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Intracellular Distribution, Assembly and Effect of Disease-associated Connexin 31 Mutants in HeLa Cells

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Abstract Mutations in connexin 31 (Cx31) are associated with erythrokeratodermia variabilis (EKV), hearing impairment and peripheral neuropathy; however, the pathological mechanism of Cx31 mutants remains unknown. This study analyzed 11 disease-associated Cx31 variants and one non-disease-associated Cx31 variant and compared their intracellular distribution and assembly in HeLa cells and their effect on these cells. The fluorescent localization assay showed no gap junction plaque formation in the cells expressing the recessive EKV-associated mutant (L34P) and four hearing impairment-associated mutants (66delD, 141delI, R180X and E183K), significantly reduced plaque formation in the cells with five EKV-associated dominant mutants (G12R, G12D, R42P, C86S and F137L) and no obvious change in the cells with two other mutants (I141V and 652del12). Immunoblotting analysis showed that 12 mutated Cx31s, like WT-Cx31, are able to form the Triton X-100 insoluble complex; however, the quantity of Triton X-100 insoluble complex in the transfected HeLa cells varied among different Cx31 mutants. Additionally, the expression of five EKV-associated dominant mutants (G12R, G12D, R42P, C86S and F137L) caused cell death in HeLa cells. However, the five hearing impairment-associated mutants did not induce cell death. The above results suggest that disease-associated mutants gain deleterious functions differentially. In summary, disease-associated Cx31 mutants impair the formation of normal gap junctions at different levels, and the diseases associated with Cx31 mutations may result from the abnormal assembly, trafficking and metabolism of the Cx31 mutants.

Key words connexin 31; erythrokeratodermia variabilis; hearing impairment; peripheral neuropathy; gap junctional intercellular communication (GJIC)

Gap junctions consist of connexin (Cx) and mediate cell-cell communication via direct intercellular exchange of small molecules (<1 kDa). To date, 19 Cx genes have been found in the mouse genome and 20 Cx genes have been found in the human genome [1]. Generally, gap junctions are formed by homomeric or heteromeric hemichannels that are assembled by the same or different kinds of connexin [2]. Mutations in connexin have been identified with various inherited diseases, including Cx32 mutation in X-linked Charcot Marie tooth disease [3,4], Cx26 and Cx30 mutations in deafness and skin diseases [5–11], Cx46 and Cx50 mutations in hereditary cataracts [12–20] and Cx31 mutation in erythrokeratodermia variabilis (EKV) and hearing impairment with/without peripheral neuropathy [21–26].

Cx31 is an important member of the connexin family, but the molecular mechanism of Cx31 in human diseases remains unclear. Diestel *et al*. [27] reported that the Cx31 mutant (G12R) was expressed at a comparable level as

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wild type Cx31 (WT-Cx31) and localized on the plasma membrane. It also showed a higher conductance than WT-Cx31 in dye couple studies. Di *et al*. [28] reported that four EKV-associated Cx31 mutants (G12R, G12D, R42P and C86S) exhibited defective trafficking to the plasma membrane and that the deafness/neuropathy-associated mutant 66delD had a primarily cytoplasmic distribution, but certain proteins were visualized at the plasma membrane in a few transfected cells. These findings suggest that the distributions of Cx31 mutants are different.

Cellular localization assays have indicated that many connexin mutants fail to assemble or localize to the cell membrane to establish normal gap junction intercellular communication (GJIC) [29,30]. Biochemical assays have also shown that many of the mutated connexins wrongly target the gap junctions and/or fail to oligomerize correctly into hemichannels [31].

In this study, the subcellular localization, effect on transfected cells and solubility in Triton X-100 of 12 Cx mutants in HeLa cells were analyzed. The study shows that different Cx31 mutants differ in terms of intracellular distribution, assembly and effect on HeLa cells.

Materials and Methods

Construct with chimeric Cx31 mutation/EGFP

The Cx31 mutants (G12R, G12D, L34P, R42P, 66delD, C86S, F137L, I141V, 141delI, R180X, E183K and 652del12) were produced by PCR using gene splicing by overlap extension (**Table 1**).

In the primary PCR, two segments of the Cx31 (C-segment and T-segment) were amplified from pEGFP-Cx31 using the primers Cx31-F and Cx31-R with the following conditions: 5 min at 95 °C; 30 cycles of 20 s each at 95 °C, 30 s at 50 °C and 45 s at 72 °C; and 10 min at 72 °C. Moreover, Cx31 was produced using the primers Cx31-F/Cx31-R in the secondary PCR under the following conditions: 5 min at 95 °C; 30 cycles of 20 s each at 95 °C, 30 s at 62 °C and 45 s at 72 °C; and 10 min at 72 °C. Furthermore, 12 mutants were generated by PCR using specific primers listed in **Table 1**, under these conditions: 5 min at 95 °C; 30 cycles of 20 s each at 95 °C, 30 s at 62 °C and 45 s at 72 °C; and 10 min at 72 °C. Primers were synthesized by Shanghai Bioasia (Shanghai, China). After the amplification, the PCR products were directly cloned into a TA cloning vector, pGEM-T (Promega, Madison, USA). The mutated Cx31 fragments were cut with two restriction enzymes (*Eco*RI and *Sal*I; TaKaRa, Dalian, China), and further cloned into the pEGFP-N1 vector (Clontech, Mountain View, USA). All mutants were sequenced, and the clones with correct base changes were chosen for subsequent study.

Transfection with Cx31/EGFP fusion constructs

HeLa cell line deficient in GJIC was purchased from CCTCC and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS (Gibco BRL, Gaithersburg, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a moist atmosphere containing 5% CO_2. Transfection was carried out using Lipofectamine 2000 reagent (InvitrogenCarlsbad, USA) according to the manufacturer's instructions. Generally, a ratio of 1 μg DNA vs. 2 μl Lipofectamine 2000 was used for the HeLa cells. 24 h post-transfection, cells were harvested for Western blotting, or fixed with cold methanol for fluorescent staining. To select HeLa cell colonies stably expressing WT-Cx31 or Cx31 mutants, the selective medium containing 800 μg/ml G418 was renewed at 4-d interval. After 2–3 weeks, single cell colonies were obtained. Under the fluorescence microscope, the cell clones displaying green fluorescence were picked for further culture.

Immunofluorescent staining

For the fluorescent staining of endoplasmic reticulum (ER) or Golgi apparatus, HeLa cells were fixed with cold methanol for 15 min, washed 3 times with 0.1% Triton X-100/PBS, 10 min each time, and then stained with con A or WGA (conjugated with Alexa Fluor 594) for 15 min, and then washed 3 times with PBS. HeLa cells were observed using a fluorescence microscope, and images were taken using a laser-scanning confocal microscope (Bio-Rad Inc., Hercules, USA).

Solubility analysis of Cx31 mutants in Triton X-100 solution

24 h after transfection, HeLa cells expressing Cx31 mutants were rinsed once with PBS and incubated on ice for 30 min with PBS containing 1% Triton X-100 and a proteinase inhibitor cocktail (Sigma, St. Louis, USA). The cells were gathered by scraping, and then centrifuged at 100,000 *g* for 30 min. The insoluble fractions were lysed in SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS) and the protein concentration was determined using a Bio-Rad DC protein assay kit. Equal amounts of each sample were separated by 10% SDS-

Table 1 Genotype and phenotype of Cx31 and its mutants

Mutant	Description	Disease	Protein domain	Primer (5′→3′)	Ref.
Cx31	Wide type	–	–	Cx31-F: CG*GAATTC*TGGGCGCCATGGACTGGAAGACAC-TCCA Cx31-R: GC*GTCGAC*TGGATGGGGGTCAGGTTGGG	
G12R	G to C at 34	EKV (dominant)	IC1	G12R-F: CC*GAATTC*TGGGCGCCATGGACTGGAAGACAC-TCCAGGCCCTACTGAGC***C***GTGTGAACAAGT Cx31-R: GC*GTCGAC*TGGATGGGGGTCAGGTTGGG	[21]
G12D	G to A at 35	EKV (dominant)	IC1	G12D-F: CC*GAATTC*TGGGCGCCATGGACTGGAAGACAC-TCCAGGCCCTACTGAGCG***A***TGTGAACAAGT Cx31-R: GC*GTCGAC*TGGATGGGGGTCAGGTTGGG	[21]
R42P	G to C at 125	EKV (dominant)	EC1	R42P-F: CTGCAGAGC***C***CGTGTGGGG R42P-R: CCCCACACG***G***GCTCTGCAG	[21]
L34P	T to C at 101	EKV (recessive)	TM1	L34P-F: CTTCCGGGTGC***C***GGTATACGTG GTGGCTG L34P-R: CACGTATAC***G***GCACCCGGAAGACGAAC	[23]
66delD	196delGAC	Peripheral neuropathy /deafness	EC1	66delD-F: CAACGTCTGCTAC(GAC)AACTACTTCCCCA 66delD-R: GGGGAAGTAGTT(GTC)GTAGCAGACGTTG	[26]
C86S	T to A at 256	EKV (dominant)	TM2	C86S-F: TTCGTCACA***A***GCCCCTCGC C86S-R: GCGAGGGGC***T***TGTGACGAA	[21]
F137L	T to C at 409	EKV (dominant)	TM3	F137L-F: AGCCTCATC***C***TCAAGCTCA F137L-R: TGAGCTTGA***G***GATGAGGCT	[22]
I141V	A to G at 423	Deafness (recessive)	TM3	I141V-F: CTTCAAGCTCATC***G***TTGAGTTCCTC I141V-R: GAGGAACTCAA***C***GATGAGCTTGAAG	[25]
141delI	421del ATT	Deafness (recessive)	TM3	141delI-F: CTTCAAGCTCATC(ATT)GAGTTCCTCTTCC 141delI-R: GGAAGAGGAACTC(AAG)GATGAGCTTGAAG	[25]
R180X	C to T at 538	Deafness (dominant)	EC2	R180X-F: CTGCTACATTGCC***T***GACCTACCGAGAAG R180X-R: CGGTAGGTC***A***GGCAATGTAGCAGTCCA	[24]
E183K	G to A at 547	Deafness (dominant)	EC2	E183K-F: GCAGAGCTCACA***A***ATGGTGAGTACGA E183K-R: GTACTCACCAT***T***TGTGAGCTCTGCTACC	[24]
652del12	del 12 nt at 652	–	IC3	652del12-F: CCTGCGAGGC(12 nt)AAGCCTCGAGGGGGTTG 652del12-R: CTCGAGGCTT(12 nt)GCCTCGCAGGACCCTG	[22]

EKV, erythrokeratodermia variabilis; EC, extracellular; IC, intracellular; TM, transmembrane; nt, nucleotide. Restriction site sequences are in italic: GTCGAC, *Sal*I site; GAATTC, *Eco*RI site. The mutated sites are in bold and italic, and the mutated or deleted codons are underlined. Ref., reference.

PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane by electro-transfer. The PVDF membrane was incubated overnight with PBS containing 5% skimmed milk and 3% BSA. The primary antibody (rabbit anti-Cx31 or anti-GFP polyclonal antibody, 1:1000; Clontech) was then added for 2 h and washed 3 times with PBS with 0.1% Trition X-100 (PBST). Next, the secondary antibody (HRP-conjugated goat anti-rabbit antibody, 1:10,000; CalbiochemSan Diego, USA) was added for 1 h, and washed 3 times with PBST. The membrane was then detected using an ECL kit (Amersham Biosciences).

Results

Localization of the Cx31 mutants

Fig. 1 showed the cellular localization of WT-Cx31 and 12 Cx31 mutants 24 h post-transfection. HeLa cells expressing EGFP exhibited green fluorescence in whole cells, both in the cytoplasm and nucleus [**Fig. 1(A)**]. HeLa cells expressing WT-Cx31/EGFP displayed punctate staining and aggregation at the plasma membrane, particularly in the regions of cell-ell contact [**Fig. 1(B)**].

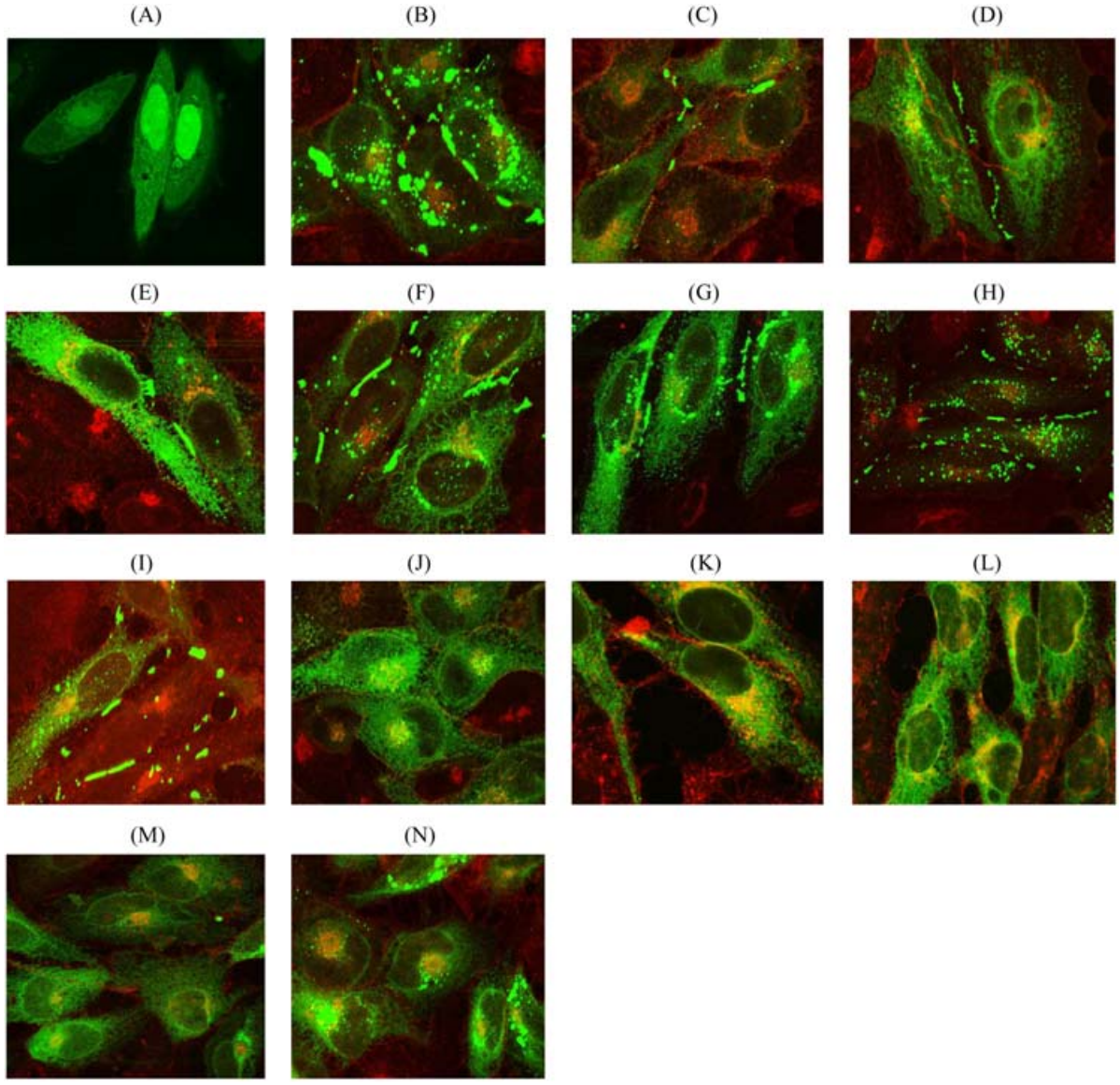

Fig. 1 Localization of EGFP, WT-Cx31, G12R, G12D, R42P, C86S, F137L, 652del12, I141V, L34P, 66delD, 141delI, R180X and E183K in HeLa cells

(A) EGFP. (B) WT-Cx31. (C) G12R. (D) G12D. (E) R42P. (F) C86S. (G) F137L. (H) 652del12. (I) I141V. (J) L34P. (K) 66delD. (L) 141delI. (M) R180X. (N) E183K. EGFP is expressed in whole cells (A). Green fluorescence represents the connexin-EGFP fusion protein, while red fluorescence indicates the Golgi apparatus (B–N). Note the clear aggregation of wild type or mutant Cx31 (B–I) at the plasma membrane between the cells. Most of the other Cx31 mutants (J–N) are evenly distributed in whole cells, and membrane localization is less apparent. Magnification, 600×.

Punctate staining and aggregation at the plasma membrane were also observed in G12R, G12D, R42P, C86S, F137L, I141V and 652del12, particularly in the regions of cell-cell contact [**Fig. 1(C–I)**]. In L34P, 66delD, 141delI, R180X and E183K strains, punctate staining and aggregation did not exhibit at the plasma membrane [**Fig. 1(J–N)**] although Cx proteins existed in the cytoplasm, mainly in ER or Golgi apparatus.

The number of cells forming gap junction channels in adjacent HeLa cells both expressing Cx31 mutants was also analyzed. The analytical results indicated that the portion of cells with gap junction plaque in the five dominant EKV mutants (G12R, G12D, R42P, C86S and F137L) was significantly lower compared with that of WT-Cx31 ($P<0.05$), while the proportions in the two mutants (I141V and 652del12) clearly did not decrease compared with

WT-Cx31 (P>0.05) (**Fig. 2**).

In G12R, G12D, R42P, C86S and F137L strains, cell and nuclei morphology changed 24 h post-transfection. G12D and F137L showed similar patterns (**Fig. 3**), and G12R, R42P and C86S were also similar (data not shown),

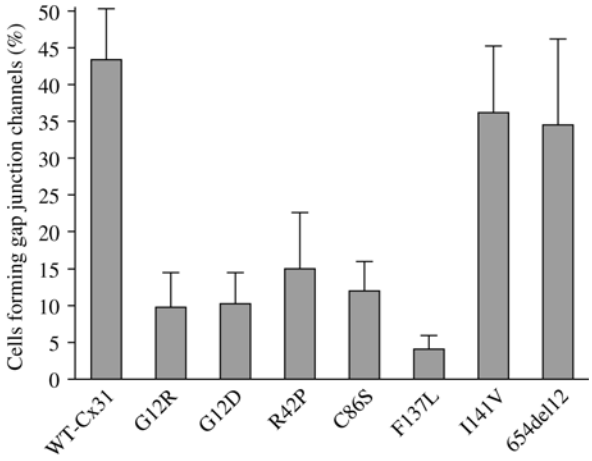

Fig. 2 HeLa cells with gap junction channels transfected with Cx31 mutants

The number of cell pairs with gap junction channels was accounted for by 250 neighbor cells expressing WT-Cx31 or Cx31 mutants. Three independent experiments were performed. The data are shown as mean±SD (n=3).

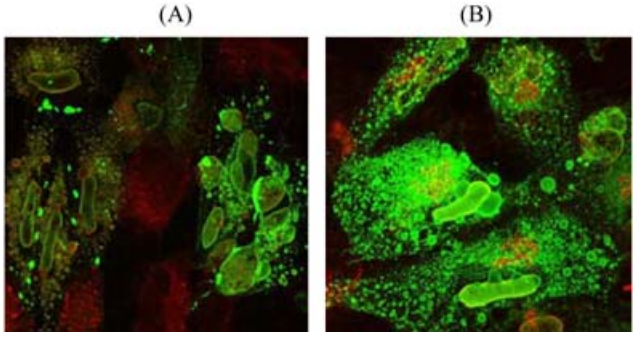

Fig. 3 Effect of Cx31 mutants on HeLa cells

(A) G12D: G12D/EGFP. (B) F137L: F137L/EGFP. Green fluorescence indicates the connexin-EGFP fusion protein, while red fluorescence indicates ER. Magnification, 600×.

which is consistent with the results of Common *et al*. [30]. Furthermore, the recessive EKV mutant, L34P, was found to be not lethal to HeLa cells.

Using the G418 screening, HeLa cell lines that stably expressed WT-Cx31, L34P, 66delD, R180X, E183K, I141V, 141delI and 652del12 were obtained (**Fig. 4**), but HeLa cell lines that stably expressed G12R, G12D, R42P, C86S or F137L could not be obtained, which is contrary to the conclusion of Common *et al*. [30] that "defective

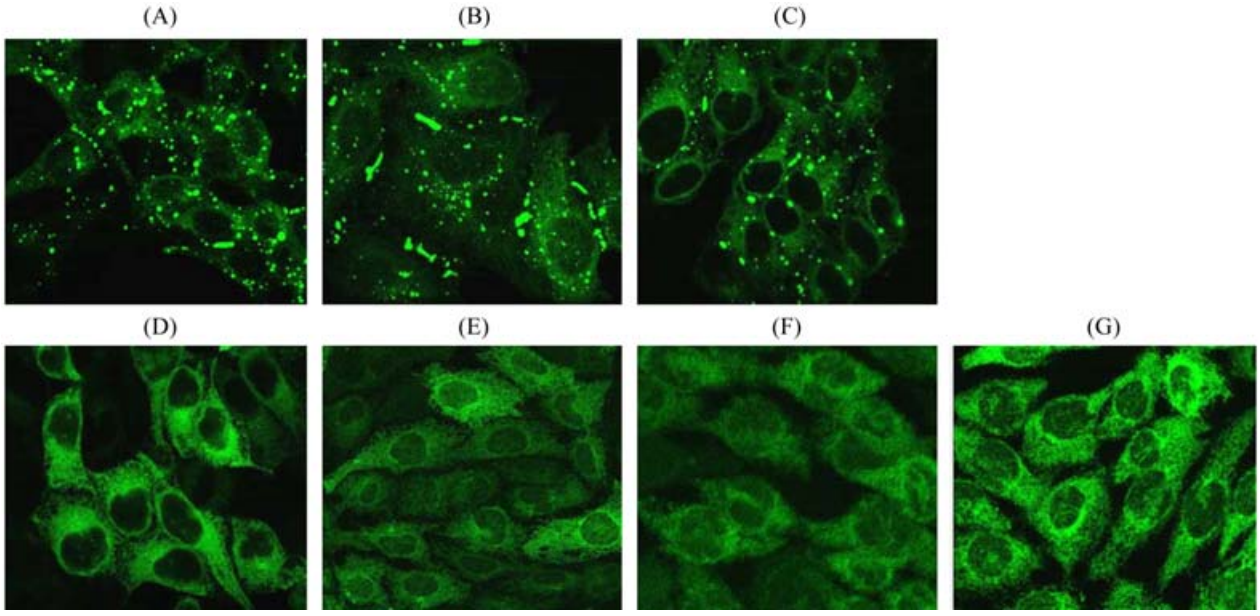

Fig. 4 Stable expression of WT-Cx31 or Cx31 mutants in HeLa cells

(A) WT-Cx31/EGFP. (B) I141V/EGFP. (C) 652del12/EGFP. (D) 66delD/EGFP. (E) 141delI/EGFP. (F) R180X/EGFP. (G) E183K/EGFP. Green fluorescence indicates the connexin-EGFP fusion protein. WT-Cx31 or mutant Cx31 (A–C) can form gap junction channels in the cell membrane, but other Cx31 mutants (D–G) can not. Magnification, 400×.

trafficking and cell death is characteristic of skin disease-associated connexin 31 mutations".

Solubility of Cx31 mutants in Triton X-100 solution

The formation of oligomers is a key step in establishing gap junctions at the cell surface. Previous studies have shown that connexin oligomers are insoluble in 1% Triton X-100, but its monomer is soluble [32]. This study examined whether the Cx31 mutants could form insoluble oligomers in 1% Triton X-100. The results revealed that all these mutants could form insoluble oligomers in 1% Triton X-100 solution (**Fig. 5**). However, the number of oligomers formed differed among the Cx31 mutants. Fewer oligomers were formed by R180X than those of other mutants.

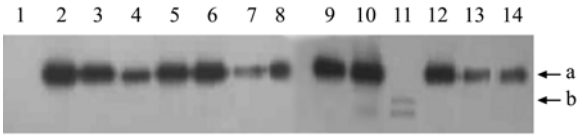

Fig. 5 Immunoblot analysis of WT-Cx31 and Cx31 mutants
HeLa cells expressing WT-Cx31 or Cx31 mutants were lysed in PBS containing 1% Triton X-100 at 4 °C and centrifuged at 100,000 *g* for 50 min. The Triton X-100 insoluble pellet was then detected using the anti-GFP polyclonal antibody. 1, control (non-transfected HeLa cells); 2, WT-Cx31/EGFP; 3, G12D/EGFP; 4, L34P/EGFP; 5, R42P/EGFP; 6, 66delD/EGFP; 7, C86S/EGFP; 8, F137L/EGFP; 9, 141delI/EGFP; 10, I141V/EGFP; 11, R180X/EGFP; 12, 652del12/EGFP; 13, E183K/EGFP; 14, G12R/EGFP. a, indicates the normal molecular weight size of Cx31/EGFP; b, indicates lower than the normal weight size of Cx31/EGFP.

Discussion

Connexin, an essential component of gap junctions, must be trafficked to the cell membrane to execute its biological function. Cellular localization and function assay suggest that mutations in connexin cause degradation in their expression, assembly, trafficking or formation of functional gap junctions, thereby damaging communication between neighboring cells.

Deschenes *et al*. [29] studied the cellular localization of nine X-linked Charcot Marie tooth disease (CMTX)-associated Cx32 mutants in PC12J cells. These Cx32 mutants were grouped into three classes: (1) mutant mRNA was transcribed, but little or no protein was detected; (2) mutant protein was detectable in the cytoplasm and at the cell surface, where it appeared as plaques and punctate staining; (3) the immunoreactivity of the mutant protein was restricted to the cytoplasm and frequently colocalized with the Golgi apparatus. Common *et al*. [30] studied four Cx30 mutants, and found that three skin disease-associated mutants failed to be trafficked to the plasma membrane, and thus could not form functional gap junctions. The deafness-associated mutant can be trafficked to the membrane, but has no channel activity.

The present study examined 11 disease-associated Cx31 mutants in HeLa cells. Three types of mutations, according to subcellular distribution, were observed. Type I, including L34P, 66delD, 141delI, R180X and E183K, is characterized by the cytoplasmic accumulation of Cx31 and the absence of cell surface expression. These mutants alter the trafficking so that the proteins accumulate in intracellular compartments, such as Golgi apparatus or other structures like ER. Type II includes five dominant EKV Cx31 mutants, G12R, G12D, R42P, C86S and F137L. The expression product of these mutants was partially trafficked to the cell surface, so they are lethal to HeLa cells. Type III is represented by I141V. I141V migrates mainly to the cell surface, which resembles that of WT-Cx31. These findings suggest that different mutations in Cx31 exhibit different subcellular distributions and none can form functional gap junction intercellular channels.

Mutations in the plasma membrane or secreted proteins that inhibit transport to the cell surface might cause disease by general mechanisms [33,34]: first, the affected protein can not be normally transported to the plasma membrane, but can be routinely degraded; second, the mutant can not be degraded, and thus accumulates within the cell and induces chronic endoplasmic reticulum stress responses, causing major changes in cell physiology, such as apoptosis, abnormal differentiation, altered proliferation, and so on. In this study, Type I Cx31 mutations do not induce chronic endoplasmic reticulum stress responses as stable cell lines were obtained. Therefore, these mutants may cause disease via the first mechanism. Although Type II Cx31 mutants can be trafficked to the cell membrane and form gap junctions, their function is abnormal because their expression can cause cell death. Therefore, the disease may result from the abnormal Cx31 function. Type III I141V mutant is found to coexist in the allele with 141delI mutant [25]. Therefore, this mutant may be recessive, and may cause a defect in normal GJIC. However, further studies will be necessary to confirm this hypothesis.

Notably, this study showed that four EKV-associated mutants (G12R, G12D, R42P and C86S) could be traf-

ficked to the plasma membrane and exhibit punctate staining; however, another mutant (66delD) could not be visualized at the plasma membrane, in contrast to the findings of Di *et al.* [28]. We believe that these differences can be explained as follows.

(1) Connexin expression may be dependent on the type of cell. Cx31 mutants were transfected into NEB1 cells by Di *et al.* [28], while Cx31 mutants were transfected into HeLa cells in this study. Owing to connexin protein deficiency, HeLa cells have been widely used to study connexin functions [35–42]. NEB1 cells, as a kind of keratinocytes, express several types of connexin. Endogenous connexins may affect the expression of transfected Cx31. However, Cx31 should be expressed in the epidermis, which contains several connexins. Therefore, the subcellular localization of Cx31 mutants in NEB1 cells may resemble the actual distribution of Cx31 mutants in patients more closely than the localization in HeLa cells. The immunolocalization of Cx31 mutants in patients provides further evidence of this.

(2) Cx31 mutants were introduced into mammalian cells via different methods. Diestel *et al.* [27] constructed G12R into an inducible vector and transfected them into HeLa cells via calcium phosphate crystals. Furthermore, Di *et al.* [28] constructed Cx31 mutants into pEGFP-N3, and microinjected them into NEB1 cells. In this study, Cx31 mutants were constructed into pEGFP-N1, and transfected them into HeLa cells by Lipofectamine 2000.

This study also investigated the border between the transmembrane and cytoplasmic domain of Cx31 using NCBI or TMpred software. Although the predicted positions of the transmembrane and extracellular and intracellular domains differ among amino acid groups, the locations of the mutated sites remain consistent. **Fig. 6** shows the positions of these sites. Disease-associated mutations were distributed in the whole structure of Cx31 except the IC2 and TM4 domains, indicating that the domains of Cx31 may play different roles in the physiological functions of Cx31. The C-terminal part of Cx31 may play a role in Cx31 oligomer formation in the cells, as the R180X mutant transfected cells contain fewer oligomers than other mutants and WT-Cx31.

In summary, this study has shown that different disease-associated Cx31 mutants exhibit different subcellular distributions, abilities in the formation of oligomers and effects on transfected HeLa cells, suggesting that diseases associated with Cx31 mutations may result from the abnormal assembly and trafficking of the mutants. Furthermore, this study has shown that deafness-associated mutations and skin disease-associated mutations have different influence on the function of Cx31. These findings may be helpful in understanding the mechanism of diseases caused by Cx31 mutations.

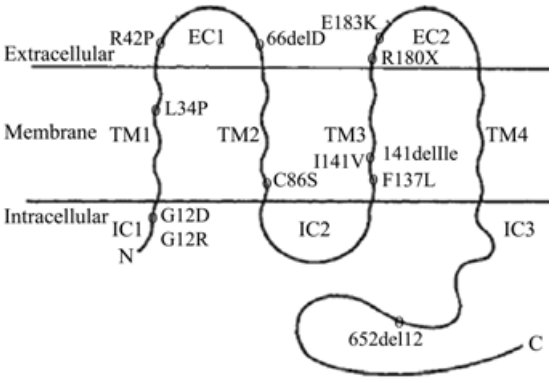

Fig. 6 Sketch of the positions of the mutations in Cx31 structure

N, amino terminal; C, carboxyl terminal; EC, extracellular domain; IC, intracellular domain; TM, transmembrane domain.

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