Pathological significance of connexin 26 expression in colorectal adenocarcinoma

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Abstract. This study was conducted to determine the level of expression and cellular localization of connexin 26 (Cx26) and the expression of p53 in colorectal adenocarcinoma as well as their relationship to clinicopathological features. Immunohistochemical staining was performed in 130 colorectal adenocarcinoma cases. A correlation between the expression levels of the two proteins and an analysis of the clinicopathological features of the samples was performed. There was a statistical significant difference in the Cx26 expression level among normal epithelium (NE), adenomas and adenocarcinomas (p<0.001). Of the 130 adenocarcinomas, 48.5% were positive for Cx26. All of the adenoma and NE samples were positive for Cx26 expression; however, the level of expression of Cx26 in adenomas was smaller than the level of expression for NE. Cytoplasmic staining for Cx26 was observed in the adenocarcinomas (23.8%), but was not observed in the adenoma and NE samples. A positive correlation between a reduction in intercellular Cx26 and tumor invasion was statistically significant (p<0.05). Expression of p53 was positive for 50% of the adeno-carcinomas, and the level of p53 was increased in a reverse proportion to the level of Cx26 intercellular staining. In conclusion, loss of intercellular and gain of intracytoplasmic Cx26 expression may play a role in colorectal carcinogenesis and tumor progression.

Introduction

One major cellular function is the maintenance of homeo-stasis. If impairment of the homeostatic state develops, various diseases, including malignant tumors, can develop. Gap junction intercellular communication (GJIC) controls proliferation, differentiation, migration, and other cellular functions and plays an important role in the maintenance of homeostasis and tumor suppression. Intercellular communi-cation primarily is mediated through the gap junction, and minute water-soluble molecules (such as small metabolites, secondary messengers and inorganic ions) can migrate directly to the cytoplasm of adjacent cells (1).

The structural unit of the gap junction is the connexon, which is composed of six connexins (Cx). The loss or dysfunction of GJIC or a mutation occurring in one of the connexin genes allows normal cells to evade normal growth control, resulting in their transformation to tumor cells (2-8). It has been previously reported that various types of malignant tumors, including skin, lung, and liver cancers (9-12), glioblastomas (13), and prostate cancers (14) are associated with the downregulation of Cx expression. Modified expression of Cx, such as intracytoplasmic localization, has been reported to be associated with a malignant phenotype (12,15,16). It has been shown that in cell lines defective in Cx expression, transfection of cDNA encoding connexin protein causes the recovery of the inter-cellular functional transmission system, resulting in the suppression of tumor growth (17,18). As the gap junctions enable cell-cell communication and are involved in the suppression of tumor development, the Cx family of proteins can be considered tumor suppressors (19,20).

It has been reported that in breast and ovarian cancer, as the clinical stage becomes more advanced, the expression of Cx26 is elevated (21). It has also been reported that Cx26 forms a heterologous gap junction with endothelial cells of the blood vessels in the vicinity of tumors, and thus facilitates the intravasation and extravasation of tumors and controls the invasion of the metastatic potential of tumor cells (22). Hence, it appears that the actual role of Cx and the gap junction may be different depending on tumor type and stage of progression.

In colorectal adenocarcinoma, it is known that the early loss and mutation of the *APC* gene appear prior to the formation of polyps. This accompanies the alteration of DNA methylation, and subsequently, in small-size adenomas, the *K-ras* gene is mutated. During the growth of an adenoma, the loss of the 18q chromosome arm occurs, and during the transition period from an adenoma to adenocarcinoma, tumor cells also undergo a sequential genetic change resulting in the mutation of the p53 gene (23). Therefore, a mutation of p53 is thought to play a major role in tumor progression, but not in the process of initiation of a colorectal adenocarcinoma.

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In the present study, through immunohistochemical methods, expression of Cx26 which is normally present on epithelial cells of the colorectum was evaluated. The specimens were classified as colorectal adenocarcinoma, adenoma, and normal epithelium, and the expression level of the protein as well as the expression pattern were evaluated. An association with the depth of invasion, nodal status, other organ metastasis, patient clinical stage, survival rate, survival length, and other previously known clinical factors related to prognosis was examined. Together with the status of p53 expression in colorectal adenocarcinoma, we characterized the role of Cx26 in tumorigenesis and in the progression of the cancer.

Materials and methods

Case selection and tissue sampling. One hundred and thirty subjects were chosen from patients diagnosed with colorectal adenocarcinoma who underwent surgery for the removal of tumors at the Department of Surgery, Chosun University Hospital from January 1992 to December 2004. Patients were selected in a non-consecutive manner when survival and the date of death could be assessed, when paraffin-embedded tissue storage conditions were adequate and when complete medical records were obtainable. In addition, for comparative analysis, 12 cases of colon adenoma that were obtained by endoscopic resection and 10 cases of normal mucosa obtained by endoscopic biopsy without specific histological abnormalities were included. In the selection of subjects, patients that received chemotherapeutic or radiation therapy prior to surgery, patients that received emergency surgery, or patients with hereditary non-polyposis colon cancer or with proof of familial adenomatous polyposis were excluded. Informed consent was obtained from each subject according to institutional guidelines, and the research protocol was approved by the Ethics Committee of Chosun University Hospital.

Histopathological analysis
Microscopic examination. Each tumor was re-examined retrospectively by analyzing the medical record and tissue slide of the patient. Age, gender, the histological pattern, the depth of invasion of the tumor, the presence or absence of metastasis to the lymph node or other organs, the expression of Cx26 as well as p53 protein were assessed. The tumor stages were determined according to the TNM staging system of the American Joint Committee on Cancer (24). The tissue samples to be observed were fixed in 10% neutral-buffered formalin, and the prepared paraffin-embedded tissues were sectioned 4–5 mm in thickness. Hematoxylin and eosion (H&E) staining was performed, and the slides were examined under a light microscope.

Immunohistochemical analysis. All the samples investigated in this study were tested using goat polyclonal Cx26 antibody (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal p53 protein antibody (DO-7) (Dako, Glostrup, Denmark). Immunolocalization was performed using the ImmunoCruz™ Staining System (Santa Cruz Biotechnology), according to the manufacturer's protocol. Briefly, 4-μm sections obtained after formalin fixation and paraffin embedding were deparaffinized in xylene and rehydrated with distilled water through a graded series of ethanol solutions. The sections were then placed in a glass jar with 10 mM citrate buffer (pH 6.0), and irradiated in a microwave oven for 15 min. The sections were allowed to cool in the jar at room temperature for 20 min. The slides were then rinsed with Tris-buffered saline (TBS). Blocking reagent was added for 10 min after quenching of the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. The slides were then washed as described above, and they were subsequently subjected to the primary antibody reaction. Cx26 (dilution 1:200) or p53 protein (dilution 1:100) was applied to the tissue sections, and they were allowed to incubate in a moist chamber overnight at 4˚C. After washing with TBS, a biotinylated antibody was added for 10 min, followed by the addition of streptavidin peroxidase for an additional 10 min. After washing out the excess complex, the localization of antibodies was visualized by incubating the sections for 15 min in the UltraVision Plus Detection System (Lab Vision, Fremont, CA, USA), and counterstaining was performed with Mayer's hematoxylin. The positive control for Cx26 was normal colon mucosa. Instead of the primary antibody, TBS was used as a negative control.

Analysis and interpretation of the immunohistochemical stains. Two pathologists who were blind to the clinical outcome of the samples performed an evaluation of the staining results. The result of Cx26 staining was classified as intercellular staining and intracytoplasmic staining, and regardless of the staining pattern, the percentage of positive tumor cells was determined, and depending on the staining level, reclassified as negative (<10% positive) and positive (≥10% positive) (25).

Regarding intracytoplasmic staining, when a positive reaction in the cytoplasm was distinctly detected, it was considered to be positive. Concerning the p53 protein, nuclear staining was considered to be reactive, and the area showing a positive result was expressed as a percentage, and, according to the intensity of the staining, was classified as 0 (0-9%), 1+ (10-39%), 2+ (40-69%), and 3+ (70-100%).

Statistical analysis. Information concerning patient survival was obtained from the hospital records and from the results of a survey conducted by the National Statistical Office. The mean follow-up time was 115 months (range, 38.2-155). For statistical analysis of the association of the expression of Cx26 as well as p53 protein and the various clinico-pathological parameters, the χ^2 test, Fisher's exact test, ANOVA, and the Student's t-test were applied. The level of significance was set at $p < 0.05$ for all cases. For statistical analysis, the Stat View software package (Abacus Concepts, Berkeley, CA, USA) was used.

Results

Clinical and histopathological findings. There were 62 male and 68 female patients (male:female ratio, 1:1.1). There were 32 cases (24.6%) of patients 50-59 years of age, 46 cases (35.4%) of patients 60-69 years of age, and 29 cases (22.3%) of patients 70–79 years of age. The stages of the

Table I. Relation between Cx26 immunoreactivity and the clinicopathologic features of the adenocarcinomas of the colorectum (%).

	No.	Intercellular staining		Cytoplasmic staining	
		+	-	+	-
Gender					
M	62	25 (40.3)	37 (59.7)	15 (24.2)	47 (75.8)
F	68	25 (36.8)	43 (63.2)	16 (23.5)	52 (76.5)
Age					
30-39	5	1 (20)	4 (80)	2 (40)	3 (60)
40-49	13	4 (30.8)	9 (69.2)	1 (7.7)	12 (92.3)
50-59	32	13 (40.6)	19 (59.4)	11 (31.3)	21 (65.6)
60-69	46	19 (41.3)	27 (58.7)	11 (23.9)	35 (76.1)
70-79	29	11 (37.9)	18 (62.1)	5 (17.2)	24 (82.8)
80-89	5	2 (40)	3 (60)	1 (20)	4 (80)
T stage[a]					
1	5	3 (60)	2 (40)	1 (20)	4 (80)
2	25	13 (52)	12 (48)	4 (16)	21 (84)
3	97	34 (35.1)	63 (64.9)	24 (24.7)	73 (75.3)
4	3	0	3 (100)	2 (33.3)	1 (66.7)
N stage					
0	86	34 (39.5)	52 (60.5)	21 (24.4)	65 (75.6)
1	41	16 (39)	25 (61)	7 (17.1)	34 (82.9)
2	3	0	3 (100)	2 (33.3)	1 (66.7)
M stage					
0	127	49 (38.6)	78 (61.4)	29 (22.8)	98 (77.2)
1	3	1 (33.3)	2 (66.7)	2 (33.3)	1 (66.7)
Clinical stage					
I	30	17 (56.7)	13 (43.3)	6 (20)	24 (80)
II	56	14 (25)	42 (75)	16 (28.6)	40 (71.4)
III	41	19 (46.3)	22 (53.7)	7 (17.1)	34 (82.9)
IV	3	0	3 (100)	2 (33.3)	1 (66.7)

[a]Statistically significant difference between loss of intercellular Cx26 expression and T stage, p<0.05.

Figure 1. Immunohistochemical staining for Cx26 in a colonic adeno-carcinoma. A decrease in intercellular Cx26 expression is noted in the adenocarcinoma (left) as compared with the normal epithelium (right) (LSAB method, counterstained with hematoxylin).

Figure 2. Immunohistochemical staining for Cx26 in a colonic adenoma. Intercellular immunoreactivity is identified but significantly decreased in the adenoma with severe dysplasia (LSAB method, counterstained with hematoxylin).

tumors (T, N and M) and the clinical stages of the 130 cases are listed in Table I.

Clinicopathologic significance of Cx26 expression
Positive cell rate of Cx26. Immunohistochemical staining for Cx26 was performed, and the number of cells showing positive expression of all of the tumor cells was expressed as a percentage (the positive cell rate). Only cases showing a >10% positive cell rate were determined to be positive, and the association between the clinicopathological parameters and the differences between different cell types were compared and analyzed. Of the 130 adenocarcinoma cases, 63 cases (48.5%) were Cx26 positive (Fig. 1) and 67 cases (51.5%) were negative. For adenoma and normal mucosa cases, 12 out of 12 adenomas were Cx26 positive (Fig. 2) and 10 out of 10 normal mucosa were Cx26 positive; the level of Cx26 positive expression for all of the cell types was statistically
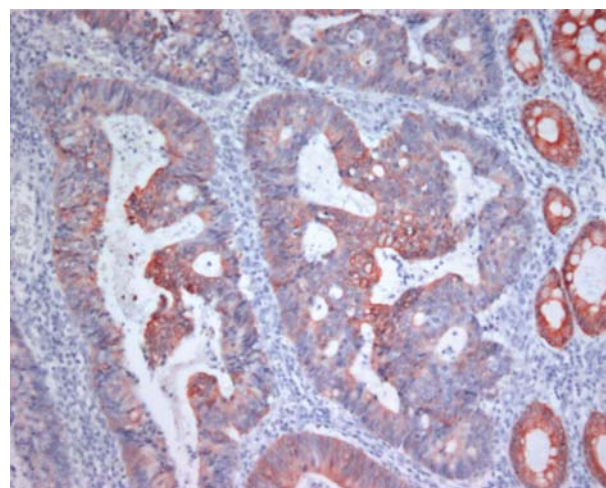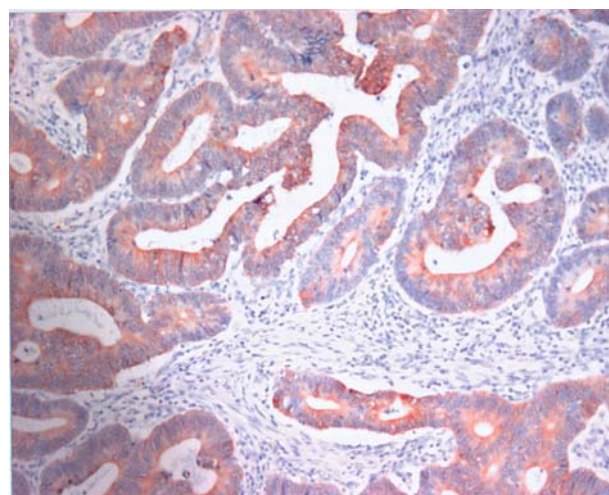
significant (p<0.0001) (Table II). The positive cell rate of adenocarcinomas showed a distribution of 0-80%, and the mean positive cell rate was 18.6%. Regarding the positive cell rate of Cx26 without distinguishing for intercellular staining and intracytoplasmic staining, the various clinicopathological parameters such as the depth of invasion, the nodal status, the presence or absence of distant metastasis, the survival length, and the tumor stage did not show a statistical significant association.

Intercellular staining of Cx26. For intercellular staining of Cx26, for the 130 adenocarcinoma cases, 50 cases (38.5%) were determined to be positive and 80 cases (61.5%) were negative; however, a significant difference according to age or gender was not found. Depth of invasion, nodal status, distant metastasis, and the clinical stage of the adeno-carcinoma and intercellular staining of Cx26 were compared.

Table II. Difference in Cx26 immunoreactivity among the normal mucosae, adenomas and adenocarcinomas of the colorectum (%).

	No.	Immunoreactivity		Cytoplasmic staining	
		+	-	+	-
Normal mucosae	10	10 (100)	0	0	10 (100)
Adenomas	12	12 (100)	0	0	12 (100)
Adenocarcinomas	130	63 (48.5)	67 (51.5)	31 (23.8)	99 (76.2)
p-value		<0.0001		<0.05	

Table III. Relation between clinicopathologic features and immunoreactivity of p53 protein in the colorectal adenocarcinomas (%).

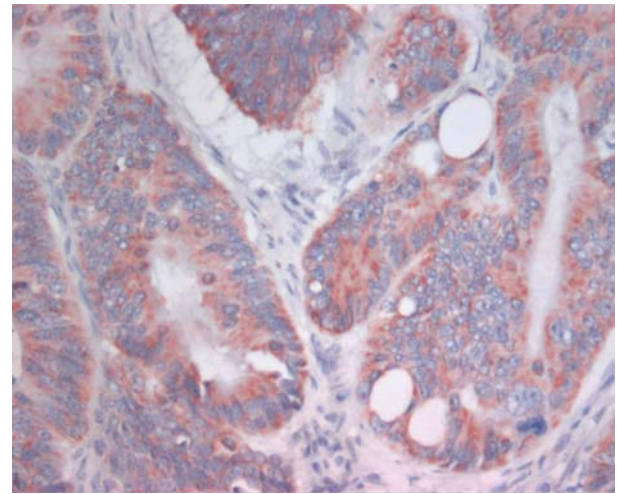|	No.	p53 protein immunoreactivity			
		0	1	2	3
T stage					
1	5	3 (60)	1 (20)	1 (20)	0
2	25	13 (52)	1 (4)	6 (24)	5 (20)
3	97	49 (50.5)	11 (11.3)	23 (23.7)	14 (14.4)
4	3	0	0	0	3 (100)
N stage					
0	86	45 (52.3)	10 (11.6)	19 (22.1)	12 (14)
1	41	20 (48.8)	3 (7.3)	11 (26.8)	7 (17.1)
2	3	0	0	0	3 (100)
M stage					
0	127	65 (51.6)	13 (103)	30 (23.8)	19 (14.9)
1	3	0	0	0	3 (100)
Clinical stage					
I	30	19 (63)	4 (13.3)	4 (13.3)	3 (10)
II	56	29 (51.8)	7 (12.5)	14 (25)	6 (10.7)
III	41	17 (41.5)	2 (4.9)	12 (29.3)	10 (24.4)
IV	3	0	0	0	3 (100)

Figure 3. Immunohistochemical staining for Cx26 in a colonic adenocarcinoma. Distinct cytoplasmic immunoreactivity is noted. (LSAB method, counterstained with hematoxylin).
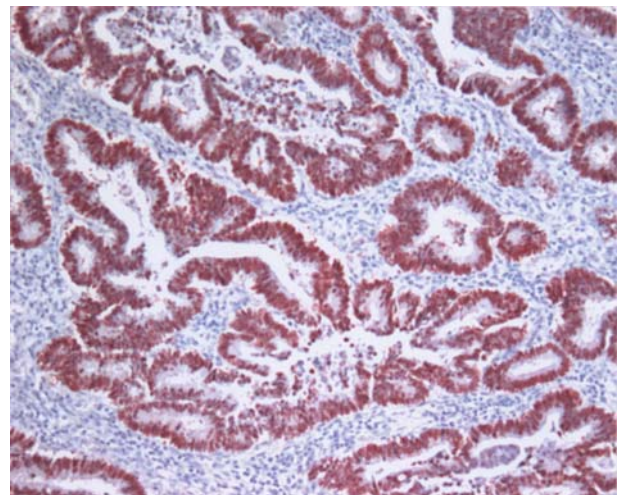

Figure 4. Immunohistochemical staining for p53 in a colonic adenocarcinoma. A strong nuclear staining is noted. (LSAB method, counterstained with hematoxylin).

The loss of Cx26 with invasion to the mucosa was detected in 2 of 5 cases (40%), invasion to the muscle layer was detected in 12 of 25 cases (48%), invasion to the pericolic adipose tissue was detected in 63 of 97 cases (64.9%), and involvement of another organ was detected in 3 of 3 cases (100%). A statistically significant difference for the depth of invasion of the tumors (pT) was observed (p=0.048). The low number of T1, N2 and M1 cases made it difficult to interpret and generalize the results. It is possible that the loss of Cx26 is associated with the progression of an adenocarcinoma, particularly for invasion of the tumors. However, the expression level of Cx26 according to lymph node metastasis (pN), metastasis to other organs (pM), and clinical stage did not demonstrate a significant difference (Table I). In the follow-up examination of patients, an association between the survival rate and the intercellular expression of Cx26 was not statistically significant.

Intracytoplasmic staining of Cx26. Intracytoplasmic staining was confirmed in 31 adenocarcinoma cases (23.8%) (Fig. 3). For all of the adenoma cases and the normal epithelium cases, intracytoplasmic staining was not detected, and thus a significant difference among the cell types was detected (p<0.001) (Table II). Gender, age, tumor invasion depth (pT), lymph node metastasis (pN), metastasis to other organs (pM), clinical stage, and other clinicopathological parameters did not show a correlation to the status of Cx26 intracytoplasmic staining (Table I). In the follow-up examination of patients, an association between patient survival rate and Cx26 intracytoplasmic staining was not statistically significant.

Clinicopathologic significance of p53 protein expression. Expression of p53 protein was found to be positive in 65 adenocarcinoma cases (50%) and negative in 65 cases (50%).

Table IV. Relation between Cx26 immunoreactivity and expression of p53 protein in the colorectal adenocarcinomas (%).

		Cx26 intercellular staining[a]		Cx26 cytoplasmic staining[b]	
		+	-	+	-
p53	0	29 (44.6)	36 (55.4)	11 (16.9)	54 (83.1)
	1	5 (38.5)	8 (61.5)	4 (30.8)	9 (69.2)
	2	10 (33.3)	20 (66.7)	9 (30.0)	21 (70.0)
	3	6 (27.3)	16 (72.7)	7 (31.8)	15 (68.2)

[a]$p < 0.05$, [b]$p > 0.05$.

Expression was classified according to the staining intensity: 13 cases (10%) were weakly positive (1+), 30 cases (23.1%) were moderately positive (2+) and 22 cases (16.9%) were strongly positive (3+) (Fig. 4). The N stage, M stage and clinical stage dependent on the staining intensity of p53 had a positive correlation, but were not statistically significant because of the limited number of samples (Table III).

Comparing the expression level of Cx26 and p53 protein, it was found that in the cases which lost intercellular expression of Cx26, p53 protein was expressed more strongly, and thus a difference among the different case groups was observed ($p=0.035$) (Table IV). The association between the survival rate of patients and expression of p53 protein was not statistically significant.

Discussion

The gap junction is a specialized cell membrane channel that mutually connects the cytoplasm of adjacent cells. Its structural unit is a connexon, and it is a hexameric hemi-channel composed of six Cxs (26). GJIC is involved in the growth and differentiation of cells and thus it plays a role in maintaining homeostasis of tissues (27). It is possible that the intercellular exchange of molecules involved in the growth and death of cells may occur through the gap junctions (28).

The connection of the gap junction is known to decrease with the progression of the level of malignancy of tumor cells. A loss of Cx expression and the deterioration of the gap junction channel in tumors have been confirmed, and the overexpression of each Cx isoform could suppress the metastatic potential of cells. Thus, Cx can be considered a tumor suppressor. The traditional gap junction connection is formed by homotypic connexons, and this connexon is composed of one type of Cx. Generally, in the carcinogenic process, reduction in Cx expression or a reduction in gap junction function are related, and the overexpression of Cx suppresses tumors, which implies the role of the homotypic gap junction which is composed of the same type of connexon (6,29). However, as tumors progress, Cx protein and the gap junction reappear. A connexon composed of heterogeneous Cx subtypes partly forms at the gap junction, resulting in a heterotypic gap junction. It has been reported that depending on the expression level of Cx subtypes, an abnormal cell transduction system between stromal cells of the host and the cancer cells is formed and induces phenotypic transformation of the host stromal cells resulting in the induction of the invasion of cancer cells (22,30). In addition, it has been speculated that by controlling the expression of genes acting on the growth and differentiation of cells, Cx controls the progression of tumors (25). In a study by Ito et al (22), Cx controled the metastatic potential of malignant melanoma. The study suggests that the heterotypic gap junction between vascular endothelial cells and melanoma is involved in metastasis, and the expression of Cx of a heterotypic gap junction accelerates stromal invasion or even facilitates metastasis. Regarding Cx26 expression in the cytoplasm during the carcinogenic process of colorectal adenocarcinoma, it is speculated that the altered type of Cx protein is present in the tumor cell cytoplasm due to a transcriptional or post-transcriptional defect of the Cx protein (25). It is possible that in the cytoplasm of the neoplastic cells, an altered form of the Cx26 protein exists. Krutovskikh et al (31) confirmed that Cx43 was expressed on the cell membrane of normal colorectal epithelial cells together with Cx26 and Cx32, and due to a mutation in the 3M domain of Cx43, instead of being present between cells, Cx protein was present within cells. Thus, Cx protein is present within the nucleus and the cytoplasm. In addition, it is thought that Cx43 present in the nucleus and cytoplasm of tumor cells plays a role in controlling cell growth. Olbina and Eckhart (32) reported that due to the mutation of Cx43, the migration of this protein to the cell membrane is suppressed; nevertheless, it could not reduce the ability of Cx to suppress tumor cell growth. It was confirmed that the control of cell growth by Cx does not always require GJIC. Therefore, it is speculated that Cx protein expressed in the cytoplasm and Cx protein expressed between cells each play a different role in cell-activated signal transduction (25). However, the role of Cx in the signal transduction system requires further clarification and additional functional studies.

In this study, expression of Cx26 was found to be substantially reduced in adenocarcinomas as compared to adenomas and this reduction was statistically significant. In a study by Kanczuga-Koda et al (25), most malignant colorectal tumors showed intracytoplasmic staining of Cx26. In this study, however, only a subset of the adenocarcinoma cases (23.8%) showed intracytoplasmic staining, and when compared to the level of staining for adenomas or normal epithelium, this level was significant. Therefore, loss or reduction of the intercellular expression of Cx26, and an alteration of its cellular localization as seen by intracyto-plasmic staining may be associated with a role of this protein in the carcinogenic process of colorectal cancer. The low level of intracytoplasmic staining and the lack of correlation between intracytoplasmic staining and any of the clinico-pathologic parameters may be explained by the subjectivity

of the interpretation of the staining results and the limited number of samples (especially high-stage samples) that could introduce bias.

The level of tumor invasion (pT) and the loss of Cx26 expression showed a significant correlation, suggesting that Cx26 may play a role in tumor progression, particularly in tumor invasion. In contrast, expression of Cx26 and lymph node metastasis (pN) or metastasis to other organs (pM) were not significantly associated. Of the 130 cases, it was assumed that cases with lymph node metastasis (44 out of 130) were 41 pN1 and 3 pN2 cases. As metastasis to other organs was limited to three cases, this small sample size may have had an influence on the findings.

A postsurgical follow-up observation was performed to examine the association of the status of Cx26 expression and its localization with patient survival. No significant association was found.

Mutation of the p53 protein, a known tumor suppressor gene, is one of the most frequently observed genetic alterations in human tumors. The product of this gene is a nuclear protein that is involved in the cell cycle, apoptosis, and chromosomal stability regulation (33). Results of this study revealed that expression of p53 protein was significantly correlated to the expression of Cx26 on the cell membrane.

In summary, for colorectal adenocarcinomas, reduction in the intercellular Cx26 staining was associated with the depth of tumor invasion, and intracytoplasmic staining of Cx26 was observed only in adenocarcinomas. This finding suggests that the alteration of the expression of Cx26 plays a role in the formation of colorectal adenocarcinomas and their progression. Furthermore, for colorectal adenocarcinomas the loss of intercellular Cx26 staining and the expression of p53 protein showed a significant correlation. Therefore, intracytoplasmic staining and loss of intercellular staining of Cx26 and strong p53 staining of a colorectal adenocarcinoma suggest the presence of a high stage lesion. Additional studies will be required to examine further the role of Cx26 in the carcinogenic process which may be useful for patient prognosis as determined by immunohistochemical staining. Additional studies examining an association between expression of p53 and loss or reduction of Cx26 expression are also suggested.

Acknowledgements

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