INTRA-GOLGI CONNEXIN26 BEHAVES IN A PRO-Oncogenic MANNER IN HEAD AND NECK CANCER CELLS

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Abstract
Downregulation of gap junctional intercellular communication is an important hallmark of malignant tumours. One of the downregulation mechanisms is translocation of gap junction (GJ) protein called connexin from cell membrane into cytoplasm, nucleus, or Golgi apparatus. Interestingly, as tumours progress and reinforce their malignant phenotype, the amount of aberrantly-localised connexin increases in different cancers including oesophageal squamous cell carcinoma, thus suggesting that such an aberrantly-localised connexin should be oncogenic. To determine the roles of aberrantly-localised connexin in head and neck squamous cell carcinoma (HNSCC), we introduced wild-type connexin26 (wtCx26) or the Golgi-retained mutant Cx26 (mtCx26) gene into human SAS lingual HNSCC cell line, which had lost the expression of connexin during carcinogenesis. The wtCx26 protein was trafficked to cell membrane and formed GJ, which successfully exerted cell-cell communication. On the other hand, mtCx26 protein was retained in the Golgi apparatus on the way to cell membrane. While the forced expression of wtCx26 suppressed various malignant phenotypes including cell proliferation, motility, and invasiveness in vitro, mtCx26 significantly reinforced these phenotypes compared with the mock control clone, indicating that an excessive accumulation of connexin protein in Golgi apparatus should be involved in cancer progression.

Key words: gap junction, connexin26, head and neck squamous cell carcinoma, intra-Golgi retention, SAS cell

Introduction
Gap junction (GJ) is a unique intercellular channel which connects directly the cytoplasm of two neighbour-

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bers in mammals\(^2,3\)). Usually, several different connexin proteins are expressed in a single cell and the combination of the expressed connexin proteins varies tissue to tissue, \(i.e.,\) while the epidermis expresses a wide variety of connexin proteins such as connexin 26, 30, 30.3, 31.1, 32, 37, 43 and 45\(^4\), the liver expresses connexin26 (Cx26) and connexin32 (Cx32) in the hepatocytes and connexin43 in the cholangiocytes\(^5,6\).

A considerable number of studies have established that GJIC suppresses tumour development, \(i.e.,\) GJIC is down-regulated in almost all tumours through whatever mechanism, including no or reduced expression, aberrant localisation, and aberrant phosphorylation or dephosphorylation of connexin protein\(^7,8\). However, a growing body of evidence indicates that an excessive accumulation of connexin protein in cytoplasm and/or some organelles enhances cancer progression such as invasion and metastasis\(^9-12\).

Notably in squamous cell carcinoma of the head and neck and the oesophagus, the expression level of Cx26 in cytoplasm correlates to the grade of malignancy or the extent of lymph node metastasis\(^13,14\). Furthermore, we have previously reported that accumulation of Cx32 in Golgi apparatus increases cancer stem cells in number and enhances the metastatic ability of the cell lines derived from human hepatocellular carcinoma\(^15\).

In the present study, to define the roles of Golgi-retained connexin protein in the malignant phenotype of human head and neck squamous cell carcinoma (HN-SCC), we transduced human SAS lingual HNSCC cell line with the retrovirus vector carrying the mutated Cx26 cDNA which encoded Golgi-retained Cx26 protein and compared their cancerous behaviours with those of the clones transduced with the wild-type Cx26 (wtCx26) or the empty vector.

### Materials and Methods

#### Vector construct

A DNA fragment coding either human wtCx26\(^16\) or the mutant Cx26 (mtCx26) protein bearing a Golgi-retention signal\(^17\) AKKFF at the C-terminus was subcloned into pQCXIN retrovirus vector (Clontech Laboratories, Mountain View, CA) as described elsewhere.

#### Cell culture and retroviral transduction

Human SAS lingual squamous cell carcinoma cell line was supplied by RIKEN CELL BANK (Tsukuba, Japan). The cells and their established subclones were cultured in RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin. PT-67 packaging cells were grown in Dulbecco modified Eagle medium (Thermo Fisher Scientific, Rockford, IL), 10% FCS, 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin. All the cells were incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\) in air.

To determine cell proliferation, \(5 \times 10^4\) cells were seeded into 60-mm dishes in triplicate in 4 ml of medium with 10% FCS. The cells were grown under the above-described conditions and counted every other day with a haemocytometer. Dead cells, as determined by trypan blue staining, were left out of the count.

SAS cells expressing Golgi-retained mtCx26 protein or the wild type were established as follows. The mtCx26/pQCXIN, the wtCx26/pQCXIN construct, or pQCXIN empty vector was transfected with FuGENE HD Transfection Reagent (Promega, Madison, WI) into the packaging cell PT-67, and stable transformants were selected with 400 \(\mu\)g/ml G418. SAS cells were then infected with virus-containing supernatant, supplemented with 4 \(\mu\)g/ml of polybrene, from PT-67 cells transfected with each of the 3 constructs. After 3 weeks of selection with 400 \(\mu\)g/ml G418, G418-resistant SAS transductants were subcloned by limiting dilution method.

#### Immunoblotting

Immunoblotting analysis was performed mostly as described previously\(^18\). Anti-Cx26 polyclonal antibody (pAb) (Thermo Fisher Scientific) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (mAb) clone 6C5 (HyTest, Turku, Finland) were diluted at 1 : 500 and 1 : 10,000, respectively. Horseradish peroxidise-conjugated anti-rabbit and anti-mouse IgG antibodies (GE Healthcare Bio-Sciences, Piscataway, NJ) were diluted at 1 : 2,000 and 1 : 5,000, respectively. Finally, the protein-antibody complex was visualized with a WEST-one Western Blot Detection System (iNtRON Biotechnology, Seoul, Korea) and exposed to X-ray film.
Biotechnology, South Korea) following the protocol provided by the manufacturer.

**Indirect immunofluorescence**

Indirect immunofluorescence was performed as described previously. \(^{19}\) Anti-Cx26 pAb (Thermo Fisher Scientific) and anti-Golgi 58K protein mAb clone 58K-9 (Sigma-Aldrich) were diluted at 1:150 and 1:50, respectively. After fixation with aceton, cells are incubated with the diluted primary antibodies. Specific signals were revealed by anti-rabbit IgG-Alexa 488 (Thermo Fisher Scientific) and anti-mouse IgG-Alexa 568 (Thermo Fisher Scientific). Nuclei were stained with diamidine phenylindole dihydrochloride (DAPI) (KPL, Gaithersburg, MD) at a concentration of 0.5 \(\mu\)g/ml.

**Scrape-loading dye-transfer assay**

The assay was performed as described in el-Fouly et al.\(^{20}\) with modification. In brief, the confluent cells on 60-mm dishes were washed with phosphate-buffered saline (PBS) containing 1 mM CaCl\(_2\) and soaked in 3 ml of dye cocktail containing 0.1% Lucifer yellow CH (Sigma-Aldrich) and 0.1% rhodamine B isothiocyanate (RITC)-dextran (Sigma-Aldrich). Several parallel scrape lines were then made with a micropipette tip, and the cells were incubated for 5 min at 37°C. After washing with PBS, the cells dye-coupled with Lucifer yellow CH were observed under a fluorescence microscope. The cells positive for RITC-dextran were considered to be primarily scraped but not dye-coupled cells.

**Anchorage-independent cell growth assay**

5 \(\times\) 10\(^4\) cells from each clone were seeded in 4 ml of RPMI1640 containing 10% FCS and 0.33% agar on 5 ml of a solidified RPMI1640 basal layer containing 10% FCS and 0.5% agar in 60-mm dishes. 14 days after seeding, colonies consisting of at least 10 cells in 4 areas (4 cm\(^2\) each) were counted in 6 dishes. Each value was converted to that for 1 ml.

**Cell migration and invasion assay**

Cell motility and invasiveness were assessed quantitatively with a Boyden chamber. For migration assay, Falcon Permeable Support with 8-\(\mu\)-m-pore filter (Corning Inc. Life Sciences, Tewksbury, MA) were settled onto the lower compartments of 6-well plates containing 4 ml of FCS-supplemented RPMI1640. 5.0 \(\times\) 10\(^5\) cells resuspended in 2 ml of serum-free RPMI1640 containing 0.01% bovine serum albumin were seeded to each upper well. After 48 h of incubation, cells were trypsinised and collected separately from the top of the membrane, the underside of the membrane, and the lower compartment. Cell motility was quantitated as the percentage of cells found at the underside of the membrane and the lower compartment over the total cell number.

For invasion assay, 8-\(\mu\)-m-pore filters of cell culture inserts were precoated with 500 \(\mu\)g Matrigel (Corning Inc. Life Sciences), dried for 24 h in an incubator and rehydrated by RPMI1640 for 1 h before inoculation of cells. The assay was done according to the same protocol as that for migration assay mentioned above except that the cells were incubated for 72 h.

**Statistical analysis**

Student’s \(t\)-test was performed for the estimation of statistical significance. \(P\) values are two-tailed.

**Results**

**The mtCx26 protein bearing the Golgi retention signal accumulates in Golgi apparatus in SAS cells**

To determine the roles of intra-Golgi connexin protein in the malignant phenotype of head and neck squamous cell carcinoma (HNSCC), the mtCx26 protein in which the Golgi retention signal\(^{17}\) was added to the C-terminus was overexpressed in human SAS lingual HNSCC cells by retroviral transduction. As shown in Fig. 1A, the control mock-transduced SAS cells express no endogenous Cx26 protein. On the other hand, the clones transduced by either wild-type or the mtCx26 construct overexpress respective corresponding proteins. Consistently with the immnoblotting, wtCx26 protein give punctuate strong fluorescent signals at a cell-cell contact area, indicating an efficient formation of GJ plaques (Fig. 1B). In contrast, the mtCx26 protein bearing the Golgi retention signal fails to locate in cell membrane and demonstrates an accumulation in Golgi apparatus as revealed by co-lo-
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ocalisation with Golgi 58 K protein, a Golgi marker.

The mtCx26 protein has no ability to exert GJIC

As mentioned above, wtCx26 protein is sorted to cell membrane and can form GJ plaques in a cell-cell contact area of SAS cells. To examine whether the wild type-mediated GJ plaques are functional in SAS cells, we performed a scrape loading dye-coupling assay. As shown in Fig. 2, while the primarily-scraped cells are co-stained by RITC-dextran and Lucifer yellow CH in all the clones examined, the clone transduced with the wild type but neither the mutant nor the mock construct is positive only for Lucifer yellow CH in a zone adjacent to RITC-dextran — positive cells. Since Lucifer yellow CH, unlike RITC-dextran, can pass through GJ, the cells in the Lucifer yellow CH-positive zone are considered to have received the dye through functional GJs. As expected, these results clearly indicate that intra-Golgi Cx26 protein cannot contribute to GJ formation in cell membrane and is thus non-functional as a GJ protein (Table 1).

wtCx26 and the mtCx26 proteins regulate cell proliferation in a mutually-reciprocal manner

To examine the effects of intra-Golgi accumulation of Cx26 protein on cell proliferation, each clone transduced with wtCx26, the mtCx26, or the mock construct was plated in 60-mm dishes in hexaplicate and population doubling time was measured. As shown in Fig. 3A, the population doubling time of the clone overexpressing wtCx26 protein is significantly longer than that of the mock clone. Interestingly, overexpression of the mtCx26 protein retained in Golgi apparatus remarkably shortens population doubling time compared with the mock clone. To examine whether overexpression of the intra-Golgi Cx26 protein affects the anchorage-independent growth capacity of SAS cells, a colony formation assay in soft agar was performed. Fig. 3B shows that the clone over-
expressing the intra-Golgi Cx26 protein is capable of forming a two-fold number of colonies compared with the mock clone while the colony forming ability of the clone transduced by wtCx26 construct is 30% of that of the mock clone. Taken together, while cell proliferation is suppressed by Cx26 protein composing GJ, GJ-independent Cx26 protein localised in Golgi apparatus enhances cell proliferation.

wtCx26 and the mtCx26 proteins regulate cell motility and invasiveness in a mutually-reciprocal manner

Cell motility is one of the malignant phenotypes leading to tumour progression. We examined the effect of intra-Golgi accumulation of Cx26 protein on cell motility by performing a serum-stimulated transwell migration assay. Motility of the clone overexpressing the mtCx26 protein is significantly upregulated compared with that of the mock clone, while the clone transduced by wtCx26 construct exhibits a much lower motility than the mock clone (Fig. 4A). We further investigated whether overexpression of the intra-Golgi Cx26 protein could affect invasiveness of SAS cells by evaluating the ability of each clone to invade the basement membrane matrix. Similarly to the other malignant phenotypes, Fig. 4B demonstrates that overexpression of the intra-Golgi Cx26 and wtCx26 proteins enhances and declines invasiveness of SAS cells, respectively.

Discussion

For the past five decades, connexin-mediated GJIC has been believed to be one of mechanisms for tumour suppression. Consistently, our present study also demonstrated that wtCx26 protein could form GJ and mediate an efficient GJIC, resulting in suppression of different malignant phenotypes manifested by SAS cells. In this context, cytoplasmic localisation of connexin protein, probably due to a defect of membrane trafficking, is considered to be one of causes for the downregulation of GJIC. Many studies have indeed reported translocation of connexin protein from cell membrane to the inside of
cells in cancers\textsuperscript{21}. Nobody would doubt that the connexin proteins localised in cytoplasm are non-functional as a GJ. Then do they play any roles while residing in cytoplasm? A growing body of studies has described suggestive observations on intracellular connexin and tumour progression. The amount of aberrantly-localised connexin protein tends to increase during cancer progression such as invasion and metastasis, and in correspondence with the grade of malignancy, suggesting the possibility that intracellular localisation of connexin proteins might be not only “loss-of-function” as a GJ but also “gain-of-function” leading to enhancement of malignant behaviours of cancer cells. In the present study, we successfully proved that Golgi-retained mtCx26 protein reinforced various malignant phenotypes including cell proliferation, anchorage-independent colony formation, cell motility, and invasiveness while wtCx26 proteins downregulated all of these parameters.

Since disorders of GJ and/or connexin proteins are implicated in various human diseases\textsuperscript{22}, connexin proteins including Cx26 have now become new pharmacological targets\textsuperscript{23}. It has long been believed that the drugs which enhance connexin expression and GJIC should exert an anti-cancer effect and reduce tumour growth\textsuperscript{24,25}. Also, in the case of benign diseases such as cardiovascular and neurosensory diseases, many drugs targeting connexin proteins are being designed so as to increase expression of connexin proteins and to reinforce GJIC\textsuperscript{22}. However, it is not clear whether all the connexin molecules increased by such agents contribute to GJIC only in the cells that need a high level of GJIC. Cx26 protein overexpressed by a drug used to treat one disease may have an adverse effect on individuals carrying another disease that deteriorates due to the intracellular Cx26 protein, e.g., HNSCC.

It is the fact that GJIC efficiently can suppress tumour development in many organs as revealed by both in vitro and in vivo experiments. Today, connexin proteins are, however, beyond GJ\textsuperscript{26}. A great variety of structures, functions, and behaviours of connexin proteins are known, \textit{i.e.}, hemichannel\textsuperscript{27}, mitochondrial connexin\textsuperscript{28}, oncogenic connexin\textsuperscript{29–30}, cytoplasmic connexin\textsuperscript{18,19}, fragmented connexin, and then, GJ. Therefore, the functions of connexin proteins in cancers are not always suppressive to either cell proliferation or tumour progression\textsuperscript{31}. It will hereafter become an important issue to elucidate so diverse functions of connexin proteins in cancer.

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