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Connexin 26 (GJB2) mutations as a cause of the KID syndrome with hearing loss

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ABSTRACT

KID syndrome (MIM 148210) is an ectodermal dysplasia characterized by the occurrence of localized erythematous scaly skin lesions, keratitis and severe bilateral sensorineural deafness. KID syndrome is inherited as an autosomic dominant disease, due to mutations in the gene encoding gap junction protein GJB2 (connexin 26, Cx26). Cx26 is a component of gap junction channels in the epidermis and in the *stria vascularis* of the cochlea. These channels play a role in the coordinated exchange of molecules and ions occurring in a wide spectrum of cellular activities. In this paper we describe two patients with Cx26 mutations cause cell death by the alteration of protein trafficking, membrane localization and probably interfering with intracellular ion concentrations. We discuss the pathogenesis of both the hearing and skin phenotypes.

1. Introduction

KID syndrome (MIM 148210) is an ectodermal dysplasia belonging to the clinically and genetically heterogeneous group of erythrokeratodermias. It is characterized by localized erythematous scaly skin lesions, keratitis [1–11] and severe bilateral sensorineural deafness, although unilateral or moderate hearing impairment has been observed. The skin lesions, described as erythrokeratoderma with occasional scarring alopecia, occur predominantly on the face, palms and soles, and have a typical reticulated pattern that is often defined as leather-like. Squamous cell carcinomas have been reported in 11% of the patients and it may probably be considered as a manifestation of the disease [7,12–14]. Additional features are corneal epithelial defects, including: scarring, and neo-vascularisation that can result in progressive decline of visual acuity and may eventually lead to blindness. To date, approx. 100 cases, the majority of which are sporadic, have been described in the world literature [15–17].

KID syndrome is mainly inherited as an autosomic dominant disease and has been associated with mutations in genes encoding gap junction protein GJB2 (connexin 26, Cx26), [18] or GJB6 (connexin30.3, Cx30.3) [19,20]. However, autosomic dominant and autosomic recessive inheritance has also been reported in a small number of families [4,12,21,22].

Eleven human connexin genes have been described, mutations of which have been associated with diseases such as Charcot-Marie-Tooth (GJB1, MIM #304040), hearing loss (GJB2, GJB3 and GJB6; MIM #121011 and 603324), cataract (GJA3 and GJA8; MIM #121015 and 600897), erythrokeratodermia variabilis (GJB3, MIM #133200: EKV) and a variant of Vohwinkel syndrome characterized by mutilating keratoderma associated with sensorineural deafness (GJB2, MIM #148350). The human GJB6 gene has also been found mutated in a family with hearing loss with partial penetrance [23].

Connexins share a common pattern of structural motifs, which includes four transmembrane (numbered from M1 to M4), two extracellular (E1, E2) and three cytoplasmic domains: the amino-terminus (NT), a cytoplasmic loop (CL) and the carboxy terminus domain (CT). The CL and CT domains are characteristic of each connexin, while the membrane spanning and the extracellular domains are highly conserved [24–26]. The interaction of six connexins leads to the formation of a functional unit across the

Abbreviations: Cx, connexin; EP, endocochlear potential; ABR, auditory brainstem responses; GFP, green fluorescent protein

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plasma membrane, called connexon or hemichannel. Finally, connexins can interact in a homomeric or heteromeric way to form the connexon [25], the functional unit.

Connexons of two opposing cells interact through their extracellular portions E1 and E2, forming a channel that is the basic unit of the functional gap junction. The gap junction is composed by several aggregated connexons [27]. This type of connection allows a rapid exchange of different molecules, from small ions to secondary messengers and metabolites between two connected cells [24–26,28].

The gated gap junctional channels possess selective properties for permeability and sensitivity, and play an important role in the response of cells to environmental stimuli in all multicellular organisms. Indeed the coordinated exchange of molecules between adjacent cells is used in a wide spectrum of cellular activities, such as transmission of neuronal signals, cell contraction, cell growth control, organ homeostasis and differentiation [24,29]. Connexins are expressed in most tissues, and their relevance is outlined by the identification of Connexins' gene mutations as the molecular cause of distinct human diseases [30].

Connexons are important for recycling potassium ions into the cochlear endolymph through the network of gap junctions that extends from the epithelial supporting cells to the fibrocytes of the spiral ligament and to the epithelial marginal cells of the *stria vascularis* [31,32]. The endocochlear potential (EP) is generated by the *stria vascularis*. Both the EP and the high K^+ and other ion concentrations of the endolymph are essential for the transduction of sound by the hair cells [33]. Maintenance of ion homeostasis is therefore essential for normal hearing, and mutations in several genes encoding connexins or other ion channels lead to hereditary deafness [31,34,35].

In this paper, we identified in two clinical cases of KID syndrome, with mutations in the Cx26 gene, which are associated to sensorineural hearing loss.

2. Materials and methods

2.1. Cell culture transfection, Western blots and histology

Cryopreserved NHEK were obtained from BioWhittaker and grown in calf skin collagen-coated dishes in serum-free keratinocyte medium at 0.05 mM Ca^{2+}, supplemented with 7.5 μg/ml bovine pituitary extract, 0.5 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.1 UG/ml hEGF. Third passage cells were used for experiments. Cells were treated for 1 and 3 days with 1.2 mM Ca^{2+}, 1 μM retinoic acid and 250 ng/ml vitamin D3. SAOS2 cells were cultured in DMEM (Dulbecco's modified Eagle medium) adding 10% FBS. Transfections have been realised using $CaPO^4$ (Invitrogen), Western blots have been realised according to Candi et al. [36]. We used the following antibodies anti-GFP (Roche; diluizione1/100), anti-actin (H-235, Santa Cruz, 1:1000); the signal has been revealed using ECL (Pierce).

Biopsy samples of the proband were taken and processed for light microscopy. Light microscopy samples were embedded in paraffin and stained with haematoxylin–eosin. Ethical approval and patient consent were obtained according to the guidelines of IDI-IRCCS Ethical Committee.

Fluorescence microscopy was performed on cells fixed with 4% paraformaldehyde, and then permeabilised with 0.25% Triton X-100 and stained 1 h with the appropriate antibody, following manufacturer instruction for concentration. Alexa fluor 488 (green) and 568 (red) antibodies were used as secondary antibodies. Nuclei were stained with DAPI 1 μg/ml.

2.2. Confocal microscopy

SAOS2 cells have been plated at density of 2.5 to 3 $\times$ 10^4 cells/cm^2 in chamber-slide and transfected using $CaPO^4$ (Invitrogen) according to the manufacturer instructions. Cells have been fixed using paraformaldehyde 4%, 10° RT, and permeabilised using PBS-Triton 0.25%. Cells have been stained using anti-Cx26 (Zymed lab; 1/100), anti-GFP (Roche; 1/100) and phalloidin-fitch (Sigma; 1/100). La fluorescence has been evaluated using Nikon, C1 on Eclipse TE200 and EZC1 software.

2.3. DNA, RNA extraction and cloning

Genomic DNA samples were extracted from patient blood and from the other family members according to standard procedures [37].

Two 3 mm skin biopsies were taken from palmar and abdomen epidermis, total RNA was extracted with Qiagen (Crawley, UK) RNAeasy mini kit and used in the RT-PCRs.

The coding region of Cx26 has been re-amplified using primers Cx26F1EcoRI (+) (5′-TGTTCCTGTGTTGTGTGAATTCGTC-3′) and Cx26RKpnI (−) (5′-TGCGTTACGGTACCTTTTTTGAC-3′), and re-cloned in pGFPN1 (Clontech) using EcoRI and KpnI restriction site. This cloning strategy creates a fusion between GFP and the connexin 26 at the C-terminus [38].

2.4. RT-PCR and direct sequencing

Reverse transcription was performed using the Superscript-II reverse transcriptase (Invitrogen), with 100 ng of total RNA using 10 pmol of oligo dT primers, buffer and enzyme concentrations were according to the manufacturer instructions. The entire coding region of the Cx26 (CJB2) gene was PCR amplified using 0.4 μM of primer 5′-ACTCAGAACACTGCCTGGTACATA-3′, for (+) strand and 5′-GGCACTGGGCCTGTGGGTTAC-3′, for (−) strand, designed from the published cDNA sequences (Accession No. AF099030). PCR fragments were resolved on 0.8% agarose gel (TAE), extracted and purified using the Qiaex II extraction kit (Qiagen).

Approximately 100 ng of purified template DNA was automatically sequenced with the BigDye Termination Reaction Kit (Applied Biosystem) on an ABI-PRISM 377 DNA sequencer (Applied Biosystem). Reverse transcription and amplification of Cx30.3 (CJB4) mRNA was performed according to Macari et al. [19].

3. Results

3.1. Clinical presentation

The first patient is a 23-year-old Caucasian woman, born without dermatological abnormalities. The patient's history revealed sensorineural deafness since the age of 2 years. Shortly after, a patchy hyperkeratosis arose in the perioral area and in the knees, with an evident palmo-plantar keratoderma. At the age of 6 the patient developed photophobia and dry eyes, suggesting ocular keratitis. Subsequently cicatricial alopecia of the scalp developed progressively.

When admitted to the hospital, the patient presented irregular patches of hyperkeratosis around the mouth (Fig. 1), the cheeks and the knees. Non-transgrediens keratoderma of the palms and soles with onychodystrophy and patches of cicatricial alopecia of the scalp were also present. Moreover, ocular examination showed a high grade bilateral ectropion, conjunctival injection and dry eyes associated with keratitis (Fig. 1B–D).

Histopathological examination clearly displayed the typical saw-tooth conformation of the epidermis. Indeed, the histology shows orthokeratotic hyperkeratosis of the stratum corneum with the characteristic basket wave conformation (Fig. 1A); also achantosis and a little perivascular infiltration of mononuclear cells were present. These features were consistent with the diagnosis of KID syndrome.

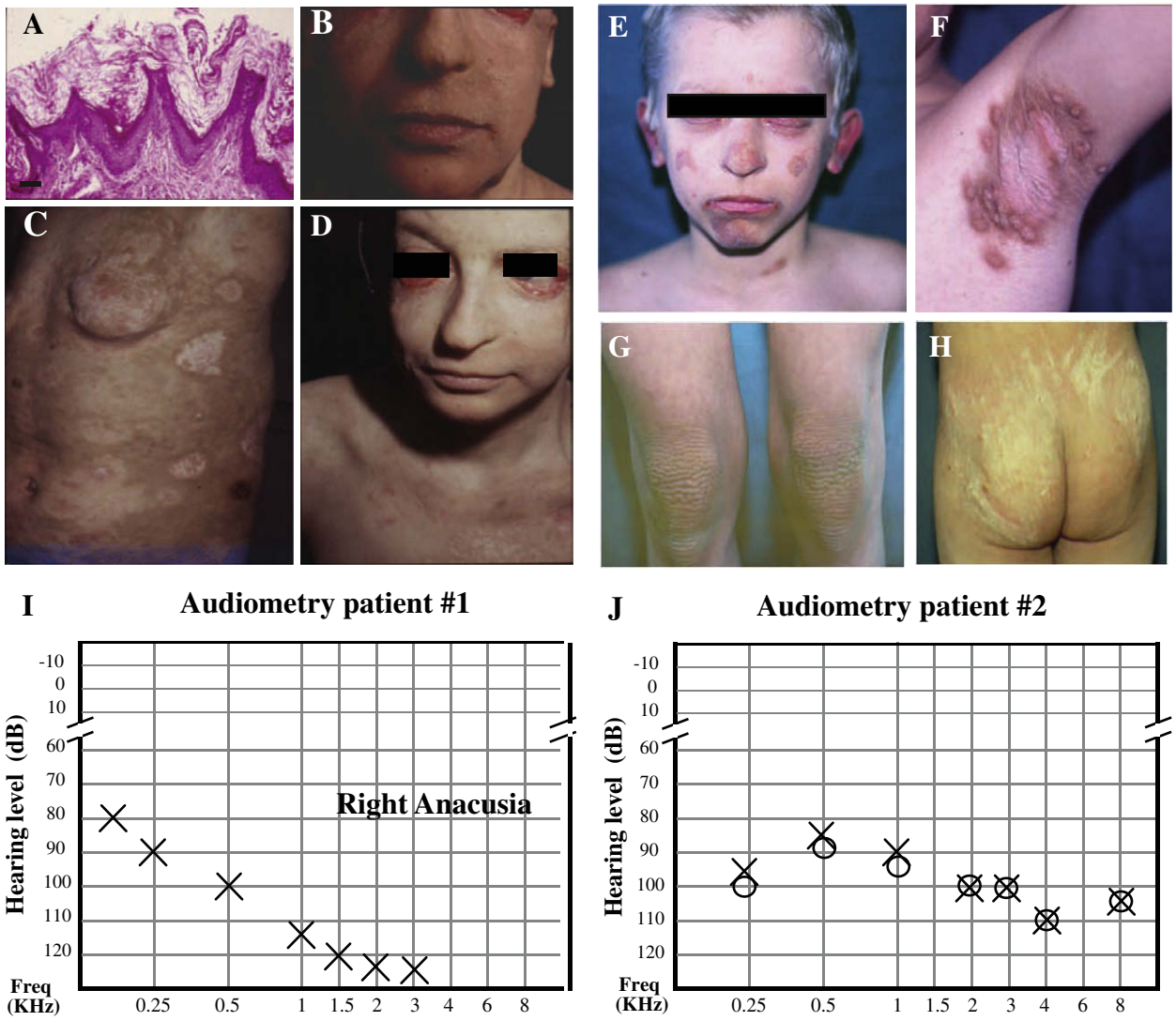

Fig. 1. Clinical presentation and histopathology. (A–D) Patient 1. (A) Histological examination of patient 1 (bar = 200 μm), showing orthohyperkeratosis, papillomatosis and light superficial lymphocyte infiltration above the granular layer, clinical findings consistent with the diagnosis of KID. (B,C) Diffused ichthyotic hyperkeratosis, characterized by thick dark brown scales and erythematous patches of body skin, changing in size and shape in course of weeks, leaving some epidermis areas completely unaffected. (D) Clinical presentation of epidermis after 10 months of retinoic treatment. (E–J) Patient 2. (E) Strongly marked erythrokeratodermia of the face, mostly localized in zygoma nose, and eyelids. (F) Hyperkeratosis and erythema of axillae, and (G) of the knees. (H) Diffused hyperkeratosis of the body. (I, J) Audiograms. (I) Audiogram of patient 1, showing a right anacusia and left deep sensorineural hearing loss. (J) Analysis of patient 2 showing bilateral severe sensorineural hearing loss.

The second patient (Fig. 1E–H) is a 12-year-old boy born with medium degree sensory-neural deafness, and admitted to our hospital with generalized xeroderma with non-homogenous hyperkeratosis, strongly affecting the face, especially the zygoma and nose. Hyperkeratosis was also present at the elbows and knees. Progressive signs of photophobia with conjunctivitis and blepharitis were present and during later childhood the patient developed keratitis and corneal ulcers in both eyes. The patient underwent a corneal graft of the right eye. After 3 years zonal erythrokeratodermia involving the nasal pyramid, zygoma, conches, elbows and knees developed; furthermore at the axillae the disease appeared as hyperkeratotic nodules. The patient had onicodystrophia of both big toes since adolescence.

Audiograms for patient 1 showed a right anacusia and left deep sensorineural hearing loss (Fig. 1I and J). Auditory brainstem responses (ABR) audiograms showed bilateral absence of evoked potentials of auditory neural pathways. In patient 2, the audiometry showed bilateral severe sensorineural hearing loss (Fig. 1I and J). In this case also no ABR was evoked. Both cases showed a relevant, compromised cochlear activity suggesting a connexon defect.

3.2. Mutation analysis

Mutations in the gene GJB2 [18] have been previously reported in KID patients. We therefore analysed the sequence of this gene to define the genetic defects responsible for the syndrome in our patients. A skin biopsy was taken from the patient's palmar epidermis, and total RNA was extracted for RT-PCR. Amplification and sequence analysis of the Cx26 led to the identification of two mutations. The first is a novel mutation, a heterozygous 32G→A transversion, leading to the G11E substitution (Fig. 2A and B). The second mutation D50N has been already reported (Fig. 2C and D) [18,39]. The complete analysis of Cx26 codifying genes did not reveal the presence of any other mutation. Since Gly11Glu is a novel mutation, and the patient reported a severe hearing loss we sequenced also GJB6 (Cx30), also associated with KID syndrome

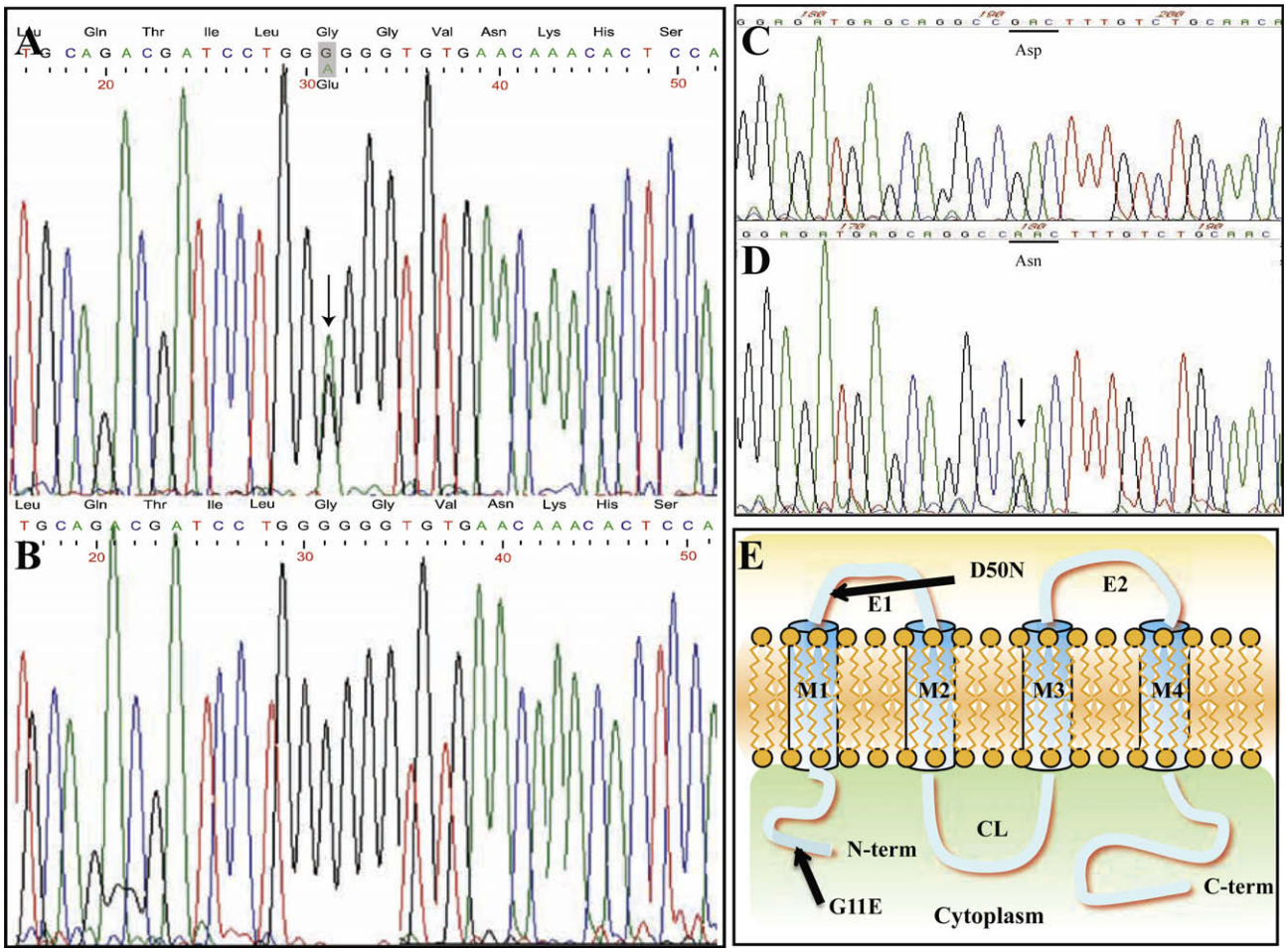

Fig. 2. Analysis of Cx26 gene. (A,B) Patient 1, Cx26 sequence chromatogram of revealing the G→A transition leading to the G11E substitution. (C,D) Patient 2, chromatogram of Cx26 sequence showing the G→A transition leading to the already described D50N substitution. (E) Schematic representation of connexin structure. The G11E substitution is localized in the cytoplasmic N-terminal domain, the D50N mutation affects the E1 extracellular domain.

[20] and to non-syndromic hearing loss [23]. We also sequenced GJB1 (Cx31) and GJB4 (Cx30.3) known to be responsible for EKV with hearing loss [19,40]. No further mutations or polymorphisms were found in these genes.

3.3. Protein trafficking and localization analysis

The mutated glycine 11 is localized in the CL domain of the protein, involved in voltage and ion gating, while the other mutated residue (aspartic acid in position 50) is localized in the M1 domain, also involved in voltage and ion gating, at the interaction between two opposing connexons (Fig. 2E).

In order to investigate the molecular mechanism leading to the epidermal and cochlear defects, we cloned the wt and mutated GJB2 cDNAs in mammalian expression vectors. We also created vectors expressing cDNAs fused with GFP at the C-termini. The GFP used does not form homo-tetramers [38], avoiding an aberrant chimerical protein localization, with subsequent erroneous connexon formation [38,41]. We then used these vectors to over-express the wt and mutated Cx isoforms and analysed their cellular localization, their ability to reconstitute the functional connexon and their effect on cell viability and ion exchange.

The GFP fused constructs were expressed by transient transfection in a SaOS cell system, and analysed by confocal imaging (Fig. 3A–C) and Western blot, at different time points (Fig. 3D). While all three constructs show high levels of expression after 16 h of transfection, at 24 h wt and D50N mutant show a modest reduction, while expression of the G11E mutant protein are significantly reduced (Fig. 3D). This suggests either a reduced protein stability of this mutant or that its over-expression results in cell death.

4. Discussion

Here, we describe two novel patients affected by KID syndrome, with distinct mutations. Both patients present skin abnormalities as well as hearing loss, suggesting a common mechanisms. In fact, as previously reported, also our two patients present mutations in the Cx26 gene, resulting in a mutated protein.

But what is the molecular mechanism through which mutated Cx26 effects its action? The in vitro expression of the recombinant mutated Cx26 protein shows an abnormal protein half-life. This could be caused by either a premature death of the expressing cells due to a toxic effect of the proteins, or by an abnormal protein degradation of the protein itself.

The first hypothesis implies the formation of a defective connexon, which results in deregulated channel properties with lethal effects for the cell itself. Premature death could, in theory, also arise from a gain of function property of the Cx26 mutated protein able to trigger the apoptotic machinery. The second hypothesis implies that the degradation has been modified by the mutation impairing the physiological ability of the E3 ubiquitin ligase to recognize the substrate, resulting in excessive protein degradation in the proteosomal system. Unfortunately, the E3 responsible for

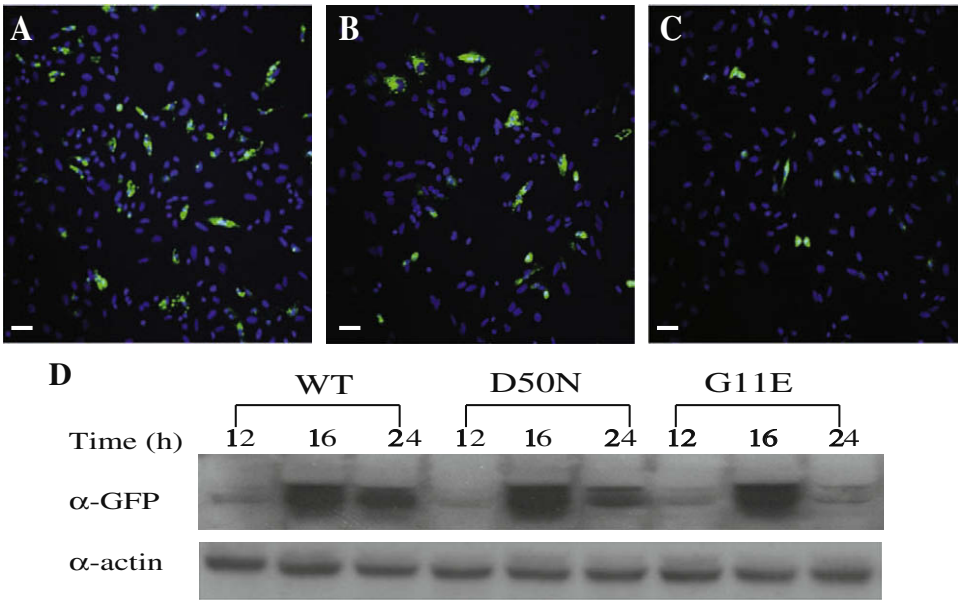

Fig. 3. WT and mutant Cx 26 expression analysis. (A–C) SAOS2 cells, transfected with wt, D50N and G11E GFP fusion constructs. The cells have been fixed and analysed 24 h after of transfection, nuclei have been stained using DAPI (blue), GFP-Cx26 is green. A decrease in total number, and in number of fluorescent cells is visible in G11E sample (C). (D) Time course Western blot demonstrating a consistent decrease of protein expression in sample transfected with the G11E GFP transgene (bar = 20 μm).

Cx26 has not been identified so far, rendering this hypothesis non-testable.

5. Conclusion

Our data demonstrate the abnormal property of the mutated Cx26 proteins in protein trafficking and membrane localization. We demonstrate also that different mutations, localized in different functional domains of connexins, give rise to different pathological phenotypes.

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