Decreased Selenium-Binding Protein 1 Enhances Glutathione Peroxidase 1 Activity and Downregulates HIF-1α to Promote Hepatocellular Carcinoma Invasiveness

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Abstract

Purpose: We aimed to characterize the role of selenium-binding protein 1 (SBP1) in hepatocellular carcinoma (HCC) invasiveness and underlying clinical significance.

Experimental Design: SBP1 expression was measured in stepwise metastatic HCC cell lines by Western blotting. The role of SBP1 in HCC was investigated using siRNA. Immunofluorescence analyses were used to detect the interaction between SBP1 and glutathione peroxidase 1 (GPX1). Nineteen fresh tumor tissues and 323 paraffin-embedded samples were used to validate in vitro findings and to detect the prognostic significance of SBP1, respectively.

Results: Inhibition of SBP1 effectively increased cell motility, promoted cell proliferation, and inhibited apoptosis only under oxidative stress; it also greatly enhanced GPX1 activity without altering GPX1 expression and downregulated hypoxia-inducible factor-1α (HIF-1α) expression. SBP1 and GPX1 formed nuclear bodies and colocalized under oxidative stress. In freshly isolated clinical HCC tissues, decreased SBP1 was linked with increased GPX1 activity and correlated with vascular invasion. Tumor tissue microarrays indicated that SBP1 was an independent risk factor for overall survival and disease recurrence; patients with lower SBP1 expression experienced shorter overall survival periods and higher rates of disease recurrence (P < 0.001). Further analyses indicated that the predictive power of SBP1 was more significant for patients beyond the Milan criteria than patients within the Milan criteria.

Conclusions: Decreased expression of SBP1 could promote tumor invasiveness by increasing GPX1 activity and diminishing HIF-1α expression in HCC; SBP1 could be a novel biomarker for predicting prognosis and guiding personalized therapeutic strategies, especially in patients with advanced HCC.
Translational Relevance
Selenium-binding protein 1 (SBP1) has been considered to be a protective agent against cancer. However, little is known about the function of SBP1 or its potential applications as a prognostic marker in hepatocellular carcinoma (HCC). Our findings indicate that SBP1 may act as a pro-oxidant rather than antioxidant through the interaction with glutathione peroxidase 1 and hypoxia-inducible factor-1α. Thus, the use of antioxidants such as selenium in patients with HCC, especially patients with advanced-stage cancer, should be completed with caution. Furthermore, determination of SBP1 expression is especially useful for personalized therapeutic strategies and decisions about individuals beyond Milan criteria who could benefit from more aggressive treatment, such as chemotherapy or liver transplantation.

Materials and Methods
Cell lines
The normal liver cell line L-02 and the HCC cell lines HepG2, Hep3B, SMMC7721, Huh 7, and PLC/PRF/5 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (CAS). HCC cell lines with stepwise metastatic potential (MHCC97L, MHCC97H, HCCLM3, and HCCLM6, which are HBV-positive cell lines with the same genetic background but different lung metastatic potentials) were established at our institute (24, 25). Cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) or RPMI-1640 (Invitrogen/GIBCO) supplemented with 10% FBS (Invitrogen/GIBCO) at 37°C in 5% carbon dioxide.

We used hydrogen peroxide (Sigma) as ROS resources to simulate oxidative stress in vitro. A concentration of 300, 100, and 50 μmol/L was used in the apoptosis assay, proliferative assay, and immunofluorescence assay, respectively.

Patients and samples
Patients with HCC (n = 342) who underwent surgical treatment at the Zhongshan Hospital at Fudan University (Shanghai, China) were enrolled in this study. Patients were divided into 2 cohorts according to their dates of surgery. To ensure accurate analysis of GPX1 activity, tumor tissue samples were freshly isolated from 19 patients during a 2-week period in 2011 (cohort 1). Tumor specimens used in tissue microarrays (TMA) analyses were consecutively chosen from 323 patients with HCC between 2003 and 2004 (cohort 2). Ethical approval for human subjects was obtained from the Institutional Review Board, and written informed consent was obtained from the patients.

Patients in cohort 1 were classified into 2 groups according to the extent of vascular invasion detected. Patients in cohort 2 were followed up every 2 months during the first postoperative year and at least every 3 to 4 months thereafter until March 15, 2009, 9 of the 323 patients were lost. The median follow-up period was 60 months (range, 2–85 months). Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last observation point. Time to recurrence was defined as the interval between the date of surgery and the date of diagnosis of intrahepatic recurrence and metastasis. Using 24 months as the cutoff value, all cases of recurrence were divided into early recurrence (n = 147) or late recurrence (n = 55; ref. 26). Preparations of tissue samples are described in the Supplementary Appendix.

Molecular and cell biology assays
Western blotting, quantitative real-time PCR (qRT-PCR), migration analysis, proliferation analysis, apoptosis assay, and immunofluorescence assay were conducted as described previously (27). Detailed information is provided in the Supplementary Appendix.

RNA interference
For siRNA-mediated SBP1 silencing, the following target siRNA sequences were used: sense, CUUUGAGGCACCAGAAAUTT and antisense, AUUUCUGUGGUGCCUCUCAGGT. The RNA duplexes were synthesized by the GenePharma Company. Transfection of the siRNAs into the SMMC7721 cell line was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.
Measurement of GPX1 activity
Measurement of GPX1 activity was conducted as described previously (28). Detailed information is provided in the Supplementary Appendix.

TMAs and immunohistochemistry
TMAs were constructed by the Shanghai Biochip Co., Ltd. The primary antibody used in immunohistochemistry was SBP1 (1:500; MBL). Immunohistochemistry was carried out using a 2-step protocol (Novolink Polymer Detection System; Novocastra). Negative control slides in which the primary antibodies were omitted were included in all assays.

To validate concordance between TMAs and whole tumor sections, we further detected the expression of SBP1 by immunohistochemistry in 50 corresponding whole tumor sections randomly chosen from the 323 cases.

Evaluation of immunohistochemical variables
Immunohistochemical scores were assessed by 2 independent pathologists without knowledge of patient characteristics, and the scores for all cases were compared with check for discrepancies. The final scores were assigned by discussion. Scores were assigned as intensity and percentage of positively staining tumor cell cytoplasm and nuclei in the whole tissue sample. Specifically, the immunostains were scored using a 4-point scale (0–++) system based on the number of positive cells and the intensity of staining.

Correlations of SBP1 expression profiles with clinical demographics, OS, and recurrence rates were evaluated. Further details about these methods are described in the Supplementary Appendix.

Statistical analysis
The software package SPSS v13.0 (SPSS Inc.) was used for statistical analyses. Univariate and multivariate Cox proportional hazards models were used to identify relevant prognostic factors. Kaplan–Meier survival curves and the proportional hazards models were used to identify relevant survival and recurrence probability between subgroups.

SBP1 inhibits proliferation and induces apoptosis only after hydrogen peroxide treatment
We used the CCK-8 assay to determine whether SBP1 might interfere with cell proliferation and we observed that SBP1 only inhibited cellular proliferation following hydrogen peroxide treatment (Fig. 2A). When cells were cultured in normal medium, SBP1 did not inhibit cell proliferation, and the proliferation rate of the control group was slightly higher than that of the SBP1-silenced group. However, if 100 μmol/L of hydrogen peroxide was added to the culture medium, SBP1 greatly inhibited cell proliferation. The inhibition of proliferation began 24 hours following treatment, and the cell counts of the control group were slightly decreased at 48 and 72 hours whereas the SBP1-silenced group cells were unaffected in the presence of hydrogen peroxide. These results indicated that SBP1 alone could not inhibit cell proliferation.

The apoptosis assay showed results similar to those obtained earlier (Fig. 2B). No significant differences in apoptosis rates were observed between the 2 groups if given normal culture medium. However, if 300 μmol/L of hydrogen peroxide was added and cells were incubated for 24 hours, the apoptosis rate of the SBP1-silenced group was dramatically reduced compared with that of the negative control group, indicating that SBP1 could somehow facilitate the hydrogen peroxide–induced apoptosis.

SBP1 and HIF-1α interactions
Figure 2C showed the interactions of SBP1, HIF-1α, and GPX1 expressions under different conditions. The hydrogen peroxide–treated groups were treated with 50 μmol/L hydrogen peroxide for 24 hours before protein extraction. The expression of HIF-1α was increased by hydrogen peroxide treatment, as shown by the control groups (SMMC7721 and SMMC7721-Mock), and an increase in SBP1 expressions could also be observed in the same groups. This is consistent with the finding that SBP1 is a target gene for HIF-1α (20). However, in the SMMC7721 groups where SBP1 expression was downregulated by siRNA treatment, the expression of HIF-1α was not elevated by hydrogen peroxide treatment. This might indicate that SBP1 could also somehow counter-regulate the expression of HIF-1α following hydrogen peroxide treatment.

The expression of GPX1, however, was not associated with either SBP1 or HIF-1α (Fig. 2C), although a slight increase could be detected following treatment with hydrogen peroxide.

SBP1 greatly inhibits GPX1 activity, not expression level in vitro
We measured the activities of GPX1 under different conditions in vitro (Fig. 2D). Compared with the control
groups, the GPX1 activities in the SBP1-silenced groups had increased by 4- or 5-fold. This dramatic increase in GPX1 activity indicates that SBP1 may greatly inhibit GPX1 activity. Given the fact that the expression levels of GPX1 in different groups were unchanged (Fig. 2C), SBP1 might inhibit GPX1 through a post-translational way.

SBP1 and GPX1 formed special bodies and colocalized in the nuclei following hydrogen peroxide treatment

Under normal conditions, GPX1 localized exclusively in the cytoplasm but SBP1 could be found both in the cytoplasm and the nucleus (Fig. 3A). However, when cells were treated with hydrogen peroxide, we observed that both GPX1 and SBP1 had established specific nuclear bodies,
and the newly formed structure were colocalized, indicating that the 2 proteins might bind to each other under oxidative stress (Fig. 3B). After silencing SBP1, no specific association between SBP1 and GPX1 could be observed, but the GPX1 nuclear bodies remained (Fig. 4). The physiologic and pathologic implications behind this phenomenon would be discussed later.

**Decreased SBP1 and increased GPX1 activity correlate with vascular invasion in HCC patients**

We further validated our in vitro findings using clinical samples obtained from patients with HCC (Fig. 5A, Fig. 5B). We observed that samples with low expression of SBP1 had relatively high GPX1 activities whereas samples with high expression of SBP1 had limited GPX1 activities. Overall, the
vascular invasion group had a lower SBP1 expression and relatively higher GPX1 activity, particularly in patients with HCC with macrovascular invasion, compared with those of the nonvascular invasion group (Supplementary Table S1).

**Immunohistochemical characteristics**

Representative photomicrographs of tumor tissues showing the various staining patterns are presented in Fig. 6A. In tumor tissues, we observed 26.01% (84 of 323) with scores of 0, 38.08% (123 of 323) with scores of +, 22.60% (73 of 323) with scores of ++, and 13.31% (43 of 323) with scores of ++++. Of the 84 patients with scores of 0 in the tumor tissues, 78.57% (66 of 84) experienced recurrent disease, as did 65.86% (81 of 123), 52.05% (38 of 73) and 53.49% (23 of 43) of patients with scores of +, ++, and ++++, respectively. With Kaplan–Meier estimates and log-rank tests considering the intensities of staining in tumor tissues, we found that the cutoff score of ++ was suitable to be the criterion (Supplementary Fig. S1); thus, we defined the samples with scores of 0 and + as negative and the samples with ++ and +++ as positive. According to the criterion...
used, 64.09% (207 of 323) of the patients with HCC were negative for expression of SBP1.

As showed in Supplementary Table S2, negative SBP1 expression in tumor tissues was significantly correlated with patient age ($P = 0.045$), $\alpha$-fetoprotein ($P < 0.001$), tumor size ($P = 0.005$), tumor number ($P = 0.019$), tumor encapsulation ($P = 0.034$), vascular invasion ($P < 0.001$), and recurrence ($P < 0.001$). Levels of SBP1 expression in tumor tissues were significantly different among patient groups according to the degree of vascular invasion ($P < 0.001$).

**SBP1 expression in tumor tissue and prognosis**

In the univariate analysis, patient sex, serum albumin (ALB), tumor differentiation, tumor encapsulation, tumor size, tumor number, and vascular invasion were associated with OS; patient sex, serum ALT, tumor encapsulation, tumor size, and vascular invasion were associated with cumulative recurrence (Supplemental Table S4). Univariate and multivariate analyses showed that SBP1 expression in tumor cells was an independent risk factor for both OS ($P < 0.001$) and recurrence ($P < 0.001$).

On the basis of Kaplan–Meier survival curves, patients who were negative for expression of SBP1 in tumor tissues experienced shorter OS periods ($P < 0.001$) and higher recurrence rates ($P < 0.001$; Fig. 6B). We investigated the predictive value of SBP1 in HCC. Of the patients with negative SBP1 expression in their tumor tissues, 69.08% (143 of 207) had recurred, and 114 patients of these patients experienced recurrence within 2 years. In patients with positive SBP1 expression in tumor cells, the recurrence rate was only 50.86% (59 of 116), and 28.45% (33 of 116) of these patients experienced recurrence within 2 years. Kaplan–Meier survival curves revealed that SBP1 was a significant prognostic factor for OS and early recurrence in HCC.

We further stratified patients by Milan criteria and investigated the predictive value of SBP1 in different
Interestingly, in the subpopulation of patients with HCC within the Milan criteria, Kaplan–Meier survival curves revealed that SBP1 was not an effective prognostic factor for OS (P > 0.05) but was for early recurrence (P = 0.039; Fig. 6C). However, in the subpopulation of patients with HCC beyond Milan criteria with positive SBP1 expression in tumor tissues, only 35.85% (19 of 53) of patients recurred within 2 years. The results indicated that even patients beyond Milan criteria could experience a relatively longer OS and lower recurrence rate with tumors positive for SBP1 expression (P < 0.001). The prognostic significance of SBP1 was retained in the subpopulation of patients with HCC beyond Milan criteria (Fig. 6D).

Discussion

As shown by our study, most HCC cell lines have a minimal SBP1 expression, with the exception of SMMC7721. Compared with other HCC cell lines, SMMC7721 has a low metastatic potential (24). In our study, the migration potential of SMMC7721 cells was inhibited by the expression of SBP1. However, SBP1 only exhibited its impact on cancer cell proliferation and apoptosis following treatment with hydrogen peroxide; these results indicated that SBP1 might exert its tumor suppressive power through modulation of the tumor redox microenvironment.

Figure 5. Relationships among clinical tumor characteristics, SBP1 and GPX1. Nineteen samples surgically removed from patients with HCC were included in this trial. All samples were preserved in liquid nitrogen immediately after resection and were analyzed within 2 weeks. These 19 samples were divided into 2 groups (N1–N9, V1–V10) based on their tumor characteristics (Supplementary Table S1). A, the vascular invasive group (VI group) had consistently low expressions of SBP1 whereas the nonvascular invasive group (NVI group) had higher and varied expressions of SBP1. B, GPX1 activity of the VI group was consistently at a relatively high level whereas the NVI group also exhibited a varied and lower activity of GPX1. Samples with high expression of SBP1 had limited GPX1 activity (e.g., N1, N7, and N8) whereas samples with lower expression of SBP1 had relatively high GPX1 activity (e.g., N4, N9, V2, and V8). C, the possible relationship among SBP1, GPX1, HIF-1α, and ROS.
GPX1 is the most important antioxidant enzyme that protects cells from ROS such as hydrogen peroxide and singlet oxygen species (29). ROS have the potential to create oxidative stress within cells that causes DNA damage, protein degradation, peroxidation of lipids, and finally leads to cell transformation or death based on ROS concentration (30). It is a well-documented fact that cancer cells are under high levels of oxidative stress compared with normal cells.
and they require defense against ROS to survive (31). Many studies have already reported that GPX1 may protect cancer cells under conditions of severe oxidative stress as it has been observed that increased GPX1 activity can inhibit apoptosis (14, 15), reduce tumor sensitivity toward ROS-generating anticancer drugs (17, 18), and promote the more malignant stages of cancer (16). Our findings showed that SBP1 could greatly inhibit the activity, but not expression, of GPX1 in cancer cells both in vitro and in vivo; the translocation of GPX1 to the nucleus in cancer cells under oxidative stress may facilitate the antioxidant functions of GPX1, whereas the formation and combination of GPX1 and SBP1 nuclear bodies might inhibit this process. The formation of this SBP1-GPX1 complex has also been validated by coimmunoprecipitation in a prior study, which suggested that this phenomenon was a direct physical interaction (19). We also noticed the expression level of SBP1 was upregulated by oxidative stress (Fig. 2C). Normally, the high level of oxidative stress in cancer cells (usually caused by tumor microenvironment or drug-induced ROS) would lead to cellular apoptosis rather than survival or transformation due to the inhibition of GPX1 activity by the upregulated SBP1. However, as the expression of SBP1 in HCC and many other cancers was reduced (mechanisms might include DNA methylation and chromatin remodeling; ref. 32), the intensive oxidative stress in the tumor microenvironment could be attenuated by the activation of GPX1, leading to cancer cell survival, proliferation, malignant transformation, and even metastasis (31).

We observed a relationship among SBP1, HIF-1α, and ROS. ROS could initiate the activation of HIF-1α (21), whereas HIF-1α could regulate the expression of SBP1 through a hypoxia response element in its promoter region (20). On the basis of this effect, ROS would elevate the expression of SBP1 through HIF-1α mediation. This was supported by our Western blotting results as showed in Fig. 2C. However, we further observed that the SBP1-silenced cancer cells had a diminished HIF-1α expression under oxidative stress, which indicated that SBP1 could somehow counter-regulate the expression of HIF-1α during cellular oxidative stress (Fig. 2C). A possible explanation for this phenomenon was that the exogenous ROS in SBP1-silenced cells was immediately degraded by GPX1, leading to diminished HIF-1α expression. It has been reported that HIF-1α can suppress the epithelial–mesenchymal transition through the p53 pathway (also, ROS is a well-known initiator of p53-mediated apoptosis; ref. 33) and inhibit malignant tumor conversion (20, 34, 35). This might also be the reason for the increased malignancy and invasive characteristics of tumors with low SBP1 expression. We illustrated the possible relationship of SBP1, GPX1, HIF-1α, and ROS (Fig. 5C).

As most of the anticancer agents kill tumor cells by generating ROS or amplifying oxidative stress (31, 36, 37), we concluded that increased SBP1 expression and decreased GPX1 activity could elevate tumor chemosensitivity. This conclusion was supported by several previous studies, which investigated SBP1 and GPX1 separately (8, 18, 38). On the other hand, the poor responses of patients with HCC to chemotherapy might be due to low SBP1 expression and high GPX1 activity, thus increasing SBP1 expression and decreasing GPX1 activity could be a novel strategy for cancer treatment. However, SBP1 and GPX1 are both selenium-containing proteins, and attempts to reduce cancer risk by simple selenium supplementation in the Selenium and Vitamin E Cancer Prevention Trial (SELECT) have already failed (39). However, recent studies have found certain forms of selenium (such as SeL) can act as pro-oxidants rather than antioxidants and have chemotherapeutic potential by inducing cancer cell apoptosis while leaving normal cells unaffected (40, 41). These certain forms of selenium might exclusively elevate the level of SBP1 rather than GPX1, which might provide a new tool in cancer treatment but requires further investigation.

Our clinical data validated the possible role of SBP1 in cancer biology. Patients with positive SBP1 expression experienced longer periods of OS and lower recurrence rates, indicating that negative SBP1 expression could be a potential biomarker predicting early recurrence/poor prognosis and guide our follow-up treatment in patients with HCC after surgery. When we further stratified patients by Milan criteria, which are widely accepted guidelines for early stage liver transplantation, the survival curves in this study show that negative SBP1 expression in the tumor cells correlated with higher early recurrence rates in patients beyond the Milan criteria. However, no significant difference was observed with regard to survival periods, thus the predictive significance of SBP1 in this subpopulation would help clinicians identify patients at high risk of early recurrence and enable them to administer rational adjuvant therapy after resection or liver transplantation. However, we noticed that SBP1 is a more effective predictor for patients with HCC beyond the Milan criteria rather than for those within the Milan criteria (Fig. 6C and D). This could be understood by the role of SBP1 in the tumor redox microenvironment considering that patients in the advanced stages of cancer often suffer from more severe hypoxia and oxidative stress than those in the early stages. On the basis of this conclusion, treatment of patients beyond the Milan criteria with SBP1 positive expression should be more aggressive, for these patients can also achieve excellent survival outcomes. Furthermore, the use of glutathione treatment in patients with cancer, especially advanced-stage cancers, should be completed with caution, for glutathione may elevate the activity of GPX1 and promote tumor development based on our findings and those of other groups (42). Taken together, our data indicate that SBP1 is a tumor biomarker with prognostic value in patients with HCC. Determination of SBP1 expression may be useful for personalized therapeutic strategies and decisions about individuals outside of the Milan criteria who could benefit from more aggressive treatment, such as liver transplantation. Currently, the outcomes of these patients are very difficult to predict using conventional clinical indices.

In conclusion, decreased expression of SBP1 could lead to higher GPX1 activity and a diminished HIF-1α expression in...
HCC; thus, SBP1 might exert its tumor suppressive function as a regulator of the tumor redox microenvironment. SBP1 could be a novel biomarker for predicting prognosis and guiding personalized therapeutic strategies, especially in patients with advanced HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Huang, C. Gu, J. Zhou, Y. He, T. Kondo, J. Fan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Huang, G. Ding, C. Gu, J. Zhou, M. Kuang, Y. Ji, Y. He, T. Kondo, J. Fan

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