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• ORIGINAL RESEARCH •

Differential expression of a novel colorectal cancer differentiation-related gene in colorectal cancer

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Abstract

AIM **To investigate SBA2 expression in CRC cell lines and surgical specimens of CRC and autologous healthy mucosa.**

METHODS **Reverse transcription-polymerase chain reaction (RT-PCR) was used for relative quantification of SBA2 mRNA levels in 4 human CRC cell lines with different grades of differentiation and 30 clinical samples. Normalization of the results was achieved by simultaneous amplification of** β-actin as an internal control.

RESULTS **In the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82%). Markedly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts ($P<0.01$). SBA2 expression was significantly ($0.01<P<0.05$) correlated with the grade of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated cases. Of the 9 cases with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($0.01<P<0.05$).**

CONCLUSION **SBA2 gene might be a promising novel biomarker of cell differentiation in colorectal cancer and its biological features need further studies.**

Subject headings colorectal neoplasms/genetics; colorectal neoplasms/pathology; DNA, complementary; cell differentiation; gene expression; polymerase chain reaction

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INTRODUCTION

Colorectal cancer (CRC) is increasing in China[1-10], and the treatment is still difficult in advanced stage[11-20]. Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel CRC differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human CRC cell line CloneA after modulation by sodium butyrate. We investigated the use of reverse transcription-polymerase chain reaction (RT-PCR) for the relative quantification of SBA2 expression in 4 human CRC cell lines with different grades of differentiation and in 30 surgical specimens of CRC and autologous healthy mucosa.

MATERIALS AND METHODS

Materials

The poorly-differentiated human CRC cell line CloneA, moderately-differentiated human CRC cell line CX1, and well-differentiated human CRC cell line CCL187 were obtained from Dana-Farber Cancer Institute, Harvard Medical School. The moderately-differentiated human CRC cell line LS174T was bought from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. These cells were cultured in Dulbecco's modified Eagle's medium containing $50mL \cdot L^{-1}$ calf serum at 37℃ with 100% humidity and $50mL \cdot L^{-1}$ CO_2. Tumor tissues were obtained from 30 patients (15 men and 15 women) at the time of surgery for removal of CRC. All cases were assessed by histopathology. Both tumor tissues and autologous healthy mucosa (at the distance >5cm from the neoplastic focus) were sampled. All specimens were snap frozen and stored in liquid nitrogen. Total cellular RNA was isolated with the TRIzol reagents (Gibco-BRL). The yield and quality of RNA preparation were determined by spectrophotometry.

Quantification of gene expression by semi-quantitative RT-PCR

Reverse transcription of 2μg of total RNA using 0.5μg oligo (dT)-15 primer was performed for 1h at 42℃ in 20μL of a reaction mixture containing 15U AMV reverse transcriptase (Promega), 5 mmol/L $MgCl_2$, 1 × RT buffer (10 mmol/L Tris-HCl, pH 9.0 at 25℃, 50 mmol/L KCl, 1 g/L Triton X 100), 1mmol/L dNTP mixture and 25U of recombinant RNasin ribonuclease inhibitor. The samples were then heated at 99℃ for 5 min to terminate the reverse transcription.

Primers used for amplification of β-actin specific sequence were residues 2217-2238 (TGTATGCCTCTG-GTCGTACCAC; sense-strand) and residues 2009-2030 (ACAGAGTACTTGCGCTCAGGAG; antisense-strand). PCR using these primers yields a 592 bp product. SBA2-specific sequence was amplified by the sense-strand primer (residues 51 - 72: GCTTGTCACGGCTTCTTACGAT) and the antisense-strand primer (residues 390 - 411: GCATAAGTGCTTCAGTGAGGAC), which yield a 361bp product.

Unless otherwise specified, 2.5μL of the reversely-

transcribed mixture was used as template DNA and amplified in a reaction volume of 25 μL containing 1 mmol/L $MgCl_2$, 1 ×RT buffer (10mmol/L Tris-HCl, pH 9.0 at 25℃, 50mmol/L KCl,1g/L Triton X100), 0.2μmo/L each of 5' and 3'primers for β-actin, 0.5μmol/L each of 5' and 3' primers for SBA2. After heat denaturation at 95℃ for 5min, 1.25U of thermus aquaticus DNA polymerase (Promega) was added to the mixture. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) in sequential cycles at 94℃ 30s; 68℃ 45s and 72℃ 1 min. After the last cycle, all samples were incubated for an additional 10 min at 72℃. Ten μL of PCR product were separated on 15g/L agarose gels, stained with ethidium bromide, and loaded onto 80g/L polyacrylamide gels, stained with DNA silver staining system (Promega). The gel was analyzed with the Electrophoresis Documentation and Analysis System 120 (Kodak 1D).

RESULTS

Linearity of PCR amplification

The yield of PCR product is proportional to the starting amount of the template only under conditions in which PCR amplification proceeds exponentially at a constant efficiency. To establish the optimal conditions for detection and quantification of SBA2 expression, the relative yield of PCR products was determined by terminating aliquots of starting reaction solution at sequential PCR cycles as shown in Figure 1. The resultant amplified sequences were analyzed and the signal intensities of the bands were plotted on a semilogarithmic scale against the cycle number to obtain amplification curves. Figure 1 shows that at higher cycle numbers (>28), the yield of the SBA2-specific product approaches the plateau while β-actin shows a tendency of saturation beyond 26 cycles. Before saturation at the plateau, however, good linearity is observed for both SBA2 and β-actin amplification over the range of cycles examined. Figure 2 compares the results obtained with successive dilutions of cDNA. The serial 1:2 dilutions were performed beginning with 5μL of reversely-transcribed mixture, as shown in the graph, and amplified 24 cycles. Thus the number of 24 cycles and the volume of 2.5μL of cDNA were chosen as the optimal parameters for the semi-quantitative RT-PCR.

If the efficiency of amplification is the same for each cycle (as indicated by the good linearity of the curves), the amount of cDNA produced can be predicted from the equation $cDNAn = (cDNA_0) \times (1+R)^n$, where cDNAn stands for the amount of product after n cycles, $cDNA_0$ is the initial amount of cDNA, and R is the efficiency of amplification. The efficiency of the amplification can be assessed from the following equation: $\log cDNAn = \log cDNA_0 + n \times \log (1+R)$. Within the exponential range, the linear regress equation for SBA2 is $y = 0.2612 \times x - 0.6179$ ($r = 0.9496$), for β-actin: $y = 0.2594 \times x - 0.4468$ ($r = 0.9754$), where y is the logarithm of cDNAn, x is the number of PCR cycles (n), r is correlation coeficient. The slope of the curve should be $\log (1+R)$ in the semi-logarithmic plot. Thus an R- value of 82% and 82% can be determined for SBA2 and β-actin respectively. Since the same amplification efficiency is observed for both the target and the internal control within the exponential range, the relative amounts of SBA2 mRNA can be determined by comparison with β-actin.

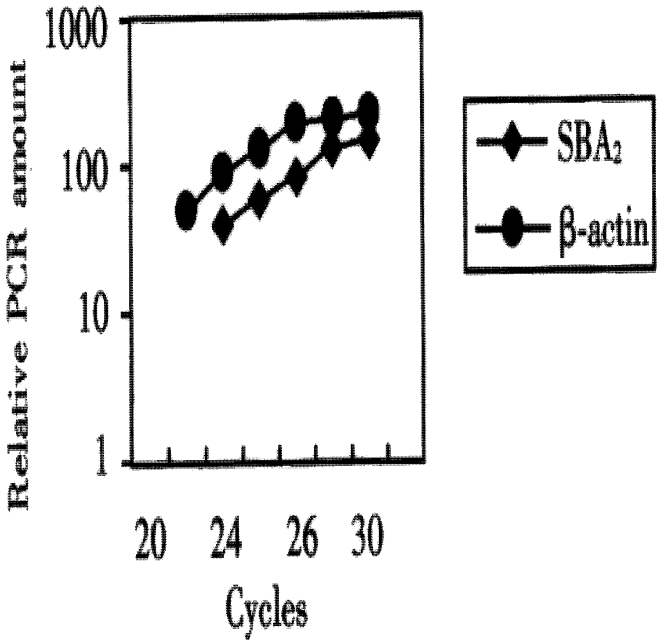

Figure 1 Detection of the exponential range by termination at sequential PCR cycles.
1-7: Cycles of 20, 22, 24, 25, 26, 28 and 30, respectively. M: Marker (100 bp DNA ladder).

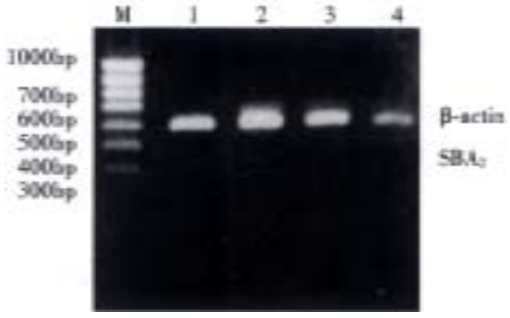

Figure 2 Detection of the exponential range by serial dilutions of cDNA.
1-4: cDNA of volume of 5, 2.5, 1.25, and 0.625μL, respectively. M: Marker (100 bp DNA ladder).

Patterns of SBA2 expression

In cultured cell lines and clinical specimens of CRC, the assay described above was used to investigate the levels of SBA2 mRNA in 4 human CRC cell lines and in 30 surgical samples of CRC and autologous healthy colonic mucosa. In the 30 samples analyzed, significantly lower levels of SBA2 mRNA were detectable in tumors, as compared with the coupled normal counterparts (Wilcoxon test, $T = 6$, $P<0.01$).

Differential expression of SBA2 in clinical samples of CRC with different grades of differentiation was similar to that observed in 4 cell lines of CRC, as shown in Figures 3 and 4, respectively. Thirty clinical samples were assigned into 3 groups, depending on the grade of differentiation. The levels of SBA2 mRNA were significantly (q test, $0.01<P<0.05$) correlated with the degree of differentiation in CRC, with relatively higher levels in well-differentiated samples and lower in poorly-differentiated ones. Mean levels of SBA2 mRNA was calculated for each group. There was a significant difference between well-differentiated group (0.384 ± 0.024) and poorly-differentiated group (0.158 ± 0.014) ($q = 3.9564$, $0.01<P<0.05$), between moderately-differentiated (0.297

±0.015) and poorly-differentiated ($q = 3.2767$, $0.01<P<0.05$), in spite of no significant difference ($q = 0.0798$, $P>0.05$) between well-differentiated and moderately-differentiated.

We also analyzed the relationship between SBA2 expression and lymph nodes metastasis. Of the 9 patients with lymph nodes affected, 78% (7/9) had reduced SBA2 mRNA expression in contrast to 24% (5/21) in non-metastasis samples ($\chi^2=5.5622$, $0.01<P<0.05$).

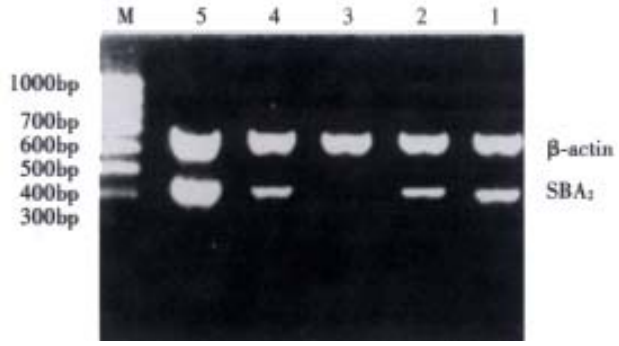

Figure 3 Relative quantification of SBA2 mRNA in colorectal tissues. 1-4: Moderately, moderately, poorly, and well differentiated CRC, respectively. 5: Healthy colorectal mucosa. M: Marker (100 bp DNA ladder).

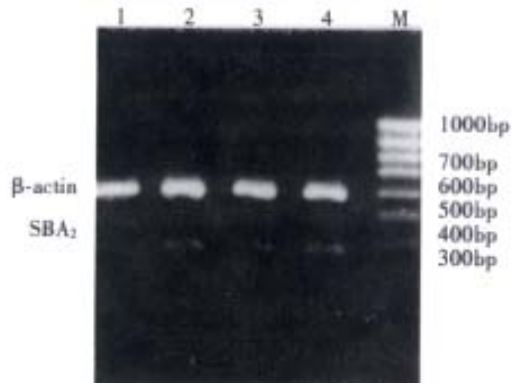

Figure 4 Relative quantification of SBA2 mRNA in cell lines of CRC. 1-4: Human CRC cell line CloneA, CX1, CCL187, and LS174T, respectively. M: Marker (100 bp DNA ladder).

DISCUSSION

We have developed an efficient protocol for relative quantification of gene expression in both clinical samples and cell lines of CRC by semi-quantitative RT-PCR. Linear evaluation of gene expression over a wide range was achieved by plotting the amplification curves of the sequence of interest and the β-actin sequence that served as an internal control. Determination of the optimal parameters of an exponential range was carried out by terminating the PCR at sequential cycles and by serially diluting the amount of cDNA. The amplification efficiency of PCR and the amount of sample cDNA have been verified by the simultaneous amplification of a sequence of a gene that serves as an internal control, such as, aldolaseA, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (G_3PD), dihydrofolate reductase[22]. However, the simultaneous amplification of a large amount of a sequence of an internal control can affect the efficiency of amplification of the sequence of interest[23]. A possible explanation of the competition is that the effect of an internal control sequence on amplification efficiency may depend on the gene sequences amplified[24]. For instance, the simultaneous amplification of a β2-microglobulin mRNA sequence suppressed the amplification efficiency of an *mdr* mRNA sequence, while amplification of β-actin sequence did not affect the amplification efficiency of a tax/rex sequence[25]. In our study, in the exponential range of amplification, fairly good linearity demonstrated identical amplification efficiency for SBA2 and β-actin (82% for both). Thus β-ctin can be used as an internal control to normalize the relative levels of SBA2 mRNA in this experiment.

Histologic types reflect the biologic nature of the cancer, exercising the most decisive influence upon the diagnosis and prognosis. A variety of pathologic changes are all closely associated with the cellular differentiation in CRC, including gross typing, growth pattern, depth of infiltration and metastasis into lymph nodes[26,27]. Classic grading categorized CRC into three discrete classes: well, moderately and poorly differentiated. Such grading, however, is largely dependent on the subjective assessment of the histopathologist. Heterogeneity of differentiation in the same cancer specimen often leads to considerable inter-and intra-observer variation in grading[28]. Colorectal neoplasia develops in a mucosa that has alterations in proliferation, maturation, and differentiation[29]. Normally, the major zone of cell proliferation is at the base of the crypts (the lowest one third) with little extension to the surface of proliferating cells. As cells migrate from the crypts to the luminal surface, they become increasingly differentiated and mature so that by the time they have reached the surface they have lost their proliferative capabilities, finally leading to apoptosis (i.e. programmed cell death)[30].

Butyrate is an important colonic fuel and induces differentiation in colonic cell lines[21]. cDNA for a novel colorectal cancer (CRC) differentiation-related gene, designated SBA2 (GenBank accession No: AF229181), has been identified in human colorectal cancer cell line CloneA after modulation by sodium butyrate. It consisted of 2470 nucleotides and an open reading frame (ORF) encoded 404 amino acid residues with a M_r of 44400. The deduced amino acid sequences showed significant homology to mouse SWiP-2 (96%), mouse WSB-2 (95%) and human WSB-1 (52%), which belong to a new family of the suppressor of cytokine signaling (SOCS). The SBA2 protein product may be a new member of SOCS protein family negatively regulating cytokine signal transduction. We have used RT-PCR for relative quantification of SBA2 mRNA levels in 4 CRC cell lines with different grades of differentiation and 30 surgical specimens of CRC and autologous healthy mucosa. Our assay showed significantly lower levels of SBA2 mRNA in tumors, as compared with coupled normal counterparts. Furthermore, differential expression of SBA2 in CRC with different grades of differentiation was observed both in clinical tissues and in cell lines SBA2 mRNA levels were correlated with the degree of differentiation in CRC, being relatively higher in well-differentiated samples and lower in poorly-differentiated ones. There was also a significant difference in the frequency of lower-level SBA2-expressing samples between patients with lymph nodes metastasis and those without metastasis. It was indicative of the close association of SBA2 with metastasis in CRC. The reduced levels of SBA2 mRNA in CRC and the correlation between SBA2 mRNA expression and the grade of differentiation in CRC suggest that SBA2 might be a

promising novel biomarker of cell differentiation of CRC, and that the biological features of SBA2 and its product in CRC and other tumors need further studies.

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