecture)和三個壁報發表時段來進行，詳細議程如下列網址所示:

http://www.zdmsociety.org/zdm7/ accommodation.html 。另外在第三天下午 16:00~17:00 大會安排所有與會者參觀麥迪遜的地標—威斯康辛州政府(Capitol Building)，在第三天晚上在開會地點舉行會議晚宴。在整個會議現場還有全球各大公司包括飼育環境設備、各種顯微鏡公司，行為觀察設備記錄....等，都在現場提供最新技術的的展現和與會者充分的討論。本人此次參與會議除聆聽一些演講和參觀別人的壁報發表外，本人也將在國科會計畫支持下所做的部分結果以壁報的方式呈現(133-P1) (如圖)。本人的壁報呈現時間在 poster viewing session　I (29, June 12:30~14:30) 。

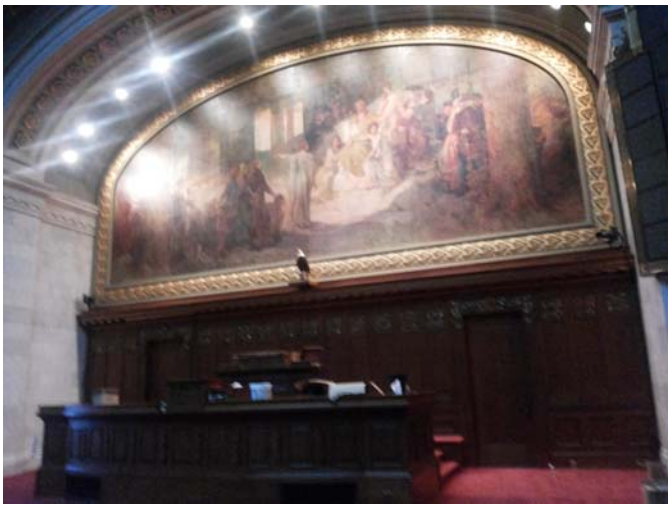
二、 與會心得

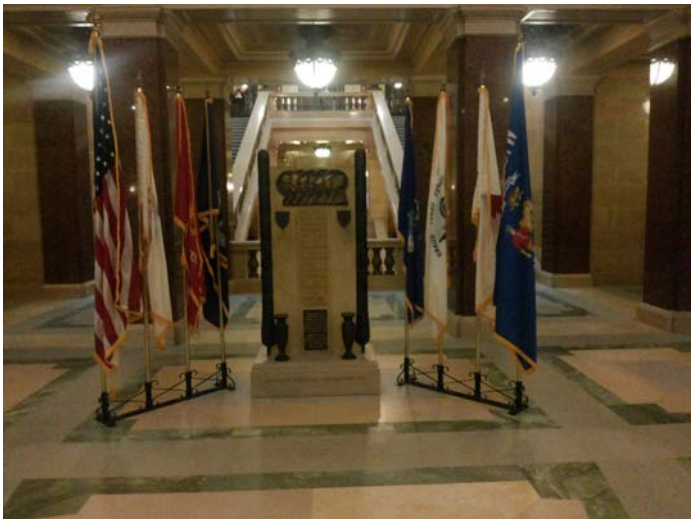
本次會議室一年一度的大型斑馬魚疾病模式國際會議，來自各國從事利用斑馬魚來研究人類疾病的研究者與會相當多，估計應該大約 300 ~ 400 人。本次會議口頭報告有 75 場，另外壁報的呈現有 119 篇，包括了斑馬魚在轉譯醫學的運用、斑馬魚在肌肉和心血管疾病的研究、斑馬魚在胃腸道疾病的研究、斑馬魚在神經系統疾病的研究、斑馬魚在血液和血管疾病的研究、斑馬魚在各種腫瘤疾病的研究、斑馬魚在骨骼疾病的研究、斑馬魚在感染和免疫系統疾病的研究、利用斑馬魚疾病模式來篩檢藥物和化學分子、斑馬魚疾病模式研究最新技術的發展如基因 knockdown 技術和行為模式觀察....等多方向的呈現。本人在研究方面主要是以聽覺和神經系統和疾病模式的建立為主，因此參與本次大會本人所著重的方向在新技術、疾病模式、行為模式分析和神經系統為主。然而，此次大會的安排讓與會者式可參與所有的十一個部份(session)、兩個專題(Keynote lecture)和三個壁報發表，因此本人也聆聽了其他部分的演講和觀看所有的壁報。因此本人參與此次大會獲益良多。在新技術方面，本人的研究最近也有利用 CRISPR/Cas9 技術進行基因剔除魚的實驗，在本次大會也遇到好幾位研究學者發展此技術，以口頭或壁報呈現，本人也與之討論，獲得不少的資訊，對本人的研究有相當的幫助。另外，cre-loxp 基因轉殖技術或其他基因 knockout 技術也在本次大會中有多方面報導，這對我將來研究方向技術的應用將有重大的幫助。另外也看到很多利用基因轉殖魚來做為藥物的篩選和行為分析平台，這也對我現在在實驗室所建立的藥物篩檢平台有很大的幫助。同時此次參與發現很多研究學者都利用斑馬魚來探討各式腫瘤的機制，也發現在斑馬魚系統中有像老鼠模式一樣，已經有免疫系統缺陷的品系被報導，這將可加速利用斑馬魚為模式動物來研究腫瘤的機制和篩選治療藥物。另外此次會議有一最大的特色是有一個 working group break out sessions，讓與會者充分討論、分享研

究所遭遇的問題和如何解決。綜合以上所述，此次會議包括的範圍很廣泛且都是很新的東西，因此本人認為參加此次會議對於本人的研究有很大的幫助。所以非常感謝國科會提供經費讓本人可以參加這國際會議。



同時，本次會議大會安排所有與會者在第三天下午參觀麥迪遜的地標—威斯康辛州政府。威斯康辛州政府是麥迪遜的地標，有著悠久的歷史和背景，在 1 小時的參觀行程中大會安排州政府理的解說員，讓與會者充分了解州政府裡的設施和其歷史背景(如附圖)。另外此活動也讓與會者充分得到一個釋放，暫時獲得休息。所謂”休息是為走更遠的路”。在此感謝大會的安排，讓與會者在會議中所獲得的資訊能夠提高到最大能量。





三、 發表論文全文或摘要

Human TMPRSS3 is a member of the type II transmembrane serine protease (TTSP). This was the first description of a serine protease involved in deafness. Previously, we have found many mutations in the TMPRSS3 genes from screening of the 230 children with non-syndromic hearing loss (14/230; 6.09%). In addition, we have investigated and confirmed the effect of mutations in TMPRSS3 in yeast and Xenopus. Therefore, identification of TMPRSS3 orthologies in different species raises interesting question. In this study, we examined the function of TMPRSS3 orthologies to human and mouse genome in zebrafish genome. First, we found tmprss4a and tmprss4b are similar to human TMPRSS3 and mouse Tmprss3 using approaches of bioinformatics. By RT-PCR, tmprss4a expresses from early to late stage and tmprss4b expresses before 9 hour post-fertilization. Here, we

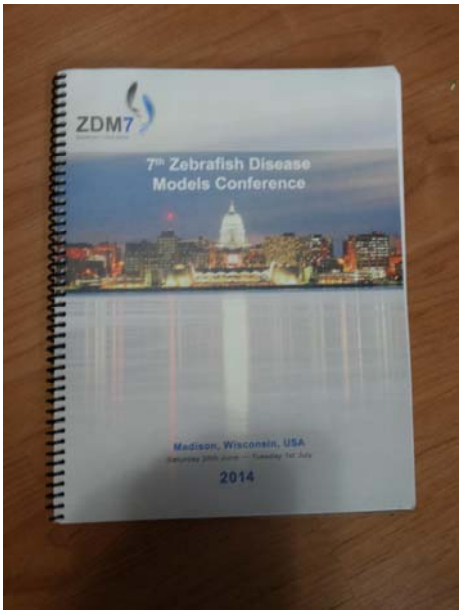
present tmprss4a and tmprss4b gene expression of embryo by whole mount in situ hybridization. The results show that zebrafish tmprss4a express ubiquitously but have the strong signal at otic vesicle at 14-16 hpf. At 24 hpf and 48 hpf, the expression of tmprss4a is not only in otic vesicle but also in lateral line with strong signal. In contrast, tmprss4b express ubiquitously at early development stages. On the contrary, there is no any significant signal at 10-22 somite and 24 hpf and 48 hpf. We also present morpholino knockdown studies targeting tmprss4a and tmprss4b. Embryos at one to two cells stage injected with tmprss4a ATG MO or tmprss4a e9i8 MO will have bending notochords, shortened anterior-posterior axes and heart edema at 72 hpf. With tmprss4b ATG MO or tmprss4b e7i6 MO, embryos show axes bending less than 90° or greater than 90° or heart edema at 72 hpf. However, function of tmprss4a and tmprss4b are direct towards hearing in zebrafish need to be investigated further.

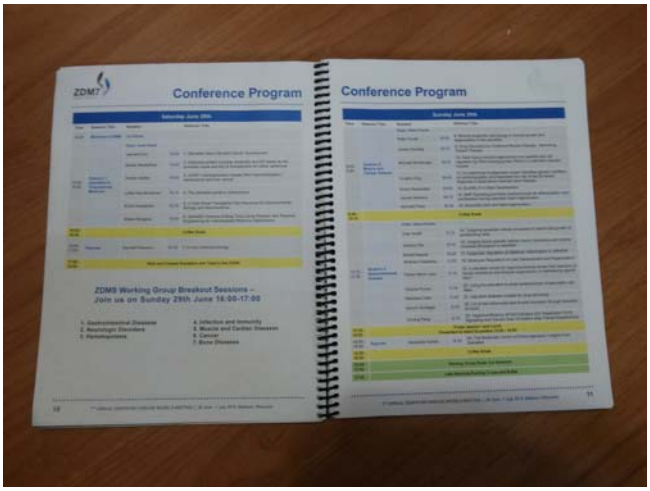
四、 建議

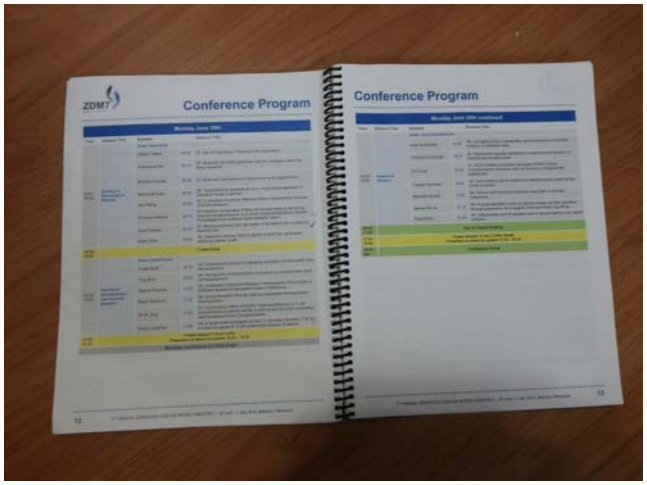
無

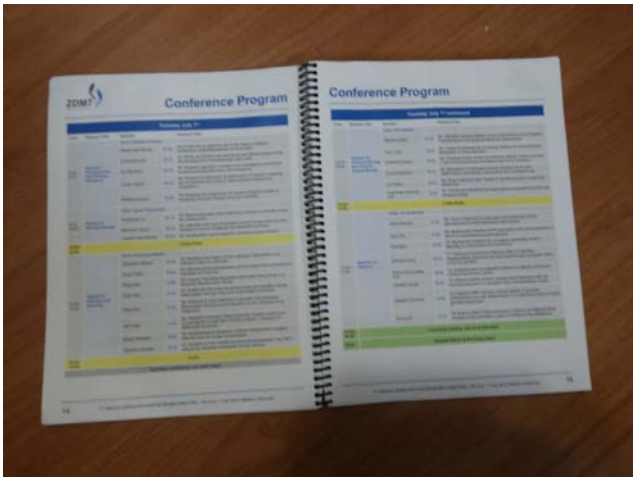
五、攜回資料名稱及內容

2014 第七屆斑馬魚疾病模式國際會議大會秩序冊，內含 6 月 28 日至 07 月 01 日所有會議行程表及所有口頭報告和壁報發表的摘要。另外攜回一份參與此次會議所有人的聯絡方式包括國家、服務機構含職稱及聯絡方式。









六、其他

感謝科技部提供經費讓研究學者參與國外國際會議，讓研究學者可獲得最新的資訊，也增加國際觀，有助於我們研究的進展。

科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/31

科技部補助計畫	計畫名稱: 利用細胞模式探討gap junction 通道蛋白造成聽障之分子機制
	計畫主持人: 楊建洲
	計畫編號: 102-2320-B-040-022- 學門領域: 醫學之生化及分子生物

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

計畫主持人：楊建洲	計畫編號：102-2320-B-040-022-
計畫名稱：利用細胞模式探討 gap junction 通道蛋白造成聽障之分子機制	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	0%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	1	1	100%		
		專書	0	0	0%		
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	2	2	100%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		
國外	論文著作	期刊論文	2	2	100%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	1	1	100%		
		專書	0	0	0%	章/本	
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（外國籍）	碩士生	2	2	100%	人次	
		博士生	2	2	100%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	獲得 102 年科技部補助大專校院獎勵特殊優秀人才

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

發表兩篇 SCI 文章，一篇投稿中。在國內和國外各發表一篇研討會論文。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

本研究對於建立台灣地區非症候群聽障的資料庫和其成因的探討有重要的影響，對於降低其發生率和後續在臨床和基礎上的研究有很大的助益。