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Expression of Survivin in Gastric Carcinoma and its Relation to Tumor Cell Proliferation and Apoptosis

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Background : Survivin, a novel antiapoptotic gene has been linked with tumor development and progression in various human carcinomas including gastric carcinomas. The aim of this study was to evaluate the expression of survivin in gastric carcinoma and its correlation with tumor cell proliferation and apoptosis. **Methods :** Expression of survivin was evaluated immunohistochemically in 84 surgically resected gastric carcinomas. Tumor cell apoptosis was evaluated with terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL), and Ki-67 immunostaining was used for evaluation of tumor cell proliferation. **Results :** Expression of survivin was noted in 53.6% of the gastric carcinomas, and was significantly associated with depth of invasion, status of lymph node metastasis or tumor stage (p=0.022, 0.034, 0.040, respectively). There was an inverse correlation between survivin expression and apoptotic index (p=0.015). But there was no significant correlation between survivin expression and Ki-67 labeling index (p=0.430). **Conclusions :** These results suggest that survivin expression may contribute to tumor development and progression by inhibiting apoptosis in human gastric carcinoma.

Key Words : BIRC5 protein; Cell proliferation; Apoptosis; Stomach neoplasms

Survivin is a member of a family of proteins that inhibit apoptosis, which acts by directly binding to, and curtailing the activity of the cell death proteases caspase-3 and caspase-7.[1,2] Survivin expression has been reported in embryonic and fetal tissues, whereas no survivin expression is detected in non-neoplastic adult human tissues. However, survivin expression resumes in transformed cell lines and malignant tissues.[3,4] Furthermore, survivin is increased in several different types of human cancers including gastric cancer, suggesting that survivin expression correlates with tumor development and progression.[5-12]

The several mechanisms by which survivin may contribute to tumor development and progression are beginning to be understood. These include surviving-mediated inhibition of apoptosis, stimulation of tumor cell proliferation, and enhanced angiogenesis through the production of angiogenic factors.[6-14] We previously reported that survivin may play a critical role in carcinogenesis by stimulating tumor angiogenesis in concert with angiogenic factor in human gastric cancer.[11]

Apoptosis is an essential mechanism for the preservation of homeostasis and morphogenesis of human tissue.[13-15] Disturbance of this process by aberrantly extending cell viability or favoring accumulation of transforming mutation is thought to contribute to carcinogenesis.[13-15]

Rapid and uncontrolled growth of malignant neoplasms compared with normal tissue may be caused by an increased proliferation of tumor cells, a smaller rate of apoptosis, or both.[16,17] Tumor cell proliferation has been accepted as a useful marker for the malignant potential of various cancers. Ki-67 is a monoclonal antibody specific for a nuclear antigen that is present in proliferating cells at the G1, S, G2 and M phases, but it is undetectable in resting cells of the G0 phase. Thus, immunostaining with Ki-67 can be used to detect the proliferative activity of tumor cells.[16,17]

Tumor development and progression results from the accumulation of alteration in genes that regulate cell proliferation and apoptosis.[13-17] Survivin expression is believed to be involved

in tumor development and progression by affecting cell proliferation and apoptosis. However, the relationship between survivin expression and cell proliferation and apoptosis in human gastric cancer has not been fully investigated. Therefore, the aim of this study was to evaluate the expression of survivin in gastric cancer and its correlation with tumor cell proliferation and apoptosis.

MATERIALS AND METHODS

Patients and tumor specimens

We investigated 84 patients with gastric cancer who underwent surgery between January 2006 and December 2007 at Chonnam National University Hospital, Gwangju, Korea. The primary selection criteria were the availability of formalin-fixed and paraffin-embedded blocks and sufficient clinical data. Patient characteristics including sex, age at the time of surgery, histologic grade, stage, and survival data, were obtained by medical records and pathologist and physician contact when necessary. None of the patients had received chemotherapy or irradiation before surgery. The histologic grade was classified according to the criteria of Lauren and the World Health Organization.[18,19] The tumors were staged at the time of surgery by the standard criteria for TNM staging using the American Joint Committee on Cancer.[20] This study group comprised 54 males and 31 females. The mean age was 58.7±10.6 (mean±SD) with a range from 33 to 79 years. The mean tumor size was 4.9±2.5 cm with a range from 0.5 to 13.0 cm.

Immunohistochemistry

Immunostaining was done using a Micro-Probe staining system based on capillary action (Fisher Scientific, Pittsburgh, PA, USA).[21] Paraffin sections, 4 μm in thickness with mounted probe on slides, were immunostained with a polyclonal antibody for survivin antigen (R&D Systems, Minneapolis, MN, USA) by the avidin-biotin peroxidase complex method. Sections were deparaffinized and heated in a microwave oven for 7 min to retrieve the antigens. The sections were then immersed in 0.6% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase activity. Primary antibody diluted, 1:500, in PBS supplemented with 5% normal horse serum and 1% bovine serum albumin was incubated with tissues overnight at room temperature. Anti-mouse immunoglobulin G (Sigma-Aldrich, St. Louis, MO, USA) labeled with biotin was used as a secondary antibody for the detection of bound primary antibody; the secondary antibody was applied for 10 min at 45℃. After multiple rinses with universal buffer, streptavidin-alkaline phosphatase detection system (Biomeda, Foster, CA, USA) was applied for 8 min. The slides were developed for 10 min with the enzyme substrate, 3,3-diaminobenzidine (DAB; Sigma-Aldrich). The slides were counterstained with hematoxylin solution (Research Genetics, Huntsville, AL, USA) for 3 min. After dehydration, the tissue was sealed with a universal mount (Research Genetics). For negative controls, the primary antibody was omitted and replaced with PBS.

Assessment of survivin expression

The immunostaining for survivin, expressed in the cytoplasm was evaluated independently by two observers without knowledge of the clinical outcomes, analyzing the intensity, area and pattern of immunostaining. If there was a discrepancy, a consensus was reached after further evaluation. The staining intensity was graded on a 0-3 scale: 0, no staining of cancer cells; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of staining area was classified as 0 for no positive staining of tumor cells, 1 for positive staining in <10% of the tumor cells, 2 for positive staining in 10-50% of the tumor cells, or 3 for positive staining in >50% of the tumor cells. The intensity rating was multiplied by the percent stain rating to obtain a staining index. The mean staining index for 84 tumors analyzed, was 4.0. Thus, a mean staining index of 4.0 was chosen as the cut-off point for discrimination of status of survivin expression. The tumors were categorized as positive expression (staining index $\geq$4) or negative expression (staining index <4).

Assessment of tumor cell proliferation

Tumor proliferative cells were visualized by staining immunohistochemically using anti-Ki-67 antibody (MIB-1; diluted 1:150; Dako, Glostrup, Denmark). A distinct nuclear immunoreactivity for Ki-67 was considered positive. The Ki-67 labeling index (KI) was determined by observing 1,000 nuclei in areas of the section with highest labeling frequency, and the percentage of KI was used for analysis.

Detection of apoptotic cells and bodies

Apoptotic cells were detected by terminal deoxynucleotidyl

transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL).[22] The TUNEL method for detection of apoptotic cells in 4 μm thick sections of formalin-fixed and paraffin-embedded tissue, was performed using the ApopTag^{TM} Plus In Situ Apoptosis Detection Kit (Intergen, Purchase, NY, USA). Briefly, each section was dewaxed and dehydrated through a graded alcohol series. After they had been digested with proteinase K (20 mg/mL; Sigma-Aldrich) for 15 min at room temperature, endogenous peroxidase was blocked by 2% H_2O_2 in PBS. TdT incubation was then performed for 120 min at 37℃. The reaction was terminated by a prewarmed working strength STOP/WASH buffer and incubated with anti-digoxygenin peroxidase for 60 min at room temperature. After the DAB color reaction, nuclear counterstaining was performed with Mayer's hematoxylin. The positive control sections were treated with 0.7 g/mL DNase I (Sigma-Aldrich) in potassium cacodylate buffer (pH 7.2) for 10 min before treatment with TdT buffer. The negative control sections were performed by substituting distilled water for working strength TdT enzymes. A quantitative method for calculating apoptotic cells was used by two independent observers. All sections were examined in high power fields (×400). The fields were selected in the highest labeled area throughout per case. The apoptotic index (AI) was the number of positive nuclei including apoptotic body among 1,000 tumor cell nuclei.

Statistical analyses

The χ^2-test and Fisher's exact test, where appropriate, were used to compare expression of the survivin with various clinicopathological variables. The correlation between survivin expression and KI or AI was analyzed for statistical significance using Student's *t* test. The statistical software program used was Statistical Package for the Social Sciences (SPSS/PC+ 15.0; SPSS, Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Expression of survivin in tissue specimens

Formalin-fixed and paraffin-embedded blocks for immunohistochemistry were selected by viewing original pathologic slides and choosing blocks that showed a junction between normal gastric epithelium and tumor. Survivin immunoreactivity was more intense in the cytoplasm of the cancer cells than in normal gastric mucosa (Fig. 1), while being undetectable in the stromal compartment of the tumors. The positive expression of survivin in cancerous tissues was 53.6% (45/84).

Correlations between expression of survivin protein and clinicopathological features

Expression of survivin protein was significantly associated with depth of invasion, status of lymph node metastasis or tumor stage (p=0.022, 0.034, 0.040, respectively) (Table 1).

Correlation between expression of survivin protein and tumor cell proliferation

Ki-67 immunoreactivity was predominantly found in the

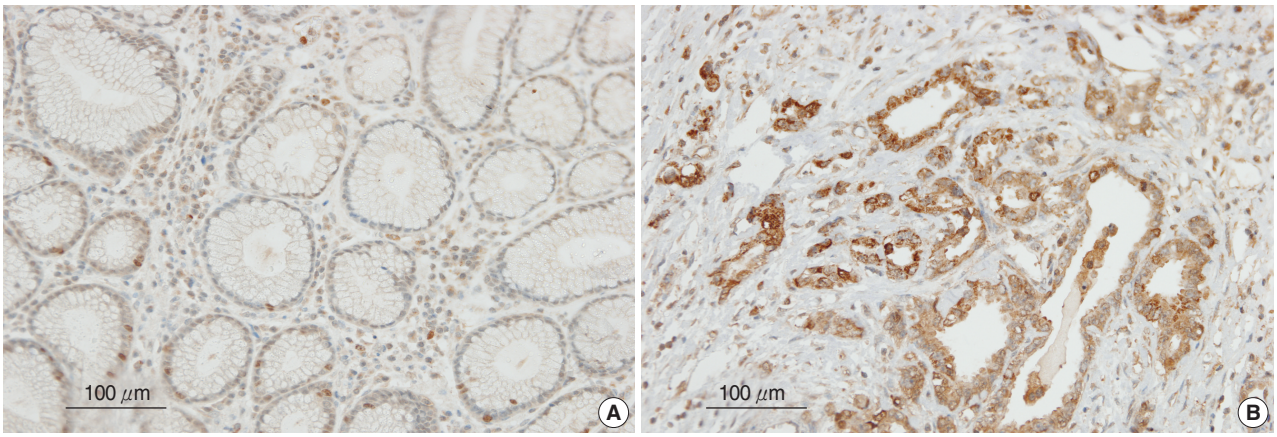

Fig. 1. Immunohistochemical staining of survivin in normal gastric mucosa (A) and gastric cancer tissue (B). Survivin immunoreactivity is more intense in the cytoplasm of the cancer cells than that of normal gastric mucosa (×200).

Table 1. The relationship between survivin protein expression and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Total (n=84)	Survivin protein expression		p
		Positive (n=45)	Negative (n=39)	
Age (years)				
<59	39	18	21	0.204
≥59	45	27	18	
Sex				
Male	53	30	23	0.466
Female	31	15	16	
Lauren Classification				
Intestinal	28	16	22	0.144
Diffuse	25	15	10	
Mixed	21	14	7	
Differentiation grade				
WD	14	5	9	0.133
MD	24	11	13	
PD	46	29	17	
TNM stage				
I	28	9	19	0.040
II	13	8	5	
III	29	20	9	
IV	14	8	6	
Depth of tumor invasion				
T1	12	4	8	0.022
T2	20	7	13	
T3	43	30	13	
T4	9	4	5	
Lymph node metastasis				
Negative	37	15	22	0.034
Positive	47	30	17	
Distant metastasis				
Negative	72	39	33	0.789
Positive	12	6	6	

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated adenocarcinoma.

nuclei of cancer cells. Positive cells were frequent in the advancing margin of the tumor. The KI for 84 tumors ranged from 18.3-85.8 with a mean KI of 54.1±16.6. There was no significant correlation between survivin protein expression and KI (p=0.430) (Table 2).

Correlation between expression of survivin protein and apoptosis

Standard morphologic criteria for identification of apoptosis are the presence of beaded or shrunken chromatin and apoptotic body with a clear halo.[24] Almost all the positively stained cells and bodies were considered to be apoptotic cells corresponding morphologically to these standard criteria. Nonspecific faint and diffuse staining in necrotic foci could be distinguished from the

Table 2. The relationship between KI and the status of survivin protein expression in gastric cancer

Survivin protein expression	Total (n=84)	KI No.	p
		Mean±SD	
Negative	39	52.5±16.7	0.430
Positive	45	55.4±16.8	

KI, Ki-67 labeling index; SD, standard deviation.

Table 3. The relationship between AI and the status of survivin protein expression in gastric cancer

Survivin protein expression	Total (n=84)	KI No.	p
		Mean±SD	
Negative	39	7.1±2.5	0.015
Positive	45	5.7±2.7	

AI, apoptotic index; SD, standard deviation.

apoptotic nuclei by simple morphological examination. The AI for 84 tumors ranged from 0.8-9.9 with a mean AI of 6.4±2.7. The mean AI value of survivin positive tumors was 7.2±2.5 and significantly lower AI than that of survivin negative tumors (5.7±2.7) (p=0.015) (Table 3).

DISCUSSION

Recently, expression of survivin, a member of a family of apoptosis inhibiting proteins, has been demonstrated to be elevated in various cancer types including gastric cancer.[5-12] The present demonstration of increased expression of survivin protein in gastric cancer tissue compared with no detectable levels in the matched normal gastric mucosa suggest that survivin expression may contribute to the evolution of gastric tumor development. At the cellular level, survivin protein immunostaining was predominantly cytoplasmic and was not detectable in the stromal compartment of the tumors. Survivin is usually found in the cytoplasm of various cancer cells and is, therefore, widely regarded as a predominantly cytoplasmic protein expressed in a cell cycle-dependent manner. Although the differential subcellular localization of functionally divergent survivin splicing variants, and immunohistochemically distinct nuclear and cytoplasmic pools have been demonstrated,[23-25] nuclear expression of survivin in this study was negligible.

Cell proliferation, migration, differentiation, apoptosis and angiogenesis are essential mechanisms in tissue homeostasis. Disturbance of these mechanisms leads to malignant transformation and progression. Previous studies have shown that survivin can

predispose to carcinogenesis via these processes tumor development and progression.[5-12] Presently, expression of survivin was significantly associated with depth of invasion, status of lymph node metastasis or tumor stage. There have been some contradictory studies showing that survivin nuclear staining is associated with favorable prognosis or survivin cytoplasmic staining being unrelated with prognosis in gastric cancer and breast cancer.[26,27] These discrepancy may be attributed to the different survivin detection methods, different scoring systems and different antibodies used in immunohistochemistry. Also, three splicing varients of survivin may have differential functions in cancer cells. In spite of some inconsistent results, the bulk of data on many human cancers overwhelmingly supports the idea that survivin cytoplasmic expression is associated with cancer progression and poor prognosis.

Angiogenesis is an essential process for the primary tumor to grow and invade into adjacent normal structures, and is controlled by a balance of angiogenic and angiostatic factors involved in multiple pathways that result in endothelial cell proliferation, differentiation and organization into a functional network of vascular channels.[28,29] Our previous study showed that survivin enhances angiogenesis through the increased production of angiogenic factor, vascular endothelial growth factor in human gastric cancer.[11]

Tumor development and progression is generally regarded as being dependent on increased proliferation rate and an apoptosis rate too low to balance cell growth.[13-17] KI, which is determined by Ki-67 immunohistochemistry, is a well-known proliferation marker that has been extensively used to estimate the growth fraction of tumors.[16,17] Presently, there was no significant difference between survivin protein expression and KI. This result in human gastric cancer is concordant with result obtained by Wakana *et al.*[30] However, another study showed that survivin expression promotes cell proliferation in human hepatocellular carcinoma.[8] Previous studies showed that immunohistochemical detection of survivin protein varies substantially in different human cancers.[5-12] These contradictory results may be related to organ-dependent differential expression of survivin. Until now, the mechanism underlying organ-dependent differential expression of survivin has remained unclear.

Apoptosis is a cellular response that regulates important processes such as tissue homeostasis, defence against certain pathogens and elimination of unwanted cells. Apoptosis is a very complex process regulated by numerous genes. There is evidence that dysregulation of the genes controlling apoptosis could be closely related to tumor development and progression.[13-15] Survivin was recently identified to be a novel antiapoptotic protein that inhibits apoptosis by binding specially to the terminal effected cell death proteases, caspase-3 and caspase-7 in vitro, thereby inhibiting caspase activity and apoptosis in cells exposed to diverse apoptotic stimuli.[1,2] Survivin is detectable during embryonic and fetal development, is undetectable in normal adult tissues,[3,4] and become prominently reexpressed in most common human cancers, including gastric cancer.[5-12] In the current study, the mean AI value of survivin positive tumors was significantly lower than that of survivin negative tumors. Wakana *et al.* reported that survivin messenger RNA expression inversely correlates with AI but there was no statistically significant difference between survivin protein expression and AI.[30] But, Li *et al.*[9] reported that survivin protein expression is inversely correlated with AI consistent with the present study. This discrepancy in survivin protein level may be because of the different detection method of survivin, different scoring system and different antibodies used in immunostaining as a quantitative marker of AI. Also, in the study of Wakana *et al.,* there was a trend (albeit statistically insignificant) towards an association between survivin protein expression and AI.[30] A larger study would probably resolve this issue. These results suggest that survivin messenger RNA or protein expression may be associated with inhibiting apoptosis in gastric cancer.

In summary, expression of survivin protein was presently shown to be significantly associated with depth of invasion, status of lymph node metastasis or tumor stage There was no significant correlation between survivin protein expression and KI. The mean AI value of survivin positive tumors was significantly lower than that of survivin negative tumors. These results suggest that survivin expression may contribute to tumor development and progression by inhibiting apoptosis in human gastric cancer.

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