Decreased Selenium-binding Protein 1 Enhances Glutathione Peroxidase 1 Activity and Down-regulates HIF-1α to Promote Hepatocellular Carcinoma Invasiveness

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Running title: Decreased SBP1 promotes HCC through GPX1 and HIF-1α

Keywords: hepatocellular carcinoma; selenium-binding protein 1; glutathione peroxidase 1; hypoxia-inducible factor-1α; oxidative stress

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Abbreviations: SBP1, selenium-binding protein 1; HCC, hepatocellular carcinoma; siRNA, small interfering RNA; GPX1, glutathione peroxidase 1; ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor-1α

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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 blot. The role of SBP1 in HCC was investigated using small interfering RNA (siRNA). Immunofluorescence analyses were used to detect the interaction between SBP1 and glutathione peroxidase 1 (GPX1). Nineteen fresh tumor tissues and 323 parafin-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; also greatly enhanced GPX1 activity without altering GPX1 expression and down-regulated hypoxia-inducible factor-1α (HIF-1α) expression. SBP1 and GPX1 formed nuclear bodies and co-localized 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: 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 HCC. Our findings indicate that SBP1 may act as a pro-oxidant rather than anti-oxidant through the interaction with GPX1 and HIF-1α. Thus the use of anti-oxidants such as glutathione in HCC patients 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 regarding individuals beyond Milan criteria who could benefit from more aggressive treatment, such as chemotherapy or liver transplantation.
Introduction

Selenium is an essential trace element with cancer-preventing activities that have been demonstrated in many epidemiological 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 anti-cancer 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) (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 anti-cancer agents (16-18). Previous studies using co-immunoprecipitation demonstrated that these two distinct selenium-containing proteins (GPX1 and SBP1) can form a physical association that facilitates their interactions (19), but their possible roles in cancer development are still unknown. SBP1 is also known to have a hypoxia response element in its promoter region and to be a target gene of hypoxia-inducible factor-1α (HIF-1α) (20), which is a fundamental mediator of cellular adaptation to micro-environmental stress, especially oxidative stress (21). The relationship between GPX1 and oxidative stress and the multifunctional role of HIF-1α in cancer biology may be associated with the anti-tumor activity of SBP1.

HCC is the sixth most common malignancy and the third leading cause of cancer death worldwide (22). Although early diagnosis and surgical treatments have
significantly improved overall patient outcomes, long-term survival is still low due to high rates of recurrence and metastasis. Resistance toward cytotoxic chemotherapy also affects survival and prognosis in HCC patients (23). We and other groups have found that the expression of SBP1 is decreased in most HCCs and is associated with poor outcomes (13), but the possible molecular mechanism of SBP1 in HCC biology, particularly in cancer invasion and metastasis, has not been studied. Given the relationship among SBP1, GPX1, HIF-1α and oxidative stress in the HCC micro-environment, in this study, we aimed to explore the role of SBP1 in cancer invasion and metastasis. We found that decreased SBP1 could promote HCC invasion/metastasis and lead to poor prognosis through the enhancement of GPX1 activity and the down-regulation of HIF-1α.

**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 DMEM or RPMI 1640 (Invitrogen/GIBCO) supplemented with 10% fetal bovine serum (Invitrogen/GIBCO) at 37°C in 5% carbon dioxide.

We employed hydrogen peroxide (Sigma, USA) as ROS resources to simulate oxidative stress in vitro. A concentration of 300 μmol/L, 100 μmol/L 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 two 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 two-week period in 2011 (cohort 1). Tumor
specimens used in tissue microarrays analyses were consecutively chosen from 323 HCC patients 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 two 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 intra-hepatic 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) (26). Preparations of tissue samples are described in the supplemental appendix.

**Molecular and cell biology assays.** Western Blotting, qRT-PCR, migration analysis, proliferation analysis, apoptosis assay and immunofluorescence assay were performed as described previously (27). Detailed information is provided in the supplement.

**RNA interference.** For small interfering RNA (siRNA)-mediated SBP1 silencing, the following target siRNA sequences were used: sense CUUGGAGGACCAAGAAUUTT and antisense AUUUCUUGGUCCUCAAAGTT. The RNA duplexes were synthesized by the Genepharma Company (Shanghai, China). Transfection of the siRNAs into the SMMC7721 cell line was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Measurement of GPX1 activity.** Measurement of GPX1 activity was performed as described previously (28). Detailed information is provided in the supplement.

**Tissue microarrays and immunohistochemistry.** Tissue microarrays (TMA) were constructed by the Shanghai Biochip Co., Ltd. The primary antibody used in immunohistochemistry was SBP1 (1:500; MBL, Japan). Immunohistochemistry was
carried out using a two-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 tissue microarrays 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 two independent pathologists without knowledge of patient characteristics, and the scores for all cases were compared to 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, overall survival, and recurrence rates were evaluated. Further details regarding these methods are described in the supplemental 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 two-sided, and a $P$ value $< 0.05$ 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 while 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 down-regulated to a minimal level (Fig. 1B, 1D) 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 employed the CCK-8 assay to determine if 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 hours and 72 hours while 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 above (Fig. 2B). No significant differences in apoptosis rates were observed between the two 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 HIF1-α (20). However, in the SMMC7721 groups where SBP1 expression was down-regulated 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 Co-localized 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 co-localized, indicating that the two 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. 4A). The physiological and pathological 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 HCC patients (Fig. 5A, Fig. 5B). We observed that samples with low expression of SBP1 had relatively high GPX1 activities while 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 HCC patients with macro-vascular invasion, compared with those of the non-vascular invasion group (Supplemental 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 (Supplemental 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 HCC patients were negative for expression of SBP1.

As showed in Supplemental Table S2, negative SBP1 expression in tumor tissues was significantly correlated with patient age ($P = 0.045$), AFP ($P < 0.001$), 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 overall survival; Patient sex, serum alanine transaminase (ALT), tumor encapsulation, tumor size, and vascular invasion were associated with cumulative recurrence (Supplemental Table S3). In the multivariate analysis, patient sex, serum ALB, tumor differentiation, tumor
encapsulation, tumor size, and vascular invasion were associated with overall survival; 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 overall survival ($P < 0.001$) and recurrence ($P < 0.001$).

Based on Kaplan–Meier survival curves, patients who were negative for expression of SBP1 in tumor tissues experienced shorter overall survival 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 two 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 two years. Kaplan–Meier survival curves revealed that SBP1 was a significant prognostic factor for overall survival and early recurrence in HCC.

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

GPX1 is the most important anti-oxidant 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 in order 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 towards ROS-generating anti-cancer 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, while 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 co-immunoprecipitation in a prior study, which suggested that this phenomenon was a direct physical interaction (19). We also noticed the expression level of SBP1 was up-regulated by oxidative stress (Fig. 2C). Normally, the high level of oxidative stress in cancer cells (usually caused by tumor micro-environment or drug-induced ROS) would lead to cellular apoptosis rather than survival or transformation due to the inhibition of GPX1 activity by the up-regulated SBP1. However, as the expression of SBP1 in HCC and many other cancers was reduced (mechanisms might include DNA methylation and chromatin remodeling (32)), the intensive oxidative stress in the tumor micro-environment 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), while HIF-1α could regulate the expression of SBP1 through a hypoxia response element in its promoter region (20). Based on this effect, ROS would elevate the expression of SBP1 through HIF-1α mediation. This was supported by our Western blot results as showed in figure 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 (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 HCC patients 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 anti-oxidants 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 overall survival 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 HCC patients 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 demonstrate 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 sub-population 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 HCC patients beyond the Milan criteria rather than for those within the Milan criteria (Fig. 6C, Fig. 6D). This could be understood by the role of SBP1 in the tumor redox micro-environment considering that patients in the advanced stages of cancer often suffer from more severe hypoxia and oxidative stress than those in the early stages. Based on 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 cancer patients, 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 HCC patients. Determination of SBP1 expression may be useful for personalized therapeutic strategies and decisions regarding 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 micro-environment. SBP1 could be a novel biomarker for predicting prognosis and guiding personalized therapeutic strategies, especially in patients with advanced HCC.
References

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Figure Legends

Figure 1. Expression of Selenium-binding Protein 1 (SBP1) in HCC cell lines and migration analysis of SBP1 by siRNA

(A); Western blot analysis of SBP1 expression in cell lines L-02, HepG2, SMMC7721, Huh7, PLC/PRF/5, Hep3B, HCCLM3, HCCLM6, MHCC97L and MHCC97H. L-02 and SMMC7721 both expressed relatively high levels of SBP1 compared with other HCC cell lines. GAPDH was used as a loading control. (B); Both Western blot and qRT-PCR results showed that SBP1 expression in SMMC7721 cells was down-regulated to a marginal level by siRNA treatment. Total protein and RNA were extracted from the cells 72 hours after transfection. (C); Transwell assays showed that the number of migrated cells in the SBP1-siRNA group increased significantly compared with those in the control group. Representative data of three independent experiments are shown. (D); Immunofluorescence showed the location of SBP1 in SMMC7721-Mock and SMMC7721-siRNA samples. (E); The wound were nearly closed 24 hours after scratch in the SBP1-siRNA group compared to the control group. All experiments were repeated at least 3 times.

Figure 2. Effects of down-regulation 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 while the SBP1-siRNA group maintained most of its proliferative ability (P<0.0001). (B); Cell apoptosis was analyzed by flow cytometry. FITC-labeled Annexin V and fluorescent dye 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 three times.

**Figure 3. Immunofluorescence images of SBP1 and GPX1 in SMMC7721-Mock cells**

(A); SMMC7721 cells were stained with anti-SBP1 monoclonal antibody (red) and anti-GPX1 polyclonal antibody (green). a–h: Cells were cultured without hydrogen peroxide treatment, and GPX1 localized exclusively in the cytoplasm (b, f) while SBP1 could be found both in the cytoplasm and the nucleus (c, g). i–p: Cells were treated with hydrogen peroxide (50 μmol/L) for 12 hours before fixation. Images showed that both GPX1 and SBP1 had established specific nuclear bodies (j, k, n, o), and most of the GPX1 nuclear bodies were co-localized with SBP1 bodies (l, p). (B); Two high magnification images are presented here to show the details of the co-localization of SBP1 and GPX1 nuclear bodies. Some had already merged together (indicated by arrowheads) while others were still in the process of co-localization (indicated by arrows). We performed the experiment at least three times and obtained similar results.

**Figure 4. Images of SBP1 and GPX1 in SMMC7721-siRNA-SBP1 cells**

SMMC7721-siRNA-SBP1 cells were stained with anti-SBP1 monoclonal antibody (red) and anti-GPX1 polyclonal antibody (green). a–h: Cells were cultured under normal conditions. i–p: Cells were treated with hydrogen peroxide (50 μmol/L) for 12
hours before fixation. SBP1 expression was down-regulated to a minimal level by
siRNA treatment (c, g, k, o) while GPX1 expression was unaffected (b, f, j, n).
Similarly, GPX1 nuclear bodies were formed under hydrogen peroxide treatment (j, n)
but were not colocalized with SBP1 bodies ( l, p). We performed the experiments at
least three times and obtained similar results.

Figure 5. Relationships among clinical tumor characteristics, SBP1 and GPX1
Nineteen samples surgically removed from HCC patients 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 to N9, V1
to V10) based on their tumor characteristics (Supplemental Table 1). (A); The
vascular invasive group (VI group) had consistently low expressions of SBP1 while
the non-vascular 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
while the NVI group also exhibited a varied and lower activity of GPX1. Samples
with high expression of SBP1 had limited GPX1 activity (for example, N1, N7 and
N8) while samples with lower expression of SBP1 had relatively high GPX1 activity
(for example, N4, N9, V2 and V8). (C); The possible relationship among SBP1,
GPX1, HIF-1α and ROS.

Figure 6. SBP1 Expression in Tumor Tissue and Prognosis
Three hundred and twenty-three HCC samples surgically removed between 2003 and
2004 were included here. (A); Representative photomicrographs of tumor tissues
showing the different staining patterns are presented here and are graded from 0 to
++. (B); Kaplan–Meier survival curves showed that patients with negative
expression of SBP1 in tumor cells experienced shorter overall survival periods ($P <
0.001$) and higher recurrence rates ($P < 0.001$) than patients with positive expression
of SBP1. (C); Kaplan–Meier survival curves revealed that SBP1 was not a significant
prognostic factor for overall survival in HCC within Milan criteria ($P > 0.05$) but was significant for the recurrence in these patients ($P = 0.039$). (D); In the sub-population of HCC patients beyond Milan criteria, SBP1 was a significant prognostic factor for overall survival ($P < 0.001$) and recurrence ($P < 0.001$).
Figure 1
Figure 3

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Figure 4

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SMMC7721-siRNA-SBP1

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SBP1

Merge

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Figure 5

A

Non-vascular Invasion

Vascular Invasion

SBP1

GAPDH

B

C

GPX1 Activity

SBP1 Inhibit Scavenge Regulate

ROS

HIF-1α

Inhibit Scavenge Activate

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Clin Cancer Res

Decreased Selenium-binding Protein 1 Enhances Glutathione Peroxidase 1 Activity and Down-regulates HIF-1α to Promote Hepatocellular Carcinoma Invasiveness

Cheng Huang, Guangyu Ding, Chengyu Gu, et al.

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