Decreased Selenium-Binding Protein 1 in Esophageal Adenocarcinoma Results from Posttranscriptional and Epigenetic Regulation and Affects Chemosensitvity

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Abstract

Purpose: The chemopreventive effects of selenium have been extensively examined, but its role in cancer development or as a chemotherapeutic agent has only recently been explored. Because selenium-binding protein 1 (SELENBP1, SBP1, hSP56) has been shown to bind selenium covalently and selenium deficiency has been associated with esophageal adenocarcinoma (EAC), we examined its role in EAC development and its potential effect on chemosensitivity in the presence of selenium.

Experimental Design: SELENBP1 expression level and copy number variation were determined by oligonucleotide microarrays, real-time reverse transcription-PCR, tissue microarrays, immunoblotting, and single-nucleotide polymorphism arrays. Bisulfite sequencing and sequence analysis of reverse transcription-PCR–amplified products explored epigenetic and posttranscriptional regulation of SELENBP1 expression, respectively. WST-1 cell proliferation assays, senescence-associated β-galactosidase staining, immunoblotting, and flow cytometry were done to evaluate the biological significance of SELENBP1 overexpression in selenium-supplemented EAC cells.

Results: SELENBP1 expression decreased significantly in Barrett’s esophagus to adenocarcinoma progression. Both epigenetic and posttranscriptional mechanisms seemed to modulate SELENBP1 expression. Stable overexpression of SELENBP1 in methylseleninic acid–supplemented Flo-1 cells resulted in enhanced apoptosis, increased cellular senescence, and enhanced cisplatin cytotoxicity. Although inorganic sodium selenite similarly enhanced cisplatin cytotoxicity, these two forms of selenium elicited different cellular responses.

Conclusions: SELENBP1 expression may be an important predictor of response to chemoprevention or chemosensitization with certain forms of selenium in esophageal tissues. Clin Cancer Res; 16(7); 2009–21.

Esophageal adenocarcinoma (EAC) is increasing in incidence in Western countries and remains a highly lethal malignancy. Despite advances in endoscopic surveillance programs, surgical therapy, and multimodality treatment, the prognosis of patients with EAC remains poor, with an overall 5-year survival of 5% to 12% (1). Serum selenium deficiency, in particular, has been associated with EAC as well as its precursor lesion, dysplastic Barrett’s esophagus (2). Organic selenium compounds, such as methylseleninic acid (MSA), seem to enhance response to chemotherapeutic agents, such as cisplatin and paclitaxel, possibly by downregulation of antiapoptotic signals (3, 4).

Selenium-binding protein 1 (Chr:1q21; SBP1, SELENBP1, hSP56) has been shown to bind selenium covalently (5, 6) and is expressed in a variety of tissues and cell lines (7, 8). Its expression is reduced markedly in multiple tumor types compared with their corresponding normal tissues, and its reduction has been associated with poor outcome in lung cancer (9), ovarian cancer (10), colorectal cancer (11, 12), and pleural mesothelioma (13). Previous studies have reported a correlation between decreased expression of a chromosome 1q21 gene cluster and resistance to preoperative EAC chemoradiotherapy (14).

Higher levels of SELENBP1 expression in nongrowing versus rapidly growing cells have been reported in both mouse and human cells (8, 15). Whereas higher SELENBP1 expression is associated with normal colonic epithelial differentiation (12), lower expression was associated with...
poor tumor differentiation in lung adenocarcinomas (9). Although clear discrepancies were observed between the levels of mRNA and protein expression, mouse selenium-binding proteins were found to be associated with the aging process in senescence-accelerated mouse models (16).

Based on such findings, we evaluated the role of SELENBP1 in the tumorigenesis of EAC as well as the effect of differential SELENBP1 expression and selenium supplementation on cell viability and chemosensitivity. We observed that SELENBP1 expression was decreased in primary EAC tumor samples. Decreased expression seemed to be regulated both by methylation of the SELENBP1 promoter and by alternative splicing of SELENBP1 mRNA. When SELENBP1 expression was reconstituted in vitro, tumor cells responded to selenium treatment with increased apoptosis, increased cellular senescence, and increased sensitivity to cisplatin.

Materials and Methods

Patients and tissues. Written patient consent and approval of the Institutional Review Board were obtained to collect specimens from patients undergoing esophagectomy at the University of Michigan Medical Center (Ann Arbor, MI). Specimens were transported to the laboratory in DMEM (Invitrogen) on ice. A portion of each sample was frozen in omithine carbamoyltransferase compound (Miles, Inc.) for cryostat sectioning. The remainder was frozen in liquid nitrogen and stored at −80°C.

Cell lines and treatment. Flo-1 was derived from a patient with EAC and has been described previously (17). OE33 was derived from an EAC and is maintained by the European Collection of Cell Cultures (Sigma-Aldrich Corp.). Het-1A (derived from SV40 large T antigen- transduced esophageal epithelial cells; ref. 18), SW480 (established from a primary colon adenocarcinoma), and H460 (derived from the pleural fluid of a patient with large cell lung carcinoma) are all maintained by the American Type Culture Collection. Cell lines were grown in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin/fungizone (Invitrogen) at 37°C in 5% carbon dioxide/95% air.

Cells were seeded at either 4,000 per well in a 96-well plate format, 50,000 per well in a 12-well format, or 325,000 per well in 60-mm plates for 24 h (T0 time point). All experiments done included a 72-h treatment with either 2.5 μmol/L MSA or 10 μmol/L sodium selenite (NaS) and/or a 12- to 24-h treatment with 20 μg/ml cisplatin [cis-diamminediplatinum(II) dichloride (CDDP)]. The demethylating agent 5-aza-2-deoxycytidine (5-Aza) and the histone deacetylase inhibitors trichostatin A (TSA) and valproic acid (VPA) were used at 5 μmol/L, 300 nmol/L, and 5 mmol/L, respectively.

RNA extraction and oligonucleotide microarray. Total RNA was isolated from EACs (37 cases) and Barrett’s esophageal samples (9 metaplasia, 15 low-grade dysplasia, and 7 high-grade dysplasia) as previously described (19) using Trizol (Invitrogen) followed by RNeasy column purification (Qiagen) per the manufacturers’ instructions. cRNA was generated and hybridized to GeneChip HG-U133A oligonucleotide microarrays (Affymetrix). Image analysis was done by the University of Michigan DNA Microarray Core Facility. A filtering algorithm was used to select genes with either increased or decreased expression in adenocarcinomas or dysplastic Barrett’s mucosa when compared with Barrett’s metaplasia samples. To normalize the microarray data, a summary statistic was calculated using the robust multichip average method (20) as implemented in the Affymetrix library of Bioconductor version 1.3, which provides background adjustment, quantile normalization, and summarization. Expression values for each sample were then compared with the mean expression value for the seven Barrett’s metaplasia samples. Fold reduction of 50% was considered significant (21).

Quantitative reverse transcription-PCR and DNA sequencing. Total RNA from treated cells was isolated and column purified using the RNeasy Mini kit (Qiagen) per the manufacturer’s instructions. RNA was eluted from the spin column using RNase-free deH2O and reverse transcribed with 5 units/μL SuperScript II reverse transcriptase (Invitrogen). Real-time PCR amplification using 20 ng of total RNA, Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and 0.2 μmol/L of both forward and reverse primers

**Translational Relevance**

The overall long-term survival for patients with esophageal adenocarcinoma remains poor. Despite improved understanding about the progression from Barrett’s esophagus to dysplasia and then adenocarcinoma, patients present commonly in advanced stage. Population-based studies have found that lower serum concentration of selenium is associated with greater incidence of esophageal cancer, including adenocarcinoma, suggesting a possible basis for selenium chemoprevention. Selenium-binding protein 1 (SELENBP1), which binds selenium covalently, is reduced in the progression from dysplastic Barrett’s esophagus to adenocarcinoma, but its function has not been well characterized. The present study shows that SELENBP1 expression is regulated at both the epigenetic and the posttranscriptional levels. Gene overexpression increased in vitro esophageal adenocarcinoma cell death in response to selenium supplementation and cisplatin. These studies suggest that differential expression of SELENBP1 may affect cellular response to selenium supplementation both in the chemoprevention and in the treatment of esophageal cancer.
immunohistochemistry and tissue microarray. Tissue microarrays (TMA) were constructed, as previously described (19, 23), with formalin-fixed, paraffin-embedded tissues from 73 patients, including 64 tumor, 8 lymph node metastases, 8 dysplastic Barrett’s mucosa, and 11 nondysplastic Barrett’s metaplasia samples. Multiple samples from representative areas of EAC, metaplasia, or dysplasia were included for 33 patients. Normal esophagus was also included from 33 patients who had undergone esophagectomy for benign indications.

Immunohistochemical staining was done on the DAKO Autostainer using DAKO LSAB+ and 3,3′-diaminobenzidine as the chromogen. Dewaxed and rehydrated sections of the TMA at 4-mm thickness were labeled with SELENBP1 antibody (mouse monoclonal antibody, clone 4D4, 1:200 dilution; MBL International). We did microwave citric acid epitope retrieval for 20 min. Slides were lightly counterstained with hematoxyn. Each sample was scored independently by two readers using a scale of 0 (no staining), 1+ (<10% staining), 2+ (10-50%) staining, or 3+ (>50%).

Protein extraction and immunoblot analysis. Total cellular protein was extracted in lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L Na3PO4, 1 mmol/L glycerol 2-phosphate disodium salt hydrate, 1 mmol/L Na2VO4, 1% Triton X-100] supplemented with Protease Inhibitor Cocktail (20 mL/1 mL lysis buffer; Sigma-Aldrich). Total cell lysates (20-40 μg) were boiled in 6× sample buffer, resolved on 8% to 16% Tris-glycine gels (Invitrogen), and transferred to Immobilon-P membranes (Millipore). Mouse monoclonal SELENBP1 (MBL International) and rabbit polyclonal poly(ADP-ribose) polymerase (PARP; Cell Signaling) antibodies were used at 1:2,000 dilution. Mouse monoclonal β-actin antibody (Abcam) was used at 1:10,000 dilution as a loading control. Both horseradish peroxidase-conjugated mouse (Southern Biotechnology Associates) and rabbit (Vector Laboratories) secondary antibodies were used at 1:10,000 to 1:25,000 dilutions. Antigen-antibody complexes were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology, Inc.).

Construction of SELENBP1 stable cell line. A SELENBP1 mammalian expression construct was created and stably transfected into Flo-1 cells. The SELENBP1 clone MGC-9270 (American Type Culture Collection) was PCR amplified using primers containing EcoRI and BamHI restriction sites for directional cloning into the pEGFP-C2 vector containing enhanced green fluorescent protein (EGFP; Clontech). The pEGFP-SELENBP1 or empty vecto construct was transfected into Flo-1 cells using FuGENE 6 transfection reagent (Roche). Selected clones were maintained in growth medium containing 200 μg/mL geneticin.

Proliferation assay. The cell proliferation reagent WST-1 (Roche) was used for spectrophotometric quantification of cell proliferation, viability, and chemosensitivity in accordance with the manufacturer’s directions. Relative proliferation rates were calculated as a percentage of the initial T0 reading within each cell line. Chemosensitivity analysis of a 3-d treatment with either MSA or NaS and/or 12- to 24-h treatment with CDDP was similarly done using WST-1 reagent. Treatments were initiated following T0 absorbance readings. Viability was calculated as a percentage of mock-treated cells at 72 h within each cell line.

Wound-healing assay. Cells were densely plated and allowed to grow to 100% confluence. Following serum starvation for 24 h, cell monolayers were wounded with sterile p200 tips. Digital images of predetermined locations in each well were taken at both ×4 and ×10 optical magnification on an Olympus CK2 inverted microscope using a SPOT Idea 1.3-megapixel camera and analyzed with SPOT Basic software (Diagnostic Instruments) immediately after wounding and, subsequently, every 24 h until the wounds were closed. The percentage of initial wound width was determined by averaging measurements per well at each time point.

Single-nucleotide polymorphism analysis. Single-nucleotide polymorphism (SNP) arrays were done at the Genetic Analysis Platform of the Broad Institute of Massachusetts Institute of Technology and Harvard in accordance with the manufacturer’s instructions as previously described (24, 25). Briefly, 73 EAC DNAs were processed for Affymetrix genome-wide 250K Styl SNP arrays. The data were annotated and visualized using Integrative Genomics Viewer software.

Bisulfite sequencing of genomic DNA. Genomic DNA was isolated using the DNeasy kit (Qiagen), and 1 μg of each sample DNA was treated with bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research Corp.) according to the manufacturers’ instructions. The 5′-untranslated region


6 http://www.broad.mit.edu/cancer/software/genepattern/
of SELENBP1 was analyzed using the Transcriptional Regulatory Element Database\(^7\) and promoter prediction software from the Berkeley Drosophila Genome Project.\(^8\) One of the highest-scored sequences (0.98) spanned −39 to +11. A 186-bp fragment (−95 to +90), which covered 158 bp upstream and 25 bp downstream (inside the first intron) of the ATG start codon of SELENBP1, was PCR amplified using bisulfite-treated DNA and primers designed by MethPrimer software.\(^9\) (Supplementary Table S1). PCR products were purified by electrophoresis on 1% agarose gels, ligated into pCR2.1pTOPO-TA vectors (Invitrogen), and introduced into TOP10 One Shot (Invitrogen) competent bacteria. Insert-containing plasmid DNA was extracted using the Quick Plasmid Miniprep kit (Invitrogen) and eluted in 30 μL buffer. A minimum of 10 clones selected at random was sequenced by the University of Michigan DNA Sequencing Core.

**Flow cytometry.** Following treatment, DNA from whole-cell nuclei was harvested and stained using modified Krishan buffer (0.1% citric acid trisodium salt dihydrate, 0.02 mg/mL RNase A, 0.3% NP40, 0.05 mg/mL propidium iodide) at the indicated time points. Apoptosis and cell cycle analyses were done by the University of Michigan Flow Cytometry Core and measured on the FACS-Calibur Cytometer (BD Biosciences) using CellQuest and ModFit Light softwares, respectively.

**Senescence assay.** Senescence-associated β-galactosidase activity was determined with the Senescence Detection kit (BioVison Research Products) per the manufacturer’s instructions. Digital images were taken at ×20 optical magnification, and stained cells were counted and expressed as a percentage of total cell number in three independent
fields per well per treatment group to obtain an average value for β-galactosidase staining activity.

**Experimental statistical analysis.** Two-way ANOVA and Student’s t test were done using SPSS for Windows 15 (SPSS, Inc.) and Microsoft Excel 2002 (Microsoft Corp.).

**Results**

**Patient demographics.** Clinical and pathologic features for the patients whose tissue samples were used for this study are provided in Supplementary Table S2.

**Downregulation of SELENBP1 expression was observed during the progression from metaplastic Barrett’s esophagus to EAC.** Affymetrix HG-U133A oligonucleotide microarray indicated diminished SELENBP1 expression in EAC relative to both metaplastic and dysplastic Barrett’s esophagus (Fig. 1A). SELENBP1 expression in EAC decreased by 25% in samples of high-grade dysplasia ($P = 0.022$) and by $\sim 50\%$ in EAC ($P = 0.005$) compared with metaplastic Barrett’s esophagus (Fig. 1B). The array results were validated using quantitative reverse transcription-PCR (RT-PCR; Fig. 1C). Reduced SELENBP1 expression in EAC was reaffirmed using a second set of patient tissues in oligonucleotide microarrays (Fig. 1D). Consistent with many primary EACs, Flo-1 cells showed minimal expression of SELENBP1 (Fig. 1C). SELENBP1 expression was decreased significantly in adenocarcinomas compared with metaplastic or dysplastic Barrett’s esophagus using immunohistochemistry of TMAs (Fig. 2A-C). Minimal-to-low SELENBP1 expression was observed more frequently in adenocarcinoma ($P < 0.001$, two-way ANOVA) and dysplastic Barrett’s esophagus ($P < 0.05$, two-way ANOVA) compared with SELENBP1 expression in samples of Barrett’s metaplasia (Supplementary Table S3). Of note, two samples on the oligonucleotide microarray with increased SELENBP1 expression (36T and 39T) had coordinately increased SELENBP1 protein expression by immunohistochemistry in TMA cores.

Immunoblot analysis of paired EAC and Barrett’s metaplasia protein extracts indicated expression of a single SELENBP1 protein band (Fig. 2D), with reduced expression in tumor samples relative to Barrett’s metaplasia in
four of the five cases analyzed. In the fifth case (36T), SELENBP1 expression was higher in the tumor compared with an adjacent noncarcinoma section, corresponding to the gene expression findings observed by both oligonucleotide microarray and quantitative RT-PCR.

Decreased DNA copy number changes indicating loss of heterozygosity were not detected by SNP array analysis. Because frequent loss of heterozygosity has been recently reported in regions near the SELENBP1 gene (14, 26), we analyzed SNP arrays of 73 EAC samples to determine if the downregulated SELENBP1 expression was due to decreased DNA copy number at 1q21-q22. No samples were found to have DNA copy number changes less than log2 ratio of $-0.737$, representing one copy if $\sim 30\%$ of the sample DNA contained contaminating normal esophagus. Nine patient samples exhibited allelic gain (log2 ratio > 0.7) or amplification (log2 ratio > 0.848) at the SELENBP1 locus (Supplementary Fig. S1). Eight tumors were examined by both SNP array and oligonucleotide microarray analyses (36T, 40T, 41T, 43T, 44T, 45T, 46T, and 50T). The differential expression of SELENBP1 in these samples could not be explained by changes in DNA copy number, suggesting transcriptional or posttranscriptional modulation.

**DNA methylation and chromatin remodeling of SELENBP1 may downregulate gene expression.** Sequence analysis of SELENBP1 revealed several CpG sites near the predicted promoter region of this gene, suggesting that DNA methylation may be another mechanisn for suppressed SELENBP1 expression in EAC. Bisulfite sequencing of genomic DNA prepared from the EAC cell line Flo-1 and the immortalized esophageal squamous cell line Het-1A indicated that methylation occurred in CpG sites at $-95$ to $+90$ in the 5′-untranslated region of SELENBP1. In contrast, very little if any methylation was detected in the colon cancer cell line SW480 (Fig. 3A). In addition, treatment of Flo-1 cells with the demethylating agent 5-Aza and/or the histone deacetylase inhibitors TSA or VPA increased SELENBP1 expression as determined by quantitative RT-PCR analysis (Fig. 3B). Combination treatments that resulted in the greatest increase in SELENBP1 mRNA expression levels were not able to produce detectable
SELENBP1 protein (Fig. 3C). Despite this, treatment of Flo-1 cells with demethylating and acetylate-enhancing agents increased sensitivity to apoptosis with cisplatin as determined by PARP cleavage (Fig. 3D). These results indicate that SELENBP1 expression may be regulated at the epigenetic level but that the level of inducible expression was still too low to detect by protein immunoblotting.

**Alternative splicing of SELENBP1 mRNA may diminish gene expression.** To determine whether the loss of SELENBP1 expression was due to internal exon deletion or mutation in the coding region of the gene, RT-PCR–amplified cDNA products from patient tumor samples and cell lines were sequenced and compared with the National Center for Biotechnology Information Genbank database for SELENBP1 using the BLAST program. Five overlapping primer pairs were designed to cover the entire coding sequence (Fig. 4A). PCR amplification of two regions denoted as the L and mid2 fragments (Fig. 4B) indicated several variants (Fig. 4C), including an exon 4 deletion (L, exon 4'), deletions of both exons 3 and 4 (L, exon 3'/4'), and an exon 7 deletion (mid2, exon 7'), suggesting the possibility of alternative splicing of the SELENBP1 transcript. Whereas deletion of exon 4 was in frame, deletion of exons 3 and 4 or of exon 7 introduced early termination codons that might translate truncated protein products.

Although truncated PCR products were detected in all tumor specimens, tumors with higher levels of exon 3'/4' and exon 7' deleted PCR products (Fig. 4C) had lower levels of full-length PCR products (Fig. 4C). These tumors also had reduced expression by both oligonucleotide microarray and quantitative RT-PCR analyses. Conversely, the two tumors that expressed the highest levels of SELENBP1 (36T and 39T) had lower levels of both exon 3'/4' and exon 7' deleted PCR products but higher levels of their respective full-length products (Fig. 4D). Although at seemingly reduced levels, alternatively spliced fragments were also detected in normal esophageal and gastric tissues (data not shown), suggesting that SELENBP1 is transcriptionally unstable. Interestingly, direct comparison of tumor sample 45T with its matching normal gastric tissue showed a marked increase in the levels of both exon 3'/4' and exon 7' deleted PCR products in tumor cDNA (data not shown). The correlative presence or absence of the alternatively spliced L (exon 3'/4') and mid2 (exon 7') fragments in tumors remains unexplained. The oligonucleotide microarray probes for SELENBP1 that were used in this study were located 3' of the mid2 fragment (Fig. 4A), and their hybridization should not have been affected by these spliced regions. It is possible that alternative splicing affects the stability of the resulting transcript. Further investigation of this gene silencing mechanism is warranted.

The intronic regions surrounding exons 3, 4, and 7 of SELENBP1 were sequenced to examine potential modifications near splice junctions. Although a base pair insertion in intron 3 and several polymorphic sites were identified (Supplementary Table S4), representing both cassette alternative exon and intron retention splicing (27), no biological correlation to the different levels of alternatively spliced fragments in the tumors was found.

**Overexpression of SELENBP1 potentiated the antiproliferative effect of MSA in vitro and increased chemosensitivity to cisplatin.** As noted earlier, a subset of tumor samples (36T and 39T) had higher expression of SELENBP1. To examine the biological significance of SELENBP1 expression in EAC, Flo-1 cells were stably transfected with either an EGFP-SELENBP1 or a corresponding empty vector plasmid, and levels of SELENBP1 expression were determined by immunoblot analysis. Clone SELENBP1.8 expressed moderate levels, whereas SELENBP1.1 expressed high levels of EGFP-SELENBP1 compared with empty vector and untransfected Flo-1 cells (Fig. 5A). Cellular proliferation and migration did not change appreciably with varying levels of SELENBP1 overexpression compared with empty vector controls as determined by WST assay (Supplementary Fig. S2) and by wound-healing assay (data not shown), respectively. Because it has been shown that SELENBP1 covalently binds selenium and that a selenium-replete form of SELENBP1 was required for protein-protein interactions (6), we evaluated whether selenium supplementation could alter the cellular response to SELENBP1 overexpression in EAC. Overexpression of SELENBP1 seemed to potentiate an antiproliferative response to selenium supplementation with MSA but not with NaS, particularly in the higher-expressing clone SELENBP1.1 (Fig. 5B).

To determine if the decrease in cell viability detected after MSA treatment of SELENBP1-overexpressing cells could enhance the cytotoxicity of CDDP, we examined the sub-G1 fraction of treated cells by flow cytometry. Although SELENBP1 overexpression itself did not have an effect, treatment of Flo-1 cells with MSA, CDDP, and the combination of MSA and CDDP resulted in slight but statistically significant increases in apoptosis in SELENBP1.1 cells when compared with empty vector–transfected controls (Fig. 5C). The proportion of apoptotic cells increased with combination treatment, but there was no apparent synergy between MSA and CDDP. The increase in apoptosis following NaS/CDDP treatment seemed to be SELENBP1 independent.

Both MSA and CDDP treatments culminated in PARP cleavage, but this measure of apoptosis was not sensitive enough to detect differences between SELENBP1- and control-transfected Flo-1 cells (Fig. 5D). WST-1 analysis revealed that overexpression of SELENBP1 enhanced cisplatin-mediated cytotoxicity. Additionally, overexpression of SELENBP1 increased cancer cell CDDP sensitivity when treated with either MSA or NaS in comparison with the empty vector–transfected control (Fig. 5B).

**SELENBP1 overexpression potentiated selenium-induced senescence.** Expression of selenium-binding proteins is associated with accelerated senescence in mice (16). To determine if SELENBP1 overexpression activated cellular senescence in human cells, we stained stably transfected Flo-1 cells for senescence-associated β-galactosidase.
Whereas baseline senescence was not consistently different between empty vector–transfected and SELENBP1-transfected Flo-1 cells, SELENBP1-transfected cells had increased levels of senescence following treatment with MSA but not NaS (Fig. 6A and B). These data suggest that MSA and SELENBP1 work together to activate this growth-inhibitory pathway.

**Discussion**

In this study, we have shown that expression of SELENBP1 is decreased in the progression from nondysplastic Barrett’s esophagus to Barrett’s esophagus with high-grade dysplasia and EAC at both mRNA and protein levels. Downregulation of SELENBP1 does not seem to be related to loss of heterozygosity at the 1q21 SELENBP1 locus as shown by SNP array analysis, suggesting that regulation is more likely a transcriptional or posttranscriptional event.

Regulation of SELENBP1 may be affected by epigenetic mechanisms. Gene hypermethylation in the epidermal differentiation complex, which is located within 700 kb of the SELENBP1 locus, has been previously reported (28, 29). Bisulfite sequencing of the EAC cell line Flo-1, which showed very low endogenous levels of SELENBP1, revealed the presence of methylated CpG sites in the upstream sequence of SELENBP1, suggesting that diminished SELENBP1 expression might be due, in part, to its promoter methylation. In support of this, significantly increased SELENBP1 mRNA expression was observed after 5-Aza–induced demethylation and TSA- or VPA-induced histone acetylation. Increased levels of corresponding protein products were not detected, suggesting that this degree of SELENBP1 reexpression was not sufficient to be detected by Western blotting.

Alternative splicing of primary transcripts is a common mechanism in higher eukaryotic organisms to increase cell type–specific and developmental stage–specific protein diversity (27). Unbalanced alternative splicing can lead to the overexpression of antagonistic splice variants of genes involved in differentiation, apoptosis, invasion, and metastasis, often correlating with poor prognosis in cancers (30). We observed an inverse correlation between the levels of two alternatively spliced PCR products (exon 3’/4’ or exon 7’) and their respective full-length sequences in EAC tissues. The expression levels of the full-length PCR products correlated with the SELENBP1 expression levels determined by oligonucleotide microarray and quantitative RT-PCR. Resulting cDNA sequences from these two alternatively spliced variants predicted altered amino acid sequences and truncated protein products. Similar NH2-terminally truncated variants have been reported in the closely related mouse homologue Selnbp2, suggesting that this particular gene is susceptible to alternative splicing (31). These truncated variants may serve to reduce directly the number of full-length transcripts that would be translated into functional protein, or they may serve as dominant-negative inhibitors of wild-type SELENBP1. The full significance and functional relevance of these splice variants in EAC tumorigenesis is yet to be determined. Based on these data, strategies to inhibit specific splicing events might serve as another mechanism for SELENBP1 reexpression in EAC.

Population-based studies suggest a correlation between higher serum selenium concentration and lower incidence for a variety of epithelial malignancies (32) as well as premalignant diseases such as Barrett’s esophagus with high-grade dysplasia (2). Comparison of the cancer chemoprotective effects of different forms of selenium in vitro has revealed marked differences between methylselenol precursors, such as MSA, and those compounds metabolized predominantly to hydrogen selenide, such as NaS (33). Although both MSA and NaS have been shown to inhibit tumor invasion (34, 35), induce apoptosis (36), and cause cell cycle arrest (33, 37), methylselenol precursors have been reported to be more effective tumor inhibitors than selenite or selenomethionine (38). Whereas MSA treatment induced cell retraction and detachment followed by p53-independent caspase-mediated apoptosis, NaS treatment resulted in cytoplasmic vacuole formation, reactive oxygen species production leading to single-strand DNA breaks, and caspase-independent cell death (36). Additionally, MSA but not NaS increased the potency of SN38 (topoisomerase I inhibitor), etoposide (topoisomerase II inhibitor), and the microtubule inhibitor paclitaxel in prostate cancer cells (39).

We observed many of these selenium compound–specific differences following treatment of Flo-1 EAC cells. MSA alone caused cell detachment and subsequent PARP...
cleavage, whereas NaS-treated cells showed characteristic vacuole formation with no apparent PARP cleavage. Although reconstitution of SELENBP1 alone did not significantly alter cellular proliferation in Flo-1 cells, addition of MSA to these cells resulted in significant inhibition compared with non–SELENBP1-expressing cells. NaS treatment did not have this differential effect. Although nontoxic doses of both selenium compounds enhanced cisplatin cytotoxicity to some extent, the effects were more pronounced with MSA treatment and were further enhanced by SELENBP1 overexpression. In addition, MSA but not NaS treatment of SELENBP1-transfected Flo-1 cells
enhanced senescence as assayed by β-galactosidase staining. These differences emphasize the importance of the chemical form of selenium in cancer chemoprevention and chemotherapy strategies.

The relationship between selenium levels and SELENBP1 expression in cancer progression is still unclear. Although levels of selenite did not affect the levels of selenium-binding proteins in rodent cells (40), the level of expression of a 58-kDa selenoprotein correlated with the level of selenite supplementation and with inhibition of DNA synthesis in mouse mammary epithelial cells (41). Huang et al. (10) have also shown that SELENBP1 expression is increased with methylselenocysteine treatment in human ovarian cancer cells. It has been reported that selenium compounds promote DNA and histone hypomethylation (42) and may act as histone deacetylase inhibitors in human prostate cancer cells (43, 44), possibly potentiating the reexpression of epigenetically silenced genes such as SELENBP1. Selenium may also exert cell growth inhibition by modifying the function of preexisting proteins. Supporting this is a recent report that selenium-replete SELENBP1 is required for the interaction between the von Hippel-Lindau protein-interacting deubiquitinating enzyme VDU1 and SELENBP1 (6). Many of the tumor-suppressive mechanisms analyzed in our studies either were enhanced by or were observed only in the presence of MSA. We have also observed increased SELENBP1 expression following MSA treatment. Our findings suggest that selenium affects both SELENBP1 activity and expression in EAC.

Recently, SELENBP1 was shown to contain hypoxia response elements in its promoter region and was found to be a target gene for the transcription factor hypoxia-inducible factor-1α (HIF-1α; ref. 45). SELENBP1 mRNA and protein expression was increased over 10-fold in the keratinocytes of transgenic mice expressing constitutive HIF-1α compared with nontransgenic littermates. These transgenic mice showed increased resistance in the transformation from papilloma to carcinoma (45). Because HIF-1α seems to be redox sensitive and the metabolic reduction of some forms of selenium resulted in reactive oxygen species production (45–47), it is plausible that
selenium may also indirectly modify SELENBP1 expression through HIF-1α.

We have found that SELENBP1 is downregulated in the progression from Barrett’s metaplasia to EAC. Although decreased expression of SELENBP1 seemed to be related to epigenetic modification, we also observed increased alternative splice products with predicted premature truncation in a significant number of tumors with diminished SELENBP1 expression. In vitro overexpression of SELENBP1 enhanced cisplatin-mediated cell death, particularly when cells were also treated with MSA. In conclusion, diminished SELENBP1 expression in EAC seemed to blunt the cellular response to some forms of selenium supplementation, and loss of SELENBP1 might be a marker for limited response to ongoing attempts to use selenium as a chemopreventive or chemosensitization agent. Alternatively, the presence of full-length SELENBP1 in cancer tissues might predict better response to chemotherapy treatment and would support selenium-mediated secondary chemoprevention strategies.

Fig. 6. SELENBP1 enhanced MSA-induced cell senescence in Flo-1 cells. Stably transfected Flo-1 cells were treated with 2.5 μmol/L MSA, 10 μmol/L NaS, or vehicle for 3 d followed by fixation and staining for senescence-associated β-galactosidase expression. A, representative digital images of treatment groups. Magnification, ×20. B, the number of blue-stained cells versus total cell count per image in three nonoverlapping areas per well was recorded for each treatment group. Although variability was high, these observations were confirmed in triplicate experiments. *, P < 0.05, Student’s t test, versus empty vector control.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Laura Lynch and Amrit Misra for their technical assistance in the experiments presented in this manuscript.

Grant Support

NIH grants 5R01CA071606 (D.G. Beer), 5P30CA046592 (T.J. Giordano), 5K08CA134931(A.J. Bass), and 5K08CA127212 (A.C. Chang) and Thoracic Surgery Foundation for Research and Education (A.C. Chang). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/20/2009; revised 01/11/2010; accepted 01/25/2010; published OnlineFirst 03/23/2010.

References

Correction: Decreased Selenium-Binding Protein 1 in Esophageal Adenocarcinoma Results from Posttranscriptional and Epigenetic Regulation and Affects Chemosensitivity

In this article (Clin Cancer Res 2010;16:2009–21), which was published in the April 1, 2010, issue of *Clinical Cancer Research* (1), the GEO accession numbers were not included. The numbers are GSE37203 and GSE37201. The authors regret this error.

Reference


Published OnlineFirst May 15, 2012.
doi: 10.1158/1078-0432.CCR-12-1479
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Clinical Cancer Research

Decreased Selenium-Binding Protein 1 in Esophageal Adenocarcinoma Results from Posttranscriptional and Epigenetic Regulation and Affects Chemosensitivity


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