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Evaluation of *ST13* gene expression in colorectal cancer patients*

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Abstract: We identified a novel gene *ST13* from a subtractive cDNA library of normal intestinal mucosa in 1993, more studies showed that *ST13* was a co-chaperone of Hsp70s. Recently we detected the *ST13* gene expression in tumor tissue and adjacent normal tissue of the same colorectal cancer patient and investigated if the *ST13* gene expression might have any prognostic value. Analysis was performed at molecular level by reverse transcription-PCR using real-time detection method. We measured two genes simultaneously, *ST13* as the target gene and *glyceraldehydes-3-phosphate dehydrogenase* as a reference gene, in primary colorectal tumor specimens and tumor-adjacent normal mucosa specimens from 50 colorectal cancer patients. The expression levels of the *ST13* gene were significantly decreased in primary tumors compared with adjacent mucosa (P<0.05). But there were no significant differences in the expression of *ST13* as compared with different Dukes' stage, tumor differentiation grade, invasion depth, lymph node metastasis and disease-specific survival.

Key words: *ST13*, Colorectal cancer, Real-time PCR
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INTRODUCTION

Today, colorectal cancer (CRC) is the third leading cause of death due to cancer and the third most common cancer expected to occur in men and women (Jemal *et al*., 2004). Colorectal cancer development and metastasis are associated with altered gene expression profiles (Vogelstein and Fearon, 1988). We reported in 1993 the identification of a novel gene *ST13* (HSU17714) from a subtractive cDNA library of normal intestinal mucosa, whose expression was down-regulated in colorectal cancer. Further studies showed that *ST13* genomic DNA had 32017 base pairs, comprised of 12 exons. Correspondingly its cDNA had 3127 base pairs, the open reading frame (ORF) was 1170 base pairs and codes for a 369-amino acid protein. The gene was mapped to chromosome 22q13 (Zheng, 1993; 1997; Zheng *et al*., 1997).

Hohfeld *et al*.(1995) reported an Hsc70-interacting protein (Hip) which bound the ATPase domain of Hsc70 in an ADP-dependent manner. P48 protein was identified as a transient component during the cell- free assembly of progesterone receptor complex in 1996 (Prapapanich *et al*., 1996a; 1996b). *ST13* showed >90% identity at the amino acid level with the rat Hip and P48. More studies showed that Hip/P48/*ST13* was associated with Hsp70 and Hsc70, affected the Hsc70/Hsp70 chaperone activities in vivo and in vitro (Nollen *et al*., 2001; Fan *et al*., 2002; Irmen and Hohfeld, 1997). Molecular chaperones of the Hsp70 family play a key role in the control of protein folding during protein biogenesis, protein transport through membranes, and when cells are exposed to proteotoxic stress (Hartl, 1996; Bukau and Horwich, 1998). Our studies showed that *ST13* might play a role in carcinogenesis and participate in regulating apoptosis (data not published).

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Northern blot and RNA dot blot analyses showed that the expression level of *ST13* gene was significantly lower in 15 cases of colorectal cancer than that in adjacent colonic mucosa. RNA dot blot analyses also showed that the ratio of low *ST13* expression in colorectal carcinoma was significantly higher in lymph node metastasis patients compared with that in mucosa (Mo *et al*., 1996). In situ hybridization analyses showed that the ratio of low *ST13* expression in colorectal carcinoma seemed higher in Duke's C or lymph node metastasis patients compared with that in mucosa, but there was no significant difference (Ye *et al*., 2001). To our knowledge, except for the Duke's stage and lymph node metastasis, the relationship between *ST13* expression and other clinicopathological factors had not been studied.

Recent reports on real-time RT-PCR showed the advantages of this method compared with conventional methods (Gelmini *et al*., 1997; Swan *et al*., 1997). This method offers the possibility to perform multiplex PCR, simultaneous detection of the expression of more than one gene in the same tube, using specific sets of primers and specific probes with different fluorogenic labels for each gene to be investigated. We use this multiplex PCR for detection of expression of our target gene *ST13* and the reference gene *GAPDH*. Measured the expression of the reference gene provides information on the integrity and amount of mRNA present in the tested samples. To clarify these disparate findings, we investigated if the expression of *ST13* might have any clinic significance or prognostic value in this study.

MATERIALS AND METHODS

Samples

Tumor and paired mucosa samples were obtained from 50 colorectal carcinoma patients undergoing primary tumor resection at the Second Affiliated Hospital of Zhejiang University during the period between Sept. 2000 to May 2001. The ethics committee of Zhejiang University approved the study. Adjacent, normal-appearing mucosa were taken at a distance of ~10 cm from the tumors. The biopsies were snap-frozen in liquid nitrogen and stored at −80 °C until used. Surgical and pathological records were reviewed for Dukes' stage, tumor differentiation grade, age, gender, and tumor localization. The growth pattern and the grade of differentiation were classified by pathologists as recommended by the WHO. The malignant tumors were classified according to Dukes' stage, as modified by others, into the pathological stages Dukes' A (TNM stage I), Dukes' B (TNM stage II), Dukes' C (TNM stage III), and Dukes' D (TNM stage IV). Clinical characteristics of the patients are presented in Table 1.

Table 1 Clinical characteristics of the patients

Variable	Number of patients (%)
All patients	50
Gender	
Male	26 (52)
Female	24 (48)
Tumor site	
Colon	26 (52)
Rectum	24 (48)
Dukes' stage	
A	8 (16)
B	19 (38)
C	21 (42)
D	2 (4)
Histology	
Well differentiated	14 (28)
Moderately differentiated	20 (40)
Poorly differentiated	3 (6)
Mucinous adenocarcinoma	13 (26)

Total RNA extraction and reverse transcription of mRNA

Isolate total RNA from TriZol (Invitrogen). Adjust concentration of total RNA to 0.2 μg/μl after RNA isolation. Take 10 μl total RNA, add 1 μl oligo (dT) (Promega), 70 °C for 10 min, put on ice for 2 min, then add 4 μl 5×1st strand buffer, 2 μl 0.1 mol/L DTT, 1 μl 10 mmol/L each dNTP (Gibcol), and 1 μl RNase inhibitor, mix well, 42 °C for 2 min. Add 1 μl SSRTII (Gibcol) to each tube, 42 °C for 50 min, then 70 °C for 15 min. Spin reagent down, add 80 μl pure water, mix well, spin down, store at −20 °C for real time PCR.

Real-time quantitative PCR

Quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). The house keeping gene *GAPDH* was used as endogenous control to compensate for the variation in RNA amount and to check the efficiency of the re-

verse transcription reaction. PCR samples were prepared as follows: 2 μl of cDNA were transferred into MicroAmp Reaction Tubes already containing 48 μl of reaction mixture. The components of the reaction mixture contained 25 μl 2×universal buffer (Roche), 6 μl target *ST13* gene primer-probe mix, 2.5 μl human *GAPDH* primer-probe mix, 14.5 μl H_2O. The condition for the PCR were 2 min at 50 °C and 10 min at 95 °C, cycling parameters were 15 s at 95 °C and 1 min at 60 °C (40 cycle), hold at 15 °C. Sequences of the used primers and probes are listed in Table 2. All of the samples were amplified simultaneously in triplicate in a one assay-run. The quantitative data were calculated according to the instructions given by Applied Biosystems.

Statistical analysis

The clinicopathological variables used in this study were as follows: Dukes' stage, differentiation grade, lymph node metastasis, and invasion depth. The obtained data were analyzed by statistical modelling using commercial software SPSS. Unless otherwise stated, the data were presented as means and SDs. To compare sets of continuous parameters measured in the same tumor tissues, Spearman's correlation coefficient® and the Wilcoxon signed-rank test were used. The statistical significance of the difference in survival of the groups was calculated using log-rank test. Relative risk was assessed by univariate and multivariate Cox proportional hazard model. Statistical values of $P \leq 0.05$ were considered to be significant.

RESULTS

Clinical characteristics of the patients

As shown in Table 1, the median age of the patients was 60 (range, 25~91) years. Twenty-six patients were male, and 24 were female. Among 50 patients, 26 patients had colon cancer, and 24 had rectal carcinoma. Of the primary carcinomas, 14 were highly, 20 were moderately, and 3 were poorly differentiated, 13 were mucinous adenocarcinoma. Primary tumor stage was Dukes' A in 8 patients, B in 19 patients, C in 21 patients, and D in 2 patients. At the end of the study, we could follow up 22 patients. The median following-up time was 50 months.

Gene expression levels in colorectal mucosa and carcinomas

The relative gene expression levels of *ST13* in the mucosa and in carcinomas are presented in Table 3. As shown, significantly lower expression levels were found in tumors as compared with mucosa (t=2.547, P=0.014). The ratio of low expression (*ST13* expression in colorectal carcinoma/colorectal mucosa≤1) samples was 62% (31/50).

Table 3 *ST13* expression in matched samples of normal colorectal mucosa (N) and primary colorectal cancer (T)

	Cases	Min	Max	$\bar{x}\pm s$
T	50	−3.56	9.5	5.1470±2.1280
N	50	−3.20	7.4	4.6653±1.7147

Gene expression levels in different Dukes' stage CRC patients

Analyses showed that there was no significant difference in different Dukes' stage group (F=1.329, P=0.227) in Table 4.

Gene expression levels in different differentiation stage CRC patients

As shown in Table 5, there was no significant difference in different differentiation stage (F=1.525, P=0.221).

Table 2 DNA sequences of the primers and probes used in the PCR

Gene	Primer/probe
GAPDH	Forward primer: 5′-CTTAGCACCCCTCCCCAAG-3′
	Reverse primer: 5′-GATGTTCTGGAGAGCCCCG-3′
	Probe: 5′-(VIC)CATGCCATCACTGCCACCCAGAAGA(TAMRA)-3′
ST13	Forward primer: 5′-CCTCGCTTGGCCATTTTGT-3′
	Reverse primer: 5′-TGGCAGCATTTGGCTTCTG-3′
	Probe: 5′-(FAM)CCAAGAGGGCCAGTGTCTTCGTCAAA(TAMRA)-3′

Gene expression levels in different invasion depth CRC patients

As shown in Table 6, there was no significant difference in different invasion depth groups (F=0.248, P=0.782).

Gene expression levels in different lymph node metastasis CRC patients

As shown in Table 7, there was no significant difference in positive and negative lymph node metastasis group (F=1.848, P=0.072).

Relationship between *ST13* expression and survival

Among the 50 CRC patients, we could conduct 22 following-up investigations. Primary tumor stage was Dukes' A in 4 patients, B in 7 patients, C in 9 patients, and D in 2 patients. The ratio of low expression cases was 68.2% (15/22). As expected, 4-year tumor-specific survival of the patients gradually decreased from those classified as Duke's A (100%), Duke's BC (75%), Duke's D (0%). As shown in Fig.1, the 4-year survival rate was 80% (12/15) for patients of group $ST13^{low}$ and 57.2% (4/7) for patients of group $ST13^{high}$. But there was no significant difference between them (P=0.209).

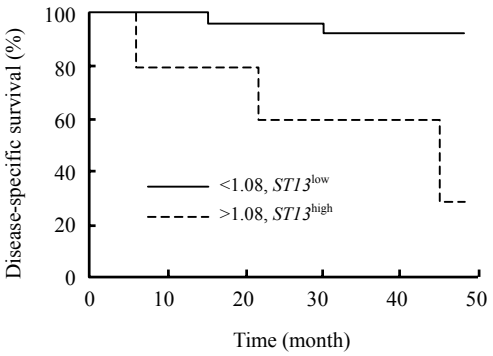

Fig.1 Kaplan-Meier curves for disease-specific survival in 22 CRC patients

ST13 expression in colorectal carcinoma/colorectal mucosa≤1.08: —$ST13^{low}$; *ST13* expression in colorectal carcinoma/colorectal mucosa>1.08: ---- $ST13^{high}$

Table 4 *ST13* expression in different Dukes' stage CRC patients

Dukes' stage	Cases	$\bar{x}\pm s$	F value	P value	Low expression samples (%)
A	8	0.9977±0.7604	1.329	0.227	5 (62.5)
B	19	1.2949±1.1309			11 (57.9)
C	21	0.7537±0.5307			14 (66.7)
D	2	1.1230±0.2778			1 (50)

Table 5 *ST13* expression in different differentiation grade CRC patients

Differentiation grade	Cases	$\bar{x}\pm s$	F value	P value	Low expression samples (%)
Well differentiated	14	0.7454±0.4618	1.525	0.221	10 (71.4)
Moderately differentiated	20	1.3240±1.0983			10 (50)
Poorly differentiated	3	0.8332±0.9181			2 (66.7)
Mucinous adenocarcinoma	13	0.8740±0.6560			9 (69.2)

Table 6 *ST13* expression in different invasion depth CRC patients

Invasion depth	Cases	$\bar{x}\pm s$	F value	P value	Low expression samples (%)
T1	2	1.4062±0.6873	0.248	0.782	1 (50)
T2	13	0.9425±0.8365			9 (69.2)
T3	35	1.0247±0.8898			21 (60)

Table 7 *ST13* expression in negative and positive lymph node metastasis CRC patients

Lymph node metastasis	Samples	$\bar{x}\pm s$	F value	P value	Low expression samples (%)
Negative	27	1.2068±1.0296	1.848	0.072	16 (59.2)
Positive	23	0.7873±0.5199			15 (65.3)

DISCUSSION

Hip/ST13 cooperates with Hsp70 in protein folding by stabilizing the ADP-bound state of Hsp70. Besides affecting the Hsp70 chaperone cycle, Hip/ST13 alone can also bind to unfolded proteins and prevent their aggregation, but refolding of protein to their active requires cooperation with other chaperones (Bruce and Churchich, 1997). Other classes of Hsp70 co-chaperones include BAG-1 (bcl-2 associated anti-death gene 1 protein) and CHIP (carboxyl terminus of Hsc70-interacting protein) cofactors (Hohfeld and Jentsch, 1997; Ballinger *et al*., 1999).

Hsp70 is molecular chaperone involved in many important biological processes. Abrogation of Hsp70 expression can induce apoptosis in tumor cells (Nylandsted *et al*., 2000; Ravagnan *et al*., 2001). Some studies showed that increased Hsp70-positive expression correlated significantly with low differentiation and was associated with worse overall survival in a series of 128 CRC patients (Lazaris *et al*., 1995), mitochondria Hsp70 (mortalin) over-expression correlated with poor survival in colorectal cancer patients (Dundas *et al*., 2005). BAG-1 is a recently identified Bcl-2 interacting anti-apoptotic protein. The percentage of CRC cases exhibiting nuclear BAG-1 positivity was significantly higher in distant metastasis-positive cases than in distant metastasis-negative cases, overall survival was significantly shorter for patients with tumors exhibiting BAG-1 positive nuclei than those without nuclear BAG-1-staining, and indicated that nuclear BAG-1 expression was the only independent prognostic variable for mortality (Kikuchi *et al*., 2002).

The real-time PCR result of the present study showed that the mean gene expression level of *ST13* was significantly low in the tumor compared with the mucosa, and that the low expression ratio was 62% in 50 CRC patients. The result was in accordance with several studies using northern blot and RNA dot blot methods which showed the low expression ratio was 60% (Mo *at al*., 1996). Our study also showed that the expression level of *ST13* gene in different clinicpathological factors group including Dukes' stage, differentiation grade, lymph node metastasis, and invasion depth had no significant difference. The result supported the in situ histochemistry study (Ye *et al*., 2001). In our study, patients with high level of *ST13* seemed to have poorer outcome (3 died in 7 cases) compared with patients having low level of *ST13* (3 died in 15 cases). But there was no significant difference between them, because we could only follow-up 22 CRC patients. Maybe we can find the expression level of *ST13* has some prognostic value if we get more patients involved in the study.

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