Research Article

Expression of selenium-binding protein 1 characterizes intestinal cell maturation and predicts survival for patients with colorectal cancer

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To identify candidate genes involved in the development of colorectal cancer, we used cDNA microarrays to analyze gene expression differences between human colorectal tumors and paired adjacent normal mucosa. We identified ~3.5-fold significant downregulation of selenium-binding protein 1 (SBP1) in colorectal tumors compared to normal mucosa ($p = 0.003$). Importantly, stage III colorectal cancer patients with low tumor-SBP1 expression had significantly shorter disease-free and overall survival as compared with those patients with high tumor-SBP1 expression ($p = 0.04$ and 0.03, respectively). We further characterized the role of SBP1 in colorectal cancer *in vivo* and *in vitro*. In normal tissue, SBP1 was maximally expressed in terminally differentiated epithelial cells on the luminal surface of crypts in the large intestine. Consistent with this *in vivo* localization, SBP1 was upregulated during *in vitro* colonic cell differentiation along the absorptive (Caco-2) and secretory (HT29 Clones 16E and 19A) cell lineages. Downregulation (approximately 50%) of SBP1 expression by small interfering RNA in colonic cancer cells was associated with reduced expression of another epithelial differentiation marker, carcinoembryonic antigen (CEA), although PCNA and $p21^{WAF1/cip1}$ expression were not altered. These data demonstrate that higher expression of SBP1 is associated with differentiation of the normal colonic epithelia and may be a positive prognostic factor for survival in stage III colorectal carcinoma.

Keywords: Colorectal cancer / Human selenium-binding protein 1 / Intestinal cell maturation / Intestinal cell differentiation / Prognostic factor

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1 Introduction

Colorectal cancer is the fourth most commonly diagnosed malignant disease worldwide, with an estimated 1 023 000 new cases and 529 000 deaths each year [1]. Although early diagnosis through screening and better treatment modalities have contributed to the decreased mortality over the past 30 years, more than one-third of patients diagnosed with early stage colorectal cancer will ultimately develop and die from incurable metastatic disease. Several clinical prognostic factors predictive of recurrence are male gender, tumor invasion to or through the serosa (T4), poorly differentiated histology, mucinous features, signet-ring features, and elevated levels of carcinoembryonic antigen (CEA) preoperatively. However, the role of these biomarkers has not been clinically validated. Currently, pathological stage at the time of presentation using the tumor–node–metastasis (TNM) system of the American Joint Committee on Cancer, which includes the depth of tumor invasion through the bowel wall (T), the extent of regional lymph node involvement (N), and the presence of distant metastasis (M), is the most commonly used staging system and the most important prognostic factor in colorectal cancer [2].

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Abbreviations: CEA, carcinoembryonic antigen; **FFPE,** formalin-fixed, paraffin-embedded; **SBP1,** selenium-binding protein 1; **TMA,** tissue microarray

Stage III colorectal cancers have metastases in local/regional lymph nodes and significantly lower 5-year survival rates of approximately 50% (SEER Surveillance Epidemiology and End Results Program, www.seer.cancer.gov, accessed: October 11, 2007). The use of adjuvant chemotherapy has increased the likelihood of cure by 30% among the patients with stage III disease, but causes significant toxicity [3 – 5]. Improving the efficacy of adjuvant chemotherapy by individualizing cancer treatment according to the tumor and the patient's characteristics, and by developing newer, less toxic therapeutic agents is needed to prevent tumor recurrence and to improve colorectal cancer-free survival.

Understanding the molecular alterations underlying colorectal cancer is critical to identify new biomarkers and therapeutic targets that ultimately lead to individualize cancer treatment instructed by the molecular signature of the tumor. Using gene expression profiling by cDNA microarrays, we found that the expression of human selenium-binding protein 1 (SBP1) was significantly downregulated in colorectal tumors as compared to adjacent normal colonic mucosa [6]. The human SBP1 (also called SELENBP1 or hSP56), which binds covalently to selenium, was cloned and characterized in 1997 [7]. The SBP1 gene is located at chromosome 1q21-22 and encodes a protein of 472 amino acids that is present as at least four different phosphorylated isoforms in membrane-enriched cellular compartments [8 – 10]. Two mouse homologs (mSP56/mSBP1 and AP56/mSBP2), displaying approximately 87% homology with the human sequence, have also been described [7]. The expression of SBP1 is high in the heart and intermediate in the liver, lung, kidney, and intestine. It has been suggested that SBP1 facilitates intracellular transport of selenium in the late stages of intra-Golgi protein transport [11] and protrusive cell motility [12]. Recent reports demonstrated that SBP1 protein is downregulated in several epithelial tumors including lung [8], prostate [13, 14], stomach [15], colorectal [6, 16], and ovarian cancer [10]. However, the mechanisms of action of SBP1 remain unclear. The objectives of this study were to determine the prognostic role of SBP1 expression in patients with stage III colorectal cancer and to dissect the role of SBP1 expression in colon cancer using *in vivo* and *in vitro* models.

2 Materials and methods

2.1 Tumor samples for RNA and protein analyses

Paired tumors and normal tissues were obtained from a bank of snap frozen tissue from colorectal cancer patients who underwent surgical resection and consented for tissue collection according to an Institutional Review Board-approved protocol at Montefiore Medical Center in New York. All study materials were coded to protect confidentiality for patients. Colorectal tumor and normal tissues were processed immediately after resection, snap-frozen, and stored at $-80^{\circ}C$ until use. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks from the same study patients were obtained for Hematoxylin and Eosin (H&E) and immunohistochemical staining.

2.2 Tissue microarray

An independent published cohort of FFPE samples from a total of 80 stage III colorectal tumors collected from patients in southern Finland was used for immunohistochemical assessment of SBP1 expression by tissue microarray (TMA) [17 – 19]. Informed consent was obtained from each patient according to a Human Investigations and Ethical Committee-approved research proposal. Cause-specific deaths have been recorded from hospital and postmortem reports. These samples were collected before adjuvant chemotherapy became standard practice [20, 21]. All of the patients underwent surgery alone as treatment and did not receive any adjuvant therapy. Complete clinical follow-up was available for these patients for at least 6 years (see Table 1). Thus, this dataset represents a unique cohort of patients to evaluate the prognostic value of biomarkers. After histological examination of hematoxylin/eosin-stained tumor sections, triplicate 0.6-mm cores of representative, paired normal, and tumor samples from 80 patients (*i. e.*, a total of six samples *per* patient) were selected to construct TMAs in fresh paraffin blocks using a Beecher Instruments tissue arrayer (Beecher Instruments, Silver Spring, MD). Each TMA slide contains a maximum of 146 spots of tissue samples. Four-micromolar sections of the TMAs were mounted on slides coated with 3-aminopropyl-triethoxy-silane and used for immunohistochemical staining of SBP1 as described below.

2.3 Cell Lines

All cell lines were cultured in MEM, supplemented with 10% FCS, 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 100 Us/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Life Technologies), and maintained at $37^{\circ}C$ in a humidified atmosphere of 95% air and 5% CO_2. For spontaneous differentiation studies, Caco-2, HT29 cl.16E, and HT29 cl.19A cells were cultured to confluence (day 0), and for 2, 5, 7, 14, or 21 days post-confluence, with medium changed every other day as previously described [22, 23]. Cells were harvested in PBS at different time points.

2.4 Small interfering RNA (siRNA) experiments in Caco-2 and SW620 cells

siRNA oligonucleotides targeted against SBP1 and a non-targeting siRNA control pool were either purchased from Dharmacon (Lafayette, CO) for Caco-2 cells, or designed

Table 1. Clinical and pathological features of 80 colorectal cancer patients in the TMA study

Categories	Tumors with low SBP1 level (<22.5%)	Tumors with high SBP1 level (≥22.5%)	Significance
No. of Patients (n = 80)	49	31	
Median age (years)	72.0	71.0	$p = 0.65$[a)]
Range	40 – 88	45 – 86	
Gender			
Female (%)	25 (51%)	16 (51.6%)	$p = 1$[b)]
Median grade (1 – 3)	2.0	2.0	$p = 0.34$[a)]
Tumor location			
Colon	25 (51%)	17 (55%)	$p = 0.39$[b)]
Rectum	24 (49%)	14 (45%)	
Median follow-up (years)	9.2	9.9	$p = 0.12$[a)]
Tumor-SBP1 expression (%)	6.67%	46.67%	$p = 6.84$ E-20[a)]*
Normal mucosa-SBP1 expression (%)	40.8%	40.0%	$p = 0.89$[a)]
Tumor/normal SBP1 Level (ratio)	0.19	1.00	$p = 5.51$ E-09[a)]*
Disease-free survival (years)	0.8	2.5	$p = 0.04$[c)]*
Overall survival (years)	1.4	3.5	$p = 0.03$[c)]*

* Indicates statistically significant differences that are defined as a two-sided p-value < 0.05.
a) Unpaired Student's t-test.
b) Fisher's exact test.
c) Log-rank analysis.

and ordered from Sigma (St. Louis, MO) for SW620 cells. The sense and antisense sequences of SBP1 siRNA were 5′-AAGGAGGGCtGAAGttGAAtt-3′ and 5′-UUCAACUUCAGCCCUCCUUtt-3′, respectively (Sigma), which were not complementary to any other known sequences in the human genome, determined using the Blast program. Control transfections were carried out using the nonspecific siRNA of green fluorescent protein (GFP), which is a reporter molecule for monitoring gene expression and protein localization *in vivo* and in real time [24]. The sense and antisense sequences of GFP siRNA were 5′-AAG CUA CCU GUU CCA UGG CCA dTdT-3′ and 5′-UGG CCA UGG AAC AGG UAG CUU dTdT-3′, respectively. Undifferentiated Caco-2 (day 0) cells expressing low endogenous SBP1 were transfected with SBP1 siRNAs or control siRNA according to the manufacturer's instruction (Dharmacon). Medium was changed the following day and transfected Caco-2 cells were allowed to differentiate for 5 or 10 days before harvest, a time course over which Caco-2 cells have previously been shown to undergo spontaneous differentiation [22]. SW620 cells expressing high endogenous SBP1 expression were transfected with siRNA targeting SBP1 or control siRNA by Lipofectamin 2000 (Invitrogen, Carlsbad, CA) and allowed to grow for 48 h before harvest. Alteration in expression of SBP and CEA was confirmed by Western blot analysis using β-actin expression as a loading control.

2.5 RNA and protein isolation

Total RNA was isolated from snap frozen tissues by homogenization in TRIzol® reagent (Invitrogen Life Technology) followed by a cleanup step using RNeasy columns (Qiagen, Valencia, CA) according to the manufacture's instructions. The quality and quantity of RNA were analyzed using an Agilent 2000 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA) or spectrophotometrically (Ultrospec 3100 Pro, Biochem, Cambridge, England). The quantity of protein was also determined spectrophotometrically.

2.6 cDNA microarray analysis

cDNA microarrays encompassing 28 704 human cDNA clones, which represented 14 685 unique genes, were prepared by the Albert Einstein College of Medicine Microarray Facility. cDNA probes were synthesized from equal amounts (50 μg) of total RNA from a sample (labeled with Cy5 dUTP) and a reference RNA (labeled with Cy3 dUTP) (Amershan) in each case as described [25]. The hybridization, data collection, and analysis were performed using GenePix Pro 3.0 (Axon Instruments, Foster city, CA) as described [25].

2.7 Quantitative real-time PCR

cDNA was synthesized by random priming of DNase-treated total RNA using TaqMan MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) as described [26]. Quantitative RT-PCR analysis was performed in an ABI Prism 7900-HT Sequence Detection System (96-well, Applied Biosystems) by the use of the PCR and the fluorogenic 5′-nuclease assay. Each PCR reaction used 50 ng cDNA as template and SYBR® Green PCR Master Mix. Primers used were: SBP1-351F, 5′-TCTCGCATC-

TATGTGGTGGA-3′, SBP1-468R, 5′-AGTGGCTGGTG-TGGAGAAAG-3′ (NP_003944); and β-actin-1265F, 5′-GATGAGATTGGCATGGCTTT-3′, β-actin-1364R, 5′-CACCTTCACCGTTCCAGTTT-3′ (NM_001101). Each sample was assayed in duplicate or triplicate. Real-time raw fluorescence data were acquired using SDS 2.0 software (Applied Biosystems) and the threshold cycle (C_t), which was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise), was determined. The comparative C_t method was used for the relative quantitative detection of SBP1 expression with the β-actin as an endogenous control for each reaction. This method is similar to the standard curve method, except that it uses the arithmetic formula $2^{\Delta\Delta C_t}$ to achieve relative quantification (Applied Biosystems User Bulletin). The $\Delta\Delta C_t$ was the difference between C_t value and initial copy number. The relative levels of SBP1 expression were derived from the ratios of sample/internal control (β-actin gene) and were presented as the fold change in gene expression normalized to SBP1 mRNA level at time 0 [26]. The presence of expected PCR products was determined by agarose gel electrophoresis and confirmed by direct sequencing of PCR products.

2.8 Western blots

Western blot analysis was performed on cell lysates of human tumor and normal tissues and cell lines. Twenty to fifty micrograms of protein from each sample were resolved by SDS-PAGE, followed by transfer to nitrocellulose membranes. The blots were then probed with antibodies at the following dilutions: anti-SBP1 antibody (mouse monoclonal, MBL™ International Corporation, Watertown, MA; 1:1000), anti-CEA antibody (Zymed, CA; 1:1000), and anti-β-actin antibody (Sigma, 1:10 000). Secondary antimouse or antirabbit antibodies were used at a dilution of 1:5000 with incubation for 1 h at room temperature. The detected signals were visualized by an enhanced chemiluminescence reaction system, as recommended by the manufacturer (Amersham, Piscataway, NJ) and quantified using ImagQuant software (Molecular Dynamics, Amersham Biosciences). Equal loading was assessed by immunoblotting for β-actin.

2.9 Immunohistochemistry

For human samples, 4 – 5-μm sections from archival FFPE human colorectal tumor tissues or the TMA were deparaffinized, rehydrated, and quenched with 1.5% H_2O_2. For antigen retrieval, slides were treated with DakoCytomation Target Retrieval Solution (Dako, Carpinteria, CA) in a steam bath at 95°C for 45 min. After equilibration in PBS for 15 min, slides were placed in an Autostainer apparatus (Dako) and incubated with anti-SBP1 antibody (mouse antihuman mAb M061-3, clone 4D4, MBL International Corporation) at 1:100 dilution at room temperature for 30 min. Immunoreactivity was detected using the Dako EnVision method according to the manufacturer's instructions. For negative controls, slides were subjected to the same procedure, including antigen retrieval, except for omission of the primary antibody. The results were reviewed independently by 2 surgical pathologists (M. L. and W. Y.), who were blinded to the clinical or pathological information of these patients. A semi-quantitative scale from 0 to 100% was used to grade the percentage of SBP1-stained epithelial cells relative to all epithelial cells in each tissue core on the TMA slides. The average score of replicate samples was used in the subsequent analyses.

2.10 Statistical analysis

All experiments were performed in duplicate or triplicate. Data of triplicates were expressed as mean ± SD. Differences in clinicopathologic variables between patient groups were computed by Fisher's exact test. Paired or unpaired, two-tailed Student's *t*-tests were used to determine the differences of SBP1 gene expression level in tumor *versus* normal intestinal tissues in patients with colorectal cancer. Normality of the distribution of continuous variables was tested by the Kolmogorov – Smirnov test. Statistical correlations were determined using a Pearson's correlation coefficient. The optimal threshold to divide tumors into high and low SBP1 expression groups was identified by a systematic approach, which calculated the mean survival, hazard ratio, and log-rank *p*-value for every possible grouping of patients considered in sets of patients from 1 to 80 [17 – 19]. Survival curves were then constructed using the method of Kaplan and Meier. All statistical tests were based on two-tailed probability. Differences at a level of $p < 0.05$ were considered statistically significant.

3 Results

3.1 SBP1 is downregulated in human colorectal tumors

To identify candidate genes involved in the development of colorectal cancer, gene expression profiles were determined using RNA prepared from 14 human colorectal tumors and their matched adjacent normal mucosa from the fresh frozen tissue bank at our single institute using a 28 704-element cDNA microarray (14 685 unique genes). Figure 1A illustrates the data only for SBP1 expression, one of the most frequently downregulated genes (the entire database is available, upon publication, at augenlichtlab.com). Downregulation was clearly seen in 8 out of 14 tumor-normal pairs (*i. e.*, 58%). Of note, there was excellent agreement between two cDNA clones of SBP1 on the human 28K microarray slides ($R^2 = 0.82$, Pearson's correlation coefficient), which had different origins: Genbank

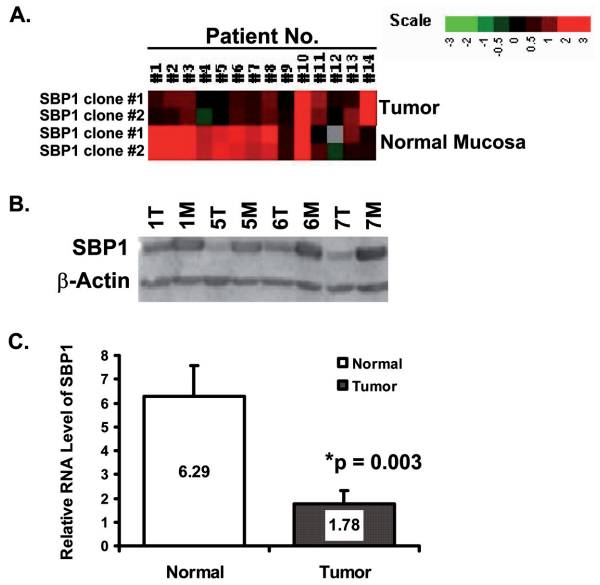


Figure 1. SBP1 expression in human colorectal tumors. (A) cDNA microarray analysis of SBP1 gene expression in tumor and normal paired samples of 14 colorectal patients. Total RNA was isolated from fresh frozen tumors (Tumor) or their adjacent nontumor colorectal tissues (Normal) in these patients. The expression of SBP1 was evaluated by two independent cDNA clones on 28K human cDNA microarray slides. (B) Western blot analysis confirmed the decreased SBP1 expression in tumors as compared to their adjacent nontumor tissues in patients with colorectal cancer. Total protein was extracted from tumors (T) and their matched nontumor tissues (M), and SBP1 expression was determined by immunoblots using anti-SBP1 antibody. β-Actin was used as a loading control. (C) Quantitative RT-PCR analysis of SBP1 expression in patients with colorectal cancer in an independent set of tumors (Tumor) and adjacent nontumor tissues (Normal). Bars represent mean ± SD of the mean. The asterisk (*) denotes a statistically significant difference (two-tailed, unpaired Student's *t*-test).

accession number T65736 cDNA was a 445-bp fragment transcribed from human lung mRNA, whereas AL531951 cDNA was a 1034-bp fragment transcribed from human fetal liver mRNA. This result was confirmed at the level of SBP1 protein expression in these samples by Western blot analysis. Representative data are shown in Fig. 1B, demonstrating SBP1 was decreased in most colorectal tumors (T) compared to normal mucosa (M). Furthermore, this finding was confirmed at the level of SBP1 RNA expression in an independent set of patient samples. Figure 1C illustrates that the expression of SBP1 RNA determined by quantitative RT-PCR was reduced by ~3.5-fold in colorectal cancer tissues compared to adjacent normal mucosa ($p = 0.003$, paired Student's *t*-test). The expression of β-actin was used as a loading control in both quantitative RT-PCR and Western blot analysis. Collectively, these data confirm that SPB1 expression is significantly downregulated in human colorectal tumors as compared to adjacent normal mucosa.

3.2 Low tumor-SBP1 expression predicts poorer survival outcomes

To determine whether downregulation of SBP1 was of prognostic value for patients with colorectal cancer, we analyzed SBP1 expression by immunohistochemistry on TMAs containing a published cohort of 80 paired tumor and normal tissues of colorectal cancer patients from a Finish institute as described in Section 2. These samples were collected before adjuvant chemotherapy became a standard of care in Europe, and these patients with Dukes C colorectal cancer did not receive any adjuvant chemotherapy after surgery [20, 21]. With a median follow-up of over 9 years, 48 patients (60.0%) relapsed, and 54 patients (67.5%) died, with 30% of elderly patients (*i.e.*, 70 years old) dying of diseases other than cancer. This cohort also contains 46% of patients with rectal cancer. Immunohistochemical staining data were available for 80 patients, whose clinical and tumor features are summarized in Table 1. Two surgical pathologists (W. Y. and M. L.), who were blinded to the clinical and pathologic data for the patients, independently analyzed the immunoreactivity of SBP1 protein. All epithelial cells present in each core were scored; this ranged from 250 to 500 cells. The percentage of positively stained epithelial cells relative to all epithelial cells for each TMA core was calculated. The average score of triplicate samples was used in the subsequent analyses.

We classified the 80 colorectal tumors with adequate IHC data as "tumor-high SBP1" (22.5%) and "tumor-low SBP1" (<22.5%) using the mean level (22.5%) of SBP1 expression in the tumors as the discriminator. This value was confirmed to be the optimal threshold to divide tumors into high and low SBP1 expression groups by a systematic approach that calculated the mean survival, hazard ratio, and log-rank *p*-value for every possible grouping of patients, as described in Statistical Analysis. As summarized in Table 1, the average percentage of SBP1 protein expression in tumor-low SBP1 and tumor-high SBP1 groups was 6.7 and 46.7%, respectively ($p < 0.0001$, Table 1); whereas the average percentages of SBP1 expression in normal epithelial cells in paired normal tissues between these two groups were not significantly different (40.8 and 40.0%, $p = 0.89$, Table 1). The 49 patients who had lower levels of tumor SBP1expresssion had median disease-free and overall survival of 0.8 and 1.4 years, respectively, whereas for the 31 patients who had higher levels of tumor-SBP1 expression the median disease-free and overall survival were 2.5 and 3.5 years, respectively. Importantly, these differences in disease-free and overall survival between patients whose tumors had lower and higher level expression of SBP1 were statistically significant ($p = 0.04$ and $p = 0.03$, respectively, by the Log-rank test; Figs. 2A and B, respectively). The two groups of patients were well balanced in other clinical and pathologic variables such as patient sex, age, tumor location (colon or rectum), histolog-

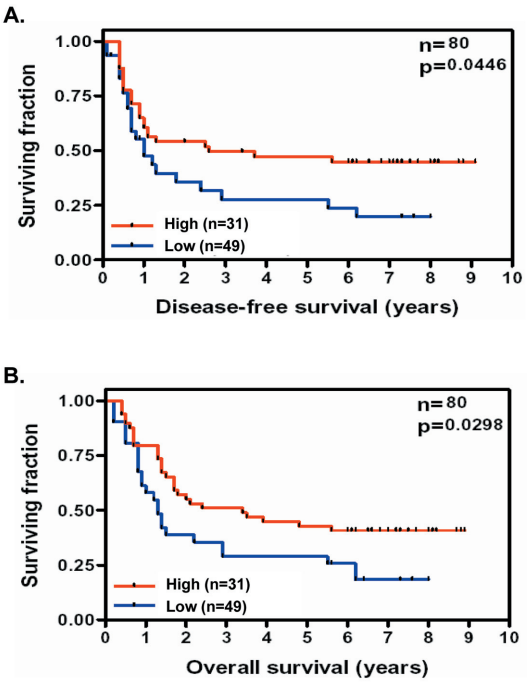


Figure 2. Kaplan–Meier estimates of disease-free survival (A) and overall survival (B) among stage III colorectal cancer patients according to the level of tumor SBP1 expression by IHC staining of TMAs. Patients whose tumors have lower SBP1 expression (<22.5%) had statistically shorter disease-free and overall survival compared to those patients whose tumors had higher SBP1 expression (≥22.5%). The differences between two groups were determined by the log-rank test and $p < 0.05$ was considered significant.

ical grade, and tumor stage (Table 1). We thus conclude that the expression level of tumor SBP1 may be an independent positive prognostic factor for patients with colorectal cancer. Consistent with the previous report in lung cancer demonstrating lower SBP1 expression was associated with poorer tumor differentiation [8], we observed a trend of lower SBP1 expression with poorer histological grade of colorectal tumor (*i. e.*, poor differentiation) in this cohort of patients, although this did not reach statistical significance (data not shown). Since SBP1 expression evaluated on limited portions of the tumors on the TMAs might not represent the histological grade of entire tumor specimen, we further characterized the role of SBP1 expression in intestinal cell differentiation.

3.3 SBP1 is upregulated during in *vivo* intestinal cell maturation and in *vitro* intestinal cell differentiation along the absorptive and secretory cell lineages

To characterize the role of SBP1 in intestinal cell differentiation, we next defined the cellular distribution of SBP1 in normal and colorectal cancer tissues by immunohistochemistry. Figure 3 illustrates that normal colonic epithelial cells expressed increasing level of SBP1 protein as they migrated along the crypt axis toward the luminal surface, with the highest expression observed in the terminally differentiated epithelial cells at the top of the crypts (Fig. 3A, longitudinal section, and 3C, cross-section). These data suggested that upregulation of SBP1 accompanies normal differentiation of intestinal epithelial cells *in vivo*. Of note, the expression of SBP1 protein in normal colonic tissues varied depending on the plane of cross-section of colonic tissue relative to the top of the crypts: the expression of SBP1 was higher in cross-sections encompassing most of the surface sections of the crypts, whereas the expression of SBP1 was lower in cross-sections that principally represent the base of the crypts (Fig. 3C). Higher power magnification revealed that SBP1 protein was expressed in both the cytoplasm and the nucleus of normal colonic epithelial cells (Figs. 3B and D), as previously reported [8, 10]. Weak staining of SBP1 was also observed in the surrounding connective tissue, consistent with previous reports of SBP1 expression in smooth muscles [9].

Because of the striking association of SBP1 expression with cell position along the crypt-luminal axis, we further characterized the expression of SBP1 in relation to cell differentiation using established *in vitro* models of colonic cell maturation. It is well known that epithelial cells in the intestinal mucosa differentiate along a number of lineages that provide various functions of absorption and secretion. Caco-2 cells are a very well-characterized model of absorptive cell differentiation. Upon contact inhibition, the cells spontaneously undergo growth arrest and begin to express classical markers of absorptive cell differentiation including CEA, alkaline phosphatase, sucrase isomaltase, and dipeptidyl-peptidase IV. Expression of these markers is maximal approximately 21 days postconfluence [22, 27–29]. We have previously characterized the changes in gene expression that occurred during Caco-2 cell differentiation using cDNA microarrays [30]. Inspection of this cDNA microarray database (available at augenlichtlab.com) identified an approximately 2.5-fold increase in SBP1 expression during this spontaneous cell differentiation of Caco-2 cells (Fig. 4A). This finding was confirmed by quantitative RT-PCR analysis (Fig. 4B) and by Western blot analysis (Fig. 4C). A significant correlation between SBP1 mRNA expression as determined by cDNA microarray (A) and real-time PCR (B) was observed ($R^2 = 0.92$, Pearson coefficient analysis).

We next examined SBP1 expression in the secretory cell lineage. HT29 cl.16E cells are a well-characterized model of secretory cell differentiation. Upon contact inhibition the cells spontaneously undergo growth arrest and differentiate into goblet cells that synthesize and secrete mucin, CEA, and alkaline phosphatase, which are maximal 21 days postconfluence [31, 32]. Parental HT29 cells do not undergo this differentiation program. There was again a robust upregulation of expression of SBP1 mRNA in these cells during

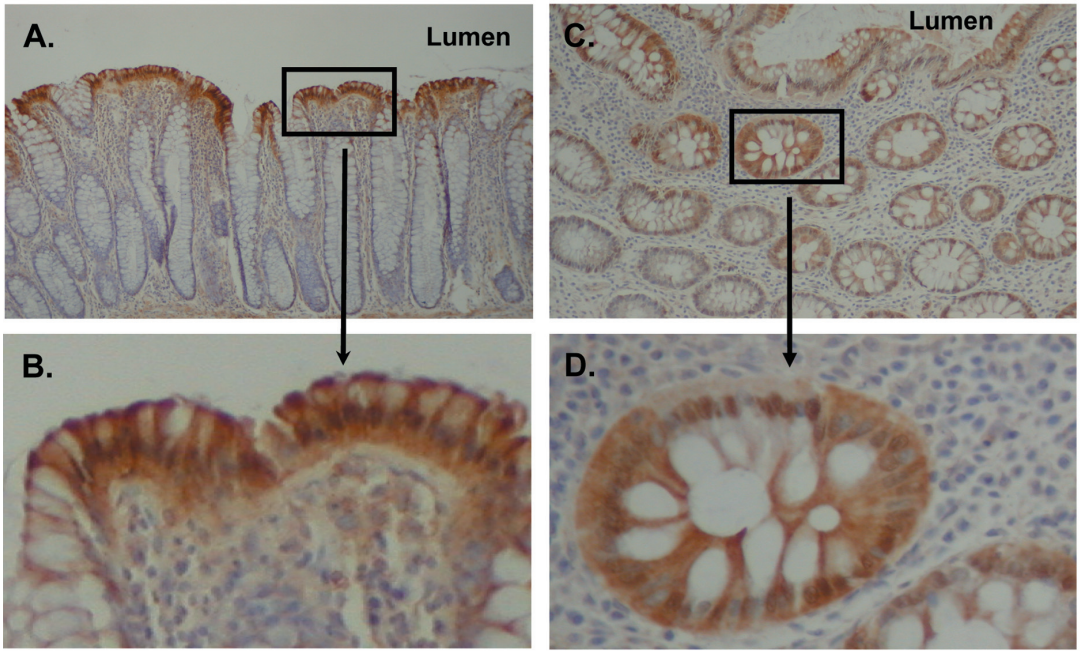


Figure 3. Immunohistochemical analysis of the SBP1 expression in normal human colonic tissues. Representative longitudinal- (A and B) and cross- (C and D) sections in relation to the crypt in normal colonic tissues showing upregulation of SBP1 expression in normal colonic epithelial cells as they migrate along the crypt-luminal axis (Magnification, 200 ×). (A) Expression of SBP1 protein is maximal at the tip of the villi. (C) The intensity of SBP1 expression varies significantly depending on the location of cross-sections in relation to the position of the crypt-lumen axis, with highest intensity at the top of the crypt and lowest intensity at the bottom of the crypt. (B) and (D) Higher power magnification of colonic epithelial cells (boxed areas in A and C, respectively) reveal that SBP1 protein is expressed in both the cytoplasm and the nucleus of colonic epithelial cells.

differentiation (Fig. 4D), which was recapitulated at the protein level by Western blot analysis (Fig. 4E). Interestingly, there was more modest induction of SBP1 in HT29 cl.19A cells, another clonal derivative of HT29 cells that differentiate into deep-crypt chloride-secreting cells over 21 days in culture [23, 33], consistent with the sequential maturation of intestinal cells as they migrate along the crypt axis in the colon. These data suggest that SBP1 expression is upregulated during intestinal cell differentiation, although the extent of upregulation may vary in different intestinal cell types.

3.4 siRNA-mediated downregulation of SBP1 is associated with decreased expression of CEA, an epithelial differentiation marker

We further explored the role of SBP1 expression during Caco-2 cell differentiation by downregulating SBP1 expression by siRNA. As shown in Fig. 4C, the very low level of SBP1 protein expression in undifferentiated Caco-2 cells at day 0 gradually increased from days 5 to 14. Thus, the transfected cells were maintained for 5 and 10 days post-transfection in order to determine the extent of SBP1 downregulation. The low level of SBP1 at day 5 was reduced by 62% by SBP1 siRNA relative to nonspecific (NSP) siRNA (see densitometrically quantified data in Fig. 5B). A similar 47% reduction of SBP1 expression was also seen at day 10 (Figs. 5A and B). The expression of CEA, a common marker of colonic cell differentiation along both the absorptive and secretory cell lineages in response to most stimuli, was also decreased by approximately 50%, suggesting a possible role of SBP1 in colonic cell differentiation. A similar downregulation of CEA following siRNA downregulation of SBP1 was observed in the human colonic cancer cell line SW620, a cell line that expresses high endogenous level of SBP1 (Figs. 5A and B). We have previously demonstrated that the cell proliferation marker PCNA was downregulated and the cell cycle inhibitor $p21^{WAF1/cip1}$ (p21) was upregulated during the differentiation of Caco-2 cells. The expression of PCNA and p21 were not altered by this level of SPB1 downregulation in Caco-2 cells (data not shown). In SW620 cells, the expression of p21 at day 2 is very low, making it difficult to determine the effect of SBP1 downregulation on this marker of cell maturation. Repeated attempts to increase the extent of SBP1 downregulation were unsuccessful due to the low efficiency of oligonucleotide transfection in these cell lines and the long half-life of SBP1 protein that exceeds the half-life of the oligonucleotides *in vitro* [9]. Thus, we could not resolve whether more efficient downregulation of SBP1 would modulate these, as

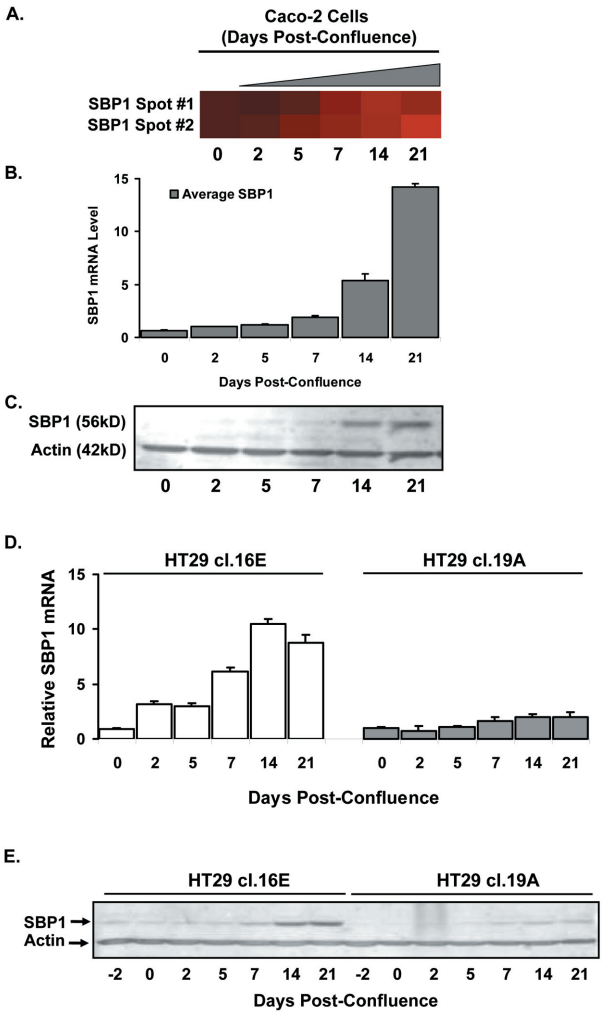
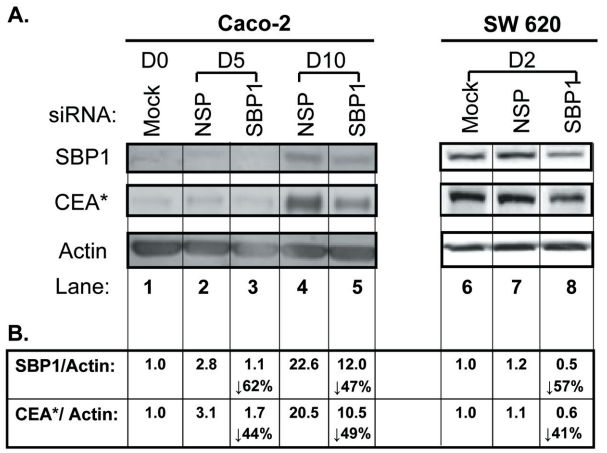


Figure 4. SBP1 expression during *in vitro* differentiation of colonic epithelial cells. Characterization of SBP1 expression during epithelial cell differentiation in human colonic tumor cell lines Caco-2 (A, B, and C) and HT29 clones 16E and 19A (D and E). (A) cDNA microarray analysis of SBP1 expression during the 21 day spontaneous differentiation of absorptive Caco-2 cells. The color scale of gene expression level was shown in Fig. 1. (B) RT-PCR analysis of SBP1 mRNA expression during the differentiation of Caco-2 cells. Data represent the average of SBP1 expression normalized to the expression level of β-actin. (C) Western blot analysis of SBP1 expression during the differentiation of Caco-2 cells. Expression of SBP1 gene was also upregulated during the differentiation of secretory cell lines HT29 cl.16E and HT29 cl.19A as assayed by RT-PCR (D) and Western blot (E) analyses. Equal loading was assessed by immunoblotting for β-actin expression.



* Less exposure time in Caco-2 cells.

Figure 5. Effect of SBP1 downregulation on colonic cell differentiation. Caco-2 and SW620 cells were mock transfected or transiently transfected with 100 nM of non-specific scrambled (NSP) siRNA or 100 nM of SBP1 siRNA as described in Section 2. Cells were allowed to differentiate for 5 and 10 days in Caco-2 cells and for 2 days in SW620 cells following transfection. The level of SBP1 and CEA expression were determined by Western blot analysis. (A) Western blots demonstrating the effect of SBP1 downregulation on the expression of CEA. Equal loading was confirmed by probing with an anti-β-actin antibody. (B) Densitometric analysis of Western blots demonstrating the average of SBP1 and CEA expression relative to β-actin and Mock transfection from at least two independent experiments.

well as other, markers of colonic cell differentiation and function. Therefore, while SPB1 is clearly a marker of differentiation that has prognostic significance in colorectal tumors, its functional role in differentiation remains to be defined.

4 Discussion

Selenium is an essential micronutrient that has been implicated as an important chemopreventive agent for several epithelial tumors, including colorectal cancer [34–36]. The mechanisms mediating the chemoprotective effects of selenium on these epithelial tumors remain to be elucidated. Interestingly, downregulation of SBP1 has now been shown in tumors from each of these sites and has been associated with poor prognosis in the patients with lung and colorectal cancer. Thus, SBP1 may mediate the effect of selenium and play a critical role in epithelial carcinogenesis and tumor progression. For colorectal cancer, there is an inverse association between higher blood selenium concentration and lower colorectal cancer risk in epidemiology studies [37]. A low selenium diet can lead to transcriptional activation of genes involving DNA damage, cell cycle control, and oxidative stress in animal models [38]. Dietary supplementation with selenium can inhibit the development of colon cancer [38, 39] and can synergistically enhance the antitumor effect of chemotherapeutic agents *in vitro* and *in vivo* models [40]. A recent study suggests that the physiological level of methylselenocysteine in the serum has a differential

modulating effect on SBP1 expression in normal and malignant ovarian cells [10], supporting that SBP1 is one of the targets of selenium.

In this study, we found that patients whose colorectal tumors have a lower SBP1 expression had a statistically significant shorter disease-free and overall survival than those patients whose tumors have a higher level of SBP1 expression, suggesting low tumor SBP1 expression is an adverse prognostic factor for patients with colorectal cancer. This is consistent with a previous study of expression profiling of colorectal tumors that reported downregulation of SBP1 mRNA expression in tumor compared to normal colon tissue [41]. Our study also extends this finding to distinct patient populations, and importantly, to analysis of the survival as well as to a link to lineage-specific differentiation. A recent report, published during the preparation of this paper [6], showed that SBP1 is associated with poorer survival in stage II but not stage III colorectal carcinomas in a Korean population [16]. In the study reported here, however, a more rigorous systematic analysis was used to validate the selection of the cut-off value between high and low expression of SBP1. While there are similarities between the Korean study and ours, there are also important differences. First, although both studies initially evaluated 14 tumor/normal pairs, we identified downregulation of SBP1 in tumors compared to matched normal controls by gene expression profiling at the RNA level, while the Korean study did so by a proteomic approach. Second, both studies used the same anti-SBP1 antibody and the scoring of SBP1 was based on the percentage of immunopositive cells to evaluate the prognostic value of SBP1 in human colorectal tumors. However, while the Korean study selected a cut-off of greater than 10% SBP1-stained cells to score as positive, we used the mean of SBP1 expression (22.5%) as the cut-off for discrimination of high *versus* low SBP1 expression, which was validated as the optimal threshold by systematic statistical analysis. Third, by this immunhistochemical method, in our population, the mean SBP1 level of tumors and matched normal controls were 6.7 and 46.7%, respectively. Considering that there is also a trend of decreasing expression with tumor stage, it is thus not clear whether selecting a 10% cut-off in the Korean study influenced the failure to detect a survival difference in stage III patients alone.

We further characterized the role of SBP1 in colonic cell differentiation *in vivo* and *in vitro*. We demonstrated that SBP1 was highly expressed in normally differentiated intestinal epithelial cells located on or near the luminal surface of the crypts of the large intestine in humans, suggesting that upregulation of SBP1 accompanies normal differentiation of intestinal epithelial cells *in vivo*. Using *in vitro* models of human colonic cell differentiation, we confirmed that upregulation of SBP1 was associated with colonic cell differentiation along both the absorptive (Caco-2) and secretory (HT29 clones16E and 19A) cell lineages, although the extent of upregulation varied among the lineages. This is consistent with a previous report in lung cancer, demonstrating that lower SBP1 expression was associated with poorer tumor differentiation [8]. The SBP1 gene is located at chromosome 1q21-22, a region which contains a number of genes constituting the epithermal differentiation complex, many of which are involved in the process of terminal differentiation of human epidermis (UniGene Hs.632460). To further understand the role of SBP1 expression in colonic cell differentiation, we determined the effect of downregulation of SBP1 on the expression of another epithelial cell differentiation marker, CEA, which is located in chromosome 19. Indeed, downregulation of SBP1 expression by siRNA is associated with a paralleled decrease in CEA expression, suggesting the potential that SBP1 might play a mechanistic role in differentiation in addition to being a marker of differentiation in at least the absorptive and secretory cell lineages. However, we could not reduce SBP1 expression by more than 50%. At this level, there was little effect on other differentiation/maturation markers tested other than CEA.

Although the function of SBP1 remains to be fully characterized, the downregulation of SBP1 at both the mRNA and protein level [8, 13, 14, 16, 41] demonstrate that SBP1 is a readily assayable biomarker present across different tumors and patient populations. Many mechanisms for the chemoprotective effect of selenium have been suggested [35, 42, 43], including decreased cell proliferation, blocked cell cycle progression, enhanced cell death, increased liver glutathione peroxidase or thioredoxin reductase activities as an antioxidant [44], modulated ER stress response [45], DNA hypomethylation [46], and modulation of surrounding adipocytes and stromal cells [47]. Of note, the genetic and epigenetic regulation of SBP1 in colorectal cancer is of particular interest since loss of heterozygosity of a microsatellite marker close to the SBP1 gene was present in 16% of 55 sporadic colorectal cancer samples [41] and there is a canonical CpG island in the 5′-untranslated region of SBP1 gene. Since selenium is a DNA hypomethylating agent, it is possible that selenium or other hypomethylating agents could modulate the expression of SBP1. Indeed, although selenium did not affect the expression of SBP1 in human lung cancer cells [8], a recent report suggests that treating ovarian tumor cells with methylselenocysteine, a new, potent organic selenium compound, increases SBP1 expression [10]. Thus, the possibility is raised that the reduction of SBP1 in tumorigenesis may abrogate the chemoprotective effect of selenium, increasing the probability of tumor formation and progression. We thus propose that SBP1 may mediate the chemopreventive effect of selenium in early intestinal cell tumorigenesis and may be useful as a serum biomaker reflecting selenium storage. Our data indicating a linkage of SBP1 expression to intestinal epithelial cell differentiation provides a framework for further study of its mechanisms of action in *in vitro* and animal models of intestinal tumorigenesis.

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