Original Paper

Reduced selenium-binding protein 1 expression is associated with poor outcome in lung adenocarcinomas

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Abstract

The effects of selenium, an essential nutrient with anti-carcinogenic properties, are mediated by selenium-binding proteins. The protein expression status of human selenium-binding protein 1 (SBP1) in human tumours and the exact function of this protein are not known. In this study, quantitative two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was used on 93 lung adenocarcinomas and ten uninvolved lung samples. Two likely isoforms of a 56 kD protein that showed a significantly decreased abundance in lung adenocarcinomas were observed. Tandem mass spectrometry and 2-D western blot analysis identified these two proteins as human SBP1. Tumour tissue microarrays were utilized to examine the cellular expression patterns of SBP1 using immunohistochemistry. The same tissue samples were examined for SBP1 mRNA expression using oligonucleotide microarrays. Two major SBP1 isoforms were detected, with an acidic isoform (457) being significantly down-regulated in lung adenocarcinomas compared with normal lung ($p = 0.02$). Two additional more acidic SBP1 isoforms were only observed in normal lung. SBP1 protein isoforms and *SBP1* mRNA levels were significantly decreased in poorly differentiated (versus moderately and well-differentiated), T2–T4 (versus T1), and bronchus-derived (versus bronchioloalveolar) tumours. Low levels of SBP1 protein (native form, 460) correlated significantly with poor survival ($p = 0.007$). The lack of SBP1 expression was not due to gene deletion. Treatment of A549 lung adenocarcinoma cells with the methylation inhibitor 5-azacytidine did not affect expression of the SBP1 protein. Analysis of the tumour proliferation status using Ki-67 suggests that down-regulated expression of SBP1 may reflect increased cell proliferation and decreased differentiation in lung adenocarcinomas.

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Keywords: selenium-binding protein 1 (SBP1); lung adenocarcinoma; 2-D PAGE

Introduction

Epidemiological studies have suggested that an increased risk for certain human diseases, including cancer of the lung, liver, colon, prostate, and pancreas, is related to insufficient intake of selenium [1–3]. Supplementation of dietary selenium results in a significant reduction in total cancer mortality and incidence [4], especially in populations with low plasma selenium concentrations [5]. Proposed mechanisms for the protective role of selenium against cancer include inhibiting carcinogen-induced covalent DNA adduct formation; impeding oxidative damage to DNA, lipids, and proteins; increasing apoptosis and inhibiting tumour cell growth; altering DNA, RNA, and protein synthesis; and increasing P53 and Cox-2 expression [1].

Mammalian selenium-containing proteins can be divided into three groups: specific selenoproteins, non-specific selenium-containing proteins, and selenium-binding proteins [6,7]. The human selenium-binding protein gene (*SBP1, SELENBP1* or *hSP56*) [8] is located at chromosome 1q21–22 and is the human homologue of the mouse *SP56* gene [9,10]. To date, the protein expression status and the function of this protein in lung cancer or other human tumours are not known.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and oligonucleotide arrays are powerful research techniques that have been widely used for the detection and identification of potential tumour-related gene products and genes [11,12]. Our previous quantitative analysis of protein expression revealed proteins showing significant changes in lung adenocarcinomas relative to normal lung tissue and associated with other clinical variables including stage, tumour differentiation, and survival [12,13]. In the present study, multiple isoforms of human selenium-binding protein 1 (SBP1) were identified as showing decreased expression in lung adenocarcinomas, and the protein and mRNA expression of SBP1 was examined in a

large series of human lung cancers using a combined proteomic and genomic-based approach.

Materials and methods

Tumour samples

Patients who underwent resections for lung cancer at the University of Michigan Hospital from May 1991 to July 2000 were evaluated for inclusion in this study. Consent was received from all patients and the protocol was approved by the University of Michigan Institutional Review Board (Medicine). All patient identifiers were coded to protect confidentiality. All lung tumours and adjacent normal lung tissues were obtained immediately at the time of surgery. A portion was embedded and frozen in OCT (Miles Scientific, Naperville, IL, USA) for cryostat sectioning and then stored at $-80\,°C$ until use. Haematoxylin-stained cryostat sections (5 μm), prepared from tumour pieces used for protein or mRNA isolation, were evaluated by the study pathologist and compared with haematoxylin and eosin (H&E)-stained sections made from formalin-fixed, paraffin wax-embedded tissue blocks from the same tumours. All samples comprised more than 70% tumour cells and none of the patients received prior chemotherapy or radiotherapy. Tumours were histopathologically divided into two broad categories: bronchus-derived (BD) if they exhibited invasive features with architectural destruction, and bronchioloalveolar (BA) if they exhibited preservation of lung architecture. A minimum of two H&E-stained slides from each tumour were evaluated to assess lymphocytic response. Tumours were divided into two broad categories based on the absence or presence of a significant peri- and intra-tumoural lymphocytic infiltrate. Sixty-four stage I lung adenocarcinomas, 29 stage III lung adenocarcinomas, and ten uninvolved lung tissue samples were examined by quantitative 2-D PAGE analysis. An additional 80 adenocarcinomas and 45 squamous lung tumours were added for immunohistochemical staining of tissue microarrays (TMAs).

2-D PAGE and mass spectrometry

Analytical 2-D PAGE protein quantification and identification by mass spectrometry were performed as previously described [12,14]. Each gel generated 1600–2200 detectable spots, of which 820 spots were selected for quantitative measurement in a total of 93 lung tumours and ten normal lung samples [12,14]. In this study, we selected two protein spots (457 and 460) on 2-D PAGE gels for further analysis. Tandem mass spectrometry (ESI MS/MS) in a Q-TOF micro (Micromass, Manchester, UK) was used for protein identification.

Affymetrix oligonucleotide microarrays

RNA isolation and oligonucleotide microarray analysis are described elsewhere [11]. In total, RNA from 86

adenocarcinomas and ten normal lung samples was used in the analysis of *SBP1* mRNA expression. Of these, samples from 76 tumours and nine normal lungs were also used in quantitative 2-D PAGE analyses. The gene symbol of *SBP1* on HuGeneFL Affymetrix oligonucleotide microarrays (U95, Affymetrix, Santa Clara, CA, USA) is *SELENBP1* and the probe set number is U29091_at.

2-D western blotting

Protein separation and 2-D western blotting were performed as described previously [12]. Individual membranes were incubated with anti-human SBP-1 antibody (mouse monoclonal antibody, M061-3, clone 4D4, MBL, Nagoya, Japan) at a 1 : 1000 dilution (1 μg/ml). Following additional washes, membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) at a 1 : 5000 dilution for 1 h, then washed, and incubated for 1 min with enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA) and exposed to film (Amersham, Piscataway, NJ, USA).

Immunohistochemical staining and tissue microarray

A TMA was constructed, as previously described by Kononen *et al* [15], using formalin-fixed, paraffin wax-embedded tissues from 148 pulmonary adenocarcinomas and 45 squamous tumours in addition to representative normal lung tissues. Three replicate tumour-rich tissue cores were sampled from each of the selected tissues. Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA, USA) using DAKO LSAB+ and diaminobenzadine (DAB) as the chromogen. Dewaxed and rehydrated sections of the TMA at 4-μm thickness were labelled with SBP-1 antibody (mouse monoclonal antibody, clone 4D4, MBL, Nagoya, Japan; 1 : 500 dilution) after microwave citric acid epitope retrieval. Slides were lightly counter-stained with haematoxylin. Each sample was then scored using a scale of negative (−), weak (+), moderate (++), and strong staining (+++).

MIB-1 (1 : 100) (DAKO, Carpinteria, CA, USA) was used to analyse nuclear expression of the Ki-67 cell proliferation antigen. The proliferative index of each tumour was determined by counting more than 1000 nuclei from each tumour and determining the percentage of labelled cells to the total cell number [16].

A549 and SKLU1 cell culture and 5-azacytidine and dexamethasone treatment

A549 and SKLU1 cell lines were obtained from the American Tissue Culture Collection (ATCC) and grown in DMEM with 10% fetal bovine serum (FBS). A549 was cultured in the presence of 5-azacytidine (2.5 or 5 μM, Sigma, St Louis, MO, USA) and
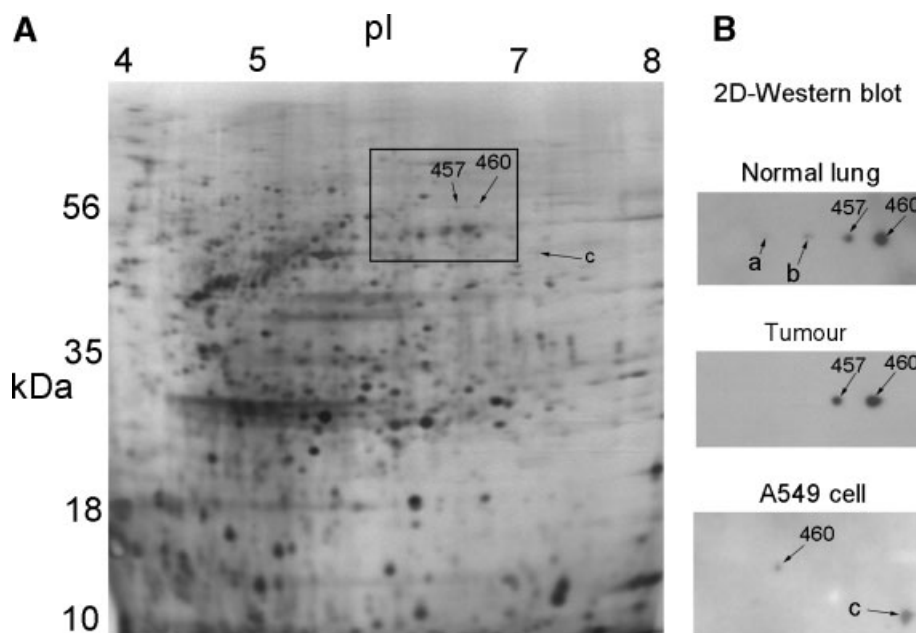
Figure 1. 2-D PAGE and 2-D western blotting of SBP1 protein. (A) Digital image of a silver-stained 2-D PAGE separation of a lung adenocarcinoma showing the region containing SBP1 protein spots separated by molecular mass (MW) and isoelectric point (pI). (B) 2-D western blot results in normal lung, a lung tumour, and the A549 cell line. Four isoforms of SBP1 protein were identified in normal lung (spots a, b, 457, and 460) and two isoforms in tumours (spots 457 and 460). Spot 460 is the proposed native form and the other three are likely phosphorylated forms. A very low abundance of native SBP1 (spot 460) and high levels of the degradation form (spot c) were found in the A549 cell line

dexamethasone (0.125 or 0.25 μM), respectively, for 3 days and then washed three times with PBS prior to protein isolation and subsequent analysis using western blotting [17].

Chromosome 1q21 LOH analysis

DNA isolation and preparation were performed as previously described [18]. Primers (forward, 5′-ttgagaact-gccttagactgc-3′; reverse, 5′-cccaagttgctggaacc-3′) were designed for D1S2347, a sequence tagged site ~20 kb centromeric from *SELENBP1*. Forward primers were end-labelled with [γ-^{32}P]ATP (NEN Life Science Products, Boston, MA, USA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). PCR was performed using *Taq* polymerase (Promega, Madison, WI, USA), and the PCR products (232–294 bp in size) were resolved on 8% denaturing polyacrylamide gels. Heterozygous individuals were then analysed for loss of one or both alleles using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, USA). Results were repeated and verified.
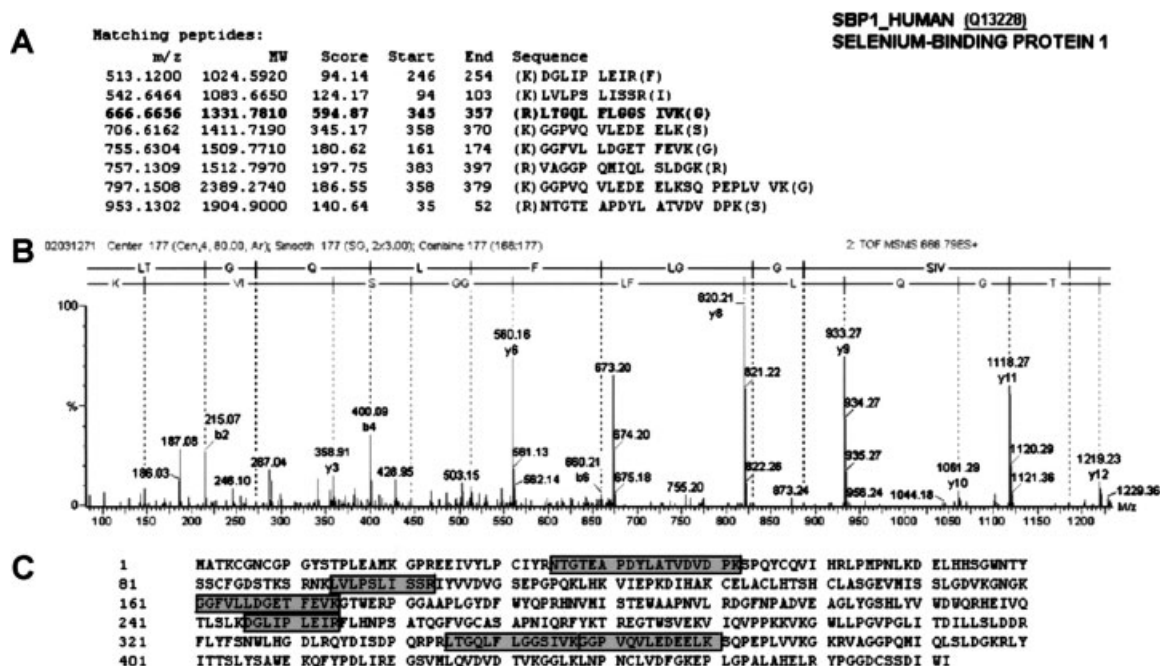
Statistical analyses

F-tests were used for testing the difference between SBP1 protein levels and in each clinicopathologically-defined group except the tumour−normal comparisons, for which T-tests were used. The Pearson correlation coefficient method was used for the correlation analysis between the levels of SBP1 protein and Ki-67 expression. Kaplan−Meier survival curves and the log-rank test were used for survival analyses.

Results

SBP1 protein identification and verification

A silver-stained 2-D PAGE gel showing the region containing the SBP1 polypeptides in a lung adenocarcinoma is shown in Figure 1A. These protein spots (457 and 460) were identified as SBP1 using tandem mass spectrometry (Figure 2). Spot 460 was judged as the native form based on molecular weight and pI. The more acidic spot (457) likely represents a phosphorylated form based on its 2-D gel location. The identity of these proteins was further verified by 2-D western blotting with an anti-human SBP1 antibody (Figure 1B). Interestingly, 2-D western blot analysis also identified two additional isoforms of SBP1 that could only be detected in normal lung samples. These immunoreactive isoforms are of similar molecular weight but are more acidic, and likely represent bi- or tri-phosphorylated forms. Due to very low abundance on 2-D gels of lung adenocarcinomas, these two isoforms could not be quantitatively analysed in all samples. The acidic isoform SBP1 (spot 457) correlated strongly with the native form SBP1 (spot 460) using Pearson correlation coefficient analysis ($r = 0.8$, $p < 0.001$). Very low expression levels of the native isoform (spot 460) and an apparent degradation form (spot c) of the SBP1 protein were found in 2-D western blot analysis of A549 cells (Figure 1B).

Quantitative 2-D PAGE analysis of the 93 lung adenocarcinomas and the ten uninvolved lung samples revealed that the level of the acidic SBP1 (457) protein was significantly decreased in lung adenocarcinomas, compared with normal lung samples ($p = 0.02$)

A

SBP1_HUMAN (Q13228)
SELENIUM-BINDING PROTEIN 1

Matching peptides:

m/z	MW	Score	Start	End	Sequence
513.1200	1024.5920	94.14	246	254	(K)DGLIP LEIR(F)
542.6464	1083.6650	124.17	94	103	(K)LVLPS LISSR(I)
666.6656	1331.7810	594.87	345	357	(R)LTGQL FLGGS IVK(G)
706.6162	1411.7190	345.17	358	370	(K)GGPVQ VLEDE ELK(S)
755.6304	1509.7710	180.62	161	174	(K)GGFVL LDGET FEVK(G)
757.1309	1512.7970	197.75	383	397	(R)VAGGP QNIQL SLDGK(R)
797.1508	2389.2740	186.55	358	379	(K)GGPVQ VLEDE ELKSQ PEPLV VK(G)
953.1302	1904.9000	140.64	35	52	(R)NTGTE APDYL ATVDV DPK(S)

B

C

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  1  MATKCGNCGP GYSTPLEAMK GPREEIVYLP CIYRNTGTEA PDYLATVDVD PKSPQYCQVI HRLPMPNLKD ELHHSGWNTY
 81  SSCFGDSTKS RNKLVLPSLI SSRIYVVDVG SEPGPQKLHK VIEPKDIHAK CELACLHTSH CLASGEVNIS SLGDVKGNGK
161  GGFVLLDGET FEVKGTWERP GGAAPLGYDF WYQPRHNVHI STEWAAPNVL RDGFNPADVE AGLYGSHLYV WDWQRHEIVQ
241  TLSLKDGLIP LEIRFLHNPS ATQGFVGCAS APNIQRFYKT REGTWSVEKV IQVPPKKVKG WLLPGVPGLI TDILLSLDDR
321  FLYFSNWLHG DLRQYDISDP QRPRLTGQLF LGGSIVKGGP VQVLEDEELK SQPEPLVVKG KRVAGGPQNI QLSLDGKRLY
401  ITTSLYSAWE KQFYPDLIRE GSVHLQVDVD TVKGGLKLNP NCLVDFGKEP LGPALAHELR YPGGDCSSDI WI
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Figure 2. Identification of SBP1 using tandem mass spectrometry (ESI MS/MS). (A) Eighteen peptides (eight shown here) of the native isoform of SBP1 (spot 460) were matched to a non-redundant database using *ProteinLynx Global SERVER* (http://www.micromass.co.uk/) with a total score of 3901.4 and coverage of 19.9%. (B) ESI MS/MS figure of a peptide in A (bold). (C) SBP1 protein sequence with matched peptides (highlighted areas). Eight peptides of the acidic isoform of SBP1 (spot 457) were matched with a total score of 1112.3 and coverage of 15.1% (figure not shown)

Table 1. SBP1 protein isoform and mRNA expression in normal lung and lung adenocarcinomas

SBP1	Normal $X^* \pm SD$	Tumour $X \pm SD$	p (T test)	Fold change Tumour/normal
457	0.17 ± 0.07	0.11 ± 0.14	0.02	0.6
460	0.11 ± 0.12	0.12 ± 0.15	0.72	1.1
Ratio 457/460	1.63 ± 0.55	1.04 ± 0.99	0.01	0.6
Sum (457 + 460)	0.28 ± 0.08	0.23 ± 0.26	0.14	0.8
mRNA	880.3 ± 254.5	742.9 ± 704.7	0.22	0.8

* Mean value.

(Table 1). The ratio of 457/460 was also significantly decreased in lung adenocarcinomas ($p = 0.01$). The native form of SBP1 (460) and the sum of spots 457 and 460 were not significantly different between tumour and normal lung, consistent with the similar mRNA levels determined using oligonucleotide microarrays (Table 1). This observation reflects the commensurate mRNA and protein expression levels between well-differentiated tumours and normal lung (Figures 3A–3C).

SBP1 is decreased in clinically aggressive tumours and related to poor outcome

Quantitative analysis of protein spots revealed decreased levels of both SBP1 isoforms (457 and 460) in poorly differentiated adenocarcinomas, compared with moderately or well-differentiated tumours (Table 2, $p < 0.038$ and 0.001, respectively, Figures 3A and 3B). The levels of these two isoforms were also

decreased in T2–T4 tumours (versus T1), bronchus-derived (versus bronchoalveolar) tumours, and tumours with a positive lymphocytic response (versus negative lymphocytic response) (Table 2). A similar relationship was observed for *SBP1* mRNA levels (Table 2 and Figure 3C) and, interestingly, *SBP1* mRNA levels were also decreased in tumours showing nuclear accumulation of P53 and in male patients (Table 2). The SBP1 protein and mRNA levels were not influenced by patient age, smoking status, tumour stage, lymph node status or K-*ras* 12th/13th codon mutations. Importantly, low levels of SBP1 protein (460) correlated significantly with poorer survival in patients with lung adenocarcinomas ($p = 0.007$) (Table 2 and Figure 3D).

Cytoplasmic and nuclear localization of SBP1 in lung tumours

To verify the 2-D PAGE results and uncover SBP1 cellular distribution, immunohistochemistry (IHC) was

Table 2. Relationship between SBP1 and clinicopathological variables in lung adenocarcinoma

Variables	n	p (457)	p (460)	p (mRNA)
Age, years				
<65	49	0.327	0.537	0.545
>65	44			
Gender:				
Male	40	0.781	0.541	0.038 down*
Female	53			
Smoking:				
Smoking	79	0.498	0.621	0.442
Non-smoking	10			
Stage:				
Stage III	29	0.643	0.172	0.079
Stage I	64			
T status				
T2–T4	44	0.042 down	0.0106 down	0.007 down
T1	49			
N status				
N0	68	0.503	0.219	0.185
N1, N2	25			
Classification				
Bronchus-derived	76	0.0418 down	0.0006 down	0.008 down
Bronchioloalveolar	14			
Differentiation				
Poor	23	0.038 down	0.001 down	0.001 down
Moderate	47			
Well	22			
Lymphocytic response				
Yes	41	0.0137 down	0.0078 down	0.006 down
No	52			
P53 nuclear accumulation				
Positive	28	0.108	0.162	0.00004 down
Negative	54			
K-ras 12th/13th codon mutation				
Positive	36	0.307	0.592	0.965
Negative	40			
Survival[†]	90	0.05	0.007	0.07

* p value of F-test (protein) or T-test (mRNA); down: decreased.
[†] Log-rank test; the median value was used as the cut-off value. Dead/alive: 43/50.

performed on formalin-fixed, paraffin wax-embedded lung TMAs (Figure 4A) containing ten normal and 193 neoplastic lung samples (148 adenocarcinomas and 45 squamous lung cancers). Higher levels of SBP1 protein were noted among pulmonary epithelial cells in normal lung tissues (Figure 4B). Positive cytoplasmic and nuclear staining for SBP1 was observed in lung adenocarcinomas (138/148, 93.2%), especially in well-differentiated tumours (Figure 4C). Reduced expression was noted in poorly differentiated tumours (Figure 4D). A much lower percentage of squamous tumours (7/45, 16%) showed positive SBP1 staining. No expression was detected in the A549 adenocarcinoma cell line. A strong correlation was found between SBP1 staining intensity using IHC and quantitative 2-D PAGE analysis of spot 457 or 460 ($r = 0.4$, $p < 0.001$).

Decreased SBP1 protein levels in highly proliferative adenocarcinomas

To assess SBP1 expression relative to cell proliferation, the proliferation index was determined in 62 of the primary lung adenocarcinomas in the tumour TMA using the MIB-1 antibody to the Ki-67 antigen. As expected, Ki-67 antigen expression and proliferation rates were higher in poorly differentiated than in well or moderately differentiated tumours [15]. There was a significant negative correlation between SBP1 protein staining assessed using IHC and Ki-67 antigen staining in lung adenocarcinomas ($r = -0.97$, $p = 0.002$) using Pearson correlation coefficient analysis. This indicates that SBP1 protein expression is decreased in highly proliferating lung tumours.

SBP1 is not increased following 5-azacytidine (5-AC) or dexamethasone treatment

To determine whether hypermethylation might cause *SBP1* gene silencing, the A549 cell line was treated with 5-AC for 3 days. These cells express very low levels of native SBP1 protein. SBP1 protein expression remained unchanged after treatment with either 5-AC or dexamethasone (Figure 5A). SKLU1 lung adenocarcinoma cells also do not express SBP1 protein (Figure 5A, lane 7).
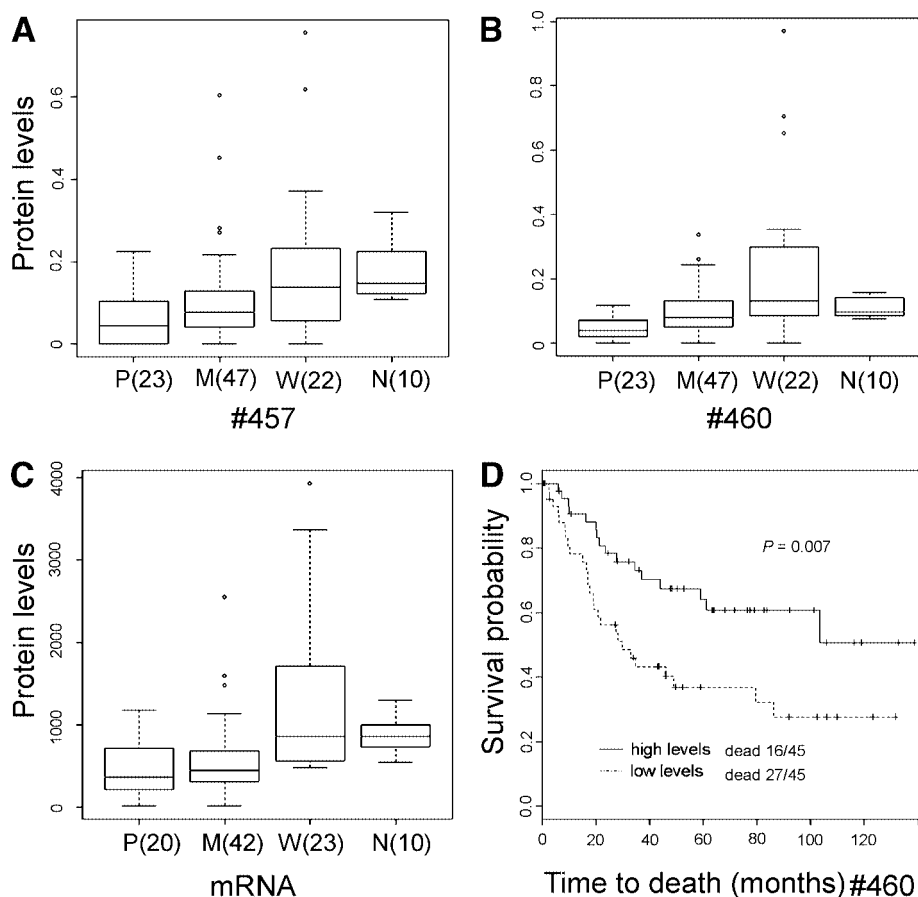
Figure 3. Lung differentiation, patient survival, and SBP1 expression. (A) Box plots showing that the levels of the acidic isoform of SBP1 (457) were significantly decreased in poor (P) or moderately differentiated (M) adenocarcinomas compared with well-differentiated (W) tumours or normal lung samples. The numbers in parentheses indicate the sample number in each differentiation group. (B) The levels of the native form of SBP1 (460) were also significantly decreased in poor or moderately differentiated adenocarcinomas compared with well-differentiated tumours or normal lung samples. (C) Decreased levels of SBP1 mRNA in poorly or moderately differentiated tumours compared with well-differentiated tumours or normal lung. (D) Kaplan–Meier survival curves showing that decreased expression of the native isoform of SBP1 (460) correlated significantly with poor patient survival. Log-rank test; the median value was used as the cut-off value. Sixteen of 45 patients died in the high-level expression group and 27 of 45 died in the low-level expression group

The *SBP1* genomic region is not deleted

To determine whether genomic deletion might explain the lower SBP1 expression in the lung adenocarcinoma, polymorphic markers for this region were used in a competitive PCR-based assay. LOH was not detected in the *SBP1* region in the six informative cases that showed no or very low expression levels of *SBP1* mRNA and/or protein expression (Figure 5B).

Discussion

Increasingly advanced genomic and proteomic techniques allow complex and high-throughput comparisons to be made for the purpose of elucidating genes that impel or suppress tumour growth, invasion, and lethality. Inherent in each technique, however, are certain advantages and limitations, as have been thoroughly described [19–21]. In the present study, complementary methods have been used to verify results observed with other techniques. Quantitative

2-D PAGE analysis of 93 lung tumours and ten normal lung specimens uncovered two protein isoforms that were significantly decreased in more aggressive tumours. Tandem mass spectrometry identified these two proteins as human isoforms of SBP1 and these results were subsequently verified by 2-D western blotting. Tumour tissue microarrays were utilized to examine the cellular localization of SBP1 using IHC and to verify the results obtained by 2-D PAGE analyses.

To date, two kinds of selenium-binding protein, SBP1 (or SP56) and SBP2 (or AP56), have been identified in mouse liver [22,23]. The mRNA levels of *hSP56* (*SP56/AP56*) were reported to be high in normal human liver, lung, kidney, colon, prostate, and pancreas [24]. Examination of the mRNA, however, cannot distinguish between *hSP56* and a putative human *AP56* due to only a 24-nucleotide difference within the coding region [25]. We identified two isoforms of SBP1 protein (acidic form 457 and native form 460), both of which have a molecular weight of 56 kD and have an approximate pI of 6.3–6.6 in human lung cancers. This protein is different from the
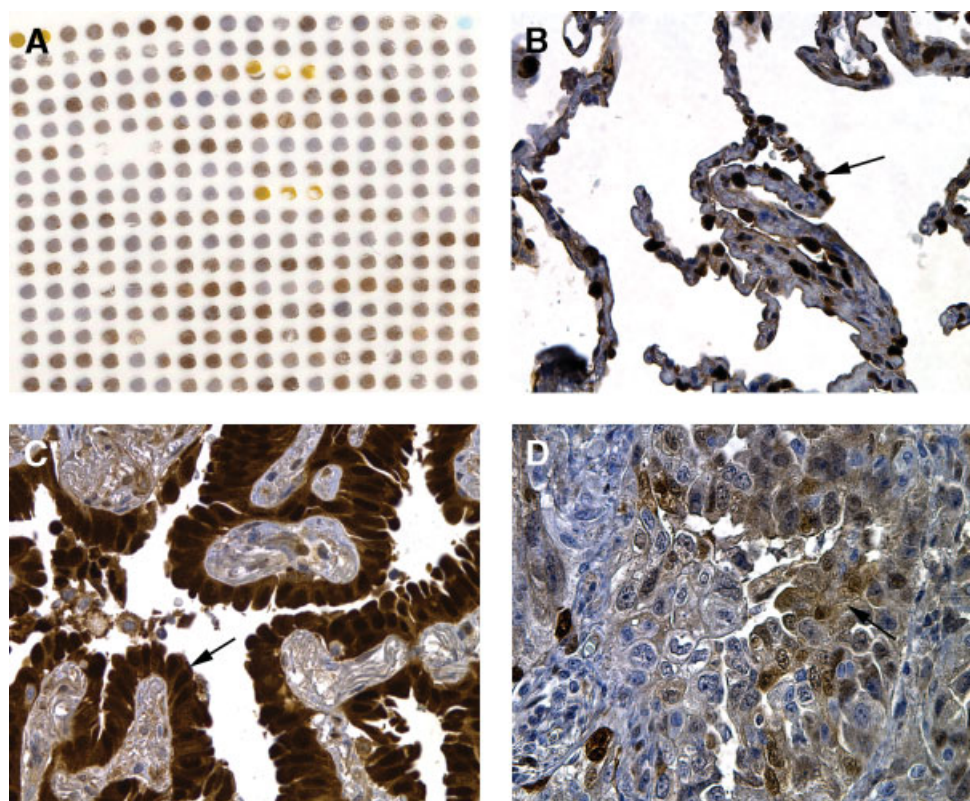
Figure 4. Immunohistochemical analysis of SBP1 with TMAs. (A) A portion of the lung TMA, stained with the anti-SBP1 antibody, showing different staining intensities (brown is positive staining and yellow is an orientation marker). The paraffin wax-embedded tissue from 148 pulmonary adenocarcinomas, 45 squamous tumours, and representative normal lung tissues are included. Three replicate tumour-rich tissue cores were sampled from each of the selected tissues ×10. (B) Higher levels of SBP1 protein expression were observed among pulmonary epithelial cells in normal lung samples (arrow indicating positive staining). (C) Higher levels of cytoplasmic and nuclear SBP1 staining in a well-differentiated adenocarcinoma (arrow). (D) Low-level staining of SBP1 in a poorly differentiated adenocarcinoma (arrow). (B–D) ×400. Only 7/45 (16%) squamous tumours showed weak positive SBP1 staining. No SBP1 protein expression was observed in the A549 adenocarcinoma cell line (not shown)

selenoprotein and selenoprotein-P that were previously identified in human lung adenocarcinoma cells and prostate cancer, respectively [26,27]. Interestingly, two additional isoforms of SBP1 were identified and were only present in normal lung samples using 2-D western blot analysis. These two isoforms have the same molecular weight but are more acidic, and likely represent bi- or tri-phosphorylated forms of SBP1. The possible structural differences between these four SBP1 isoforms are unknown.

The mechanism by which selenium binds to this protein and the exact function of SBP1 are unknown. It has been proposed that SBP1 (or SBP56) may participate in the late stages of intra-Golgi protein transport [28], and have a growth-inhibitory role in mouse liver [23]. *SBP1* mRNA is expressed in the slow-growing, androgen-sensitive human prostate cancer cell line LNCaP, but is not expressed in either of two more rapidly growing, androgen-insensitive human prostate cancer cell lines, PC-3 and DU145 [24]. The levels of *selenoprotein-P* mRNA, however, are significantly decreased in a subset of prostate cancers and in the LNCaP and PC-3 cell lines compared with normal prostate tissue [27]. In our study, the acidic form of SBP1 (457) and the ratio of 457/460 were significantly decreased in lung adenocarcinomas compared

with normal lung. The native SBP1 protein and mRNA levels were not significantly different between tumour and normal lung, most likely a result of the similar protein levels observed in normal lung and well-differentiated tumours (Figures 3A–3C). Low levels of SBP1 protein are observed in tumours from patients with poor survival. When comparing subgroups of tumours, both SBP1 protein and mRNA levels were significantly decreased in poorly differentiated, T2–T4, and bronchus-derived tumours, indicating that down-regulated expression of SBP1 occurs in larger and potentially more aggressive lung adenocarcinomas.

Two isoforms of SBP2 protein have been previously identified in mouse liver [29,30]. SBP2 expression is dramatically decreased after treatment with ciprofibrate, which increases cell proliferation. Dexamethasone, an inhibitor of cell proliferation in lung cells, can significantly increase SBP2 protein expression in liver [29]. However, dexamethasone did not increase SBP1 expression in the A549 cell line (Figure 5A). Furthermore, treatment of this cell line with 5-AC (Figure 5B) did not result in elevated expression of SBP1. This suggests that SBP1 may not be silenced by gene methylation. We also examined tumours that showed lack of SBP1 expression for potential genomic
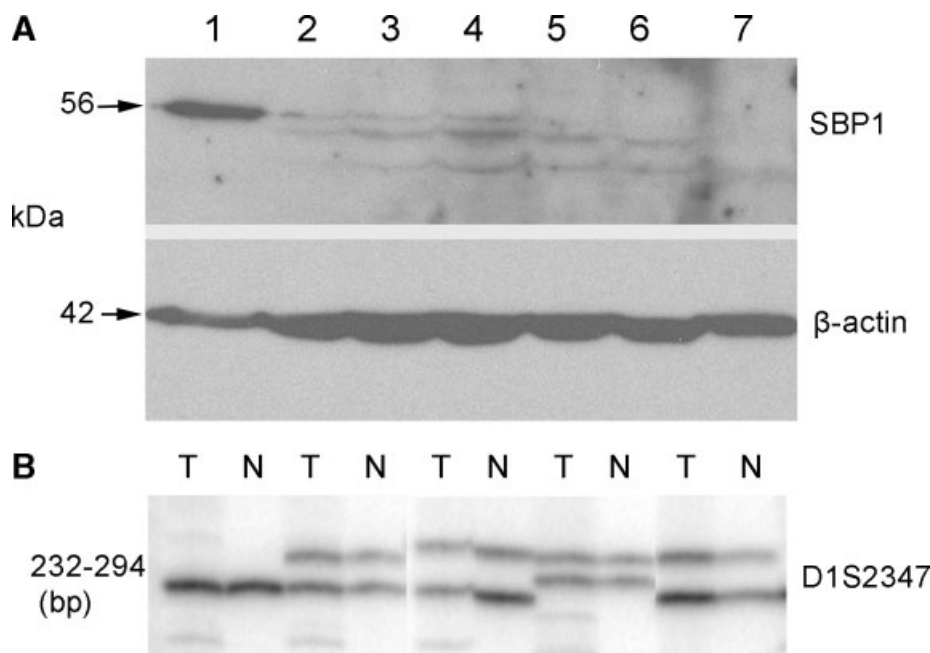
Figure 5. SBP1 induction assays and gene LOH analysis. (A) Expression of SBP1 protein following treatment with varying concentration of dexamethasone (DX) and 5-azacytidine (5-AC) in the A549 cell line. Top panel, increased SBP1 protein expression was not detected in A549 cells following treatment with either DX or 5-AC (arrow). Two apparent smaller degradation bands were only found in the A549 cell line. Lane 1: normal lung tissue; 2: control (no treatment); 3: DX 0.125 μM; 4: DX 0.25 μM; 5: 5-AC 2.5 μM; 6: 5-AC 5 μM; 7: SKLU1 cell line, which does not express SBP1 protein; bottom panel, β-actin was used as a loading control. (B) LOH analysis of the SBP1 gene region in lung adenocarcinomas. LOH was not detected in informative cases from 12 patient samples. T indicates tumour and N indicates the patient's corresponding normal lung DNA

deletion and did not detect these alterations. We did find, however, that there is a significant negative correlation between SBP1 protein expression and Ki-67 antigen staining, suggesting that lower SBP1 expression is present in highly proliferative lung tumours.

Immunohistochemical analysis using an anti-human SBP1 antibody showed positive cytoplasmic and nuclear protein staining in lung adenocarcinomas and in normal pulmonary epithelial cells. Much lower levels of expression were detected in squamous lung tumours (16%, 7/45 positive staining), which is consistent with a recent report on mRNA levels [31] indicating that *SBP1* expression is also tissue-specific. The reason for decreased SBP1 in tumours with a positive lymphocytic response may reflect a higher percentage of BD tumours (78.1%, 32/41) and a lower percentage of BA tumours (4.9%, 2/41) in the positive than in the negative lymphocytic response group (55.8%, 29/52 for BD; 17.3%, 9/52 for BA, respectively). The selenium compound selenodiglutathione can induce p53 protein levels in ovarian cells (A2780) that contain wild-type p53 [32]. We found that only *SBP1* mRNA, and not protein, was increased in tumours that did not show p53 nuclear protein accumulation (an indication of mutant p53), suggesting that SBP1 may induce wild-type p53 expression in lung tumours. We also found that *SBP1* mRNA was decreased in male lung cancer patients; however, the protein levels did not change significantly. This is different from the mouse SBP2 protein, which is expressed at higher levels in males than in female mice [29].

To our knowledge, this is the first report that multiple isoforms of human SBP1 are expressed in lung tissue and that low levels of SBP1 protein are related to worse survival in patients with lung adenocarcinomas. Both the SBP1 protein and mRNA were significantly decreased in poorly differentiated and bronchus-derived tumours. Low levels of SBP1 expression were present in tumours with a higher proliferation status and in squamous lung tumours. SBP1 may have clinical potential as a marker for patient outcome, but understanding the regulation of phosphorylation of the different isoforms, the basis for reduced SBP1 expression, and its role in lung cancer will require further study.

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