

研究快报

Connexin31 显性听力下降相关突变体研究*

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摘要 Cx31 突变可以导致常染色体显性听力下降、常染色体隐性听力下降、周围神经疾病伴听力丧失, 以及变性红皮肤病角化病, 其导致不同疾病的机理一直是研究的重点. 利用定点突变技术 (site-directed mutagenesis, SDM) 构建 connexin31 显性听力下降相关突变体 Cx31R180X, Cx31E183K 并插入到真核表达载体 pEGFP-N1, 转染 HeLa 细胞, 转染 Cx31R180X-pEGFP-N1 和 Cx31E183K-pEGFP-N1 Cx31 突变体质粒的 HeLa 细胞质膜上没有出现斑块状染色和聚集现象, 分别用内质网染料 conA 和高尔基体染料 WGA 进行免疫荧光染色, 结果显示 Cx31 显性听力下降相关突变体蛋白主要分布在细胞质内, 且大部分定位在内质网和高尔基体上. 同时分别用抗 Cx31 和抗 GFP 抗体进行蛋白质印迹检测, 证实 Cx31R180X, Cx31E183K 在转染的 HeLa 细胞中都有表达. 研究发现 Connexin31 显性耳聋相关突变体 Cx31R180X, Cx31E183K 不能正常地形成间隙连接通道, 这与 connexin31 EKV (变性红皮肤病角化病) 相关突变体能够运输到细胞膜上形成间隙连接通道的报道不相同, 提示 connexin31 不同部位突变导致不同疾病的致病机理可能不一样, 从而为解释 “one connexin two diseases” 提供分子水平的依据.

关键词 Cx31, 定点突变技术, 突变体, 绿色荧光蛋白

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间隙连接 (gap junction) 是在相邻细胞间形成的一类聚合通道, 这类通道允许小分子物质如离子、第二信使以及代谢过程中产生的某些分子通过^[1,2]. 几乎所有的细胞与组织均利用间隙通道来传递信息. 间隙连接通道在细胞增殖, 胚胎发育和肿瘤抑制过程中均起着十分重要的作用^[3]. 形成间隙连接的间隙连接蛋白 (connexin) 是一个多基因家族, 该家族不同蛋白质之间具有高度相似性: 均由 4 个跨膜结构域, 2 个胞外环, 1 个胞内环和位于胞质部分的氨基端与羧基端组成. 其中跨膜区与胞外环高度保守, 而胞质部分则相对保守性较低^[4-6].

间隙连接蛋白 31 (Cx31) 是间隙连接蛋白家族中的一员. 1998 年夏家辉院士等^[7]首次在中国湖南怀化和浙江两个显性听力下降家系中检测到 Cx31R180X 和 Cx31E183K 两个突变, 几乎同时 Richard 等^[8]在变异性红皮肤病角化病中检测到 G12R 和 G12D 两种突变. 目前, 发现 Cx31 的突变可导致显、隐性神经性耳聋 (dominant/recessive hearing impairment)^[7-9], 显、隐性红皮肤病角化病 (dominant/recessive EKV)^[10], 或者显性神经性耳聋 - 周围神经病 (dominant hearing impairment combined with peripheral neuropathy)^[11]. 但是 connexin31 基因不同

位点的突变导致不同疾病的分子机理一直不是很清楚.

定点突变技术 (site-directed mutagenesis, SDM) 可以非常方便、快捷地在目的区域引入所需要的突变. 我们利用 mega-primer 法引入 Cx31 显性听力下降相关突变, Cx31R180X 和 Cx31E183K, 并插入到真核表达载体 pEGFP-N1 中, 转染 HeLa 细胞, 激光共聚焦显微镜下观察突变体表达定位情况, 并与已报道的其他 connexin31 基因相关疾病突变体表达定位情况加以比较, 以进一步探明 Cx31 引起不同疾病的致病机理.

1 材料与方法

1.1 仪器与材料

1.1.1 仪器. Pioneer 多肽合成系统 (ABI 公司), 基质辅助激光解析飞行时间质谱 (ABI 公司), Q-TOF (四极杆 - 飞行时间质谱, MicroMASS 公司), 高

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效液相色谱(Waters公司),激光共聚焦(Radiance2100)显微镜

1.1.2 材料. Fmoc-AA-OH(9-芴氧羰基保护氨基酸), HATU, DIPEA, DMF(二甲基甲酰胺), Piperidine(哌啶), TFA(三氟乙酸)(ABI公司); Fmoc-Ala-Wang resin(南开合成公司); 弗氏佐剂(Sigma公司); sulfolink coupling gel(Pierce公司); Pyrobest酶(TaKaRa公司); pEGM-T(Promega公司); pEGFP-N1(Clontech公司); HeLa细胞(CCTCC公司); anti-GFP polyclonal antibody(Clontech公司); anti-rabbit IgG/HRP二抗(CalBiochem.); ECL plus detection kit(Pharmacia公司); ConA(Molecular Probes公司); WGA(Molecular Probes公司).

1.2 方法

1.2.1 MAP多肽法制备抗Cx31抗体. MAP(multiple antigenic peptide)多肽法与KLH法是常用的制备多克隆抗体方法.与KLH法相比,MAP多肽法具有技术较新、操作简单、制备的抗体效价更高等特点^[12].运用DNASTar软件中protean分析人源Cx31序列,选择强免疫原性肽,进行Blast分析,确定Cx31 250~266位氨基酸为合适片段,运用MAP多肽合成法合成多肽片段,超滤纯化后免疫新西兰雄兔,采血检测,并纯化,并用蛋白质印迹(Western blotting),细胞免疫荧光染色来证实得到的抗人Cx31抗体的特异性.

1.2.2 Mega-primer法构建突变体表达质粒.所用到的扩增引物: Cx31peF, 5' CG gaa ttc GGC GCC ATG GAC TGG AAG ACA CTC CA 3' (小写为EcoR I切点,下划线为Kozak序列); Cx31peR, 5' CC gtc gac GGG ATG GGG GTC AGG TTG GG 3' (小写为Sal I切点).突变引物: Cx31R180X, 5' CTA CAT TGC CTG ACC TAC CGA G 3' (T为突变点); Cx31E183K, 5' GCC CGA CCT ACC AAG AAG AAA ATC 3' (A为突变点).PCR产物加A尾后,克隆到T载体中,测序证实序列正确,EcoR I和Sal I酶切并回收目的片段,亚克隆至pEGFP-N1,最后得到表达质粒Cx31R180X-pEGFP-N1和Cx31E183K-pEGFP-N1.

1.2.3 细胞免疫荧光染色.脂质体LipofectAmine 2000转染Cx31R180X-EGFP、Cx31E183K-EGFP、Cx31-EGFP的HeLa细胞用于荧光免疫共定位.操作步骤:细胞吸去培养基后用PBS洗1次,然后用甲醇(-20℃)固定15 min,再用PBS洗4次,每

次10 min,最后用刀豆素A(ConA, 1 mg/L)或麦胚素(WGA, 5 mg/L)染色30 min,用PBS洗4次,每次10 min.

1.2.4 突变体表达的检测.Cx31R180X-pEGFP-N1、Cx31E183K-pEGFP-N1、Cx31-pEGFP-N1转染HeLa细胞,用脂质体LipofectAmine 2000进行转染,具体方法见LipofectAmine 2000说明书,转染后24~48 h检测.加G418至600 mg/L筛选10~15天,至单克隆长出.挑选单克隆,用抗Cx31多克隆抗体和抗GFP抗体蛋白质印迹检测正常和显性听力下降相关突变体的表达情况.

2 结果

2.1 抗血清蛋白质印迹检测

蛋白质印迹结果显示:免疫前血清中没有检测到抗Cx31抗体,免疫血清中检测到抗Cx31抗体,并且效价可达1:2 000(结果没显示).免疫血清经sulfolink coupling gel-半抗原纯化后,再经蛋白质印迹检测,结果显示(图1):抗Cx31抗体效价在1:1 000时,效果较为理想.

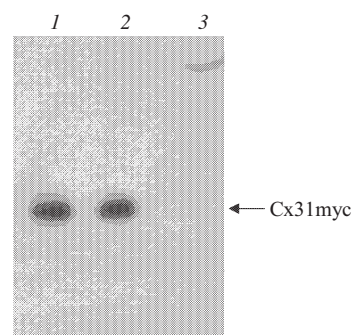


Fig.1 Western blotting analysis of connexin31 polyclonal antibody

1,2: Cx31-pcDNA3.1/myc-his (-) B/HT1080 cell lysate; 3: the HT1080 cell lysate. Arrow shows the Cx31-myc band (32 ku).

2.2 Cx31R180X、Cx31E183K突变体EGFP融合表达载体的构建

先用PCR法扩增出Cx31R180X和Cx31E183K的mega-primer,低熔点凝胶分离,电洗脱回收.以此PCR产物做引物扩增出突变体全长,克隆至T载体中,测序证实成功引入突变,再酶切插入pEGFP-N1中EGFP基因的上游,得到融合表达载体Cx31R180X-pEGFP-N1和Cx31E183K-pEGFP-N1(图2).

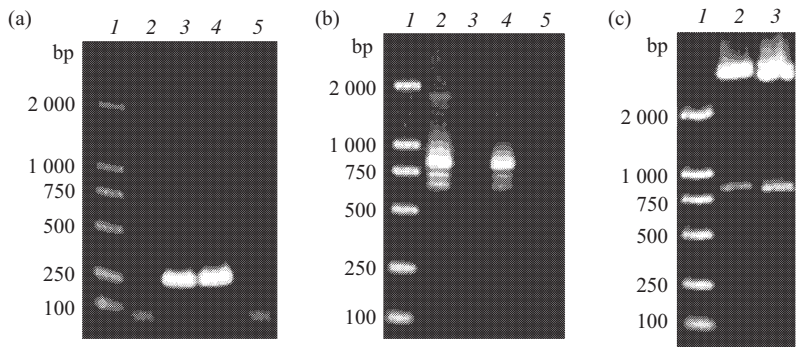


Fig.2 Expression plasmids construction of the dominant hearing impairment associated connexin 31 mutants Cx31R180X and Cx31E183K

(a) amplification of the mega-primer. 1: DL2000; 2,5: negative control; 3: Cx31E183K megaprimer; 4: Cx31R180X mega-primer. (b) amplification of connexin 31 mutants. 1: DL2000; 3,5: negative control; 2: Cx31E183K mutant; 4: Cx31R180X. (c) *EcoR* I and *Sal* I enzyme digestion of the Cx31 mutant constructs. 1: DL2000; 2: Cx31E183K; 3: Cx31R180X.

2.3 细胞免疫荧光染色

转染野生型 Cx31-pEGFP-N1 的细胞间隙连接处和胞质中，有明显而特异的绿色荧光信号，而转染 Cx31R180X-pEGFP-N1 和 Cx31E183K-pEGFP-N1 Cx31 突变体质粒的 HeLa 细胞胞质中可见明显特异的荧光信号，但质膜上却未出现斑块状

染色和聚集现象. 用 WGA 和 ConA 分别对转染后的 HeLa 细胞高尔基体和内质网进行染色，发现 Cx31 显性听力下降相关突变体蛋白分布在细胞质内，且主要在内质网和高尔基体上(图 3，图 4).

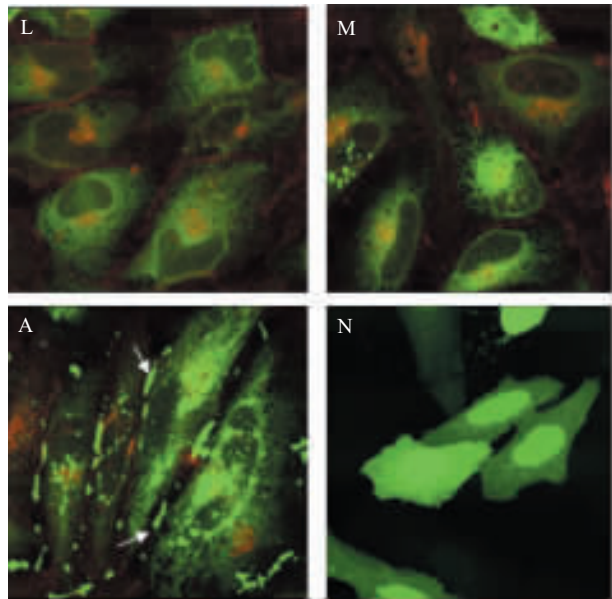


Fig.3 Localization of the R180X and E183K mutants of Cx31 in HeLa cells by confocal microscopy

L: R180X; M: E183K; A: WT-Cx31. N: EGFP; Green fluorescence indicates the connexin EGFP fusion protein. Red fluorescence shows Golgi dyed with WGA. Note the conspicuous aggregation of wild-type Cx31 at the plasma membrane between the cells. In contrast, the majority of the mutant R180X and E183K were cytoplasmic and with less apparent membrane localization. Arrows show the gap junction.

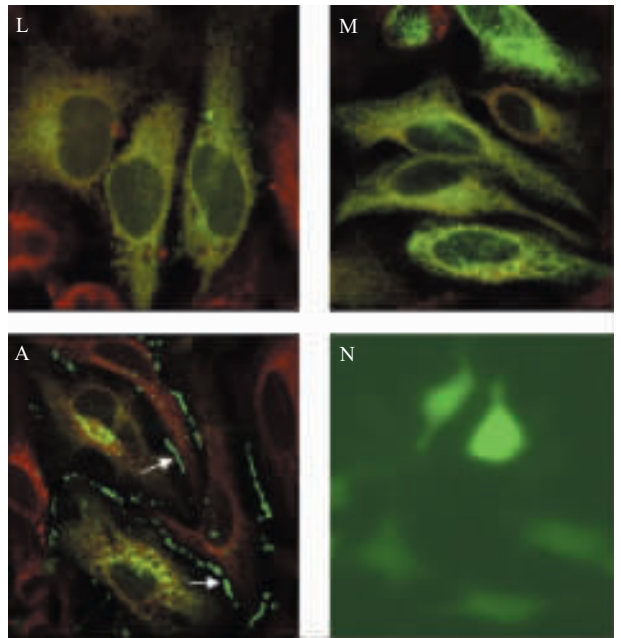


Fig.4 Localization of the R180X and E183K mutants of Cx31 in HeLa cells by confocal microscopy

L: R180X; M: E183K; A: WT-Cx31; N: EGFP. Green fluorescence indicates the connexin EGFP fusion protein. Red fluorescence shows ER dyed with conA. Note the conspicuous aggregation of wild-type Cx31 at the plasma membrane between the cells. In contrast, the majority of the mutant R180X and E183K were cytoplasmic and with less apparent membrane localization. Arrows show gap junction.

2.4 蛋白质印迹检测突变体在转染的 HeLa 中的表达

分别采用 Cx31 和 GFP 抗体来检测 Cx31 的表达情况(图 5). 图 5 中 1, 2, 3 道为用制备的 Cx31 多克隆抗体检测结果, 因为是以 Cx31250~266 位氨基酸作为抗原免疫新西兰兔, 而 R180X 无义突变缺失了 179 位以后的氨基酸, 故用此抗体不能检测到 R180X 突变体蛋白. 图 5 中 4, 5, 6 道为用 GFP 抗体检测结果, R180X 突变是无义突变, 突变蛋白丢失了 179 位后的氨基酸, 故其分子比正常蛋白质要小. 野生型的 Cx31 用这两种抗体都能检测到特异性目的条带.

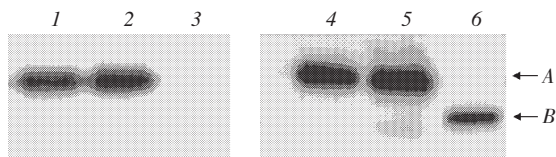


Fig.5 Western blotting analysis of Cx31 R180X and E183K variants expressed in HeLa cells with Cx31 and GFP antibody respectively

1 and 4: WT-Cx31-EGFP; 2 and 5: E183K-EGFP; 3 and 6: R180X-EGFP; 1, 2, 3 blotted with anti-Cx31 antibody; 4, 5, 6 blotted with anti-GFP antibody. A shows the normal size Cx31-EGFP band; B shows the truncated R180X-EGFP band.

3 讨 论

connexin31(即 Cx31, 又称为 GJB3) 是间隙连接蛋白家族中的一员. 1998 年 7 月, Wenzel 等^[13]克隆出人类 Cx31 基因, 同年夏家辉院士等^[7]和 Richard 等^[8]同时报道了 connexin31 为常染色体显性听力下降和变性红皮肤病角化病的致病基因, 但是他们报道的突变位点并不相同. Steel^[14]撰写评论“*One connexin two diseases*”来解释这一现象. 一直以来人们对 connexin31 基因不同位点导致不同疾病的分子机理了解不多. 通过突变体研究, Diestel 等^[15]和 Rouan 等^[16]分别证实 Cx31 的两个显性变性红皮肤病角化病(EKV)突变 (G12R 和 G12D) 能够表达至细胞质膜并形成间隙连接通道. 而 Di 等^[17]则证实包括 G12R 和 G12D 在内的 4 个 EKV 突变 (G12R, G12D, R42P 和 C86S) 不能运输至细胞质膜, 因而不能形成间隙连接通道. 同时, Gottfried 等^[18]发现 Cx31 的隐性 EKV 突变 L34P 表达的蛋白质不能运输至细胞表面形成间隙连接通道, 从而导致 L34P 蛋白积累在细胞质内.

目前为止, 还没有关于 connexin31 显性听力下降相关突变体转运及定位情况的报道. 我们利用定点突变体构建技术构建 Cx31R180X, Cx31E183K 两种 connexin31 显性听力下降相关突变体, 发现这两种显性听力下降相关突变体不能运输至细胞膜表面, 因而不能形成间隙连接通道. 这与 Diestel 等^[15]有关 Cx31 EKV 相关突变体能够运输到细胞膜上并形成间隙连接通道的报道不相同, 从而为解释 “one connexin two diseases”^[14]提供分子水平的参考依据.

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Defective Membrane Localization of Dominant Hearing Impairment Associated Connexin31 Mutants*

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Abstract Connexins form gap junctions that mediate the transfer of ions, metabolites, and second messengers between contacting cells. Connexin31(Cx31) is an important member of connexin β family. Mutations in Cx31 are associated with erythrokeratoderma variabilis (EKV), hearing impairment and peripheral neuropathy. The pathological mechanism for Cx31 mutants in these diseases remains unknown. Using Site-directed mutagenesis (SDM) to construct two dominant hearing impairment associated Cx31 mutant expression plasmids, R180X-pEGFP-N1 and E183K- pEGFP-N1. A number of functional analysis were described to investigate the effect of two dominant hearing impairment-associated Cx31 mutants, R180X and E183K, on connexon trafficking. The majority of the mutant R180X and E183K were cytoplasmic, they can not traffic to normal intracellular localization and can not form functional gap junction channels. But not observed with wild-type Cx31. This may provide an insight into the pathological mechanism of Cx31 mutants.

Key words connexin31, site-directed mutagenesis, mutant, EGFP

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