BTB/POZ Domain-Containing Protein 7: Epithelial-Mesenchymal Transition Promoter and Prognostic Biomarker of Hepatocellular Carcinoma

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Epithelial-mesenchymal transition (EMT) is a critical step in the metastasis of hepatocellular carcinoma (HCC). BTB/POZ domain-containing protein 7 (BTBD7) regulates EMT-associated proteins implicated in HCC progression. However, the role(s) of BTBD7 in HCC have not been identified. Using highly metastatic HCC HCCLM3 cells, immortalized L02 hepatocytes, metastatic HCC animal models, and three independent cohorts of HCC patient specimens, we aimed to determine the involvement of BTBD7 in HCC metastasis. We show that BTBD7 messenger RNA and protein was highly expressed in HCC cells and tumor tissues, with such expression being associated with: enhanced cell motility, venous invasion, and poor prognosis. BTBD7 promoted HCC angiogenesis and metastasis in vitro and in vivo, but did not influence cell proliferation or colony formation. BTBD7 enhancement of HCC invasion and EMT phenotype occurred through activation of a RhoC-Rock2-FAK-signaling pathway, resulting in matrix metalloproteinase-2/9 production and microvessel formation. Applying a predictive risk score model, Cox regression analysis revealed that high BTBD7 expression integrated with high microvessel density was a powerful independent predictive factor of HCC clinical outcome.

Conclusion: The present study identifies BTBD7 as a novel candidate prognostic factor and a potential therapeutic target of HCC. (HEPATOLOGY 2013; 57:2326-2337)

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. Overall survival (OS) of HCC patients remains unsatisfactory because of a high incidence of recurrence and metastasis after hepatic resection. Understanding the mechanisms of HCC recurrence and metastasis should improve HCC treatment and outcomes.

Epithelial-mesenchymal transition (EMT) is an important physiological process contributing to HCC recurrence and metastasis. Expression of several EMT-associated genes is correlated with HCC recurrence and metastasis. HCCs with EMT features consistently exhibit more venous invasion, metastases, and a poorer prognosis than those without EMT characteristics. Therefore, for the treatment of HCC, there exists significant clinical potential in targeting EMT-associated factors.

Members of the BTB (bric-a-brac tramtrack broad complex) (also known as POZ) gene family, characterized by a conserved BTB/POZ protein-protein interaction motif, have been implicated in human cancer. The promoter region of BTB/POZ domain-containing protein 7 (BTBD7) contains binding sites for notable transcription factors, including alpha-fetoprotein (AFP)-1, CAAT enhancer-binding protein beta, GATA,
and activator protein 1. These genetic structures are suggestive of biological roles for BTBD7 in liver tissue. Moreover, BTBD7 was recently identified as a branching morphogenesis-associated gene, which regulates Snail2, fibronectin, and E-cadherin, which are mediators of EMT in HCC. Although there is evidence suggesting possible BTBD7 involvement in EMT of human malignancies, the roles of BTBD7 in human HCC remain unknown. In the present project, we analyzed the expression and functions of BTBD7 in EMT-related HCC recurrence and metastasis and evaluated the significance of BTBD7 in the prediction of HCC prognosis.

Materials and Methods

**HCC Cell Lines.** MHCC97L and HCCLM3 cell lines were purchased from the Liver Cancer Institute of Fudan University (Shanghai, China). HepG2, Bel7404, and L02 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

**HCC Patients and Follow-Up.** HCC was diagnosed according to World Health Organization criteria. HCC was staged based on (1) tumor-node metastasis (TNM) classification (6th Edition) of the International Union Against Cancer and (2) Barcelona Clinic Liver Cancer (BCLC) staging scores. All research protocols strictly complied with REMARK guidelines for reporting prognostic biomarkers in cancer.

Three independent cohorts of HCC subjects were enrolled in this study (Supporting Fig. 1). In training cohort 1 (n = 78), fresh matched specimens of HCC and adjacent nontumorous liver tissue (ANLT) were randomly collected from HCC patients undergoing hepatic resection between July 2004 and March 2005 in the Department of Hepatobiliary Surgery of Xiangya Hospital (Changsha, China). In training cohort 2 (n = 326), paraffin-embedded specimens were randomly collected from HCC patients undergoing curative resection in Xiangya Hospital from September 2001 to June 2004. The validation cohort 3 (n = 112) was comprised of randomly selected paraffin-embedded specimens from HCC patients undergoing resection in Hunan Provincial Tumor Hospital (Changsha, China) between October 2005 and March 2006. Baseline demographics and clinicopathological characteristics of the three cohorts are summarized in Table 1, and clinical outcomes of HCC patients in cohorts 2 and 3 are summarized in Supporting Table 3.

### Table 1. Clinicopathological Characteristics of Three Cohorts of HCC Patients

| Clinicopathologic Variables | Training Cohort 1 (78 cases) | Training Cohort 2 (326 cases) | Validation Cohort 3 (112 cases) |
|----------------------------|-----------------------------|-------------------------------|---------------------------------|
| Age, years                 |                            |                               |                                 |
| ≤60                        | 50 (64.1)                   | 189 (58.0)                    | 62 (55.4)                       |
| >60                        | 28 (35.9)                   | 137 (42.0)                    | 50 (44.6)                       |
| Sex                        |                            |                               |                                 |
| Male                       | 67 (85.9)                   | 289 (88.7)                    | 104 (92.9)                      |
| Female                     | 11 (14.1)                   | 37 (11.3)                     | 8 (7.1)                         |
| HBsAg                      |                            |                               |                                 |
| Negative                   | 6 (7.7)                     | 47 (14.4)                     | 15 (13.4)                       |
| Positive                   | 72 (92.3)                   | 279 (85.6)                    | 97 (86.6)                       |
| Albumin, g/L               |                            |                               |                                 |
| ≤35                        | 9 (11.5)                    | 22 (6.8)                      | 14 (12.5)                       |
| >35                        | 69 (88.5)                   | 304 (93.2)                    | 98 (87.5)                       |
| Child-Pugh classification  | A 72 (92.3)                 | 322 (98.8)                    | 107 (95.5)                      |
|AFP, ng/mL                  | ≤20                         | 22 (28.2)                     | 169 (51.8)                      |
|                           | >20                         | 56 (71.8)                     | 257 (48.2)                      |
| Liver cirrhosis            | Absent                      | 10 (12.8)                     | 31 (9.5)                        |
|                           | Present                     | 68 (87.2)                     | 295 (90.5)                      |
| Tumor encapsulation        | Absent                      | 46 (59.0)                     | 167 (51.2)                      |
|                           | Present                     | 32 (41.0)                     | 159 (48.8)                      |
| Tumor size, cm             | ≤5                          | 29 (37.2)                     | 134 (41.1)                      |
|                           | >5                          | 40 (62.8)                     | 192 (58.9)                      |
| Tumor number               | Single                      | 38 (48.7)                     | 179 (54.9)                      |
|                           | Multiple                    | 40 (51.3)                     | 147 (45.1)                      |
| Satellite nodules*         | Absent                      | 30 (38.5)                     | 143 (43.9)                      |
|                           | Present                     | 48 (61.5)                     | 183 (56.1)                      |
| Venous invasion            | Absent                      | 45 (57.7)                     | 237 (72.4)                      |
|                           | Present                     | 33 (42.3)                     | 54 (15.6)                       |
| Tumor differentiation      | I–II                        | 52 (66.7)                     | 237 (72.7)                      |
|                           | III–IV                      | 26 (33.3)                     | 89 (27.3)                       |
| BCLC stage†                | A 36 (46.1)                 | 154 (47.2)                    | 48 (42.8)                       |
|                           | B 23 (29.5)                 | 85 (26.1)                     | 32 (28.6)                       |
|                           | C 19 (24.4)                 | 87 (26.7)                     | 32 (28.6)                       |
| TNM stage                  | I                           | 40 (51.3)                     | 237 (72.7)                      |
|                           | II                          | 23 (29.5)                     | 64 (19.6)                       |
|                           | III                         | 15 (19.2)                     | 25 (7.7)                        |

Median follow-up time is 38.5 months (range, 2-63). Abbreviation: HBsAg, hepatitis B surface antigen.

Satellite nodules are defined as tumors <2 cm located within 2 cm of the primary lesion.

HCC cases of BCLC stage D, who are not usually considered candidates for hepatic resection, are excluded from the study.
Patient follow-up was terminated on June 26, 2011. Median follow-up was 38.5 months (range, 2-63). Deaths from causes other than HCC were censored. HCC-related death was defined if there was a computed tomography scan increase of >25% in the sum of recurrent tumor index lesions’ cross-sectional areas or new onset of, or increase in, either recurrence, vascular invasion, or metastasis within the last 6 months of life, irrespective of immediate cause of death. OS was defined as the time interval between HCC resection and death or the last observation. Patients alive at the end of follow-up were censored. Recurrence-free survival (RFS) is calculated from the HCC resection to the first radiological evidence of recurrence. Patients that died with no sign of recurrence were censored (Supporting Materials).

**Quantitative Reverse-Transcriptase Polymerase Chain Reaction.** SYBR Green fluorescent-based quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed as previously described. Primers are detailed in Supporting Table 1. Relative quantitative (qRT-PCR) was performed as previously described. Primers are detailed in Supporting Table 1. Relative messenger RNA (mRNA) expression levels were calculated by the 2^ΔΔCt method, based on threshold cycle values, and were normalized to the internal control of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin.

**Western Blotting.** Total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA). Blocked membranes were incubated with the primary antibodies (Abs), followed by horseradish-peroxidase–conjugated secondary Abs (KPL, Gaithersburg, MD). Bands were visualized using the enhanced chemiluminescence kit (Santa Cruz Biotechnology, Santa Cruz, CA). Target signals were quantified by BandScan software (Bio-Rad, Hercules, CA) and defined as the ratio of target protein relative to α-tubulin. Abs are listed in Supporting Table 2.

**Immunohistochemistry and Microvessel Density Scoring.** Procedures of immunohistochemistry (IHC) and microvessel density (MVD) scoring are detailed in the Supporting Materials.

**Construction of BTBD7 Short Interfering RNA Lentivirus.** A BTBD7/RNA interference lentiviral vector of pLKO.1-BTBD7 was constructed as previously described (see the Supporting Materials for details).

**Construction of BTBD7 and RhoC Plasmids.** Complementary DNA containing open reading frames of BTBD7 and RhoC were amplified by PCR and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA), as previously described (see the Supporting Materials for details).

**Immunofluorescence Staining.** Immunofluorescence (IF) staining was performed in triplicate as previously described (see the Supporting Materials for details).

**Invasion and Migration Assays.** Wound-healing assay and Matrigel invasion chamber assay were performed in triplicate as previously described.

**RhoGTP Activation Assay.** Detection of Rho family small guanosine triphosphatase (GTPase) activation is described in the Supporting Materials.

**Gelatin Zymography.** HCCLM3 culture media was analyzed for secreted matrix metalloproteinase (MMP)-2 and MMP-9 by gelatin zymography. Transparent bands of the gel with Coomassie blue background were scanned and sorted according to the presence of MMP-2 and MMP-9 enzymatic activities (see the Supporting Materials for details).

**Cell Viability, Colony-Forming, Cell-Cycle, and Apoptosis Assays.** Cell viability was measured using a Vi-Cell Automated Cell Viability Analyzer (Beckman Coulter, Fullerton, CA). Colony-forming, cell-cycle, and apoptosis analysis are detailed in the Supporting Materials.

**Animal Model.** 5 × 10^6 HCCLM3 cells transfected with Si-BTBD7 were suspended in 100 μl of serum-free DMEM and Matrigel (1:1; BD Biosciences). The solution was injected subcutaneously (SC) into the flanks of male BALB/c mice (3-4 weeks old). SC tumors were harvested 4 weeks after injection and cut into 1.0-mm^3 pieces. One piece was then implanted into the left liver lobe of a nude mouse (n = 8 in each group). Transplanted mice were sacrificed 35 days later, at which point the weight and volume of transplanted tumors were calculated. The number and grade of metastatic intrahepatic and lung nodules were detected using microscopy. Metastases were graded based on the number of tumor cells present at the most populated section in each metastatic lesion (grade I, ≤20; grade II, 20-50; grade III, 50-100; and grade IV, >100 tumor cells). In addition, IHC staining against BTBD7, CD34, E-cadherin, fibronectin, Twist1, Ki67, RhoC, MMP-2, MMP-9, Rock2, and focal adhesion kinase (FAK) were performed on serial sections of each transplantation tumor and intrahepatic metastasis lesions. Tumor cell proliferation and apoptosis assays in lesions are detailed in the Supporting Materials.

**Statistical Analysis.** Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL). Quantitative values are presented as mean ± standard deviation or median (range). Paired t tests and Student t tests were used for paired and unpaired continuous data, respectively. The chi-square test was
applied for categorical data. Pearson’s correlation was used to analyze the relationship between BTBD7 expression and RhoC, Rock2, and FAK expression in cohort 1. Cumulative OS and RFS were evaluated using Kaplan-Meier’s method and the log-rank test. Cox’s proportional hazards regression model was used to determine whether BTBD7 expression combined with MVD is an independent prognostic indicator. \( P < 0.05 \) was considered to be statistically significant.

**Results**

**BTBD7 Overexpression Was Significantly Correlated With HCC Metastasis.** In a cohort of 78 paired HCC specimens, BTBD7 mRNA was up-regulated in tumors, compared with that in ANLT (0.0269 ± 0.0022 versus 0.0045 ± 0.0012; \( P < 0.01 \)). Patients with recurrences of HCC exhibited higher BTBD7 mRNA expression than those without recurrences (0.0489 ± 0.0085 versus 0.0248 ± 0.0032; \( P < 0.01 \)). BTBD7 mRNA expression levels were higher in intrahepatic metastasis lesions than those in primary lesions (0.0562 ± 0.0037 versus 0.0278 ± 0.0049, \( P < 0.05 \); Fig. 1A). qRT-PCR results were further verified by IHC (Fig. 1B) and western blotting (Fig. 1C) in these paired specimens. There was a significant association between BTBD7 expression and satellite nodules, venous invasion, MVD, and HCC stage (Table 2). These data are indicative of BTBD7 involvement not only in HCC pathogenesis, but also in its aggressiveness.

Expression of RhoC, Rock2, and FAK proteins in tumor tissues were significantly higher than that in ANLT (0.89 ± 0.18 versus 0.39 ± 0.15, 0.76 ± 0.33 versus 0.37 ± 0.22, and 0.69 ± 0.29 versus 0.35 ± 0.16; \( P < 0.01 \); Fig. 1C). BTBD7 protein expression was positively correlated with RhoC, Rock2, and FAK expression in paired HCC specimens (\( r = 0.741, 0.794, \) and 0.785, \( P < 0.001 \), respectively; Fig. 1D), implicating the RhoC-Rock2-FAK cascade as a downstream effector of BTBD7 in HCC.

**BTBD7 Enhanced HCC Cell Metastasis.** HCCLM3—the cell line having the highest metastatic potential—had the highest BTBD7 expression, in comparison to the other HCC cell lines (HepG2, Bel7404, and MHCC97L) and immortalized hepatocytes (L02) (\( P < 0.01 \); Supporting Fig. 2A,B). Subsequently, we (1) depleted BTBD7 expression using short interfering RNA in HCCLM3 cells and (2) over-expressed BTBD7 in L02 cells (Supporting Fig. 2C,D). HCCLM3 cells with BTBD7 depletion exhibited a significant reduction in number of invaded cells (\( P = 0.007 \); Fig. 2A). Wound-healing assays showed that a cell migration was significantly suppressed by Si-BTBD7 in HCCLM3 cells (Supporting Fig. 3A). Conversely, ectopic expression of pcDNA3.1-BTBD7 markedly stimulated invasion and migration of L02 cells (\( P = 0.021 \); Fig. 2A and Supporting Fig. 3B). Neither BTBD7 depletion in HCCLM3 cells nor BTBD7 transfection in L02 cells affected (1) cell viability and colony forming, or (2) cell cycle and apoptosis (data not shown).

**BTBD7 Promoted HCC Metastasis and Angiogenesis In Vivo.** Compared to that in the Si-Control group, BTBD7 knockdown resulted in significantly decreased tumor size (2.33 ± 0.64 versus 1.23 ± 0.29 cm²; \( P < 0.05 \)), intrahepatic metastatic nodules (14.0 ± 2.1 versus 5.0 ± 3.2; \( P < 0.001 \); Fig. 2D), and pulmonary metastatic nodules (54.6 ± 4.8 versus 19 ± 3.5; \( P < 0.01 \); Fig. 2E). In transplanted and intrahepatic metastatic tumors, BTBD7 staining and MVD decreased significantly in the Si-BTBD7 group, as compared to that in the Si-Control group (Supporting Fig. 4). However, in transplantation tissues, no significant association was found between BTBD7 expression and the cell proliferation marker, Ki67, or apoptosis index (data not shown).

**BTBD7 Facilitated Invasion and Metastasis of HCC by EMT.** Having the highest metastatic potential, HCCLM3 cells are characterized by specific expression patterns of EMT markers, including E-cadherin and fibronectin.14 Intriguingly, BTBD7 depletion in HCCLM3 cells inhibited the formation of typical pipe-like structure within the Matrigel, whereas BTBD7-overexpressing L02 cells changed from an epithelial to a fibroblastic-like morphology (Fig. 2B).

BTBD7 modulation exhibited no significant effects on mRNA expression of albumin, hepatocyte nuclear factor 4 (HNF-4), P450, or \( \beta \)-catenin in both HCCLM3 and L02 cells (Supporting Fig. 5). However, BTBD7-depleted HCCLM3 cells showed an up-regulated cohesive epithelial marker of E-cadherin and down-regulated mesenchymal markers of fibronectin and Twist1. Conversely, BTBD7-transfected L02 cells exhibited decreased E-cadherin, but increased fibronectin expression (Fig. 2C). Moreover, these EMT-associated changes in protein expression were also detected in transplanted tumors and intrahepatic metastasis lesions of nude mice (Supporting Fig. 4).

**BTBD7 Promotes HCC Metastasis by Enhancing Activity of RhoC and MMPs.** Mechanisms of cancer cell invasion involve actin polymerization-dependent mediators of cell migration.15 F-actin polymerization and stress fiber disassembly was reduced in BTBD7-depleted
HCCLM3 cells, whereas L02 cells ectopically expressing BTBD7 exhibited increased actin cytoskeleton rearrangements (Fig. 3A). Activation of GTPase plays a critical regulatory role in actin cytoskeleton rearrangements in HCCLM3 cells. BTBD7-depleted HCCLM3 cells exhibited impaired RhoC GTPase activity (fold ratio = 0.19), but normal Cdc42, Rac1, and RhoA, as compared to control, whereas RhoC remained unchanged. Conversely, enhanced RhoC GTPase activity was noted in BTBD7-transfected L02 cells, compared to controls (fold ratio = 2.56; Fig. 3B).

MMP-9 and MMP-2 activation of RhoC can trigger production of angiogenic switch molecules and matrix remodeling. MMP-2 and MMP-9 production was reduced after BTBD7 silencing in HCCLM3 cells, but was increased after BTBD7 overexpression in L02 cells (Fig. 3C). RhoC-overexpressing BTBD7-depleted HCCLM3 cells were similar to cells in the Si-Control group in terms of actin cytoskeleton rearrangements, cell motility, and invasion (Fig. 3D,E), suggesting that RhoC and MMP-2/9 are likely downstream effectors of BTBD7 in HCC cells.

Fig. 1. Characteristics of BTBD7 expression in 78 HCC specimens from training cohort 1. (A) BTBD7 mRNA expression in HCC tissues is higher than that in ANLT. Patients with recurrence exhibit higher BTBD7 mRNA expression in tumor tissues (T) than those without recurrences. Intrahepatic metastatic lesions (ML) exhibit higher BTBD7 mRNA levels than primary lesions (PL). (B) Representative IHC staining of BTBD7 protein in liver tissues. a: NL; b: T and ANLT; c: PL; d: ML (×400). (C and D) Relationship between BTBD7 and RhoC, Rock2, and FAK protein expression in paired T and ANLT, PL and ML, and NL tissues. α-Tubulin is used as an internal loading control. NL, ANLT, T, PL, ML, R, and NR represent normal liver tissue, adjacent nontumorous liver tissue, tumor tissue, primary lesion, metastasis lesion, HCC with recurrence, and HCC without recurrence, respectively (unless otherwise indicated). *P < 0.05; **P < 0.01; ***P < 0.001, unless otherwise indicated.
Signaling Pathways Involved in HCC Invasion Induced by BTBD7. In BTBD7-depleted HCCLM3 cells, ectopic expression of RhoC significantly increased mRNA and/or protein expression of Rock2, phosphorylated Rock2, FAK, and phosphorylated FAK (Fig. 4A,B). To clarify the roles of Rock2 and FAK in BTBD7-regulated signaling, we treated RhoC-overexpressing Si-DTBD7 HCCLM3 cells with either the Rock2-specific inhibitor (TAE226) or the FAK-specific inhibitor (H1152). Inhibition of Rock2 or FAK significantly blocked RhoC-induced invasiveness of Si-DTBD7 HCCLM3 cells (Fig. 4C). Although RhoC transfection markedly increased MMP-2 and MMP-9 expression in Si-DTBD7-infected HCCLM3 cells (Fig. 4A,B), inhibition of Rock2 and FAK signaling significantly suppressed MMP-2 and MMP-9 activities (Fig. 4D). IHC staining was performed on serial sections of transplantation tumors and intrahepatic metastasis lesions in nude mice. Protein expression of RhoC, Rock2, FAK, MMP-2, and MMP-9 was significantly lower in the Si-BTBD7 group than that in the Si-Control group (Fig. 4E), thereby implicating BTBD7 as an upstream regulator of these important HCC invasiveness-related signaling molecules.

Correlations Between BTBD7 Expression Combined With MVD and HCC Prognosis. In both training cohort 2 and validation cohort 3, positive BTBD7 expression rate was significantly higher in HCC tissues than that in ANLT (79.7% versus 21.5% and 82.6% versus 18.7%, \( P < 0.001 \), respectively). BTBD7 staining was mainly located in the nuclei, and expression was graded as low and high according to the percentage of positive-staining hepatocytes. MVD count determined by CD34 staining (range, 0.0-554.2; median = 65.5) was classified as low (\(<65.5\)) and high (\(>65.5\)) using the median value. The

| Clinicopathologic Variables                  | n  | BTBD7 Expression mRNA* | Protein mRNA | Protein | P Value |
|---------------------------------------------|----|------------------------|--------------|---------|---------|
| Age, years                                  |    |                        |              |         |         |
| ≤60                                         | 50 | 2.49 ± 0.27            | 0.92 ± 0.21  | 0.617   | 0.794   |
| >60                                         | 28 | 1.96 ± 0.12            | 1.15 ± 0.12  | 0.452   | 0.656   |
| Sex                                         |    |                        |              |         |         |
| Male                                        | 67 | 3.05 ± 0.29            | 0.93 ± 0.22  | 0.438   | 0.463   |
| Female                                      | 11 | 2.56 ± 0.37            | 1.08 ± 0.33  | 0.637   | 0.584   |
| HBSAg                                       |    |                        |              |         |         |
| Negative                                    | 6  | 2.32 ± 0.34            | 0.89 ± 0.15  | 0.513   | 0.714   |
| Positive                                    | 72 | 2.93 ± 0.36            | 0.96 ± 0.26  | 0.059   | 0.067   |
| Albumin, g/L                                |    |                        |              |         |         |
| ≤35                                         | 9  | 1.73 ± 0.22            | 0.76 ± 0.14  | 0.068   | 0.093   |
| >35                                         | 69 | 1.96 ± 0.17            | 0.72 ± 0.15  | 0.023   | 0.106   |
| AFP, ng/mL                                   |    |                        |              |         |         |
| ≤20                                         | 22 | 1.69 ± 0.26            | 0.83 ± 0.16  | 0.034   | 0.026   |
| >20                                         | 56 | 2.03 ± 0.43            | 1.02 ± 0.22  | 0.006   | 0.004   |
| Child-Pugh classification                    |    |                        |              |         |         |
| A                                           | 72 | 2.36 ± 0.31            | 0.89 ± 0.14  | 0.002   | 0.116   |
| B                                           | 6  | 1.97 ± 0.35            | 0.91 ± 0.23  | 0.077   | 0.062   |
| Liver cirrhosis                             |    |                        |              |         |         |
| Absence                                     | 10 | 1.92 ± 0.36            | 0.95 ± 0.33  | 0.059   | 0.067   |
| Presence                                    | 68 | 2.45 ± 0.29            | 0.76 ± 0.27  | 0.077   | 0.062   |
| Tumor encapsulation                         |    |                        |              |         |         |
| Absence                                     | 46 | 2.89 ± 0.32            | 1.03 ± 0.15  | 0.006   | 0.004   |
| Presence                                    | 32 | 3.46 ± 0.37            | 0.77 ± 0.19  | 0.002   | 0.003   |
| Tumor size, cm                              |    |                        |              |         |         |
| ≤5                                          | 29 | 1.99 ± 0.33            | 0.89 ± 0.37  | 0.012   | 0.019   |
| >5                                          | 49 | 2.73 ± 0.38            | 0.79 ± 0.26  | 0.002   | 0.116   |
| Tumor number                                |    |                        |              |         |         |
| Single                                      | 38 | 1.89 ± 0.21            | 0.75 ± 0.18  | 0.059   | 0.026   |
| Multiple                                    | 40 | 2.36 ± 0.16            | 1.05 ± 0.22  | 0.006   | 0.004   |
| Satellite nodules                           |    |                        |              |         |         |
| Absent                                      | 30 | 1.93 ± 0.26            | 0.73 ± 0.19  | 0.002   | 0.003   |
| Present                                     | 48 | 2.83 ± 0.25            | 1.06 ± 0.25  | 0.002   | 0.003   |
| Venous invasion                             |    |                        |              |         |         |
| Absent                                      | 45 | 1.94 ± 0.19            | 0.77 ± 0.16  | 0.002   | 0.003   |
| Present                                     | 33 | 2.89 ± 0.22            | 1.02 ± 0.23  | 0.002   | 0.003   |
| Tumor differentiation                       |    |                        |              |         |         |
| I–II                                        | 52 | 2.31 ± 0.18            | 1.05 ± 0.37  | 0.077   | 0.062   |
| III–IV                                     | 26 | 2.67 ± 0.23            | 1.12 ± 0.35  | 0.002   | 0.003   |
| MVD\(^{†}\)                                 |    |                        |              |         |         |
| ≤65.5                                       | 47 | 2.56 ± 0.17            | 0.69 ± 0.33  | 0.029   | 0.034   |
| >65.5                                       | 31 | 3.09 ± 0.32            | 1.01 ± 0.39  | 0.001   | 0.003   |
| BCLC stage                                  |    |                        |              |         |         |
| A                                           | 36 | 2.04 ± 0.16            | 0.99 ± 0.18  |         |         |
| B–C                                         | 42 | 3.62 ± 0.34            | 1.06 ± 0.49  | 0.002   | 0.003   |
| TNM stage                                   |    |                        |              |         |         |
| I                                           | 40 | 1.85 ± 0.21            | 0.84 ± 0.16  | 0.002   | 0.003   |
| II–III                                      | 38 | 2.76 ± 0.22            | 1.01 ± 0.32  | 0.002   | 0.003   |

Abbreviation: HBSAg, hepatitis B surface antigen.

\*ΔCt = 1 × 10^{-2}.

\(^{†}\)Considering the data of MVD count do not fit the normal distribution, the median value of 65.5 (but not the mean value) is used as the cutoff for low and high MVD.
patients were subsequently divided into low-, medium-, and high-risk groups based on the combination of BTBD7 expression with MVD count (Fig. 5A).

Immuno-scoring (IS) of BTBD7 in HCC tumor tissues was significantly different among three different TNM stages (Fig. 5B). High BTBD7-expressing groups exhibited significantly higher MVD, as compared with low BTBD7-expressing groups (175.6 ± 15.5 versus 52.5 ± 14.4 and 156.7 ± 33.6 versus 47.3 ± 12.2, P < 0.01, respectively; Fig. 5C). Moreover, there was a significant association between BTBD7 protein expression and the presence of satellite nodules, venous invasion, MVD, BCLC, and TNM stage (Supporting Table 4). Furthermore, a significant correlation was indentified between BTBD7 expression and CD34 (representing MVD) in both cohorts (r = 0.656 and 0.712, P < 0.01, respectively).

Based on the importance of BTBD7 and MVD in HCC angiogenesis, BTBD7 expression combined with MVD was evaluated as an independent predictor of OS and RFS in cohort 2 (Table 3). The 1-, 3-, and 5-year OS rates in the high-risk group were significantly lower than those in the low-risk group (61.2% versus 90.7%, 35.6% versus 74.9%, and 19.4% versus 69.8%, respectively). The ratio of recurrence after surgery in high-, medium-, and low-risk groups, respectively, was 58/127 (45.7%), 42/145 (29.0%), and 5/54 (9.3%). The 1-, 3-, and 5-year RFS rates were
markedly lower in the high-risk group than those in the low-risk group (55.2% versus 84.7%, 21.6% versus 74.8%, and 5.6% versus 70.3%, respectively). Differences in OS and RFS were significant, as compared with each other, in all three groups ($P < 0.001$; Fig. 5D).

The value of BTBD7 integrated with MVD as a clinical predictor of HCC prognosis was subsequently evaluated in another independent cohort. In the validation cohort 3, 43 of 112 patients (38.4%) were categorized as high risk, 50 of 112 patients (44.6%) as medium risk, and 19 of 112 patients (17.0%) as low risk. The 1-, 3-, and 5-year OS rates in the high-risk group were significantly lower than those in the low-risk group (59.7% versus 94.3%, 34.7% versus 89.6%, and 30.5% versus 89.6%, respectively). In the high-, medium-, and low-risk groups, HCC patients developed recurrence after surgery in 21 of 43 (48.8%), 15 of 50 (30.0%), and 2 of 19 (11.7%) cases, respectively. The 1-, 3-, and 5-year RFS rates were markedly lower in the high-risk group, compared to the low-risk group (45.3% versus 89.9%, 11.7% versus 90.3%, and 9.8% versus 90.2%, respectively). There was a significant difference in OS and RFS...
between each of the two groups ($P < 0.01$; Fig. 5E). The predictive value of BTBD7 integrated with MVD was consistent between training cohort 2 and validation cohort 3.

**Discussion**

BTBD7 protein is linked to focal extracellular matrix (ECM) formation through its local regulation of E-cadherin and cell motility. Here, we show that BTBD7 expression was greatest in HCC cells having the highest metastatic potential: HCCLM3 cells. HCCLM3 cells required BTBD7 expression to maintain their characteristic mobility and invasiveness. Depletion of endogenous BTBD7 expression in HCCLM3 cells suppressed cell migration and invasion, whereas ectopic BTBD7 expression in immortalized hepatocytes L02 had the opposite effect. These findings implicate BTBD7 in HCC invasion and metastasis.

We found that BTBD7 depletion could cause a rapid regression of EMT features, but had no significant effect on proliferation of HCCLM3 cells. Importantly, an animal model confirmed that BTBD7 was associated with tumor invasion and MVD formation. Moreover, these findings were mirrored in human HCC samples in which BTBD7 expression was significantly increased in primary and recurrent HCC tissues. The focal overexpression of BTBD7 was correlated with frequent tumor venous invasion, which is a central pathological feature of intrahepatic metastasis in HCC. Although BTBD7 overexpression was previously shown to promote Bel7404 cell proliferation, Bel7404 are quite different from HCCLM3 cells in terms of BTBD7 abundance, invasion and metastasis abilities, and proliferation regulation.

EMT, which is characterized by down-regulation of the epithelial cell adhesion molecule, E-cadherin, and up-regulation mesenchymal markers fibronectin and Twist1, is associated with enhanced cell motility, invasive phenotypes, and, consequently, metastasis in human malignancies. In HCC, E-cadherin, fibronectin, and Twist1 are intensively involved in various steps of metastasis, such as loss of cell-cell/cell-ECM adhesion, invasion of ECM, and angiogenesis. Here, we show that knockdown of BTBD7, which acted as an upstream activator of EMT, induced E-cadherin expression, but restrained fibronectin and Twist1 expression in HCC cells, thereby suppressing metastasis and recurrence.
The processes characterizing EMT, which include cytoskeleton rearrangement, production of matrix-remodeling factors, and epithelial cell migration out of epithelial sheets into adjacent stroma, are believed to contribute to cancer invasion and metastasis. The altered phenotypes of HCCLM3 and L02 cells induced by BTBD7 depletion or transfection prompted a potential mechanism of BTBD7-mediated cell motility. Interestingly, BTBD7 knockdown did not exert any significant effects on the expression of β-catenin and HNF-4, which are important factors in hepatocyte differentiation and proliferation.

We have previously shown that RhoC promotes HCC metastasis through enhancement of cell-matrix deadhesion, stress fiber generation, and microvessel formation. In the present study, exogenous RhoC expression in BTBD7-depleted HCCLM3 cells produced increased expression of BTBD7, Rock2, phosphorylated Rock2, FAK, phosphorylated FAK, MMP-2, and MMP-9. Inhibition of either Rock2 or FAK significantly repressed activities of MMP-2 and MMP-9, and blocked the RhoC-induced invasiveness of Si-DTBD7 HCCLM3 cells. RhoC, Rock2, FAK, MMP-2, and MMP-9 protein were widely distributed in both orthotropic transplantation tumors and intrahepatic metastasis lesions. Although we consider RhoC as a downstream molecular target of BTBD7, RhoC promotes BTBD7 expression, in turn, through a kind of positive-feedback process. Our results suggest that BTBD7 can activate a RhoC-Rock2-FAK-signaling cascade, which is involved in HCC cell motility, microvessel formation, and tumor progression.

HCC patients with high MVD, a marker of angiogenesis in tumor tissues, normally have a poor prognosis. The association study of BTBD7 and clinicopathologic characteristics revealed that BTBD7 expression was significantly correlated with venous invasion of HCC (a widely accepted marker of HCC.

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**Fig. 5.** BTBD7 expression in combination with MVD count is a powerful independent predictor of poor clinical outcome in HCC. (A) Classification of BTBD7 nuclear staining and MVD in HCC tissues. (a) 1+: 0%-25%, (b) 2+: 26%-50%, (c) 3+: 51%-75%, (d) 4+: 76%-100% positive-staining cells. 1+ and 2+ are considered as low, whereas 3+ and 4+ are considered as high BTBD7 expression. (e) low (≤65.5) and (f) high (>65.5) MVD count (range: 0.0-554.2; median = 65.5). Patients are divided into three risk groups: I: low risk (BTBD7low/MVDlow); II: medium risk (BTBD7low/MVDhigh or BTBD7high/MVDlow); and III: high risk (BTBD7high/MVDhigh). (B) Comparison of BTBD7 IS of HCC tissues among three different TNM stages in training cohort 2 and validation cohort 3. (C) High-BTBD7-expression groups have higher MVD in HCC tissues than low-BTBD7-expression groups in cohorts 2 and 3. (D) OS and RFS of HCC patients in training cohort 2. (E) Evaluation of BTBD7 expression combined with MVD in validation cohort 3 as an independent predictor of poor HCC clinical outcome.
Table 3. Univariate and Multivariate Analysis by Cox's Proportional Hazards Regression Model in Training Cohort 2

| Variable                                      | OS (Univariate) | OS (Multivariate) | RFS (Univariate) | RFS (Multivariate) |
|-----------------------------------------------|-----------------|-------------------|------------------|--------------------|
|                                               | HR (95% CI)     | P Value           | HR (95% CI)      | P Value            |
| Gender (male versus female)                   | 1.013 (0.723-1.105) | 0.112             | NA               | 1.068 (0.887-1.260)  | 0.102 |
| Age, years (>60 versus ≤60)                   | 1.202 (0.974-1.436) | 0.064             | NA               | 1.134 (0.848-1.543)  | 0.095 |
| HBsAg (positive versus negative)              | 1.154 (0.874-1.457) | 0.088             | NA               | 1.019 (0.716-1.488)  | 0.275 |
| Albumin, g/L (≥35 versus >35)                 | 1.213 (0.832-1.695) | 0.076             | NA               | 1.218 (0.939-1.571)  | 0.075 |
| Child-Pugh classification (B versus A)        | 1.588 (1.287-1.992) | 0.018             | 1.054 (0.893-1.473) | NS                 |
| Liver cirrhosis (presence versus absence)     | 1.386 (1.173-1.624) | 0.025             | 1.262 (0.922-1.532) | NS                 |
| Serum AFP level, ng/mL (>20 versus ≤20)       | 1.232 (1.945-2.829) | 0.003             | 1.373 (0.967-1.915) | NS                 |
| Tumor diameter, cm (>5 versus ≤5)             | 1.715 (1.384-2.037) | 0.009             | 1.087 (0.901-1.512) | NS                 |
| Tumor number (multiple versus single)         | 1.653 (1.205-1.972) | 0.022             | 1.875 (1.397-2.396) | 0.005              |
| Tumor encapsulation (none versus complete)    | 1.354 (1.090-1.587) | 0.041             | 1.137 (0.715-1.464) | NS                 |
| Venous invasion (presence versus absence)     | 3.123 (2.433-4.054) | <0.0001           | 2.261 (1.507-3.132) | 0.003              |
| Tumor differentiation (II/IV versus I/II)     | 2.415 (1.986-2.813) | <0.0001           | 1.206 (0.761-1.695) | NS                 |
| Satellite nodules (presence versus absence)   | 1.814 (1.401-2.221) | 0.006             | 1.794 (1.237-2.191) | 0.016              |
| BCLC stage (B + C versus A)                   | 1.679 (1.375-1.996) | 0.013             | 2.557 (2.092-1.962) | 0.004              |
| TNM stage (II/III versus I)                   | 1.729 (1.332-2.117) | 0.008             | 1.645 (1.137-2.067) | 0.015              |
| BTBD7 expression level (high versus low)      | 2.916 (2.368-4.153) | <0.0001           | 3.987 (2.880-4.975) | <0.0001            |
| MVD (>65.5 versus ≤65.5)                      | 2.252 (1.856-2.734) | 0.004             | 3.152 (2.613-4.016) | <0.0001            |
| Combination of BTBD7 and MVD*                 |                 |                   |                  |                    |
| II versus I                                   | 2.423 (2.035-3.024) | <0.0001           | 3.320 (2.153-4.176) | <0.0001            |
| III versus I                                  | 4.817 (2.367-5.138) | <0.0001           | 5.563 (3.928-7.357) | <0.0001            |
| III versus II                                 | 1.703 (1.307-2.176) | 0.011             | 2.682 (1.219-4.23) | <0.0001            |

Abbreviations: HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; NA, not available; NS, not significant.

*I (low-risk group): BTBD7<sub>L</sub>/MVD<sub>L</sub>; II (medium-risk group): BTBD7<sub>II</sub>/MVD<sub>L</sub> or BTBD7<sub>L</sub>/MVD<sub>II</sub>; III (high-risk group): BTBD7<sub>H</sub>/MVD<sub>II</sub>.
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