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SURVIVIN EXPRESSION IN COLORECTAL: REAL TIME POLYMERASE CHAIN REACTION AND CORRELATION WITH CLINICOPATHOLOGICAL FEATURES

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ABSTRACT

Colorectal cancer develops in a multi-step process and is associated with genetic alterations. Blocking apoptosis is a key factor to unlimited cell proliferation and immortalization. Survivin expression inhibits the programmed cell death process, apoptosis. This molecule could play a potential role in cancer development. Survivin is an attractive target for clinical trials to develop cancer treatment. RNA was prepared from colorectal tumor and adjacent non-tumor tissues and then transcribed to complementary DNA. Human survivin mRNA levels were quantitatively measured by real time polymerase chain reaction (PCR) in tumor and adjacent non-tumor tissues. Expression levels of survivin mRNA in adenocarcinomas were significantly higher than in non-tumor mucosa ($p < 0.0001$). The expressions of survivin mRNA in adenocarcinomas were related to the degree of differentiation (survivin; $p=0.034$). No difference was found with other clinicopathological features. These findings indicate survivin role in colorectal carcinogenesis. Survivin could be used as a potential diagnostic and prognostic marker in colorectal cancer. Successful inhibition of this molecule could lead to the development of a new drug for cancer therapy.

Key words: Survivin, Colorectal cancer, Real time PCR

INTRODUCTION

Colorectal cancer is one of the most common cancers in the western world. It develops in a multi-step process and is associated with genetic alteration. Adenomatous polyposis emerges due to mutations in the Adenomatosis Polyposis Coli (APC) gene. Accumulation of additional mutations in tumor suppressor genes or cell cycle control genes can contribute to increasing the risk of polyps progressing toward adenocarcinoma (Vogelstein and Kinzler, 1993; Vogt, 1993). This complex genetic process could block apoptosis, a programmed cell death, and ultimately leads to unlimited cell proliferation.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family (Altieri, 2001). It is a bifunctional protein involved in regulation of cell proliferation and suppression of apoptosis. Survivin is expressed in embryonic tissues but undetectable in most normal differentiated tissues (Ambrosini *et al*., 1997; Adida *et al*., 1998). Survivin is also upregulated in most tumors and it's over expression, which may be due to promoter mutation, may allow abnormally divided cells to overcome cell cycle checkpoints during mitosis (Ambrosini *et al*., 1997). However, the inhibition mechanism of apoptosis by survivin is not clear. A high survivin expression level was detected in different human tumors: breast cancer, gastric cancer, lung cancer, pancreatic cancer and colon cancer. These studies found no correlation with clinicopathological features but a prognostic value of survivin expression (Kawasaki *et al*., 1998; Kami *et al*., 2004; Meng *et al*., 2004; Span *et al*., 2004; Atikcan *et al*., 2006).

In this study, expression levels of survivin mRNA in colorectal tissues was quantitatively investigated by real time polymerase chain reaction

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(PCR). Additionally, survivin expression was correlated with the clinicopathological features of the cancer tissue samples.

MATERIALS AND METHODS

Patient selection and sample collection

Patients with histologically confirmed colon and rectal tumors were prospectively and consecutively recruited into the study. Patients who had pre-operative adjuvant chemo or radiotherapy were excluded. Consent was obtained according to the hospital human ethics committee approval. The clinical diagnostic, staging, surgical, and pathological data were collected in a standardized manner, using a computerized database. At the time of surgery, the tissue from the tumor and normal colorectal mucosa from the proximal resection margin were promptly sampled from the resected specimen. These samples were preserved in RNA*later* within 10 minutes of surgery to prevent degradation of the RNA and then stored at –20ºC.

RNA extraction

Total RNA was extracted from the 10-25 mg of tested tissue by disruption and homogenization using High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany). DNase I was used to digest DNA contamination. The quality of the RNA was checked by agarose gel electrophoresis to rule out degraded RNA.

cDNA preparation

Purified total RNA was reverse transcribed in a total volume of 25 µl using random primer at a ratio of 2 to 1 RNA and transcriptor reverse transcriptase (Roche Diagnostics, Mannheim, Germany). The reaction mixture was incubated for 10 min at 25ºC first, 60 min at 50ºC, and followed by enzyme inactivation step for 5 min at 85ºC. The cDNA samples were stored at –20ºC until use.

Real time quantification PCR

The rotor gene system (Corbett Research, Sydney, Australia) was used to run real-time quantification polymerase chain reaction. Absolute quantification assay was chosen to analyze survivin (target), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH, endogenous control) mRNA expression. PCR was performed in a total volume of 20 µl reaction mixture containing 1x TaqMan universal master mix with AmpErase uracil N-glycosylase (Applied Biosystems, Foster City, USA) 600nM of each primer, 200nM TaqMan probe, and 2 µl of unknown cDNA or 2 µl of standard template. All the samples (unknown and standard) were run in duplicate and accompanied by a non-template control. Thermal cycling conditions included 2 min at 50ºC and 10 min at 95ºC, followed by 45 cycles at 95ºC for 15 sec and 60ºC for 60 sec.

Primers and probes

The primer set and probe for amplification of survivin (GeneBank accession number NM_001168), and GAPDH, (GenBank accession number NM_002046) mRNA were designed using GenScript web site/design tool (www.genscript.com/ssl-bin/app/primer) (Table 1). The primers for the genes placed in different exons, were checked by conventional PCR to ensure these did not amplify genomic DNA. The probes contained 6-carboxyflourscein (FAM) as a fluorescent reporter dye, and 6-carboxytetramethyl-rhodamine (TAMRA) as the quencher for its light emission spectrum. These probes were purchased from Sigma Genosys (Wollands, TX, USA). During the extension phase of PCR, the probe hybridized to the target sequence and was then cleaved due to the 5' to 3' exonuclease activity of Taq polymerase. The increase in fluorescent signal of the reporter was proportional to the amount of specific PCR products, providing highly accurate and reproducible quantification.

Standard curve

For absolute quantitation assay, a standard curve was constructed from a known concentration of DNA sample. A segment of 290 bp survivin gene was generated by conventional RT-PCR. The PCR-product was checked on agarose gel for unspecific amplification, purified by High Pure PCR Product Kit (Roche Diagnostics, Mannheim, Germany), and then accurately quantified by ultraviolet-spectrophotometer. The concentration of the known DNA sample was converted to molecules per microliter. This known standard sample was adjusted to 0.8×10^{10} molecule/µl and then serially diluted by 1/10 down to 0.8x10. Two µl of each dilution (10^6 to 10^1) in duplicate was used as a template for real time PCR.

For each sample, the expression level of survivin, and GAPDH mRNA were quantified as a copy number (per reaction) using the standard curve (Fig. 1). Expression level of survivin for each sample was normalized by dividing by the copy number of GAPDH (Normalization = copy number of target $gene_{sample}$ / copy number of $GAPDH_{sample}$ x100).

Statistical Analysis

The expression difference between the tumor and matched non-tumor samples was done using *t*-test. Correlation between expression level and clinicopathological features of the patients was analyzed by Mann-Whitney tests. The significance

Table 1. PCR primers and probes used for quantitation of survivin and GAPDH

Primer	Sequence	Amplicon size, bp
Survivin		120
Upper	5'-AAGGACCACCGCATCTCTAC-3'	
Lower	5'-CAAGTCTGGCTCGTTCTCAG-3'	
Probe	5'-(FAM) CAGCCCTCCAAGAAGGGCCA (TAMRA)-3'	
GAPDH		79
Upper	5'-ATGGGTGTGAACCATGAGAA-3'	
Lower	5'-GTGCTAAGCAGTTGGTGGTG-3'	
Probe	5'-(FAM) CCTCAAGATCATCAGCAATGCCTCC (TAMRA)-3'	

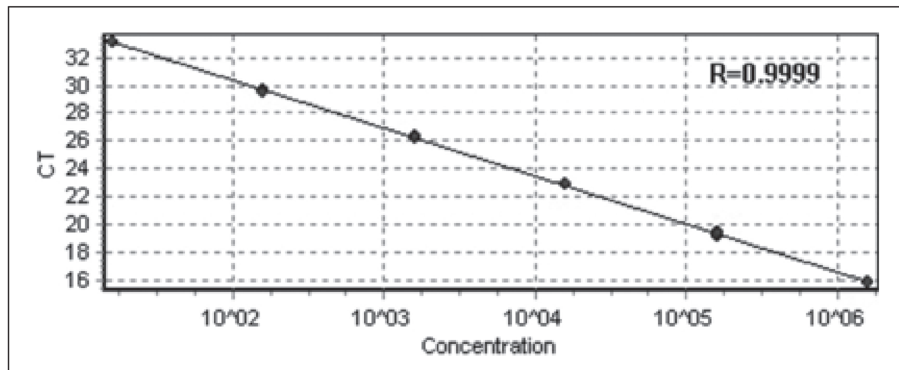

Fig. 1. Standard curve, generated by rotor gene, of serially dilution of known concentration of survivin segment ($1.6x10^1$-$1.6x10^6$). The threshold cycle (CT) of each dilution was plotted against concentration.

level was taken at $p \leq 0.05$. All statistical tests were performed with the Statistical Package for Social Sciences (SPSS version 14.0, Chicago, IL).

RESULTS

Fourty seven patients (28 Males and 19 Females) with colorectal adenocarcinomas were recruited in this study. The age of the patients was 37 to 90 years (mean 66.45).

Expression level of survivin mRNA was analyzed quantitatively by real time PCR. All the tumor tissue samples expressed survivin mRNA and the expression of survivin mRNA in tumor tissues was 100% higher when compared with the expressions in non-tumor tissues. The level of survivin expressed in tumor tissue samples was more than four times in comparison with non-tumor tissues (Fig. 2).

The expression level of survivin mRNA in tumor tissues was higher than in matched adjacent non-tumor tissue samples and statistically significant ($p<0.0001$, t-test).

At the same time, expressions of survivin mRNA were higher in poorly differentiated samples than in well and moderate differentiated samples. A significant relationship was found between differentiation and tumor expression of survivin ($p=0.034$). Other clinical data revealed no significance was found with the expression of survivin gene (Table 2).

DISCUSSION

The expression of survivin mRNA was investigated in colorectal tissue cancer. The molecule has a characteristic function which is essential for cancer cells to continue proliferation indefinitely. Survivin interacts either with caspases (-3, -7, -9) and/or p^{53} to block the programmed cell death (apoptosis) (Sah *et al.*, 2006).

Real time PCR technique was used to quantitate the expression levels of hTERT and survivin mRNA. This technique is specific, and sensitive enough to detect mRNA molecules even in a single cell (Bustin, 2000; De Kok *et al.*, 2000). Also, quantitative evaluation makes statistical analysis more appropriate.

Survivin was detected in colorectal adenocarcinomas and adjacent non-tumor tissues. Expression level of survivin mRNA in tumor tissues was 100% higher than in non-tumor tissues. It

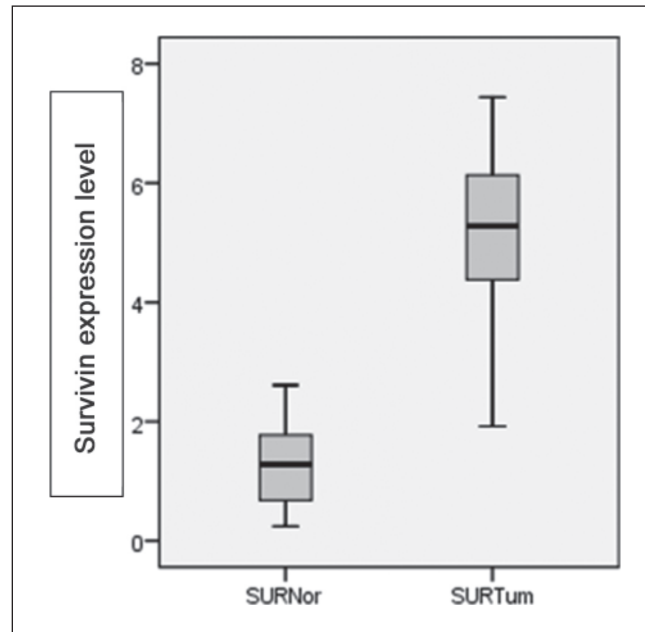

Fig. 2. Expression level of survivin mRNA (mean±SD) in colorectal tissue samples.

Table 2. Expression level of survivin mRNA and correlation with clinicopathological features for the patients with colorectal cancer

Variable	No (%)	Expression of Survivin				P value*
		Mucosa		Tumor		
		Mean	Range	Mean	Range	
Gender						n.s
Male	28 (60)	1.330	0.24–2.61	5.186	1.92–7.16	
Female	19 (40)	1.147	0.39–2.21	5.175	2.82–7.44	
Age						n.s
<65	20 (43)	1.322	0.24–2.21	5.346	1.92–7.16	
≥65	27 (57)	1.207	0.31–2.61	5.060	3.03–7.44	
Site						n.s
Colon	31 (66)	1.167	0.24–2.21	5.183	1.92–7.44	
Rectum	16 (34)	1.429	0.47–2.61	5.180	3.03–7.09	
Differentiation						0.034
Well+Moderate	38 (81)	1.275	0.24–2.61	5.042	2.25–7.44	
Poor	9 (19)	1.176	0.38–2.19	5.773	1.92–7.16	
Stage						n.s
I+II	29 (62)	1.333	0.31–2.61	5.022	2.25–6.89	
III+IV	18 (38)	1.132	0.24–2.19	5.440	1.92–7.44	

*Mann-Whitney test.

has been reported that expression and localization of survivin in normal colonic mucosa was restricted to the bottom of crypts (Gianani *et al*., 2001). In the literature, while survivin expression in adenocarcinomas was up-regulated, a contradictory result of survivin expression in normal mucosa was reported (Kawasaki *et al*., 1998; Gianani *et al*., 2001). Detection of survivin was approached either by immunohistochemistry staining, in situ hybridization and/or RT-PCR. Some studies showed no survivin was detected in normal colonic mucosa (Kawasaki *et al*., 1998; Kawasaki *et al*., 2001; Sarela *et al*., 2001). Others studies found survivin expressed in normal mucosa (Sarela *et al*., 2000;

Gianani *et al*., 2001; Zhang *et al*., 2001). These discrepancies were explained by either the specimen contamination with a component of non-epithelial tissue that possibly hindered the detection of survivin, or the difference in the sensitivity of the methods used for detection (Zhang *et al*., 2001). We found expression of survivin mRNA in normal mucosa was consistent with the previous reports (Sarela *et al*., 2000; Gianani *et al*., 2001; Zhang *et al*., 2001). This low level of survivin mRNA could represent expression of colonic stem cells at the bottom of basal crypt. This expression may be essential for these colonic stem cells to maintain normal proliferation activities before their differentiation and migration to the top of crypt.

Survivin which is expressed in different types of cancer is a potential target for anti-cancer treatment development strategies. Correlation between survivin expression and clinico-pathological parameters could lead to the prediction of cancer development. In colorectal cancer, survivin was detected in 53% to 100% of tumor samples (Kawasaki *et al*., 1998; Sarela *et al*., 2000; Gianani *et al*., 2001). It has been reported that survivin expression increases during development of colorectal tumorigenesis (Kawasaki *et al*., 2001) but no correlation was found with clinico-pathological features (Kawasaki *et al*., 1998; Sarela *et al*., 2000; Sarela *et al*., 2001). However, survivin expression has been reported as a prognostic factor of poor outcome in colorectal carcinoma (Kawasaki *et al*., 1998; Sarela *et al*., 2001), as well as not being a specific marker of colorectal adenocarcinomas (Gianani *et al*., 2001).

In this study, survivin mRNA was detected in all tumor samples and expression level was four times higher than in normal tissues. The degree of differentiation or tumor grade was considered as low grade (well and moderately differentiated) and high grade (poorly differentiated and undifferentiated). This stage independent prognostic factor showed a significant increase of survivin mRNA was found in tumor samples with poor differentiation ($p=0.034$). This is the first study reporting a significant statistical relationship between expression of survivin mRNA and the degree of tumor differentiation in colorectal cancer. It has been reported that the expression of survivin in breast cancer also correlated with histological grade, stage and lymph metastasis (Sohn *et al*., 2006). These results indicate the diagnostic and possibly prognostic role of survivin in the pathology of colorectal cancer and the possibility of targeting survivin as a goal for anti-cancer therapy.

In conclusion, Survivin mRNA expression in colorectal tissues was quantitatively explored by real time PCR. The expression levels of survivin in adenocarcinomas were different from adjacent non-tumor mucosa, and correlated with the degree of differentiation.

These findings indicate the significant importance of survivin molecule in pathology of cancer and could be used as a potential diagnostic and prognostic markers as well as an indication for possible cancer treatment.

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