Gastric Cancer (2003) 6: 217–224
DOI 10.1007/s10120-003-0255-2

Original article

Correlation between survivin mRNA expression and lymph node metastasis in gastric cancer

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Abstract

Background. **Correlations between the malignant potential and prognosis of cancer and abnormal control mechanisms of apoptosis have been discovered in a variety of cancers. Survivin is a member of the inhibiting apoptosis protein family that is abundant in embryonic and carcinoma tissues. We measured the expression of survivin mRNA in gastric cancer to determine whether levels of survivin mRNA expression could serve as an index of malignancy.**

Methods. **Expression of survivin mRNA was measured in samples of both gastric cancer and noncancerous tissue from 107 patients. Survivin mRNA was detected by the real-time polymerase chain reaction (PCR) method, and then the relationship between the survivin mRNA level and histological diagnosis was analyzed.**

Results. **Expression of survivin mRNA was observed in 105 of 107 cancerous tissues and in 101 of 107 noncancerous tissues. The Mean value of survivin mRNA expression in cancerous tissue was 5.18 ± 1.30, significantly higher ($P < 0.01$) than that in noncancerous tissue, at 4.21 ± 1.48. No significant differences were found in the values of survivin mRNA expression according to histological classification or according to increasing depth of tumor invasion. However, survivin mRNA expression was significantly higher ($P < 0.01$) in patients displaying lymph node metastasis (5.48 ± 1.01) than in patients without the metastasis (4.70 ± 1.55).**

Conclusion. **These results indicate that increased survivin mRNA expression begins in the early stages of gastric carcinogenesis. Moreover, the level of survivin mRNA expression may indicate the potential for lymph node metastasis in patients with gastric cancer.**

Key words Survivin mRNA · Gastric cancer · Noncancerous gastric tissue · Lymph-node metastasis · Real-time PCR

Offprint requests to: K. Miyachi
Received: December 20, 2002 / Accepted: July 28, 2003

Introduction

Apoptosis plays an important role in the developmental morphogenesis [1] and differentiation of fetal human organs and in homeostatic maintenance in mature human organs by removing senescent or unneeded cells [2]. When the functional control of apoptosis is disturbed, homeostatic maintenance becomes impossible, and numerous disorders can result [3]. In the case of such dysfunction, cells that would otherwise be removed by apoptosis remain and multiply, creating the potential for increasing numbers of cells carrying abnormal genes. This suggests an important connection between the abnormal functional control of apoptosis and the neoplastic transformation of cells into a carcinoma. Furthermore, cancerous cells may continue growing by inhibiting the apoptosis induced by natural defense products such as Fas and tumor necrosis factor (TNF), and the apoptosis induced by cancer therapies such as anticancer drugs and radiation [4–7]. The roles of various genes and proteins in the induction and control of apoptosis have been investigated. Research has demonstrated that damaged DNA is put together with the cancer suppressor gene *p53* and that the activated p53 protein sends apoptosis-inducing signals [8,9]. In another pathway, mitochondrial membranes are broken down by an apoptosis-inducing factor, and apoptosis-inducing signals (i.e., cytochrome C) are subsequently discharged from the mitochondria [9]. In this pathway, *bcl-2* acts to stabilize the mitochondrial membrane and thus suppresses apoptosis [10]. Therefore, abnormalities of *p53* and high levels of *bcl-2* expression may indicate dysfunctional control of apoptosis and could, therefore, represent possible indices of malignancy. In addition, malignant potential is reportedly higher for gastric cancer patients displaying these changes in *p53* and *bcl-2* [11,12]. Recently, survivin was discovered as a member of the inhibiting apoptosis protein (IAP) family [13]. Increased levels of survivin expression have been re-

ported in carcinomas [13–15]. The worst prognoses have been noted in patients with hematopoietic disease, neuroblastoma, renal tumor, and mammary cancer in whom survivin expression was high [16–19]. Previous studies have also reported a relationship between survivin expression and both the prognosis and the cell proliferation kinetics in patients with gastric cancer [20,21]. In previous clinical studies of cancer, survivin expression has been examined by immunostaining and immunoblotting for the protein; little investigation has been performed at the genetic level [15,22–24]. In the present study, we examined the expression of survivin mRNA in gastric cancer by the real-time polymerase chain reaction (PCR) amplification method, and then compared semiquantitative values for survivin mRNA expression with histopathological findings from the cancers, to determine whether the level of survivin mRNA expression could serve as an index of malignancy.

Subjects, materials, and methods

Gastric tissue

Samples were obtained from 107 stomachs surgically resected at the First Department of Surgery, Dokkyo University School of Medicine, between September 1999 and August 2002. Tumors were located in the upper third of the stomach in 23 patients, in the middle third in 47, and in the lower third of the stomach in 37. Total gastrectomy was performed in 21 patients, proximal partial gastrectomy in 2, and distal partial gastrectomy in 84. We obtained 107 cancerous tissue samples, and 107 noncancerous tissue samples that were found more than 3 cm away from cancerous tissue. Informed consent was obtained from all 107 subjects in this study. For each tissue sample, one-half was used for histological diagnosis under standard hematoxylin-eosin staining and the other for survivin mRNA measurements.

Histological diagnosis was performed according to the second English edition of the *Japanese classification of gastric carcinoma* [25]. Of the 107 cancerous samples, 52 were differentiated (pap, tub1, and tub2) adenocarcinomas and 55 were undifferentiated (sig, por, and muc) adenocarcinomas. When samples were classified by depth of tumor invasion, 28 were T1, 33 were T2, 31 were T3, and 15 were T4. When samples were classified on the basis of lymph node metastasis, 41 were N0, 27 were N1, 19 were N2, and 20 were N3. In addition, hepatic metastasis was recognized in 6 patients and peritoneal metastasis was recognized in 22 patients.

Real-time PCR

Survivin mRNA was detected using a real-time PCR amplification method [26–29]. Total RNA was extracted from each 5-mg tissue sample by acid guanidine thiocyanate-phenol-chloroform extraction, using RNAzolB (Sawady, Tokyo, Japan) and collected by ethanol precipitation [30]. Complementary DNA was synthesized using a survivin 466–485 primer (5′-AGA GGC CTC AAT CCA TGG CA-3′) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), with an exon 8 primer (5′-CCT GAT GTC ATC ATA TTT GGC AGG-3′) as an internal control. The amplification reaction mixture was prepared using TaqMan Universal Master mix (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primer set for amplifying the survivin mRNA was designed according to data from Gene Bank NM001168, using primers for exon 1 (survivin [SVN]-forward [F]): 5′-AGA ACT GGC CCT TCT TGG AGG-3′ and exons 2–3 (SVN-reverse [R]): 5′-CTT TTT ATG TTC CTC TAT GGG GTC-3′. The probe for these exons (SVN-probe [P]): 5′-AGC GGA TGG CCG AGG CTG GCT TC-3′) was designed to target an internal region between the SVN-F and SVN-R primers. This primer set did not detect survivin-beta mRNA. The primer set for amplification of the G3PDH mRNA was designed according to data from Gene Bank M33197, using primers for exon 7: 5′-TGC ACC ACC AAC TGC TTA GCA CCC-3′ and exon 8: 5′-CTT GAT GTC ATC ATA TTT GGC AGG-3′. The probe for G3PDH-P was based on exons 7–8: 5′-TGA CCA CAG TCC ATG CCG TCA CTG C-3′. Each real-time amplification reaction proceeded for 50 cycles (95 °C for 30 s, 60 °C for 40 s, and 72 °C for 30 s) and was tracked by the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems) (Fig. 1).

The following examination was performed in order to confirm whether the amplified product was indeed survivin. The mRNA amplification products were purified using a commercial kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced using another commercial kit (Thermo Sequence Cy5 Dye Terminator kit; Amersham Pharmacia Biotech, Uppsala, Sweden) and an automated laser fluorescence DNA sequencer (ALF Express; Amersham Pharmacia Biotech). The resulting sequences were compared with target mRNA sequences.

The semiquantitative value for survivin mRNA expression was determined as the index value of the logarithm of the light emission value of the sample RNA relative to that of standard RNA (for example, a value of "5.84" represents $10^{5.84}$ copies per μg total mRNA). Values for results are given as means ± SD unless otherwise stated.

Statistical analysis

Semiquantitative values for survivin mRNA expression in cancerous and noncancerous samples were examined

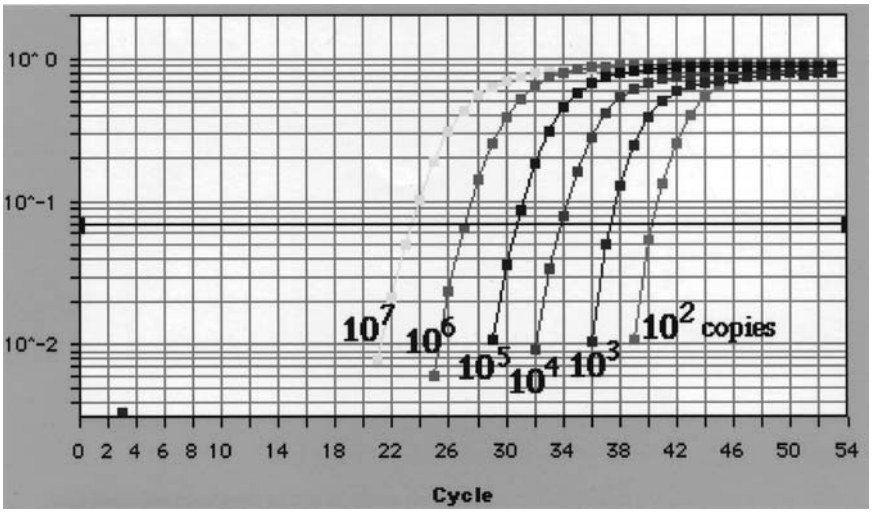

Fig. 1. Real-time polynerase chain reaction (PCR) for serial dilutions of a survivin cDNA clone was performed using a one-step method, with a FAM-labeled reporter primer, a TAMRA-labeled quencher primer, and TaqMan Universal Master Mix (PE Applied Biosystems). Reaction mixtures were incubated, and this was followed by 50 cycles of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 30 s

by using the Wilcoxon signed rank test. The semiquantitative values for survivin mRNA expression were compared with histological classification, depth of tumor, and lymph node metastasis, by using the Mann-Whitney *U*-test. High and low levels of survivin mRNA expression were compared with histological classification, depth of tumor, and lymph node metastasis, using the χ^2 test.

P values of less than 0.05 were judged to be statistically significant.

Results

Expression of survivin mRNA

Survivin mRNA was detected in 105 of 107 (98.1%) cancerous tissues and in 101 of 107 (94.4%) noncancerous tissues: The mean value of survivin mRNA expression in cancerous tissues was 5.18 ± 1.30, significantly higher ($P < 0.01$) than that in noncancerous tissues, at 4.21 ± 1.48 (Figs 2, 3). As survivin mRNA was expressed in most cancerous tissue samples, we divided these samples into these with relatively high and low levels of expression, with the cutoff value being 5.56, the median value of survivin mRNA expression in cancerous tissues.

Relationship between expression of survivin mRNA and histological type

The mean value for survivin mRNA expression in differentiated gastric cancers was 5.17 ± 1.40, not significantly different from that in undifferentiated gastric cancers, at 5.20 ± 1.20 (Table 1). Of the 52 differentiated cancers, 27 (51.9%) showed high survivin mRNA expression. Of the 55 undifferentiated cancers, 27 (49.1%) showed high survivin mRNA expression. No apparent relationship was found between high survivin mRNA expression and the histological type of gastric cancer; increased survivin expression was thus recog-

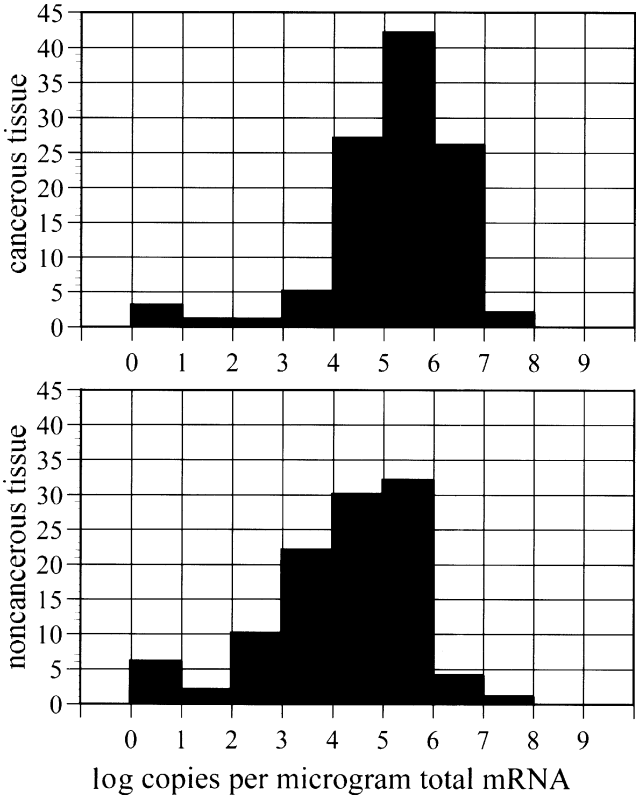

Fig. 2. Distribution of semiquantitative values of survivin mRNA expression in gastric cancerous and noncancerous tissues. Expression of survivin mRNA was observed in 105 of 107 (98.1%) cancerous tissues and in 101 of 107 (94.4%) noncancerous tissues

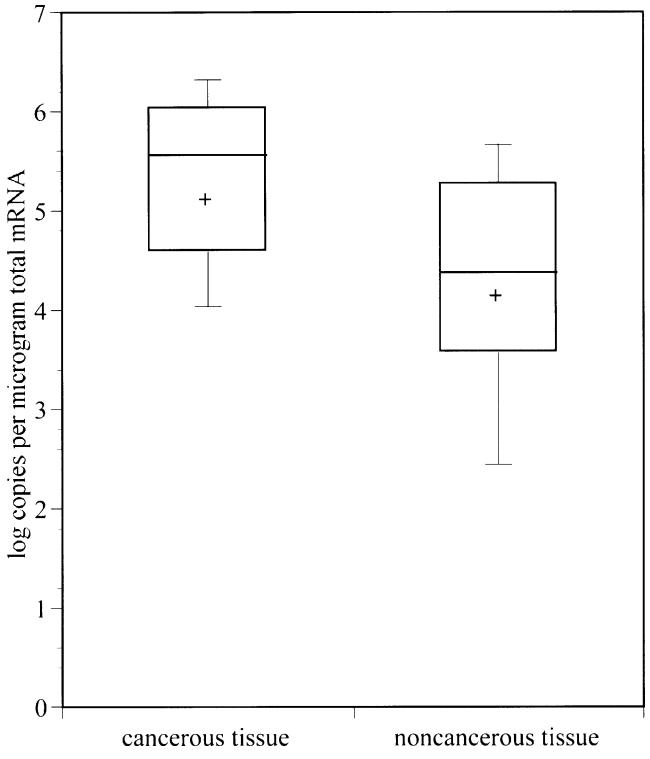

Fig. 3. Proportion of semiquantitative values of survivin mRNA expression in gastric cancerous and noncancerous tissues. The median values for survivin mRNA expression were 5.56 in cancerous tissue and 4.38 in noncancerous tissue. The interquartile ranges were 4.60 to 6.03 in cancerous tissue and 3.58 to 5.27 in noncancerous tissue. The mean value for survivin mRNA expression in cancerous tissue was 5.18 ± 1.30, significantly higher than that in noncancerous tissues at 4.21 ± 1.48 ($P < 0.01$; Mann-Whitney U-test)

nized to be associated with carcinogenesis regardless of the histological type of the gastric cancer.

Relationship between expression of survivin mRNA and depth of primary tumor

With increasing depth of tumor invasion, the values for survivin mRNA expression were 5.04 ± 1.30 in T1, 5.01 ± 1.80 in T2, 5.39 ± 0.82 in T3, and 5.42 ± 0.61 in T4 (Table 2). High survivin mRNA expression was shown by 50.0% (14/28) in T1, 48.5% (16/33) in T2, 51.6% (16/31) in T3, and 53.3% (8/15) in T4. No significant differences were found in the values for survivin mRNA expression according to increasing depth of tumor invasion. This indicates that the increased expression of survivin mRNA begins in the early phase of the gastric carcinogenesis rather than increasing with the progression of the tumor.

Relationship between expression of survivin mRNA and lymph node metastasis

The value for survivin mRNA expression in the 66 patients with lymph node metastasis was 5.48 ± 1.01, significantly higher ($P = 0.002$) than that in the 41 patients without lymph node metastasis, at 4.70 ± 1.55 (Table 1). Of the 66 patients with lymph node metastasis, 38 (57.6%) showed high survivin mRNA expression, a significantly higher proportion ($P < 0.05$) than the 16 of 41 (39.0%) patients without lymph node metastasis (Table 1).

Table 1. Correlation of survivin mRNA expression and histopathological findings in gastric cancer

		Expression of survivin mRNA					
		High	Low	*P* value[a]	Positive ratio of high group	Expression of survvin mRNA (mean ± SD)[c]	*P* value[b]
Histological classification	Differentiated type	27	25	0.919	51.9%	5.17 ± 1.40	0.813
	Undifferentiated type	27	28		49.1%	5.20 ± 1.20	
Lymph node metastasis	Existence	38	28	0.095	57.6%	5.48 ± 1.01	0.002
	Absent	16	25		39.0%	4.70 ± 1.55	
Lymphatic invasion	Existence	45	37	0.154	54.9%	5.38 ± 1.06	0.016
	Absent	9	16		36.0%	4.52 ± 1.75	
Liver metastasis	Existence	4	2	0.689	66.7%	5.77 ± 0.54	0.147
	Absent	50	51		49.5%	5.15 ± 1.32	
Venous invasion	Existence	36	36	>0.999	50.0%	5.21 ± 1.30	0.550
	Absent	18	17		51.4%	5.13 ± 1.31	
Peritoneal metastasis	Existence	8	14	0.212	36.4%	5.03 ± 1.36	0.598
	Absent	46	39		54.1%	5.22 ± 1.29	

[a] χ^2 test with Yates' correction
[b] Mann-Whitney *U*-test
[c] Survivin mRNA expression as log copies/μg total mRNA

Table 2. Relationship between expression of survivin mRNA and depth of primary tumor invasion

Depth of invasion	Expression of survivin mRNA		*P* value[a]	Positive ratio of high group	Expression of survivin mRNA (mean ± SD)[c]	*P* value[b]
	High	Low				
T1	14	14		50.0%	5.04 ± 1.30	
T2	16	17	>0.999	48.5%	5.01 ± 1.80	0.659
T3	16	15	>0.999	51.6%	5.39 ± 0.82	0.210
T4	8	7	>0.999	53.3%	5.42 ± 0.61	0.575

[a] χ^2 test with Yates' correction; T0 vs T2, T3, T4
[b] Mann-Whitney *U*-test; T0 vs T2, T3, T4
[c] Survivin mRNA expression as log copies/μg total mRNA

Table 3. Correlation of expression of survivin mRNA and lymph node metastasis

Lymph node metastasis	Expression of survivin mRNA		*P* value[a]	Positive ratio of high group	Expression of survivin mRNA (mean ± SD)[c]	*P* value[b]
	High	Low				
N0	16	25		39.0%	4.70 ± 1.55	
N1	18	9	0.047	66.7%	5.53 ± 1.73	0.005
N2	8	11	>0.999	42.1%	5.41 ± 0.79	0.058
N3	12	8	0.203	60.0%	5.49 ± 0.79	0.029

[a] χ^2 test with Yates' correction; N0 vs N1, N2, N3
[b] Mann-Whitney *U*-test; N0 vs N1, N2, N3
[c] Survivin mRNA expression as log copies/μg total mRNA

Table 4. Relationship between expression of survivin mRNA and lymph node metastasis according to depth of tumor invasion

Depth of tumor	Expression of survivin mRNA	Lymph node metastasis		Positive ratio of lymph node metastasis	*P* value[a]
		N0	N1–3		
T1	High	11	3	21.4%	0.581
	Low	13	1	7.1%	
T2	High	3	13	81.3%	0.091
	Low	9	8	53.3%	
T3	High	1	15	93.8%	0.534
	Low	3	12	80.0%	
T4	High	1	7	87.5%	>0.999
	Low	0	7	100.0%	
T2–4	High	5	35	87.5%	0.088
	Low	12	27	69.2%	

[a] χ^2 test with Yates' correction

Of the 54 patients demonstrating high survivin mRNA expression, those with lymph node metastasis accounted for 38 (70.3%) and those without lymph node metastasis accounted for the remaining 16 (29.7%). In the other 53 patients, with low survivin mRNA expression, 28 had lymph node metastasis (52.8%) and 25 had no lymph node metastasis (47.2%). With increasing degree of lymph node metastasis, values for survivin mRNA expression were 5.53 ± 1.31 in N1, 5.41 ± 0.79 in N2, and 5.49 ± 0.79 in N3 (Table 3). High survivin mRNA expression was seen in 66.7% (18/27) in N1, 42.1% (8/19) in N2, and 60.0% (12/20) in N3. No apparent relationship was found between high survivin mRNA expression and degree of lymph node metastasis in the patients with gastric cancer.

Relationship between expression of survivin mRNA and lymph node metastasis according to the depth of tumor invasion

At each invasion depth from T1 to T4, lymph node metastasis was more prevalent in patients who also showed high survivin mRNA expression (Table 4). However, no significant relationship was found between

the level of survivin mRNA expression and lymph node metastasis at any invasion depth, suggesting that high survivin mRNA expression is related only to lymph node metastasis, independent of tumor progression.

Relationship between expression of survivin mRNA and lymphatic invasion

The mean value for survivin mRNA expression in patients with lymphatic invasion was 5.38 ± 1.06, significantly higher ($P = 0.016$) that that in patients without lymphatic invasion, at 4.52 ± 1.75 (Table 1). Of the 54 patients displaying high survivin mRNA expression, 45 (83.3%) revealed lymphatic invasion, not significantly different from the 37/53 (69.8%) of patients demonstrating low survivin mRNA expression and lymphatic invasion.

Relationship between expression of survivin mRNA and liver metastasis

The mean value for survivin mRNA expression in the six patients with liver metastasis was 5.77 ± 0.54, not significantly higher than that in patients without liver metastasis, at 5.15 ± 1.32 (Table 1). High survivin mRNA expression was seen in four of the six patients with liver metastasis (66.7%) and in 50 of the 101 patients without liver metastasis (49.5%); again, not a significant difference.

Relationship between expression of survivin mRNA and venous invasion

The mean value for survivin mRNA expression in the patients with venous invasion was 5.21 ± 1.30, not significantly higher than that in the patients without venous invasion, at 5.13 ± 1.31 (Table 1). Of the patients displaying high survivin mRNA expression, 66.7% (36/54) also revealed venous invasion, not a significant difference from the 67.9% (36/53) of the patients who showed both low survivin mRNA expression and venous invasion.

Relationship between expression of survivin mRNA and peritoneal metastasis

The mean value for survivin mRNA expression in the 22 patients with peritoneal metastasis was 5.03 ± 1.36, not significantly different from that in the patients without peritoneal metastasis, at 5.22 ± 1.29 (Table 1). High survivin mRNA expression was seen in 8 of the 22 patients with peritoneal metastasis (36.4%) and in 46 of the 85 patients without peritoneal metastasis (54.1%); again, not a significant difference.

Discussion

The aim of our present study was to investigate quantitative values for the expression of survivin mRNA, indicative of a gene encoding a novel inhibitor of apoptosis, in gastric cancer. Expression of survivin mRNA was detected by the real-time PCR method in 98.1% of cancerous gastric tissues and in 94.4% of noncancerous gastric tissues. The level of survivin mRNA expression was significantly higher in gastric cancer tissue than in noncancerous gastric tissue. However, immunohistochemical analyses of gastric tissue have previously demonstrated that survivin expression could be detected in 34.5% to 82.0% of cancerous gastric cancer tissues, with no survivin expression found in the neighboring normal tissues [20,21]. Similar studies using immunohistochemical analyses of colorectal, mammary, and skin tissues [14,19,31] have also demonstrated survivin expression in cancerous tissues and none in noncancerous tissues.

A few studies using immunohistochemical analyses have demonstrated survivin expression in noncancerous colon and lymph node tissues [32,33]. Survivin expression has also been demonstrated in noncancerous tissue using the reverse transcription-polymerase chain reaction (RT-PCR) method. By this method, survivin mRNA expression was identified in 85.5% of cancerous lung samples and in 12% of paired noncancerous lung samples [24]. Similarly, survivin mRNA expression was detected in 63.5% of cancerous colon samples and in 29.1% of noncancerous colon mucosa samples [23]. In our study, the level of survivin mRNA expression in noncancerous tissue was low in comparison with that in cancerous tissue, but the expression was, nonetheless, observed in 94.4% of the noncancerous tissue samples. This result was very high compared to findings in other reports. Our result was attributed to the increased number of PCR cycles used for the real-time PCR method (50 cycles) in the present study, compared to PCR cycle conditions in other studies using conventional RT-PCR methods (30 to 35 cycles). Using real-time PCR methods, the number of amplification cycles required for the detection of a signal of standard RNA was measured using a known number of existing copies. The result was compared with the results of experimental preparations. Based on this, the quantity of survivin mRNA included in preparations was determined from the number of cycles with confirmed survivin expression. The number of amplifications required for the detection of survivin is more important than the quantity of amplified material created. This study therefore sought to demonstrate the presence of survivin mRNA in noncancerous tissue.

Recently, survivin mRNA has been reported to serve important functions in normal tissues. Survivin is re-

quired for the proliferation of cells during conversion in the G2/M-phase and mitotic progression [34,35]. In addition, the expression of survivin is reported in normal endometrium, where survivin has been demonstrated in the secretory and proliferative phases [36]. Moreover, survivin expression has been identified in normal colonic mucosa in the bases of colonic crypts, which are regarded as proliferation loci [32]. These results show that survivin participates in proliferative functions, supporting the high incidence of survivin mRNA expression in both cancerous and noncancerous gastric tissues shown in the present study.

As survivin mRNA was present in most of our tissue samples, we needed to determine a value above which expression was considered high (i.e., overexpression). We selected the median value of survivin mRNA expression observed in cancerous gastric tissue. Only 15 samples (14.0%) of noncancerous tissue exceeded this cutoff value; this proportion was equal to or less than that determined in other studies using the RT-PCR method. Thus, expression values beneath the cutoff value represent levels at which the expression of survivin is necessary for fundamental cell proliferation in normal tissue.

Of the 107 cancerous gastric tissue samples, 54 showed high survivin mRNA expression and 53 showed low expression. Survivin mRNA expression in these two groups was then compared with the pathological findings. Survivin mRNA expression was independent of both histological type and depth of tumor invasion in patients with gastric cancer. High survivin mRNA expression may therefore occur in the early stages of carcinogenesis of gastric cancer through abnormal control mechanisms, irrespective of the histological type of gastric cancer. This is supported by studies in which survivin expression was observed in precancerous lesions such as Bowen's disease and hypertrophic actinic keratosis preceding cutaneous cancer, and in adenomas, which are the precancerous lesions preceding large-bowel cancer [18,37].

In our study, the proportion of patients with high survivin mRNA expression also displaying lymph node metastasis was significantly higher than the proportion of patients with low survivin mRNA expression and lymph node metastasis. This connection between high survivin expression and lymph node metastasis may be related to an earlier finding that survivin expression is connected with microvessel density [38]. Angiogenesis is essential for cancerous tissue to receive adequate nutrition necessary for its continuous growth. Tumors exhibiting high numbers of microvessels have been shown to possess high metastatic potential [39,40]. Vascular endothelial growth factor (VEGF) has been nominated as one of the triggers of angiogenesis. VEGF expression has also demonstrated a strong association with the presence of lymph node metastasis [41,42]. Moreover, VEGF stimulation reportedly causes increased expression of survivin [43]. Conversely, survivin may prevent new blood vessels formed by VEGF from disappearing by apoptosis. Thus, VEGF and survivin appear to function cooperatively to increase and maintain newly formed blood vessels. In patients with high survivin expression, newly formed blood vessels are apparently retained, providing greater blood flow to the cancerous tissue and promoting metastasis. In the present study, the value for survivin mRNA expression in the patients with lymphatic invasion was significantly higher that that in the patients without lymphatic invasion. However, no significant differences were found between survivin mRNA expression and vessel invasion.

The relationship between survivin expression and metastasis may also arise from an essential function of survivin: when survivin inhibits apoptosis, the proportion of cancerous cells in a tissue increases with continued growth. Previous studies have indicated that when abnormal cells that would otherwise be removed by apoptosis continue to grow, their potential for invasion and metastasis increases [44,45]. However, in the present study, no relationship was found between the formation of peritoneal metastasis, a condition related to the invasion ability of the cancer, and survivin expression. Therefore, the increasing likelihood of cancer metastasis from increased angiogenesis seems to describe the relationship between the increased lymph node metastasis and the increased expression of survivin found in the present study.

These results indicate that survivin mRNA expression starts to increase in the early stages of carcinogenesis. Moreover, the level of survivin mRNA expression may indicate the potential for lymph node metastasis.

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