수의학박사 학위논문

The Roles of Connexin32 on Cell Proliferation during Gastric Carcinogenesis

위암 발생 과정 중 세포의 증식에 있어서 Connexin32의 역할

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ABSTRACT

The Roles of Connexin32 on Cell Proliferation during Gastric Carcinogenesis

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Gap junctional channels are present at cell-cell contact area. Cell communication through gap junctions (GJs) regulates basic processes such as cell growth and differentiation, and maintains

homeostasis. Gap junction intercellular communication (GJIC) reflects the flux of small (< 1 kDa) and hydrophilic molecules, such as glucose, glutamate, and adenosine trisphosphate (ATP), as well as ions, through these channels via passive diffusion. Six connexins cluster into a connexon, and two connexons dock together to form a gap junction channel. Gap junctions cluster together to form plaques and the gap junction complex is made up of connexins. Individual Cxs are defined and named based on their molecular weight and differ in both function and expression pattern. Abnormality of GJIC is associated with numerous diseases, specifically, the downregulation and dysfunction of GJIC are typical features of various cancers and diseases.

To explore the role of Cx32 in gastric carcinogenesis, we investigated altered expression and localization of Cx32 protein in human gastric cancer, using a tissue array-based approach and *Helicobacter pylori*-induced murine gastric tumors and preneoplastic changes (mucous metaplasia). We examined the relationship between expression of Cx32 and cell proliferation marker Ki67 or cell-cycle regulatory proteins $p21^{Cip1}$ or $p27^{Kip1}$ using immunohistochemistry. Also, the relationship between

expression of Cx32 and that of adhesion protein was investigated. In addition, we examined cell proliferation, cell cycle distribution, and levels of the cell-cycle regulatory proteins $p21^{Cip1}$ and $p27^{Kip1}$ after Cx32 overexpression in the human gastric cancer cell line AGS.

In immunohistochemical analyses, the frequency of Cx32 loss of expression was significantly higher in human adenocarcinomas than in normal stomach. As tumor cells were less differentiated, Cx32 expression levels and intercellular and intracytoplasmic staining were also significantly lower. Cx32 expression in foveolar surface cells in the gastric pit was strong, whereas that in basal cells was weak. Deep pyloric glandular cells showed punctuate Cx32 staining in the membranes and/or cytoplasm. In tumor cells this intercellular expression was lost, and Cx32 expression varied according to the differentiation status of tumor cells. Some adenocarcinomas showed mild to moderate Cx32 expression in the cytoplasm as intracytoplasmic dots whereas others showed loss of staining. In mucous metaplasia of the mouse stomach, Cx32 was mainly expressed in the cytoplasm of epithelial cells. An examination of Ki67-positivity in relation to the pattern of Cx32 expression

in human and murine gastric tissue showed that the frequency of Ki67-positive cells was increased as Cx32 localization shifted from a membranous to cytoplasmic pattern, and was further increased with loss of expression. We then investigated the correlation between expression of the typical cell-cycle regulatory proteins $p21^{Cip1}$ or $p27^{Kip1}$ and that of Cx32. As the Cx32 expression changed from normal membranous expression to cytoplasmic expression or was lost, the $p21^{Cip1}$- and $p27^{Kip1}$-positive cell rate decreased (negative staining). The relationship between Cx32 expression and E-cadherin or β-catenin expression showed significant correlations using immunohistochemistry. As Cx32 expression was turning from normal membranous expression to cytoplasmic expression and was eventually lost, the E-cadherin and β-catenin expression also changed from the normal membranous expression to cytoplasmic expression and was eventually lost or showed nuclear expression, respectively. To further examine the direct relationship between Cx32 expression and cell proliferation, AGS cells were transfected with a Cx32 expression plasmid or control vector. Cell proliferation was decreased in AGS cells overexpressing Cx32 compared to AGS cells wild-type or AGS

cells transfected with control vector. The percentage of G_1-phase cells was significantly greater and that of S-phase was less in AGS cells overexpressing Cx32 vector than in AGS cells wild-type or AGS cells transfected with control vector. Real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses revealed $p21^{Cip1}$ and $p27^{Kip1}$ levels were greater in AGS cells overexpressing Cx32 vector compared to control groups.

In conclusion, our immunohistochemical analysis demonstrated that the altered Cx32 expression, specifically the loss of Cx32 expression and the gain of intracytoplasmic Cx32, was observed not only in adenocarcinoma and adenoma but also in mucous metaplasia. And we found a correlation between Cx32 expression pattern and cell proliferation. Our *in vitro* study of the effects of Cx32 overexpression showed that Cx32 inhibited the proliferation of gastric cancer cells through cell cycle arrest and upregulation of $p21^{Cip1}$ and $p27^{Kip1}$. Together, these results suggest that Cx32 play an important role in gastric carcinogenesis.

Keywords : connexin32, cancer, cell proliferation, gap junction intercellular communication, stomach, AGS cell

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ABBREVIATIONS

ADP : adenosine di-phosphate

ATP : adenosine tri-phosphate

BrdU : bromodeoxyuridine

cAMP : cyclic adenosine monophosphate

cdc25A : cell division cycle 25 homolog A

CDK : cyclin-dependent kinase

Cx : connexin

ER : endoplasmic reticulum

ERAD : ER-associated-degradation

ERGIC : ER-Golgi intermediate compartment

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

GFP : green fluorescence protein

GJ : gap junction

GJIC : gap junctional intercellular communication

HCC : hepatocellular carcinoma

Her-2 : human epidermal growth factor receptor-2

H&E : hematoxylin and eosin

IL-6 : interleukin-6

MCP-1 : monocyte chemotactic protein-1

MLN : metastases to lymph nodes

mRNA : messenger ribonucleic acid

$p21^{Cip1}$: cyclin-dependent kinase inhibitor $p21^{Cip1}$

$p27^{Kip1}$: cyclin-dependent kinase inhibitor $p27^{Kip1}$

PT : primary tumor

PAS : periodic acid Schiff

PBS : phosphate-buffered saline

PKC : protein kinase C

RT-PCR : reverse transcription-polymerase chain reaction

Skp2 : S-phase kinase associated protein-2

TCF : T-cell factor

TGN : *trans*-Golgi network

TPA : 12-O-tetradecanoylphorbol- 13-acetate

Tr-Cx43 : truncated carboxy-terminal part of Cx43

Ub : ubiquitination

LITERATURE REVIEW

Introduction

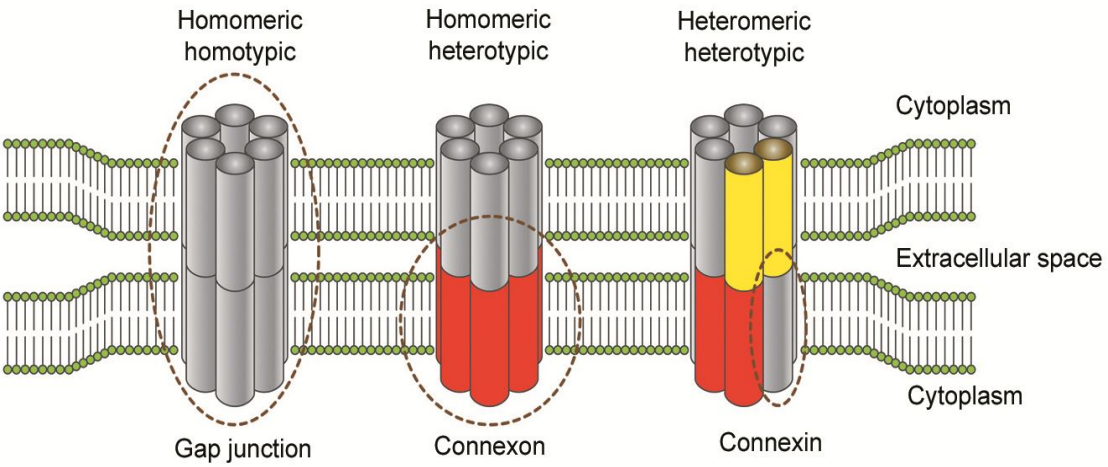
Gap junctions (GJs) are classically defined as clusters of a few to hundreds of tightly packed intercellular channels that function, in the simplest assessment, to allow small the exchanges of molecules between adjoining cells (Alexander and Goldberg 2003). This exchange forms a critical system for intercellular communication in cell and tissue biology and GJs are found in nearly every mammalian cell type (Goodenough, Goliger and Paul 1996, Saez et al. 2003). In vertebrates, GJs are formed largely by the connexin (Cx) family of proteins. The Cxs oligomerize to form a hexameric hemichannel termed a connexon, and two connexons dock in opposing membranes to form the GJ channel (Text figure 1) (Kumar and Gilula 1996, Wei, Xu and Lo 2004). In mammals, Cxs are encoded by at least 20 different genes, which exhibit tissue and cell-type specificity, but also show overlapping patterns of expression (Goodenough et al. 1996, Willecke et al. 2002, Laird 2006). All Cx subtypes share a common structure, consisting of four trans-membrane domains with two extracellular loops, an

intracellular loop, and the cytoplasmic C− and N−termini. Identical or different Cxs can oligomerize to form homomeric or heteromeric connexons, respectively. The channels assembled from Cx family members serve a common purpose of allowing the intercellular exchange of small metabolites, second messengers and electrical signals, but the diversity of function of channels is attributed to a subset of Cxs that are expressed in specific cell types (White 2003). Not all channels are the same, although they share the property of excluding molecules that exceed 1 kDa in size (Bennett and Verselis 1992, Moreno 2004, Bukauskas and Verselis 2004). Importantly, small molecules that differ in size, shape, and charge can be included or excluded from passage through distinctly different GJ channel subtypes, which result in a wide variety of transjunctional selectivity for specific molecules (Bukauskas and Verselis 2004, Goldberg et al. 1998, Goldberg, Lampe and Nicholson 1999). Collectively, secondary messengers and small metabolites are recognized as be the molecular constituents that directly pass from one cell to another through these channels. Important transjunctional molecules include cyclic adenosine mono−phosphates (cAMP), inositol triphosphate,

adenosine, adenosine di-phosphate (ADP), and adenosine tri-phosphate (ATP), to name only a few (Goldberg, Valiunas and Brink 2004, Goldberg et al. 1998, Goldberg et al. 1999). The intermixing of Cx subunits within the same channel becomes even more important as we attempt to understand the mechanisms associated with diseases linked to autosomal recessive and dominant mutations of Cxs.

Given the large number of Cxs, it is not surprising to learn that their cellular and tissue distribution is overlapping, yet distinct. Three fundamental principles come to the forefront: First, many tissues and cell types express two or more members of the Cx family. For example, keratinocytes express at least Cx26, Cx30, Cx30.3, Cx31, Cx31.1, and Cx43 (Salomon et al. 1994, Wiszniewski et al. 2000, Kretz et al. 2003, Goliger and Paul 1994, Di et al. 2005). Likewise, cardiomyocytes express Cx40, Cx43, and Cx45 (Moreno 2004, Beyer et al. 1995, Gros and Jongsma 1996) and hepatocytes express Cx26 and Cx32 (Paul 1986, Zhang and Nicholson 1989, Hennemann et al. 1992, Zhang and Nicholson 1994, Laird 2006). Collectively, co-expression of multiple Cx family members within the same cell type allows for possible

compensatory mechanisms to overcome the loss or mutation of one Cx family member (Hombach et al. 2004, Kelsell et al. 1997). However, a second principle to note from the Cx distribution patterns is that even though two or more Cxs may be co-expressed in the same cell, the resulting channels that form cannot always compensate for the loss or mutation of a Cx family member (Richard et al. 2002, Richard et al. 2004, White 2003, Laird 2006). The third notable observation, from the examination of Cx expression patterns in mammals, is that the most ubiquitously expressed Cx is Cx43. The Cx43 is now known to be endogenously expressed in at least 35 distinct tissues encompassing over 35 cell types, including cardiomyocytes, keratinocytes, astrocytes, and smooth-muscle cells, among many others (Richard et al. 2002, Dahl et al. 1995, Saitoh et al. 1997, Darrow et al. 1995, Kamibayashi et al. 1993, Nagy et al. 1999, Laird 2006).



Text figure 1. From connexin (Cx) to gap junction complex.

Six Cxs (Cx is represented as a cylinder) cluster into a connexon, and two connexons dock together to form a gap junction channel. Many gap junctions cluster together to form plaques. The gap junction complex made up of Cxs and their associated proteins is shown. (Source: Kidney international 2008 73, 547–555)

Life Cycle of Connexins

Remarkably, Cx proteins have a short half-life of only a few hours. This short lifespan has been well documented (Laird, Puranam and Revel 1991, Fallon and Goodenough 1981), and confirms that Cxs are pre-programmed for continuous biosynthesis and degradation. Some physiological changes may not demand dramatic changes in GJ status. However, the general ability of cells to govern their overall level of GJIC through alteration of expression levels, coupled to assembly and degradation events allows for an exquisite level of regulation that extends beyond the rapid channel opening and closing events associated with channel gating (Bukauskas and Verselis 2004, Harris 2001).

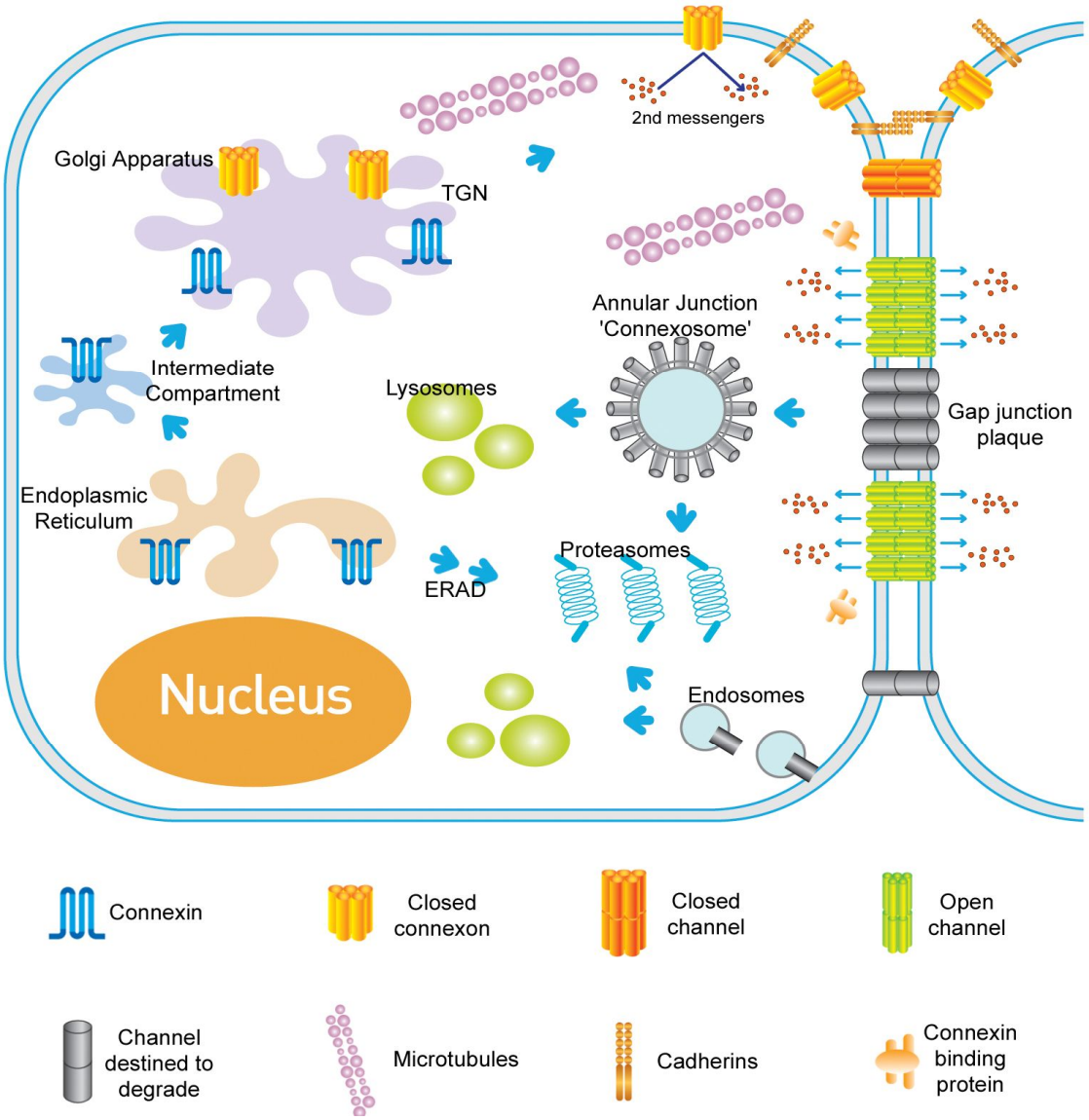
Similar to other classical integral membrane proteins, Cxs are thought to co-translationally thread into the endoplasmic reticulum (ER) via the translocon and encoded start and stop transfer sequences (Falk and Gilula 1998, Laird 2006). The Cx oligomerization then occurs during the residence time within the ER (Ahmad et al. 1999, Falk and Gilula 1998) (Text figure 2). Upon exiting the ER, properly folded Cxs are passed through the ER-Golgi intermediate compartment (ERGIC) prior to

entering the *cis*-Golgi network. For at least some members of the Cx family, complete oligomerization is delayed until the Cx passes through the intermediate compartment and reaches the distal elements of the Golgi apparatus, namely the *trans*-Golgi network (TGN) (Musil and Goodenough 1993, Koval et al. 1997, Laird 2006, Lauf et al. 2002). Cx26 is an exception to this rule, as it can reach the cell surface via a Golgi-independent pathway (Martin, Errington and Evans 2001, Evans et al. 1999). Pleomorphic vesicles and transport intermediates are thought to deliver closed connexons to the cell surface, a process that is facilitated by microtubules (Lauf et al. 2002, Thomas et al. 2005, Jordan et al. 1999). Connexons may function as hemichannels and exchange small molecules with the extracellular environment. Alternatively, they may diffuse laterally in a closed state to sites of cell-cell apposition and dock with connexons from an opposing cell, under the guidance of specific N- and E-cadherin-based adhesion events (Laird 2006, Wei et al. 2005). Recent studies have suggested that N-cadherin and Cx43 in fact may co-assemble, which suggests the occurrence of direct cross-talk between adherens junctions and GJs (Wei et al. 2005). In conjunction with cadherin-based

cell adhesion, GJ channels cluster into plaques and then open and exchange secondary messengers. New GJ channels are recruited to the margins of GJ plaques, while older channels are found in the center of the plaques (Laird 2006, Gaietta et al. 2002). One interpretation of these findings is that the older channels in the middle of a plaque are destined for internalization and degradation. Several Cx-binding proteins have been identified, and one or more of these binding proteins likely regulates plaque formation and stability, possibly by acting as a scaffold for cytoskeletal elements. GJ plaques and fragments of GJ plaques are internalized into one of two adjacent cells as a double-membrane structure commonly referred to as an annular junction. These structures represent the products of one cell internalizing either the entire GJ or a fragment of it (Severs et al. 1989, Sasaki and Garant 1986). Since the term 'annular junction' is descriptive in nature and does not implicitly refer to these structures originating from GJs, these structures have been renamed connexosomes (Laird 2006). This new nomenclature was validated by a study that used fluorescent-tagged Cxs. Anti-Cx43 antibody microinjection studies, together with live imaging of green

fluorescence protein (GFP)-tagged Cx43, revealed that the origin of annular junctions was in fact from pre-existing GJ plaques at cell-cell interfaces (Jordan et al. 2001). Other pathways for Cx internalization may exist, where connexons disassemble and enter the cell by classical endocytic pathways (Fujimoto et al. 1997). Proteasomes are involved either directly or indirectly in regulation of Cx degradation (Laing and Beyer 1995, Leithe and Rivedal 2004a, Leithe and Rivedal 2004b), as compelling evidence has been reported for a delay in the degradation of Cx43 in the presence of proteasome inhibitors (Laing and Beyer 1995). However, other drug-based studies convincingly argue for degradation of Cxs in lysosomes (Leithe and Rivedal 2004a, Leithe and Rivedal 2004b, Laird 2006). This apparent discrepancy may be resolved by the observation that a subpopulation of Cx43 and Cx32 can apparently be reverse-translocated from the ER into the cytosol in proteasomal-inhibitor-treated cells, a process that is inhibited by cytosolic stress (VanSlyke and Musil 2002). Consequently, a scenario can be envisioned whereby proteasomes are responsible for ER-associated-degradation (ERAD), whereas lysosomes only degrade those Cxs that cycle through the plasma membrane

(Laird 2006).



Text figure 2. Life cycle of a connexin.

Connexins are typically co-translationally inserted into the endoplasmic reticulum (ER). If properly folded, Cxs are in all

likelihood spared from ER-associated-degradation (ERAD), whereas in other cases they may be targeted for ERAD. Cxs pass through the Golgi apparatus. On exiting the trans-Golgi network (TGN), Cxs enter a variety of transport intermediates of different sizes and shapes that are used for delivery of the Cx cargo to the cell surface. Cx transport is mediated in part by microtubules. Once inserted into the plasma membranes, connexons freely diffuse within the lipid bilayer and dock with connexons from adjacent cells to form gap-junction (GJ) channels. The clustering of individual GJ channels appears to be a continually active and dynamic process. GJs formed from the outer margins of the plaques, while older channels become localized at the center of the plaques. After old and new channels are distinctly separated within the GJ plaque, double-membrane vesicular structures termed 'annular junctions' can be identified; these are now referred connexosomes. Internalized GJs are targeted for degradation in lysosomes, although some evidence suggests a role for proteasomal degradation. (Source: Biochem J 2006 394, 527-543)

Connexins and Cancer

The hypothesis, that GJIC is involved in cancer and more generally in the control of growth in an old and recurrent story. This assumption is almost as old as the first description of GJs in the 1960s, and was described much before the molecular structure of GJs was known. The hypothesis was first proposed by W. Loewenstein and his colleagues, who reported that the cancer cells they were studying did not communicate through GJs. What they observed was an apparent lack of ionic coupling between hepatoma cells derived from chemically induced liver tumors of rats or between transplanted human hepatoma cells (Loewenstein and Kanno, 1966, 1967). This lack of cell coupling was also observed in thyroid tumors in rodents and in carcinoma of the stomach in humans (Jamakosmanovic and Loewenstein, 1968, Kanno and Mitsui, 1968). The molecules expected to pass through GJs could also inhibit the proliferation of cells transformed by the polyoma virus (Stoker, 1964). All of these observations were supported a general hypothesis for a lack of GJs in cancers.

In fact, loss of Cxs and GJIC is common marker of malignancy. Cx43, the best-known Cx, shows diminished expression in

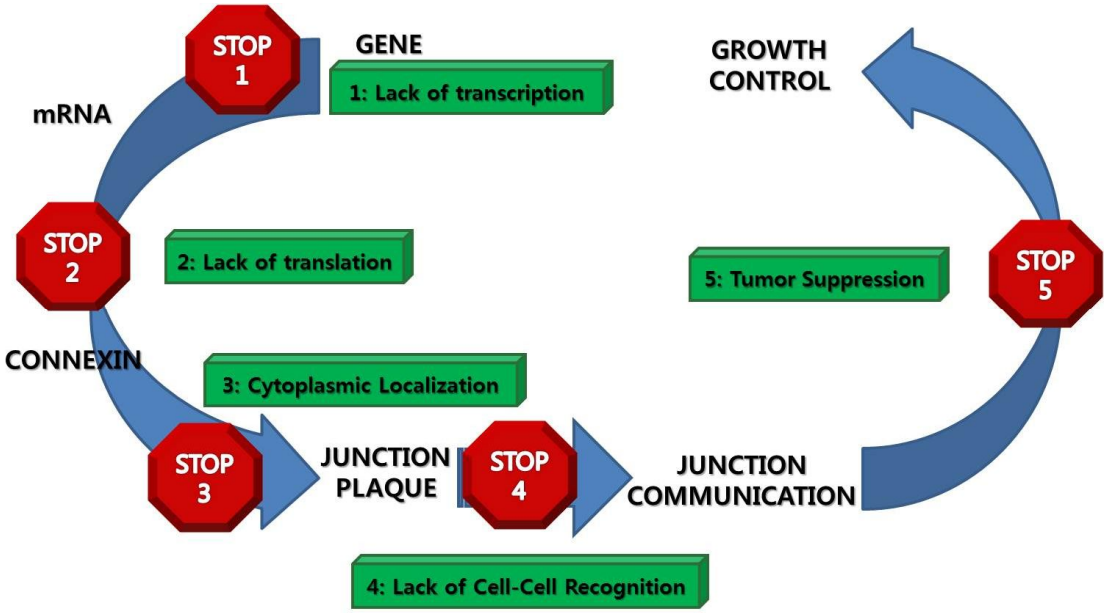
human tumors when compared with adjacent normal tissues, such as breast and prostate (Laird et al. 1999, Tsai et al. 1996). The general observation is that Cxs and GJIC are lost in cancer cells, which suggests that GJICs play a tumor-suppressing role (Mesnil et al. 2005). Multiple tumor-promoting agents, such as 12-O-tetradecanoylphorbol- 13-acetate (TPA) or cigarette components, and oncogenes such as Ras and Src, have long been known to regulate Cx expression and phosphorylation and to inhibit GJIC (Brissette et al. 1991, Leithe and Rivedal 2004a, Tai et al. 2007, Peterson-Roth, Brdlik and Glazer 2009). In contrast, restoration of GJIC and Cx43 in cancer cells has been shown to inhibit their growth (Fernstrom et al. 2002, Zhang et al. 2001, Xu et al. 2008). Extensive studies have reported that a loss, or at least a diminution, of coupling capacity is common between cancer cells and their surrounding normal counterparts (Text figure 3).

In addition to their potential role in tumorigenesis, Cxs are also thought to be involved in tumor metastasis, although the data are equivocal. The metastatic process involves multiple steps that include cell migration, invasion, angiogenesis, intravasation, and extravasation. Emerging evidence suggests

that GJIC and Cxs contribute to cell adhesion, migration, and metastasis of breast cancer. Overexpression of Cx43 is also indicated to affect angiogenesis in both *in vitro* and in vivo studies, through regulation of various angiogenesis-linked proteins such as the Monocyte chemotactic protein-1 (MCP-1) and interleukin-6(IL-6), which inhibit *in vitro* endothelial cell tubulogenesis and migration (McLachlan et al. 2006). Metastatic homing of cancer cells to the lung appears to be facilitated by GJIC through Cx43 expression and increased adhesion to endothelial cells (Elzarrad et al. 2008). Paradoxically, Cxs have been suggested to contribute to metastasis of breast cancer to the lymph nodes. Expression of Cx26 and Cx43 was increased in metastases to lymph nodes (MLNs) when compared with matched primary breast tumors (PTs), while Cx26- and Cx43-negative PTs developed Cx26- and Cx43-positive MLNs (Kanczuga-Koda et al. 2006). Bone marrow studies provide additional evidence that Cxs and GJIC may play different and context-dependent roles in metastasis. Upon entry into the marrow, breast cancer cells reach the endosteum to form Cx43 GJs with bone marrow stroma cells, an event that is partly mediated by the Tac1 gene, a potential

metastatic marker (Moharita et al. 2006).

In summary, Cxs appear to have a dynamic role in the metastatic process, involving multiple factors. A specific series of events leads to metastasis: tumor cells leave the PT, travel to distant sites, and some establish as secondary tumors. One might surmise that the detachment of tumor cells would necessitate the loss of intercellular junctions. In contrast, the other stages or steps in the metastatic process, such as intravasation, endothelial attachment, and vascularization, might be enhanced by or even require increased cell–cell contact. This diversity in roles of Cxs and GJIC in tumor progression demands futher attention (Kandouz and Batist 2010).



Text figure 3. Possible mechanisms leading to an inhibition of gap-junctional intercellular communication (GJIC) capacity in cancer.

Various experimental data have shown that each step leading to the establishment of GJIC can be altered, thereby inducing the loss of junctional communication. Four major events (STOPS) appear to be the cause of a lack of junctional communication (lack of transcription of connexin (Cx) genes; lack of translation of connexin mRNA; lack of membrane addressing leading to an accumulation of Cx proteins in the cytoplasm; and

lack of cell cell recognition preventing the establishment of junctional intercellular communication). Recent data indicate that the reestablishment of GJIC by connexin cDNAs may be not sufficient for inducing cell growth control in cancer cells (STOP 5). In this last case, the expression of specific Cxs seems to be a more critical event than the recovery of junctional communication. In other words, Cxs could control cell growth in a manner that is independent of cell-cell communication. (Source : Antioxidant & Redox Singaling, 2009, 11, 323-338)

Connexins and Aberrant Localization

Some cancers are also characterized by an aberrant localization of Cx proteins (Mesnil et al. 2005, Nakashima et al. 2004). Since GJs are composed exclusively of Cx protein, aberrant localization of Cx protein should logically lead to downregulation of GJIC in the form of ‘loss of function’. However, a few reports have presented immunohistochemical evidence showing that tumors with higher-grade malignancy tend to exhibit a more intense staining of Cx protein in cytoplasm (Krutovskikh et al. 1994, Mehta et al. 1999). In contrast, metastasized tumors, despite the lack of Cx expression in the primary tumor, show restored expression of a certain Cx proteins, but these are localized within the cytoplasm rather than on the plasma membrane (Kanczuga-Koda et al. 2006). Krutovskikh et al. examined 20 surgical samples of human hepatocellular carcinoma (HCC) for the expression pattern of Cx32 protein and found that stronger signals of Cx32 protein were found in the cytoplasm of poorly differentiated HCC than in well differentiated HCC (Krutovskikh et al. 1994). Mehta et al. immunostained 20 primary and 20 metastatic lesions of human prostate cancer, along with normal counterparts, to detect Cx32

and Cx43 proteins (Mehta et al. 1999). While both Cx43 and Cx32 gave punctate signals in cell-cell contact areas of acinar epithelial cells in both normal tissues and well differentiated adenocarcinoma tissues, both Cx proteins were localized in the cytoplasm in poorly-differentiated and undifferentiated carcinoma tissues and no GJ plaques were formed. More interestingly, Kanczuga-Koda et al. clearly showed that both Cx26 and Cx43 proteins were expressed much more frequently in the cytoplasm in tumors that had metastasized to lymph nodes when compared to the primary lesions of human breast cancers (Kanczuga-Koda et al. 2006). More recently, experimental data have shown that altered Cx32 expression specifically the loss of intercellular Cx32 and the gain of intracytoplasmic Cx32 plays an important role in gastric carcinogenesis (Jee et al. 2011).

The overexpression of Cx32 protein in the cytoplasm of human hepatoma cells enhances cell motility and invasiveness and ultimately induces in vivo metastasis without forming GJs (Li et al. 2007). The effects of cytoplasmic localization of Cx32 protein on tumor progression of HCC were assessed by retroviral transduction of human HuH7 HCC cells with the Tet-

off Cx32 construct, which directed overexpression of the Cx32 protein within the cytoplasm. Subcellular fractionation confirmed that no Cx32 protein was present in the cell surface protein fraction of the transformed cells. Interestingly, the population doubling time of the HuH7 Tet-off Cx32 cells was significantly shorter than mock-transformed HuH7 Tet-off cells. To verify whether overexpression of the cytoplasmic Cx32 protein enhanced the in vivo metastatic capability, the cells were xenografted into a subserosal area of the liver of SCID mice. Overexpression of cytoplasmic Cx32 protein induced metastases of the intrahepatic xenografts without enhancing their tumorigenicity (Li et al. 2007).

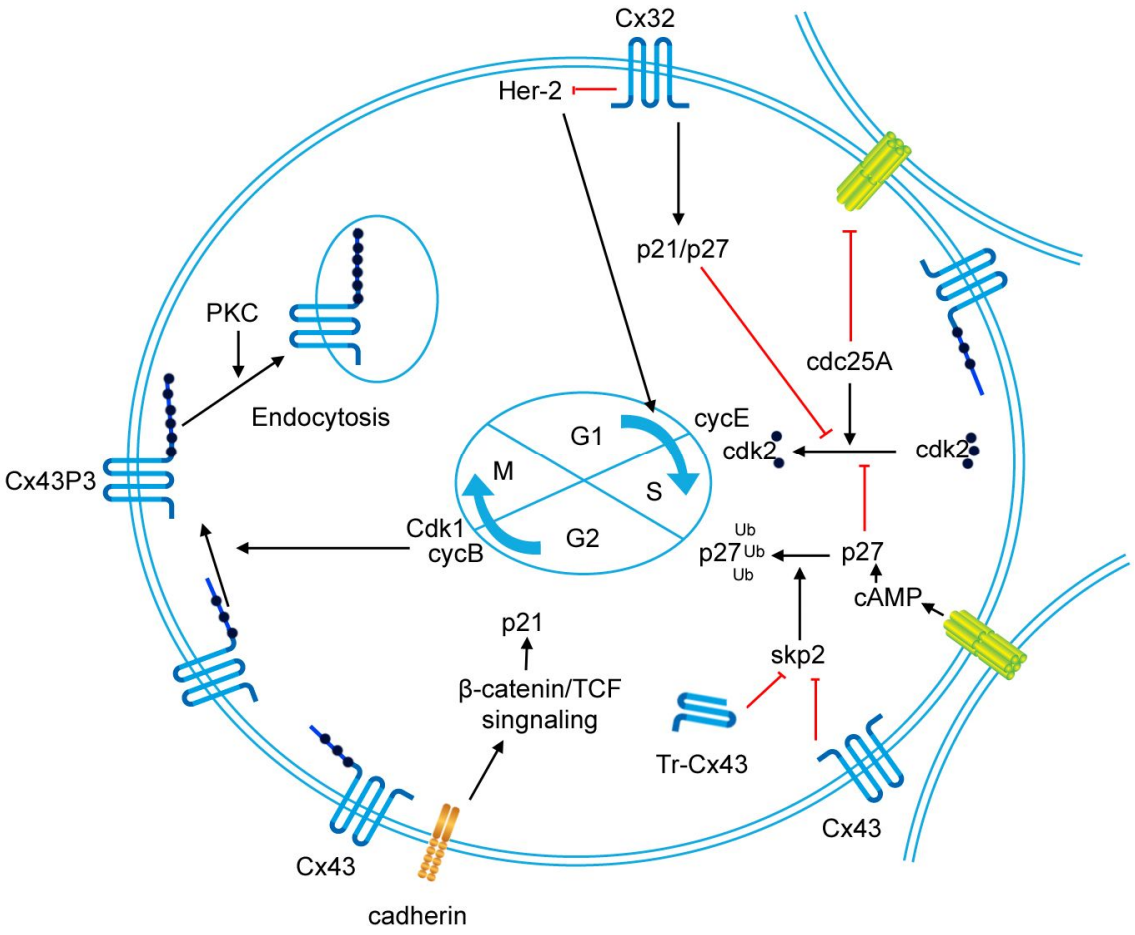
Taken together, these results suggest that translocation of Cx protein from the plasma membranes to the cytoplasm could lead not only to downregulation of GJIC but could also promote tumor progression (Omori et al. 2007).

Connexins and Cell Proliferation

A link between GJs and cell growth has also been established. This has been confirmed by investigations ranging from inhibition of GJIC by carcinogens to decreasing growth by reinduction of GJIC through Cx cDNA transfection or chemical treatment. Overexpression of Cx43 decreased the proliferation of human lung cancer-derived cells (Xu et al. 2008). Transgenic mice also exhibited higher tumor susceptibility when defective for specific Cxs: Cx32-deficient mice tended to develop liver tumors, while lung neoplasia was prevalent in Cx43-deficient mice (Temme et al. 1997, Avanzo et al. 2004).

Any change in cell proliferation is expected to show a related change in cell-cycle gene expression. Consequently, other similar strategies revealed that transfection of Cx could influence expression of genes involved in cell cycle regulation. Interestingly, the proliferation of transfected cells was associated with a decreased expression of cell cycle regulatory genes, such as cyclin A, D1, D2, and cyclin-dependent kinases (CDK) 5 and CDK6. These genes are now established as critical for cell cycle progression, which could explain the increased duration of the G_1- and S-phases observed in the

transfected cells (Chen et al. 1995). A longer G_1 phase was also observed following Cx43 transfection in osteosarcoma cells, probably due to reduced degradation of $p27^{Kip1}$, an inhibitor of CDK activity. Inhibition of CDK, results in accumulation of the hypophosphorylated form of Rb, a characteristic of the G_1 phase (Zhang et al. 2001). A similar effect has been observed after Cx transfection of liver (Cx32) and lung (Cx43) carcinoma cells, where a prolongation of the G1-phase was linked to $p27^{Kip1}$ accumulation and to a decreased amount of cyclin D1 (Koffler et al. 2000). An increased amount of $p27^{Kip1}$ and a decrease of Skp2 (S-phase kinase associated protein-2) were also reported following Cx43 expression (Zhang et al. 2001, Zhang, Nakayama and Morita 2003). The real impact of connexins in cell-growth regulation is not yet understood, even if some molecular process can be drawn (Text figure 4).



Text figure 4. Connexins and cell-growth regulation.

In this cell, molecular events associating connexins and regulation of cell growth are shown. Cell-cycle phases are noted from G_1 to M at the center of the cell (Source : Involvements of connexins in carcinogenesis. In: Connexins: a guide, Humana Press, 2009). ●, phosphorylation of the

carboxy-terminal part of Cx43; cAMP, cyclic adenosine monophosphate; cdc25A, cell division cycle 25 homolog A; Cdk, cyclin-dependent kinase; Cx, connexin; Cx43P3, hyperphosphorylated form of Cx43; cyc, cyclin; Her-2, human epidermal growth factor receptor-2; p21, cyclin-dependent kinase inhibitor $p21^{Cip1}$; p27, cyclin-dependent kinase inhibitor $p27^{Kip1}$; PKC, protein kinase C, skp2, S-phase kinase-associated protein 2; TCF, T-cell factor; Tr-Cx43, truncated carboxy-terminal part of Cx43; Ub, ubiquitination of $p27^{Kip1}$.

Objectives

This study was focused to investigate (1) altered expression and localization of Cx32 in human gastric cancer using a tissue array-based approach, *Helicobacter pylori*-induced murine gastric tumors, and murine preneoplastic changes (mucous metaplasia), (2) the relationship between Cx32 and cell proliferation marker Ki67 or cell-cycle regulatory proteins $p21^{Cip1}$ or $p27^{Kip1}$ using immunohistochemistry in gastric tissues, (3) and the relationship between adhesion proteins E-cadherin or β-catenin expression and Cx32 expression, using immunohistochemistry in gastric tissues. (4) And we examined cell proliferation, cell cycle distribution, and levels of the cell cycle-regulatory proteins $p21^{Cip1}$ and $p27^{Kip1}$ after Cx32 overexpression in the human gastric cancer cell line AGS.

CHAPTER I.

Altered expression and localization of connexin32 in human and murine gastric carcinogenesis

ABSTRACT

Intercellular communication via gap junctions, composed of protein subuints called connexins (Cxs), plays a key role in controlling cell growth, differentiation and carcinogenesis. Impaired gap junctional intercellular communication has been reported in various cancers and diseases. We investigated Cx32 expression patterns and semiquantitatively assessed Cx32 expression in cancers and preneoplastic lesions. To determine if cell proliferation is correlated with Cx32 expression, we evaluated Ki67 expression in a gastric cancer mouse model. In human and mouse normal stomach and gastric adenocarcinoma tissues were used for immunohistochemical analyses. Cx32 was detected at cell–cell (intercellular) contact points in normal cells and exhibited punctate intercellular and intracytoplasmic staining in cancer cells. The frequency of Cx32 loss of expression was significantly higher in human adenocarcinomas than in normal stomach. As tumor cells were less differentiated, Cx32 expression levels and intercellular and intracytoplasmic staining were also significantly lower. The Cx32 expression pattern in the mouse gastric cancer model was similar in

several important respects to that of human. In mucous metaplasia of the mouse stomach, Cx32 was mainly expressed in the cytoplasm of epithelial cells. There was also an inverse correlation between Cx32 expression and cell proliferation in mouse tumors. However, there was no difference in the levels of Cx32 mRNA between normal and cancerous tissues. These findings suggest that altered Cx32 expression, a loss of intercellular Cx32 and a gain of intracytoplasmic Cx32 in the form of punctuate "dot", plays an important role in the formation of gastric adenocarcinomas.

INTRODUCTION

Cell communication through gap junctions regulates basic processes such as cell growth and differentiation, and maintains homeostasis (Loewenstein and Rose 1992, Vinken et al. 2008). Gap junction intercellular communication (GJIC) reflects the flux of small (< 1 kDa) and hydrophilic molecules, such as glucose, glutamate, and adenosine trisphosphate (ATP), as well as ions, through these channels via passive diffusion; in contrast large polypeptides and nucleic acids are excluded (Vinken et al. 2008, Alexander and Goldberg 2003). Gap junctional channels are composed of two connexon complexs in adjacent cells, each of which is composed of six connexin (Cx) proteins. Individual Cxs are defined and named based on their molecular weight and differ in both function and expression patterns (Vinken et al. 2008, Willecke et al. 2002).

Cxs have been previously reported in various types of malignant tumors, including breast, liver, colon and esophageal tumors (Monaghan et al. 1996, Conklin et al. 2007, Nakashima et al. 2004, Kanczuga-Koda et al. 2005b, Hong and Lim 2008, Inose et al. 2009). Decrease or loss of Cxs expression has also

been reported (Jamieson et al. 1998, Conklin et al. 2007). In normal human breast tissue, Cx26 is localized to the membranes of luminal cells, whereas epithelial cells of benign lesions and lobular carcinoma do not express Cx26 (Jamieson et al. 1998, Monaghan et al. 1996). In addition, Conklin *et al.* reported that Cx43 is downregulated at various stages of breast cancer progression, including ductal carcinomas in situ, infiltrating ductal carcinoma and infiltrating lobular carcinomas (Conklin et al. 2007). Although some reports have implicated reduced or absent Cx expression in cancer pregression, others have suggested that altered subcellular localization of Cx32, specifically, intracytoplasmic expression, is involved (Nakashima et al. 2004, Hong and Lim 2008, Kanczuga-Koda et al. 2005b). Generally Cx is distributed primarily along membranes of normal cells, but in hepatocellular carcinoma Cx32 is mostly detected in both the cytoplasm and on the cell membranes of tumor cells (Nakashima et al. 2004). In colorectal cancers, Cx26-positive adenocarcinoma cells exhibit primarily cytoplasmic Cx26 (Kanczuga-Koda et al. 2005b, Hong and Lim 2008). There is some evidence from experimental models that Cx is reduced or absent in tumors. In

the mouse, real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses have shown that Cx26, Cx32, Cx40 and/or Cx46 levels are decreased in urethane or 4-nitroquinoline 1-oxide-induced lung tumors compared to normal lung (Avanzo et al. 2006, Udaka, Miyagi and Ito 2007).

Gastric adenocarcinoma is one of the most prevalent malignant neoplasms in the world, particularly in Eastern Asia (Parkin, Laara and Muir 1988). The development of gastric cancer is believed to occur over a long period of time and involve a number of events subsequent to exposure to carcinogens and/or *Helicobacter pylori* (Correa and Houghton 2007, Correa 1992). In humans, chronic infection with *H. pylori* generally increases the risk of gastric carcinoma by five- to six-fold. *H. pylori* causes chronic gastritis and duodenal and gastric ulcer, and is linked gastric cancer and lymphoma (Marshall 2003).

Cx26 and Cx32 are present in normal gastric glands, but their expression patterns are different. Cx32 is mainly found along the membranes of foveolar cells and Cx26 is predominantly found within the cytoplasm of parietal cells (Fink et al. 2006,

Radebold et al. 2001, Uchida et al. 1995). In an investigation of Cx32 expression in gastritis, intestinal metaplasia and gastric carcinoma, Uchida et al. showed that Cx32 expression was significantly reduced in epithelial cells with gastritis and intestinal metaplasia compared to normal gastric mucosa, and was undetectable in carcinoma cells (Uchida et al. 1995). However, these studies were limited by the relatively small number of cases investigated. Moreover, the potential association between altered GJIC and gastric adenocarcinoma has not yet been clearly defined. In the present study, we investigated altered patterns of Cx32 expression in human gastric cancer using a tissue array–based approach. As a comparison, we also evaluated Cx32 expression patterns in *H. pylori*–induced gastric tumors. Preneoplastic changes (mucous metaplasia) and correlations between the index of Cx32 expression and the cell proliferation index were also investigated.

MATERIALS AND METHODS

Preparation of tissue-arrayed human gastric cancers

Tissue-arrayed slides containing gastric adenocarcinomas (n = 97) and normal gastric tissues (n = 60) were purchased from SuperBioChips Laboratories (Seoul, Korea, www.tissue-array.com) and ISU ABXIS Co., Ltd. (Seoul, Korea, tissuearray.pentagen.com). These samples were collected by biopsy or during the partial or total gastrostomy of gastric cancer patients. Each tissue array spot was classified based on the degree of tumor cells differentiation of the adenocarcinomas, 16 were well differentiated, 16 were moderately differentiated, 37 were poorly differentiated, and 4 were mucinous; there were also 24 signet ring cell carcinomas. Tumors were also classified according to the Lauren's and TNM classification schemes. Briefly, adenocarcinomas were classified into intestinal and diffuse types according to Lauren's classification, and were further divided into non-invasive or invasive (invading the lamina propria, submucosa, muscularis propria, subserosa, serosa or adjacent structures), and non-metastatic or

metastasized (to regional lymph nodes or distant organs) (American Joint Committee on Cancer 2002).

Mice and experimental design

The experimental protocol for mouse gastric cancer induction was as described previously (Han et al. 2002). Male C57BL/6J mice (5 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). The mice were housed five per cage in a clean rack maintained at room temperature (22° C-26° C) under a 12-h light/dark illumination cycle. Mice were given the 200 ppm N-methyl-N-nitrosourea (Sigma Chemical Co., St. Louis, MO, USA) solutions *ad libitum* in light-shielded bottles every other week for 10 weeks and then inoculated with mouse-adapted *H. pylori* (SS1) three times every other day for six days. After fasting for 24 hours, mice were administered a 0.1-mL suspension of *H. pylori* containing $1x10^9$ colony-forming units (CFU)/mL (OD_{450} 2.4). The mice were sacrificed 38 weeks after the last *H. pylori* infection. This experiment was approved by the Institutional Animal Care and Use committee of Seoul National University (SNU-060112-1

and SNU-060507-1) and was carried out in accordance with institutional guidelines.

Histopathologic examination

Immediately after sacrifice, mouse stomachs were opened along the greater curvature. The stomachs were fixed in neutral-buffered 10% formalin and processed by standard methods. Tissues were embedded in a low-melting-point paraffin, sectioned into 4 μm slices, and stained with hematoxylin and eosin (H&E).

Mucin-containing cells were detected by periodic Acid Schiff (PAS) staining of replicate serial paraffin sections.

Immunohistochemical staining for Cx32 and Ki67

Cx32 and Ki67 expressions in mouse and human gastric tissues was detected by immunohistochemistry using mouse anti-rat Cx32 (Chemicon International Inc., Temecula, CA, USA) and rabbit anti-human Ki67 (Novocastra, Newcastle, UK) antibodies. Immunoreactive proteins were detected using a MOM immunodetection kit (Vector Laboratories, Burlingame,

CA, USA) or a Universal DakoCytomation LSAB+ kit (DakoCytomation, Carpinteria, CA, USA) in mouse gastric tissues as described by the manufacturers. Briefly, after dewaxing and rehydrating, the sections were subjected to antigen retrieval using an electric Pressure Cooker (Cell Marque, Rocklin, CA, USA), and endogenous peroxidase activity was quenched with hydrogen peroxide. The slides were then washed with phosphate-buffered saline (PBS) and blocked using the blocking serum provided in the staining kit. Thereafter, slides were incubated overnight at 4° C with primary anti-Cx32 (1:200) or anti-Ki67 (1:1000) antibody, followed by incubation with biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin. 3,3' -Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) was used as the chromogen. The sections were counterstained with Mayer's hematoxylin (DakoCytomation, Carpinteria, CA, USA) and examined under a light microscope. In human gastric tissues, Cx32 staining was performed using the BOND-MAX automated immunostainer (Leica Microsystems, Wetzlar, Germany) with bond polymer refine detection kit (Leica Microsystems, New York City, NY, USA)

as described by the manufacturers.

Immunohistochemistry scoring and analysis

A scoring system was established such that tumor cells in which Cx32 staining was detected in 10% or fewer cells were defined as negative and those in which staining was detected in more than 10% of cells were defined as positive (Kim et al. 2010, Madore et al. 2010). Positively stained tissues were categorized based on localization of Cx32 expression: cell membrane and/or cytoplasm. The intensity of Cx32 staining in human and mouse stomach tissues was scored semiquantitatively based on the percentage of stained cells and their intensities of staining, as described previously (Grizzle WE 1998). Staining intensity was rated on a 0–to–3 scale: 0, negative; 1, weak positive staining; 2, moderate positive staining; 3, strong positive staining. The final semiquantitative score was obtained by multiplying the percentage of stained cells. Thus, a score of 1 was obtained if 100% of cells stained with an intensity of +1 or if 50% of cells stained with an intensity of +2. For example, if 10% of tumor cells stained at 0,

10% of cells stained at +1, 30% of cells stained at +2, and 50% of cells stained at +3, the total score would be 2.2. All samples were evaluated and scored without knowledge of sample-identifying information.

RNA isolation and quantitative real-time RT-PCR

A quantitative messenger ribonucleic acid (mRNA) expression analysis of target genes was performed using a real-time RT-PCR system. Total RNA was extracted from mouse gastric tissue with TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The extracted RNA sample (500 μg) was reversed transcribed using an ImProm-II^{TM} reverse transcription system kit (Promega, Madison, WI, USA) and the following reaction conditions: 25° C for 5 minutes (annealing), 42° C for 60 minutes (extension), and 70° C for 15 minutes (inactivation). The cDNA was analyzed by real-time PCR using SYBR Green PCR master mix (Applied Biosystems Inc., Foster City, CA, USA) and the following primer pairs: Cx32 forward 5'-ATCTGCTCTACCCCGGCTATG-3', reverse 5'-

GCAGGCTGAGCATCGGTC GCT-3' and GAPDH forward 5' -GACCTCAACTACATGGTCTA-3', reverse 5' -ACTCCAC GACATACTCAGCA-3' (Udaka et al. 2007). Targets were amplified and mRNA was quantified using an ABI 7000 sequence detection system and the manufacturer's software (Applied Biosystems Inc., Foster City, CA, USA). The amount of mRNA was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. After it was confirmed that the dynamic ranges for Cx32 (target) and GAPDH (normalizer) amplification were similar, the differences (△Ct) between the Ct values of the target and normalizer were calculated. The △Ct value of normal stomach was chosen as the reference (baseline), and was used to calculate the comparative △△Ct value (difference between tumor sample △Ct and baseline △Ct). △△Ct values were converted to absolute values using the transformation $2^{\triangle\triangle Ct}$.

Statistical analysis

All scores were presented as means ± SEMs. The presence/absence of Cx32 expression was analyzed using the Chi-square test. Intensity scores were analyzed using

Student's t-tests or one-way ANOVAs. The relationship between Ki67 and Cx32 expression was analyzed using linear regressions and Spearman's rho correlation tests. Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). *P*-values less that 0.05 were considered statistically significant.

RESULTS

Cx32 expression in human gastric cancer and normal tissue

To evaluate Cx32 expression and localization in human gastric tumors, we compared gastric carcinoma tissues and normal gastric tissues using tissue-arrayed slides. In the gastric cancer groups, we found a loss of Cx32 protein compared to normal human gastric tissues. Table 1 summarizes the Cx32 protein loss frequencies in normal human gastric tissues and gastric cancer tissues. In tumor tissues, there was a loss of Cx32 expressions in 27.8% (27/97) of samples whereas only 3.33% (2/60) of normal human gastric tissues showed loss of Cx32, a difference that was significant ($P < 0.01$). Cx32 expression was also analyzed in gastric adenocarcinomas subdivided into categories based on WHO and Lauren's classifications. The frequency of Cx32 loss was 0% (0/16) in well-differentiated adenocarcinomas, 6.25% (1/16) in moderately differentiated adenocarcinoma, 29.7% (11/37) in poorly differentiated adenocarcinomas, 25.0% (1/4) in mucinous adenocarcinomas, and 58.3% (14/24) in signet ring

cell carcinomas. Cx32 protein loss frequencies were significantly increased in poorly differentiated adenocarcinomas and signet ring cell carcinomas compared to normal tissues (all $P < 0.05$). The frequency of Cx32 expression was also significantly lower in less-differentiated tumor cells ($P < 0.001$). Among tumor Lauren classifications, the frequency of Cx32 loss was 8.70% (4/46) in intestinal type and 45.1% (23/51) in diffuse type, a difference that was statistically significant ($P < 0.001$).

An evaluation of Cx32 protein localization in normal and gastric cancer tissues revealed that, in normal stomach tissues, Cx32 protein was prominantly localized to cell-cell contact sites (intercellular expression) in the membranes of foveolar cells (Fig. 1A), but was expressed in cell membranes and/or cytoplasm (Fig. 1B). In tumor tissues, the Cx32 protein distribution was altered compared to that in normal cells, and varied according to the differentiation status of tumor cells. Some adenocarcinomas showed mild-to-moderate Cx32 expression in the cytoplasm (Fig. 1C and D), whereas others showed loss of staining in the cell membrane and weak positive staining in the cytoplasm (Fig. 1E). Signet ring cell carcinomas

were negative for Cx32 (Fig.1F).

We then analyzed the expression of Cx32 protein according to intercellular and intracytoplasmic localization (Table 1), and found significant differences between normal tissues and cancerous tissues ($P < 0.01$). Both intercellular and intracytoplasmic expression were significantly lower in well-differentiated, moderately-differentiated, poorly-differentiated, mucinous adenocarcinoma and signet ring cell carcinoma compared to normal tissues respectively (all $P < 0.05$). Intercelluar and intracytoplasmic expression of Cx32 was also significantly lower in less-differentiated tumor cells ($P < 0.05$), and was eventually lost entirely in least-differentiated tumor cells. As a consequence, overall Cx32 expression was significantly lower in less-differentiated tumor cells. We found no significant difference in intercellular expression between intestinal and diffuse tumor types. However, an examination of expression patterns according to tumor invasion status showed a significant loss of total and intercellular Cx32 expression as the tumor invaded from the mucosa to the serosa ($P = 0.005$ and $P = 0.047$ respectively). Consistent with those results, an analysis of staining, intensity showed that Cx32 scores were

significantly higher in normal stomach tissues (2.05 ± 0.07) than in tumor cells (1.27 ± 0.06; $P < 0.01$), and were lower in less differentiated than more differentiated tumor cells ($P < 0.01$).

Table 1. Loss of Cx32 expression and Cx32 cellular localization in normal human gastric tissues and adenocarcinoma

Classification	Loss of Cx32 expression (%)	Cx32 cellular localization	
		Intercellular	Intracytoplasmic
WHO			
Normal (n = 60)	2 (3.33%)†	42 (70.0%)‡	16 (26.7%)
WD (n = 16)	0 (0%)	3 (18.7%)*	13 (81.3%)
MD (n = 16)	1 (6.25%)	4 (25.0%)*	11 (68.8%)
PD (n = 37)	11 (29.7%)*	1 (2.70%)*	25 (67.6%)
Mucinous (n = 4)	1 (25.0%)	0 (0%)*	3 (75.0%)
SRC (n = 24)	14 (58.3%)*	1 (4.17%)*	9 (37.5%)
Lauren			
Intestinal(n = 46)	4 (8.70%)§	8 (17.4%)¶	34 (73.9%)
Diffuse (n = 51)	23 (45.1%)	1 (1.96%)	27 (52.9%)

WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; Mucionus, mucinous adenocarcinoma; SRC, signet ring cell carcinoma. †$P < 0.001$ vs. cancer, ‡$P < 0.01$ vs. cancer, §$P < 0.01$ vs. diffuse type, ¶$P > 0.05$ vs. diffuse type, and *$P < 0.05$ vs. normal.

Fig 1

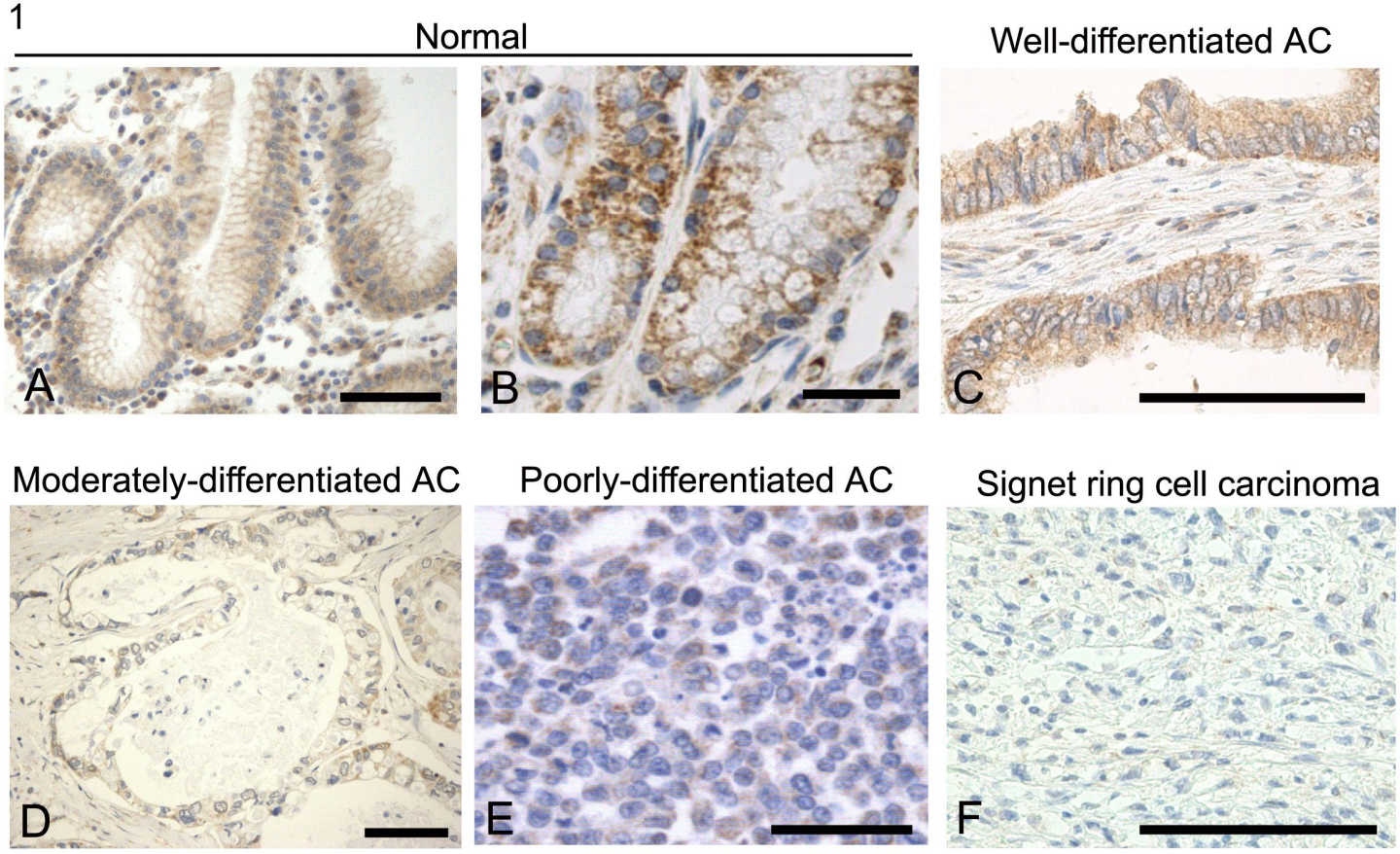

Normal
Well-differentiated AC
A
B
C
Moderately-differentiated AC
Poorly-differentiated AC
Signet ring cell carcinoma
D
E
F

Figure 1. Immunohistochemical detection of Cx32 expression in human tissue-arrayed normal stomach (A and B) and gastric adenocarcinoma (C-F).

Distribution and intensity of Cx32 expression was altered in tumor cells: intercellular expression of Cx32 in foveolar cells (A) and intracytoplasmic expression (B). Cx32 is strongly detected (C), faintly detected (D and E) or absent (F). Bar = 50 μm.

Cx32 expression in mouse *H. pylori* induced gastric tumors

We next analyzed Cx32 protein expression in normal and gastric cancer tissues in the *H. pylori*–induced gastric cancer mouse model. The Cx32 protein loss frequency by group is summarized in Table 2. The Frequency of Cx32 loss was 0% (0/10) in normal stomach, 10% (1/10) in adenoma and 50% (5/10) in adenocarcinoma. Compared to normal tissues, the loss of Cx32 expression in adenocarcinoma was significant (P= 0.033) whereas that for adenomas was not. Cx32 expression decreased significantly as tumor became progressively more malignant, reflecting the transition from normal to adenomas and then to adenocarcinomas (P = 0.013).

To determine if the decrease in total and intercellular Cx32 expression observed in human gastric cancer tissues also occurred in mouse gastric cancer models and to determine of Cx32 subcellular localization, we examined Cx32 expression in normal murine stomach tissues (Fig. 2A and B) and gastric cancer tissues (Fig. 2E and F).

Mouse gastric tumors were diagnosed as adenoma or

adenocarcinoma on the basis of cytologic criteria as well as the presence or absence of invasion to the submucosa. Adenomas consisted of acini and tubules lined with cuboidal or polygonal cells without submucosal invasion. Adenocarcinomas showed invasion into the submucosa, and their tumor cells were arranged in acini, tubule types or solid patterns (Fig. 2E). Neoplastic cells that showed acinar or solid types had high nuclei/cytoplasm ratios and showed frequent mitotic figures. Tumor cells that were arranged in tubules or cysts had abundant cytoplasm, and some formed branching structures (Fig. 2F).

We also investigated the localization of Cx32 expression in normal mouse stomach and tumor tissues. In normal stomachs, the membranes of foveolar cells showed strong Cx32positivity. Cx32 was strongly expressed on the surface of gastric pit, but was not expressed or was expressed weakly in the base area (Fig. 2C). Cx32 staining was observed in the cell membranes and cytoplasm of deep glandular cells of the pylorus (Fig. 2D). In adenomas, epithelial cells arranged in acinar or tubular patterns exhibited strong Cx32 expression in the form of dots in cell membranes and cytoplasm. Strong punctate Cx32

expression was also evident in the cell membranes and cytoplasm of epithelial cells that constituted the branched tubules or cysts of adenocarcinomas, (Fig. 2G). However, Cx32 staining was lost in solid and acinar type cancers (Fig. 2H). We also analyzed the location of Cx32 protein expression in mouse tissues. We found no significant difference in the intercellular expression of Cx32 between adenomas and normal tissues. However, the intercellular expression of Cx32 was significantly decreased in adenocarcinomas compared to normal tissues (P = 0.003). Consistent with this, intercellular Cx32 expression significantly decreased as the tumor progressed to a more malignant form (P = 0.02). An analysis of staining intensity scores in mouse tissues reinforced these findings. There was no significant difference in scores between adenomas (2.33 ± 0.07) and normal tissues (2.42 ± 0.06), but Cx32 intensity scores of adenocarcinoma tissues (0.78 ± 0.09) was decreased compared to normal tissues (P< 0.01). Moreover, Cx32 expression intensity scores decreased as mouse tumor cells became less differentiated (P < 0.01).

Table 2. Cx32 expression in normal murine gastric tissues, adenocarcinoma and mucous metaplasia

Classification	Loss of Cx32 expression (%)	Cx32 cellular localization	
		Intercellular	Intracytoplasmic
Normal (n=10)	0 (0%)	10 (100%)	0 (0%)
Adenoma (n=10)	1 (10%)	8 (80%)	1 (10%)
Adenocarcinoma (n=10)	5 (50%)*	3 (30%)*	2 (20%)
Mucous metaplasia (n=10)	2 (20%)	4 (40%)	4 (40%)

* $P < 0.05$ vs. normal tissue.

Fig 2

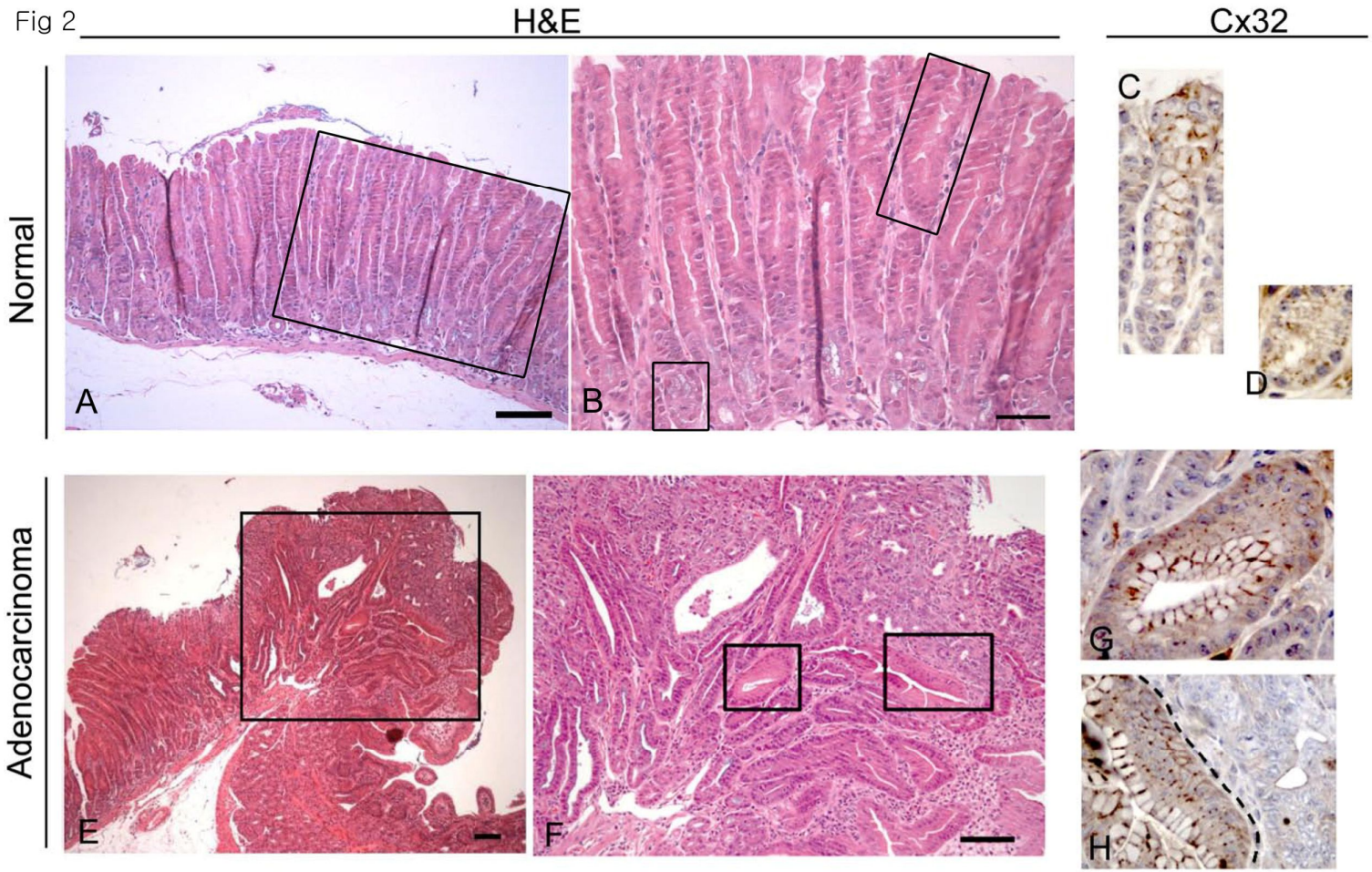


Figure 2. Histology and immunohistochemical detection of Cx32 in pyloric regions of normal mouse stomach (A, B, C and D) and gastric adenocarcinoma (E, F, G and H).

Immunohistochemistry shows that Cx32 expression in surface cells in the gastric pit is strong, whereas that in basal cells is weak (C). Deep pyloric glandular cells show punctuate Cx32 staining in membranes and/or cytoplasm (D). In neoplastic cells arranged in irregularly glandular patterns lined by stratified epithelial cells, both intercellular and intracytoplasmic punctate staining was strongly observed (G). In cancer cells that showed nests or acinar patterns (dashed line), Cx32 is absent (H). Bar = 100 μm.

Cx32 expression in mouse *H. pylori* induced gastric preneoplastic lesions

To examine Cx32 expression in preneoplastic lesions, we analyzed Cx32 in mucous metaplasia of mouse tissues using immunohistochemistry. The frequency of Cx32 loss was 20% (2/10) in mucous metaplasia compared to normal stomach tissues (Table 2). In normal pyloric foveolar cells, Cx32 protein was observed in the form of dots in the lateral aspects of cell membranes (Fig. 3C). However, in mucous metaplasia, Cx32 protein was observed in the form of dots in the base of metaplastic epithelial cell cytoplasm (Fig. 3F and G). Although differences in intercellular expression of Cx32 between normal stomach tissues and mucous cells were insignificant, the Cx32 intensity scores of mucous metaplastic epithelial cells (1.66 ± 0.07) were significantly lower than those of normal stomach tissues (2.42 ± 0.06; $P < 0.01$).

Fig 3

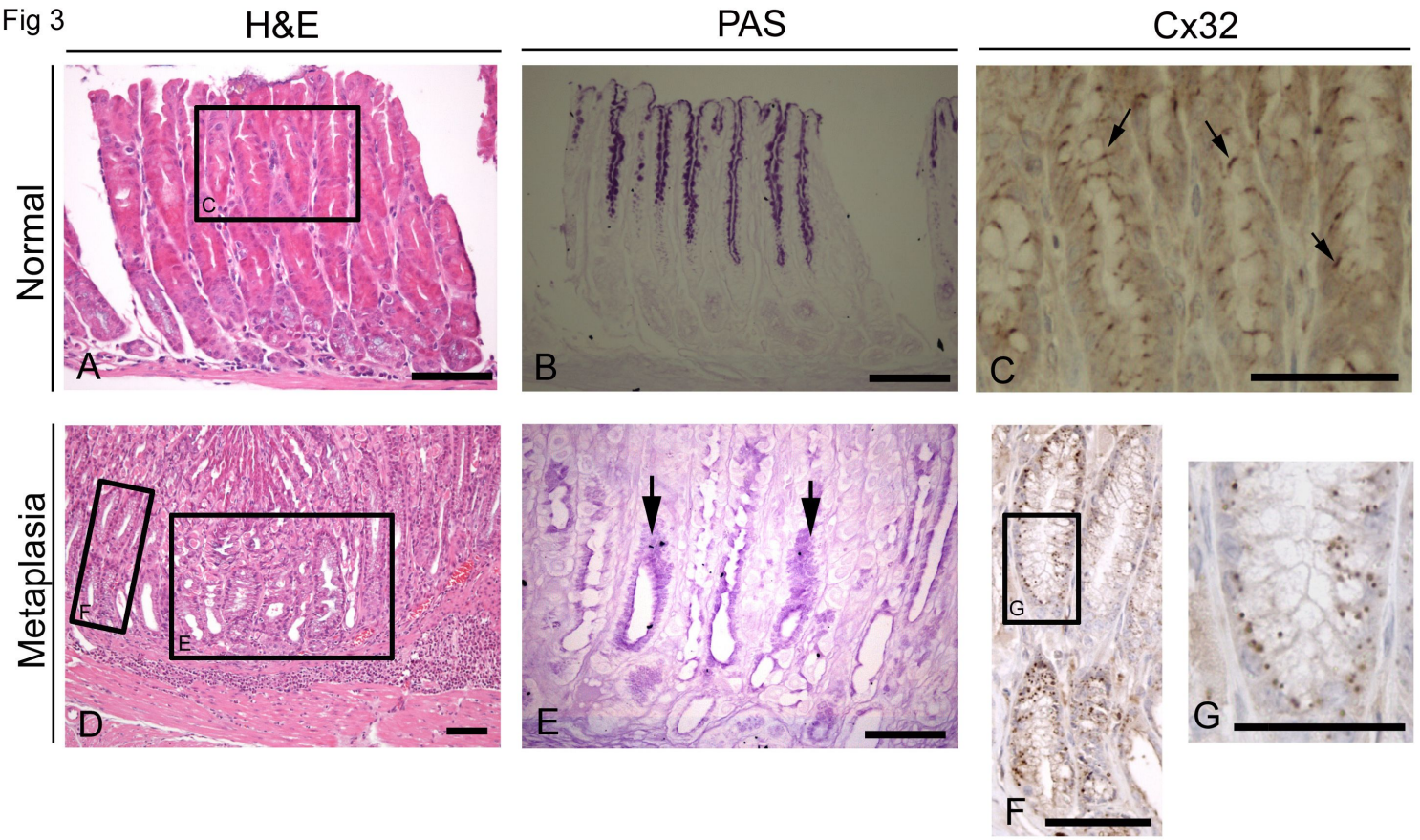


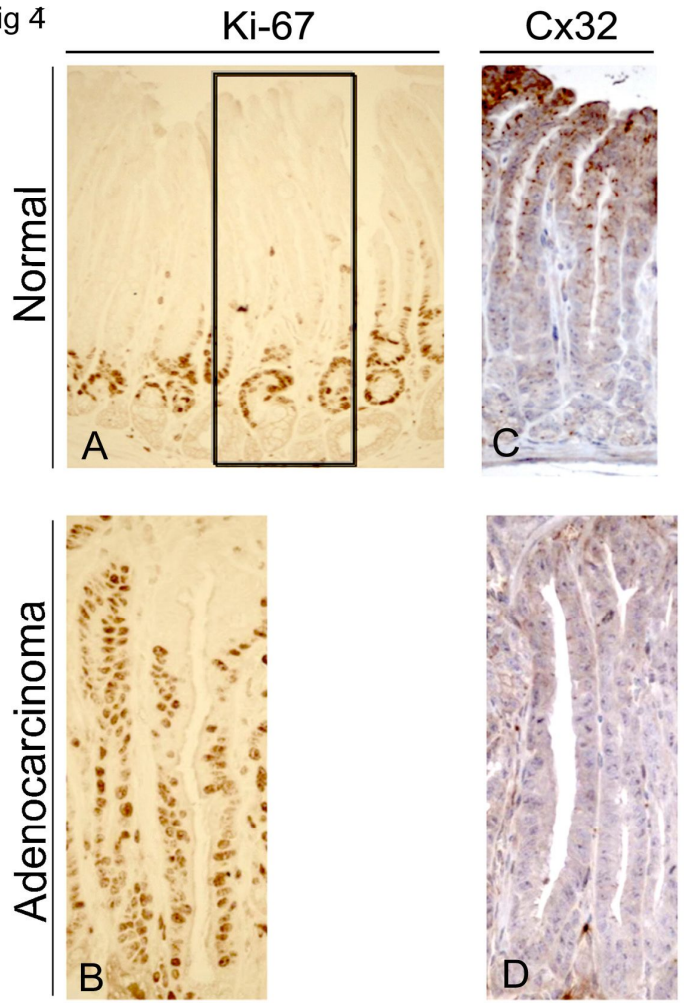
Figure 3. Histology and immunohistochemical detection of Cx32 in normal mouse stomach and preneoplastic lesions.

Normal foveolar epitheliums (A) showed PAS-positive (B) and Cx32 punctate immunostaining in the lateral portion of membrane (C, arrow). Mucous metaplasia showed a loss of parietal cells and foamy changes (D). These mucous cells were positive for neutral mucin based on PAS staining (E, arrow). In mucous metaplasia, Cx32 is detected mainly as dots form in the base of cytoplasm (F); a higer magnification is shown in G. Bar = 50 μm.

Correlation between cell proliferation and Cx32 expression in mouse stomach tissue

Serial sections of tissues were co-immunostained for Cx32 and the proliferative marker Ki67 to examine the relationship between cell proliferation and Cx32 expressions. Ki67 expression was detected in nuclei of the upper-mid portion of the fundus and in nuclei of the sub-basal zone of the pylorus in normal stomach tissues (Fig. 4A). Compared to normal tissues, Ki67-positive cells in adenocarcinomas were increased and irregularly distributed (Fig. 4B). Among Ki67-positive cells, Cx32 expression was negative (Fig. 4C and D). As shown in Fig 4E, a plot of the ratio of Ki67-positive cells against Cx32 intensity reveals an inverse correlation (Kendall $\tau = -0.644$ $p = 0.01$; Spearman $r = -0.697$, $p = 0.03$; linear regression $r^2 = 0.45$, $p = 0.03$). That is the large the number of Ki67-positive cells, the lower the intensity of Cx32 staining. Thus, Cx32-postivity is associated with decreased neoplastic proliferation.

Fig 4


Ki-67
Cx32
Normal
Adenocarcinoma
A
B
C
D

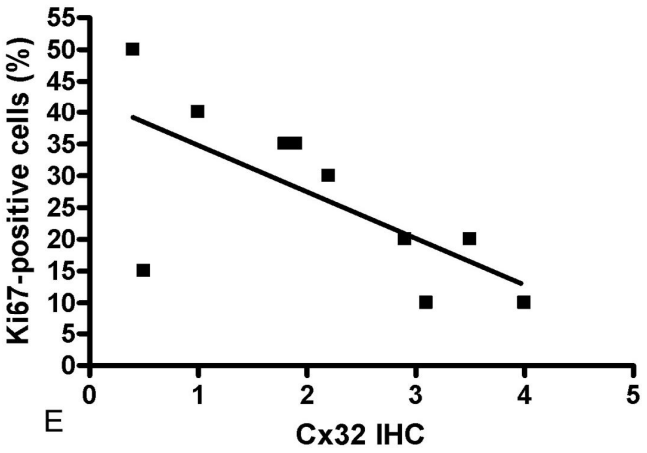

Ki67-positive cells (%)
55
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Cx32 IHC
E

Figure 4. Correlation between Ki-67 and Cx32 expression in mouse tissue.

Ki67-positive cells were found in the basal zone of normal pylorus (A) and were irregularly distributed in adenocarcinoma (B). Foveolar cells stained strongly for Cx32 protein in the cell membrane (C). Reduced intracytoplasmic expressin was observed in adenocarcinoma (D). A tendency for Ki67-positive cells to exhibit no staining for Cx32 is reflected in the inverse correlation between Ki67 staining and Cx32 (E: Kendall $\tau = -0.644$ $p = 0.01$; Spearman $r = -0.697$, $p = 0.03$; linear regression $r^2 = 0.45$, $p = 0.03$).

Semiquantitiative analysis of Cx32 mRNA in mouse stomach tissue

To investigate differences in the levels of Cx32 mRNA between normal murine stomach tissues and gastric cancer tissues, we used real-time RT-PCR as described in Materials and Methods. Our results showed that the relative amount of Cx32 mRNA in normal tissues was 0.99 ± 0.02 (n=6) and that of adenocarcinomas was 0.97 ± 0.01 (n = 5), a difference that was not statistically significant.

DISCCUSION

We have provided evidence of altered expression of Cx32 in human gastric cancers using immunohistochemistry. The results obtained from human gastric adenocarcinomas were similar in many respect to those obtained in the *H. pylori*-induced mouse gastric cancer model. We also investigated Cx32 expression in preneoplastic lesions and related Cx32 expression with cell proliferation.

We found that Cx32 is localized at cell-cell contact areas in normal epithelial cells, whereas in tumor cells, this intercellular expression is lost and Cx32 protein staining is detected as intracytoplasmic dots. Similar to these results, immunohistochemical analysis of Cx expression in hepatocellular carcinoma and colorectal carcinoma showed the presence of Cx protein in the form of dots in both cell membrane and cytoplasm (Nakashima et al. 2004, Kanczuga-Koda et al. 2005b). Our comparative study using mouse gastric tissues also provided convincing evidence of altered Cx32 expression, showing a significant loss of Cx32 expression in adenocarcinomas compared to normal tissues. As human gastric

tumors progressed to more undifferentiated stages, the intercellular and intracytoplasmic expression and intensity of Cx32 decreased significantly, and was eventually lost. These results are consistent with the idea that Cx32 plays an important role in the transformation of glandular epithelial cells and progression of gastric cancers.

Some studies have reported results that diverge from those reported here. Inose *et al.* reported that Cx26 was not detectable in normal esophageal squamous cells but was expressed in the cytoplasm of cancer cells (Inose et al. 2009). An investigation of Cx32 and Cx43 expression in normal tissues and human prostate cancer tissue showed that while normal prostate epithelial cells expressed only Cx32, prostate cancer cells expressed both Cx32 and Cx43 (Mehta et al. 1999). Thus, the expression pattern of Cx may vary according to tumor type and Cx isoform.

Our results showed that there was a tendency for Cx32 expression to decrease compared to normal tissues as the tumor invaded from the submucosa to the serosa. Hong *et al.* reported a significant positive correlation between reduced intercellular Cx26 and tumor invasion (Hong and Lim 2008).

Although more studies are needed, this suggests that Cx32 may play a role in preventing the invasion of gastric cancer to the submucosa or serosa. In esophageal cancer, abnormal expression of Cx26 protein may correlate with tumor metastasis and poor prognosis (Inose et al. 2009). Our observation that altered Cx32 expression is significantly correlated with serosal invasion suggests that Cx32 protein has a supportive effect on gastric cancer growth rate.

The dysfunction or loss of membrane proteins such as Cx, E-cadherin and β-catenin is related to tumor malignancy and tends to occur in undifferentiated carcinomas (Shimoyama and Hirohashi 1991b, Jinn, Ichioka and Marumo 1998, Shimoyama and Hirohashi 1991a, Ogawa et al. 1999, Takayama et al. 1996). E-cadherin maintains intercellular adherence junctions and β-catenin connects E-cadherin to the cytoskeleton. Given that cell-cell adhesion is necessary for GJIC, correlations between Cx and E-cadherin or β-catenin have been described, but thses relationships remain unclear. In human lung cancer, a concurrent reduction in E-cadherin and Cx43 expression is significantly associated with differentiation and progression, and exogenous expression of Cx43 in human pulmonary

carcinoma cells significantly induces E-cadherin expression (Xu et al. 2008). On the basis of results obtained using Cx32-null mice treated with *N*-nitrosodiethylamine and Phenobarbital, Schwarz *et al.* suggested that the loss of Cx32 function and mutation of β-catenin were important during hepatocarcinogenesis (Schwarz et al. 2003).

Because neoplastic or preneoplastic cells showed alterated Cx expression, we investigated the association between Cx and cell proliferation in mouse gastric tumors by immunohistochemically examining the expression of the Ki67 nuclear antigen, which is expressed in all proliferating cells during late G1, S, M and G2 phases. Our demonstration that Cx32 expression was low in Ki67-positive cells is in consistent with the previous demonstration of a possible inverse relationship between Cx43 expression and cell proliferation in canine bone tumors (Sanches et al. 2009). In addition, knocking down Cx32 expression in rat hepatoma cell line using RNA interference has been showed to increase proliferation, providing support for a causal relationship between Cx32 expression and proliferative activity (Edwards et al. 2008). Taken together with the observation, that expression of

proliferation markers in various cancer samples correlates with mitotic activity and survival (Bouzubar et al. 1989, Wang, Luo and Wang 2006). And the inverse relationship between proliferation and Cx expression suggests that Cx32 expression might be an indicator of carcinogenesis potential, and as such may serve as an additional cancer biomarker.

No statistically significant differences were seen in Cx32 mRNA expression in adenocarcinomas compared to normal stomach tissues in the mouse gastric cancer model. This agrees with a previous report that showed no significant differences in Cx mRNA levels between normal tissues and tumors of liver or bone (Krutovskikh et al. 1994, Sanches et al. 2009). However, in mouse lung tissues, differences in expression of Cx26, 32, and 40 between normal tissues and tumors have been reported depending on tumor size (Udaka et al. 2007, Avanzo et al. 2006). One possible interpretation of the present result is that aberrant localization of Cx32 protein, and not a reduction in expression per se, could be responsible for the observed dysfunction of the GJIC. Expression of Cx protein or mRNA does not necessarily reflect the functional activities of Cxs. Further functional examinations, such as dye transfer studies,

are required to confirm the presence of functional channels. Another possible interpretation is that the Cx mRNA is transcribed but not translated. A number of studies have indicated that Cxs, in particular, may express large amounts of message without actually producing any protein (Vinken et al. 2009). For example, Anderson *et al* reported that Cx43 downregulation during skeletal muscle development is accomplished by two related microRNAs, miR-206 and miR-1, that inhibit the expression of Cx43 protein during myoblast differentiation without altering Cx43 mRNA levels (Anderson, Catoe and Werner 2006). Our immunohistochemistry findings may not necessarily have reflected the amount of mRNA translated. Further investigations are required to determine whether microRNAs are upregulated and serve to inhibit the translation of mRNAs to produce Cx32 protein. Lastly, while we used total mass to extract mRNA for quantitative real-time RT-PCR, only malignant tumor cells are estimated by immunohistochemistry when viewing the histological results. A clearer definition of this relationship will require additional studies that can precisely determine the amount of Cx mRNA in tumor and normal tissues through microdissection or an in situ

RT-PCR that would prevent contamination from non-malignant cells.

Various experimental data have shown that each step leading to the establishment of GJIC can be altered and induce the loss of junctional communication. Possible mechanisms include lack of transcription of Cx genes; lack of translation of Cx mRNA; lack of membrane trafficking, leading to an accumulation of Cx proteins in the cytoplasm; and lack of cell-cell recognition preventing the establishment of junctional intercellular communication (Cronier et al. 2009). Our results support the hypotheses that an accumulation of Cx protein in cytoplasm and/or lack of translation of Cx mRNA lead to altered Cx expression. Additional theories have been introduced to explain altered Cx expression. For examples, the presence of phosphorylated Cx43 in lung tumors suggests an association of Cx43 phosphorylation with lung tumorigenesis (Udaka et al. 2007). Additional studies will be needed to confirm the role of Cx during carcinogenesis.

In conclusion, the disappearance or reduction in intercellular Cx32 staining in gastric adenocarcinoma was associated with cell proliferation, and intracytoplasmic staining of Cx32 was

observed not only in adenocarcinoma and adenoma but also in mucous metaplasia. These findings suggest that the altered Cx32 expression, specifically, the loss of intercellular Cx32 and the gain of intracytoplasmic Cx32 plays an important role in the formation of gastric adenocarcinomas.

CHAPTER II.

Connexin32 inhibits gastric carcinogenesis through cell cycle arrest and altered expression of $p21^{Cip1}$ and $p27^{Kip1}$

ABSTRACT

Gap junctions and their structural proteins, connexins (Cxs), have been implicated in carcinogenesis. Not only connexins but also cell adhesion-associated proteins play a role in differentiation, proliferation and homeostasis. Aberrant expression of adhesion proteins and connexins has been described in diverse tumors. Also, abnormal expressions of cell cycle-regulatory proteins are frequently observed in various cancers. To explore the involvement of Cx32 in gastric carcinogenesis, immunochemical analysis of Cx32 and proliferation marker Ki67 using tissue-microarrayed human gastric cancer and normal tissues was performed. And we evaluated the relationship between expression of cell cycle-regulatory proteins such as $p21^{Cip1}$ and $p27^{Kip1}$ or adhesion protein E-cadherin and β-catenin and Cx32 expression in human gastric cancers using immunohistochemistry. In addition, after Cx32 overexpression in the human gastric cell line AGS, cell proliferation, cell cycle analyses, and $p21^{Cip1}$ and $p27^{Kip1}$ expression levels were examined by bromodeoxyuridine assay, flow cytometry, real-time RT-PCR, and western blotting.

Immunohistochemical study noted a strong inverse correlation between Cx32 and Ki67 expression pattern as well as their location. Moreover, as the Cx32 expression changed from normal membranous expression to cytoplasmic expression or was lost, the $p21^{Cip1}$- and $p27^{Kip1}$-positive cell rate decreased (negative staining). We found a statistically significant positive correlation between Cx32 and E-cadherin or β-catenin expression in gastric tissues. *In vitro*, overexpression of Cx32 in AGS cells inhibited cell proliferation significantly. G_1 arrest, up-regulation of cell cycle-regulatory proteins $p21^{Cip1}$ and $p27^{Kip1}$ were also found at both mRNA and protein levels. Taken together, Cx32 plays some roles in gastric cancer development by inhibiting gastric cancer cell proliferation through cell cycle arrest and cell cycle regulatory proteins.

INTRODUCTION

Gastric cancer is one of the most important causes of cancer-related death worldwide and remains a major public health concern in eastern Asian countries, including Korea and Japan (Hohenberger and Gretschel 2003, Smith et al. 2006). In Korea, gastric cancer has been the most common type of cancer for the last ten years, causing 56.8 deaths per 100,000 individuals annually (Jung et al. 2011). The development of gastric cancer in response to exposure to carcinogens and/or *Helicobacter pylori* is believed to occur over a long period of time and involve a number of events (Correa 1992, Correa and Houghton 2007). Disruption of the balance between cell proliferation and apoptosis is an important driving force of gastric cancer development (Kwon et al. 2012, Crabtree et al. 2004). Although our understanding of gastric cancer has improved considerably, the precise mechanisms underlying gastric cancer progression remain incompletely understood.

Gap junction channels, which are localized to cell-cell contact sites, are composed of connexins (Cxs) and mediate the intercellular flux of metabolites, nutrients, and second

messengers (Vinken et al. 2008, Laird 2006, Mroue, El-Sabban and Talhouk 2011). This gap junction intercellular communication and Cxs play important roles in organ/tissue homeostasis and cell differentiation (Alexander and Goldberg 2003, Laird 2006, Vinken et al. 2008). Individual Cxs are defined and named based on their molecular weight and differ in both function and expression patterns (Mroue et al. 2011, Vinken et al. 2008, Willecke et al. 2002). Cx26 and Cx32 are the main types of stomach Cxs (Radebold et al. 2001, Uchida et al. 1995), whereas colonic and rectal epithelial cells primarily express Cx26 (Kanczuga-Koda et al. 2005b).

Abnormal patterns of Cx expression, such as decreases, loss or abnormal subcellular localizations, have been reported in various human tumors (Conklin et al. 2007, Hong and Lim 2008, Huang et al. 1998, Kanczuga-Koda et al. 2005b, Nakashima et al. 2004, Uchida et al. 1995). Recently, we reported that localization of Cx32 expression altered from cell membranes to the cytoplasm or its expression was altogether lost in gastric cancer in relation to the degree of tumor cell differentiation (Jee et al. 2011). Moreover, decreased expression of several types of Cxs has been reported in chemically induced mouse

lung tumors (Avanzo et al. 2006, Udaka et al. 2007).

Accumulating evidence has demonstrated a role for Cxs in cell proliferation. A comparison of the cellular proliferation with the Cx43 levels has demonstrated a possible inverse correlation in canine bone tumors (Sanches et al. 2009). Consistent with this, knocking down Cx32 expression was shown to increase cell proliferation in rat hepatoma cell line (Edwards et al. 2008), and Cx43 overexpression was found to decrease proliferation of human lung cancer-derived cell lines (Xu et al. 2008). It is generally recognized that tumors develop and progress through uncontrolled cell growth due to abnormalities in the cell cycle (Hunter and Pines 1994, Ford HL 2004).

In this study, we examined the expression of Cx32 and that of the proliferation marker Ki67 in tissue-microarrayed human gastric tissues and investigated the correlation between their expression patterns. And we evaluated the relationship between expression of cell cycle-regulatory proteins such as $p21^{Cip1}$ and $p27^{Kip1}$ or adhesion protein E-cadherin and β-catenin and Cx32 expression in human gastric cancers using immunohistochemistry. We then examined cell proliferation, cell cycle distribution, and cell cycle-regulatory proteins $p21^{Cip1}$

and $p27^{Kip1}$ expression levels after Cx32 overexpression in the human gastric cancer cell line AGS.

MATERIALS AND METHODS

Immunohistochemical staining

Tissue-microarrayed slides containing a total of 105 gastric adenocarcinoma and 62 normal gastric tissues, purchased from SuperBioChips Laboratories (Seoul, Korea) and ISU ABXIS Co., Ltd. (Seoul, Korea) were used for immunohistochemistry.

Table 3 summerizes the antibodies used in this study. The degrees and pattern of Cx32, Ki67, E-cadherin, β-catenin, Ki67, $p21^{Cip1}$ and $p27^{Kip1}$ expressions were studied immunohistochemically in replicate sections of tissue-microarrayed slides, using mouse anti-rat Cx32 (Chemicon International Inc., Temecula, CA, USA), rabbit anti-human Ki67 (Dakocytomation, Glostrup, Denmark), mouse anti-human E-cadherin (BD Transduction Laboratories^{TM}, CA, USA), mouse anti-mouse β-catenin (BD Transduction Laboratories^{TM}, CA, USA), $p21^{Cip1}$ (Santa Cruz biotechnology Inc., CA, USA), and $p27^{Kip1}$ (Oncogene, Cambridges, Mass., USA) antibodies. Immunoreactive proteins were detected using a Bond Polymer Refine Detection kit and BOND-MAX automated immunostainer (Leica Microsystems, New York City, NY, USA). Briefly, after

dewaxing and rehydrating, the sections were subjected to an antigen-retrieval procedure, and endogenous peroxidase activity was quenched with hydrogen peroxide. The slides were then washed with phosphate-buffered saline (PBS) and blocked using the blocking serum provided in the staining kit. Thereafter, slides were incubated with primary anti-Cx32 and anti-Ki67, anti-E-cadherin, anti-β-catenin, anti-$p21^{Cip1}$, or anti-$p27^{Kip1}$ antibodies followed by incubation with a post-primary blocker and polymer, as described by the manufacturers. 3,3' -diaminobenzidine was used as the chromogen. The sections were counterstained with Mayer' s hematoxylin (DakoCytomation, Carpinteria, CA, USA) and examined under a light microscope.

Table 3. Antibodies used in the immunohistochemistry

Antibody	Dilution	Expression in non-neoplastic mucosa	Altered expression in cancer
Connexin32	1:200	Membranous	Cytoplasmic or loss
Ki67	1:100	Nuclear	Nuclear expression increase
E-cadherin	1:500	Membranous	Cytoplasmic or loss
β-catenin	1:800	Membranous	Cytoplasmic or Nuclear
$p21^{Cip1}$	1:200	Nuclear	Nuclear expression decrease
$p27^{Kip1}$	1:50	Nuclear	Nuclear expression decrease

Immunohistochemistry scoring and analysis

For immunohistochemical analyses, a total of approximately 1,000 tumor or normal cells from each microarrayed spot were evaluated. Cx32 expression patterns were classified into three categories: normal membranous expression, cytoplasmic expression or loss (Jee et al. 2011). A spot with less than 10% Cx32 was positivity regarded as negative (loss). To determine the Ki67-labeling index, epithelial cells were separated into positive cells and negative cells. The Ki67-labeling index (%) was determined by dividing the number of positive cells by the total number of cells, and multiplying by 100. E-cadherin expression was applied according to the systems as follows: no staining; cytoplasmic expression; normal membranous expression and β-catenin expression was applied according to the systems as follows: nuclear; cytoplasmic expression; normal membranous expression (Almeida et al. 2010, Zhang et al. 2010, Kim, Han and Lim 2011, Jawhari et al. 1997). $p21^{Cip1}$ expression was graded as negative ($\leq$10%) or positive ($\geq$10% of tumor cells stained) (Natsugoe et al. 1999, Liu et al. 2001). The $p27^{Kip1}$ immunoreactivity was considered as high if the percentage of positive cells was over 30%, low if the

percentage of positive cells was 5-30%, or negative if the percentage of positive cells was less than 5%.

Cell culture and Cx32 transfection

The AGS human gastric cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), and antibiotic-antimycotic (Invitrogen Biotechnology, Grand Island, NY, USA) at 37° C in a humidified atmosphere of 5% CO_2.

A cDNA insert containing the entire coding region of human Cx32 (NM_000166) (Kumar and Gilula 1986) was subcloned into the *Xho* I - *Hind* III site of the expression vector pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA, USA). The sequence of the resulting Cx32 expression plasmid (pEGFP-N1-Cx32) was confirmed by DNA sequencing. AGS cells were transfected with pEGFP-N1-Cx32 or pEGFP-N1 (control vector) using Metafectene Pro transfection reagent according to the manufacturer's instructions (Biontex Laboratories, Martinsried, Germany). After selection for 14 days with 1 mg/ml of G-418 (Sigma-Aldrich Co., St. Louis, MD, USA), a

single-cell clone was established and screened for Cx32 expression by immunoblotting.

Cell proliferation and cell cycle analysis

Cell proliferation was measured using bromodeoxyuridine (BrdU)-based cell proliferation assay (Millipore, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, during the final 2 hours incubation, BrdU was added to cells (2 x 10^5 cells/mL) grown on culture dishes. After fixing and washing, cells were incubated with a mouse anti-BrdU monoclonal antibody, followed by incubation with a peroxidase-conjugated goat anti-mouse IgG secondary antibody and a peroxidase substrate. Thereafter, absorbance at 450/550nm was monitored using a spectrophotometer microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For cell cycle analysis, AGS cells wild-type, AGS cells transfected with control vector, and AGS cells transfected with Cx32 vector were trypsinized, and then fixed and incubating at -20° C. After washing, the cells were incubated in phosphate-buffered saline containing RNase for 30 min at 37° C. Then, a solution of propidium iodide was added to the cell suspension,

and the cells were analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences, San Jose, CA, USA).

Western blotting

For preparation of total protein lysates, AGS cells of three groups were lysed (1 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, and NP-40) and microcentrifuged. The supernatants were separated by electrophoresis on polyacrylamide gels, transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), and probed with antibodies against Cx32, $p21^{Cip1}$, or $p27^{Kip1}$. The membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GE Healthcare UK Limited, Buckinghamshire, UK) or horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA), and immunoreactive bands were identified. Equal protein loading was ensured by reprobing membranes with an antibody directed against β-actin (Cell Signaling Technology Inc., Danvers, MA, USA).

RNA isolaton and quantitative real-time RT-PCR

The total RNA from transfected cells was extracted by RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. About 1 μg of total RNA from each sample was subjected to cDNA synthesis using QuantiTeck Reverse Transcriptation Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The cDNA was analyzed by real-time polymerase chain reaction (PCR) using Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and the following primer pairs: $p21^{Cip1}$ forward 5'-TCCAGCGACCTTCCTCATC CAC-3', reverse 5'-TCCATAGCCTCTACTGCCACCATC-3'; $p27^{Kip1}$ forward 5'-CGCTCGCCAGTCCATT-3', reverse 5'-ACAAAACCGAACAAAA CAAAG-3'; β-actin forward 5'-CCACACTGTGCCCATCTACG-3'; reverse 5'-AGGATCT TCATGAGGTAGTCAGT CAG-3'. Targets were amplified and mRNA was quantified using a Rotor-Gene Q and the manufacturer's software (Qiagen, Hilden, Germany). The amount of mRNA was calculated using β-actin as the endogenous control.

Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 18.0; SPSS Inc., Chicago, IL, USA). Proliferation indexes (Ki67 labeling indexes) were expressed as means ± standard deviations; data were compared using a two-tailed Student's *t*-test. The relationship between Ki67, E-cadherin, β-catenin, $p21^{Cip1}$, or $p27^{Kip1}$and Cx32 expression was analyzed using Chi-square and Spearman's rho correlation tests. In tests on AGS cells, the data were expressed as means ± standard deviations of at least three independent experiments (n = 3); the data were compared using an unpaired two-tailed Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Cx32 expression in human gastric cancer and normal tissue

We recently investigated Cx32 expression in human normal as well as gastric cancer tissues (Jee et al. 2011). As previously found, normal gastric mucosa predominantly showed intercellular Cx32 expression (Fig. 5A), whereas cytoplasmic expression (Fig. 5B) and loss of expression (Fig. 5C) were often noted in cancer tissues. The expression of Cx32 at intercellular junctions gradually decreased, whereas cytoplasmic expression or loss of expression increased in proportion to the degree of neoplastic cell differentiation.

The relationship between Cx32 and Ki67 expression in human gastric cancer and normal tissue

Nuclear Ki67 expression was evident in both normal (Fig. 5D) and cancer tissues (Fig. 5E and F). Our results showed that 10.15% ± 7.57% of cells in normal tissues were Ki67−positive

compared with 18.99% ± 17.41% in gastric cancer tissue. Thus, the percentage of Ki67-positive cells was significantly increased in gastric cancer ($P < 0.01$).

An examination of Ki67-positivity in relation to the pattern of Cx32 expression in normal gastric tissues and carcinoma tissue showed an inverse correlation between Cx32 and Ki67 expression (Spearman rho = −0.421; $P < 0.01$) (Fig. 6). This correlation held for normal tissue (Spearman rho = −0.269; $P = 0.034$) and cancer tissue (Spearman rho = −0.430; $P < 0.01$) analyzed separately. Specifically, the frequency of Ki67-positive cells was increased as Cx32 localization shifted from a membranous to cytoplasmic pattern, and was further increased with loss of expression.

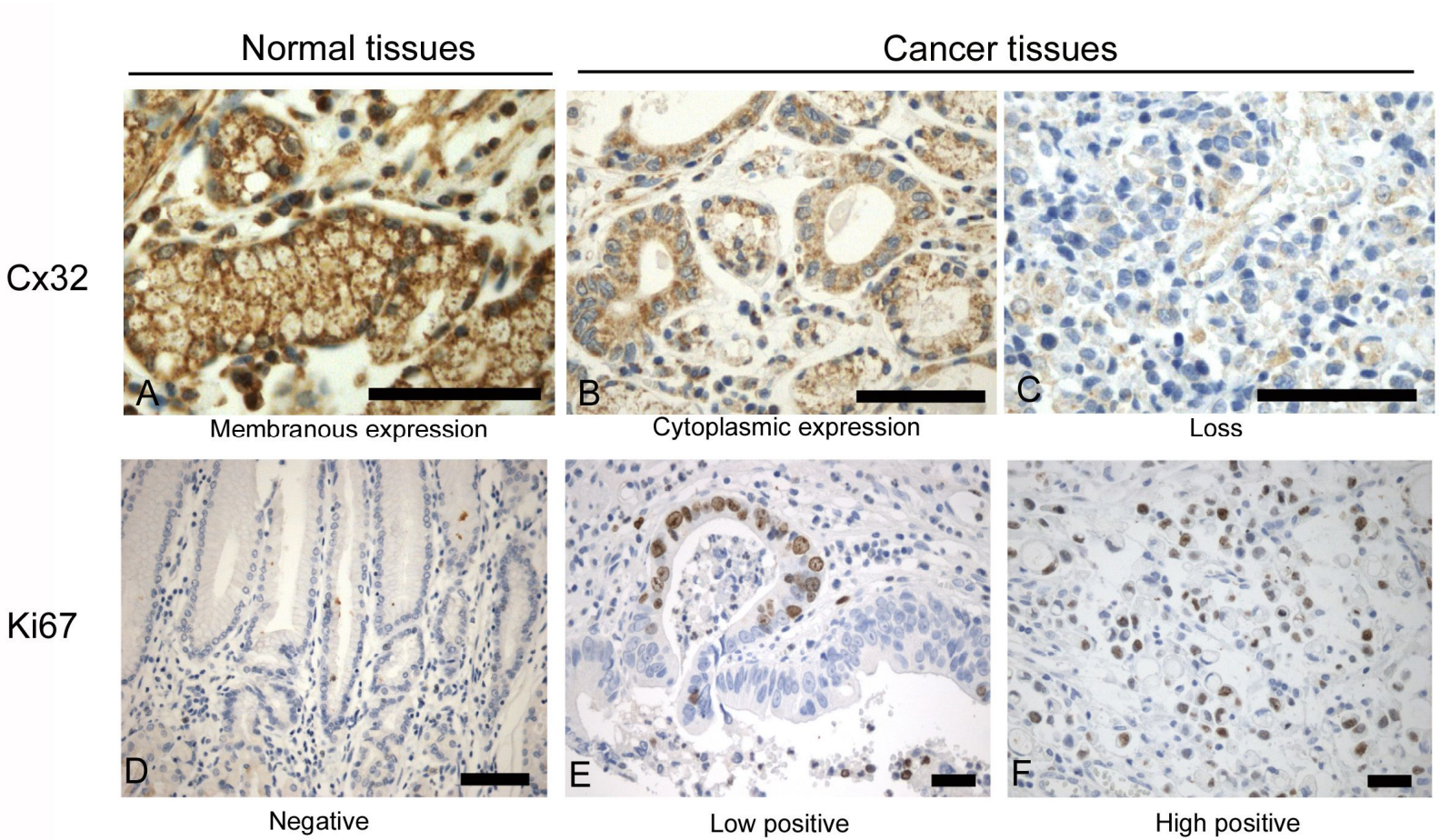

Normal tissues
Cancer tissues
Cx32
Ki67
A
B
C
D
E
F
Membranous expression
Cytoplasmic expression
Loss
Negative
Low positive
High positive

Figure 5. Immunohistochemical stainingfor Cx32 and Ki67 in normal gastric tissues (A and D) and gastric cancer tissues (B, C, E, and F).

A, normal gastric mucosa showed intercellular Cx32 expression; B, The cancer cells in moderately differentiated adenocarcinoma showed intracytoplasmic Cx32 expression; C, The cancer cells in poorly differentiated adenocarcinoma showed negative staining; D, normal gastric mucosa showed negative staining for Ki67 E, The cancer cells in moderately differentiated adenocarcinoma showed nuclear expression; F, The cancer cells in signet ring cell carcinoma showed nuclear expression; All bar = 30 μm.

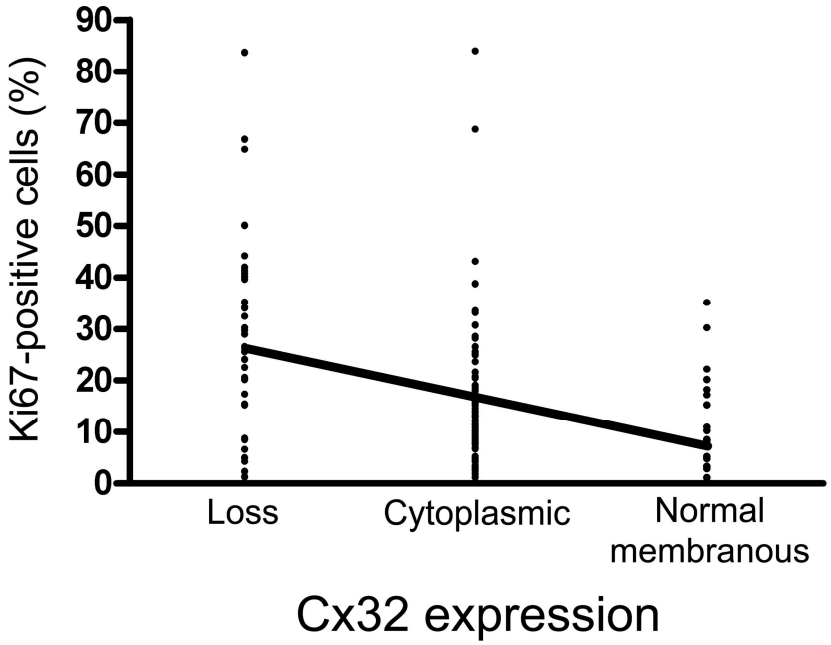


Figure 6. The relationship between Ki67 and Cx32 expression in normal gastric tissues and cancer tissues.

There is an inverse correlation between Ki67 positivity and Cx32 expression (Spearman $r = -0.421$; $P < 0.01$).

The relationship between the cell adhesion proteins, E-cadherin and ß-catenin, and Cx32 expression

Firstly, we investigated the expression and distribution of E-cadherin, a well-known cell adhesion molecule, in gastric normal tissue and cancer tissue. E-cadherin was expressed at the membrane of most of the epithelial cells in normal tissue (Fig. 7D), while it showed only cytoplasmic expression without membranous expression in epithelial cells (Fig. 7E) or it was not expressed at all (Fig. 7F), indicating different pattern of expression. The loss rate of E-cadherin expression in gastric normal tissue was 4.84% (3/62), whereas it was 13.3% (14/105) in gastric cancer tissue. The loss rate of E-cadherin membranous expression in gastric normal tissue was 14.5% (9/62), whereas it was 83.8% (88/105) in gastric cancer tissue. The loss rates of E-cadherin membranous expression in normal tissue and cancer tissue were significant ($P < 0.01$) and the result was not different from the previous result of Almeida *et al* (Almeida et al. 2010).

We investigated the association between the Cx32 expression

and E-cadherin or β-catenin expression (Table 4). In gastric normal tissues and cancer tissues, a positive correlation was found between the expression and the expression location of Cx32 and E-cadherin (Spearman rho = 0.471, $P < 0.01$). In other words, as the Cx32 expression was turning from normal membranous expression to cytoplasmic expression and lost eventually, the E-cadherin expression was also turning from normal membranous expression to cytoplasmic expression and then lost eventually.

Next, we investigated β-catenin, another marker well known as a cell adhesion molecule. The immunohistochemical staining test showed that it was expressed at the intercellular boundary in gastric normal epithelial cells (Fig. 7G). In some tumor epithelial cells, the expression at the intercellular boundary was decreased whereas cytoplasmic expression was increased (Fig. 7H). However, in other tumor regions, clustered tumor cells only with nuclear expression were found without any membranous expression (Fig. 7I). We investigated the location of the β-catenin expression in normal tissue and tumor tissue. The loss rate of normal membranous expression of β-catenin was 4.84% (3/62) in gastric normal tissue, whereas it was 60%

(63/105) in gastric cancer tissue. The rate of nuclear expression was 0% (0/62) in normal tissue, while it was 35.2% (37/105) in tumor tissue. The result was not different with that of previous studies (Kim et al. 2011, Jawhari et al. 1997). There was a significant difference in the loss rate of membranous expression and the rate of nuclear expression between gastric normal tissue and tumor tissue ($P < 0.05$).

Next, we investigated the correlation in the expression and the location of expression between Cx32 and β-catenin and the result showed that there was a significant correlation in the expression and the location of expression between Cx32 and β-catenin in gastric normal tissue and tumor tissue (Spearman rho = 0.519, $P < 0.01$) (Table 4). In other words, as the Cx32 expression was turning from normal membranous expression to cytoplasmic expression and lost eventually, the β-catenin expression was also turning from the normal membranous expression to cytoplasmic expression or nuclear expression.

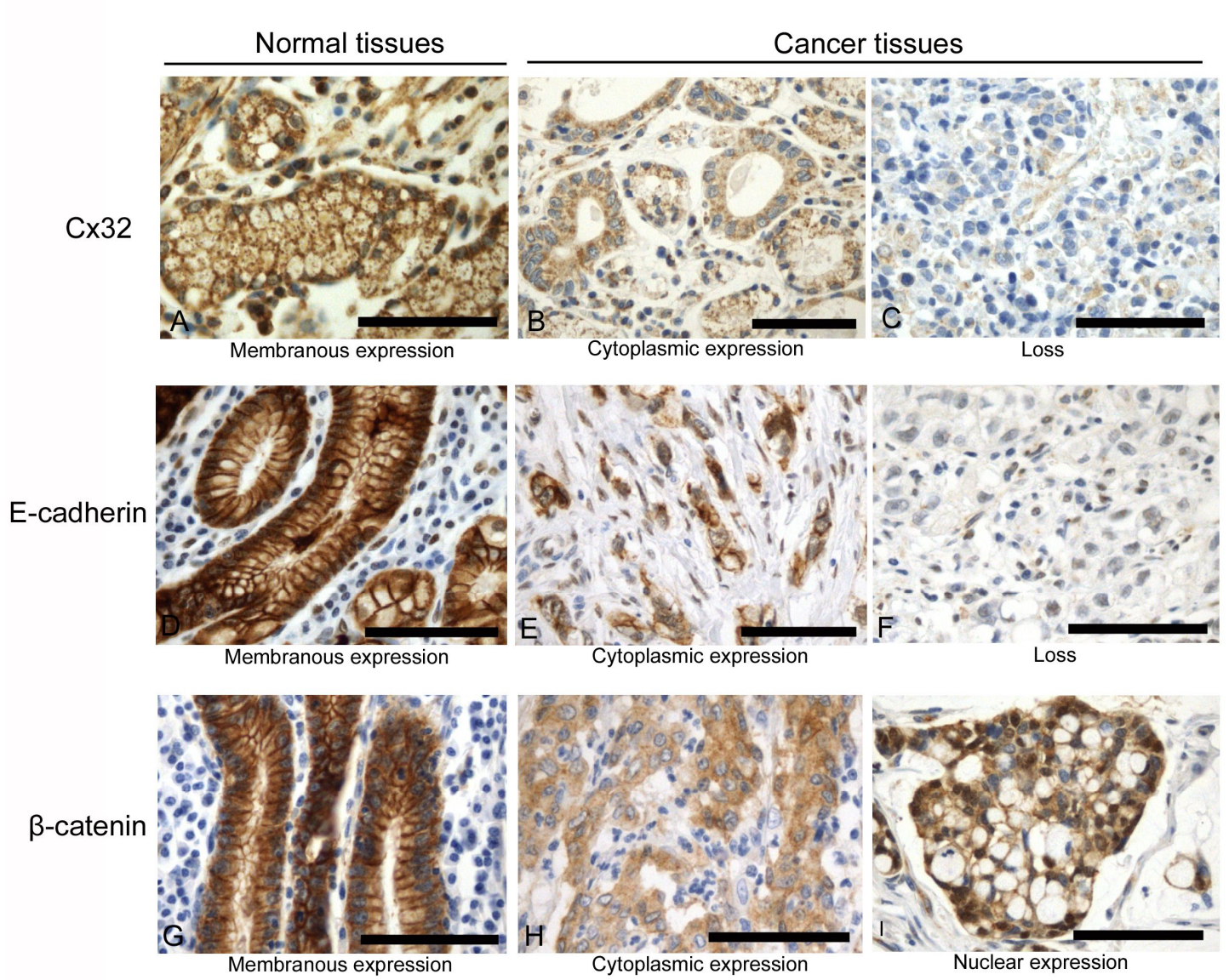

Normal tissues
Cancer tissues
Cx32
E-cadherin
β-catenin
A
B
C
D
E
F
G
H
I
Membranous expression
Cytoplasmic expression
Loss
Membranous expression
Cytoplasmic expression
Loss
Membranous expression
Cytoplasmic expression
Nuclear expression

Figure 7. Immunohistochemistry for adhesion proteins Cx32, E-cadherin, β-catenin in gastric normal tissues (A, D, and G) and cancer tissues (B, C, E, F, H, and I).

A, Cx32 protein in normal stomach: intercellular expression; B, Cx32 protein in moderately differentiated adenocarcinoma: intracytoplasmic expression; C, Cx32 protein in poorly differentiated adenocarcinoma: negative staining; D, E-cadherin protein in normal tissue: strong membranous expression; E, E-cadherin protein in poorly differentiated adenocarcinoma: cytoplasmic and partial membranous expression; F, E-cadherin protein in signet ring cell carcinoma: negative staining; G, β-catenin protein in normal tissue: strong membranous and cytoplasmic expression; H, β-catenin protein in poorly differentiated adenocarcinoma: loss of membranous expression; I, β-catenin protein in signet ring cell carcinoma: nuclear expression and loss of membranous expression; All bar = 30 μm.

Table 4. The expression of Cx32 in relation to that of adhesion proteins E−cadherin and β−catenin in human normal gastric tissues and carcinomas

	n	Cx32 expression			*P*−value
		Membranous	Cytoplasmic	Loss	
E−cadherin					< 0.01
Membranous	70	40	26	4	
Cytoplasmic	80	8	48	24	
Loss	17	3	8	6	
β−catenin					< 0.01
Membranous	101	47	46	8	
Cytoplasmic	29	3	19	7	
Nuclear	37	1	17	19	

The relationship between cell cycle-regulatory proteins and Cx32 expression

For the next, we examined $p21^{Cip1}$, and $p27^{Kip1}$ expressions in normal and cancer tissues to investigate the correlation between expression of the typical cell cycle-regulatory proteins and the Cx32 expression. The immunohistochemical staining test showed that $p21^{Cip1}$ showed nuclear expression in both normal tissue (Fig. 8A) and tumor tissue (Fig. 8B and C). The $p21^{Cip1}$ positive cell rate was 100% (62/62) in gastric normal tissue, while it was 63.8% (67/105) in tumor tissue. The positive cell rate was significantly lower in tumor cell than in normal tissue ($P < 0.05$) and this result was not different with that of previous studies (Liu et al. 2001).

The correlation between $p21^{Cip1}$ expression and Cx32 expression as well as the expression location was investigated and the result showed a positive correlation (Spearman rho = 0.475, $P < 0.01$) (Table 5). As the Cx32 expression was turning from normal membranous expression to cytoplasmic expression or lost, $p21^{Cip1}$ was not expressed (negative staining).

In most of the normal tissues, $p27^{Kip1}$ showed nuclear expression, but cytoplasmic expression was also found in some of them (Fig. 8D). Some tumor cells showed nuclear expression (Fig. 8E) or negative staining (Fig. 8F). The $p27^{Kip1}$ positive cell rate was 96.8% (60/62) in normal tissue, while it was 61.9% (65/105) in tumor tissue, indicating a significant difference between normal tissue and tumor tissue ($P < 0.01$). Among the 65 cases in tumor tissue, the expression was high in 40 cases (38.1%) and low in 25 cases (23.8%). The result was not significantly different with that of previous studies (Kim et al. 2000).

The correlation between $p27^{Kip1}$ expression and Cx32 expression as well as the expression location was investigated and the result showed a positive correlation (Spearman rho = 0.470, $P < 0.01$) (Table 5), which indicated that, as the Cx32 expression was turning from normal membranous expression to cytoplasmic expression or lost, the $p27^{Kip1}$ positive cell rate was decreased.

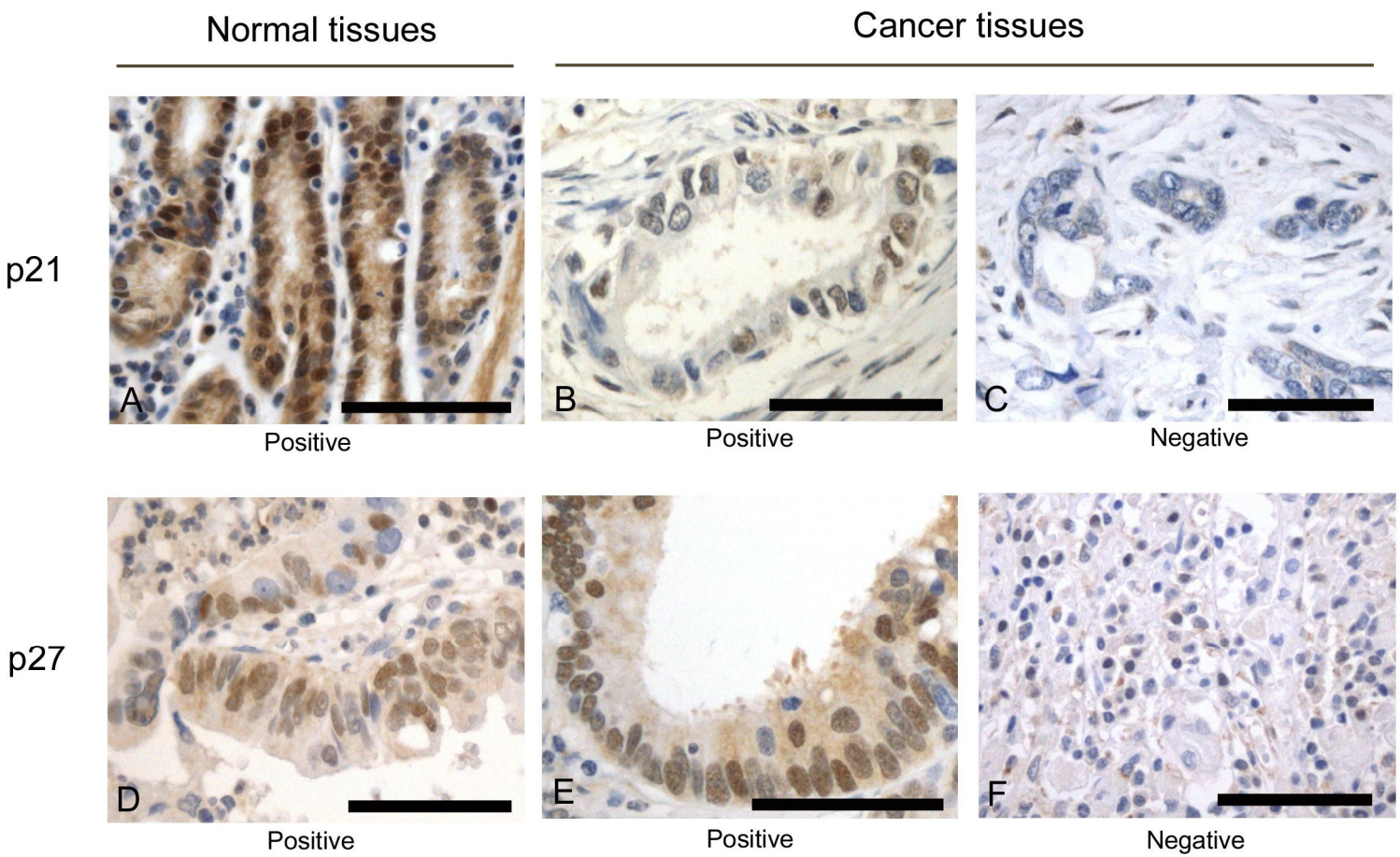

Normal tissues
Cancer tissues
p21
p27
A
B
C
D
E
F
Positive
Positive
Negative
Positive
Positive
Negative

Figure 8. Immunohistochemistry for $p21^{Cip1}$, and $p27^{Kip1}$ in gastric normal tissues (A and D) and cancer tissues (B, C, E, and F).

A, $p21^{Cip1}$ protein in normal stomach: nuclear expression; B, $p21^{Cip1}$ protein in moderately differentiated adenocarcinoma: nuclear expression; C, $p21^{Cip1}$ protein in poorly differentiated adenocarcinoma: negative staining; D, $p27^{Kip1}$ protein in normal tissue: strong nuclear expression; E, $p27^{Kip1}$ protein in well differentiated adenocarcinoma: strong nuclear expression; F, $p27^{Kip1}$ protein in poorly differentiated adenocarcinoma: negative staining; All bar = 30 μm.

Table 5. The expression of Cx32 in relation to that of cell cycle-regulatory proteins in human normal gastric tissues and carcinomas

	n	Cx32 expression			*P*-value
		Normal membranous	Cytoplasmic	Loss	
$p21^{Cip1}$					< 0.01
Negative	38	1	16	21	
Positive	129	50	66	13	
$p27^{Kip1}$					< 0.01
Negative	42	3	23	16	
Low positive	48	9	25	14	
High positive	77	39	34	4	

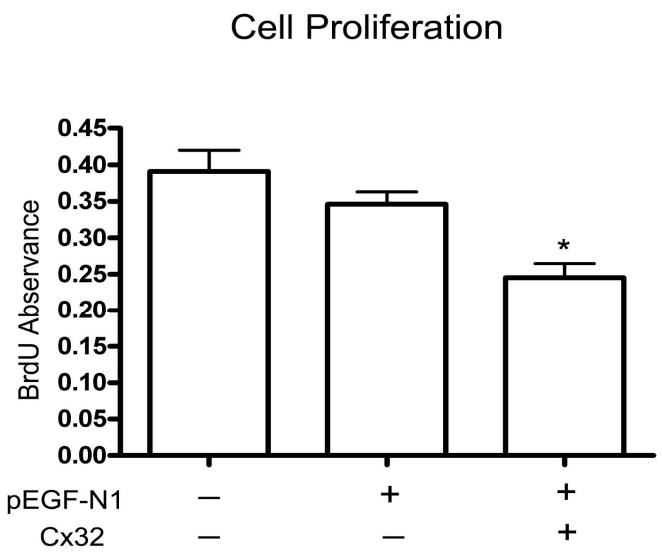
Cell proliferation and cell cycle distribution following overexpression of Cx32 in the AGS gastric cancer cell line

Because a negative correlation was found between cell proliferation and Cx32 expression in human gastric cancer and normal tissues, we performed *in vitro* experiments to further examine the direct relationship between Cx32 expression and cell proliferation. The value of BrdU absorbance decreased approximately 30−40% in the AGS cells overexpressing Cx32 compared to that in wild−type AGS cells or AGS cells transfected with a control vector ($P < 0.05$). There was no significant difference in the value between wild−type AGS cells and AGS cells transfected with a control vector ($P > 0.05$) (Fig. 9A). These results suggested that Cx32 overexpression inhibits cell proliferation in AGS cells.

Having demonstrated that Cx32 overexpression negatively regulated cell proliferation, we next quantified the cell cycle distribution of AGS cells wild−type, AGS cells transfected with control vector, and AGS cells transfected with Cx32 vector (Fig 9B). The percentage of G_1−phase cells was significantly

greater in AGS cells transfected with Cx32 vector (68.06% ± 3.93%) than in AGS cells wild-type (40.54% ± 1.80%) or AGS cells transfected with control vector (46.91% ± 2.78%) ($P < 0.05$). In addition, the percentage of S-phase cells was significantly less in AGS cells overexpressing Cx32 (11.32% ± 0.24%) than in AGS cells wild-type (26.44%±3.04%) or AGS cells transfected with control vector (22.13% ± 2.86%) ($P<0.05$). The percentage of cells in G_2-M phase did not differ among groups ($P > 0.05$), and there were no significant differences in G_1- and S-phase populations between AGS cells wild-type and AGS cells transfected with control vector ($P > 0.05$). These results suggest that Cx32-dependent inhibition of cell proliferation is related to G1 arrest in AGS cells.

A


Cell Proliferation
BrdU Abservance
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
pEGF-N1 — + +
Cx32 — — +

B

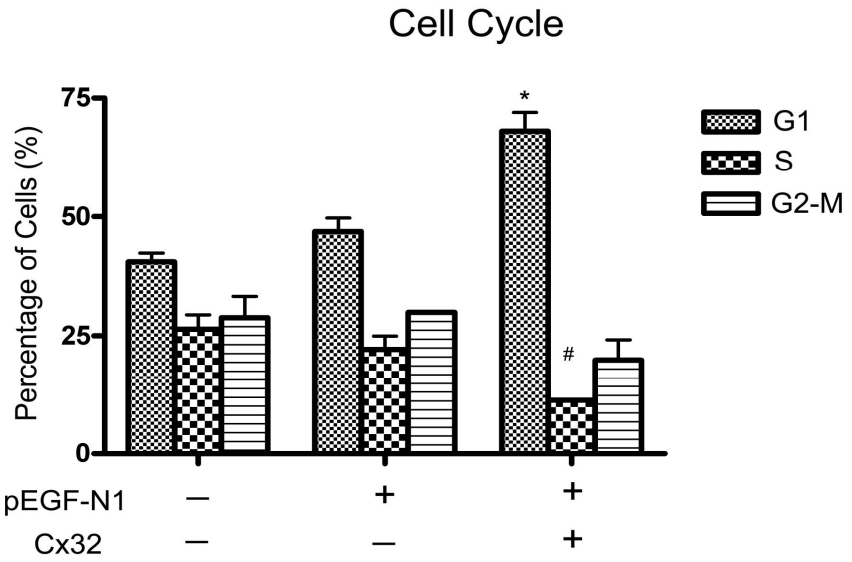

Cell Cycle
Percentage of Cells (%)
75
50
25
0
*
#
G1
S
G2-M
pEGF-N1 — + +
Cx32 — — +

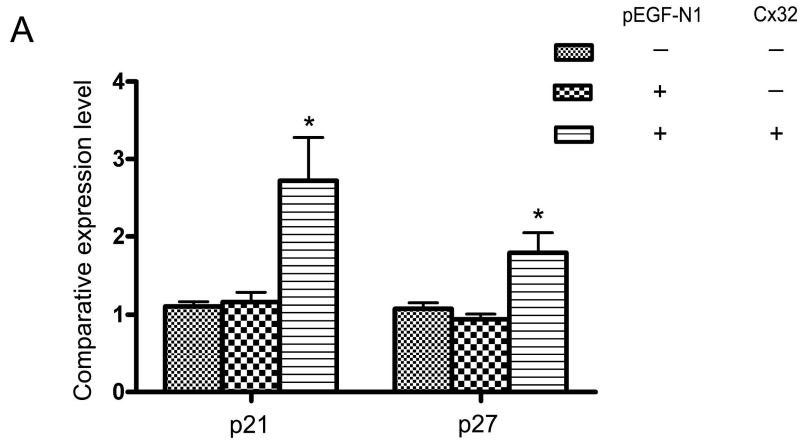
Figure 9. Cell proliferation and cell cycle distribution analyses showed that Cx32 overexpression inhibited cell proliferation through G_1 arrest in AGS cells.

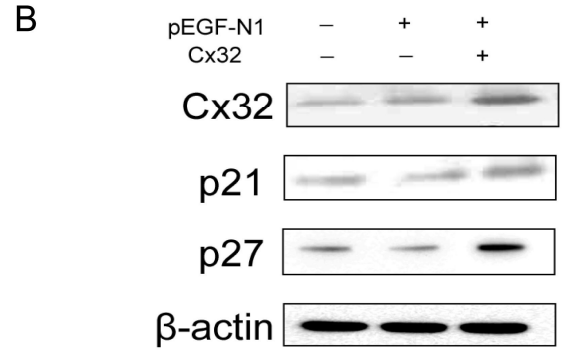
A, Bromodeoxyuridine assay. The bars represent the means ± standard deviations of difference of absorbance. Cell proliferation was decreased in AGS cells overexpressing Cx32 compared to the control groups. $*P < 0.05$; relative to control groups. B, cell cycle distribution. The bars represent the means ± standard deviations of the percentage of cell-cycle stage. The percentage of G1-phase cells was significantly greater in AGS cells transfected with Cx32 vector (68.06% ± 3.93%) than in wild-type AGS cells (40.54% ± 1.80%) or AGS cells transfected with control vector (46.91% ± 2.78%)($P < 0.05$). In addition, the percentage of S-phase cells was significantly less in AGS cells overexpressing Cx32 (11.32% ± 0.24%) than in wild-type AGS cells (26.44% ± 3.04%) or AGS cells transfected with control vector (22.13% ± 2.86%)($P < 0.05$). $*P < 0.05$; Vs relative to the control groups in G1-phase. $^{\#}P < 0.05$; relative to control groups in the S-phase.

$p21^{Cip1}$ and $p27^{Kip1}$ expression following overexpression of Cx32 in the AGS gastric cancer cell line

Because Cx32 overexpression affected the cell-cycle distribution, we sought to determine whether the expression of cell-cycle regulatory proteins differed among the three groups, focusing on changes in $p21^{Cip1}$ and $p27^{Kip1}$ (Fig. 10). Stable cell lines were screened for the amount of Cx32 protein by immunoblotting. Western blot analyses revealed that Cx32 expression in AGS cells transfected with Cx32 vector was approximately 2- to 3-fold greater compared to wild-type AGS cells or AGS cells transfected with control vector. The expression of $p21^{Cip1}$ at the mRNA level increased 2- to 2.5-fold and the expression of $p21^{Cip1}$ protein was approximately 30-50% greater in AGS cells overexpressing Cx32 compared to that of wild-type AGS cells and AGS cells transfected with control vector ($P < 0.05$). $p27^{Kip1}$ expression at the mRNA level increased 60-90% and the content of $p27^{Kip1}$ protein was also approximately 2- to 3-fold greater in AGS cells overexpressing Cx32 than the control cell lines ($P < 0.05$). The

levels of all proteins in wild-type AGS cells and AGS cells transfected with control vector were not significantly different ($P > 0.05$). Our results thus show that Cx32 overexpression in AGS cells induced an increase in $p21^{Cip1}$ and $p27^{Kip1}$ expressions.


A
pEGF-N1
Cx32
–
–
+
–
+
+
Comparative expression level
0
1
2
3
4
*
*
p21
p27


B
pEGF-N1
Cx32
–
+
+
–
–
+
Cx32
p21
p27
β-actin

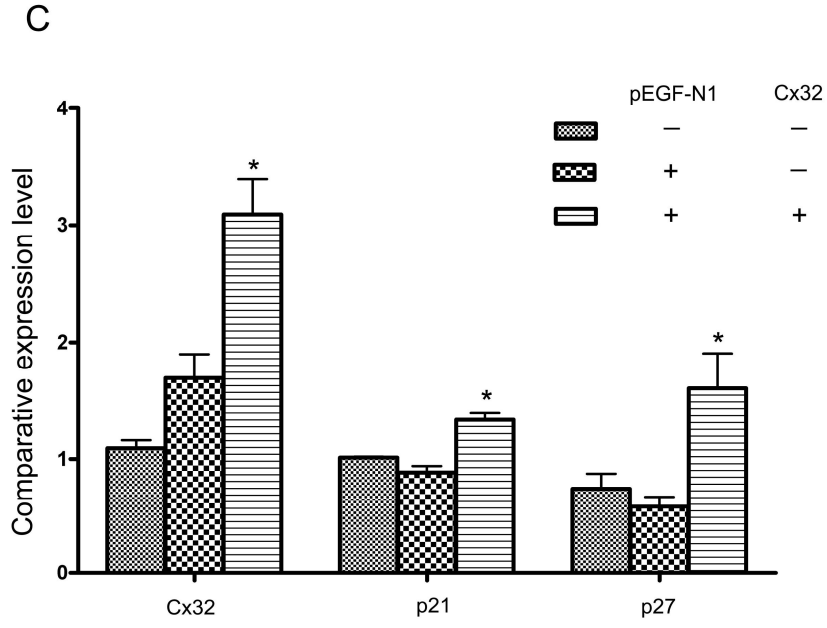

C
pEGF-N1
Cx32
–
–
+
–
+
+
Comparative expression level
0
1
2
3
4
*
*
*
Cx32
p21
p27

Figure 10. Real-time RT-PCR and Western blotting analyses showed that Cx32 overexpression increased the expression of $p21^{Cip1}$ and $p27^{Kip1}$ at mRNA and protein levels in AGS cells.

A, The bars represent the means ± standard deviations of ratio of $p21^{Cip1}$ and $p27^{Kip1}$/β-actin in the real-time RT-PCR data. Expression of $p21^{Cip1}$ and $p27^{Kip1}$ was significantly higher than control groups; B, Cx32, $p21^{Cip1}$, and $p27^{Kip1}$ protein levels were analyzed by Western blotting using β-actin as the loading control; C, The density graph indicates the ratios of Cx32, $p21^{Cip1}$ and $p27^{Kip1}$/β-actin in the Western blot data. Expression of Cx32, $p21^{Cip1}$, and $p27^{Kip1}$ was significantly higher than control groups. $*P < 0.05$ relative to the control groups.

DISCUSSION

Similar to other previous studies, we recently found that Cx32 expression is often shifted from an intercellular to an intracytoplasmic location, or is even lost, in human gastric cancer (Jee et al. 2011). Although altered expression of Cxs has been reported in various malignant tumors, including gastric cancer, the exact role of Cx32 in gastric carcinogenesis has not yet been clearly defined. Because tumor growth reflects an imbalance between cell proliferation and apoptosis, we investigated whether altered Cx32 expression might impact gastric cancer development, placing special emphasis on disruption of normal cell proliferation.

Recent studies showed that Cxs and adhesion proteins cadherins and catenins play important roles in carcinogenesis and their expressions were investigated in many tumors. However, there are only a few publications on Cxs and cadherins or catenins (Jinn et al. 1998, Xu et al. 2008) and it is still unclear that whether Cx32 and adhesion proteins affect each other or not in gastric carcinogenesis. Thus, we have investigated relationship between Cx32 and E-cadherin or β-catenin using immunohistochemistry. In this study, we showed

statistically significant positive correlations between E-cadherin or β-catenin expression and Cx32 expression. Immunohistochemical analyses of Cxs and adhesion molecules in endometriod adenocarcinomas and of Cx43 and E-cadherin in gastric cancer were similar to our results (Wincewicz et al. 2010, Tang et al. 2010). Thus, Cxs and adhesion proteins expression correlated with each other and concurrent reduction or loss and/or aberrant intracellular localization of two proteins may contribute to the gastric carcinogenesis. These alterations may be one of the key mechanisms through which changes toward dedifferentiation and progression of gastric cancers are mediated.

Furthermore, we confirmed that overexpression of Cx32 restored the level of E-cadherin and β-catenin. This result indicates that potential connections exist between Cx32 and E-cadherin or β-catenin in gastric epithelial cells. Murine skin papilloma cells that are transfected with E-cadherin showed Cx43 translocation from cytoplasm to cell-cell contact membranes owing to actin filaments (Hernandez-Blazquez et al. 2001). It means that E-cadherin is instrumental in regulating intracellular trafficking of Cx43. And the intracellular loop of

the Cx43 protein chain interacts with cadherins (Nambara, Kawasaki and Yamasaki 2007) and Cx43 is a target of Wnt signaling and β-catenin (van der Heyden et al. 1998). Another experiment in mouse epidermal cells showed that cells with E-cadherin transfection expressed GJIC in calcium-dependent manner and it suggests that calcium-dependent regulation of GJIC is directly controlled by a calcium-dependent cell adhesion molecule, E-cadherin (Jongen et al. 1991). These studies suggest that abnormal expression of E-cadherin or ß-catenin may affect Cx expression and localization and that GJIC mediated via them might be involved in gastric carcinogenesis. Because individual Cxs differ in function and expression and correlation between Cx32 and adhesion proteins has not yet been clearly defined, additional studies will be needed to confirm the role of Cx32 in gastric carcinogenesis. Future studies, including functional and site-directed mutagenesis, should reveal whether these interactions are direct or reflect precipitation of macromolecular/subcellular complexes.

The involvement of Cxs in the regulation of tumor cell proliferation has been suggested by a number of recent studies. A study to knock down Cx32 expression in rat hepatoma cells

has shown that the magnitude of cell proliferation is inversely proportional to the level of Cx32 expression (Edwards et al. 2008). Furthermore, the incidence of hepatic and pulmonary neoplasms was found to be higher in Cx32-deficient and Cx43-deficient mice, respectively, than in their wild types littermates (Avanzo et al. 2006, Temme et al. 1997). An immunohistochemical analysis of *H. pylori*-associated mouse gastric tumors showed an inverse relationship between Cx32 and Ki67 expression (Jee et al. 2011). Our *in vitro* Cx32 overexpression study is in full agreement with these studies.

Cx expression per se can reduce the proliferation of cancer cells, an effect that is independent of localization to the plasma membrane and formation of gap-junction plaques by transfected Cxs (Huang et al. 1998). Other studies reinforce this interpretation, showing that some Cx mutants that are incapable of plasma membrane insertion are nonetheless capable of down-regulating the cell proliferation (Krutovskikh et al. 2000, Olbina and Eckhart 2003). In canine mammary tumors, malignant tumors showed increased cytoplasmic staining for both Cx26 and Cx43, whereas hyperplastic and benign neoplastic glands showed only membranous expression.

Moreover, the expression and distribution of Cx26 and Cx43 were inversely correlated to cell proliferation in malignant tumors (Torres et al. 2005). Consistent with these observations, we found a relationship between Cx32 localization and cell proliferation in our tissue microarray-based immunohistochemical study. Thus, Cxs clearly play some role in proliferative aspects of gastric cancer development but further studies are need to decipher how actually or what pathways are involved. Depending upon such mechanisms, Cxs might be good target for cancer therapy.

Our investigation of the Cx32 effect on the cell cycle showed that Cx32 inhibits cell cycle progression. Similarly, overexpression of Cx43 has been shown to suppress the proliferation of human osteosarcoma U2OS cells through inhibition of cell cycle transition from G_1- to S-phase (Zhang et al. 2001). In addition, the forced expression of Cx43 and Cx32 was reported to decrease the growth of neoplastic mouse lung and rat liver epithelial cells *in vitro* in association with a reduction in exit of cells from G_1-phase (Koffler et al. 2000). These data suggest that Cx32 regulates cell proliferation, at least in part, through G_1-phase arrest. We then investigated the

expression levels of $p21^{Cip1}$ and $p27^{Kip1}$ following Cx32 in AGS cells. We found that the degrees of $p21^{Cip1}$ and $p27^{Kip1}$ expression were significantly increased both at the mRNA and protein levels. In rat glioma cells, tolbutamide was shown to increase Cx43 protein synthesis, an effect that was accompanied by up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$ (Sanchez-Alvarez et al. 2006). As shown in Zhang *et al.*, increased synthesis as well as post-transcriptional reduced degradation of $p27^{Kip1}$ was evident in human osteosarcoma cell line U2OS (Zhang et al. 2001). Cx43 overexpression can inhibit cell proliferation in association with a decrease in the stability of S-phase kinase-associated protein 2 (Skp-2), which is involved in cell-cycle regulation. This study raises another intriguing possibility that Cxs might have other roles, such as the direct transcriptional regulation of various genes that might include $p21^{Cip1}$ and $p27^{Kip1}$ (Giepmans 2004). The regulation mechanism of Cxs in $p21^{Cip1}$ and $p27^{Kip1}$ expression might vary according to tumor type and Cx isoform. Hence, additional studies are needed for better understanding of $p21^{Cip1}$ and $p27^{Kip1}$ expression mechanisms by Cxs in gastric cancer.

In conclusion, our immunohistochemical analysis of patient-

matched normal and cancerous gastric tissues demonstrated an inverse relationship between Cx32 expression and proliferation, and our *in vitro* study of the effects of Cx32 overexpression showed that Cx32 inhibited the proliferation of gastric cancer cells through cell cycle arrest and up-regulation of $p21^{Cip1}$ and $p27^{Kip1}$.

GENERAL DISCUSSION AND CONCLUSION

To explore the role of Cx32 in gastric carcinogenesis, we investigated altered expression and localization of Cx32 in human gastric cancer using a tissue array-based approach, *H. pylori*-induced murine gastric tumors, and preneoplastic changes (mucous metaplasia) in murine gastric tissues. We examined the relationship between expression of Cx32 and cell proliferation marker Ki67 or cell-cycle regulatory proteins $p21^{Cip1}$ or $p27^{Kip1}$ using immunohistochemistry. In addition, we evaluated the relationship between E-cadherin or β-catenin expression and Cx32 expression. In addition, we examined cell proliferation, cell cycle distribution, and change of levels of the cell-cycle regulatory proteins $p21^{Cip1}$ and $p27^{Kip1}$ following Cx32 overexpression in the human gastric cancer cell line AGS.

In immunohistochemical analyses, the frequency of Cx32 loss of expression was significantly higher in human adenocarcinomas than in normal stomach. As tumor cells were less differentiated, Cx32 expression levels and intercellular and intracytoplasmic staining were also significantly lower. In normal stomach, Cx32 expression in foveolar surface cells in the gastric pit was strong, whereas that in basal cells was weak.

Deep pyloric glandular cells showed punctuate Cx32 staining in the membranes and/or cytoplasm. In tumor cells this intercellular expression was lost, and Cx32 expression varied according to the differentiation status of tumor cells. Some adenocarcinomas showed mild to moderate Cx32 expression in the cytoplasm, whereas others showed loss of staining in the cell membrane and weak positive staining as intracytoplasmic dots or negative staining. As human gastric tumors progressed to more undifferentiated stages, the intercellular and intracytoplasmic expression and intensity of Cx32 decreased significantly and was eventually lost. In mucous metaplasia of the mouse stomach that is meant to be pre-neoplastic lesions, Cx32 was mainly expressed in the cytoplasm of epithelial cells. Similar to these results, immunohistochemical analysis of Cx expression in hepatocellular carcinoma and colorectal carcinoma showed the presence of Cx protein in the form of dots in both cell membrane and cytoplasm (Nakashima et al. 2004, Kanczuga-Koda et al. 2005a). These results are consistent with the idea that altered expression of Cx32 plays an important role in the transformation of glandular epithelial cells and progression of gastric cancers.

Because tumor growth reflects an imbalance between cell proliferation and apoptosis, we investigated whether altered Cx32 expression might impact gastric cancer development, placing special emphasis on disruption of normal cell proliferation. An examination of Ki67-positivity in relation to the pattern of Cx32 expression in human and murine gastric tissue showed that the frequency of Ki67-positive cells increased as Cx32 localization shifted from a membranous to cytoplasmic pattern and was further increased with loss of expression. Our demonstration that Cx32 expression was low in Ki67-positive cells is consistent with the previous demonstration of a possible inverse relationship between Cx43 expression and cell proliferation in canine bone tumors (Sanches et al. 2009). Taken together, the inverse relationship between proliferation and Cx expression suggests that Cx32 expression might be an indicator of carcinogenesis potential, and as such may serve as an additional cancer biomarker.

We then investigated the correlation between expression of the typical cell-cycle regulatory proteins $p21^{Cip1}$ or $p27^{Kip1}$ and that of Cx32. As the Cx32 expression changed from normal membranous expression to cytoplasmic expression or was lost,

the $p21^{Cip1}$- and $p27^{Kip1}$-positive cell rate decreased (negative staining). Thus, Cx32 expression and expression pattern are associated with not only cell proliferation but also cell-cycle regulatory proteins.

We have also investigated the relationship between Cx32 and E-cadherin or β-catenin, using immunohistochemistry. In this study, we showed statistically significant positive correlations between E-cadherin or β-catenin expression and Cx32 expression. As Cx32 expression was turning from normal membranous expression to cytoplasmic expression and was eventually lost, the E-cadherin expression also changed from the normal membranous expression to cytoplasmic expression and was eventually lost. And as Cx32 expression changed, the β-catenin expression was also turning from the normal membranous expression to cytoplasmic expression or nuclear expression. Immunohistochemical analyses of Cxs and adhesion molecules in endometriod adenocarcinomas and of Cx43 and E-cadherin in gastric cancer showed similar results to ours (Tang et al. 2010, Wincewicz et al. 2010). Thus, Cxs and adhesion protein expression correlated with each other, and concurrent reduction or loss and/or aberrant intracellular localization of

two proteins may contribute to gastric carcinogenesis. These alterations may be one of the key mechanisms through which changes toward dedifferentiation and progression of gastric cancers are mediated.

To further examine the direct relationship between Cx32 expression and cell proliferation, gastric cancer cell line AGS cells were transfected with a Cx32 expression plasmid or control vector. Cell proliferation was decreased in AGS cells overexpressing Cx32 compared to wild-type AGS cells or AGS cells transfected with control vector. The percentage of G_1-phase cells was significantly greater and that of S-phase was less in AGS cells overexpressing Cx32 vector than in wild-type AGS cells or AGS cells transfected with control vector. Real-time RT-PCR and Western blot analyses revealed $p21^{Cip1}$ and $p27^{Kip1}$ levels were greater in AGS cells overexpressing Cx32 vector compared to control groups. The involvement of Cxs in the regulation of tumor cell proliferation has been suggested by a number of recent studies. Studies using RNAi to knock down Cx32 expression in rat hepatoma MH_1C_1 cells have shown that the magnitude of cell proliferation is inversely proportional to the level of Cx32 expression (Edwards et al.

2008). Similarly, overexpression of Cx43 has been shown to suppress the proliferation of human osteosarcoma U2OS cells through inhibition of cell-cycle transition from G_1- to S-phase (Zhang et al. 2001). As shown in Zhang *et al.*, increased synthesis as well as post-transcriptional reduced degradation of $p27^{Kip1}$ was evident in human osteosarcoma cell line U2OS (Zhang et al. 2001). These data suggest that Cx32 plays some roles in gastric cancer development by inhibiting gastric cancer cell proliferation through cell cycle arrest and cell cycle regulatory proteins.

In conclusion, our immunohistochemical analysis demonstrated that that the altered Cx32 expression, specifically the loss of Cx32 expression and the gain of intracytoplasmic Cx32, was observed not only in adenocarcinoma and adenoma but also in mucous metaplasia. In addition we found a correlation between Cx32 expression pattern and cell proliferation. Our *in vitro* study of the effects of Cx32 overexpression showed that Cx32 inhibited the proliferation of gastric cancer cells through cell cycle arrest and upregulation of $p21^{Cip1}$ and $p27^{Kip1}$. Together, these results suggest that Cx32 play an important role in gastric carcinogenesis.

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국문초록

위암 발생 과정 중 세포의 증식에 있어서 connexin32의 역할

지 향

수의학과 수의병리학 전공

서울대학교 대학원

한 세포와 인접하는 세포 사이에는 세포막을 관통하는 세포 간 channel이 존재하며, 이는 세포 간 신호전달을 담당한다. Glucose와 glutamate, adenosine trisphosphate (ATP), 이온 같은 물질의 확산을 통해 세포의 성장과 분화, 항상성을 유지한다. 이 channel은 각각의 세포막에 묻혀있는 connexon이라는 단백질 입자가 결합하여 형성되며, 이 connexon은 6개의 connexin (Cx)으로 구성되어 있다. 각각의 connexin은 분자량에 따라 이름이 지어졌으며, 기능과 발현 양상이 각기 다르다. 이 세포 간 신호전달의 이상은 질병과 매우 밀접한 관련이 있으며, 특히 세포 간 신호전달이 저해될 경우 암 발생을 유발한다.

본 논문은 위암 발생에 있어 세포 간 신호전달의 구성 요소인 Cx32의 역할을 알기 위해, 사람 위암 조직과 *Helicobacter pylori* 감염에 의한 마우스 모델에서 위암 조직과 전암 병변에서 이 단백질의 발현율과 발현 패턴을 조사하였다. 그리고 Cx32와 세포 증식과의 관계를 알기 위해, 세포 증식 인자인 Ki67 또는 세포주기 조절 단백질인 $p21^{Cip1}$ 또는 $p27^{Kip1}$의 발현과 Cx32 발현 패턴 관계를 조사하였으며, 세포부착단백질 E-cadherin과 β-catenin의 발현 패턴과 Cx32 발현 패턴 관계도 조사하였다. 그리고 위암 세포주 AGS 세포에 Cx32 cDNA를 주입하여 세포 증식과 세포 주기 분포, 세포주기 조절 단백질 $p21^{Cip1}$과 $p27^{Kip1}$의 발현 정도를 대조군과 비교하였다.

면역조직화학검사에서 사람과 마우스 위암 조직과 정상 조직에서의 Cx32 소실률을 비교한 결과 Cx32 소실률은 정상조직에 비해 위암조직에서 유의성 있게 높았으며, 분화도에 따라 분류한 뒤 소실률을 조사한 결과 분화도가 낮을수록 소실률은 유의성 있게 증가하였다. Cx32는 주로 정상 위 소와 세포의 막에서 강하게 발현되었으며 (세포 사이 발현), 유문선 세포에서는 세포막과 세포질에서 Cx32의 발현이 관찰되었다. 종양에서 Cx32는 정상과 비교하여 변화된 발현을 보였으며, 분화도에 따라 다양하게 발현되었다. 일부 종양 상피세포에서는 세포질에서 발현되었으며, 일부 종양세포에서는 전혀 발현되지 않았다. 위암의 전암 병변인 점막화생을 보인 상피세포에서는 주로 세포질 발현이 관찰되었다. Cx32 발현율과 발현 위치와 Ki67 양성률과의 관계를

알아본 결과, 사람과 마우스 조직 모두에서 Cx32 발현이 세포막 발현에서 세포질 발현으로, 발현의 소실로 진행될수록 Ki67 양성률은 증가하였다. 그리고 Cx32 발현 패턴과 $p21^{Cip1}$ 또는 $p27^{Kip1}$ 양성율의 관계를 조사한 결과, Cx32 발현이 세포막 발현에서 세포질 발현으로, 발현되지 않을수록 $p21^{Cip1}$과 $p27^{Kip1}$의 양성률은 감소하였다. 또한 Cx32 발현 패턴과 세포 부착 단백질 E-cadherin의 발현 패턴 관계는 Cx32 발현이 세포막 발현에서 세포질 발현으로, 발현되지 않을수록 E-cadherin도 세포막 발현에서 세포질 발현으로, 발현의 소실로 변화되었다. 그리고 다른 잘 알려진 세포 부착 단백질 β-catenin의 경우, Cx32와의 관계는 Cx32 발현이 소실될수록 β-catenin는 세포막 발현에서 세포질 발현으로, 핵 내 발현이 관찰되었다. 다음으로 위암세포주 AGS 세포를 이용하여 Cx32의 역할을 알아보았다. Cx32 cDNA가 주입된 AGS 세포는 대조군과 비교하여 세포의 증식이 유의성 있게 억제되었으며, G_1기 비율이 높았으며 S기 비율은 감소되어 세포 주기 분포에서도 영향을 주었다. Real-time 역전사 중합효소 연쇄 반응(reverse transcription polymerase chain reaction)과 Western blot 검사를 실시하여, $p21^{Cip1}$과 $p27^{Kip1}$의 발현 수준을 검사한 결과 Cx32 cDNA가 주입된 AGS 세포는 대조군과 비교하여 $p21^{Cip1}$과 $p27^{Kip1}$ 발현은 증가하였다.

면역조직화학검사 결과를 바탕으로 Cx32의 변화된 발현, 즉, 세포질에서의 발현 또는 소실은 위암과 전암 병변에서 관찰되었다. 그리고

Cx32 발현과 위치변화는 세포 증식과 관련이 있으며, 위암세포주 AGS 세포를 이용한 생체 외 실험에서도 Cx32는 세포 주기 분포를 변화시키고 $p21^{Cip1}$과 $p27^{Kip1}$ 발현을 증가시켜 세포 증식에 영향을 주었다. 이와 같은 결과를 바탕으로 Cx32는 위암 형성에 중요한 역할을 한다고 생각된다.

주요어 : connexin32, 암, 세포증식, 세포 간 신호전달, 위, AGS cell

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