Abstract. The aim of the present study was to examine the regulation of selenium binding protein 1 (SELENBP1) expression in colorectal cancer (CRC). Samples of cancer tissue and adjacent normal mucosa were collected from 83 CRC patients, and analyzed for SELENBP1 expression by 2D-DIGE, immunoblotting, RT-PCR and immunostaining. Expression levels of SELENBP1, carcinoembryonic antigen (CEA) and alkaline phosphatase (AKP) were determined in cultures of human colon cancer cell lines (SW480, SW620 and HT29) following treatment with i) sodium butyrate (NaB, 2 mM), a differentiation inducer; ii) Trichostatin A (TSA, 0.3 µM), a histone deacetylase inhibitor; or iii) 5'-aza-2'-deoxycytidine (5-Aza-dC, 5 µM), a DNA methylation inhibitor. SELENBP1 expression was found to be downregulated (2.54-fold) in the CRC samples as determined by 2D-DIGE and confirmed by immunoblotting and RT-PCR. SELENBP1 expression was correlated with the degree of differentiation, but not with TNM stage or lymph node metastasis, and was higher in benign polyps (1.97±0.57) than in CRC tissues (0.96±0.59). In the CRC cell lines, NaB treatment led to the upregulation of SELENBP1, CEA and AKP when compared with the untreated cells (2.24- to 4.82-fold). SELENBP1 was also upregulated in cells treated with TSA alone (1.25- to 3.64-fold), or in combination with 5-Aza-dC (1.32- to 4.13-fold). In CRC, the downregulated SELENBP1 expression was reactivated by inducing differentiation. Therefore, SELENBP1 is a potential pharmacological target for individualized CRC treatment.

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers and common causes of cancer-related mortality. The identification of differentially expressed genes and proteins between cancer and adjacent non-tumor mucosa provides valuable information with which to identify the molecular and physiological changes involved in tumor development and differentiation. In our previous proteomic studies, we demonstrated that selenium binding protein 1 (SELENBP1) is one of the significantly downregulated proteins in CRC (1). SELENBP1 has been found to be downregulated in multiple cancer types, such as lung, ovarian, colorectal, esophageal, gastric and liver cancers (2-8), and its reduced expression has been associated with poor outcome (4). However, little is known concerning its function and regulatory mechanism.

The two well-recognized epigenetic alterations, DNA methylation and histone modification, are known to play a key role in the development and progression of various types of cancers, including CRC (9). DNA methylation at CpG islands in promoter regions often leads to silencing of tumor-suppressor genes (10,11). Histone methylation patterns associated with CRC were uncovered by Enroth et al (12) in a genome-wide search in tumor samples. Pohl et al (22) demonstrated that in colon cancer cells the SELENBP1 promoter is hypermethylated which presumably leads to downregulation of this protein. They also demonstrated that overexpression of SELENBP1 prevents cancer cell migration in vitro and inhibits tumor growth in nude mice. Due to its patterns of expression in cancer cells, SELENBP1 has also been suggested as a potential marker for predicting progression (8,13).

In the present study, we attempted to establish the role of SELENBP1 in CRC by studying tissue samples from patients with sporadic colorectal cancer. In addition, SELENBP1 expression was studied in five different colorectal cancer cell lines (HCT116, HT29, LS174T, SW480, SW620) and its regulation by epigenetic modifications was examined in three of these. The results support the notion that SELENBP1 is associated with CRC differentiation and shed light on its possible regulation by histone modification.

Materials and methods

Tissue samples. Tissue samples were obtained from The First Hospital of China Medical University between 2008 and 2012. The procedures were approved by the Ethics Committee of the
First Hospital of China Medical University. All tissue specimens were obtained by experienced surgeons and analyzed by experienced pathologists. Informed consent was obtained from patients. Samples of cancer tissues and adjacent normal mucosa were collected from sporadic CRC patients who had not received any preoperative chemotherapy or radiotherapy. Normal mucosa was obtained 10 cm from the cancer tissue with normal visualization. The level of differentiation of the cancer tissues was determined as either well, moderately or poorly differentiated, and the TNM stage was estimated.

Fresh samples were obtained by surgical resections, immediately frozen in liquid nitrogen, and then stored at -80°C until use. In addition to fresh frozen samples, 10% formalin-fixed tissues were also prepared at the same time for immunohistochemical study.

**Proteomic analysis.** Proteomic analyses were performed as previously described (1). Briefly, protein extracts from cancer, normal mucosa and an internal pool were extracted from fresh frozen tissues, and then labeled with different fluorescent dyes. Each paired CRC and normal mucosa sample together with the internal pool (the mixture of all extracts of the 6 paired tissues) were resolved by electrophoreses on a single gel. After isoelectric focusing on immobilized pH gradient (IPG) and SDS-PAGE, proteins were visualized by fluorescence scanning. Changes in the amount of each protein were calculated by DeCyder 6.5 software (GE Healthcare). Proteins showing significant changes in abundance were excised from the gel, subjected to trypsin digestion and prepared for MALDI-TOF-MS analysis. Swiss-Prot database was used for sequence comparisons.

**Western blot analysis.** After tissue or cell sonication lysis, protein extracts were quantified using the BCA method. Samples equivalent to 50–90 µg proteins were resolved by 8–12% SDS-PAGE and transferred to a PVDF membrane. Primary antibodies for SELENBP1 (Abcam) and carcinoembryonic antigen (CEA) (Sigma-Aldrich) were diluted 1:1,000 and incubation was carried out overnight at 4°C. The anti-GAPDH antibody (Santa Cruz Biotechnology) was used as a loading control. After washing, membranes were incubated for 2 h with 1:4,000 diluted HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), washed and developed with ECL-Plus (GE Healthcare).

**Real-time reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was extracted from the tissue samples or the cells with TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 500 ng total RNA using a reverse transcription system (Promega). SYBR® Premix Ex Taq™ II (Takara) was used for real-time quantitative PCR analysis. The PCR reaction consisted of DNA denaturation (95°C, 30 sec) and 45 cycles of an amplification step (95°C, 5 sec; 62°C, 30 sec). The dissociation curve was generated for every run to validate the specificity of amplification. β-actin was chosen as the internal control. The sequences of the primers (Takara Biotechnology, Dalian, China) were as follows: SELENBP1-129048F (5'-TGG TGC TGC CCA GTC TCA TC-3') and SELENBP1-129048R (5'-AGT TCG CAC TTG GCA TGG A-3'); β-actin F, 5'-TGG CAC CCA GCA CAA TGA A-3' and β-actin R, 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'. For calculation of the relative mRNA level, the ΔACT method was used as described in the manufacturer's technical note (Roto Gene 6000).

**Immunohistochemistry.** Consecutive paraffin wax-embedded tissue sections (3-5 µm) were obtained, and the sections were dewaxed and rehydrated. The slides were pretreated for 2 min with citrate buffer (pH 6.0) in an autoclave to retrieve the antigen. To quench endogenous peroxidase activity, the slides were incubated in fresh 3% H2O2 and then washed for 4 min with water. For the specific staining of SELENBP1, the slides were first incubated for 1 h at room temperature with normal goat serum and then incubated at 4°C overnight with rabbit anti-human SELENBP1 (diluted 1:100; Abcam). After rinsing with PBS, the slides were incubated with 1:1,000 HRP-conjugated goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology) at 37°C for 30 min, rinsed with PBS, followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. Slides containing samples of normal mucosa were incubated with PBS instead of the primary antibodies to serve as negative controls. Immunostaining was graded by three pathologists as: 0, lack of staining; 1, weak staining; 2, moderate staining and 3, strong staining.

**Cell culture and treatments.** Human colon cancer cell lines, HCT116, HT29, LS174T, SW480 and SW620, were obtained from the laboratory stocks of the Department of Cell Biology, China Medical University. All of the cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (HyClone®, Thermo Scientific) and incubated at 37°C in a humidified 5% CO2 atmosphere. Human colonic epithelial cell line, HCoEpiC (ScienCell, San Diego, CA, USA), was cultured in high-glucose DMEM medium (HyClone®, Thermo Scientific) under the same conditions as above.

The cultures of HT29, SW480, SW620 cells (in two 25-cm² flasks) were treated with sodium butyrate (NaB) to induce differentiation. Cells were allowed to adhere for 24 h, and then the medium was replaced with medium containing 2 mM NaB (Sigma Aldrich) and incubated for 72 h, with changes of the NaB-containing medium every 24 h.

For analysis of epigenetic modifications, cells were treated with 5'-aza-2'-deoxycytidine (5-Aza-dC) and/or Trichostatin A (TSA; Sigma Aldrich). HT29, SW480 and SW620 cells were incubated for 24 h prior to treatment with the drugs as follows: i) 5-Aza-dC (5 µM) for 72 h, medium changes every 24 h; ii) TSA (0.3 µM) for 24 h; iii) 5-Aza-dC (5 µM) for 48 h followed by TSA (0.3 µM) for an additional 24 h. Control cells were incubated without NaB or 5-Aza-dC or TSA, with medium changes every 24 h. The experiments were repeated three times.

**Alkaline phosphatase (AKP) activity assays.** Following treatment of HT29, SW480 and SW620 cells with NaB for 72 h, the cells were harvested and lysed. AKP activity was assayed in the cell lysates using an AKP kit (Pointe Scientific Inc. Canton, MI, USA) according to the manufacturer's protocol, using para-nitrophenol phosphate as a substrate. Reaction production was detected by the absorbance at 400–415 nm.
Statistical analysis. All data are expressed as means ± SD. Measurement data that did not follow a Gaussian distribution were transformed into a logarithm. 2D DIGE spots with intensity changes, Western blot analysis and RT-PCR results were analyzed by paired two sample t-test or one-way ANOVA. Western blot data were analyzed by a paired two-sample t-test. Immunohistochemistry staining intensity score was analyzed by Wilcoxon signed-rank test or Kruskal-Wallis test. Statistical significance was defined as P<0.05.

Results

Tissue samples. A total of 83 sporadic CRC patients who had not received preoperative chemotherapy or radiotherapy were included in the present study. Forty-two patients were males, 41 were females, and their mean age was 61.3±12.4 years. All were cases of adenocarcinoma. From all patients, samples of cancer tissue along with paired adjacent normal mucosa were collected. There were 33 benign polyps including 20 hyperplastic polyps and 13 adenomatous polyps, and 26 lymph node metastasis (LNM). Of the 83 tissue samples, the majority were considered moderately differentiated (44/83, 53.0%), while 17 (20.5%) were well differentiated and 22 (26.5%) were poorly differentiated. According to TNM stage, 6 were stage I, 35 were stage II, 38 were stage III and 4 were stage IV.

Proteomic analysis of CRC tissues and normal mucosa. In the 2D-DIGE analysis, cancer tissues and normal mucosa were compared in the collected samples. The spot intensities of more than 20 proteins from the CRC samples were found to be <1.5-fold that of the normal mucosa (P<0.05), and these were identified by MALDI-TOF-MS analysis and Swiss-Prot database similarity search with a score of at least 64. In 6 CRC tissue samples, one particular protein spot was significantly less intense than this spot in the corresponding normal mucosa samples, by an average of 2.54-fold (Fig. 1). Subsequent MALDI-TOF-MS analysis and Swiss-Prot database similarity search identified it as SELENBP1 with a score of 148 (Fig. 1).

Differential expression of SELENBP1 in the CRC tissues. To validate our proteomic analysis, 24 paired fresh frozen CRC and normal mucosa samples were randomly selected, and SELENBP1 expression was analyzed by western blot analysis and real-time RT-PCR. Western blot analysis revealed that SELENBP1 expression relative to the internal control was 1.68±0.41 and 0.95±0.23 in the normal and CRC tissues, respectively (P<0.01), indicating a 1.77-fold downregulation in CRC. RT-PCR analysis revealed that SELENBP1 expression relative to the internal control was 4.75±3.36 and 2.15±1.90 in the normal and CRC tissues, respectively (P<0.01), indicating a 2.21-fold downregulation in CRC (Fig. 2).

SELENBP1 expression in human colon carcinoma cell lines. SELENBP1 expression in the HCoEpiC cultured cells was compared with that in five human colon cancer cultured cell lines, by western blot analysis and real-time RT-PCR. In all
cell lines except for LS174T, protein and mRNA levels of SELENBP1 were significantly lower than the levels in the HCoEpiC cells (Fig. 3).

**Immunohistochemical staining of SELENBP1 in CRC tissues.** SELENBP1 protein expression was compared between the samples of CRC tissues and the corresponding normal mucosa by immunohistochemical staining. SELENBP1 was expressed in both the cytoplasm and nucleus. SELENBP1 expression scores in CRC tissues (n=83) and their paired normal mucosa (n=83) were 1.25±0.51 and 2.02±0.57, respectively (P<0.01). Of the 83 patients, in 56 patients (67.5%), SELENBP1 expression scores in CRC were lower than those in the normal mucosa; while in 18 patients (21.7%) SELENBP1 expression scores in CRC were similar to those in the normal mucosa.

The SELENBP1 staining scores in well-differentiated (n=17), moderately differentiated (n=44) and poorly differentiated (n=22) CRC tissues were 1.75±0.53, 1.29±0.41 and 0.89±0.49, respectively, with a significant difference (P<0.05). Among the CRC samples analyzed, there were only 6 and 4 that were classified as TNM stages I and IV respectively; therefore, early (I and II, n=41) and advanced (III and IV, n=42) stages were pooled for analysis. However, SELENBP1 staining scores were not significantly different between TNM stages I/II (1.36±0.92) and III/IV (1.13±1.03). The staining scores of SELENBP1 were similar in the benign polyps (1.97±0.57, n=33) and their paired normal mucosa (2.06±0.59, n=28). However, the staining score in the benign polyps was significantly higher than the score in CRC (0.96±0.59, n=28) (P<0.01).

There was no significant difference between the SELENBP1 expression score in LNM (1.27±0.67, n=26) and the score in the primary cancer tissues (0.96±0.63, n=23) (P>0.05) (Fig. 4). **SELENBP1 expression is correlated with degree of differentiation.** SELENBP1 expression was compared between CRC samples showing different levels of differentiation. Of the fresh frozen CRC samples analyzed, 10 were well differentiated, while 14 were moderately differentiated and 10 were poorly differentiated. Western blot and real-time RT-PCR analyses
showed that the expression levels of SELENBP1 exhibited significant differences (P<0.01) (Fig. 5).

**SELENBP1 expression in NaB-induced differentiated cells.** To analyze the correlation between the degree of differentiation and SELENBP1 expression, cultured cell lines were induced to differentiate by NaB treatment. Expression levels of SELENBP1 and CEA, and enzyme activity levels of AKP were assessed before and following treatment with NaB for 72 h. Western blot analysis showed that SELENBP1 protein levels were similar in all cell lines prior to NaB treatment, but were higher by ~3.73-, 2.25- and 3.12-fold, in the SW480, SW620 and HT29 cells, respectively, following NaB treatment (P<0.05) (Fig. 6). RT-PCR analysis also revealed that SELENBP1 mRNA levels were higher by ~3.19-, 4.82- and 2.24-fold, respectively, in these three cell lines (P<0.05) following NaB treatment (Fig. 6).

Similarly, following NaB treatment, SW480, SW620 and HT29 cells showed upregulation of CEA protein by ~2.54-, 2.58- and 3.67-fold, respectively (P<0.05), and upregulation of AKP activity by ~2.50-, 2.32-, and 3.55-fold, respectively (P<0.05) (Fig. 7).

**Epigenetic modifications and SELENBP1 expression.** To investigate whether SELENBP1 is regulated by epigenetic modification, cultured SW480, SW620 and HT29 cells were incubated with the DNA demethylating agent 5-Aza-dC and/or the histone deacetylase inhibitor, TSA.

Following treatment with TSA alone, SELENBP1 protein levels were higher by 1.82- and 2.12-fold on average in the SW480 and SW620 cells, respectively, compared with these levels in the corresponding untreated control cells (P<0.05), while in the HT29 cells the change was not significant (Fig. 8). SELENBP1 mRNA levels were also higher by
3.64- and 3.01-fold on average in the SW480 and SW620 cells, respectively, when compared with the levels in the untreated control cells (P<0.05). Although SELENBP1 protein did not significantly increase in the HT29 cells, the mRNA level was significantly higher by 1.25-fold following TSA treatment (P<0.05) (Fig. 8).

Treatment with 5-Aza-dC alone did not significantly alter SELENBP1 protein and mRNA levels in any of the three cell lines when compared with these levels in the untreated controls (P>0.05) (Fig. 8). However, following the combined treatment of 5-Aza-dC and TSA, SELENBP1 protein levels were higher by 1.82- and 1.83-fold on average in the SW480
and SW620 cells, respectively, when compared to the levels in the corresponding untreated control cells (P<0.05). In the HT29 cells, SELENBP1 protein levels increased slightly, but insignificantly. SELENBP1 mRNA levels were higher by 4.13-, 3.16- and 1.32-fold on average in the SW480, SW620 and HT29 cells, respectively, when compared with the levels in the corresponding untreated control cells (P<0.05) (Fig. 8).

Discussion

CRC is one of the most commonly diagnosed malignant diseases worldwide. Although great progress has been achieved in ensuring early diagnosis and treatment, many patients still succumb to CRC due to cancer progression and metastasis. Understanding the molecular alterations underlying CRC is critical for identifying new biomarkers and therapeutic targets that may ultimately lead to individualized cancer treatment.

Proteomic analysis is one of the most effective and high-throughput methods by which to screen novel biomarkers for cancer (3,4,14,15). In the present study, proteomic analysis showed SELENBP1 to be one of the significantly downregulated proteins in CRC. Furthermore, western blot analysis and real-time RT-PCR of CRC patient tissue samples confirmed this finding. SELENBP1 protein and mRNA levels were lower in established cancer cell lines when compared with levels in the HCoEpiC cell line. SELENBP1 was demonstrated to be significantly downregulated not only in CRC (4,5), but also in

Figure 8. SELENBP1 expression after treatment with histone modifying agents. Cultured cells of the indicated cell lines (SW480, SW620 or HT29) were processed untreated (Control) or treated with 0.3 µM trichostatin alone (TSA), or with 5 µM 5’-aza-2’-deoxycytidine alone (5-Aza-dC) or with both (5-Aza + TSA), and SELENBP1 expression was measured by immunoblotting (A-C) and real-time RT-PCR analyses (D and E). (A) SELENBP1 and GAPDH (loading control) levels in the culture extracts of the indicated cell lines were visualized by immunoblotting. (B and C) SELENBP1-specific band intensities in the above immunoblots were quantified by densitometry and expressed relative to those of GAPDH (loading control). (D and E) Real-time RT-PCR for SELENBP1 mRNA levels, were expressed relative to those of the internal control.
lung, ovarian, esophageal, gastric and liver cancer by different research groups (2,3,6-8).

Immunohistochemical staining showed slightly higher expression of SELENBP1 in TNM stages I/II than in stages III/IV, but the difference was not significant. The difference in immunohistochemical staining for SELENBP1 was similar between normal mucosa and benign polyps, but was higher in benign polyps than in cancer tissues. The difference in SELENBP1 staining between primary cancer tissues and lymph node metastasis was not significant. These results are similar to those reported by Kim et al (4). SW480 and SW620 cells were obtained from the same patient, but from primary and lymph node metastasis. The similarity of SELENBP1 expression levels in the two cell lines also confirmed the results.

However, in contrast to the report by Kim et al (4), our IHC analysis showed significantly differential expression of SELENBP1 among well, moderately and poorly differentiated CRC tissues from freshly obtained samples. SELENBP1 had the strongest expression in terminally differentiated epithelial cells on the luminal surface of crypts in normal mucosa (Fig. 4). Li et al (5) also demonstrated that SELENBP1 was upregulated during differentiation and that its downregulation by small interfering RNA in colonic cells was associated with reduced expression of the differentiation marker, CEA.

Sodium butyrate (NaB) has been widely used as a differentiation inducer in CRC research (16-18). In the present study, NaB treatment led to reactivation of expression of SELENBP1 protein and mRNA, concomitant with upregulation of the differentiation indicators, CEA and AKP (5,17,18). One of the differentiation characteristics of colon epithelium is the appearance of or a significant increase in brush border hydrolase enzyme activities, such as AKP. Cell lines with high expression of CEA were found to exhibit differentiation-associated morphologic changes and decreased cell growth and de novo tumor formation in nude mouse xenografts (18).

In the present study, SELENBP1 expression was not affected by treatment with 5-Aza-dC alone, but was significantly upregulated following treatment with TSA alone or combined with 5-Aza-dC. Since NaB and TSA are both HDAC inhibitors (19-21), we hypothesized that the downregulation of SELENBP1 was mainly due to histone deacetylation that occurred following treatment with these two agents and during differentiation. Pohl et al (22) showed that treatment with 10 or 30 μM 5-Aza-dC for 72 or 96 h, significantly reactivated SELENBP1 in the cancer cell line HCT116. Further research concerning the relationship between SELENBP1 and histone deacetylation is warranted.

Cancer is a type of differentiation disorder disease. Inducing cancer cells to re-differentiate is a concept that is being pursued for cancer therapy (23). Although 5-Aza-dC is routinely applied at a concentration of 5 μM for epigenetic modification research (24-26), as a cancer therapeutic, the correct dosage and the associated side-effects of a differentiation inducer must be determined.

Collectively, our results indicate that SELENBP1 expression is downregulated in CRC, is associated with differentiation, and can be reactivated by NaB and TSA. Therefore, SELENBP1 is a potential new target for pharmacological intervention for CRC.

Acknowledgements

The present study was supported by the General Project of Liaoning Province Department of Education (L2010600).

References


