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A Quantitative Study to Evaluate Expression Level and Correlation of Human Telomerase Reverse Transcriptase and Survivin Genes in Colorectal Cancer Patients

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ABSTRACT
Colorectal cancer is developed in a multi-step process and associated with genetic alterations. Blocking apoptosis is the key factor to unlimited proliferation and immortalization the cells. Telomerase activation acquires the cell immortalization state whereas survivin expression inhibits the programmed cell death process, apoptosis. These two molecules could play a potential role in cancer development and are attractive targets for clinical trials to develop cancer treatment. In this study, RNA was prepared from colorectal tumor and adjacent non-tumor tissues and then transcribed to complementary DNA. Human Telomerase Reverse Transcriptase (hTERT) and survivin mRNA levels were quantitatively measured by real time Polymerase Chain Reaction (PCR) in tumor and non-tumor tissues. Expression levels of both hTERT and survivin mRNA in adenocarcinomas were significantly higher than in non-tumor mucosa ($p<0.0001$). The expressions of hTERT and survivin mRNA in adenocarcinomas were correlated ($p = 0.012$). The high levels of hTERT mRNA were often associated to the high levels of survivin mRNA in colorectal carcinomas ($p = 0.039$). The expressions of hTERT and survivin mRNA in adenocarcinomas were related to the degree of differentiation (hTERT; $p = 0.037$, survivin; $p = 0.034$). No difference was found with other clinicopathological features. These findings indicate the role of hTERT and survivin in colorectal carcinogenesis. HTERT and survivin could be used as a potential diagnostic and prognostic marker in colorectal cancer. Successful inhibition of these molecules could lead to the development of a new drug for cancer therapy.

Key words: Survivin, colorectal cancer, hTERT, telomerase

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.

Telomerase is a complex of ribonucleoprotein composed of Human Telomerase Reverse Transcriptase Protein (hTERT) and human telomerase RNA (TER). Telomerase adds telomeric repeats at the chromosome ends in order to compensate for telomeres loss during cell division and to overcome the end replication problem (Nugent and Lundblad, 1998). It is expressed in almost all human tumors, stem and germ cells but is low or undetectable in normal tissues (Kim *et al.*, 1994). While TER component is expressed in normal and tumour cells, expression of hTERT component is restricted to cancer cells and closely related to telomerase activity. It is widely believed that hTERT is a rate-limiting determinant of telomerase and plays a key role in carcinogenesis (Feng *et al.*, 1995; Meyerson *et al.*, 1997; Nakayama *et al.*, 1998). Expression of telomerase or hTERT has been proposed as a valuable biomarker for cancer (Kim *et al.*, 1994) and used most recently in correlation with clinicopathological parameters to predict tumor progression. However, the mechanism by which telomerase hTERT is up-regulated and expressed in cancer is not fully understood.

Survivin, a recently discovered protein 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 (Adida *et al.*, 1998; Ambrosini *et al.*, 1997). 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. The expression of high survivin 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 (Span *et al.*, 2004; Meng *et al.*, 2004; Atikcan *et al.*, 2006; Kami *et al.*, 2004; Kawasaki *et al.*, 1998).

In this study, expression levels of mRNA of hTERT and survivin in colorectal tissues were quantitatively investigated by real time Polymerase Chain Reaction (PCR) in order to find a relationship between expression of hTERT and survivin. In addition, expressions of both hTERT and survivin were correlated with the clinicopathological features of the 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 min of surgery to prevent degradation of the RNA and then stored at -20°C.

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 25°C first, 60 min at 50°C and followed by enzyme inactivation step for 5 min at 85°C. The cDNA samples were stored at -20°C 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 hTERT (target), 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) 600 nM of each primer, 200 nM 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 50°C and 10 min at 95°C, followed by 45 cycles at 95°C for 15 sec and 60°C for 60 sec.

Primers and probes: The primer set and probe for amplification of hTERT (GenBank accession number AF015950), survivin (GenBank 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 they did not amplify genomic DNA. The probes contained 6-carboxyfluorescein (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 hTERT gene was generated by conventional

Table 1: PCR primers and probes used for quantitation of hTERT, survivin and GAPDH

Primer	Sequence	Amplicon size (bp)
hTERT		93
Upper	5'-TATGTCACGGAGACCACGTT-3'	
Lower	5'-GTGCTGTCTGATTCCAATGC-3'	
Probe	5'-(FAM) CAACTTGCTCCAGACACTCTTCCGG (TAMRA)-3'	
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'	

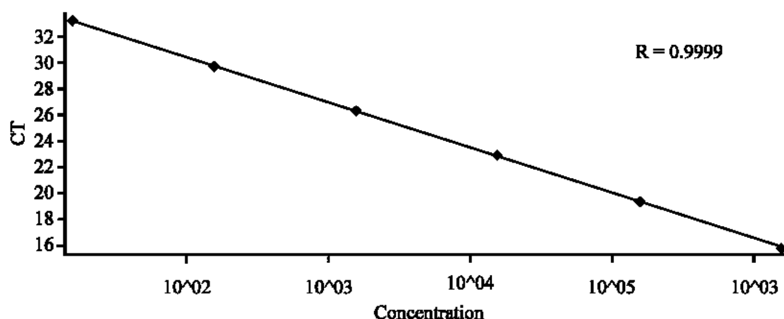

Fig. 1: Standard curve, generated by rotor gene, of serially dilution of known concentration of hTERT segment (1.6×10^{1}-1.6×10^{6}), the threshold cycle (CT) of each dilution was plotted against concentration

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^{-1} and then serially diluted by 1/10 down to 0.8×10. Two microliter of each dilution (10^{6}-10^{1}) in duplicate was used as a template for real time PCR.

For each sample, the expression level of hTERT, survivin and GAPDH mRNA were quantified as a copy number (per reaction) using the standard curve (Fig. 1). Expression level of hTERT and survivin for each sample was normalized by dividing by the copy No. of GAPDH:

$$\text{Normalization} = \frac{\text{Copy No. of target gene sample}}{\text{Copy No. of GAPDH sample}}\times100$$

Statistical analysis: For both hTERT and survivin genes, expression difference between the tumor and matched non-tumor samples were done using t-test. Correlation between expression level and clinicopathological features of the patients was analyzed by Mann-Whitney tests. Association between low and high expression of both hTERT and survivin genes was analyzed by Fisher's exact test. The significance level was taken at $p\leq0.05$. All statistical tests were performed with the Statistical Package for Social Sciences (SPSS version 14.0, Chicago, IL).

RESULTS

Forty 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 levels of mRNA of hTERT and survivin were analyzed quantitatively by real time PCR. All the tumor tissue samples expressed hTERT and survivin mRNA. The expression levels of hTERT and survivin mRNA in tumor were higher than in matched adjacent non-tumour tissue samples and statistically significant ($p<0.0001$, t-test) (Table 2). In tumor tissues, a significant correlation was found between expression of hTERT mRNA and survivin mRNA ($r = 0.364$, $p = 0.012$).

In order to study the possible expression association between hTERT and survivin in colorectal adenocarcinomas, the mean values of the expression levels of tumor hTERT (1.18) and survivin (5.19) were considered as cut-off value to categorize expression levels as either low or high. Of

Table 2: Expression level of survivin and hTERT mRNA in colorectal tissue samples

Tissue sample	No.	hTERT	Survivin
Normal mucosa	47	0.486±0.284 (0.00-1.02)	1.256±0.631 (0.24-2.61)
Tumor	47	1.179±0.791 (0.11-2.92)	5.182±1.284 (1.92-7.44)
		p<0.0001	p<0.0001

Values are as Mean±SD, Values in brackets are range

Table 3: Correlation between expression of survivin and hTERT mRNA in colorectal adenocarcinomas tissues

	Survivin expression				
	Low		High		
hTERT expression	No.	%	No.	%	p-value[a]
Low	14	29.8	10	21.3	0.039
High	6	12.8	17	36.2	

[a]Fisher's exact test

Table 4: Correlation of expression of hTERT and survivin mRNA in tumor tissues with clinicopathological features of the patients with colorectal cancer

		Expression of tumor					
		hTERT mRNA			Survivin mRNA		
Variable	No. (%)	Mean	Range	p-value*	Mean	Range	p-value*
Gender							
Male	28 (60)	1.232	0.111-2.919	n.s	5.186	1.922-7.158	n.s
Female	19 (40)	1.100	0.150-2.539		5.175	2.816-7.439	
Age							
<65	20 (43)	1.218	0.171-2.919	n.s	5.346	1.922-7.158	n.s
≥65	27 (57)	1.150	0.111-2.747		5.06	3.030-7.439	
Site							
Colon	31 (66)	0.966	0.111-2.919	n.s	5.183	1.922-7.439	n.s
Rectum	16 (34)	1.592	0.200-2.539		5.18	3.030-7.088	
Differentiation							
Well+Moderate	38 (81)	1.059	0.111-2.539	0.037	5.042	2.247-7.439	0.034
Poor	9 (19)	1.687	0.337-2.919		5.773	1.922-7.158	
Stage							
I+II	29 (62)	1.056	0.150-2.919	n.s	5.022	2.247-6.886	n.s
III+IV	18 (38)	1.377	0.111-2.747		5.44	1.922-7.439	

*Mann-Whitney test

47 tumor samples, the expression of hTERT mRNA was low in 24 (51%) samples and high in 23 (49%) samples whereas survivin mRNA expression was low in 20 (43%) samples and high in 27 (57%) samples. Thirty-six percent of tumor samples which expressed high hTERT mRNA were found to express high level of survivin mRNA in comparison with 13% of the tumor samples which expressed low survivin (p = 0.039, Fisher's exact test) (Table 3).

At the same time, expressions of both hTERT and 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 both hTERT (p = 0.037) and survivin (p = 0.034). Other clinical data revealed no significance with the expression of both genes (Table 4).

DISCUSSION

The expressions of hTERT and survivin mRNA were investigated in colorectal tissue cancer. These molecules have a characteristic function which is essential for cancer cells to continue proliferation indefinitely. As telomerase immortalizes the cells by maintaining telomere length at the chromosome ends and overcoming the end replication problem (Nugent and Lundblad, 1998), 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, a recently developed 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.

The expression of hTERT mRNA was detected in tumor and adjacent non-tumor colorectal tissues. Seventy percent of 47 adenocarcinoma tissue samples expressed higher hTERT mRNA than adjacent non-tumor tissue samples. Telomerase activity has been detected in colorectal carcinomas but not in normal mucosa (Fang *et al*., 1999; Yan *et al*., 1999; Cheng *et al*., 1998). However, telomerase activity or hTERT expression has been demonstrated in normal mucosa (Shoji *et al*., 2000; Kanamaru *et al*., 2002). A quantitative study found expression level of hTERT mRNA was higher in adjacent non-tumor colorectal tissues than in tumor colorectal tissues and 21% of 57 colorectal tumor samples expressed higher hTERT mRNA than non-tumor samples (Gertler *et al*., 2002).

Furthermore, expression level of hTERT mRNA was low in normal mucosa and increased in the process of multistage tumorigenesis of colorectal cancer (Kanamaru *et al*., 2002; Niiyama *et al*., 2001).

The study found expression of low hTERT mRNA in non-tumor colorectal mucosa was consistent with the previous reports (Shoji *et al*., 2000; Kanamaru *et al*., 2002; Gertler *et al*., 2002; Niiyama *et al*., 2001). This could suggest that telomerase activation occurs early in colonic mucosa as it progresses toward transition to adenoma. This indicates that genetic changes may first occur in the cell before any cytological changes could be observed by the pathologist.

However, the presence of hTERT mRNA in adjacent non-tumor colorectal mucosa could be explained by alternative splicing of hTERT transcript which has been reported to regulate telomerase activity (Ulaner *et al*., 2000). Infiltrating activated lymphocytes or cancer cells may also affect the outcome of hTERT mRNA. As telomerase is a common feature of abnormal epithelial cell proliferation during cancer development, the expression of hTERT mRNA in non-tumor tissues may not be sufficient to activate telomerase.

Expression of telomerase in human cancer has been considered a diagnostic and prognostic biomarker. In colorectal cancer, it has been reported that the expression of telomerase correlated with either the depth of tumor (pT) (Shoji *et al*., 2000; Kawanishi-Tabata *et al*., 2002), tumor grade (Kanamaru *et al*., 2002; Gertler *et al*., 2002) or with its stage (Ghori *et al*., 2002). Other studies found no correlation between telomerase expression and prognosis despite a steady increase in telomerase expression during cancer development (Fang *et al*., 1999; Niiyama *et al*., 2001).

In this study, hTERT mRNA was increased during development of adenocarcinomas. A significant association was found between telomerase hTERT and the degree of tumor differentiation ($p = 0.037$) but the study failed to confirm the statistical difference between hTERT mRNA expression and tumor stage. However, no difference was found between hTERT expression and the age, gender, or site.

These findings support previous reports (Kanamaru *et al.*, 2002; Gertler *et al.*, 2002) and indicate the important role of telomerase in colorectal carcinogenesis as a diagnostic and possibly prognostic marker as well as its potential for use as cancer therapy.

Survivin was detected in colorectal adenocarcinomas and adjacent non-tumor tissues. Expression level of survivin mRNA in tumor tissues was higher than in non-tumor tissues. It 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 (Gianani *et al.*, 2001; Sarela *et al.*, 2000; 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 (Gianani *et al.*, 2001; Sarela *et al.*, 2000; 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 clinicopathological parameters could lead to the prediction of cancer development. In colorectal cancer, survivin was detected in 53-100% of tumor samples (Kawasaki *et al.*, 1998; Gianani *et al.*, 2001; Sarela *et al.*, 2000). It has been reported that, survivin expression increases during development of colorectal tumorigenesis (Kawasaki *et al.*, 2001) but no correlation was found with clinicopathological features (Kawasaki *et al.*, 1998; Sarela *et al.*, 2000, 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. 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.

The expressions of hTERT and survivin mRNA in tumor tissues were 70 and 100% higher, respectively, when compared with the expressions in non-tumor tissues. A significant correlation was found between expression of hTERT and survivin mRNA in colorectal tumor. This correlation was further tested in term of expression association between hTERT and survivin by using a relationship of low and high expression level of both hTERT and survivin mRNA. A significant association was found between expression of hTERT and survivin ($p = 0.039$, Fisher's exact test)

which indicates that expression of high level of hTERT is more likely related to the high level of survivin expression in colorectal carcinomas. This is a new and important finding which linked expression behavior of both hTERT and survivin mRNA in colorectal cancer. This association between expression of hTERT and survivin could be the key to better understanding the mechanism by which these genes are activated in the cell during transformation to an abnormal state. Additionally, this finding confirms a study addressing the relationship between survivin and telomerase activity by reporting that survivin expression in colon cancer cells enhanced telomerase activity (Endoh *et al.*, 2005). These findings indicate the expression behavior of telomerase and survivin in colorectal carcinogenesis.

In conclusion, hTERT and survivin mRNA expression in colorectal tissues were quantitatively explored by real time PCR. The expression levels of hTERT and survivin in adenocarcinomas were different from adjacent non-tumor mucosa and correlated with the degree of differentiation. At the same time, expression of hTERT was positively correlated with survivin expression in adenocarcinomas. These findings indicate the role of both hTERT and survivin as potential diagnostic and prognostic markers as well as an indication for possible cancer treatment.

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