HBP21, a chaperone of heat shock protein 70, functions as a tumor suppressor in hepatocellular carcinoma

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Abstract

Inactivation of tumor suppressor genes, caused by genetic and epigenetic alterations, is one of the key issues in the development and progression of cancer. To identify and characterize cancer related genes in hepatocellular carcinoma (HCC) pathogenesis, transcriptome sequencing has been applied to compare expression profiles between tumor and non-tumor tissues. Among the down-regulated genes, heat shock binding protein 21 (HBP21) was selected for further study. In this study, down-regulation of HBP21 was frequently detected in primary HCCs (87/120, 72.5%), which was significantly associated with advanced clinical stage (P = 0.049), poor differentiation (P = 0.018) and poor prognosis (P = 0.026). Further study found that down-regulation of HBP21 in HCC was mainly caused by allele loss and promoter methylation. Functional study found that HBP21 could inhibit tumor cell growth rate, foci formation and colony formation in soft agar, and tumor formation in nude mice when it was transfected into HCC cells. Molecular study found that HBP21 could promote cell apoptosis, especially under adverse conditions such as heat and chemotherapeutic agent treatment. As a chaperone of heat shock protein 70 (HSP70), HBP21 could inhibit interaction between HSP70 and Bax, increased Bax protein translocation from cytoplasm to mitochondria, and subsequently increased the release of cytochrome c into cytoplasm, and finally induced apoptosis. Clinically, HBP21 could be used as a prognostic biomarker for HCC outcome prediction and might be also as a novel therapeutic agent in HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and ranks as the third-leading cause of cancer-related deaths worldwide (1). It generally believed that the incidence of HCC is associated with chronic hepatitis B and C viral infection, alcohol addiction or dietary exposure to aflatoxin (2). Like many other cancers, the hepatocellular phenotypes progressively evolves into dysplastic hepatocytes, and finally into HCC with accumulation of genetic and epigenetic alterations (3). Despite under intensive investigation, the detailed underlying molecular mechanisms involved in the pathogenesis of HCC remains to be elucidated. With advanced in next-generation sequencing technologies recently, transcriptome sequencing has been used to delineate changes of genes at the messenger RNA (mRNA) levels. As is reported, many oncogenes and tumor suppressor genes (TSGs) were up-regulated or down-regulated in tumor tissues compared with their matched non-tumorous tissues (4). Thus, studying the changes at the mRNA levels of genes in HCCs compared with adjacent non-tumor tissues favors the identification of up-regulated or down-regulated genes and corresponding pathways involved in the development of HCC. In previous study, we conducted an integrative transcriptome sequencing analysis (Genbank accession no. GSE33294) on three pairs of tumor and corresponding non-tumor tissues from HCC cases to identify differentially expressed genes (5). Heat shock binding protein
21 (HBP21), encoding a tetratricopeptide repeat (TPR) containing protein (6), is one of the significantly down-regulated genes. TPR proteins can act as interaction scaffolds in the formation of protein–protein complexes involved in numerous cellular functions such as transcription, protein translocation and degradation (7). Previous studies have shown that HBP21 can interact with heat shock protein 70 (HSP70) which is up-regulated to resist to various adverse conditions and inhibit apoptosis in cancer cells (8). The role of HBP21 in HCC has not been explored yet so far. In the present study, the tumor suppressive roles of HBP21 in HCC have been investigated and discussed.

RNA extraction and quantitative real-time PCR
Total RNA was isolated using TRIzol reagent (Life Technologies), and reverse transcription was performed using a reverse-transcription PCR kit (Roche, Basel, Switzerland) according to manufacturer’s instruction. Quantitative real-time PCR was carried out using SYBR Green PCR master mix (Roche) on an ABI Prism 7900HT System (Applied Biosystems, Carlsbad, CA). Sequences of primers used are listed in Supplementary Table S1, available at Carcinogenesis Online.

Bisulfite treatment and methylation analysis
Genomic DNA was isolated from tissues and cell lines by phenol-chloroform method. DNA samples (1 µg) were bisulfite modified using the EpiTECT Bisulfite Kit (Qiagen, Hilden, Germany). Methylation-specific PCR and bisulfite genomic sequencing were carried out as described using primers listed in Supplementary Table S1, available at Carcinogenesis Online (10).

Loss of heterozygosity detection with SNPs
Loss of heterozygosity (LOH) study was performed in 72 primary HCCs with three SNPs: rs3741323 (T/G = 0.465/0.535 in Chinese), rs45474901 (T/C = 0.480/0.520) and rs573971 (A/G = 0.580/0.420). The PCR products were sequenced (primer sequences are listed in Supplementary Table S1, available at Carcinogenesis Online) to detect LOH in tumor tissues, compared with the corresponding non-tumor tissues.

Functional assays for tumorigenicity
In vitro tumorigenicity was assessed by XTT cell proliferation assay (Roche Diagnostics, Indianapolis, IN), foci formation assay and colony formation in soft agar as described previously (11). For XTT cell proliferation and foci formation assays, cells were seeded at a density of 1000 per well. For soft agar assay, cells were seeded at a density of 3000 (PLC8024) and 10000 (Huh7) per well. The results are analyzed as mean ±SD of three independent experiments. For in vivo tumor formation experiments, the cell number used for injection was 5 x 10⁶ (empty vector- and HBP21-transfected cells), 8 x 10⁶ (shCtl- and shHBP21-transfected cells). Tumor volume was measured weekly over a 4–8 week period (Formula: Volume = 0.5 x Length x Width²). All experiments conducted on animals were approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR).

Heat or cisplatin treatment
Approximately 2 x 10⁶ PLC8024 cells and 5 x 10⁶ Huh7 cells were seeded into each well of the six-well plate with 2ml of Dulbecco’s modified Eagle medium with 10% fetal bovine serum. After 24h of incubation, they were exposed to heat stress or cisplatin. Heat treatment was performed by sealing the tops of culture plates with parafilm, submerging the culture plates in a water bath set at 45°C for 3h. PLC8024 and Huh7 were incubated at 37°C for 1h with 50 µM Z-VAD(OMe)-FMK (Abcam, Austin, TX). After this incubation, 16 or 10 µg/ml of cisplatin was added to PLC8024 or Huh7 which were incubated for further 24h. Chemotherapy-induced cytotoxicity was determined by XTT Cell Proliferation Assay (Roche Diagnostics) according to the manufacturer’s instructions. The relative number of viable cells as compared with the number of cells without cisplatin treatment was expressed as percentage cell viability using the following formula: cell viability (%) = A₅₇₀ of treated cells/A₅₇₀ of untreated cells. Two independent experiments were performed with three repeats.

Detection of apoptosis by flow cytometry
After heat or cisplatin treatment, cells were harvested and double stained with phycoerythrin (PE)-conjugated Annexin-V and 7-aminomethylcoumarin D (7-AAD) provided by the BD apoptosis detection kit (BD Biosciences, San Jose, CA). Cells were run on a FACS Calibur (BD Biosciences) and analyzed using BD Cytomics software. Apoptotic cells were detected by forward scatter, side scatter, PE Annexin V and 7-AAD.
Annexin-V positive cells were counted. Data were analyzed by cellQuest Pro software (BD Biosciences). All the results are expressed as mean ± SD of three independent repeats.

### Co-immunoprecipitation and western blotting analysis

For co-immunoprecipitation assay, 5 μg of total cell lysate was immunoprecipitated with 2 μg of HSP70 or mouse isotype antibodies at 4°C for 2 h. Immunocomplexes were then precipitated using 50 μl of protein G-agarose, which was provided in the immunoprecipitation kit (Roche Diagnostics). Extensive washing and immunocomplexes denaturation steps were carried out according to the manufacturer's instruction. Denatured immunocomplexes were analyzed by western blotting. About 5% of the whole lysate (input) was used as a positive control. Western blotting analysis was performed with the standard protocol. Antibodies used in this study are as follows: HBP21, HSP70 and mouse isotype IgG (Abcam, Austin, TX), poly (ADP ribose) polymerase, cytochrome c (cyto-c), caspase-3 and-9, Bax, COX IV and β-actin (Cell Signaling Technology, Danvers, MA). Subcellular fractionation was performed as described previously (12).

### Statistical analysis

Statistical analysis was conducted using SPSS version 17.0 (Chicago, IL). Pearson’s chi-square test was used for categorical variables and independent Student’s t-test for continuous data. The mRNA level of HBP21 in paired HCC and non-tumor tissues was compared with a paired Student’s t-test. The Pearson chi-square test was used to analyze the association of HBP21 expression with clinicopathologic parameters. Kaplan-Meier plots and log-rank tests were used for survival analysis. Fisher’s exact test was used to analyze the association of LOH, aberrant methylation and the expression of HBP21. A P value less than 0.05 was considered statistically significantly.

### Results

#### Down-regulation of HBP21 in HCC

qRT-PCR was applied to compare the HBP21 expression levels between HCC and their paired non-tumor tissues in 120 HCC samples. The results showed that HBP21 was significantly downregulated in tumor tissues compared with adjacent non-tumor tissues (P < 0.0001, paired Student’s t-test; Figure 1A). Compared with their paired non-tumor tissues, down-regulation of HBP21 was detected in 87/120 (72.5%) of HCC tissues. Expression of HBP21 in seven HCC cell lines and two immortalized liver cell lines was tested by RT-PCR and absent HBP21 expression was detected in all tested cell lines (Figure 1B). The down-regulation of HBP21 at the protein level was determined by immunohistochemical analysis in 10 HCCs and the results showed that down-regulation of HBP21 was observed in 9 HCC tissues including six cases without detectable expression (Figure 1C). A clinopathologic association study in 120 HCCs showed that the down-regulation of HBP21 was significantly associated with advanced clinical stage (P = 0.049, Pearson chi-square test, Table 1) and poor differentiation (P = 0.018; Pearson chi-square test, Table 1). Intriguingly, in a Kaplan–Meier survival analysis comparing patients with different HBP21 expression, lower HBP21 expression was significantly associated with shorter overall survival (log rank, 4.974; P = 0.026, Figure 1D).

#### Down-regulation of HBP21 is associated with allele loss and promoter methylation

As LOH is a good tool to detect allele loss in cancer, three SNP sites located at the upstream or downstream of the HBP21 gene were applied to test the LOH status of the gene in 72 HCCs. Heterozygosity of either SNP site was detected in 32 HCCs (informative cases) including 25 cases with HBP21 down-regulation and 7 HCCs without HBP21 down-regulation. LOH was detected in 12/25 (48%) of cases with HBP21 down-regulation, but not in 7 cases without HBP21 down-regulation, suggesting that the down-regulation of HBP21 in HCC was significantly correlated with LOH (P = 0.025, Figure 1E, Supplementary Table S2, available at Carcinogenesis Online).

Down-regulation of tumor suppressors in cancers is also often associated with aberrant methylation and histone deacetylation in their promoter regions. To determine whether the down-regulation of HBP21 was associated with aberrant methylation or histone deacetylation, two HCC cell lines PLC8024 and HuH7 were treated with 5-aza-dC, a DNA methyltransferase inhibitor or trichostatin A, a histone acetylation agent to investigate the effects of DNA methylation and histone acetylation on HBP21 expression. As shown in Figure 1F, qRT-PCR analysis showed that HBP21 expression was increased with 200 μM 5-aza-dC, however, HBP21 expression was not significantly affected by trichostatin A treatment, implying that aberrant promoter methylation, not histone modification, is responsible for HBP21 down-regulation in HCC. One CpG-island was then predicted by a publicly available database CpG Island Searcher (http://www.cgislands.com) at the promoter region (−2064 nt to −1590 nt) of the HBP21 gene (Supplementary Figure 1A, available at Carcinogenesis Online). Bisulfite genomic sequencing was conducted to investigate the methylation status of the CpG-island in two HCC cell lines (PLC8024 and HuH7), one HCC clinical sample with HBP21 down-regulation, and PLC8024 treated with 5-aza-dC. The results showed that a high density of methylation was detected in two HBP21-absent cell lines and one HCC clinical sample, whereas lower density of methylation was observed in the HBP21-expression non-tumor tissue and PLC8024 treated with 5-aza-dC.

To further confirm whether the methylation status of GC sites is correlated with HBP21 down-regulation, methylation-specific PCR using methylation- and unmethylation-specific primers was performed in 32 paired HCC and non-tumor samples. Interestingly, methylated PCR product of HBP21 was detected in all tumor and their paired non-tumor specimens. Unmethylation product was detected in all non-tumor specimens, but not in 15/25 (60%) of cases with HBP21 down-regulation and 1/7 of cases with HBP21 expression, respectively. Statistical analysis showed that HBP21 down-regulation in HCC was significantly correlated with its promoter methylation. Taken together, our data showed that down-regulation of HBP21 was significantly associated with allele loss (12/25) and promoter methylation (15/25) of HBP21 (P = 0.038, Fisher’s exact test, Figure 1G). These findings indicate that both allele loss and promoter methylation are responsible to the down-regulation of HBP21 in HCC.

### HBP21 has strong tumor suppressive ability

To determine the tumor suppressive effects of HBP21 on HCC pathogenesis, HBP21 was cloned into a lentivectors and stably transduced into two HCC cell lines, PLC8024 and HuH7. Empty vector was used as a control. mRNA and protein expressions of HBP21 in these transfectants were evaluated by RT-PCR and western blotting analysis (Figure 2A). The tumor-suppressive ability of HBP21 was assessed by cell proliferation, foci formation, colony formation in soft agar and tumor formation in nude mice. XTT assay showed that the cell growth rates in HBP21-transfectants were significantly slower compared with control cells (P < 0.01, Student’s t-test, Figure 2B). In addition, HBP21 could significantly inhibit the abilities of foci formation (P < 0.01, Student’s t-test, Figure 2C) and colony formation in soft agar (P < 0.01, Student’s t-test, Figure 2D). To further assess the in vivo tumor-suppressive
Figure 1. Down-regulation of HBP21 in HCC and the down-regulation is associated with LOH and promoter methylation. (A) Relative expression level of HBP21 was detected by qRT-PCR in 120 HCCs. The results are expressed as mean ± SD. (B) Expressions of HBP21 in seven HCC cell lines and two immortalized liver cell lines were detected by RT-PCR. Normal control is pooled non-tumor liver tissues (five specimens). 18S was used as loading control. (C) Representatives of immunohistochemical images showing that expression of HBP21 was only detected in non-tumor tissues but not in HCC tumor tissues. Original magnification, 100×. (D) Kaplan–Meier overall survival curve of HCC patients correlated with HBP21 expression. HBP21(+), patients with normal expression of HBP21; HBP21(−), patients with down-regulation of HBP21. (E) Representative of LOH at SNP sites rs3741323, rs45474901, rs573971 in tumor tissues (e.g. 278T) with HBP21 down-regulation compared with paired non-tumor tissues (e.g. 278N). (F) Detection of HBP21 expression in PLC8024 and Huh7 cells by qRT-PCR after demethylation (5-Aza-dC) and histone acetylation (trichostatin A) treatment. (G) Association study indicates that the down-regulation of HBP21 was significantly associated with the LOH and aberrant methylation of the HBP21 gene (P < 0.05, Fisher’s exact test).
Table 1. Association of HBP21 downregulation with clinicopathologic features in 120 primary HCCs

<table>
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<th>Total</th>
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<th>P value</th>
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</tr>
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<td>16 (76.2%)</td>
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<td>21</td>
<td>5 (23.8%)</td>
<td>16 (76.2%)</td>
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<tr>
<td>Positive</td>
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<td>25 (27.5%)</td>
<td>66 (72.5%)</td>
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<td>Serumα-fetoprotein level, ng/ml</td>
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<td>&lt;400</td>
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<td>22 (34.4%)</td>
<td>42 (65.6%)</td>
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<td>8 (17.0%)</td>
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<td>37 (74.0%)</td>
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<td>18 (28.1%)</td>
<td>46 (71.9%)</td>
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<td>24 (70.6%)</td>
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<td>20 (66.7%)</td>
<td>58 (70.7%)</td>
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<td>30 (29.4%)</td>
<td>72 (70.6%)</td>
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<tr>
<td>Capsule</td>
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<td>14 (29.8%)</td>
<td>33 (70.2%)</td>
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<td>24 (36.9%)</td>
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<td>38 (84.4%)</td>
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<td>17 (27.4%)</td>
<td>45 (72.6%)</td>
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<tr>
<td>Present</td>
<td>55</td>
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<td>39 (70.9%)</td>
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<td>I</td>
<td>74</td>
<td>22 (29.7%)</td>
<td>52 (70.3%)</td>
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<td>2 (9.1%)</td>
<td>20 (90.9%)</td>
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Statistical significance (P < 0.05) is shown in bold.

*Partial data are not available, and the statistic was based on available data.

ability of HBP21, HBP21-8024 or HBP21-Huh7 cells were subcutaneously injected into the right dorsal flank of nude mice, respectively. Empty vector-transfected cells were injected into the left dorsal flank of the tested nude mice as controls. Although tumor formation was observed in all five mice injected with HBP21-8024 and Vec-8024 cells, the average volume of tumors induced by HBP21-8024 cells was significantly smaller than that induced by Vec-8024 cells (P < 0.05, Student’s t-test, Figure 2E). For Huh7 cells, tumor formation was observed in 2/5 and 5/5 mice injected with HBP21-Huh7 and Vec-Huh7 cells, respectively. The volume of tumors induced by HBP21-Huh7 was significantly smaller than that induced by Vec-Huh7 cells (P < 0.01, Student’s t-test).

**Knockdown of HBP21 abolishes its tumor suppressive effect**

To further confirm the tumor suppressive effect of HBP21, two shRNA (shHBP21-2 and shHBP21-3) specifically targeting HBP21 and shCtl were transfected into HBP21-8024 and HBP21-Huh7 cells. The silencing effect was detected by western blotting analysis, and the result showed that both shRNAs could effectively decrease HBP21 expression (Figure 3A). Functional assays showed that knockdown of HBP21 could significantly increase the frequency of foci formation (P < 0.01, Student’s t-test, Figure 3B) and colony formation in soft agar (P < 0.01, Student’s t-test, Figure 3C). For in vivo study, shHBP21 or shCtl were stably transfected into HBP21-8024 and HBP21-Huh7 cells, respectively. shCtl- and shHBP21-transfected cells were subcutaneously injected into the left and right dorsal flank of nude mice, respectively. For HBP21-8024 cells, tumor formation was observed in 5/5 and 4/5 mice induced by shHBP21- and shCtl-transfected cells, respectively. The average volume of tumor induced by shHBP21-transfected cells was obviously larger than that induced by shCtl-transfected cells (Figure 3D). For HBP21-Huh7 cells, tumor formation was observed in 2/3 and 0/3 mice injected with shHBP21- and shCtl-transfected cells, respectively.

**HBP21 promote apoptosis induced by cisplatin and heat treatment**

As HBP21 is the cochaperone of HSP70 and the chemoresistant effect of HSP70 is via blocking the cytoplasm-to-mitochondria translocation of Bax (13), we studied the effect of HBP21 on apoptosis induced by chemotherapeutic agent cisplatin. HBP21- and vector-transfected cells were exposed to different concentrations of cisplatin (0, 2, 4, 6, 8 and 10 μg/ml) for 72h. The XTT assay showed that the viabilities of HBP21-transfected cells were decreased significantly compared with vector-transfected cells after cisplatin treatment (Figure 4A). Next, we studied whether the decreased viability in HBP21-transfected cells was attributed to higher rate of apoptosis. After 24 h treatment with cisplatin, the apoptotic index was evaluated by flow cytometry. As seen...
in Figure 4B, the apoptotic index of HBP21-transfected cells was increased dramatically compared with vector-transfected cells. Furthermore, the decreased apoptotic index was observed in HBP21 knockdown cells (shHBP21-8024). Consistently, the typical molecular indicators of apoptosis, cleaved caspase-3, caspase-9 and poly (ADP ribose) polymerase were dramatically increased in HBP21-transfected cells compared with vector-transfected cells (Figure 4C). Moreover, the response of HBP21 to the heat shock stress was also explored. HBP21- and vector-transfected cells were incubated either at 37°C or 42°C for 3h. The heat shock effect was tested by western blotting at 6h and flow cytometry at 24h after treatment. As shown in Supplementary Figure S2, available at Carcinogenesis Online, the apoptotic index was significantly increased in HBP21-transfected cells (P < 0.05, Student’s t-test). Also, increased cleaved caspase-3, caspase-9 and poly (ADP ribose) polymerase were observed in HBP21-transfected cells compared with vector-transfected cells after heat treatment.

HBP21 promotes cytoplasm-to-mitochondria translocation of Bax

We next investigated whether HBP21 could promote the cytoplasm-to-mitochondria translocation of Bax. After cisplatin treatment, mitochondrial and cytosolic fractions from HBP21- and vector-transfected cells were prepared. Bax and cytochrome c
were determined by western blotting. As shown in Figure 5A, cisplatin treatment increased the Bax in mitochondria fraction and the release of cytochrome c. The interaction of HBP21 with HSP70 has been demonstrated by yeast two-hybrid screening and glutathione-S-transferase pull-down assays (6). In the present study, cell lysates from HBP21- and vector-transfected cells were immunoprecipitated with HSP70 antibody, and western blotting was used to detect the amount of Bax protein in HSP70 immunoprecipitates. The result showed that Bax could be specifically co-immunoprecipitated by HSP70 antibody, however, no difference in the amount of immunoprecipitated Bax protein was observed between HBP21- and vector-transfected cells (Figure 5B, Supplementary Figure S3, available at Carcinogenesis Online). Interestingly, with cisplatin treatment, the amount of Bax protein in HSP70 immunoprecipitates was decreased in HBP21-transfected cells compared with vector-transfected cells (Figure 5C, Supplementary Figure S3, available at Carcinogenesis Online). These data suggested that HBP21 might inhibit the interaction of HSP70 with Bax, and therefore promote the cytoplasm-to-mitochondria translocation of Bax, subsequently induce apoptosis by releasing cytochrome c and activating caspase-9 and caspase-3.

Discussion

Recently, the development of RNA sequencing technology has provided a novel approach for mapping and delineating transcriptomes (14). In our previous work, an integrative transcriptome sequencing analysis had been conducted on three patient-derived HCC and their adjacent non-tumor tissues to identify differentially expressed genes (5). Among the down-regulated genes, HBP21 has been investigated as a candidate TSG. In this study, down-regulation of HBP21 was detected in 87/120 (72.5%) of primary HCCs, which was significantly associated with advanced clinical stage (P = 0.049), poor differentiation (P = 0.018) and poorer prognosis (P = 0.026), suggesting that HBP21 might play important suppressive roles in HCC development.

TSGs are widely considered to be ‘recessive’. The inactivation of a TSG can be caused by both genetic (e.g. deletion and mutation) and epigenetic (e.g. methylation and micro RNA) alterations (10,15,16). In the present study, we demonstrated that down-regulation of HBP21 was correlated with allele loss and promoter hypermethylation. LOH was detected in almost half of informative (12/25) HCCs with HBP21 down-regulation. In the same cohort of samples, promoter hypermethylation was detected in 15/25
Both LOH and promoter methylation were observed in 8/25 cases. Interestingly, absent expression of HBP21 was detected in all tested HCC cell lines and immunohistochemical data also showed that absent expression of HBP21 was observed in 6/10 HCCs. Full-length of HBP21 in 10 HCCs was analyzed by sequencing and no mutation was identified (data not shown).

The tumor-suppressive role of HBP21 in HCC development was characterized by both in vitro and in vivo functional assays. Our Figure 4. HBP21 promotes the cisplatin-induced apoptosis. (A) Cell viabilities of HBP21 and vector-transfected cells were detected by XTT assay after treatment with cisplatin at indicated concentration for 48h. Data represent the mean ± SD of three independent experiments with three repeats. *P < 0.05, **P < 0.01, independent Student's t-test. (B) Apoptotic indexes of vector-transfected, HBP21-transfected and shHBP21-transfected cells were detected by fluorescence-activated cell sorting-based Annexin V/AAD double staining after treatment with cisplatin at indicated concentration for 24h. Results represent the mean ± SD of three independent experiments with three repeats. *P < 0.05, **P < 0.01, independent Student's t-test. (C) The activation of caspase-9, caspase-3, poly (ADP ribose) polymerase were compared between vector- and HBP21-transfected cells in PLC8024 (left panel) or Huh7 cells (right panel) by western blot analysis. Cells were incubated with control (left), 10 or 16 µg/ml cisplatin for 24h (middle), and 50 µM Z-VAD(OMe)-FMK for 1h before cisplatin treatment (right). β-actin was used as a loading control.
results demonstrated that HBP21 was able to inhibit cell proliferation, abilities of foci formation and colony formation in soft agar, as well as tumor formation in nude mice. These tumor suppressive abilities could be effectively inhibited when HBP21 expression was silenced by shRNA. Molecular study found that the tumor suppressive effect of HBP21 was mainly through the promotion of cell apoptosis. HBP21 is first discovered as a chaperone of HSP70 (6), which is a cytoprotective factor to protect cells from apoptosis induced by heat shock, serum starvation or chemotherapeutic agents (8). Expression profiling in multistage HCC carcinogenesis identified HSP70 as an early molecular marker for HCC development (17). The roles of TPR proteins in HSP70 chaperone functions have been well studied (7,18). Among TPR chaperones, Hip has been identified as anti-apoptotic protein in leukemia cells (19), on the contrary, DNAJC25 has been reported as a TSG in HCC with proapoptotic property (20). In this study, HBP21 has been demonstrated as another HSP70 chaperone with proapoptotic ability.

To further explore the proapoptotic mechanism of HBP21, the interaction of HBP21 with HSP70 as well as its effect on anti-apoptotic ability of HSP70 was investigated. A recent study shows that HSP70 confers resistance to apoptosis by blocking Bax translocation to mitochondria (13), which is required for cytochrome c release and cisplatin-induced apoptosis. Here we found that HBP21 was able to bind HSP70 and inhibit its interaction with Bax, and subsequently promote the cytoplasm-to-mitochondria translocation of Bax. This effect was more obvious when cells under the stress, such as heat and cisplatin treatment. Taken together, our study indicates that down-regulation of HBP21, caused by allele loss and aberrant methylation, is a common event in HCC. HBP21 can promote cell apoptosis induced by adverse conditions and play important roles in HCC pathogenesis. A better understanding of molecular mechanism of HBP21 in promoting tumor cell apoptosis may provide novel therapeutic strategies in HCC treatments.

Figure 5. HBP21 promotes cytoplasm-to-mitochondria translocation of Bax by disturbing the interaction of HSP70 with Bax. (A) The levels of Bax in the mitochondria fraction and cytochrome c in the cytosolic fraction were compared by western blotting between HBP21- and vector-transfected cells after treatment with/without cisplatin for 24 h. (B) The interaction of HSP70 with Bax was determined by co-immunoprecipitation with anti-HSP70 antibody (IP: HSP70) or IgG (IP: IgG) in vector- and HBP21-transfected cells. Total cell lysate (Input) was used as a positive control. (C) The interaction of HSP70 with Bax was detected by Co-IP in vector-, HBP21- cells and shHBP21-transfected cells after 10 or 16 μg/ml cisplatin treatment for 24 h.
Supplementary material

Supplementary Tables S1 and S2 and Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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