BRIT1 Inhibits Invasion and Metastasis by Regulating SERPINA5 in Hepatocellular Carcinoma

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Research article

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Abstract

Purpose: BRIT1 (also known as MCPH1) is a DNA repair and tumor suppressor gene, whose alterations might be potentially associated with tumor progression and metastasis in HCC. SERPINA5, also known as Protein C inhibitor, has been reported to be decreased in a variety of human cancers and its decrease is associated with cellular invasion and tumor metastasis. However, little is known about the underlying mechanism of SERPINA5 downregulation and whether BRIT1 deficiency contributed to regulation control of SEPINA5 and invasion and metastasis in HCC.

Methods: The expression of BRIT1 and SERPINs in HCC cells or human tumor tissue microarrays were determined by using quantitative real-time PCR, Western blot analysis, or immunohistochemistry. Migratory and invasive capacity of HCC cells were assessed by using *in vitro* wound-healing and Transwell-based assays or *in vivo* mouse xenograft tumor model. Dual luciferase reporter assay was performed to measure promoter activity of the SERPINA5 gene. HCC datasets analyzed in this study was performed by using Oncomine and cBioPortal Cancer Genomics with default parameters.

Results: In this study we showed that BRIT1 protein was low expressed in HCC cells and tumor tissues. BRIT1 deficiency increased the capacity of migration, invasion and metastasis of HCC *in vitro* and *in vivo*; its expression level was positively correlated with that of SERPINA5 in HCC cells and tissue microarray. With the transcription factor E2F1, BRIT1 can upregulated the promoter activity of the SERPINA5 gene in HCC.

Conclusion: These findings suggest that BRIT1 deficiency might induce tumor invasion and metastasis of HCC through regulatory control of SERPINA5 with the help of E2F1, and provide convincing evidence that BRIT1/SERPINA5 might be a novel biomarker for HCC metastasis.

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers and the leading cause of cancer death worldwide, especially in China and the Southeast Asia [1–4]. HCC patients are usually diagnosed in high-grade tumors or at advanced stages, and most patients have died of intrahepatic or distant metastasis [5, 6]. Thereby, the identification and characterization of genes involved in the metastasis can provide insights into the pathogenesis of tumor progression and may lead to novel therapy for HCC. Recently, several studies provided the evidence showing the intrinsic contribution of genomic instability to metastasis [7, 8], indicated the genes for genomic instability may play a role in metastasis.

BRIT1 (also known as MCPH1) was previously identified as a transcriptional repressor of human telomerase reverse transcriptase [9], and its coding gene is also responsible for type 1 microcephaly [10, 11]. BRIT1has been demonstrated to function as a key player in DNA damage response (DDR) at multiple levels, e.g. regulating cell cycle checkpoint by controlling the expression of BRCA1 and CHK1 [12, 13], acting as an early DDR mediator by co-localizing with γ-H2AX, MDC1, 53BP1, NBS1, p-ATM, etc [14–17], modifying chromatin structure by interaction with the components of SWI/SNF complex [18], and

repairing double-strand DNA breaks through homologous recombination (HR) and non-homologous end joining (NHEJ) [19–24]. BRIT1 has also been shown to interact with the transcription factor E2F1, leading to alterations in the expression of BRCA1, RAD51, CHK1, p73, and caspases, etc [25]. Its mutations, deletions, and/or aberrant expressions are related to microcephaly [10, 11, 26–32], premature chromosomal condensation [23, 29, 33–36], and genomic instability [17, 20, 22–24, 37]. BRIT1's functions in DNA repair and checkpoint control make it a potential tumor suppressor gene [17, 21, 38, 39]. Indeed, our studies based on BRIT1 knockout mouse model have demonstrated that it genuinely acts as a tumor suppressor by maintaining genomic integrity [22, 24], and its deficiency enhances the incidence of tumor formation in p53 null mice [24]. Moreover, the deletions of BRIT1 loci (8p23) are associated with tumor progression and metastasis in a wide range of cancers including HCC [40–44]. Thus, potentially, BRIT1's aberrations might be associated with the metastasis of liver cancer.

SERPINA5, also called protein C inhibitor (PCI) or plasminogen activator inhibitor type 3, (PAI3), belongs to the serine protease inhibitor superfamily which is multifunctionally involved in thrombosis, inflammation, hemostasis, and metastasis [45–49]. In human, SERPINA5 is mainly expressed in the liver as well as several other organs including kidneys and reproductive tissues [50–52]. SERPINA5 has been demonstrated to be decreased in a variety of human cancer [53–57], and its decrease is associated with cellular invasion and tumor metastasis [54, 55, 57]. Particularly, in HCC, SERPINA5 was reported to be downregulated in 64% (37/58) of tumor specimens, and its low level was correlated with intrahepatic metastasis [58]. However, the molecular mechanism underlying the downregulation of SERPINA5 in HCC is not yet fully investigated.

In this study, we aimed to investigate whether and how BRIT1 acts as a metastatic modulator in HCC. Our study demonstrated that BRIT1 was pathologically decreased in HCC specimens where BRIT1 low expression negatively correlated with tumor grade and vascular invasion, accompanying with SERPINA5 significantly downregulated in high-grade HCC patients. BRIT1 knockdown promoted migration and invasion, and ectopic expression of BRIT1 inhibited the metastatic ability of HCC cells *in vitro* and *in vivo*. Moreover, the potential of BRIT1 inhibiting metastasis was attributed to the downregulation of SERPINA5 by BRIT1. Together, our findings not only advanced the molecular understanding of HCC metastasis, but may also provide potential markers of BRIT1/SERPINA5 for metastatic HCC.

Methods

Cell lines, antibodies, and siRNAs

Human immortalized hepatocytes LO2 and HCC cells Hep3B, HepG2, Huh7, and SNU449 were purchased from the Cellcook Biotech (Guangzhou, Guangdong, China) or the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). The short tandem repeat (STR) typing data of these cell lines were provided with purchase. BRIT1-overexpressed cells Hep3B.BRIT1 were constructed by using pMSCVpuro-BRIT1 vector (Addgene #16205) (pMSCVpuro as empty control) as described previously [17]. BRIT1-specific siRNA transiently knockdown cells siSNU449 were used as a BRIT1-low control compared to parental

SNU449 cells. LO2, Hep3B, Hep3B.BRIT1, and HepG2 cells were cultured in DMEM, while SNU449 and siSNU449 cells were in RPMI 1640 (Hyclone, GE Healthcare Life Sciences, Utah, USA), both supplemented with 10% FBS (PAN-Biotech GmbH, Germany) and penicillin-streptomycin (Solarbio, Beijing, China), and maintained at 37˚C in an atmosphere of 5% CO_2. Primary antibodies used in this study were anti-BRIT1 (#4120, Cell Signaling Technology, UAS), anti-SERPINB2 (ab47742, Abcam, USA), anti-SERPINA5 (ab172060, Abcam, USA), anti-b-Actin (ab8226, Abcam, USA), and anti-GAPDH (E12-052, EnoGene, China). The confirmatory BRIT1-specific siRNAs were described previously [17] and listed below: AGGAAGUUGGAAGGAUCCAdTdT (sense), UGGAUCCUUCCAACUUCCUTT (antisense). The negative control siRNAs (Ctrl siRNA) were as follows: UUCUCCGAACGUGUCACGUTT (sense), ACGUGACACGUUCGGAGAATT (antisense). All siRNAs were synthesized by GenePharma (Shanghai, China), and each siRNA was resuspended in nuclease-free water and stock solutions were stored at -20°C until use.

siRNA transfection assay

BRIT1-proficient SNU449 cells were transiently transfected with either BRIT1 siRNA or Ctrl siRNA at final concentration of 30 pM by using Lipofectamine RNAi MAX (HB-TRLF-1000, Hanbio, China) according to the manufacturer's instructions. siRNA transfection was first performed in 24-well plates (Corning Costar, Cambridge, MA, USA). As instructed, 1.5 μl of siRNA duplex (30 pM) and 1 μl transfection reagent were individually mixed in 50 μl Opti-MEM medium, the siRNA-transfection reagent combined complex (100 μl) was added into 24-well plates and incubated with 500 μl of diluted cells in opti-MEM with 10% FBS ($1x10^5$ cells/ well) for 24 h at 37°C under 5% CO_2. For measuring siRNA knockdown efficiency, the siRNAs-treated cells were cultured for up to 72 h and harvested at the indicated time intervals. For examining cell proliferation, the siRNAs-treated cells after 24 h transfection were resuspended and seeded into 96-well plates, and then assessed by using Cell Counting Kit-8 (CCK8) at the indicated time points. For monitoring cell migration and invasion, the siRNA-transfected cells from 24-well plates were planted into Transwell cambers, and the other steps were done as described below.

Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed as described previously [59]. Briefly, total RNAs were prepared and reverse transcribed to cDNA by using First Strand cDNA Synthesis kits (Roche, USA). PCR reactions were performed using a Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The baseline cycles and threshold (CT) were autocalculated in PCR baseline-subtracted mode using the system's software. Gene expression levels were normalized against that of GAPDH and determined relative to controls using the $2^{-\Delta\Delta CT}$ method. The qRT-PCR primer sequences for the selected SERPIN genes and GAPDH were provided upon request.

Cell proliferation assay

Cell proliferation assay was performed by using a CCK8 kit (Transgene, Beijing, China). Briefly, cells (3×10^3 cells/well) were seeded into 96-well plates and incubated for 1, 2, 3, 4, and 5 days. At the indicated time point, 100 μl fresh medium plus10 μl CCK8 solution was added to each well pre-emptied. The plate was then incubated for additional 2 hours before measuring the absorbance at 450 nm wavelength using a microplate reader (M5e, MD Co. USA). The assays for each time point were performed in triplicate and the experiments were biologically repeated at least three times.

Tissue microarrays

HCC tissue microarrays (TMA) were purchased from Alenobio (cat# LV2084) (Xi'an, Shaanxi, China). This TMA contained 83 HCCs, 5 cholangiocellular carcinomas, 2 adenosquamous carcinomas, 1 each of mixed carcinoma, carcinoma sarcomatodes, squamous cell carcinoma, and papillary carcinoma, and 10 normal liver tissues. The TMA was generated in duplicate so as to compare the staining in different areas of the same sample. Tumor characteristics of these TMA samples were available including age, gender, tumor type, histological grade, tumor stage, and metastasis status (Supplementary Table S1).

Immunohistochemistry

Immunohistochemical analysis of BRIT1 and SERPINA5 was performed on the TMA slides with rabbit anti-BRIT1 antibody (E2A4831, EnoGene, China, 1:80) and rabbit anti-SERPINA5 antibody (ab172060, Abcam, USA, 1:500), respectively. Briefly, the TMA slides of 4 μm were deparaffinized in graded alcohols. Antigen retrieval was carried out by pressure cooking slides for 5 min at full pressure in 0.01 M citrate buffer (pH6.0). Endogenous peroxidase activity was inhibited by immersing the sections in 3% H_2O_2. The slides were then incubated with primary antibodies at 4ºC for overnight. Bound antibodies were detected with EliVision^{TM}plus (Bioz, Palo Alto, CA, USA) and visualized with diaminobenzine (Sigma). The slides were counterstained with Mayer's haematoxylin. Negative controls, without primary antibody and positive controls of normal tissue, were included in each batch of immunohistochemistry. The TMA sections were scanned by using high resolution digital images (Olympus BX51, Japan) at 20 x magnifications. The cytoplasmic and/or nuclear staining were quantitated as a H-score, i.e. a percentage of the positive cells in relation to the total number of tumor cells (0-100%) weighted with the staining intensity (0-3) [60].

Wound healing assay

Wound healing assay was used to determine the migratory capacity as described previously [61]. Cells (5×10^5 cells/well) were planted into the wells of 6-well plates and cultured 24 h until the culture was subconfluent. After that, a wound track was scored in each well, and the complete medium was replaced with 1% FBS-containing medium to reduce the influence of cell proliferation on cell migration to the least degree. Replicated wells were terminated and cell movements were examined under inverted microscopy at 0, 12, 24, 36, 48, 60, and 72 h after wounding. The experiments were duplicated for statistical analysis.

Transwell migration and invasion assays

Cell migration and invasion were also assayed by using Transwell chambers (8 μm pore, 353097, Corning, New York, USA) as described previously [62]. For migration assay, about 5×10^4 cells in 1% FBS-containing medium were seeded into the upper chambers. The lower chambers contained the medium with 10% FBS to stimulate cell migration. For invasion assay, the upper chambers were pre-coated with Matrigel (354480, Corning Costar, Cambridge, MA, USA) to mimic the basement membrane, and the other steps were the same as migration assay. At 12, 24, 36, and 48 h after incubation, the cells located on the lower surface of the chambers were fixed with cool methanol for 10 min, stained with 0.1% DAPI (Solarbio, Beijing, China) for 20 min, and counted using a fluorescent microscope (Olympus, Tokyo, Japan). The number of migratory or invasive cells was showed as the average of 5 random fields under the microscope at ×200 magnification. The experiments were repeated at least three times.

Western blotting analysis

Cultured cells were collected and lysed with a RIPA Buffer (Boster, Wuhan, China) with 0.1% Protease Inhibitors Cocktail (B14001, Biotool, USA). Protein concentrations were measured by using conventional BCA assay kit (PC0020, Solarbio, China). Around 30 μg of total lysates were subjected to electrophoresis on 8%-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and then wet-transferred onto a Polyvinyl difluoride (PVDF) membrane (Immobilon-P, 0.45 μm, Merck Millipore, USA) at 100 V for 1.5 h. The membranes were blocked with 5% non-fat dry milk in TBS-T for 1 h at room temperature, and then incubated with the indicated primary antibodies (anti-BRIT1, #4120, Cell Signaling Technology, UAS, SERPINA5, ab172060, Abcam, USA, anti-b-Actin, ab8226, Abcam, USA, GAPDH, E12-052, EnoGene, China) at 4°C for overnight. After that, the membranes were extensively washed with TBS-T, and incubated with secondary antibodies conjugated with goat anti-mouse IgG H&L (ab6789, Abcam, USA) or goat anti-rabbit IgG H&L (ab6721, Abcam, USA) for 1 h at room temperature. Following an additional wash with TBS-T, chemiluminescent reaction was recorded using ECL prime western blot detection kit (WBKLS0100, Millipore, USA), as per manufacturer's guidelines. The signals were captured, and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad).

Promoter activity analysis

SERPINA5 promoter region spanning -1445 nt to +49 nt (+1 representing the first nucleotide of AUG in coding sequencing, the position 94581369 in the human chromosome 14 reference GRch38.p12 primary assembly (NC_000014.9)) was constructed as original template (promoter region P1), and the subcloned promoter regions including P2 (-1102nt to -31nt), P3 (-822 nt to +49nt), P4 (-563 nt to +49 nt), P5 (-340 nt to +49 nt), and P6 (-365 nt to -31 nt) subsequently constructed. E2F1 wild-type (E2F1 wt) and its N-terminus (E2F1 mutant) expression plasmids were constructed according to the literature [63]. For promoter activity analysis, Hep3B cells were transfected with P5 promoter-Luc (firefly luciferase) and pRL (Renilla luciferase)-TK (a constitutively active plasmid for controlling transfection efficiency containing only a portion of the herpes simplex virus TK promoter and lacking E2F1 binding sites) with or without BRIT1 plasmid, E2F1 wt/mutant plasmids. Firefly and Renilla luciferase activity were assayed by using

dual-luciferase reporter assay system, and the firefly luciferase activity representing the SERPINA5 promoter activity were normalized with Renilla activity.

Data analysis

HCC datasets (including BRIT1's or SERPINA5's copy number variation and mRNA expression level, and the association of BRIT1 or SERPINA5 with recurrence, vascular invasion, or metastasis) from several other cohorts were retrieved from Oncomine (https://www.oncomine.org/) or the cBioPortal for Cancer Genomics(www.cbioportal.org). The promoter region of the SERPINA5 gene was retrieved from UCSC Genome Browser on human Dec 2013 (GRCh38/hg38) assembly (https://genome.ucsc.edu). The E2F1 binding sites in the SERPINA5 promoter region was predicted by using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), a virtual laboratory for the study of transcription factor binding sites in DNA sequences [64].

Statistical analysis

Each experiment was performed in triplicate for all data. Data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using the Student's *t* test and ANOVA. *P* values less than 0.05 were considered to be statistically significant.

Results

BRIT1 is aberrantly expressed in high grades of HCC and its deficiency is associated with predisposition to metastasis.

We initially investigated the expression level of BRIT1 in HCC and its association with patients' pathological properties since previous studies reported that human chromosome 8p32, the proximal locus of *BRIT1* gene, was frequently deleted in HCC [41-44]. We performed the immunohistochemical staining of BRIT1 in HCC tissue microarray (TMA). This cohort of patients consisted of 83 HCCs, 5 cholangiocellular carcinomas, 2 adenosquamous carcinomas, 1 each of mixed carcinoma, carcinoma sarcomatodes, squamous cell carcinoma, and papillary carcinoma, and 10 normal liver tissues, detailed characteristics of HCC patients (n=83) in this cohort were summarized in Supplementary Table S1. As shown in Fig. 1, BRIT1 was typically stained intensively in the nucleus in the normal liver tissue (Fig. 1A). In HCC samples, BRIT1 was expressed in the nucleus of cancer cells (Fig. 1B-1D), or almost in the cytoplasmic portions (Fig. 1E), and even was diminished and lost in the cancerous cells (Fig. 1F). To quantify the staining data of BRIT1 in HCC, we exploited H-score and performed two types of analysis (continuous and categorized). Initially, H-score of BRIT1 staining for each patient was treated as continuous data. We also dichotomized patients' H-score, and a cut-off value of 82.5 from the lower quadrantile of H-score in normal liver was used to determine the low or high of BRIT1 staining in HCC patients. As shown in Table 1, BRIT1 expression was dramatically decreased in high grades of patients by using continuous data ($P<0.05$), dichotomized data also confirmed the difference of BRIT1 staining

between low and high grades of HCC patients (G1 vs G2, or G1 vs G3/4, $P<0.05$), indicating BRIT1 was low expressed in high grades of HCC patients.

We further assess whether BRIT1 loss is associated with the recurrence of HCC. We retrieved HCC datasets by using Oncomine, which exhibited BRIT1 copy number was lower in HCC (Fig. 2A) and HCC patients with recurrence tended to be BRIT1 low in a Guichard HCC cohort [65] (Fig. 2B), suggesting that BRIT1 low might be significantly associated with recurrence. In clinic, tumor recurrence may occur at the original site (local recurrence) or at the distant site (metastasis). We examined whether BRIT1 low is associated with metastasis. Although the data about HCC samples with metastasis were limited, there was emerging evidence that BRIT1 low was indeed associated with a high possibility of metastasis. For instance, BRIT1 low was correlated with vascular invasion, a first step for metastasis (Fig. 2C & 2D). Altogether, this result indicated that BRIT1 low was strongly associated with HCC metastasis.

BRIT1 inhibits the capabilities of migration and invasion

To investigate the potential role of BRIT1in metastasis, we exploited BRIT1-deficient HCC cells Hep3B and BRIT1-proficient HCC cells SNU-449 (Supplementary Fig. S1), with which we constructed a BRIT1-overexpressing cell line Hep3B.BRIT1 (vs Hep3B) and a BRIT1 transiently knockdown cell line siSNU449 (vs SNU449). We examined the BRIT1 role in cell growth by using the above 2 pairs of cell lines, Hep3B vs Hep3B.BRIT1 and SNU449 vs siSNU449. BRIT1-proficient cells Hep3B.BRIT1 exhibited a low rate of cell growth, when compared to parental Hep3B cells, this was also the case for the pair of cells SNU449 vs siSNU449 (Supplementary Fig. S2). We next wanted to know whether BRIT1 acts as an important player in metastasis. To this end, we employed the *in vitro* cell models to assess the effect of BRIT1 on migration and/or invasion by using scratch-wound and Transwell-based assays. As demonstrated by scratch-wound assay, when compared to parental Hep3B cells, Hep3B.BRIT1 (overexpression of BRIT1) significantly slowed down cell migration, up to 2.6 times slower than parental cells (e.g. the distance for Hep3B.BRIT1retained 82% at 72h, while for parental Hep3B, it was just 32% at 72h) (Fig. 3A). This was also the case for SNU449 cells (high level of BRIT1 vs siSNU449), showing 5 times slower when compared to its BRIT1-knockdown counterpart siSNU449 cells (60% vs 12%) (Fig. 3B). Then, we confirmed the above findings by using Transwell migration and invasion assays in the above 2 cell models. As shown in Fig. 4, BRIT1 overexpression significantly inhibited the capacity of migration and invasion in Hep3B.BRIT1 cells, while BRIT1 knockdown in siSNU449 cells obviously increased the number of migrated and invaded cells when compared to that in SNU449. Last, metastasis inhibition induced by BRIT1 overexpression was validated by using *in vivo* HCC xenograft mouse model. Hep3B.BRIT1 and parental Hep3B cells (2×10^6 cells/mouse) were administered into NOD/SCID mice by tail vein injection and the mice were observed for 2-4 months. Compared to control mice whose body weights were steadily increased during the experiments (9/9, 100%), 5 out of 9 (56%) Hep3B-xenografted mice showed the decrease of body weights, while only 22% (2/9) Hep3B.BRIT1-xenografted mice decreased body weights (Fig. 5A). Accordingly, more Hep3B-xenografted mice exhibited abnormal organ conditions than Hep3B.BRIT1-xenografted mice macroscopically (Fig. 5B). Regarding the lung, its relative weight in Hep3B-xenografted mice was much heavier than those in Hep3B.BRIT1-xeografted mice, in accordance

with that, Hep3B -xenografted mice exhibited more cancerous emboli and micrometastatic foci in the lung than those in Hep3B.BRIT1-xeografted mice (Fig. 5B & 5C). Taken together, these findings together indicated that BRIT1 inhibited the capacity of migration and invasion *in vitro* and *in vivo*.

SERPINA5 contributes to the inhibition of migration induced by BRIT1.

To investigate the underlying mechanism of migration inhibition induced by BRIT1, we screened a cluster of SERPIN genes encoding the inhibitors of serine proteases that may control the critical steps of tumor invasion and metastasis [66, 67]. We focused on two clusters of SERPINs, SERPINAs (including SERPINA1, A2, A3, A5) and SERPINBs (including SERPINB1, B2, B3, B7), SERPINE1, and SERPINE2, and examined their mRNA level in Hep3B and BRIT1-overexpressing Hep3B.BRIT1 cells by using qRT-PCR. As shown in Fig. 6A, compared to parental Hep3B cells, the mRNA levels of several SERPIN genes especially SERPINA5 were altered in Hep3B.BRIT1 cells. We then examined the protein level of SERPINA5 in 2 pairs of HCC cell models: Hep3B vs Hep3B.BRIT1 and SNU449 vs siSNU449. The protein level of SERPINA5 was significantly increased in Hep3B.BRIT1 cells compared to the parental Hep3B cells (Fig. 6B). In contrast, when we knocked down the BRIT1 gene in SNU449 cells by BRIT1-specific siRNA, the SERPINA5 protein was significantly decreased in siSNU449 cells. This result suggested that SERPINA5 might be upregulated by the overexpression of BRIT1. Since BRIT1 has been reported to function as a co-factor of transcription factor E2F1 to upregulate the expression of its target genes [25], we analyzed the proximal promoter region of the SERPINA5 gene and found the minimal region containing the E2F1-binding site (P5) can mimic the activity of the whole promoter region (Fig. 6C). Subsequently, we used the minimal promoter region P5 to assess whether BRIT1 can enhance the promoter activity of the SERPINA5 gene. As shown in Fig.6D, BRIT1 overexpression enhanced the promoter activity of the SERPINA5 gene, and the increase of promoter activity was dependent on the presence of transcription factor of E2F1. Taken together, these results indicated that BRIT1 may modulate the expression level of SERPINA5 in HCC cells.

SERPINA5 is downregulated in HCC samples.

To assess the protein level of SERPINA5 in HCC samples, we performed the IHC staining of SERPINA5 by using the same TMA as done for BRIT1. As shown in Fig. 7, SERPINA5 was mainly stained in the cytoplasm of normal and cancerous liver cells and the extracellular regions in liver tissue. The protein level of SERPINA5 was diminished or even lost in higher grades of HCC samples, which was demonstrated by using continuous and dichotomized data of SERPINA5 staining (Fig. 7 & Table 2). To further verify the level of SERPINA5 in HCC, we retrieved the data from other datasets. As shown in Supplementary Fig. S3, SERPINA5 was also decreased in several datasets of HCC samples such as the cohorts of Chen Liver (n=197), Roessler Liver (n=43), and Roessler Liver 2 (n=445). Altogether, these results indicated that SERPINA5 was decreased in HCC samples, especially in high grades of patients.

SERPINA5 is positively correlated with the level of BRIT1 in HCC samples.

As described above, SERPINA5 was regulated by and large by BRIT1 *in vitro* (Fig. 6), therefore, we further investigated whether the expression of SERPINA5 was correlated with the protein level of BRIT1 in HCC

samples. To this end, we analyzed the protein levels of BRIT1 and SERPINA5 obtained from TMA. We sorted the BRIT1 H-scores of individual samples in descending order and separated the samples into 4 categories in quartile: strong (top 25%, H-score>150), moderate (H-score: 82.5-150), low (H-score: 25-82.5), and negative (lower 25%, H-score<25). As shown in Table 3, continuous data analysis demonstrated that SERPINA5 expression was positively correlated with the expression of BRIT1 when compared strong to negative group (P=0.0219). Spearman's rho analysis (correlation coefficiency) also demonstrated that the level of SERPINA5 exhibited a slight increase with the elevated of BRIT1 (P=0.044) (Table 4). To verify this association of BRIT1 and SERPINA5, we retrieved the datasets of HCC cell lines, and found that SERPINA5 levels were in parallel with BRIT1 in these cell lines (Supplementary Fig. S4A). Also, we retrieved HCC data from Oncomine for meta-analysis. BRIT1 was downregulated in 5 independent HCC datasets, where SERPINA5 was in parallel decreased and exhibited the similar trend to BRIT1 (Supplementary Fig. S4B). Taken together, these findings suggested that SEPINA5 was indeed associated with the level of BRIT1 in HCC samples.

Discussion

HCC is a highly aggressive human malignancy worldwide, usually featured as intrahepatic and distant metastasis at advanced stages. It would be a great endeavor for prognosis prediction and tumor management to further understand the molecular mechanisms governing metastasis. In this study, we demonstrated that BRIT1 protein was downregulated in high-grade patients. BRIT1 deletion resulted in high level of migration and invasion of HCC cells, whereby its ectopic expression mitigated the capability of migration and invasion *in vitro* and suppressed metastasis by using xenograft tumor model *in vivo*. Furthermore, we identified SERPINA5 is expressed in parallel with BRIT1, and its downregulation largely contributes to the inhibition of invasion caused by BRIT1. Further, our findings indicated that BRIT1 might regulate the expression of SERPINA5 by modulating the E2F1 function to control the transcription level of SERPINA5. Together with the inhibition of SERPINA5 for metastasis, our findings provided the convincing evidence to support the notion that BRIT1 might modulate the remodeling of extracellular matrix of cancer cells to inhibit the metastasis.

Previously, we [22, 24] and others [17, 38] have identified BRIT1 acts as a tumor suppressor via maintaining genomic integrity by using *in vitro* cultured cells and our unique *in vivo* knockout mouse model. Our recent report also indicated that BRIT1 dysfunction may provide an opportunity to target HCC by using PARP inhibitors in combination with PI3K/mTOR inhibitor [68]. BRIT1's locus 8p22-p23 has also been reported to be frequently deleted in HCC and the deletion is strongly associated with the predisposition of metastasis in HCC [42–44, 69]. In light of that, the pathological relevance of BRIT1 in tumor metastasis is apparently predictable. In this study, we actually found that low expression of BRIT1 was negatively correlated with the tumor grade of HCC, and the samples with low BRIT1 were prone to undergo vascular invasion or metastasis (Figs. 1& 2). Meanwhile, BRIT1 knockdown promoted the capabilities of cell migration and invasion in SNU449 cells, while ectopic expression of BRIT1 can lead to the inhibition of migration and invasion (Figs. 3 & 4). The *in vivo* xenograft model also confirmed that BRIT1 low enhanced the probabilities of cell metastasis (Fig. 5). Our study provided convincing evidence

that BRIT1 was associated with metastasis. This association was substantiated by a plenty of reports on BRIT1 expression in other cancers. For instance, BRIT1 CNA by comparative genomic hybridization (CGH) has identified its CN loss in 72% (39/54) of breast cancer cell lines, and 40% (35/87) of ovarian cancers [17]. Moreover, BRIT1 protein was decreased in 29% of breast cancer specimens and was associated with high-grade tumors [70], and BRIT1 has been regarded as a breast cancer susceptibility gene [38], these data supporting the involvement of BRIT1 in cancer progression. More importantly, BRIT1 loss of heterozygosity was associated with intrahepatic multiple tumors, which is possibly a sign of intrahepatic metastasis [71]. These data support our notion that BRIT1 might be a metastatic inhibitor for HCC.

Also, we demonstrated that SERPINA5 was downregulated in high-grade HCC tumors and proposed that SERPINA5 might be a candidate contributor. SERPINA5 is a SERPIN family member, belonging to a serine related peptidase. It inhibits several serine proteases including protein C and various plasminogen activators in coagulation, which is also involved in degradation of the components of extracellular matrix, as a result, may participate into the remodeling control of extracellular matrix. Previous reports demonstrate that SERPINA5 can inhibit the metastasis by modulating fibronectin-integrin β1 signaling pathway in HCC [58]. SERPINA5 also involved in one of the tumor progression and metastasis-related SERPIN genes in gastric cancer [72]. Although SERPINA5 has a paradoxically association with a poor prognosis in breast cancer [73, 74], it tends to inhibit the metastasis of breast cancer by using xenograft tumor model [57, 75–77]. These studies support our findings reported in this study. Our data showed that BRIT1 can upregulate the expression of the SERPINA5 gene determined by qRT-PCR and Western blotting analysis and verified with siRNA knockdown assay (Fig. 6). SERPINA5 protein level was decreased in high-grade tumors, the way similar to the decrease of BRIT1 (Figs. 7 & Table 2). These findings indicated that SERPINA5 might be a key player in metastasis induced by BRIT1. In accordance with that, SERPINA5 has been reported to exactly regulate tumor metastasis [77]. Therefore, our study provides a deep insight of how BRIT1 and SERPINA5 interaction to modulate the each other to control metastasis of HCC.

Given the molecular mechanisms underlying metastasis inhibition by BRIT1 in HCC, our result demonstrated that BRIT1 upregulated the expression of SERPINA5, and E2F1 can enhanced the activity of the promoter of the SERPINA5 gene (Fig. 5). As previously reported, BRIT1 functions as a co-transcription factor to control several genes' expression involved in cell death and DNA repair, and acts through transcription factor E2F1 to control this phenomenon [25]. Consistently, E2F1 has been reported to bind with the regulatory region of the mouse SERPINA5 gene [78], and also may transcriptionally control SEPINA5 by analyzing CHEA Transcription Factor Targets [79] using analysis tool The harmonizome [80]. E2F1 wild-type protein (437 aa) contains a number of conserved domains, at least including a cyclin A binding domain (aa 67–108), a NLS (aa 85–91), a DNA binding domain (aa 120–191), and a transactivation domain (aa 368–437) [81]. According to previous report [25], the N-terminus portion of E2F1 (aa 1-109), the E2F1 mutant used in this study, can bind to BRCT domains, but lack DNA binding domain. Thereby, our data showed mutant E2F1 lacking binding to promoter, cannot activate promoter activity of SERPINA5 irrespective of the presence of BRIT1, supporting E2F1 at least may be one of the major transcription factors that pass the act of BRIT1 to SERPINA5.

Frankly speaking, however, besides SERPINA5, other genes may be also involved in metastasis control induced by BRIT1 since there are still other genes including other SERPINs can be controlled by BRIT1. In this study, we mainly focused on the SERPINA5 function in BRIT1-induced inhibition of metastasis, while other genes will be further investigated in the future.

Conclusions

In summary, we provided convincing evidence that BRIT1 low or loss in HCC presents a migratory potential by cooperating with transcription factor E2F1 to modulate a potential matrix remodeling protein SERPINA5 in HCC.

Abbreviations

BRIT1, Breast cancer 1 (BRCA1) C terminus-repeat inhibitor of human telomerase expression 1

CCK8, Cell Counting Kit-8

DDR, DNA damage response

E2F1, E2F transcription factor 1

HCC, hepatocellular carcinoma

HR, homologous recombination

MCPH1, microcephalin 1

NHEJ, non-homologous end joining

PAI3, plasminogen activator inhibitor type 3

PCI, protein C inhibitor

SERPINA5, serpin family A member 5

TMA, tissue microarray

Declarations

Ethics approval and Consent to participate

The present study was authorized by the institutional research ethics committee of Shandong First Medical University.

Consent for publication

Not applicable.

Availability of data and materials

Data mining performed in this study was using Oncomine and cBioPortal Cancer Genomics.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

XZhao and YL designed the project. XZhao, MX, JF, NL, JW, XZhang, and YL performed experiments and data analysis. XZhao and YL wrote the manuscript. All authors discussed the results and revised the manuscript.

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Authors' information (optional)

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Tables

Table 1. Correlation of BRIT1 expression with clinicopathological data in TMA cohort a

Parameter (n) [b]	BRIT1 expression				
	Continuous		Categorized [c]		
	Mean	P value	High (%)	Low (%)	P value
Age					
<50 (36)	84.17	0.2603	15 (37.50)	21 (50.00)	0.2542
>50 (46)	95.27		25 (62.50)	21 (50.00)	
Gender					
M (71)	92.10	0.3069	34 (85.00)	37 (88.09)	0.6810
F (11)	79.41		6 (15.00)	5 (11.91)	
Tumor grade					
G1 (11)	134.30	G1 vs G2, **0.0332** [d]	9 (22.50)	2 (4.76)	Total, 0.0594
G2 (49)	85.05	G2 vs G3, 0.4085	22 (55.00)	27 (64.29)	G1 vs G2, **0.0268**
G3/4 (22)	80.35	G1 vs G3/4, **0.0130**	9 (22.50)	13 (30.95)	G1 vs G3/4, **0.0261**
Tumor stage					
T1/2 (36)	90.35	T1/2 vs T3, 0.4207	21 (52.50)	15 (35.71)	Total, 0.2454
T3 (42)	93.93	T1/2 vs T4, 0.1332	18 (45.00)	24 (57.14)	T1/2 vs T3, 0.1730
T4 (4)	53.75	T3 vs T4, 0.1922	1 (2.50)	3 (7.14)	T1/2 vs T4, 0.2036
		T1/2 vs T3/4, 0.4980			T1/2 vs T3/4, 0.1258

[a] Only the data of HCC samples (n=83) from the TMA were included in this table.

[b] Total HCC samples used here was 82 (1 sample was ruled out due to tissue loss).

[c] A cutoff value of H-score (82.5) from a lower quartile of normal liver tissue (n=10) was used to categorize BRIT1 expression into low and high groups.

[d] *P*<0.05 is significant and are shown in bold.

Table 2. Correlation of SERPINA5 expression with clinicopathological data in TMA cohort [a]

Parameter (n) [b]	SERPINA5 expression				
	Continuous		Categorized [c]		
	Mean	P value	High (%)	Low (%)	P value
Age					
<50 (36)	76.01	0.0547	18 (41.86)	18 (46.15)	0.6956
>50 (46)	96.72		25 (58.14)	21 (53.85)	
Gender					
M (71)	89.61	0.2167	38 (90.48)	33 (82.50)	0.2894
F (11)	74.77		4 (9.52)	7 (17.50)	
Tumor grade					
G1 (11)	122.27	G1 vs G2, **0.0305** [d]	9 (20.93)	2 (5.13)	Total, 0.0813
G2 (49)	91.25	G2 vs G3/4, **0.0260**	25 (58.14)	24 (61.54)	G1 vs G2, 0.0625
G3/4 (22)	62.22	G1 vs G3/4, **0.0018**	9 (20.93)	13 (33.33)	G1 vs G3/4, **0.0261**
TNM stage					
T1/2 (36)	90.97	T1/2 vs T3, 0.1285,	21 (48.84)	15 (38.46)	Total, 0.4047
T3 (42)	94.52	T1/2 vs T4, 0.0543,	21 (48.84)	21 (53.85)	T1/2 vs T3, 0.4617
T4 (4)	53.75	T2 vs T3/4, 0.0826	1 (2.33)	3 (7.69)	T1/2 vs T4, 0.2036

[a] Only the data of HCC samples (n=83) from the TMA were included in this table.

[b] Total HCC samples used here was 82 (1 sample was ruled out due to tissue loss).

[c] A cutoff value of H-score (80) from a lower quartile of normal liver tissue (n=10) was used to categorize BRIT1 expression into low and high groups.

[d] *P*<0.05 is significant and are shown in bold.

Table 3. Correlation of SERPINA5 expression with BRIT1 level in TMA cohort

Parameter (H-score)	n [a]	SERPINA5 expression	
		Continuous	
		Mean	P value
BRIT1			
Strong (>150) [b]	20	102.27	S vs M, 0.2459
Moderate (82.5-150)	20	88.28	S vs L, 0.2063
Low (25-82.5)	21	87.19	M vs L, 0.4639
Negative (<25)	21	68.81	S vs N, **0.0219** [c]

[a] For HCC samples used here, n=82.

[b] The quartile H-score values (lower quartile, 25, median, 82.5, upper quartile, 150) of BRIT1 were used to categorize BRIT1 expression into 4 groups (Negative, low, moderate, and strong).

[c] *P*<0.05 is significant and are shown in bold.

Table 4. Correlation of SERPINA5 with BRIT1 by using Spearman's analysis

		BRIT1	SERPINA5
BRIT1	Correlation Coefficiency	1.000	.223*
	Significant (2-tailed)	.	.044**
	n	82	82
SERPINA5	Correlation Coefficiency	.223*	1.000
	Significant (2-tailed)	.044**	.
	n	82	82

Note: *, Spearman's ρ value, **, *P* value.

Figures

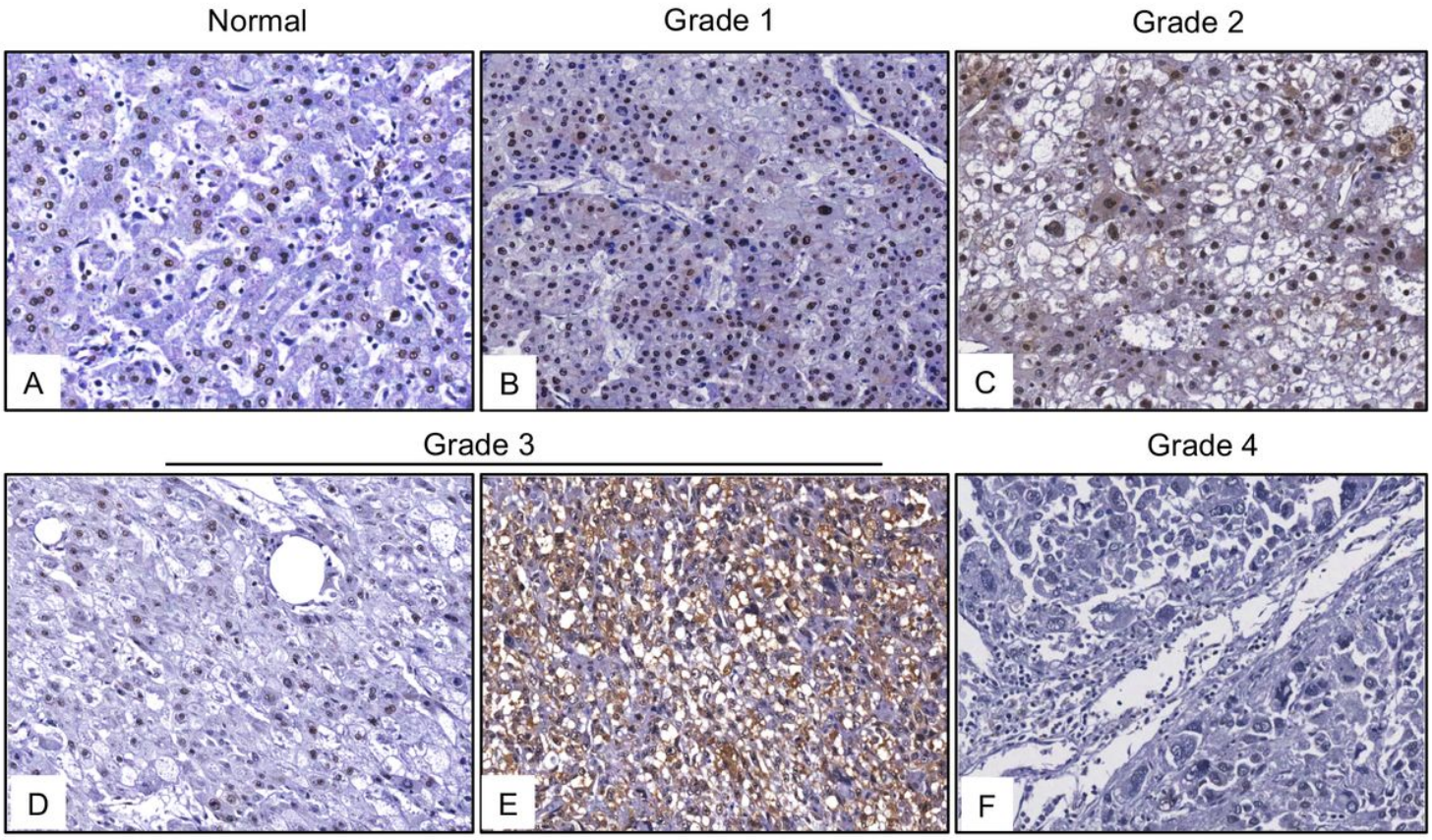


Figure 1

Immunohistochemical analysis of BRIT1 in normal and malignant liver tissue samples. The TMA slides of normal liver (n=10) and HCC (n=83) were immunohistochemically stained with anti-BRIT1 antibody. (A) Normal liver tissues showing strong nuclear expression of BRIT1 (×20). (B and C) Grade 1 (B) and grade 2 (C) HCC tissues showing nuclear staining of BRIT1 (×20). (D and E) Grade 3 HCC tissues showing low nuclear expression (D) or low nuclear/strong cytoplasmic expression (E) of BRIT1 (×20). (F) Grade 4 HCC tissues with loss of nuclear expression of BRIT1 (×20).

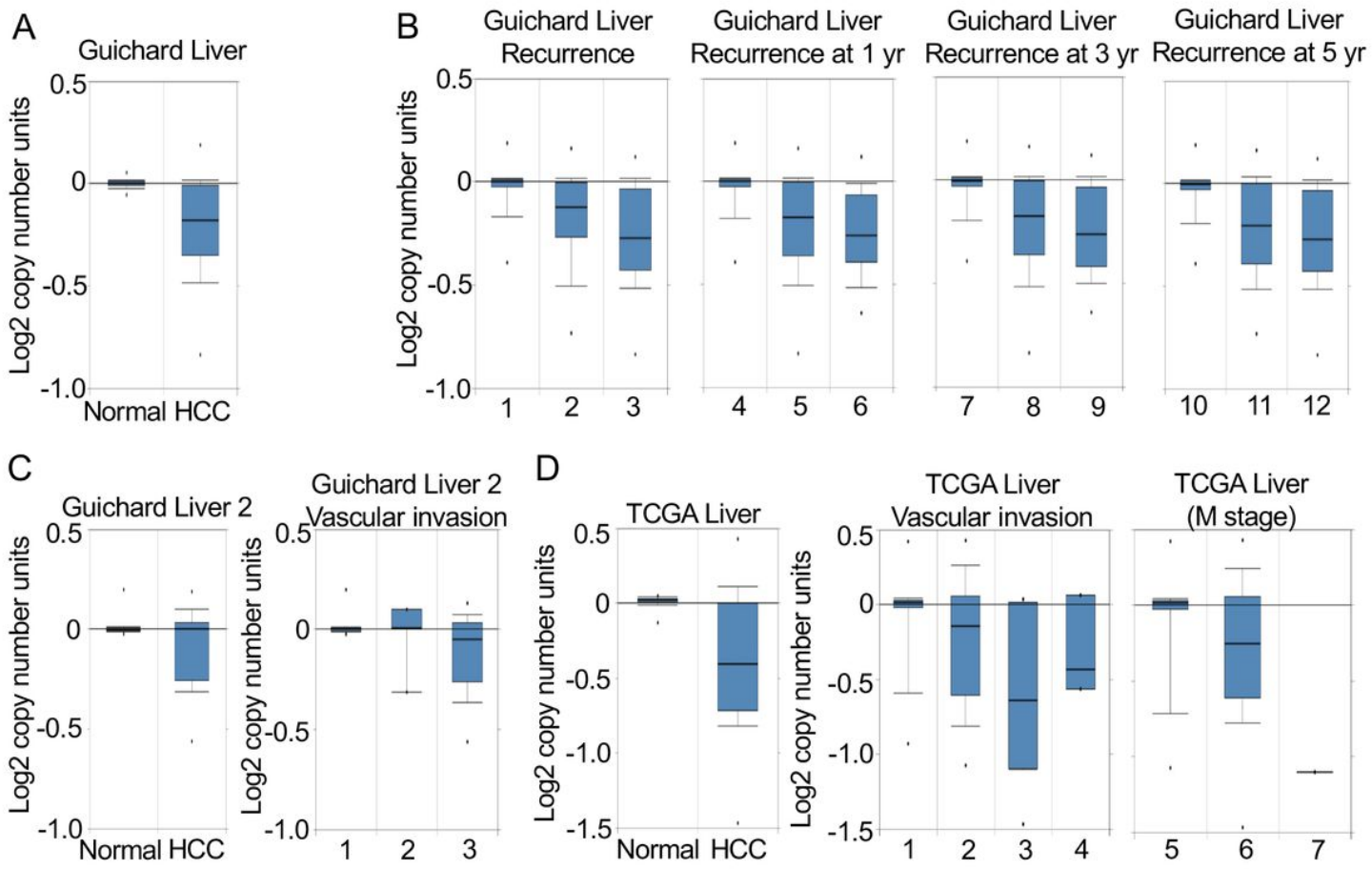


Figure 2

BRIT1 low is associated with the recurrence, vascular invasion, and metastasis in HCC. (A) BRIT1 copy number is remarkably decreased in the Guichard cohort of HCC. The data was retrieved from the cohort of Guichard Liver through Oncomine. Normal samples, n=86, HCC, n=99. Fold change=-1.154, P=5.26E-17. (B)HCC samples with low BRIT1 are prone to be recurrent. For total recurrence in the Guichard cohort: 1, normal and recurrence-not-determined samples, n=111, 2, HCC without recurrence, n=27, 3, HCC with recurrence, n=47. For 1-year recurrence: 4, normal and recurrence-not-determined samples, n=112, 5, HCC without recurrence at 1 year, n=48, 6, HCC with recurrence at 1 year, n=25. For 3-year recurrence: 7, normal and recurrence-not-determined samples, n=118, 8, HCC without recurrence at 3 years, n=23, 9, HCC with recurrence at 3 years, n=44. For 5-year recurrence: 10, normal and recurrence-not-determined samples, n=124, 11, HCC without recurrence at 5 years, n=14, 12, HCC with recurrence at 5 years, n=47. (C) BRIT1 low is associated with vascular invasion in the Guichard liver 2 cohort of HCC. The data was retrieved from the cohort of Guichard Liver 2 through Oncomine. Normal, n=26, HCC, n=26, Fold change=-1.069, P=0.007.1, normal and invasion-not-determined samples, n=27, 2, HCC without vascular invasion, n=6, 3, HCC with vascular invasion, n=19. (D) BRIT1 low is associated with vascular invasion and metastasis in the TCGA cohort of HCC. TCGA Liver: Normal, n=59, HCC, n=97, Fold change=-1.303, P=2.80E-15. For vascular invasion: 1, normal and invasion-not-determined samples, n=162, 2, HCC without vascular invasion, n=36, 3, HCC with microvascular invasion, n=11, 4, HCC with macrovascular invasion, n=3. For

metastasis: 5, normal and metastasis-not-determined samples, n=120, 6, HCC at M0, n=35, 7, HCC at M1, n=1.

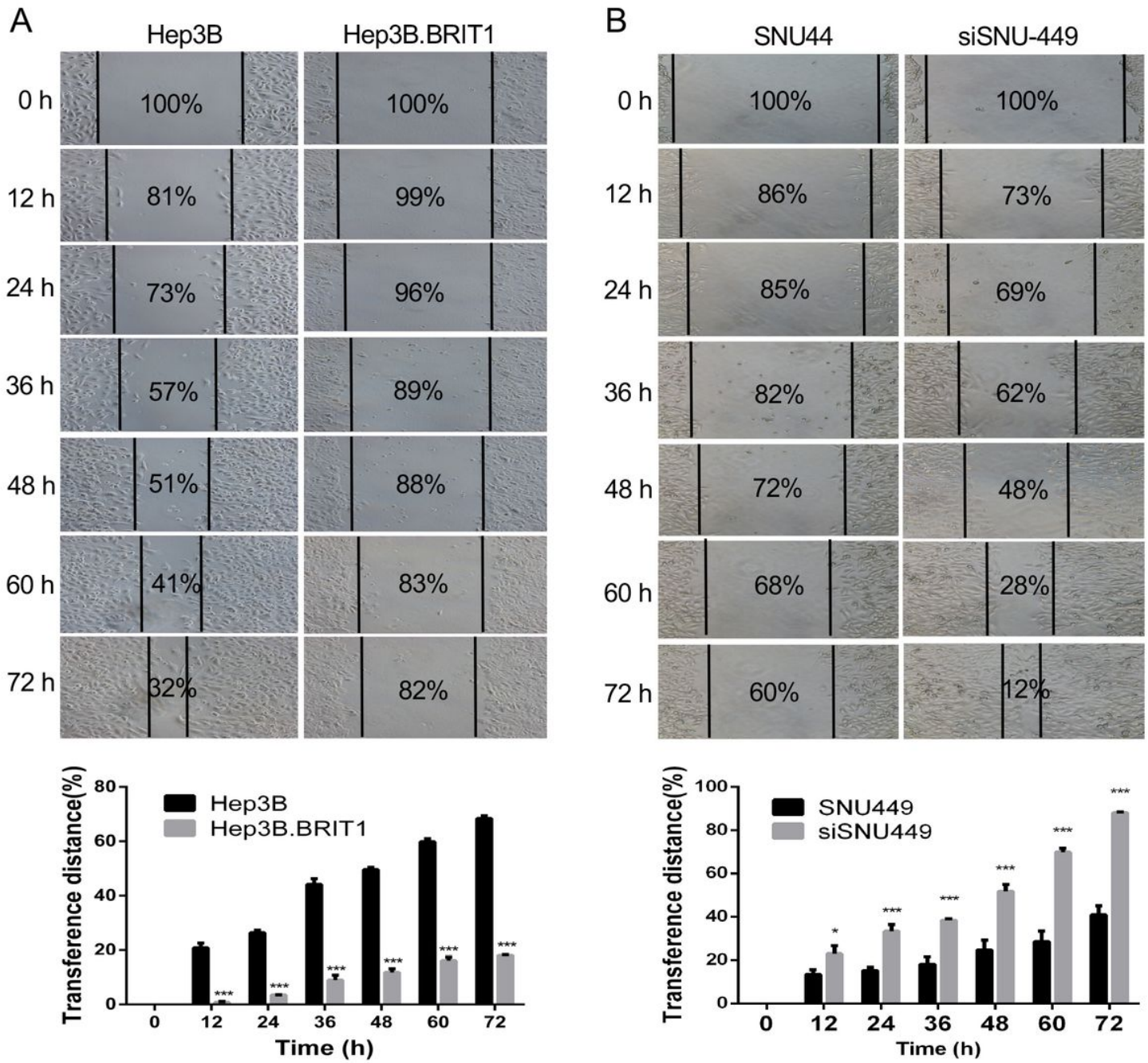


Figure 3

BRIT1 results in the inhibition of cell migration determined by wound-healing assay. (A) Wound-healing assay demonstrates ectopic expression of BRIT1 inhibits the migratory ability of Hep3B cells. (B) BRIT1 knockdown enhances cell migration of SNU449 cells determined by wound-healing assay. The experiments were biologically repeated three times. *, P<0.05, **, P<0.01, ***, P<0.001, compared to parent cells Hep3B or SNU449.

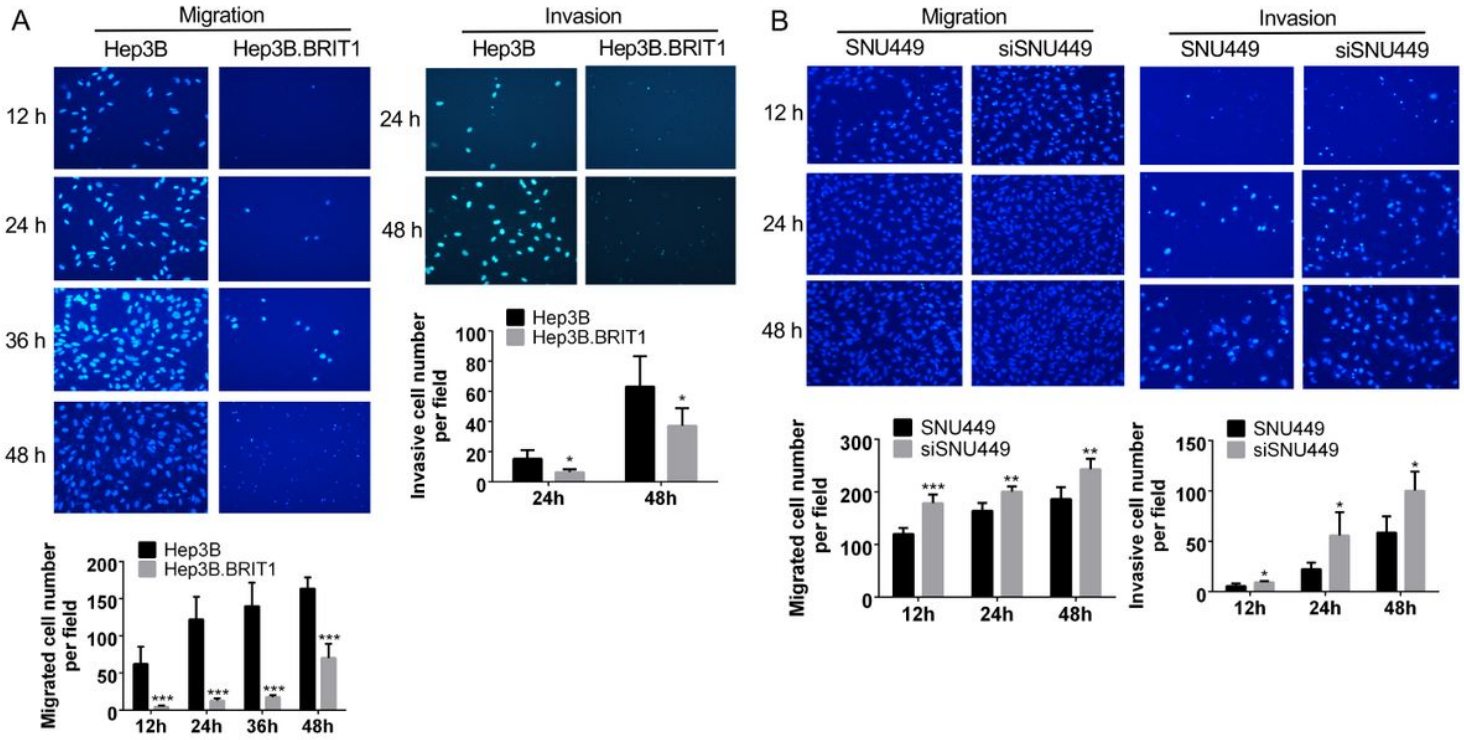
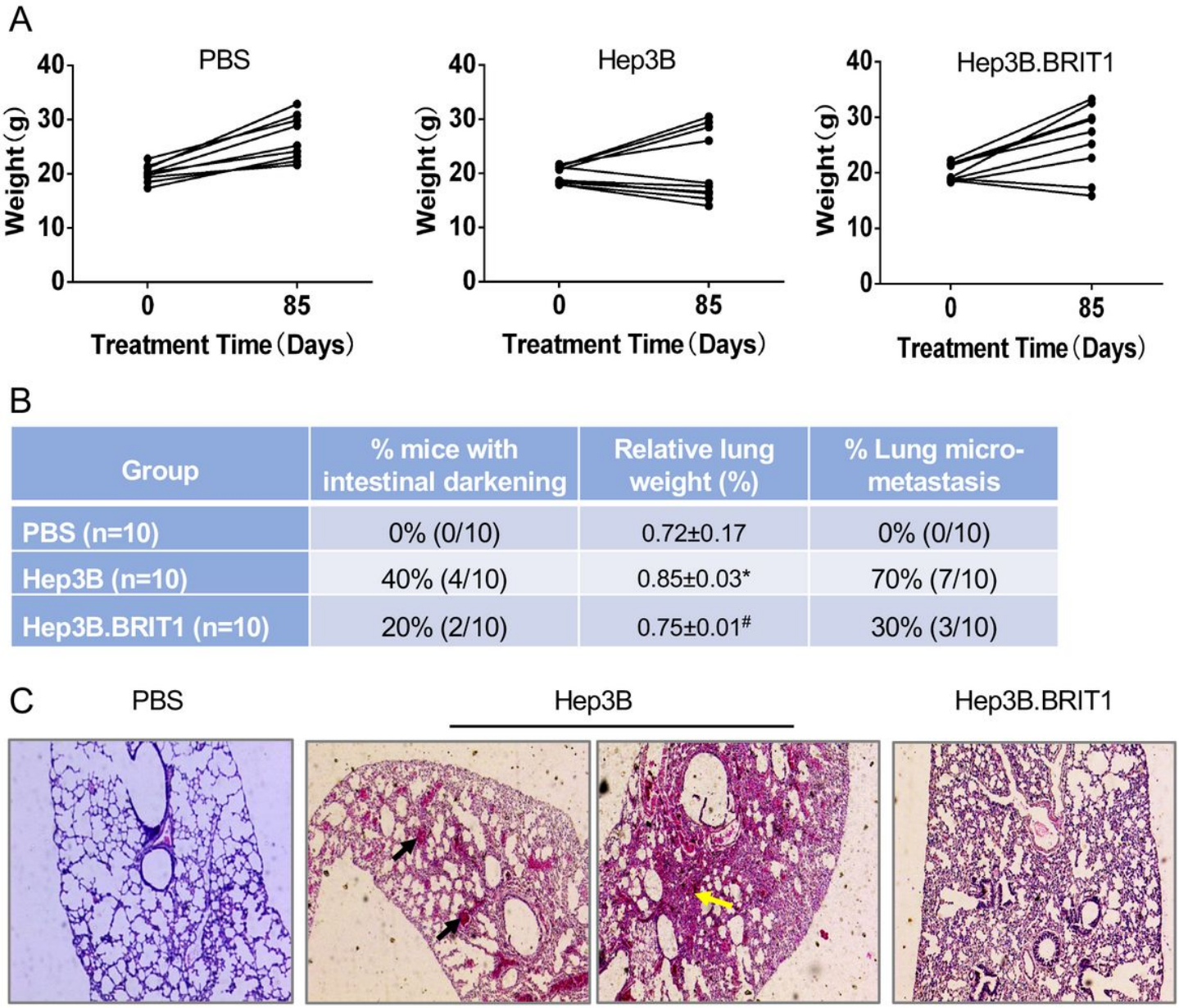


Figure 4

BRIT1 inhibits cell migration and invasion by using Transwell chamber assays. Two pairs of cells, Hep3B vs Hep3B.BRIT1 and SNU449 vs siSNU449, were used to perform Transwell migration and invasion assays at the indicated time points as described in the Methods section. (A) Ectopic expression of BRIT1 inhibits cell migration and invasion of Hep3B cells determined by Transwell chamber assay. (B) BRIT1 knockdown promotes cell migration and invasion in SNU449 cells determined by Transwell chamber assay. All experiments were biologically repeated three times. *, P<0.05, **, P<0.01, ***, P<0.001, compared to parent cells Hep3B or SNU449.



Group	% mice with intestinal darkening	Relative lung weight (%)	% Lung micro-metastasis
PBS (n=10)	0% (0/10)	0.72±0.17	0% (0/10)
Hep3B (n=10)	40% (4/10)	0.85±0.03*	70% (7/10)
Hep3B.BRIT1 (n=10)	20% (2/10)	0.75±0.01#	30% (3/10)

Figure 5

BRIT1 inhibits the in vivo metastatic ability in xenograft HCC mouse model. Hep3B, Hep3B.BRIT1 cells, or PBS (as a cell-free control) (n=10 for each group) were administered into NOD/SCID mice by tail vein injection. The treated mice were then monitored for about 3 months (85 days). (A) The change of total body weight of mice before administration and after sacrifice (n=9 for data collection in each group). (B) BRIT1 mitigates the extent of metastasis-related symptoms. *, P<0.05 compared to PBS group, #, P<0.05 compared to Hep3B group. (C) Representative results of the histological examination of mouse lungs for metastatic nodules from PBS, Hep3B and Hep3B.BRIT1. Black arrows, cancerous emboli, yellow arrow, micrometastasis.

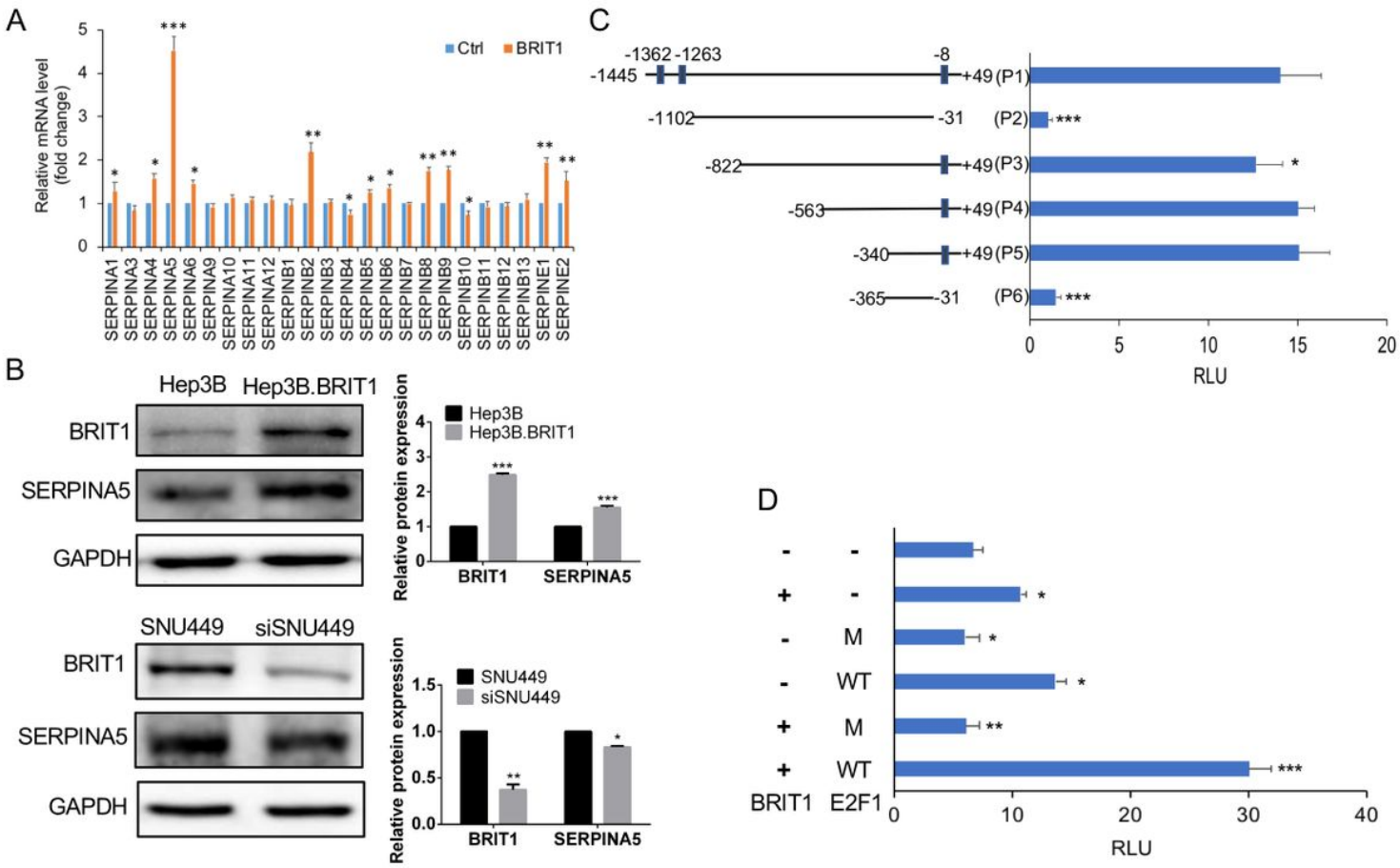


Figure 6

BRIT1 controls the upregulation of SERPINA5. (A) Ectopic expression of BRIT1 modulates the expression of multiple SERPIN genes. Hep3B.BRIT1 and Hep3B cells were used to examine the expression of SERPIN genes by using qRT-PCR. *, P<0.05, **, P<0.01, ***, P<0.001, compared to parent Hep3B cells. (B) BRIT1 regulates the upregulation of SERPINA5 gene determined by Western blots. *, P<0.05, **, P<0.01, ***, P<0.001, compared to parent Hep3B or SNH449 cells. (C) Promoter analysis demonstrates E2F1-binding site-containing proximal promoter region mimics the promoter activity of SERPINA5 gene. The promoter region of SERPINA5 was amplified from Hep3B cells according to the sequence of SERPINA5 deposited in UCSC genome browser. The putative transcription start site of SERPINA5 was regarded as +1, the position 94581369 in the human chromosome 14 reference GRch38.p12 primary assembly (NC_000014.9). Blue bars represent the potential E2F1-binding sites, which are located in the positions -8 bp, -1263bp, and -1362 bp. *, P<0.05, **, P<0.01, ***, P<0.001, compared to the activity of P1. (D) BRIT1 modulate the promoter activity of SERPINA5 gene partly dependent on E2F1. BRIT1, pMSCV-puro-BRIT1 vector, E2F1, E2F1-plasmid, M, mutant E2F1, WT, wild-type E2F1. *, P<0.05, **, P<0.01, ***, P<0.001, compared to the activity of parent cells Hep3B or SNU449.

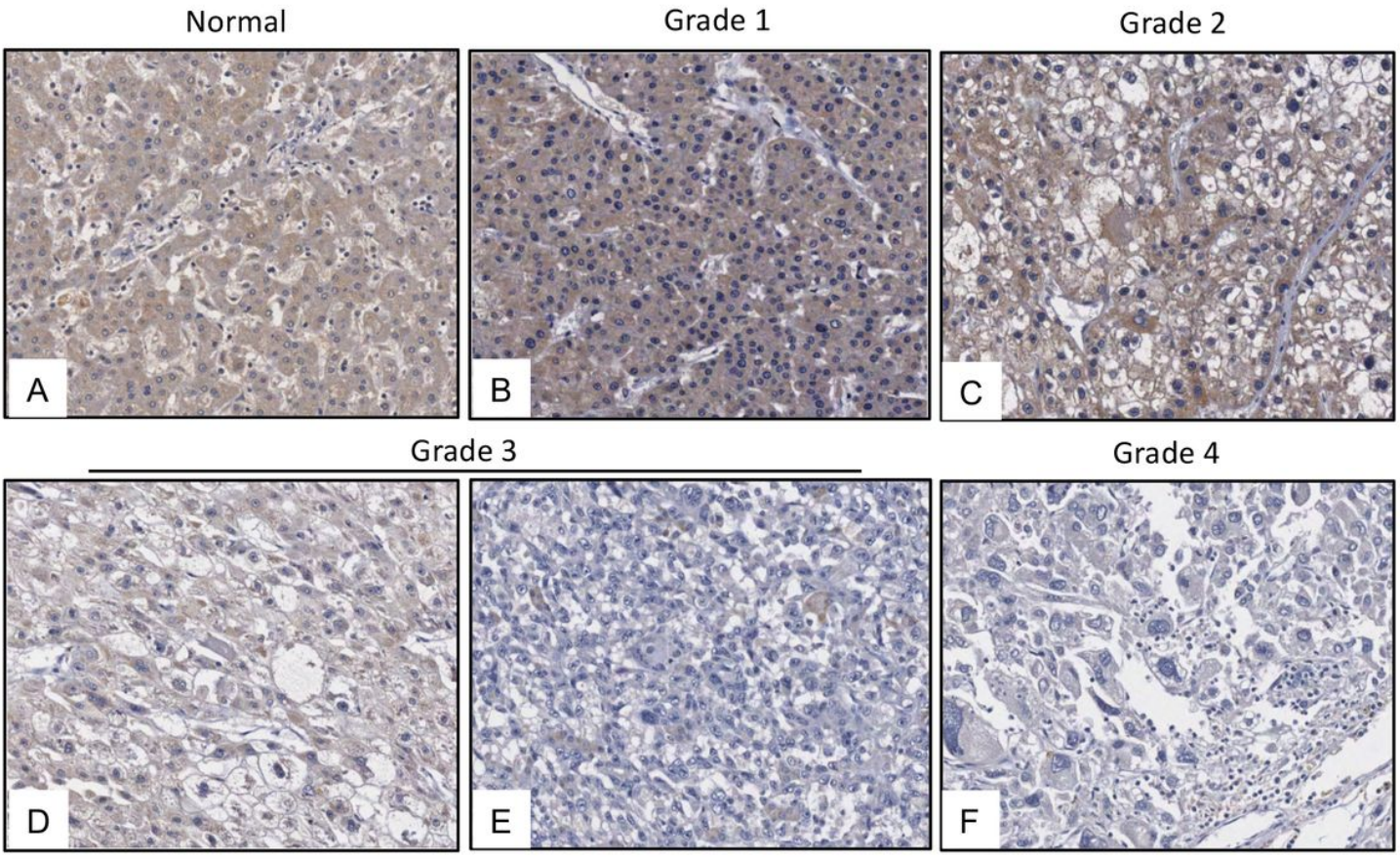


Figure 7

Immunohistochemical analysis of SERPINA5 in normal and malignant liver tissue samples. The TMA slides used here was same as that described in Figure 1. (A) Normal liver tissues showing strong cytoplasmic expression ofSERPINA5 (×20). (B and C) Grade 1 (B) and grade 2 (C) HCC tissues showing strong cytoplasmic staining of SERPINA5 (×20). (D and E) Grade 3 HCC tissues showing low to lack of cytoplasmic expression of SERPINA5 (×20). (F) Grade 4 HCC tissues with loss of cytoplasmic expression of SERPINA5 (×20).

Supplementary Files

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