Human Cancer Biology

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.

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Introduction

Selenium is an essential trace element with cancer-preventing activities that have been shown in many epidemiologic studies (1–3). The cellular biochemistry of selenium is a complex system that involves the expression of a wide range of selenium-containing proteins (4–6). Of these proteins, a 56-kDa molecule termed selenium-binding protein 1 (SBP1) was found to be the possible mediator of selenium’s anticancer functions (7, 8). SBP1 is expressed in various cell types, including liver, heart, and kidney (2), and previous studies have established a solid connection between SBP1 and cancer. Decreased SBP1 was found in a vast number of human cancers, such as colorectal cancer (9), lung adenocarcinomas (10), ovarian cancer (11), gastric cancer (12), and hepatocellular carcinoma (HCC; ref. 13). However, the molecular mechanism underlying the tumor suppressive functions of SBP1 remains unclear.

Glutathione peroxidase 1 (GPX1) is also an important selenium-containing protein, in which selenium is a constituent of the amino acid selenocysteine. GPX1 is a ubiquitously expressed antioxidant enzyme that scavenges organic hydroperoxides and protects cells from reactive oxygen species (ROS) and hydrogen peroxide–induced or -dependent apoptotic injury (14, 15). Elevated GPX1 activity was reported to protect cancer cells from oxidative stress and anticancer agents (16–18). Previous studies using...
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 glutathione 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.

Translational Relevance

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, CUUUGAGCCACCAAGAAUIT and antisense, AUUUCUGGGUUGCCUCACAGT. The RNA duplexes were synthesized by the Genpharma 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.

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 log-rank (Mantel–Cox) test were used to compare patient survival and recurrence probability between subgroups (26). All statistical tests were 2-sided, and a P value less than 0.05 was considered statistically significant.

Results

SBP1 is minimally expressed in most human HCC cell lines and inhibits cell migration

We detected the expression levels of SBP1 in current HCC cell lines (Fig. 1A). SBP1 was highly expressed in normal liver cells whereas barely detected in the highly metastatic HCC cell lines (MHCC97L, MHCC97H, HCCLM3, and HCCLM6). HCC cell lines with low metastatic potential also expressed marginal levels of SBP1 with the exception of SMMC7721. SMMC7721 was the only HCC cell line that expressed a high level SBP1, and we chose this cell line for further study. The expression of SBP1 in SMMC7721 72 hours after siRNA transfection was downregulated to a minimal level (Fig. 1B and D) compared with SBP1 expression in the negative control.

Cell migration of SBP1-silenced SMMC7721 cells and negative control cells was assessed via Transwell chambers (Fig. 1C) and wound-healing assays (Fig. 1E). The results showed significant differences indicating that SBP1 greatly inhibits cancer cell migration.

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 inhibits 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.

Figure 2. Effects of downregulation of SBP1 on cell proliferation, apoptosis, and GPX1 activity. A, cell proliferation was detected by CCK-8 assay. When both SBP1-siRNA and the control group were cultured under normal conditions, the proliferation rate of the control group was higher than that of the SBP1-siRNA group ($P < 0.05$). However, when both groups were treated with 100 μmol/L hydrogen peroxide, the cell proliferation in the control group was significantly suppressed after 48 hours whereas the SBP1-siRNA group maintained most of its proliferative ability ($P < 0.0001$). B, cell apoptosis was analyzed by flow cytometry. Fluorescein isothiocyanate (FITC)-labeled Annexin V and fluorescent dye propidium iodide (PI) were used to stain cells. No obvious differences were shown between the SBP1-siRNA group and the control group when cultured using normal medium. A significant decrease in the apoptosis rate of the SBP1-siRNA group was observed when cells were treated with hydrogen peroxide (300 μmol/L) for 12 hours. C, Western blot analysis showed the different HIF-1α and GPX1 expression levels in SMMC7721 cells following SBP1-siRNA transfection and different hydrogen peroxide treatment. GAPDH was used as a loading control. D, GPX1 activity of the SBP1-siRNA group was significantly higher than that of the control groups ($P < 0.001$), and a slight increase could be observed between the hydrogen peroxide-treated and -untreated groups. All experiments were repeated at least 3 times.

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 ALB, 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
subpopulations. 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.
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 within 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
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Huang, G. Ding, C. Gu, J. Zhou, M. Kwang, Y. Ji, Y. He, T. Kondo, J. Fan

References


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