• GASTRIC CANCER •

Expression level of wild-type survivin in gastric cancer is an independent predictor of survival

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Abstract

AIM: *Survivin* is a novel antiapoptotic gene in which three splicing variants have been recently cloned and characterized. Survivin has been found to be abundantly expressed in a wide variety of human malignancies, whereas it is undetectable in normal adult tissues. We aimed to study the expression of three survivin splicing variants in gastric cancer, and to evaluate the prognostic significance of the expression of survivin variants in gastric cancer.

METHODS: Real time quantitative RT-PCR was performed to analyze the expression of survivin variants in 79 paired tumors and normal gastric mucosa samples at the mRNA level. Proliferative and apoptotic activity was measured using Ki-67 immunohistochemical analysis and the TUNEL method, respectively.

RESULTS: All the cases tested expressed wild-type survivin mRNA, which was not only the dominant transcript, but also a poor prognostic biomarker ($P = 0.003$). Non-antiapoptostic survivin-2B mRNA was correlated with tumor stage ($P = 0.001$), histological type ($P = 0.004$), and depth of tumor invasion ($P = 0.041$), while survivin-$\triangle$Ex3 mRNA showed a significant association with apoptosis ($P = 0.02$).

CONCLUSION: Wild-type survivin mRNA expression levels are of important prognostic value and significant participation of survivin-2B and survivin-$\triangle$Ex3 is suggested in gastric cancer development.

INTRODUCTION

Apoptosis, also called programmed cell death, plays an important role in the development and homeostasis of tissues. Deregulation of apoptosis is involved in carcinogenesis by abnormally prolonged cell survival, facilitating the accumulation of transforming mutations and promoting resistance to immunosurveillance[1]. Several studies have consistently shown that survivin could mediate suppression of apoptosis. Surprisingly, in a single copy of *survivin* gene, three alternatively splicing transcripts have been identified. In addition to wild-type survivin, two novel survivin variants (survivin-2B, survivin-$\triangle$Ex3), which have different antiapoptotic properties, have been generated. Survivin-2B has lost its anti-apoptotic potential, whereas its anti-apoptotic potential is preserved in survivin-$\triangle$Ex3[2,3]. Their different functions in carcinogenesis are largely unknown.

Gastric carcinoma is one of the most frequent human malignancies[4]. As shown by our group[5], 34.5% of gastric cancers expressed survivin protein and a positive correlation between accumulated p53 and survivin expression in neoplasia was found. In this study, we investigated the distribution of survivin variants in paired tumors and normal gastric mucosa samples at the mRNA level and assessed the potential relationship between the expression of survivin variants and proliferative activity, apoptosis or prognostic significance.

MATERIALS AND METHODS

Patients and specimens

Matched pairs of tumors and normal gastric mucosa samples were obtained from 76 patients with gastric cancer and 1 patient with malignant lymphoma at the Department of General and Gastroenterological Surgery, Osaka Medical College Hospital during 2000-2002. The specimens resected at surgery were immediately frozen in liquid nitrogen and stored at -80 ℃ until total RNA extraction. Clinicopathological parameters were assigned according to the principles outlined by Japanese Classification of Gastric Carcinoma[6]. Samples included stage I cases ($n = 22$), stage II cases ($n = 11$), stages III cases ($n = 20$), stage IV cases ($n = 26$). There were 62 (78.5%) males and 17 (22.5%) females, and the mean age of the patients was 65.2 years (SD, 9.6 years; range, 40-87 years). No Patients received chemotherapy or radiation therapy either before or after surgery. The mean follow-up period was 19.7 mo (SD, 14.9 mo; range, 1.5-87 mo). Formalin-fixed paraffin-embedded blocks of primary tumors were taken from pathological archives. Two to four μm thick serial sections of 2-4 thicken were prepared from the cut surface of the blocks at the maximum cross-section of the tumors.

Total RNA extraction

Total RNA was extracted by an acid guanidinium-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Afterwards the total RNA was purified using DNase 1 (GIBCO-BRL, Gaithersburg, MD, USA). Extracted total RNA pellets were dissolved with RNase free diethyl pyrocarbonate (DEPC)-treated water.

Reverse transcription

Complementary DNA (cDNA) was synthesized with 5 μg of total RNA and 20 pmoL oligo (dT)$_{18}$ primer using an Advantage

RNA-for-PCR kit (CLONTECH, Inc., Palo Alto, USA), with the exception of 200 U SurperScript™ II RNase H⁻ reverse transcriptase (Invitrogen, Inc., Carlsbad, USA) in a final 20 μL reaction volume. RT reactions were performed at 50 ℃ for 120 min. Finally, cDNA solution was diluted to a total volume 100 μL.

Quantitative real time RT-PCR

Quantitative real time RT-PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany). As an internal control, housekeeping gene G6PDH mRNA expression was measured at the same time. DEPC-treated H_2O was used as a negative control and MKN-74 was used as a positive control. Then, 1μL of cDNA mixture was subjected to amplification in 10 μL reaction mixture. The PCR conditions were initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 10 s, annealing at 62 ℃ for 10 s for survivin, survivin-2B and survivin-△Ex3 (or at 63 ℃ for G6PDH), extension at 72 ℃ for 10 s, respectively. A standard curve using fluorescent data was generated from serial tenfold dilution of specific plasmids from 10^7 to 10^2 copies, respectively. The number of gene copies was calculated by the LightCycler Software Version3.5.3 according to the Fit Points Above Threshold method. Primer pairs and hybridization probes for survivin, survivin-2B, survivin-△Ex3 and G6PDH mRNA were as follows. The sequence of the common forward primer for survivin variants was 5'-CCACCGCATCTCTACATTCA-3'. To distinguish the 3 splice variants of survivin, the sequences of reverse primers for survivin variants were designed to correspond to exon/exon borders of the complementary strand (5'-TATGTTCCTCTATGGGGTCG-3' for survivin, 5'-AGTGCTGGTATTACAGGCGT-3' for survivin-2B, 5'-TTTCCTTTGCATGGGGTC-3' for survivin-△Ex3). The sequences of common hybridization probes for survivin variants were 5'-CAAGTCTGGCTCGTTCTCAGTGGG-3'-FITC and LCRed640-5'-CAGTGGATGAAGCCAGCCTCG-3'. The sequence of the forward primer for G6PDH was 5'-TGGACCTGACCTACGGCAACAGATA-3'. The sequence of the reverse primer for G6PDH was 5'-GCCCTCATACTGGAAACCC-3'. The sequences of hybridization probes for G6PDH were 5'-TTTTCACCCCACTGCTGCACC-3'-FITC and LCRed640-5'-GATTGAGCTGGAGAAGCCCAAGC-3.

PCR products were additionally checked by electrophoresis on 30 g/L agarose gels (BIO-RAD, Inc., Hercules, USA) containing ethidium bromide and visualized under UV transillumination.

Sequence analysis

To confirm the identity of the PCR products, their bands were excised from agarose gels and isolated by a QIAquick gel extraction kit (Qiagen, Tokyo, Japan), ligated into a pGEM-T-cloning vector (Promega), and cloned according to standard protocols. Plasmid DNA was recovered using a Plasmid Mini kit (Qiagen, Tokyo, Japan). Cycle was sequenced and analyzed in an ABI-PRISM310 (Applied Biosystems) using T7 or SP6-site specific primers. The sequences of the PCR products were compared with those in GenBank, which were found to be identical (Data not shown).

Ki-67 immunohistochemical staining and assessment of proliferative index

Immunohistochemical staining was performed by the standard avidin-biotin-peroxidase complex technique using L.V. Dako LSAB kit (DAKO, Copenhagen, Denmark) as described previously[5,7,8]. The antibodies used were monoclonal mouse antibody Ki-67 (MIB-1, diluted 1：50; Immunotech, Marseilles, France). The labeling index (LI) of Ki-67 was determined in those tumor areas with positive stained nuclei. Five random areas within a section were chosen and counted under 200-fold magnification using a point counting technique. The average percentage of positivity was recorded as the Ki-67 LI for each case[7].

Histochemical detection of apoptosis and determination of AI

Apoptotic cells in tissue sections were detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL), using an Apop Tag *in situ* detection kit (Oncor, Gaithersburg, MD). The staining procedures were based on a method described previously[5,6,8]. The apoptotic index (AI) was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells according to the criteria described elsewhere[5,7]. Five areas were randomly selected for counting under 400-fold magnification.

Statistical methods

All statistical analyses were performed by the SPSS11.0 software package for Windows (SPSS Inc., Chicago, IL). Differences in the numerical data between the two groups were evaluated using the Mann-Whitney U test. The χ^2 test was further used to compare the distribution of individual variables and any correlation between AI or Ki-67 index and expression of survivin variants. The correlation between AI and expression of survivin variants for each case was also analyzed by Spearman's rank correlation test. Survival curves were calculated using the Kaplan-Meier method and analyzed by the log rank test. A two-tailed P value less than 0.05 was considered statistically significant.

RESULTS

Expression of survivin variants in clinical materials and cell lines

Among the 79 tumor samples, survivin expression was detected in all tumor samples (79/79), survivin-2B expression was demonstrated in 78.5% (62/79) of the samples and survivin-△Ex3 expression was detected in 64.6% (51/79) of the samples (Figure 1). In contrast, survivin expression was detectable in 46 (58.2%) of the normal mucosa samples, while survivin2B expression and survivin-△Ex3 were detected in 23 (29.1%) and 12 (15.2%) of the mucosa samples respectively.
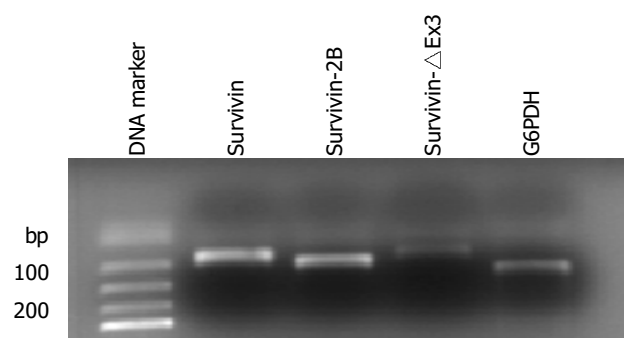

Figure 1 Amplification, separation and visualization of survivin (185 bp), survivin-2B (214 bp) survivin-△Ex3 (184 bp), and G6PDH (256 bp) mRNA (40 cycles) in a typical case.

The relative amounts of survivin variant mRNA were determined by dividing the amount of survivin variant mRNA by that of G6PDH mRNA for each sample. In tumor samples, the relative levels of survivin-2B and survivin-△Ex3 was further normalized by matched survivin. Because three alternatively spliced variants are derived from a common hnRNA precursor pool, these ratios seemed to be independent of any possible bias imposed by variations in housekeeping gene expression levels[9,10]. Although there was a significant difference in G6PDH expression between normal tissues and cancer samples at the same amount of total RNA used ($P<0.0001$, Mann-Whitney U test, Figure 2A), the survivin variant/G6PDH ratio in our cancer tissues were significantly higher than that in non-neoplastic tissues ($P<0.0001$, Mann-Whitney U test, Figure 2B).

Table 1 Relationship among expression levels of survivin variants and clinicopathologic parameters, proliferative activity or apoptosis

Parameters	No. of patients (%)	Sur/G6PDH	Sur-2B/G6PDH	Sur-△Ex3/G6PDH	Sur-2B/sur	Sur-△Ex3/sur
Gender (median)						
Male	62 (78.5)	$P = 0.445$	$P = 0.598$	$P = 0.079$	$P = 0.125$	$P = 0.408$
Female	17 (22.5)					
Age yr (median)						
<64	39	$P = 0.462$	$P = 0.108$	$P = 0.177$	$P = 0.129$	$P = 0.262$
>64	40					
Histological type						
Differentiated	47	$P = 0.976$	$P = 0.004$	$P = 0.151$	$P = 0.053$	$P = 0.231$
Undifferentiated	32					
Depth of invade						
Not invasion	27	$P = 0.336$	$P = 0.042$	$P = 0.266$	$P = 0.354$	$P = 0.555$
Invasion	40					
Clinical stage						
Stage I+II	(22+11)	$P = 0.075$	$P = 0.001$	$P = 0.139$	$P = 0.018$	$P = 0.863$
Stage III+IV	(20+26)					
Ki67 (median)						
Low	39	$P = 0.791$	$P = 0.197$	$P = 0.567$	$P = 0.401$	$P = 0.512$
High	40					
Apop Tag (median)						
Low	39	$P = 0.421$	$P = 0.070$	$P = 0.0020$	$P = 0.408$	$P = 0.099$
High	40					

Two-tailed *P* values were calculated by the Mann-Whitney U test. Differentiated type: Papillary type, Tubular type. Undifferentiated type: Poorly differentiated type, Signet ring cell type, Mucinous type. Not invasion: No invasion of the serosa. Invasion: invasion of the serosa.
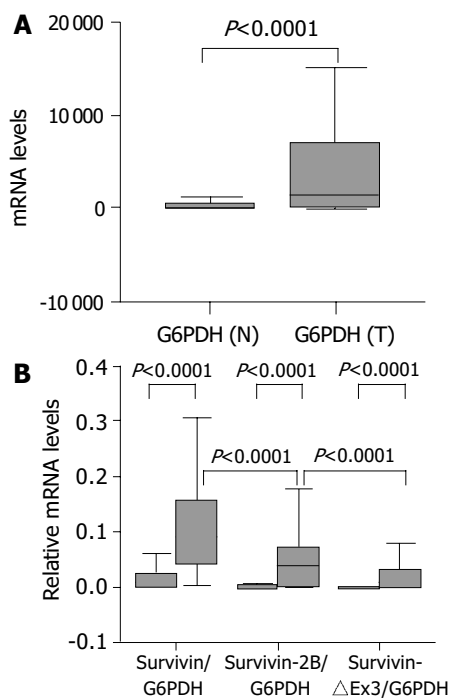
Figure 2 A: RT-PCR amplification of G6PDH in paired samples of normal gastric mucosa and gastric carcinoma. B: Relative (G6PDH-normalised) mRNA levels of survivin variants in paired samples of normal gastric mucosa and gastric carcinoma. Two-tailed *P* values were calculated by the Mann-Whitney *U* test.

Relationship between expression levels of survivin variants and clinicopathological parameters

The expression level of survivin-2B was significantly ($P = 0.001$, Table 1, $P<0.0001$, χ^2 test, Figure 3A) decreased in advanced (III+IV) stage compared with early (I+II) tumor stage. Furthermore, the level of survivin-2B was inversely correlated with the grade of tumor differentiation ($P = 0.004$, Table 1, $P = 0.023$, χ^2 test, Figure 3B) and depth of tumor invasion ($P=0.042$, Table 1, $P = 0.021$,

χ^2 test). A stage-dependent decrease of survivin-2B was also confirmed by the ratio of survivin-2B/survivin ($P = 0.018$, Table 1, $P = 0.022$, χ^2 test, Figure 3C).

None of the investigated clinicopathological parameters showed a statistically significant correlation with the expression of other types of survivin variants besides survivin-2B.

Correlation between expression levels of survivin variants and AI

Apoptotic cells and bodies were detected in all cases of gastric carcinoma tested (Table 1, Figure 4A). The mean AI was 1.07% (SD, 0.41%; range, 0.38-2.38%) with a median of 0.95%. The mean AI in survivin-△Ex3-low expression samples was significantly higher than that in survivin-△Ex3-high expression samples ($P = 0.02$, Table 1, $P = 0.028$, χ^2 test, Table 1). There was no correlation between apoptotic index and clinicopathological parameters described. Spearman's rank correlation test demonstrated a significant negative correlation between apoptotic index and expression level of survivin-△Ex3 (Γ = -0.257, $P = 0.022$).

### Association of expression levels of survivin variants with PI

Proliferative cells were detected by intense nuclear immunoreactivity to the Ki-67 antigen, as illustrated in Figure 4B. The mean proliferative index (PI) in gastric tumors tested was 48.46% (SD, 9.54%; range, 28.49-82.98%) with a median of 46.87%. No significant relationship was identified between PI of tumor cells and clinicopathological parameters, as demonstrated in Table1. In addition, no significant association was found between PI and any type of survivin variants.

### Prognostic value of survivin mRNA expression

Postoperative survival of the 79 gastric cancer patients was analyzed according to the expression level of survivin variants. Results are shown in Figure 5. Each survivin variant value was divided by the median value. The survival curve of patients with high-survivin expression was significantly lower than that of those with low-survivin expression ($P = 0.003$, log-rank test). No prognostic significance for either survivin-2B or survivin-△Ex3, however, was found in the current study.
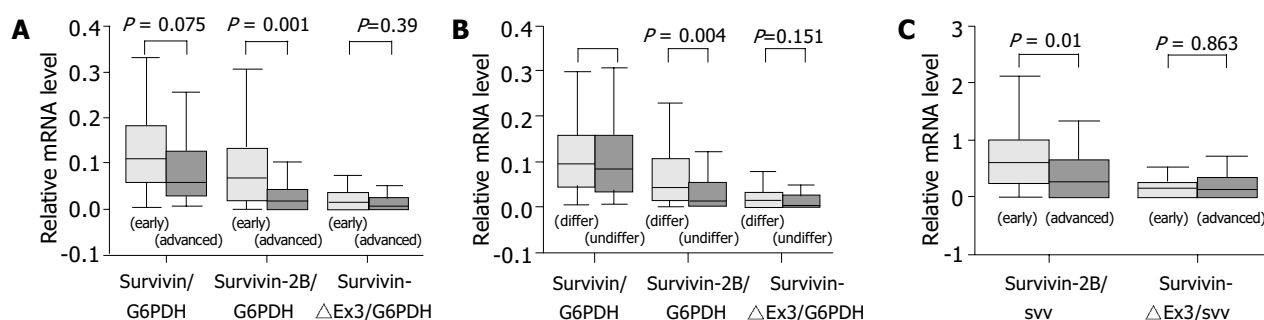
Figure 3 Relative mRNA levels of survivin variants. A: Significant decrease of relative (G6PDH-normalised) mRNA levels of survivin-2B in advanced stages ($P = 0.001$, Mann-Whitney U test). B: Significant decrease of relative (G6PDH-normalised) mRNA levels of survivin-2B in undifferentiated type ($P = 0.004$, Mann-Whitney U test). C: Significantly lower ratio of survivin-2B/survivin in advanced stages than that in early stages ($P = 0.018$, Mann-Whitney U test).
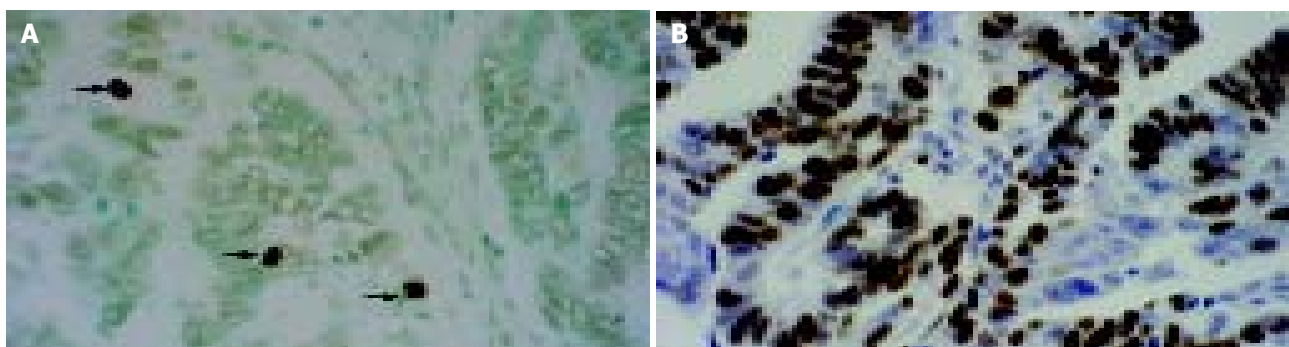
Figure 4 Histochemical staining for Ki-67 and apoptotic tumor cells. A: TUNEL *in situ* labeling for detecting apoptotic cells and bodies (arrows, magnification ×400) in adenocarcinoma. B: Ki-67 nuclear immunohistochemical staining revealing proliferating tumor cells in adenocarcinoma (magnification ×400).
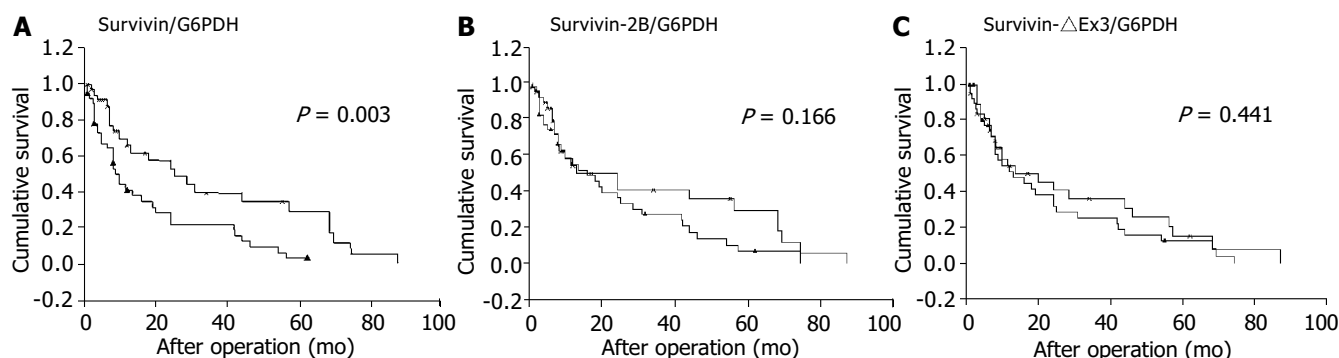
Figure 5 postoperative survivals of 79 gastric carcinoma patients. A: Shorter survival time of patients with high expression of wild-type survivin mRNA compared to patients with low expression of wild-type survivin expression ($P = 0.003$, log-rank test). B: No significance in expression of survivin-2B mRNA ($P = 0.166$, log-rank test). C: No significance in expression of survivin-△ Ex3 mRNA ($P = 0.441$, log-rank test).

DISCUSSION

In this study, the prognostic significance of the expression of three survivin splicing variants in gastric carcinoma was examined for the first time. Interestingly, only increased wild-type survivin mRNA expression was prominently correlated with reduced survival time, whereas neither survivin-2B nor survivin-△Ex3 showed an association with prognosis. In accordance with recent studies, we did not find a correlation between mRNA expression levels of anti-apoptotic variants (survivin or survivin-△Ex3) and the investigated clinicopathological parameters[9,10]. There is no doubt, however, survivin plays a role in enhancing the malignant behavior of tumors. In cell culture systems, over-

expression of survivin was consistently associated with inhibition of cell death[2]. Strong survivin mRNA expression showed chemo-radio resistance[11-13] and a correlation with recurrence in patients[14]. In retrospective trials, increased survivin mRNA or protein expression was reported to be a prognostic indicator of tumor progression in different types of human cancer[15-19]. Our observations further confirmed that survivin mRNA was a poor prognostic biomarker. This suggests that a relatively straightforward detection of survivin protein or mRNA in clinical patients may provide an initial marker of aggressive diseases, potentially requiring in-depth follow-up protocols or alternative treatment regimens in the future.

The notion that survivin inhibits apoptosis has been established, but the mechanism (s) by which this occurs has not been conclusively determined. The actual molecular interaction partners of survivin variants, however, have remained elusive[2]. Although there is a good agreement that deregulation of cell death/survival pathways contributes to malignant transformation, the potential predictive/prognostic impact of apoptosis regulatory molecules and AI has remained controversial[8]. This study failed to show the relationship between apoptotic index and survivin expression in gastric carcinoma although it was shown in previous[5,20] studies showed in gastric cancer that survivin expression was inversely correlated with apoptosis. These controversial findings were attributed in part to the dual function by which survivin regulates cell death and cell division. In recent studies, frameshift carboxyl terminus of survivin-△Ex3 was found to contain a bipartite localization signal, which mediates its strong nuclear accumulation and might interfere with degradation of survivin-△Ex3 protein by ubiquitin tagging. Survivin-△Ex3, therefore, could evade cell cycle-specific degradation by the ubiquitin-proteasome pathway, known for survivin. In contrast, cytoplasm has been found to be the most probable location of both survivin and survivin-2B[21,22]. Other unique features in survivin-△Ex3 were also identified, including a mitochondrial localization signal and a BH2 domain, which might inhibit apoptosis by association with bcl-2, and suppress Caspase-3 activity by BIR-dependent pathway[23]. Consequently, survivin-△Ex3 may play a more crucial role in the regulation of apoptosis.

Although mRNA levels of survivin-2B and survivin-△Ex3 have been found in different tumors[3,10,24,25], little is known about the protein levels of endogenous survivin-2B and survivin-△Ex3, due to the lack of survivin variant-specific antibodies. Our results showed that a low mRNA expression level of survivin-2B was inversely correlated to the advanced (III+IV) stage, undifferentiated tumor histology and deep tumor invasion. There are at least two reasonable explanations for this finding. After losing its antiapoptotic potential, survivin-2B was hypothesized to be a naturally occurring antagonist of survivin from competitive binding to heterologous interaction partners, or from the formation of inactive survivin: survivin-2B heterodimers with respect to recently observed dimer formation of survivin[21]. Downregulation of survivin-2B might weaken its functional antagonism, and moreover, could permit the generation of more survivin or survivin-△Ex3 because three survivin variants come from the same pool of a hnRNA precursor. Thus, the decrease in survivin-2B may result in the development, invasion and anaplasia of carcinomas.

In addition, our data showed that all gastric carcinoma samples expressed wild-type survivin at a significant level compared to the matched normal gastric mucosa samples. Similarly, the expression of survivin-2B or survivin-△Ex3 was significantly elevated in carcinoma tissue. Among three survivin variants, survivin was the dominant transcript. In this regard, our results were consistent with those of two recent TR-PCR studies[10,26] on gastric cancer in which a few gastric mucosa samples (17-23%) of the first-degree relatives also expressed survivin mRNA, but the normal control subjects did not. All these findings may help explain why survivin variants played important roles in the early stage of carcinogenesis of gastric cancer.

In summary, survivin mRNA is a poor prognostic biomarker and that non-antiapoptotic survivin-2B was correlated to tumor stage, histological type and depth of tumor invasion. Even more striking is the observation for the first time that neither survivin nor survivin-2B show a close association with the apoptosis index, as observed in survivin-△Ex3, although further experimental work is necessary to elucidate the functional properties of different splicing variants in more detail.

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