INVESTIGATING THE FUNCTION OF CX26-I30N AND D50Y MUTATIONS IN SQUAMOUS CELL CARCINOMA CELL LINE SCC-25

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Büşra YAVUZ

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ABSTRACT

INVESTIGATING THE FUNCTION OF CX26-I30N AND D50Y MUTATIONS IN SQUAMOUS CELL CARCINOMA CELL LINE SCC-25

Gap junctions are responsible for cell homeostasis and provide cell-cell and cell-ECM communications in multicellular organisms. Gap junction hemichannels consist of connexin (Cx) proteins which functions are crucial for human physiology. Moreover, Cx mutations are associated with various genetic diseases. For example, more than 10 missense mutations in the Cx26 gene cause keratitis-ichthyosis-deafness (KID) syndrome. Among these, Cx26-I30N and Cx26-D50Y cause to form abnormal hemichannels and allow excessive calcium influx into the cell. Furthermore, KID patients have higher squamous cell carcinoma (SCC) incidence and SCC is seen at an earlier age in these patients. Immunohistochemistry stainings have demonstrated aberrant E-cadherin and β-catenin spread in KID patients' samples.

In this study, we aimed to effects of Cx26-I30N and Cx26-D50Y mutations associated with KID syndrome in human epithelial SCC cell line SCC-25. For this purpose, stable MSCV, Cx26-WT, Cx26-I30N and Cx26-D50Y cell lines were generated and RT-qPCR, Western blotting, immunostaining and MTT assays were done for expression level, localization and viability analysis. For these 4 different cell conditions, a significant increase was detected at Cx26 mRNA levels, but not at protein levels. No difference was found in Cx43 level, which is abundant in epithelial tissue, and was localized in the perinuclear area like Cx26. Additionally, E-cadherin protein levels increased and their localization changed in parallel with the mutations from the perinuclear area to the plasma membrane. Moreover, significant decreases in viability were observed in cells grown in high extracellular Ca^{2+} medium compared to control in contrast to cells grown in Ca^{2+}-free medium.

ÖZET

SKUAMÖZ HÜCELİ KARSİNOM HÜCRE HATTI SCC-25'DE CX26-I30N VE D50Y MUTASYONLARININ FONKSİYONUNUN ARAŞTIRILMASI

Oluklu bağlantılar çok hücreli canlılarda, hücre homeostazından sorumludur ve hücre-hücre ve hücre-ECM iletişimini sağlar. Oluklu bağlantı yarıkanalları, insan fizyolojisi için çok önemli olan işlevleri olan connexin (Cx) proteinlerinden oluşur. Ayrıca, Cx mutasyonları çeşitli genetik hastalıklarla ilişkilidir. Örneğin, Cx26 genindeki 10'dan fazla hatalı mutasyon, Keratit-İktiyozis-Sağırlık (KID) sendromuna neden olur. Bunlar arasında Cx26-I30N ve Cx26-D50Y anormal yarı kanallar oluşturarak hücre içine aşırı kalsiyum girişine izin verir. Ayrıca, KID hastalarında skuamöz hücreli karsinom (SCC) insidansı daha yüksektir ve bu hastalarda SCC daha erken yaşta görülür. İmmünohistokimya boyamaları, KID hastalarının örneklerinde anormal E-cadherin ve β-catenin yayılımı göstermiştir.

Bu çalışmada, insan epitelyal SCC hücre hattı SCC-25'te KID sendromu ile ilişkili Cx26-I30N ve Cx26-D50Y mutasyonlarının etkilerini araştırmayı amaçladık. Bu amaçla stabil MSCV, Cx26-WT, Cx26-I30N ve Cx26-D50Y hücre hatları oluşturuldu ve ekspresyon seviyesi, lokalizasyon ve canlılık analizi için RT-qPCR, Western blot, immün boyama ve MTT testleri yapıldı. Bu 4 farklı hücre durumu için Cx26 mRNA seviyelerinde önemli bir artış tespit edildi, ancak protein seviyelerinde tespit edilmedi. Epitel dokusunda bol olan ve Cx26 gibi perinükleer bölgede lokalize olan Cx43 düzeyinde fark bulunmadı. Ek olarak, mutasyonlara paralel olarak E-cadherin protein seviyeleri artmış ve lokalizasyonları perinükleer alandan plazma membranına doğru değişmiştir. Ayrıca, Ca^{2+}'suz ortamda büyütülen hücrelerin aksine, kontrole kıyasla yüksek hücre dışı Ca^{2+} ortamında büyütülen hücrelerde canlılıkta önemli düşüşler gözlendi.

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CHAPTER 1

INTRODUCTION

1.1. Gap Junctions

Gap junctions are one of the four intercellular connection type in multicellular organisms. Between the neighbouring cells, gap junctional intercellular communication (GJIC) can form that has various properties such as cellular homeostasis, autocrine and paracrine signaling[1–3]. Moreover, this linkage system mediates cell to extracellular matrix (ECM) communication[4].

Gap junctions are made of hemichannels called connexons between two neighbouring cells to connect the cytoplasm of cells to each other[3]. The docking structure among cells called connexon contains gaps in its structure is approximately 2-3 nm in diameter[5]. These selective pores supply the trafficking of small molecules, roughly below 1 kDa[4]. These exchanged molecules are ions (K^+, Ca^{2+}), which are essential for the maintenance of the intracellular electric coupling, secondary messengers (Ca^{2+}, cAMP, cGMP, and inositol 1,4,5-triphosphate (IP_3)), microRNAs, and small metabolites (glucose, ATP)[6–9]. In addition, the uncoupled hemichannels, in the plasma membrane of a cell, mediate the influx or efflux of molecules required for the cell. This flow of the molecules occurs between cytosol and the environment of cells[10]. The transmission of the small particles serves to regulate cellular processes such as cell proliferation, cell death, cellular expression and suppression of the proteins[11].

The gap junctional hemichannels consist of distinct protein family members in eukaryotic cells which are called innexins in invertebrates and connexins in vertebrates[12]. A hemichannel (connexon) is formed from six connexin proteins which are located in the plasma membrane of a cell. The connexon channels from two adjacent cells join to generate gap junction channels between them[13,14]. All the hemichannels have gating systems that are responsible for the conformational changes between the opening and closing states of the pores according to the homeostasis of the cells and these systems are regulated by the changes from the electrical charge, pH, Ca^{2+} and the expression levels of connexin proteins[1,3].

1.2. Connexins

Connexins are integral membrane proteins. Human genome contains 21 isoforms of connexin genes and 20 different connexin genes have been characterized in mice genome up to date. Nineteen of the connexin genes which are identified in the human and mice genome, are derived from a common ancestral gene found in the mammalian ancestor[15–17].

Connexins traverse the plasma membrane four times, in other words, connexin protein structure has four membrane-spanning domains which demonstrate hydrophobic biochemical properties (M1-M4)[4,10] (Fig 1.1.). These transmembrane subunits are connected by a cytoplasmic loop (CL) and two extracellular loops (E1 and E2) that are hydrophilic[12]. The extracellular loops are responsible for the cell-cell recognition and docking process and have highly conserved amino acid sequences with three cysteine residues that form disulfide bonds in the same connexon structure[18]. Additionally, both the amino-terminal (NT) and the carboxyl-terminal (CT) face the cytoplasmic region of the membrane[4,10,12,17]. The amino-terminus domain of connexin is highly conserved among the member of the protein family, whereas, the carboxy-terminus and the cytoplasmic loop vary for each member[14,19,20].

There are two different Cx nomenclature in the literature. One of the nomenclatures of connexin proteins is generated according to the molecular weight of the protein that is frequently originated from the differences in the length of the C-terminal region[4,10,12]. For example, connexin 43 (Cx43) is a Cx isoform which has 43 kDa mass. On the other hand, connexins form three group based on the homology of the amino acid sequences and these are alpha connexins, beta connexins and a third group including gamma, delta and epsilon connexins which indicate intermediate homology[21]. Depending on these homology clusters, connexins are named with Greek symbols, for instance, GJA1 (Gap Junction Alpha 1) is used for Cx43[4,22].

A hemichannel comprises six connexin proteins and two connexons generate a gap junctional channel on the plasma membrane. Therefore, the oligomerization of different connexin proteins causes the creation of diverse hemichannels and gap junction combinations. A hemichannel that has one type of Cx proteins is called homomeric, on the other hand, if it is composed of two different Cx proteins, is called a heteromeric channel. A gap junction channel is homotypic when two neighboring cells have the same Cx configuration. However, when the channel is made from different Cx isoforms, it is called heterotypic (Fig 1.1.)[23,24].

Connexins are generally phosphoproteins, and the phosphorylation process plays an important role in the alteration of the biochemical characteristics and the regulation of channel formation[25,26]. Moreover, the cytosol-facing carboxyl group of Cx proteins contains multiple interaction sites for proliferation pathway proteins such as MAPK, Src,

β-catenin and PKC, indicating that Cx proteins are crucial to the processes including cell division[27].

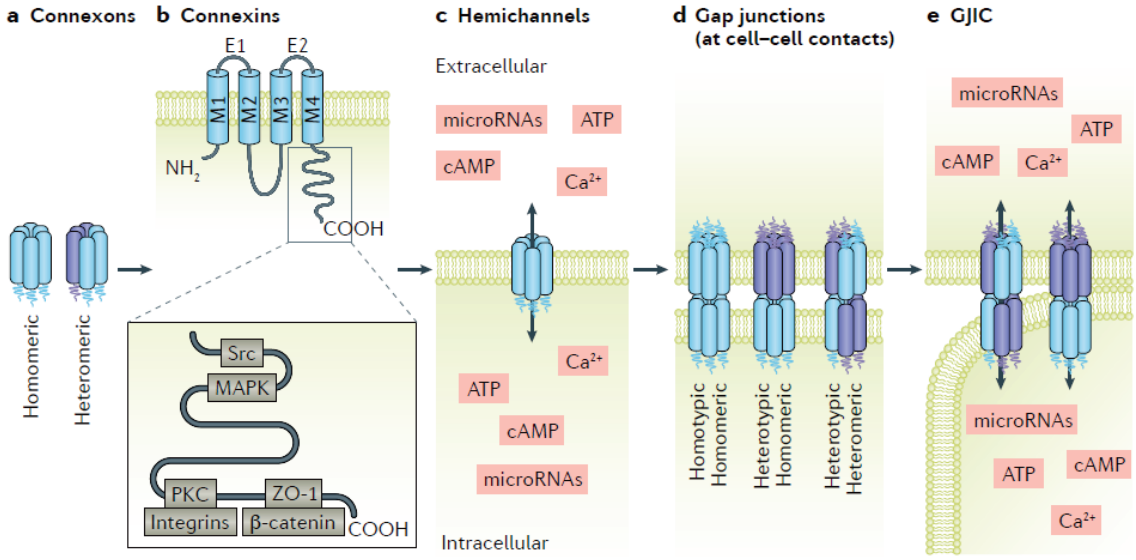

Figure 1.1. Connexins, connexons and gap junctions.[27]

1.2.1. Life Cycle of Connexins

Gap junctions are dynamic cellular channel systems, for this reason, connexins that are the small set unit of these channels, undergo rapid synthesis and degradation continuously[28]. The half-life of connexin proteins is between 1.5 - 5 hours in most tissue types and shorter than the other plasma membrane proteins[13,29]. Therefore, regulation of Cx protein level and transport is important for the proper formation of the intercellular communication network[30].

Connexins are generally co-translationally inserted into the endoplasmic reticulum (ER) membranes[24,31]. Furthermore, a large amount of newly synthesized proteins, incomplete or misfolded connexins, pursue ER-associated degradation (ERAD) pathway[32,33]. Other connexins which have correct conformation, are transported to the cell surface via Golgi apparatus and trans-Golgi network system in vesicles along microtubules[33,34]. However, depending on the connexin isoform, the oligomerization of hexameric structure (i.e. connexon) can occur in either ER-Golgi intermediate compartment (ERGIC) (e.g. Cx26 and Cx32), or trans-Golgi region (e.g. Cx43)[35]. In the plasma membrane, the hemichannels sometimes interact with another one found in the neighbouring cell membrane and form a gap junction, but sometimes do not and function as hemichannels[10].

The degradation process of gap junctions is initiated by the formation of annular gap junctions (AGJ), in other words, connexosome. This endocytic pathway can occur in three different ways. In the first way, connexosomes which are clathrin-dependent vesicles, are internalized and can fuse with early endosomes. Then, late endosomes will be formed after the maturation and fuse with lysosome for degradation. The second way is a direct fusion of AGJ and lysosome[36]. The last way of degradation is the fusion between AGJ and autophagosome which involves ubiquitinated connexosome retained via phagophore, and lysosome[37].

Post-translational modifications of connexin proteins that have been identified so far, are phosphorylation, ubiquitination, SUMOylation, acetylation, methylation, hydroxylation and nitrosylation[38]. Phosphorylation, ubiquitination and SUMOylation have critical effects on connexin levels[37]. Several stages of the Cx life cycle are regulated by the phosphorylation process. Furthermore, kinases which are important parts of cell cycle pathways, are responsible for Cx phosphorylation. For instance, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), casein kinase 1, protein kinase A, c-Src and v-Src catalyze the phosphorylation of Cx43 protein[25]. This process regulates Cx43 transport from the Golgi apparatus to the cell membrane, the formation of connexon, and gap junctional assembly, the gating of gap junction channels, and the internalization and degradation of gap junctions via ubiquitination of connexins[29]. Additionally, SUMO (small ubiquitin-related modifier) is a ubiquitin-like protein and responsible for the SUMOylation process, but in contrast to ubiquitination, it links to the gap junctions and stabilizes them by increasing the size of the channels[37,39] (Fig 1.2.).

1.3. Physiological Signifigance of Connexin Channels

Gap junctions and thus connexin proteins undertake a crucial role in human physiology, playing roles in many critical processes from embryo development to the adults[40]. One of the interesting examples of the gap junctional effects in tissues is how our brain perceives the voices. Connexin proteins such as Cx26, Cx30, Cx31, Cx32 and Cx43 help this process by passing the K ions from cells that vibrate hair cells of the Corti organ in the inner ear. As a result of this, these motion impulses are sent to the brain so that connexins have a critical role in the hearing mechanism[41].

Connexins determine the fate of cells by adjusting ion balances, such as Ca, K ions, between cells and the extracellular matrix[1]. For instance, connexin channels play a role in cell death mechanisms where inner and outer ion balances are very important. This can be observed while Cx26 and Cx32 have an impact on chemical-induced necrosis

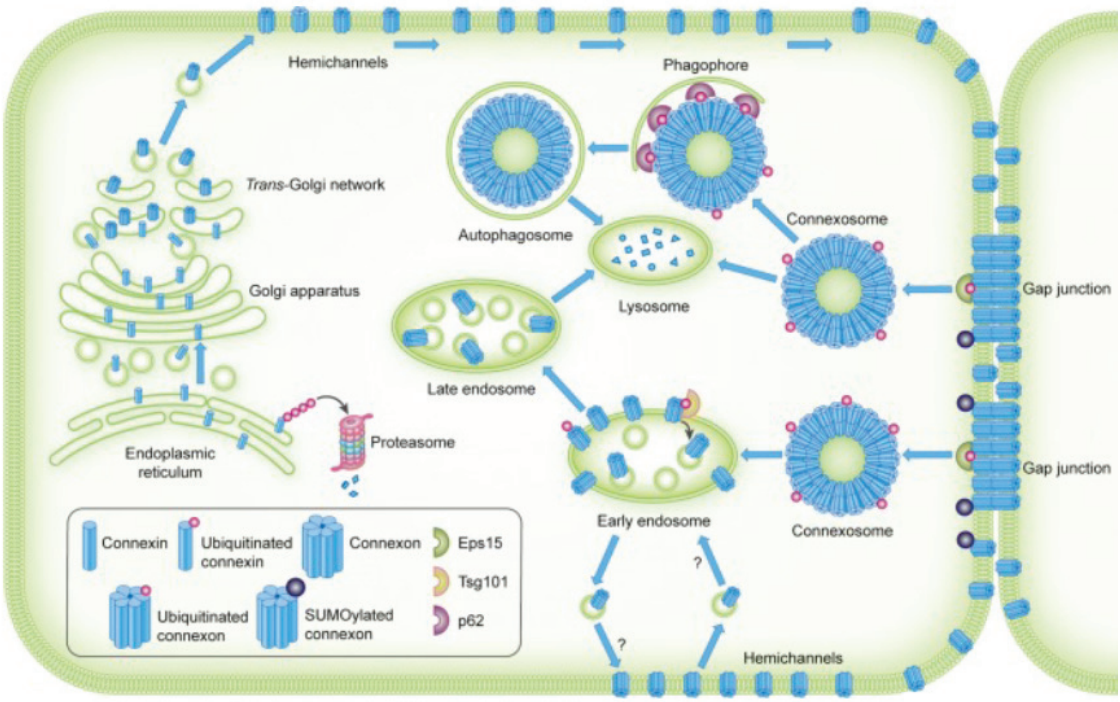

Figure 1.2. Life cycle of connexin proteins.[37]

on liver cells, expression, and phosphorylation of Cx43 on apoptosis[42]. In addition to this, gap junctions take part in the proliferation and differentiation processes. In the adult liver, connexins also involve in glycogenolysis and secretion mechanisms and Cx26 patterns regulate the transcription of glucagon[42]. Moreover, they have various effects on the onset, development, progression, regression, and recurrence of diseases such as cancer and hearing loss[43,44].

One of the reasons why connexins are so effective in human physiology is that they are present in almost every tissue apart from differentiated skeletal muscle, erythrocytes, and mature sperm cells[40] (Table 1.1.). However, abnormal or incomplete expressions of connexin proteins, which are normally responsible for ionic conduction, metabolic coupling, or seconder messenger diffusion in most tissues, cause many physiological disorders. For instance, mutations in Cx26, Cx46, and Cx40 genes cause hearing loss, cataract, and arrhythmia, respectively[45].

1.3.1. Connexin-related Genetic Diseases

Since the gap junctional network is used to rapidly regulate ions and metabolites between cells, damage to this network can have many pathological consequences. Dysfunctional connexins are generally related to congenital and acquired human disorders in

Table 1.1. Expression patterns of human connexins in different tissues.
(Source: modified from Rackauskas M, et al., 2010)

Human Connexins	Gene Name	Expression Patterns of Connexins in Different Tissues
Cx23	GJE1	Transcription and translation have not been demonstrated in humans Human fetal cochlea.[46]
Cx25	GJB7	Human genome Transcript in placenta only.[15]
Cx26	GJB2	Breast; skin; cochlea; liver; endometrium; glial cells; airway epithelium; somniferous tubules; pancreas.
Cx30.2	GJC3	Cochlea Transcripts in skeletal muscle, liver, and heart.[47]
Cx30	GJB6	Skin; brain; cochlea; airway epithelium; exocrine gland.
Cx30.3	GJB4	Skin
Cx31	GJB3	Skin; airway epithelium; cochlea; placenta.
Cx31.1	GJB5	Skin
Cx31.9	GJD3	Liver
Cx32	GJB1	Liver; skin; Schwann cells; oligodendrocytes; endometrium; gland cells.
Cx36	GJD2	Retina; pancreatic beta cells; neurons throughout the central nervous system.
Cx37	GJA4	Vascular smooth muscle; endothelium; ovaries; skin.
Cx40	GJA5	Skin; nervous system; endothelium; heart.
Cx40.1	GJD4	Human genome Transcript in pancreas, kidney, skeletal muscle, liver, placenta, and heart.[47]
Cx43	GJA1	Most widely expressed connexin, present in at least 34 tissues and 46 cell types.
Cx45	GJC1	Human pancreatic ductal epithelial cells; SA and AV nodes of the heart; neurons; oligodendrocytes, astrocytes, vascular system, skin; osteoblasts; retina; uterus.
Cx46	GJA3	Lens; alveolar epithelium.
Cx47	GJC2	Brain, spinal cord, oligodendrocytes.
Cx50	GJA8	Lens
Cx59	GJA9	Human genome Transcript in skeletal muscle, with lower expression in heart and testis.[15]
Cx62	GJA10	Horizontal cells of the retina.

several tissues such as skin, eyes, and ears and systems such as the cardiovascular system and nervous system[48,49]. The first identified genetic disease that is caused by Cx mutations, is peripheral neuropathy X-linked Charcot-Marie-Tooth (CMTX) syndrome[50]. GJB1 gene which encodes Cx32 protein has mutations that cause Cx32 protein accumulation in ER and Golgi apparatus or loss of gap junction channels leading to progressive degeneration of peripheral nerve sites[51,52]. As a result of CMTX, the distal muscles of patients begin to weaken, and atrophy occurs[53].

Pelizaeus–Merzbacher-like disease, (PMLD), another myelin-related hereditary nervous system disease, affects the central nervous system due to mutation in Cx47 (GJC2) gene[54]. This hypomyelinating leukodystrophy is an autosomal recessive disorder and has a negative effect on the development of myelin sheath in the white matter of the brain[52]. Further, mutations in Cx46 (GJA3) and Cx50 (GJA8) genes are responsible for zonular pulverulent cataracts (CZP1), as these genes regulate the ion balance of the cells in the lens[52,55]. CZP1 has an autosomal dominant inheritance and result in the loss of lens transparency[56].

Cx37 (GJA4), Cx40 (GJA5) and Cx43 (GJA1) are found in the structure of blood vessels and one of the causes of heart diseases, for instance, some atherosclerosis cases are due to mutations in these genes[57]. Furthermore, Cx43 is also related to skin and cochlear (inner ear) defects in addition to Cx26 (GJB2), Cx30 (GJB6), Cx31 (GJB3) and Cx30.3 (GJB4)[52]. Oculodentodigital dysplasia (ODDD) is a frequent and autosomal dominant disorder while nonsyndromic sensorineural hearing loss (SNHL) is less frequent, and both are linked to Cx43 mutations[58–60]. Either Cx30.3 or Cx31 mutations are responsible for erythrokeratodermia variabilis (EKV) which is an autosomal dominant skin disease when a mutation in Cx31 gene causes autosomal recessive deafness[61–63]. Additionally, inherited Cx26 and Cx30 mutations cause both non-syndromic and syndromic sensorineural hearing loss with skin defects[49]. Whereas mutation in Cx26 gene leads to Vohwinkel syndrome (VS), Bart–Pumphrey Syndrome (BPS), palmoplantar keratoderma (PPK), keratitis–ichthyosis–deafness syndrome (KID), and hystrix-like ichthyosis deafness syndrome (HID), Cx30 gene mutations cause Clouston (i.e. hidrotic ectodermal dysplasia (HED)) and rarely KID syndromes[52,64] (see Table 1.2.).

Table 1.2. Connexin types and related genetic diseases.
(Source: modified from Zoidl and Dermietzel, 2010).

Gene	Disease	Expression Pattern
Cx26 (GJB2)	- Deafness (OMIM; 220290 and 601544) - Keratitis–Ichthyosis–Deafness syndrome (KID) (OMIM; 148210) - Vohwinkel syndrome (VS) (OMIM; 124500) - Bart–Pumphrey syndrome (BPS) (OMIM; 149200) - Palmoplantar keratoderma with deafness (PPK) (OMIM; 148350) - Hystrix-like ichthyosis deafness syndrome (HID) (OMIM; 602540)	Cochlea Skin Liver Placenta Breast
Cx30 (GJB6)	- Deafness (OMIM; 604418) - Clauston syndrome (OMIM; 129500) - KID syndrome (OMIM; 148210)	Cochlea Skin Brain
Cx30.3 (GJB4)	- Erythrokeratodermia variabilis (EKV) (OMIM; 133200)	Cochlea Skin
Cx31 (GJB3)	- Erythrokeratodermia variabilis (EKV) (OMIM; 133200) - Deafness (OMIM; 603324)	Cochlea Skin Placenta
Cx32 (GJB1)	- X-linked Charcot-Marie-Tooth (CMTX) syndrome (OMIM; 302800)	Liver Oligodendrocytes Schwann cells
Cx43 (GJA1)	- Oculodentodigital dysplasia (ODDD) (OMIM; 164200) - Syndactyly, type III (OMIM; 186100) - Hypoplastic left heart syndrome (OMIM; 241550) - Atrioventricular septal defect (OMIM; 606215)	Most of tissues
Cx47 (GJC2)	- Pelizaeus–Merzbacher-like disease (PMLD)	Brain
Cx37 (GJA4) Cx40 (GJA5)	- Atherosclerosis	Myocardium
Cx46 (GJA3) Cx50 (GJA8)	- Cataract (OMIM; 116200)	Lens

1.4. Connexins in Skin

Skin is mainly consisting of two layers, epidermis and dermis from top to bottom which have epithelial and mesenchymal characteristics, respectively[65]. Stratum corneum, stratum granulosum, stratum spinosum, and stratum basale are the four distinct layers of the epidermis from the bottom to the top. The main element of these layers are keratinocytes and gap junctional proteins have a critical impact on their growth and differentiation processes[45].

There are ten different Cx isoforms expressed in the skin with Cx43 being the most abundant type in the whole skin tissue. In addition, Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40 and Cx45 are the other connexin proteins which are located between keratinocytes[66]. Furthermore, these Cx isoforms are expressed in different locations in the epidermis. For example, Cx26 is a crucial element in the granular and the basal layers of the skin whereas, Cx40 is in the granular and the spinous layers in adult human skin[4] (Figure 1.3.). One of the most important functions of Cx isoforms is wound healing on the epidermis. Cx26, Cx30, and Cx43 mediate the healing process via the regulation of keratinocyte proliferation and differentiation. In addition, any mutations in these connexins cause epidermal diseases[67].

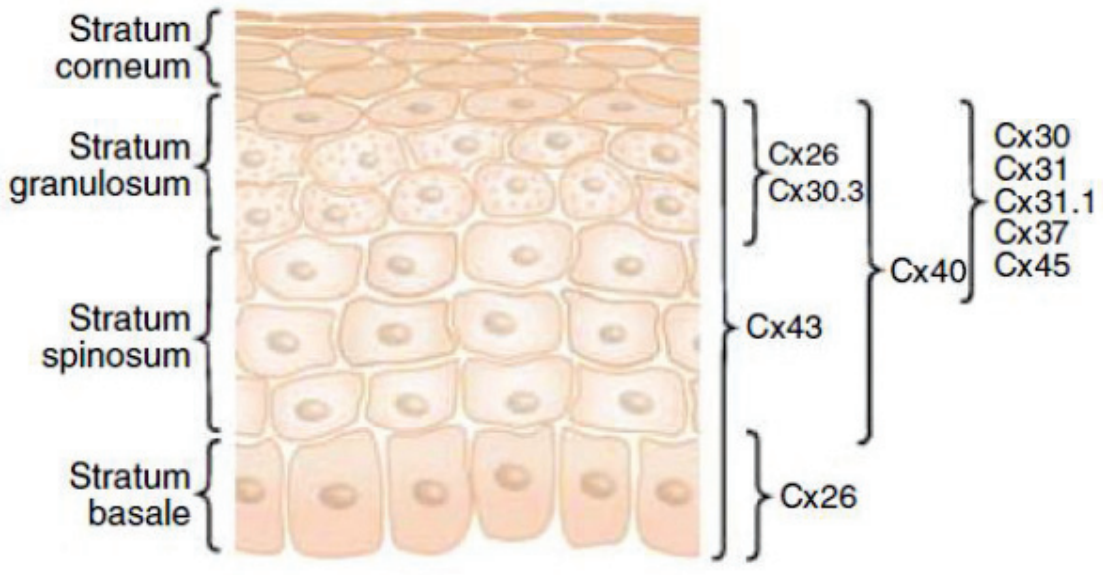

Figure 1.3. Connexin isoforms in ephitelium.[4]

1.5. Keratitis-Ichthyosis-Deafness (KID) Syndrome

The keratitis-ichthyosis-deafness (OMIM 148210) syndrome is a rare hereditary disorder that has mostly autosomal dominant inheritance besides seldom autosomal recessive inheritance[68–71]. This ectodermal dysplasia causes several anomalies in the skin, cochlea, and cornea[69,72]. Hyperkeratosis in skin especially in palms and soles, unilateral or bilateral sensorineural hearing loss, and epithelial defects and neovascularization in the cornea are the hallmarks of the KID syndrome[73,74]. It is a congenital disease that originates mostly from sporadic mutations in Cx26 and Cx30 genes[69,71].

More than ten missense mutations in GJB2 gene encoding Cx26 and two missense mutations in GJB6 gene encoding Cx30 were identified so far, resulting in KID syndrome[75,76]. Aberrant hemichannels which are composed of gain-of-function induced Cx26 proteins, cause leaky connexon structures[77,78]. One reason for the instability of Ca^{2+} and ATP concentrations in a cell is due to Cx mutations[78]. The Ca^{2+} gradient of the skin barrier, which increases from the stratum basale to the stratum granulosum layer, is destroyed due to abnormal hemichannels. Since the calcium ion gradient, which is crucial in keratinocyte proliferation and differentiation processes, is disrupted in KID patients, skin anomalies occur[78–80].

The missense mutations on Cx26 gene is mostly detected in the amino terminus and rarely in the first transmembrane loop segment of Cx26[81]. The mutant proteins show abnormal chemical and voltage gating properties and also abnormal docking process of gap junctions[82]. For instance, one KID syndrome mutation, G45E, increases hemichannel activity and causes abnormal cell death in KID patients who has high fatality rates within the first year[83]. Moreover, the change in the 50th amino acid position, which is highly common in KID patients, causes hyperactive hemichannel formation. For example, D50N and D50Y mutations were detected in a Japanese family with KID syndrome[84]. Besides, a KID syndrome patient had I30N mutation in the GJB2 gene and this mutation affected the first transmembrane loop of Cx26 that caused attachment problem between hemichannels[85]. Furthermore, it has been shown that I30N and D50Y mutations increased intercellular calcium concentration by supporting the formation of aberrant hemichannels instead of intercellular plaque formation, and this result is consistent with abnormal calcium signals in epidermis cells in people who suffered from KID syndrome[85].

1.6. KID Syndrome and Squamous Cell Carcinoma (SCC) Relationship

Squamous cell carcinoma (SCC) is a non-melanoma skin cancer type[86]. SCC which has an ability to evolve in any location on the human body arises from keratinocytes in the epidermis and adnexal structures[87]. Some chronic medical conditions, hereditary skin conditions, especially ultraviolet (UV) exposure and sunlight increase the risk of developing SCC. Furthermore, light-skinned populations have a higher risk for developing SCC than dark-skinned populations[88]. A scaly skin patch called actinic keratosis (AK), a lip inflammation called actinic cheilitis (AC) and a skin lump called keratoacanthoma (KA), caused by ultraviolet ray exposure, are various morphologic skin lesion types for SCC[87]. Both regional and distant metastatic potential of SCC is relatively low[89].

KID syndrome is also related to SCC[90]. Ichthyosis is a skin disorder characterized by dryness of the skin and a hallmark of carcinoma[91]. It was reported that KID patients aged 13 to 46 years with Cx26 mutations were tended to have SCC[74,90–92].

The studies have shown that Cx26 is a tumor suppressor and leads to cancer cells via apoptosis. However, it is demonstrated that at least 10% of patients with KID syndrome, who have mutations in the Cx26 gene develop SCC at early ages[93–95].

Altered working of the hemichannels involve in mutant Cx26 proteins are responsible for the Ca^{2+} gradient impairment in skin layers. Furthermore, upregulation of E-cadherin and β-catenin staining were observed in the histologic samples of SCC tissues in KID patients[96,97]. Additionally, some researches showed that bacterial, fungal, and viral infectious induce SCC in KID syndrome patients.[20,92]

1.7. Aim of the Project

KID syndrome caused by Cx26 mutations may cause an increased incidence of SCC in patients at an early age, however, the reason for this predisposition has not yet been clearly determined by molecular mechanism[95]. In the light of the previous study, it was seen that the I30N and D50Y mutations, which cause KID syndrome, formed abnormal hemichannels and allowed excessive calcium influx into the cell, and as a result, it was predicted to induce the hyperproliferative cell phenotype[85].

In this project, we aimed to enlighten the differences between I30N and D50Y mutations in Cx26 gene, on biological processes of the SCC. Throughout this study, we aimed to investigate the expression of mutant Cxs produced by SCC-25 cell line, as well as the expression and localization of the epithelial tumor suppressor protein, E-cadherin.

CHAPTER 2

MATERIALS & METHODS

2.1. Maintenance of SCC-25 Cell Line

Squamous cell carcinoma SCC-25 cell line (ATCC, Cat# CRL-1628) was used to perform experiments. SCC-25 cells were grown in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 nutrient mixture (GIBCO, Cat# 31330038) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Cat# 16000044), 1% Penicillin/Streptomycin (P/S) (GIBCO, Cat# 15140122) and 400 ng/ml Hydrocortisone (Sigma, Cat# H0888). Cells were incubated at 5% CO_2 and 37^{o}C conditions. The medium was changed every 48-72 hours as essential for all experiments.

SCC-25 cells were infected with pMSCV vectors that contain only GFP, Cx26-WT, Cx26-I30N (isoleucine to asparagine transition on 30th amino acid position) and Cx26-D50Y (aspartic acid to tyrosine transition on 50th amino acid position) mutated genes. The selection of the stable cell lines was provided with 2 µg/ml puromycin 72 hours post-infection.

Every 7-10 days, SCC-25 cells were trypsinized with 0.25% Trypsin-EDTA solution (Biological Industries, Cat# 03-053-1A) and incubated for 10 minutes at 37^{o}C. Then, cells were collected and centrifuged for 3.5 minutes at 1200 rpm at room temperature. After removal of the supernatant, the pellet, in other words, cells were dissolved in the medium and were split by 1:3.

2.2. Semi-quantitative RT-PCR and Gene Expression Analysis

Total RNA isolation was performed from fresh SCC-25 cells which were washed with 1× Phosphate Buffered Saline (PBS) twice, by using Pure-link RNA Mini Kit (Invitrogen, Cat#12183018A) following the manufacturer's instructions. RNA concentrations were measured by Nanodrop. cDNAs were synthesized by using cDNA Synthesis Kit (ThermoScientific, Cat# K1622) from 1µg RNA. Then, SYBR Green based RT-qPCR

was performed with cDNA samples by using FastStart Essential DNA Green Master Mix (Roche, Cat# 42729200) in Roche LightCycler® 96. RT-qPCR results were analyzed by Delta-Delta Ct method. As a housekeeping gene control, GAPDH was used. The durations and temperatures of the cycles and primer pairs are given in the tables below (Table 2.1. and Table 2.2.).

Table 2.1. Cycles used for RT-qPCR.

Stage	Temperature	Duration	Cycle
Preincubation	95°C	10 min	1
Amplification	95°C	30 sec	45
	60°C		
	72°C		
Melting	95°C	10 sec	1
	65°C	60 sec	
	72°C	1 sec	

Table 2.2. Forward and reverse primers used in RT-qPCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Connexin 26	ctgcagctgatcttcgtgtc	aagcagtccacagtgttg
Connexin 43	gtgcctgaacttgccttttc	ccctcccagcagttgagtagg
E-cadherin	cagcacgtacacagccctaa	ggtatgggggcgttgtcatt
GAPDH	gaaggtgaaggtcggagtca	aatgaaggggtcattgatgg

2.3. Western Blot and Protein Expression Analysis

2.3.1. Total Protein Isolation

Fresh SCC-25 cells were washed with 1× PBS twice and centrifuged for 3.5 min at 1200 rpm. 200 µl lysis buffer (100 mM Tris-HCl, 1mM EDTA, 0.1% Triton X) was added onto the pellets. Lysates were passed through an insulin syringe 8-10 times which

is followed by an incubation of 20 minutes on ice. Samples were centrifuged at 14,000 rpm for 20 minutes at +4°C and then supernatants were transferred into new Eppendorf tubes which were saved at -80°C.

2.3.2. Bradford Assay

BSA standards were prepared as 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml. 5× Bradford Reagent was diluted to 1× using autoclaved dH_2O to a total volume of 1 ml. Afterwards, 10 µl protein standards and protein samples were loaded into every cuvette with 1 ml of 1× Bradford Reagent. The concentrations of protein samples were measured at 595 nm on spectrophotometry.

2.3.3. Western blot analysis

SDS gel was prepared with two different densities for resolving and stacking gels at 15% and 5% acrylamide/bisacrylamide concentrations, respectively. A set of protein samples that had equal concentrations were loaded in the gel after incubation at 95^oC for 5 minutes with 5x loading dye. The samples were run in SDS gel at 32 mA for 2 hours and transferred to a nitrocellulose membrane at 350 mA for three and a half hours. Then, the nonspecific proteins on the membrane were blocked with the incubation of 5% non-fat milk (includes 36% protein in dry milk powder) solution in 1× Tris Buffer Saline-Tween 20 (TBS-T) for 2 hours with shaking. After the blocking step, the membrane was incubated with 1:1000 diluted primary antibody in blocking solution with shaking, overnight at 4^oC. The next day, the membrane was washed with TBS-T solution three times in the order of 10-20-10 minutes. After washing, the membrane was incubated with 1:1000 diluted secondary antibody in 5% non-fat milk solution for 2 hours and then washed again three times for 5 minutes with shaking at room temperature. The specific proteins on the membrane were visualized by the incubation of chemiluminescent substrate (BIORAD, Cat# 1705061) with FUSION SL VILBER LOURMAT imaging system.

Primary antibodies used for this experiment were anti-GJB2 (Cx26) goat monoclonal antibody (mAb) (Abcam, Cat# ab59020), E-Cadherin (24E10) rabbit mAb (Cell Signaling, Cat# 3195S), and anti-γ-Tubulin mouse mAb (Sigma, Cat# T6557). HRP-conjugated secondary antibodies used were anti-goat (Pierce, Cat# 31460), anti-rabbit (Thermo, Cat# 31460), and anti-mouse (Dako, Cat# 00071312). After the visualization

process, protein levels were normalized to γ-tubulin using ImageJ (NIH) software.

2.4. Immunostaining and Fluorescence Imaging

For immunostaining experiments, SCC-25 cells ($2.5\text{x}10^5$) cells ($3\text{x}10^5$) were seeded on coverslips in 6 well plates and cultured for 24 hours. After 2 days, cells were washed with 1x PBS three times and incubated in 4% Paraformaldehyde (PFA) at $+4^oC$ overnight for fixation of the cells. The following day, permeabilization was performed with 0.1% Triton X-100/ 1x PBS at room temperature for 15 min. Then, cells were blocked with 5% bovine serum albumin (BSA) in 0.1% Triton X-100/ 1x PBS for an hour at room temperature. Cells were incubated with diluted primary antibodies in 5% BSA solution for 1 hour and washed with 1x PBS three times for 10 minutes at room temperature. After washing, cells were incubated with Alexa Flour-conjugated secondary antibodies diluted in 5% BSA solution for 45 minutes at room temperature in dark conditions. After three washes with 1x PBS for 10 minutes in dark, coverslips were dipped into dH_2O and mounted onto the slides and fixed with nail polish.

Primary antibodies used for this experiment were 1:200 diluted rabbit anti-Cx26 antibody (Abcam, Cat# ab65969), 1:200 diluted rabbit anti-Cx43 antibody (Invitrogen, Cat# 71-0700), and 1:300 diluted anti E-Cadherin (24E10) rabbit mAb (Cell Signaling, Cat# 3195S). Secondary antibodies used for this experiment were 1:250 diluted Alexa488-conjugated goat anti-rabbit secondary antibody (Abcam, Cat# ab150081) with DAPI (Sigma, Cat# D95242-10MG) at 1:1000 dilution or 1:250 diluted Alexa555- conjugated goat anti-rabbit secondary antibody (Invitrogen, Cat# A21428) with Alexa Fluor 488 phalloidin (Invitrogen, Cat# A12379) at 1:500 dilution and DAPI at 1:1000 dilution.

Images of the slides were taken with Olympus IX83 microscope and analysed using ImageJ (NIH) software.

2.4.1. Fluorescence Intensity Analysis

A pipeline was designed and used in CellProfiler software to detect E-Cadherin localizations in SCC-25 cell lines obtained by immunostaining. This pipeline was enabled the detection of perinuclear E-cadherin signals by overlapping the DAPI-identified nucleus and red antibody-labeled E-cadherin as the objects. On the other hand, it was enabled the detection of cytoplasmic E-cadherin signals as a result of overlapping the cytoplasm

determined with Phalloidin and E-cadherin marked with antibody in red. After the analysis, these data were obtained numerically and graphics were created.

2.5. MTT Assay

After seeding of SCC-25 cells ($3x10^4$) on 12 well plates, cell cultures were incubated for 7 days. Then cells were incubated in 500 µl medium with MTT labeling reagent (Amresco, Cat# 0793) (final concentration of 0.5 mg/ml) to each well on the 1^{st}, 4^{th}, 7^{th} days for 4 hours. After 4 hours, the medium was removed and 500 µl DMSO was added to each sample. Each well was divided into 3 replicas of 100 µl and absorbance was measured at 570, 650 and 670 nm. The absorbance value of 650 nm and 670 nm were subtracted from 570 nm for analysis.

2.6. Statistical Analysis

All experiments were performed in triplicates and analysis of their results are shown as mean ± standard deviation. Statistical differences in all experimental designs were determined by two-tailed unpaired t-test. Statistical significance was indicated with p-values as $p<0.05$ (*), $p<0.01$(**), $p<0.005$ (***) and $p<0.001$(****) levels.

CHAPTER 3

RESULTS

3.1. The expressions of Cx26 and Cx43 were verified in SCC-25 cell line.

Cx43 proteins are known to be abundant in the epithelium layer of human skin tissue under normal conditions[4]. Additionally, Cx26 proteins, which play a role in the development of KID syndrome, may be expressed in the wild-type human cancerous epithelium cell line, SCC-25. Cx26 and Cx43 levels in SCC-25 cells need to be determined. Therefore, the presence of Cx26 and Cx43 expression levels were controlled initially in SCC-25 cell line by qPCR for mRNA level using specific primers, and by Western blotting and immunostaining for protein level and localization, respectively using specific antibodies.

Cx26 and Cx43 expressions in the SCC-25 cell line were verified at mRNA (Figure 3.1.A; n=4) and protein levels (Figure 3.1.B and 3.1.C; n=3). Relative mRNA levels were normalized to TBP and relative protein level was normalized to γ-tubulin. Endogenous Cx proteins were found throughout the cytoplasm (Figure 3.1.D).

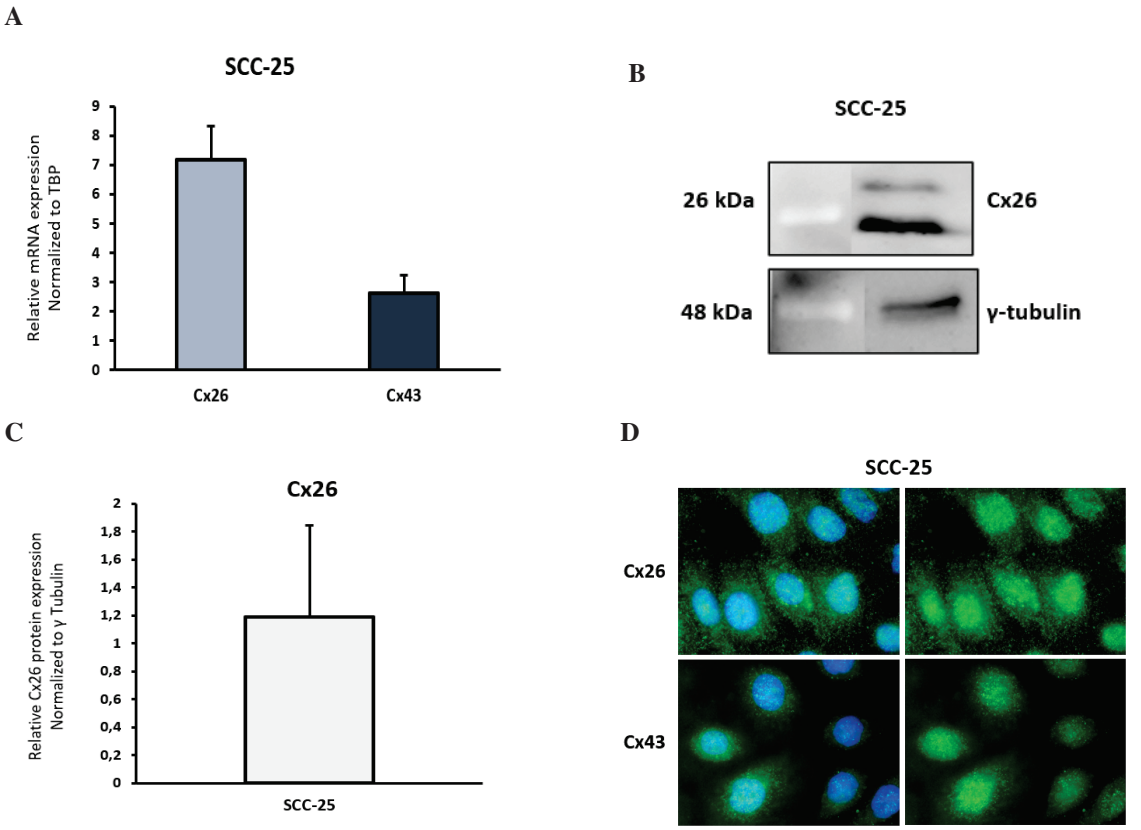

Figure 3.1. Localization and expression levels of Cx26 and Cx43 in SCC-25 cells. mRNA levels of Cx26 and Cx43 (Figure 3.1.A) (n=4), a representative image of Cx26 protein (Figure 3.1.B), and quantification of protein levels (Figure 3.1.C) (n=3) of the cells. Cx26 and Cx43 localizations (Figure 3.1.D) (n=3) were represented with green colour and nuclei were represented with blue colour (scale bar: 10 µm.). Error bars represent the standard deviation.

3.2. The expressions and localizations of Cx26 levels were verified in infected SCC-25 cells.

Cx26 levels in infected SCC-25 cells were tested to see whether overexpression was successful or not. Overexpression levels of Cx26 in SCC-25 cells infected with MSCV constructs were checked with RT-qPCR for mRNA expression and Western blot for protein expression. Samples were normalized to GAPDH and γ-tubulin at the mRNA and protein levels, respectively. In both of these experiments, expression levels of the cells were normalized to the only vector-infected SCC-25 cells (MSCV).

SCC-25 cells which contain WT-Cx26, I30N mutated-Cx26, and D50Y mutated-Cx26 cells, there was a significant 550, 88, and a 52-fold increase in mRNA level (Figure 3.2.A), respectively, however, no increase at protein level was observed (Figure 3.2.C)

compared to vector (MSCV) only infected cells. In other respects, there were significant increases between SCC-25 cells including Cx26-I30N and Cx26-D50Y genes and Cx26-WT gene at mRNA levels.

The distribution of Cx26 protein in SCC-25 cells was observed similarly for the MSCV, WT, I30N and D50Y infected cell types (Figure 3.2.D). It was seen that Cx26 proteins were located in cytosol instead of the plasma membrane.

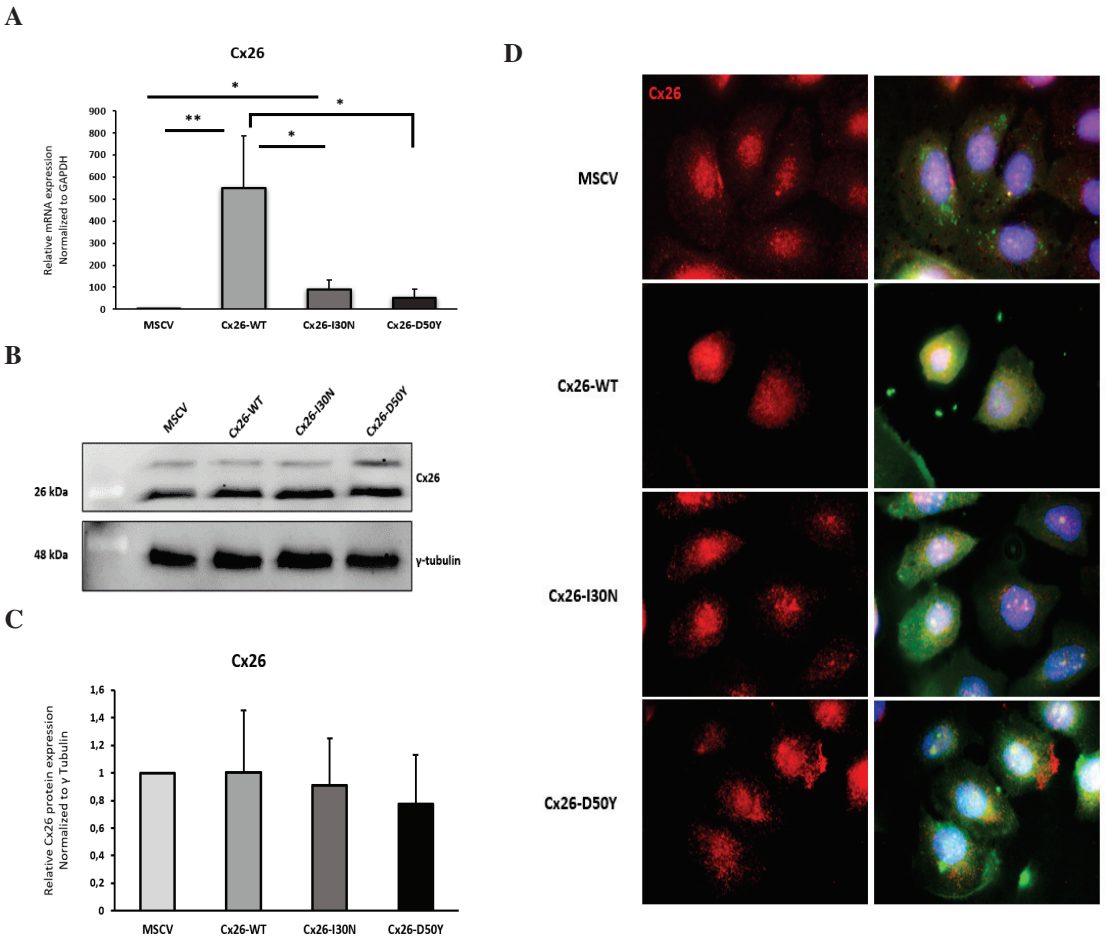

Figure 3.2. Relative Cx26 expressions and localizations of infected SCC-25 cells. mRNA levels of Cx26 (Figure 3.2.A) (n=3, *p<0,05; **p<0,01) were demonstrated by normalizing both GAPDH and control MSCV infected cells. Representative image (Figure 3.2.B) and quantitative comparison (Figure 3.2.C) (n=3) of Cx26 protein levels in infected cells. Cx26 protein localizations were shown (Figure 3.2.D) (n=3). Cx26 was represented with red colour, GFP with green colour, and nuclei with blue colour (scale bar: 10 µm.). Statistical analysis was performed using an unpaired t-test and the error bars represent the standard deviation.

3.3. Cx43 expression levels and localizations were revealed in infected SCC-25 cells.

Cx43 is found in most tissues in the human body, including epithelial tissue[49]. On the other hand, it is known that Cx26 mutations found in patients with KID syndrome can cause changes in localizations of Cx43 levels of cells[98]. Therefore, the effect of Cx26 overexpression on Cx43 was investigated by RT-qPCR and immunostaining assays. No changes were seen in both mRNA levels (Figure 3.3.A) and protein distributions (Figure 3.3.B) for these four different types of cells. In Cx43 immunostaining assay, it was seen that Cx43 were present in cytoplasms of infected SCC-25 cells.

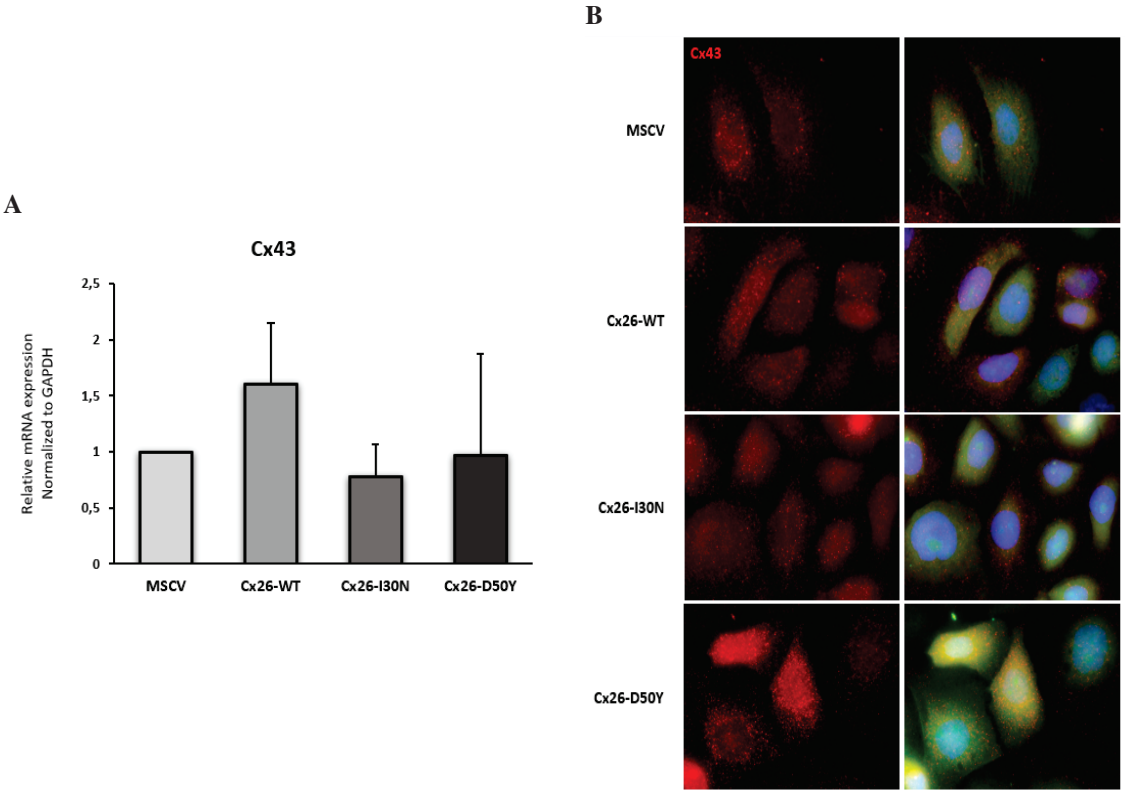

Figure 3.3. Relative mRNA expressions and localizations of Cx43 of infected SCC-25 cells. mRNA level of Cx43 by normalizing both GAPDH and control MSCV infected cells (Figure 3.3.A) and localizations of Cx43 protein (Figure 3.3.B) (n=3) were shown. Cx43 protein were was represented with red colour, GFP with green colour, and nuclei with blue colour (scale bar: 10 μm.).

3.4. E-cadherin levels and localizations revealed in infected SCC-25 cells.

It was shown that E-cadherin anomalies were found in primary culture samples taken from patients with KID syndrome carrying Cx26 mutations[90]. Therefore, E-cadherin levels and localizations were examined in SCC-25 cells, containing Cx26-I30N and Cx26-D50Y by comparison with Cx26 overexpression and MSCV infected.

E-cadherin mRNA levels of infected SCC-25 cells were observed by normalizing to GAPDH, and no changes were seen among the groups (Figure 3.4A; n=3). However, there was a significant change in E-cadherin protein levels between I30N-Cx26 infected and MSCV infected cells (Figure 3.4B; n=6, *p<0,05). An increasing trend was also seen in Cx26-D50Y cells, but this was not significant (p=0,37).

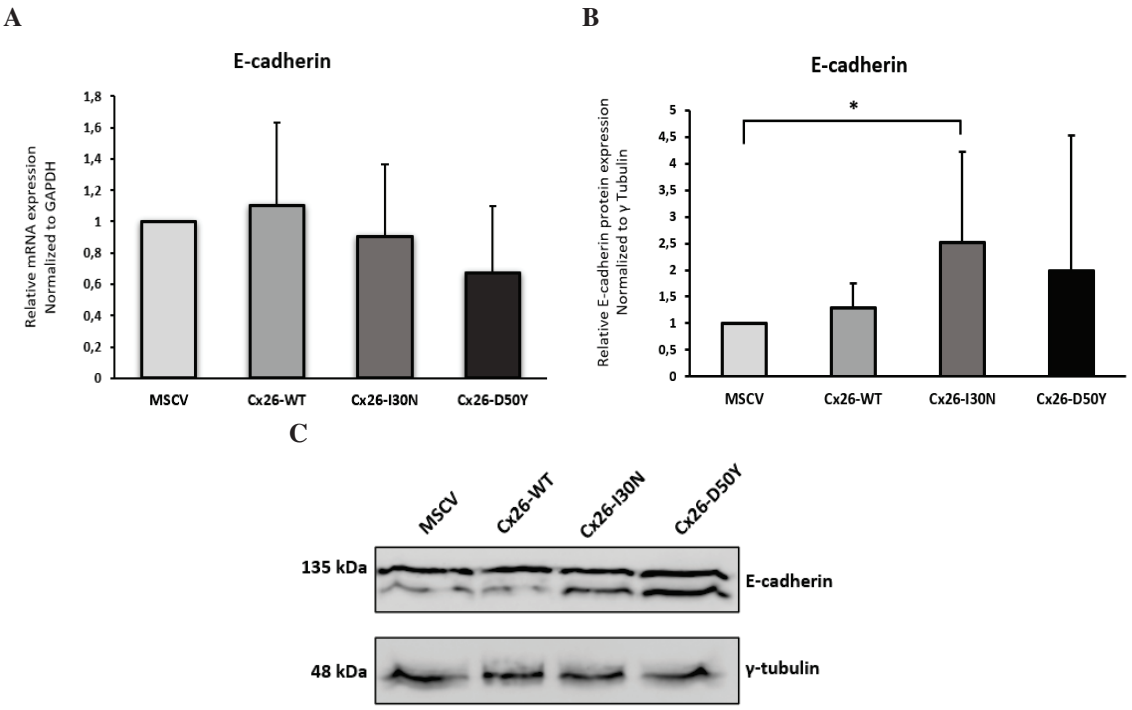

Figure 3.4. Relative E-cadherin levels of infected SCC-25 cells. mRNA levels of E-cadherin were normalized to GAPDH and MSCV (Figure 3.4.A) (n=3). Representative image of E-cadherin (Figure 3.4.C) and quantitative data of the protein (Figure 3.4.B) were shown. E-cadherin protein levels were normalized to γ-tubulin and MSCV (n=6; *p<0,05). Statistical analysis was performed using an unpaired t-test and the error bars represent the standard deviation.

The distributions of E-cadherin in infected SCC-25 cells were determined with immunostaining assay (Figure 3.5.A & Figure 3.5.B n=4). All images from four different cell groups were taken with the same exposure time for the red channel. Based on this experiment, E-cadherin was mostly observed in and around the nucleus in WT-Cx26 and MSCV infected cells, whereas, in cells carrying mutant Cx26, it was observed to be nearly uniformly distributed in the cytoplasm. It was also observed that E-cadherin proteins formed plaques at the intercellular junctions in cells containing mutant Cx26. The majority of the cell types imaged were observed as in Figure 3.5.A. However, plaque formation was seen in approximately 20% of cells containing MSCV and Cx26-WT. In addition, approximately 1 out of 3 cells containing Cx26-I30N or Cx26-D50Y were encountered with signal accumulation in the perinuclear area (Figure 3.5.B). E-cadherin images taken at equal exposure time were used in E-cadherin intensity analysis to compare to the difference in E-cadherin distributions within and between cells (Figure 3.5.C, ****p<0,001). The analysis was performed using CellProfiler software with 20 images for each cell type. Significant changes were seen at E-cadherin levels between around nuclei and only cytoplasms for the infected SCC-25 cells. Compared to MSCV, a significant decrease was found between intensities in the perinuclear area and the cytoplasm for SCC-25 cells including D50Y-Cx26 (Figure 3.5.D and Figure 3.5.E, *p<0,05; **p<0,01). Besides that, a significant change was observed between MSCV and Cx26-I30N infected cells for E-cadherin intensities of only the perinuclear area. Nonetheless, no significant changes were seen in SCC-25 cells including WT-Cx26 compared to SCC-25 cells including MSCV (Figure 3.5.D and Figure 3.5.E, *p<0,05; **p<0,01).

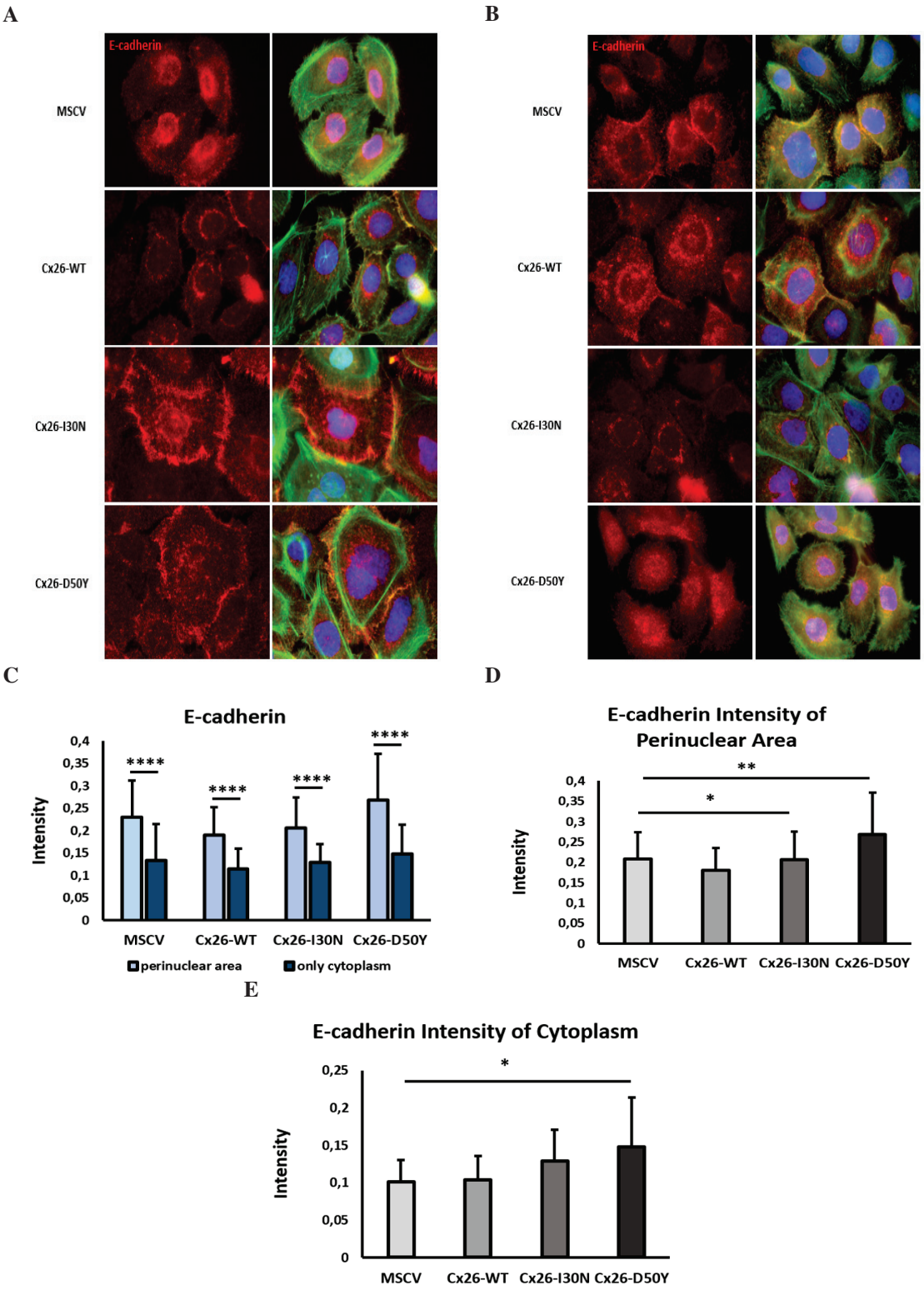

Figure 3.5. Localizations and intensities of E-cadherin levels of infected SCC-25 cells. E-cadherin localization (Figure 3.5.A and 3.5.B) (n=4) was represented with red colour, filaments with green colour, and nuclei with blue colour (scale bar: 10 µm.). The comparisons of E-cadherin intensities (Figure 3.5.C) (signals from perinuclear area (Figure 3.5.D) & signals from cytoplasm (Figure 3.5.E)) were analyzed according to immunostaining experiments with CellProfiler software (*p<0,05; **p<0,01; ****p<0,001). Statistical analysis was performed using an unpaired t-test and the error bars represent the standard deviation.

3.5. The effect of mutations and Ca^{2+} content of medium on the viability of cells were revealed in SCC-25 cells.

It has been reported that mutant Cx26 proteins have negative effects on cell viability, such as apoptosis and necrosis, by forming aberrant hemichannels[99]. Additionally, in some studies, it has been indicated that cells containing mutant Cx26 increase their viability as a result of treatment with high extracellular Ca^{2+}[98]. Hence, the viability of mutant (I30N and D50Y) Cx26-containing SCC-25 cells and WT-Cx26 and vector-infected SCC-25 cells were compared using the MTT assay. These MTT experiments were done under 2 different nutrient media conditions which were including high Ca^{2+} concentrations (3.2 mM) or Ca^{2+}– free.

There were significant viability changes in infected cells incubated in high extracellular Ca^{2+} containing media by contrast with cells incubated in Ca^{2+}– free media (n=4). No significant difference in cell viability was found in infected SCC-25 cells grown in Ca^{2+}– free media compared to day one (Figure 3.6.A). Contrariwise, the viability of Cx26-WT, Cx26-I30N and Cx26-D50Y cells cultured in media containing high concentrations of Ca^{2+} demonstrated significant increases at day 4 ((Figure 3.6.B); *p<0,05; **p<0,01). When values were normalized to day 1, no significant increase was observed at day 7 for cells incubated in the same conditions compared to control cells.

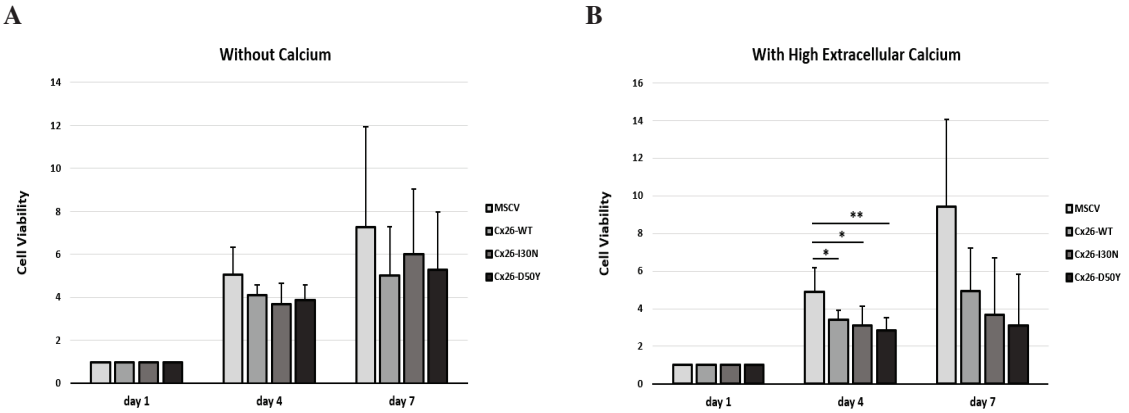

Figure 3.6. Comparison of the cell viabilities of infected SCC-25 cells. Quantification of cell viabilities of infected SCC-25 in culturing Ca^{2+}– free media (Figure 3.6.A) and high Ca^{2+} media (Figure 3.6.B) (*p<0,05; **p<0,01). Graphs includes values normalized to day one (Figure 3.6.A and 3.6.B) (n=4). Statistical analysis was performed using an unpaired t-test and the error bars represent the standard deviation.

CHAPTER 4

DISCUSSION AND CONCLUSION

Squamous cell carcinoma is a skin cancer type which is caused by DNA damage from UV exposure[100]. In addition, patients with KID syndrome, which is characterized by hearing and skin problems[68], are predisposed to SCC[84]. In studies conducted to date, it has been reported that KID syndrome occurs in patients with mutations in the Cx26 or Cx30 genes[66,68]. In our study, we aimed to investigate the cellular processes of SCC such as cell viability regarding the differences between I30N and D50Y mutations in the Cx26 gene which are responsible for KID syndrome in patients. Wild-type and four different infected SCC-25 cells were used in this research, MSCV-infected is including only vector, WT-Cx26 infected is Cx26-overexpressed, I30N mutated-Cx26 infected has isoleucine (I) to asparagine (N) translocation in Cx26 gene, and D50Y mutated-Cx26 infected has aspartate (D) to tyrosine (Y) translocation in Cx26 gene. In order to reveal the potentials of these mutations on SCC, mRNA and protein levels, and localizations of Cx26 and Cx43 were investigated.

Cx43 is found in all layers of epithelial tissue except the stratum corneum and interacts with other Cx isoforms[4]. In other respects, studies have found differences when in conversion of non-functionals channels to hyperactive channels when wild type Cx43 is coexpressed with mutant Cx26 in patients with KID syndrome[98]. Therefore, while investigating mutant Cx26 expressions, Cx43 expressions were also controlled in our study, but for this, basal Cx26 and Cx43 expression levels in SCC-25 cells were examined first. As a result of RT-qPCR and Western blot experiments, it was determined that Cx26 and Cx43 were expressed in SCC-25 cells. However, Cx43 protein was not detected in SCC-25 cells by Western blot, which may be due to the low amount of this protein in WT-Cx26, I30N-Cx26 and D50Y-Cx26 cells.

The localization of Cx proteins in cells can provide information about the fate of cells. There is a strong evidence that connexins and their localizations control cellular growth or growth inhibition. In some researches, connexins found in nuclear envelope or perinuclear area of cells instead of the plasma membrane[101–103]. Krutovskikh and colleagues showed that A431 cells (epidermoid carcinoma) carrying the Cx43T154Y mutant are located in the intracytoplasmic and nuclear areas, while the cells carrying the Cx43S158Y mutant are located in the perinuclear cytoplasmic and nuclear areas. In this study, it was determined that while the intercellular connection was lost in the

cells containing Cx43T154Y mutant, intense communication was observed in the cells containing Cx43S158Y mutant[103]. Moreover, lack of intercellular communication and paracrine signalling between cells indicated cancerous formation in many situations[104]. In our reserch, we observed that Cx26 and Cx43 were localized in cytosolic space, not in cell membrane in all SCC-25 cells (MSCV, WT-Cx26, I30N-Cx26 and D50Y-Cx26). According to these informations, perinuclear Cx26 and Cx43 localizations in SCC-25 cells may affect cell growth, but it is not known whether this effect is positive or negative.

Structural and biochemical changes occur in I30N and D50Y mutations in connexin proteins, which are among the connexin mutations that cause KID syndrome, due to amino acid sequence changes. Amino acid changes at position 30 in the first transmembrane domain and at position 50 in the first extracellular loop on Cx26 gene, prevent connexon formation and the docking process of hemichannels to the cell membrane[105]. For these reasons, we determined mRNA and protein levels of Cx26 and Cx43 and their localizations in infected SCC-25 cells with only vector (MSCV), WT (overexpression) and mutant (I30N and D50Y) Cx26 constructs. Cx26 overexpression was achieved at mRNA level but not in protein level. Besides that, there were significant decreases in Cx26 expression for cells including Cx26-I30N and Cx26-D50Y at mRNA level, but not at protein level. The reason for no differences in protein levels may be misfolding of the proteins or failure to insert into the membrane. On the other hand, Cx26 and Cx43 localizations were detected in the perinuclear area, as in wild-type SCC-25 cells. As seen in various disease-related Cx26 disorders[106], this localization of Cxs in the cell may be due to accumulation of them in the Golgi apparatus through misfolding or trafficking defects. The possibility of Cx proteins accumulating in Golgi in these SCC-25 cells will be tested by immunostaining assay for Cxs, marked with Golgi.

E-cadherin is a calcium-dependent adherens junction protein that controls cell proliferation, migration, and death, and also works as a transcription factor for various genes[107]. For instance, the disruption of E-cadherin and β-catenin connection in the membrane of epithelium, increases the migration potential of cells[108]. In previous studies, upregulation of E-cadherin and β-catenin has been seen in samples from patients with KID syndrome carrying Cx26 mutations[90,91]. Thus, E-cadherin levels in cells were investigated. Compared to vector infected cells, there was no difference in E-cadherin mRNA levels of mutant cells, while a significant increase in protein level was observed in I30N mutant cells. It will be necessary to control β-catenin levels in I30N mutant cells to support these results.

E-cadherin protein localization provides information about the growth and differentiation processes of cells in different cell types. Previous studies showed that Cx43, E-cadherin and β-catenin interactions were found to affect the Wnt-pathway[109]. Furthermore, it is possible for these components to be localized in the plasma membrane, in the cytoplasm or in the nucleus. Additionally, E-cadherin and β-catenin, which are connected

to each other in the membrane under healthy conditions, induce carcinogenesis through the Wnt-pathway by changing the localization of E-cadherin or increasing β-catenin expression[108]. It has been observed that the E-cadherin localization is in a nuclear manner in Merkel cell carcinoma[110]. As a result of our immunostaining assays, we observed that E-cadherin is located in the perinuclear area in MSCV and Cx26 overexpressed cells, while E-cadherin is located in the cytoplasm and especially in the membrane in cells containing mutant Cx26 (both I30N and D50Y). This can be interpreted as E-cadherin proteins can be involved in adherent junctions in mutant Cx26-containing SCC-25 cells, while in WT-Cx26-containing SCC-25 cells, they may act as transcription factors. In addition, to fully understand the E-cadherin localizations in these cells, β-catenin levels and localizations in the cells should be examined for carcinogenesis process.

Ca^{2+} concentrations in the layers of epithelial tissue form a gradient, and this gradient plays an important role in keratinocyte proliferation and differentiation[75–77]. Disturbances in this Ca^{2+} gradient have been observed in patients with KID syndrome due to mutant Cx proteins[4]. In other words, aberrant hemichannels have a negative effect on the normal keratinocyte life balance in epithelial tissue. In addition, studies have shown that extracellular Ca^{2+} facilitates closure by rearranging the open state of hemichannels, with a mechanism that requires Ca^{2+} to bind to the open state of Cx26 channel in human cells[111]. It may cause the cellular homeostasis that is important for the cellular life cycle to deteriorate by preventing the influx or efflux of small molecules into the cell. Considering all these, viability was tested by MTT analysis in 4 different cell types grown in high Ca^{2+} and Ca^{2+}– free media. MTT assay showed that there was an inconsistent rate of viability in all four different infected cell types which were grown in the Ca^{2+}– free medium. Contrary to the cells grown in the Ca^{2+}– free medium, it was observed that the viability of the mutant Cx26-containing cells was significantly slowed in the cells growing in the high concentrations of Ca^{2+}-containing medium. Based on these results, E-cadherin as calcium-dependent proteins, may have a direct effect on the viability of cells containing mutant-Cx26 containing cells; therefore, Western blot experiments can be repeated with cells grown in media with high Ca^{2+} concentrations.

In this study, all the results show that the presence of mutant Cx26 proteins in SCC-25 cells did not affect the Cx43 level but affected the E-cadherin localizations and cell viability in the presence of high extracellular Ca^{2+} in the cell environment. The relationship between mutant Cx26 proteins and Wnt-β-catenin pathway of SCC cells should be enlightened.

CHAPTER 5

CONCLUSION

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