• BRIEF REPORTS •

Expression of survivin and its significance in colorectal cancer

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Abstract

AIM: To study the expression of survivin,a novel member of inhibitors of apoptosis protein (IAP) and its significance in colorectal carcinoma.

METHODS: Survivin mRNA expression was evaluated by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in 52 colorectal carcinoma samples and 48 adjacent normal colorectal tissue samples. PCR product was sequenced to verify the desired result. Expressions of survivin protein, proliferating cell nuclear antigen labelling index (PI) and apoptotic index (AI) were detected immunohistochemically in 52 human colorectal carcinomas.

RESULTS: The expression of survivin mRNA was detected in a significantly greater proportion of colorectal carcinoma samples than in adjacent normal colorectal tissues (67.3% vs 25%; $P<0.01$). There was no relationship between survivin mRNA expression in colorectal carcinomas and sex, tumor size, histological types, lymphnode metastasis, distant metastasis and Dukes' stage. The PCR product shared 99% of homology with human counterparts. Survivin expression was observed immunohistochemically in 27 of 52 cases of colorectal carcinoma (51.9%). The AI was significantly lower in survivin positive group than in survivin negative group ($0.67\pm0.18\%$ vs $1.14\pm0.42\%$; $P<0.001$), while the PI was greater in survivin positive group than in survivin negative group ($51\pm22\%$ vs $27\pm18\%$, $P<0.001$).

CONCLUSION: Survivin is a special tumor marker independent of histopathological characteristics. It may play an important role during human colorectal tumorigenesis by inhibiting apoptosis and accelerating proliferative activity of colorectal tumor cells.

INTRODUCTION

Apoptosis is an evolutionarily conserved genetic program of cellular suicide and also central to the homeostasis of adult tissues by maintaining the balance between cell production and cell elimination. There is compelling evidence that defects in apoptosis contribute to human diseases such as malignancies. In the evolution of colorectal cancer (CRC) the imbalance between cell proliferation and cell apoptosis may be an important potential factor. A large number of evidences show that many molecules such as p53 and bcl-2 are involved in the regulation of apoptosis during colorectal tumorigenesis. The inhibitor of apoptosis proteins(IAPs), which are widely expressed in all kinds of malignacies, is encoded by the highly-conservative anti-apoptosis gene family and plays an important role in the regulation of apoptosis. IAP molecules contain 1-3 copies of a -70 amino acid zinc-finger fold, and designate the baculovirus IAP repeat (BIR) and their mechanism of action has been directly attributed to the binding of Caspases,thus preventing enzyme activity of the Caspases[1,2]. Among molecules of the IAP family, survivin, a novel anti-apoptosis gene is prominently expressed in CRC and several other malignancies[3,13-18]. The role of survivin in blocking apoptosis is unclear and its ability to inhibit Caspase-3 activity has been widely debated[3-5]. Survivin may paly an important role in the development of CRC. The present study was to examine the expression of survivin in CRC and to evaluate the association between expression of survivin and clinicopathological characteristics of patients with CRC. We also investigated the significance of survivin expression in CRC.

MATERIALS AND METHODS

Patients
Specimens used for this study were obtained from 52 carcinomas and 48 adjacent normal tissures from the resection margins at the First Affiliated Hospital of Suzhou Univercity. The suspected de novo cancers and patients with a known familial colon cancer syndrome history were excluded. All patients had received neither chemotherapy nor radiation therapy before tumor resection. The diagnosis and histopathologic classification were determined according to the General Rules for Pathologic Studies of National CRC Cooperation Group. In CRC patients, there were 31 males and 21 females, and the mean age of the patients was 61.1 years. According to histological differentiation grade, 52 cases of CRC were classified as well, moderately and poorly differentiated subgroups which included 6, 39 and 7 cases respectively. Seven, 19, 17 and 9 cases were staged as Dukes' A, B, C and D respectively. After washed with physiological saline, all cancer tissue samples were cut into two pieces by sterile blade.One piece was fixed with formalin for H&E, the other was frozen in liquid nitrogen immediately after the lesion was resected and stored at -80 ℃ for subsequent assay.

Semi-quantitative RT-PCR
Briefly, total RNAs were extracted from colorectal cancer and normal mucosae, using TRIzol reagent (Life Technologies, Rockville, MD). Prepared RNA (2 μg) was mixed with the random hexamer (100 ng) ,incubated for 5 min at 70 ℃, and then quickly chilled at 4 ℃ for 5 min. RNA samples were reverse-transcriped at 37 ℃ for 60 min into first-strand cDNA in reverse transcription solution [50 mmol/L Tris-HCl (pH8.3), 40 mmol/L KCl, 8 mmol/L

$MgCl_2$, 0.5 mmol/L each dNTP, 225 mg/mL BSA, 5 mmol/L DTT, 25 units of RNasin (Promega, Madison, WI), and 200 units of Mo-MLV reverse transcriptase (Life Sciences, Rockville, MD)] with a total volume of 40 μL. The cDNA samples were incubated at 95 ℃ for 5 min to inactivate the reverse transcriptase and then chilled. The samples were amplified by adding of 50 μL of PCR mixture [50 mmol/L Tris-HCl (pH 8.3), 40 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 10 pmoL of each sense and antisense primer, and 1.5 units of Taq polymerase(MBI,)]. Amplification of $β_2$-microglobulin ($β_2$-MG) gene was performed 28 cycles, each for 30 s at 94 ℃, for 30 s at 58 ℃, and 60 s at 72 ℃, followed by an extension at 72 ℃ for 7 min. Amplification of survivin gene was performed 32 cycles,each for 45 s at 94 ℃, 45 s at 61 ℃, and for 60 s at 72 ℃, followed by an extension at 72 ℃ for 7 min. The products were electrophoresed on 15 g/L agarose gels and then scanned. Specific primers for survivin gene, targeting a 528-bp fragment, were: 5'-TTGAA TCGCG GGACC CGTTG G-3' (sense) and 5'-CAGAG GCCTC AATCC ATGGC A-3'(antisense). As internal standards, $β_2$-microglobulin ($β_2$-MG) gene was used. Primer pairs specific to $β_2$-MG gene, targeting a 330-bp fragment, were used sense: 5'-CTCGC GCTAC TCTCT CTTTC-3' and antisense: 5'-CATGT CTCGA TCCCA CTTAA-3'.

Sequencing of survivin
The PCR products of survivin were isolated and purified using UNIQ-10 purification kit (MBI) and then ligated with pGEM easy T vector by T4 DNA ligase and transformed *E. coli* JM109. Recombinant vectors were identified through *Eco*RI digestion and agarose gel electrophoresis. Positive plasmids were sequenced by an ABI PRISM 377 DNA sequencer (Perkin-Elmer Company).

Immunohistochemistry
Immunohistochemical staining of survivin was performed by SP method. For PCNA, SABC method was performed. TUNEL assay was used to detect apoptosis. In brief, paraffin sections (4 μm thick) were dewaxed, dehydrated, and then immersed in a 10^{-3}mol/L sodium citrate buffer (pH 6.0). For detection of survivin, antigen retrieval was performed by boiling the sections for 20 min in a pressure cooker. For detection of PNCA, the sections were heated in a microwave oven for 10 min to retrieve the antigens. Endogenous peroxidase was inactivated by incubating the sections with 30 mL/L hydrogen peroxide.Nonspecific reactions were blocked by bovine serum. The sections were incubated with a primary antibody overnight at 4 ℃. The antibody used was an 8E2 monoclonal antibody(Chemcon, USA) at 1:5 dilution. Rinsed with PBS, the sections were incubated for 30 min at 37 ℃ with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase, respectively. After three rinse with PBS, the sections were incubated with diaminobenzidine substrate, then rinsed with distilled water and counterstained with hematoxylin. As a negative control, PBS was used instead of the primary antibody. PCNA (Maxim, USA) and TUNEL(Roche, USA) assay were performed according to the manufacturer's instructions.

Scoring criteria
The mean percentage of positive tumor cells for the expression of survivin was determined in at least five areas at 400-fold magnification, and cases with less than 10% positively stained cells were defined as negative. Cases with 10-29% positively stained cells were defined as "+", 30-59% as "++", and 60% or more as "+++".These scorings were performed in a blind fashion.

PI and AI were expressed as the ratio of positively stained tumor cells to all tumor cells. If possible, five areas were randomly selected for counting under 400-fold magnification. Otherwise, the whole section underwent assessment.

Statistical analysis
All results were analyzed by software of SPSS 10.00. $P<0.05$ was considered statistically significant.

RESULTS

Expressions of survivin mRNA in colorectal carcinoma samples and adjacent normal mucosa samples
Fifty two CRC samples and forty eight normal mucosa samples were available for analyses. The expressions of survivvin mRNA were detected in a significantly greater proportion of CRC than in normal mucosa samples (67.3% *vs* 25% $P<0.01$). Survivin mRNA was not detected in normal tissue when the associated cancer was survivin negative (Figure 1).
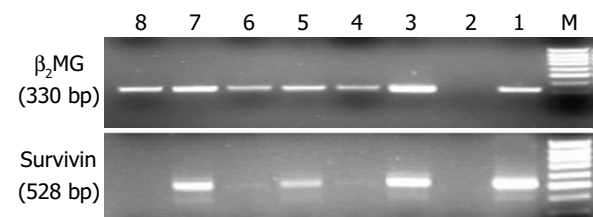
Figure 1 RT-PCR products of paired samples from normal colonic mucosae (lanes 4, 6, 8) and CRC (lanes 3, 5, 7) from three different resection specimens. Lane 1: positive control (U937 cell line) , lane 2: negative control, lane M: DNA mark. Expression of $β_2$MG confirmed the fidelity of reverse transcription.

Relationship between expressions of survivin mRNA and clinicopathological characteristics of patients with CRC
No relationship was found between survivin mRNA expression in CRC and sex, tumor size, histological types, lymphnode metastasis, distant metastasis and Dukes' stage (Table 1).

Table 1 Correlation between expression of survivin mRNA and clinicopathological characteristics of patients with CRC

| Variable	Total No $β_2$MG	Survivin	P Valur
Sex			
Male	31	0.74±0.63	>0.05
Female	21	0.77±0.56	
Tumor size			
≥5 cm	20	0.71±0.59	>0.05
<5 cm	32	0.78±0.61	
Histological type			
High	7	0.62±0.51	>0.05
Middle	39	0.75±0.59	
Low	6	0.92±0.74	
Lymphnode metastasis			
Positive	22	0.80±0.65	>0.05
Negative	30	0.72±0.57	
Distant metastasis			
Positive	9	0.93±0.18	>0.05
Negative	43	0.72±0.65	
Duke stage			
A	7	0.55±0.57	>0.05
B	19	0.73±0.62	
C	17	0.77±0.73	
D	9	0.93±0.18	

Correlations between expression of survivin, AI and PI
The expression of survivin protein was observed in cytoplasm of malignant tumor cells. Survivin expression was observed immunohistochemically in 27 of 52 cases of colorectal carcinoma (51.9%). To examine whether the expression of survivin correlated with AI and PI, tissure samples obtained from
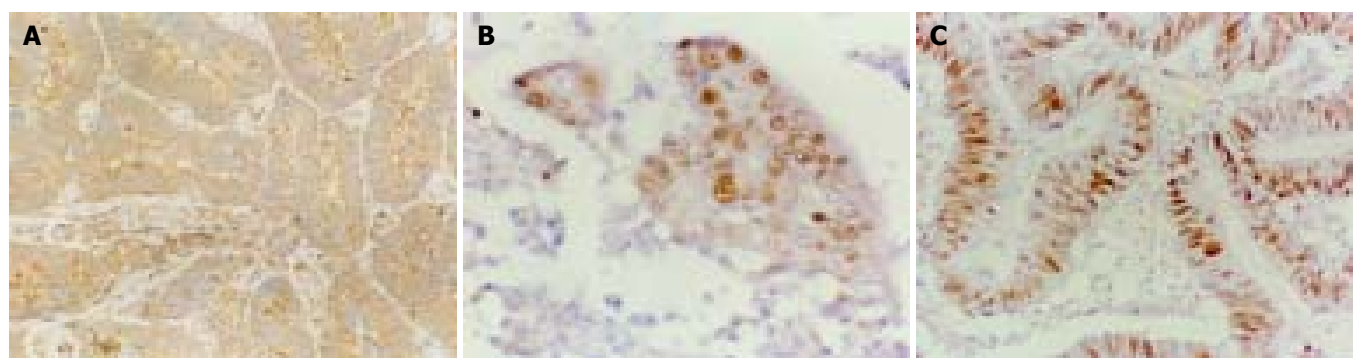
Figure 3 Immunohistochemical analysis of apoptosis,proliferation,and survivin in CRC. Survivin expression was restricted to the cytoplasm of cancer cells (brown staining, ×400 fold). Apoptotic cells and proliferative cells were detected in the nucleoli of cancer cells (brown staining, ×400 fold).

carcinoma were divided into two groups according to the expression of survivin and compared to AI and PI (Figure 2). Survivin positive tumors had significantly lower values for AI than survivin negative tumors ($P<0.001$), and PI in survivin positive tumors was higher than in survivin negative tumors ($P \leqslant 0.001$, Figure 3).
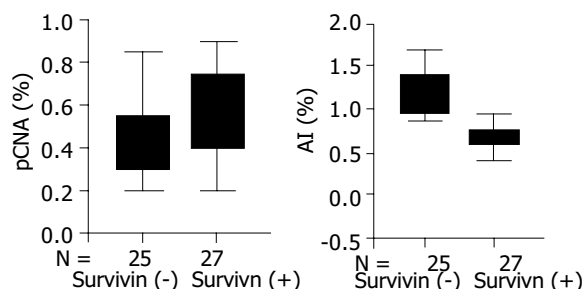
Figure 2 Correlations between survivin expression and AI and PI. $P<0.001$ as PI in survivin positive tumors (51±22%) compared with survivin negative tumors (27±18%), $P<0.001$ as AI in survivin positive tumors (0.67±0.18%) compared with survivin negative tumors (1.14±0.42%).

DISCUSSION

Hybridization screening of a human P1genomic library with the cDNA of effector cell protease recepor-1 (EPR-1) yielded a new gene spanning -15 kb containing four exons and three introns, and located to band 17q25 by Ambrosini et al. in 1997[6].Survivin is the smallest member of the IAP gene family,structurally characterized by a single BIR module and a -COOH-terminus α-helix coiled domain, substituting a canonical ring finger[6]. Prominently expressed in a variety of apoptosis-regulated organs during embryonic and fatal development[7], survivin is undetectable in most normal adult tissues,and becomes abundantly reexpressed in a variety of human cancers. The PCR based approach used in our study is exquisitely sensitive and may detect gene transcripts even in a single cell or cell cluster with a comparable specificity. The present study showed that expression of survivin mRNA was detected in a significantly greater proportion of CRC samples than in adjacent normal colorectal tissues (67.3% vs 25%, $P<0.01$). The data were consistent with a study by Adida et al.[8]. Furthermore, the prevalence of survivin mRNA expression in the present study correlated well with the expression of survivin protein (unpublished data). Recent studies demonstrated that the novel splice variants survivin-△Ex3 and survivin-2B were expressed in malignancies[9,10]. Survivin-△Ex3, which lacks exon 3, exhibited a pronounced anti-apoptotic activity, whereas survivin-2B containing a part of intron 2 as an additional cryptic

exon, largely lost its anti-apoptotic activity. These alternative splice variants have not been explored so far. In gastric carcinomas, Mahotka et al.[10] showed that survivin-2B might act as a naturally occurring antagonist of survivin and might play a role in tumor progression. We found that CRC also expressed survivin-△Ex3, survivin-2B and survivin. The latter was a dominant transcrip (Figure 1), and shared 99% of homology with human counterparts by sequencing.

The expression of survivin mRNA was independent of clinical pathological characteristics. The result was consistent with previous studies[8,11]. However, in endometrial carcinoma, survivin expression correlated with clinical stage, histological grade, invasive behavior and survival rate[12]. The expression of survivin has been proved as a prognostic mark in non-small cell lung cancer[13], breast carcinoma[14], oesophageal carcinoma[15] and hepatocellular carcinoma[16]. In CRC, Adida et al.[8] reported that the predictive value of survivin expression was limited to patients with stage II CRC, those with survivin negative tumors had a five year survival rate of 94.4% compared with 44.8% for patients with survivin positive tumors. Recent studies showed the phenomena of chemo-radioresistance were prevalent in survivin positive tumors and molecular manipulation of survivin expression might enhance chemotherapy and radiation therapy[17-22]. These findings indicate that survivin may play an important role in chemoresistance of cancer cells. However, the clinical importance of survivin expression remains unclear in patients with cancer.

We found that survivin positive tumors had significantly lower values for AI than survivin negative tumors, and PI was higher in survivin positive tumors than in survivin negative tumors ($P<0.001$ for both). These findings indicate that the overexpression of survivin not only inhibits cell death but also reflects the presence of a greater number of proliferating cells. By inhibiting apoptosis and promoting proliferation, survivin plays an important role in colorectal tumorigenesis.

There has been a considerable interest in survivin biomedical researches. The exploitation of survivin might provide important predictive and prognostic clues in diagnosis, and offer new therapeutic alternatives for cancer. Further studies are required to identify the potential role and mechanism of survivin in the development of CRC.

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