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MTBP inhibits migration and metastasis of hepatocellular carcinoma

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Abstract

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Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide with increasing incidence. Despite curative surgical resection and advanced chemotherapy, its survival rate remains low. The presence of microvascular invasion and occult metastasis is one of the major causes for this poor outcome. MDM2 Binding Protein (MTBP) has been implicated in the suppression of cell migration and cancer metastasis. However, clinical significance of MTBP, particularly in human cancer, is poorly understood. Specifically, clinical relevance of MTBP in human HCC has never been investigated. Here we demonstrated that expression of MTBP was significantly reduced in human HCC tissues compared to adjacent non-tumor tissues. MTBP expression was negatively correlated with capsular/vascular invasion and lymph node metastasis. Overexpression of MTBP resulted in the suppression of the migratory and metastatic potential of HCC cells, while its downregulation increased the migration. Consistent with the previous report, MTBP endogenously bound to alpha-actinin 4 (ACTN4) and suppressed ACTN4-mediated cell migration in multiple HCC cell lines. However, MTBP also inhibited migratory potential of PLC/PRF/5 HCC cells whose migration was not altered by manipulation of ACTN4 expression. These results suggest that mechanisms behind MTBP-mediated migration suppression may not be limited to the pathway involving ACTN4 in certain cellular contexts. Additionally, as a potential mechanism for reduced MTBP expression in tumors, we found that MTBP expression was increased following the treatment with histone deacetylase inhibitors (HDIs). Our study, for the first time, provides clinical relevance of MTBP in the suppression of HCC metastasis.

Keywords

Hepatocellular carcinoma; MTBP; ACTN4; Migration; Metastasis

Introduction

The ability of cancer cells to metastasize is the leading cause of poor prognosis in cancer patients. Metastasis involves multiple steps [1], and thus the mechanisms underlying metastasis appear complicated and remain unsolved. Identifying factors participating in the metastasis suppression would significantly contribute to the improvement of prognosis in patients with cancer.

Hepatocellular carcinoma (HCC) is one of the most common types of cancer. Despite advances in the treatment of HCC, 60–100 % of the patients suffer from recurrence, and the 5-year survival rate remains 30–60 % even after curative resection or treatments [2, 3]. Although only a minority of HCC shows macro-metastasis, one of the major causes for this poor outcome is microvascular invasion and occult metastasis [4–6]. Understanding the mechanisms behind metastasis suppression in HCC would greatly help in the discovery of novel strategies targeting HCC and other types of cancer.

We previously demonstrated that MDM2 Binding Protein (MTBP) functions as a metastasis suppressor. MTBP inhibited invasion and migration of several osteosarcoma cells and mouse embryonic fibroblasts (MEFs) regardless of their p53 status [7, 8]. Using orthotopic (intra-femoral) tumor cell transplantation models, overexpression of MTBP in multiple p53-mutated osteosarcoma cell lines significantly reduced the number of metastatic pulmonary

nodules with little effect on primary tumor growth [8]. In mice with a deletion of the *MTBP* gene, we found that *MTBP* haploinsufficiency significantly increased metastasis of HCC, sarcoma, and other types of cancer without affecting loss of heterozygosity (LOH) of the *p53* allele [7]. MTBP also inhibited migration of MEFs null for both *p53* and *Mdm2* [8]. Thus, MTBP suppresses cell migration and metastasis in a p53-independent manner.

Furthermore, we recently identified α-actinin-4 (ACTN4) as an MTBP-interacting protein by performing co-immunoprecipitation (co-IP) and mass spectrometry [8]. ACTN4 is an actin-crosslinking protein that promotes filopodia/microspike formation, migration, and metastasis of many cancer types [9–11]. Endogenous MTBP interacted and partially colocalized with ACTN4 [8]. MTBP inhibited not only actin-crosslinking function of ACTN4 in vitro, but also ACTN4-mediated filopodia formation and migration in osteosarcoma cells [8]. Thus, MTBP suppresses cell migration and filopodia formation by inhibiting ACTN4 function. However, it remains unclear whether or not MTBP inhibits cell migration solely through inhibition of ACTN4.

Clinically, reduced MTBP expression in head and neck carcinoma was associated with reduced patient survival, and MTBP expression levels served as an independent prognostic factor in tumors having p53 mutation [12]. On the other hand, MTBP was found to be overexpressed in B-cell lymphoma and triple negative breast cancer where MTBP contributed to tumor progression by cooperating with Myc [13–15]. These observations suggest that MTBP plays an important role in tumor progression but the clinical relevance of MTBP in human cancer may be dependent on types of cancer. In this study, we attempted to determine clinical and functional significance of MTBP in HCC. We demonstrated that reduced MTBP expression was associated with capsular/vascular invasion and lymph node metastasis in human HCC tissues. Also, MTBP expression was negatively correlated with migratory potential of HCC cells.

Materials and methods

Patients and tissue samples

We obtained archived formalin-fixed, paraffin-embedded samples from 102 HCC patients who underwent primary HCC resection between January 2005 and August 2008 at the Department of General Surgery in Xijing hospital, Xi'an, China. The patients ranged from 12 to 79 years old, with a mean age of 50.12 ± 15.02 years. No patient received preoperative radiation therapy or chemotherapy. The histopathological features were assessed according to the WHO classification system [16] and the cancer staging criteria set by the International Union Against Cancer/ Union International Contre le Cancer (UICC) [17]. Separately, for quantitative RT-PCR (qRT-PCR), another 20 paired surgical specimens of HCC and adjacent non-tumor liver tissues were obtained from patients who received primary HCC resection at the Xijing hospital. Fresh specimens were immediately frozen in liquid nitrogen after surgical removal and stored at −80 °C until the analysis. The study was approved by the Hospital's Protection of Human Subjects Committee, and informed consent was obtained from all patients (#XJYYLL-2008402).

Cell lines

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS in a humidified incubator at 37 °C and 5 % CO2. Following are cell lines used in this study, and their p53 status are described in the parenthesis: Huh7 (Y220C); SNU-475 (N146D/N239D/ G262D); HepG2 (wild-type); PLC/PRF/5 (R249S); SK-hep-1 (wild-type); SNU-449 (K139R/A161T); Hep3B (null).

Tumor transplantation assays

For tail vein injection assays, cells (500,000) in 50 μl of Hank's balanced salt solution (HBSS) were injected into lateral tail veins of nude mice (Harlan, Indianapolis, IN, USA). For subcutaneous injections, cells (1,000,000) were suspended in 4.5 mg/mL growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA) in HBSS and injected into the flank of nude mice. Mice were monitored daily for the development of tumors and signs of labored breathing or lethargy. Mice injected with empty vector-infected cells (control) and those with MTBP-overex-pressing cells were killed on the same day. The sizes of subcutaneous tumors were measured in three dimensions, while the number of metastatic pulmonary nodules (>0.5 mm) were determined by gross examination at a full necropsy. Mice were maintained under specific pathogen-free conditions, and experimental procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee.

Antibodies

For western blotting, antibodies for rabbit polyclonal anti-ACTN4 (210-356-C050, Enzo Life sciences, Plymouth Meeting, PA, USA), mouse monoclonal anti-ACTN4 (sc-134236, Santa Cruz Biotechnology) goat polyclonal anti-MTBP (K20, Santa Cruz Biotechnology, Dallas, Texas, USA), mouse monoclonal anti-MTBP (B-5, sc-137201), mouse monoclonal anti-β-tubulin (sc-55529, Santa Cruz Biotechnology), rabbit polyclonal anti-GAPDH (sc-27117, Santa Cruz), rabbit polyclonal anti-actin (AAN01, Cytoskeleton, Denver, CO, USA), mouse monoclonal anti-β-actin (A5441, Sigma, St. Louis, MO, USA), and mouse monoclonal anti-β-tubulin (T0198, Sigma, St. Louis, MO, USA) were used. For immunohistochemistry (IHC), rabbit anti-MTBP (N-13, sc-47174, Santa Cruz Biotechnology) was used. For immunoprecipitation studies, rabbit monoclonal anti-ACTN4 (GTX62422, GeneTex Inc., Irvine, CA) and rabbit polyclonal anti-MTBP (hC2) were used. The hC2 antibody was generated against C-terminal region of human MTBP as previously described [8].

Immunohistochemistry (IHC)

Sections (4 μm thick) from specimens that were formalin-fixed and paraffin-embedded were processed as described previously [18]. Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide for 30 min followed by heating in a microwave oven for epitope retrieval. The sections were then blocked in 10 % normal horse serum and 0.3 % Triton X-100 in PBS for 1 h and incubated overnight at 4° C with goat anti-human MTBP polyclonal antibody (diluted 1:500, N-13, sc-47174). The slides were washed in PBS 3 times for 5 min each. The tissues were incubated in biotin-labeled donkey anti-goat secondary

antibody for 30 min and then rinsed with PBS. The signal was detected using 3,3-diaminobenzidine as the chromogen. Negative control slides using normal goat immunoglobulin G (IgG, Vector Labs, Burlingame, CA, USA) were included in all of the assays. All stained sections were blindly evaluated by two independent investigators. Scoring was based on intensity and extensity. The percentage of positive tumor cells was determined semi-quantitatively by assessing the whole tumor section, and each sample was scored on a scale of 0–3 with 0 corresponding to less than 25 % of positive tumor cells; 1 to 26–50 %; 2 to 51–75 %; and 3 to 76–100 %. The intensity of immunostaining was determined as 0 (negative staining), 1 (weakly positive staining), 2 (moderately positive staining), and 3 (strongly positive staining). The immunoreactive score of each section was calculated by the average of these two parameters and presented 0–1 vs 2–3 similar to the previous report [18].

Filopodia assays

Cells were plated onto poly-D-lysine/laminin-coated glass coverslips (BD Biosciences), fixed, and permeabilized with 4 % formaldehyde in 100 mM PIPES (pH6.8), 10 mM EGTA, 1 mM $MgCl_2$, and 0.2 % Triton-X 100 for 15 min at room temperature. Following phosphate buffered saline (PBS) washing, cells were blocked in 1 % BSA in PBS plus 0.1 % Tween 20 (PBS-T) for 30 min and further incubated with rhodamine-phalloidin (Invitrogen, San Diego, CA, USA). Samples were mounted in the ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA, USA), and examined for filopodia formation under a Nikon epifluorescence microscope (Nikon, Melville, NY, USA).

In vitro cell migration assays

Migration assays were performed using 24-well transwell chambers (6.5 mm diameter, 8 mm pore size; Corning Inc., Corning, NY, USA). MTBP was overexpressed using an *MTBP*-encoding adenoviral vector or downregulated using a human *MTBP*-specific siRNA [8]. ACTN4 was overexpressed with a lentiviral vector encoding human *ACTN4* cDNA and downregulated using a lentiviral vector encoding an *ACTN4*-specific short hairpin (sh) RNA [8]. Empty vectors were used as controls. Cells (10,000) in 100 μl of 0.2 % fetal bovine serum-containing DMEM was added into the upper compartment of the chamber, while 10 % fetal bovine serum in DMEM was added to the lower compartment as chemoattractant. Cells were then allowed to migrate across the membrane by incubating at 37 °C in CO_2 incubator for 14 h. The non-migrating cells were removed from the upper face of the filters using cotton swabs, while migrating cells to the lower face of the filters were fixed and stained with Diff-Quik Stain Set (Dade Behring, Newark, DE, USA). Stained cells in the entire fields were counted under an inverted microscope.

qRT-PCR

Total RNAs from human tissues were isolated using Trizol reagent (Life Technologies, Carlsbad, CA) and reverse transcribed with the Reverse Transcription Kit (TaKaRa) starting with 2 μg total RNA from each sample according to the manufacturer's instructions. The mRNAs expression of *MTBP* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* were analyzed by qRT-PCR using a real-time LightCycler rapid thermal cycler (Roche

Molecular Biochemicals LightCycler System). Specific *MTBP* primers: 5′-ACTGAGAATCAGCCGGACTT-3′ (forward) and 5′-C TGCACTGCAAAGAACCACT-3′ (reverse), as well as internal control *GAPDH* primers: 5′-GCACCGTCAAGGC TGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAG TGGAT-3′ (reverse), were used for qRT-PCR. The *MTBP* mRNA levels were normalized to those of *GAPDH*. For human *MDM2* mRNA quantification, we used TaqMan gene expression assays (Hs00242813_m1, Life Technologies).

Co-immunoprecipitation (co-IP)

Cells were lysed with Cell Lytic M (Sigma) buffer containing protease inhibitor cocktail. Approximately 200 μg of whole cell lysates were incubated with protein-specific antibodies overnight at 4 °C, followed by precipitation of the antibody-protein complex using protein A/G plus-agarose (Santa Cruz Biotechnology). In each experiment, an isotype negative control was used. After washing with Cell Lytic M buffer, precipitates were analyzed by western blotting.

Statistical analysis

Experiments were performed independently at least three times with values expressed as mean ± standard deviation (S.D.). For differences between groups in the analyses of qRT-PCR using clinical samples, Mann–Whitney *U* test was used, while for correlation between clinical features and MTBP IHC results, Chi square tests were used. All other experimental results were analyzed using Student's *t* test. Statistical analysis was performed with Graph Pad Prism software (San Diego, CA, USA). In each analysis, values of $p < 0.05$ were considered to be statistically significant.

Results

Reduced MTBP expression is associated with capsular/vascular invasion and lymph node metastasis in HCC

To investigate clinical relevance of MTBP in HCC, *MTBP* mRNA expression relative to *GAPDH* (control) was compared between 20 pairs of HCC and adjacent non-tumor liver tissues. Quantitative RT-PCR (qRT-PCR) results demonstrated that mRNA expression of *MTBP* in HCC was significantly lower than that in adjacent non-tumor liver tissues (Fig. 1a).

Next, we performed IHC for MTBP using 102 paraffin embedded HCC tissues. We validated the MTBP antibody for IHC using tissues where MTBP was knocked down with a corresponding reduction in the MTBP IHC signal (data not shown). Using the titers and conditions described in the Materials and methods, subcellular localization patterns were not readily distinguished (i.e., uniform instead of localized in specific cellular structures). Representative images of MTBP staining suggested that MTBP expression was lower than that in adjacent non-tumor tissues, consistent with the results of qRT-PCR (Fig. 1b). Summary of MTBP staining results together with clinicopathological parameter revealed that reduced MTBP protein expression in HCC tissues was correlated with the presence of capsular/vascular invasion and lymph node metastasis (Table 1).

MTBP inhibits metastasis and migration of HCC cells

Results above suggested that reduced MTBP expression was associted with HCC metastasis. We therefore examined whether overexpression of MTBP suppressed metastasis and migration of HCC cells. We first performed intravenous (tail vein) injections of SK-hep-1 cells and found that MTBP overexpression inhibited lung metastasis (Fig. 2a). We then confirmed that MTBP did not alter primary tumor growth by subcutaneous injection assays (Fig. 2b), supporting the previous studies demonstrating that MTBP functions as a metastasis suppressor [8].

Previous studies also demonstrated that MTBP suppresses migration of MEFs and osteosarcoma cells [7, 8]. We therefore examined effects of MTBP on the migratory potential of several HCC cell lines using transwell migration assays. Different levels of MTBP expression were obtained by infecting varying multiplicity of infections (MOIs) of an *MTBP*-encoding adenovirus. Approximately twofold increase in MTBP protein levels by 5 MOI significantly inhibited the migration of SNU-475 and Huh7 cells (Fig. 2c), while its downregulation increased the migratory potential of SNU-475 and PLC/PRF/5 cells (Fig. 2d). We confirmed that MTBP overexpression did not change cell proliferation during the course of the experiments (data not shown). Similar results were obtained in other HCC cell lines tested regardless of their p53 mutation status (wild-type: SK-hep-1; mutant: PLC/PRF/5, SNU-449; null: Hep3B; Supplementary Fig. S1). These results suggest that MTBP regulates migratory potential of HCC cells in a p53-independent manner, similar to other cellular types.

MTBP inhibits HCC cell migration through different mechanisms depending on cellular contexts

In our previous studies, we demonstrated that MTBP attenuates ACTN4-mediated filopodia formation and cell migration, using osteosarcoma cells [8]. We first confirmed that MTBP endogenously bound to ACTN4 in both Huh7 and PLC/PRF/5 HCC cells (Fig. 3a). We then tested the effects of MTBP overexpression on ACTN4 function in HCC cells. We found that MTBP overexpression inhibited ACTN4-mediated filopodia formation (Fig. 3b; lanes 2 and 4) and migration (Fig. 3c; lanes 2 and 4) of SNU-475 cells. It should be noted that overexpression of MTBP did not significantly alter the expression levels of ACTN4 (Fig. 3b; lanes 1 and 3). Coupled with our previously published data showing that MTBP inhibits actin bundling in vitro [8], the data suggest that MTBP alters ACTN4 function rather than its expression.

Since migration of all available HCC cell lines was inhibited by MTBP (Fig. 2, Supplementary Fig. S1), we also examined if migration of these HCC cells was altered by ACTN4. Overexpression of ACTN4 increased the migration of most of HCC cell lines examined, including SK-hep-1, Huh7, and SNU-449, whereas it failed to alter the migratory potential of PLC/PRF/5 cells (Supplementary Fig. S2a). Also, downregulation of ACTN4 decreased migratory potential of SNU-449 cells, but not PLC/PRF/5 cells (Supplementary Fig. S2b). These results suggest that effects of ACTN4 on HCC cell migration may vary with differences in cellular contexts.

Since migration of PLC/PRF/5 cells was not altered by ACTN4, yet still suppressed by MTBP, we hypothesized that MTBP suppressed migration of PLC/PRF/5 cells in an ACTN4–independent manners. To test this hypothesis, we simultaneously manipulated expression of MTBP and ACTN4 in PLC/PRF/5 cells, followed by migration assays. Overexpression of MTBP decreased migratory potential of PLC/PRF/5 cells, whether or not cells were overexpressed or downregulated for ACTN4 (Fig. 4a, b). Also, down-regulation of MTBP still increased migration of PLC/PRF/ 5 cells downregulated for ACTN4 (Fig. 4c). These results strongly suggest that MTBP inhibits migration of PLC/PRF/5 cells independent of ACTN4, and different mechanisms may be involved in the suppression of HCC cell migration by MTBP, depending on cellular contexts.

MTBP expression can be transcriptionally silenced in HCC

Mechanisms for reduced MTBP expression in HCC are unknown. Mutations and/or deletion of the human *MTBP* gene are rare events (http://cbioportal.org), suggesting mechanisms involved in the transcriptional or translational levels. To address this, several HCC cells were treated with inhibitors for histone deacetylases, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), or an inhibitor for DNA methylation, 5-aza-2′-deoxycytidine (5-aza-dC). Treatments with SAHA and TSA resulted in approximately twofold increase in mRNA and protein expression of MTBP in both Huh7 and SNU-475 HCC cell lines; whereas, the effects of 5-aza-dC were not obvious (Fig. 5a, b). We also tested the possibility of MTBP protein degradation via 26S and 20S proteasomes by treating Huh7 and PLC/PRF/5 HCC cell lines with proteasome inhibitors MG132 and MG115, respectively. These treatments did not substantially increase MTBP protein levels in either cell line (Fig. 5c). Thus, treatment of HCC cells with DNA methylation inhibitors and proteasome inhibitors did not significantly increase MTBP expression, whereas histone deacetylase inhibitors (HDIs) resulted in approximately twofold increase in MTBP expression. These results suggest that reduced MTBP expression in HCC can be due to epigenetic gene silencing. However, we cannot exclude the possibility that MTBP expression is regulated via other transcriptional and translational mechanisms.

To furthermore examine whether observed changes in the *MTBP* mRNA expression by the TSA treatment are dependent on MDM2, we downregulated MDM2 in p53-mutated HCC cell lines, Huh7 and Hep3B, using a lentiviral vector encoding an *MDM2*-specific shRNA (Open Biosystems, V2LHS_151656). These cells were then treated with TSA for 24 h and examined for *MTBP* mRNA expression (Fig. 5d). There was no difference in the *MTBP* mRNA expression between MDM2-expressing and MDM2-knockdown cells, and the treatment with TSA resulted in approximately twofold increase in the *MTBP* mRNA expression regardless of MDM2 status, suggesting the MDM2-independency of MTBP expression.

Discussion

The role of MTBP in tumor progression has been exemplified in multiple cancer types; however, clinical relevance of MTBP in human cancer is poorly investigated, and also whether it is suppressive or progressive for tumor progression remains controversial. In the

current study, we demonstrate the metastasis suppressive function of MTBP in HCC, where reduced expression of MTBP is observed in *70 % of human HCC tissues compared to adjacent non-tumor tissues and is correlated with local invasion and lymph node metastasis. MTBP expression levels are also negatively correlated with migratory potential of HCC cells regardless of their p53 status. These results showing the role of MTBP in metastasis suppression are consistent with the previous following two find-ings: (1) Vlatkovic et al. [12] show that reduced MTBP expression is correlated with poor prognosis in patients with head and neck carcinoma. (2) We demonstrate that MTBP suppresses osteosarcoma metastasis using an orthotopic mouse model [8]. On the other hand, in B-cell lymphoma and triple negative breast cancer, MTBP appears to be overexpressed and rather contributes to tumor progression by enhancing Myc function [13–15]. The paradox of data in different tissues leads us to conclude that there are context-specific issues that we cannot yet be fully defined. Numerous other examples have been reported in the literature regarding different activities of several proteins for tumor progression, depending upon tissues (e.g., KISS-1 is a metastasis suppressor in all other tissues except HCC, in which it is a metastasis-promoting gene) [19–21]. Further pertinent studies are required to completely understand the context-dependent nature of MTBP function.

Regulation of MTBP expression is poorly understood. Specifically, it is unknown how MTBP expression is regulated in tumor tissues. According to online databases (e.g., cBioPortal), mutations and/or deletions of the human *MTBP* genes are rare events. Based upon those data, we focus our attention on transcriptional regulation and proteasomal degradation in HCC cells. We show that HDI treatment of HCC cells significantly increases *MTBP* mRNA expression, whereas a DNA methylation inhibitor and proteasome inhibitors fail to do so. Additionally, we demonstrate that the effects of HDIs on MTBP expression are independent of MDM2. These results raise the possibility that *MTBP* mRNA expression is epigenetically silenced in tumors. However, there is still a possibility that MTBP expression is regulated via other transcriptional and translational mechanisms and is dependent on cellular contexts or types of cellular damages. Also, functional characterization of MTBP proteins harboring cancer-associated mutations is necessary to understand expression kinetics of MTBP mutants in tumors and if they still suppress cancer metastasis.

Migratory and metastatic potentials of cancer cells are regulated by multiple pathways. In this study, we demonstrate that MTBP inhibits migration of HCC cells, as well as pulmonary metastasis using intravenous (tail vein) injection assays. It is important to mention that an orthotopic/ spontaneous metastasis assay is conceptually ideal, but it is often technically fraught with artifacts. For example, the intrahepatic injection often results in leakage into the peritoneal cavity, which makes it challenging to ascribe metastases from the liver to lymph nodes or lungs. Additionally, ethical considerations require euthanasia prior to the time when metastasis development is observable. With these reasons, we choose to perform tail vein injections. In the future, a reliable and reproducible orthotopic metastasis assay for liver cancer needs to be developed.

Our current findings suggest the involvement of ACTN4 in the mechanisms behind MTBP-mediated migration suppression [8]. This is consistent with most of HCC cell lines examined in this study except for PLC/PRF/5, because MTBP suppresses migration of this

cell line even when ACTN4 is downregulated. These results suggest that MTBP may inhibit migration of PLC/PRF/5 cells in an ACTN4-independent manner. Moreover, other mechanisms for migration suppression by MTBP may exist depending on cellular contexts. Further investigations are required to identify novel metastasis-associated signaling pathways regulated by MTBP.

MTBP has been reported to have other biological functions in addition to metastasis suppression [8, 13, 22]. MTBP is involved in replication origin firing [23], mitotic checkpoint [22], and c-Myc activity [13, 14]. It is important to determine whether or not MTBP regulates cancer metastasis using some of the key factors involved in these cellular activities.

The functional correlation between MTBP and MDM2 remains unclear. Only a few reports demonstrate the functional association of these proteins [24, 25]. However, the majority of experiments in these studies are performed through overexpression of MTBP and/or MDM2 using a few specific cell lines. In addition, multiple studies rather show that MTBP does not alter the MDM2-p53 activity, suggesting that MTBP is not an upstream regulator of MDM2 in certain cellular contexts [7, 8, 13]. Conversely, it is possible that MDM2 inhibits MTBP function, since MDM2 overexpression induces cellular phenotypes similar to those induced by MTBP downregulation. These include increased metastatic potential [8, 26], enhanced chromosome instability [22, 27], and inhibited DNA replication origin firing [23, 28]. It would be critical to determine whether MDM2 antagonizes the MTBP functions or if MDM2 induces these cellular phenotypes independent of MTBP. Nonetheless, functional and expressional interactions between MTBP and MDM2, cellular context-dependent nature of MTBP function, and the underlying mechanisms need to be rigorously addressed as future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Chen X, Xu Z, Wang Y. Recent advances in breast cancer metastasis suppressor 1. Int J Biol Markers. 2011; 26(1):1–8. [PubMed: 21279956]
2. Guillot C, Hall J, Herceg Z, Merle P, Chemin I. Update on hepatocellular carcinoma breakthroughs: poly(ADP-ribose) polymerase inhibitors as a promising therapeutic strategy. Clin Res Hepatol Gastroenterol. 2014; 38(2):137–142. [PubMed: 23953496]
3. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012; 379(9822):1245–1255. [PubMed: 22353262]
4. Uka K, Aikata H, Takaki S, Shirakawa H, Jeong SC, Yamashina K, Hiramatsu A, Kodama H, Takahashi S, Chayama K. Clinical features and prognosis of patients with extrahepatic metastases from hepatocellular carcinoma. World J Gastroenterol. 2007; 13(3):414–420. [PubMed: 17230611]

5. Chun JM, Kwon HJ, Sohn J, Kim SG, Park JY, Bae HI, Yun YK, Hwang YJ. Prognostic factors after early recurrence in patients who underwent curative resection for hepatocellular carcinoma. J Surg Oncol. 2011; 103(2):148–151. [PubMed: 21259248]
6. Chua TC, Morris DL. Exploring the role of resection of extrahepatic metastases from hepatocellular carcinoma. Surg Oncol. 2012; 21(2):95–101. [PubMed: 21397495]
7. Iwakuma T, Tochigi Y, Van Pelt CS, Caldwell LC, Terzian T, Parant JM, Chau GP, Koch JG, Eischen CM, Lozano G. Mtbp haploinsufficiency in mice increases tumor metastasis. Oncogene. 2008; 27(13):1813–1820. [PubMed: 17906694]
8. Agarwal N, Adhikari AS, Iyer SV, Hekmatdoost K, Welch DR, Iwakuma T. MTBP suppresses cell migration and filopodia formation by inhibiting ACTN4. Oncogene. 2013; 32(4):462–470. [PubMed: 22370640]
9. Barbolina MV, Adley BP, Kelly DL, Fought AJ, Scholtens DM, Shea LD, Stack MS. Motility-related actinin alpha-4 is associated with advanced and metastatic ovarian carcinoma. Lab Investig. 2008; 88(6):602–614. [PubMed: 18362906]
10. Honda K, Yamada T, Endo R, Ino Y, Gotoh M, Tsuda H, Yamada Y, Chiba H, Hirohashi S. Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J Cell Biol. 1998; 140(6):1383–1393. [PubMed: 9508771]
11. Koizumi T, Nakatsuji H, Fukawa T, Avirmed S, Fukumori T, Takahashi M, Kanayama H. The role of actinin-4 in bladder cancer invasion. Urology. 2010; 75(2):357–364. [PubMed: 19969329]
12. Vlatkovic N, El-Fert A, Devling T, Ray-Sinha A, Gore DM, Rubbi CP, Dodson A, Jones AS, Helliwell TR, Jones TM, Boyd MT. Loss of MTBP expression is associated with reduced survival in a biomarker-defined subset of patients with squamous cell carcinoma of the head and neck. Cancer. 2011; 117(13):2939–2950. [PubMed: 21692053]
13. Odvody J, Vincent T, Arrate MP, Grieb B, Wang S, Garriga J, Lozano G, Iwakuma T, Haines DS, Eischen CM. A deficiency in Mdm2 binding protein inhibits Myc-induced B-cell proliferation and lymphomagenesis. Oncogene. 2010; 29(22):3287–3296. [PubMed: 20305689]
14. Grieb BC, Gramling MW, Arrate MP, Chen X, Beauparlant SL, Haines DS, Xiao H, Eischen CM. Oncogenic protein MTBP interacts with MYC to promote tumorigenesis. Cancer Res. 2014; 74(13):3591–3602. [PubMed: 24786788]
15. Grieb BC, Chen X, Eischen CM. MTBP is overexpressed in triple negative breast cancer and contributes to its growth and survival. Mol Cancer Res. 2014; 12(9):1216–1224. [PubMed: 24866769]
16. Akiba J, Nakashima O, Hattori S, Tanikawa K, Takenaka M, Nakayama M, Kondo R, Nomura Y, Koura K, Ueda K, Sanada S, Naito Y, Yamaguchi R, Yano H. Clinicopathologic analysis of combined hepatocellular-cholangiocarcinoma according to the latest WHO classification. Am J Surg Pathol. 2013; 37(4):496–505. [PubMed: 23388123]
17. Farges O, Fuks D, Le Treut YP, Azoulay D, Laurent A, Bachellier P, Nuzzo G, Belghiti J, Pruvot FR, Regimbeau JM. AJCC 7th edition of TNM staging accurately discriminates outcomes of patients with resectable intrahepatic cholangiocarcinoma: By the AFC-IHCC-2009 study group. Cancer. 2011; 117(10):2170–2177. [PubMed: 21523730]
18. Gao J, Chen C, Hong L, Wang J, Du Y, Song J, Shao X, Zhang J, Han H, Liu J, Fan D. Expression of Jagged1 and its association with hepatitis B virus X protein in hepatocellular carcinoma. Biochem Biophys Res Commun. 2007; 356(2):341–347. [PubMed: 17359939]
19. Schmid K, Wang X, Haitel A, Sieghart W, Peck-Radosavljevic M, Bodingbauer M, Rasoul-Rockenschaub S, Wrba F. KiSS-1 overexpression as an independent prognostic marker in hepatocellular carcinoma: an immunohistochemical study. Virchows Arch. 2007; 450(2):143–149. [PubMed: 17216189]
20. He XJ, Ma YY, Yu S, Jiang XT, Lu YD, Tao L, Wang HP, Hu ZM, Tao HQ. Up-regulated miR-199a-5p in gastric cancer functions as an oncogene and targets klotho. BMC Cancer. 2014; 14:218. [PubMed: 24655788]
21. Katase N, Lefeuvre M, Tsujigiwa H, Fujii M, Ito S, Tamamura R, Buery RR, Gunduz M, Nagatsuka H. Knockdown of Dkk-3 decreases cancer cell migration and invasion independently of the Wnt pathways in oral squamous cell carcinoma derived cells. Oncol Rep. 2013; 29(4):1349–1355. [PubMed: 23354949]

22. Agarwal N, Tochigi Y, Adhikari AS, Cui S, Cui Y, Iwakuma T. MTBP plays a crucial role in mitotic progression and chromosome segregation. Cell Death Differ. 2011; 18(7):1208–1219. [PubMed: 21274008]

23. Boos D, Yekezare M, Diffley JF. Identification of a heteromeric complex that promotes DNA replication origin firing in human cells. Science. 2013; 340(6135):981–984. [PubMed: 23704573]

24. Boyd MT, Vlatkovic N, Haines DS. A novel cellular protein (MTBP) binds to MDM2 and induces a G1 arrest that is suppressed by MDM2. J Biol Chem. 2000; 275(41):31883–31890. [PubMed: 10906133]

25. Brady M, Vlatkovic N, Boyd MT. Regulation of p53 and MDM2 activity by MTBP. Mol Cell Biol. 2005; 25(2):545–553. [PubMed: 15632057]

26. Polanski R, Warburton HE, Ray-Sinha A, Devling T, Pakula H, Rubbi CP, Vlatkovic N, Boyd MT. MDM2 promotes cell motility and invasiveness through a RING-finger independent mechanism. FEBS Lett. 2010; 584(22):4695–4702. [PubMed: 21034743]

27. Bouska A, Eischen CM. Murine double minute 2: p53-independent roads lead to genome instability or death. Trends Biochem Sci. 2009; 34(6):279–286. [PubMed: 19447627]

28. Frum RA, Singh S, Vaughan C, Mukhopadhyay ND, Grossman SR, Windle B, Deb S, Deb SP. The human oncoprotein MDM2 induces replication stress eliciting early intra-S-phase checkpoint response and inhibition of DNA replication origin firing. Nucl Acids Res. 2014; 42(2):926–940. [PubMed: 24163099]

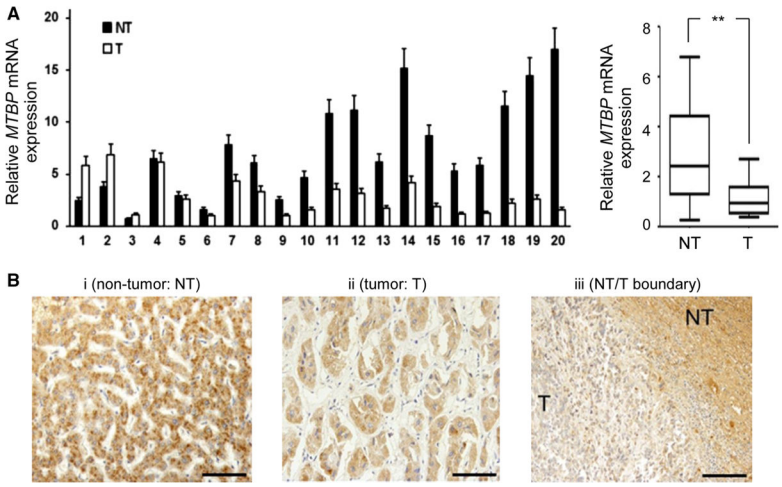

Fig. 1.

Decreased MTBP expression in HCC tissues compared with non-tumor liver tissues. **a** qRT-PCR. The mRNAs from 20 pairs of non-tumor (NT) and HCC (T) tissues were extracted, and qRT-PCR was performed. Data are shown as relative values of *MTBP* mRNA normalized by that of *GAPDH* to one of the tumor samples (*left*). Summary of qRT-PCR and statistical analysis (*right*, **P < 0.01; Mann–Whitney *U* test). **b** Representative MTBP IHC results using (*i*) NT (*scale bar* 50 μm), (*ii*) T (*scale bar* 50 μm), and (*iii*) NT and T boundary (*scale bar* 200 μm) liver tissues

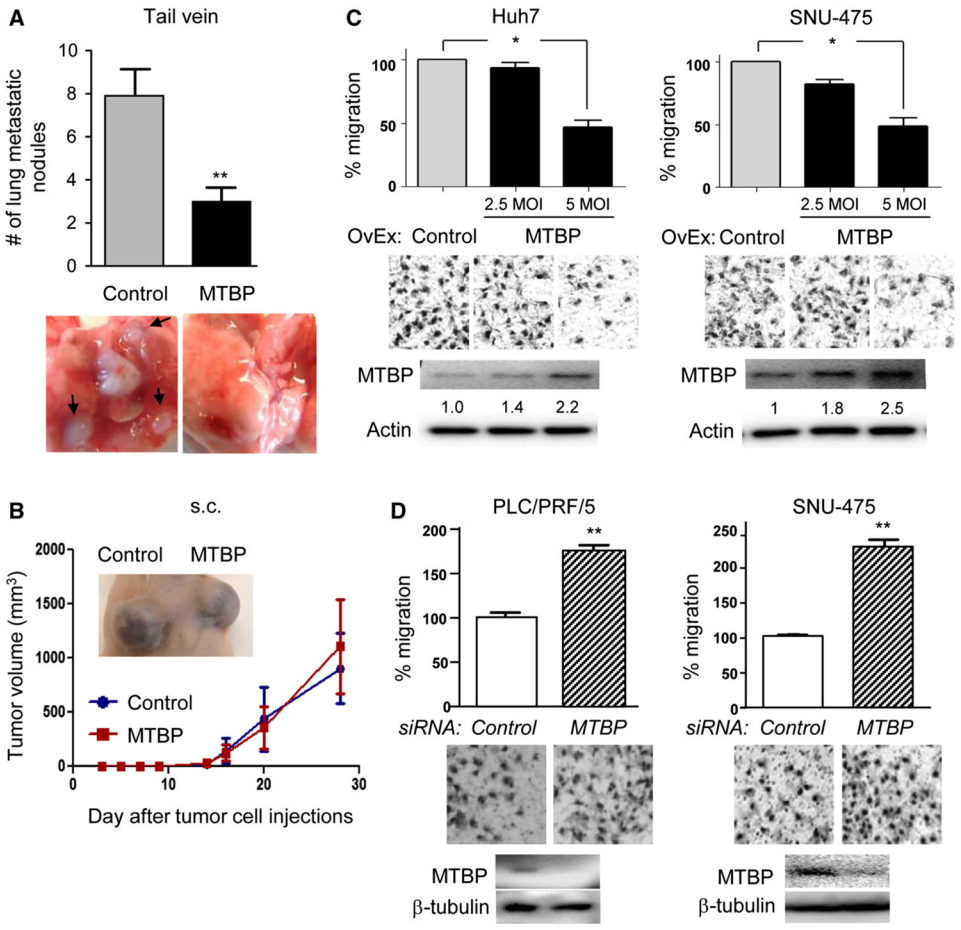

Fig. 2.

MTBP suppresses HCC metastasis and migration. **a** and **b** SK-hep-1 HCC cells infected with either empty (control, *grey*) or *MTBP*-encoding (*black*) lentiviral vectors were either intravenously (tail vein, n = 10, **a**) or subcutaneously (s.c., n = 6, **b**) injected into nude mice. Seven weeks after tail vein injections, mice were examined for the number of metastatic lung nodules (*arrows*). Tumor sizes following s.c. injections were measured twice a week. **c** MTBP overexpression (OvEx) inhibits the migratory potential of Huh7 (*left*) and SNU-475 (*right*) cells by transwell migration assays. Cells infected with empty (control, *grey*) or different (2.5 and 5) MOIs of an *MTBP*-encoding adenovirus (MTBP, *black*) were plated on the *upper* chambers and migrating cells in entire fields were counted 14 h later. Relative cell migration (%) compared to the number of migrating cells in control (*top*) and representative staining images (*middle*). Western blotting results below the images (*bottom*). Relative MTBP protein levels to controls normalized by actin loading control were shown below the MTBP blots. **d** MTBP downregulation increases the migratory potential of PLC/PRF/5 (*left*) and SNU-475 (*right*) cells by transwell migration assays. One day after non-target (control, *white*) or *MTBP*-specific siRNA transfection (*oblique*), migration assays were performed as in **c**. *Error bars* mean ± SD from three independent experiments. *P <0.05 and **P < 0.01; Student's *t* test

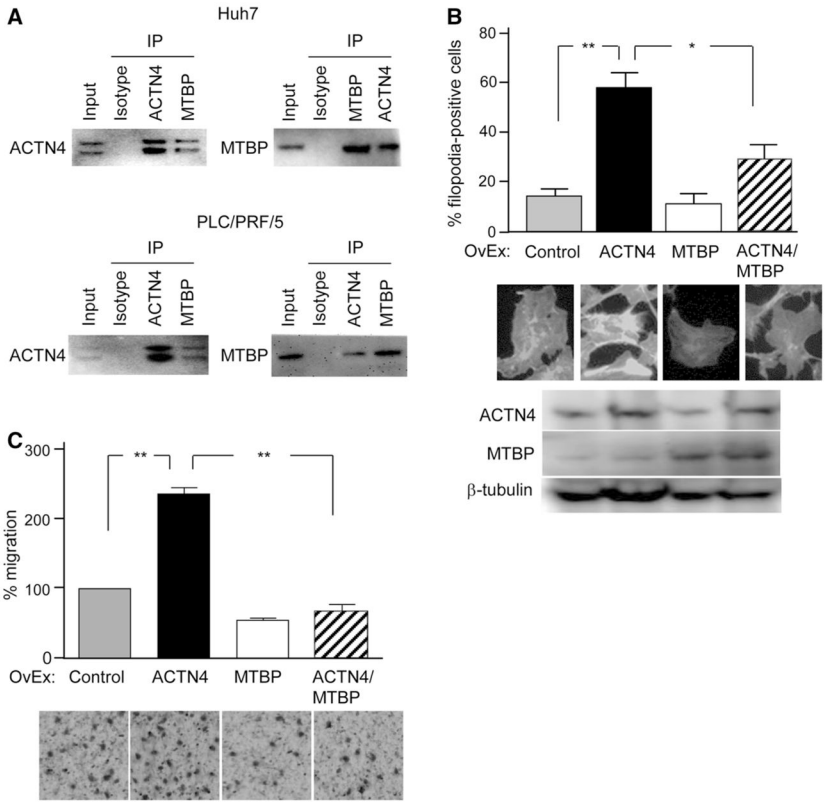

Fig. 3.

MTBP binds to and suppresses ACTN4-mediated HCC migration. **a** Endogenous interactions between MTBP and ACTN4 in Huh7 and PLC/PRF/5 cell lines by co-immunoprecipitation (co-IP) studies using indicated antibodies for MTBP and ACTN4. Anti-rabbit IgG was used as an isotype control. **b** and **c** SNU-475 cells stably infected with lentiviral vectors encoding empty (control, *lanes* 1, 3) or *ACTN4* cDNA (*lanes* 2, 4) were infected with empty (*lanes* 1, 2) or *MTBP*-encoding (*lanes* 3, 4) adenoviral vectors. **b** Phalloidin staining was performed 48 h later. Cells (n = 50) were examined for filopodia formation. The percentage of cells positive for filopodia formation (*top*), representative phalloidin staining images (*middle*), and representative western blotting for ACTN4 and MTBP (*bottom*). *Grey* control, *black* ACTN4 overexpression (OvEx), *white* MTBP overexpression, *oblique* both MTBP and ACTN4 overexpression. **c** Tran-swell migration assays were performed as in Fig. 2c. *Graphs* showing relative cell migration (%) compared to the number of migrating cells in control. Representative images are placed below the graphs. *Error bars* means ± SD from three independent experiments. *P < 0.05 and **P < 0.01; Student's *t* test

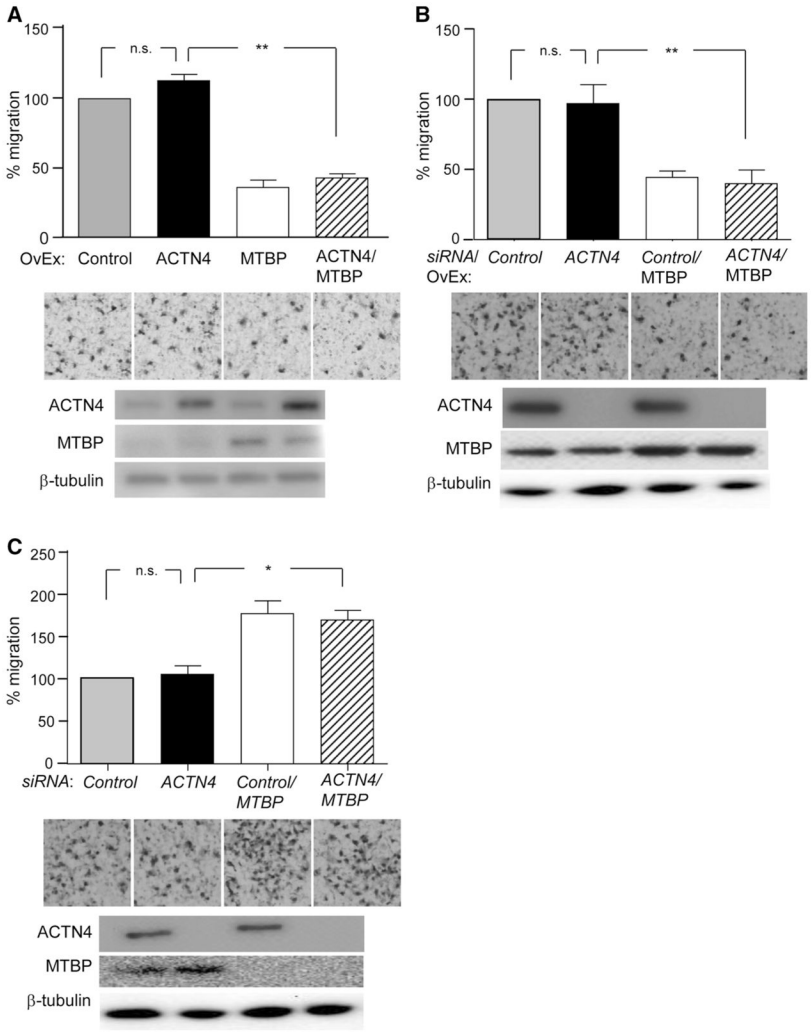

Fig. 4.

MTBP inhibits the migratory potential of PLC/ PRF/5 cells in an ACTN4–independent manner. **a** Transwell migration assays were performed using PLC/ PRF/5 cells overexpressed for ACTN4 and/or MTBP. Summarized graph (*top*), representative images (*middle*), and western blotting results (*bottom*). **b** and **c** Migration assays were performed using PLC/PRF/5 cells downregulated for ACTN4 with or without manipulation of MTBP expression (**b** MTBP overexpression; **c** MTBP downregulation). Summarized graph (*top*), representative staining images (*middle*), and western blotting results (*bottom*). *Error bars* mean ± S.D. from three independent experiments. *P <0.05 and **P < 0.01; Student's *t* test. n.s. not significant

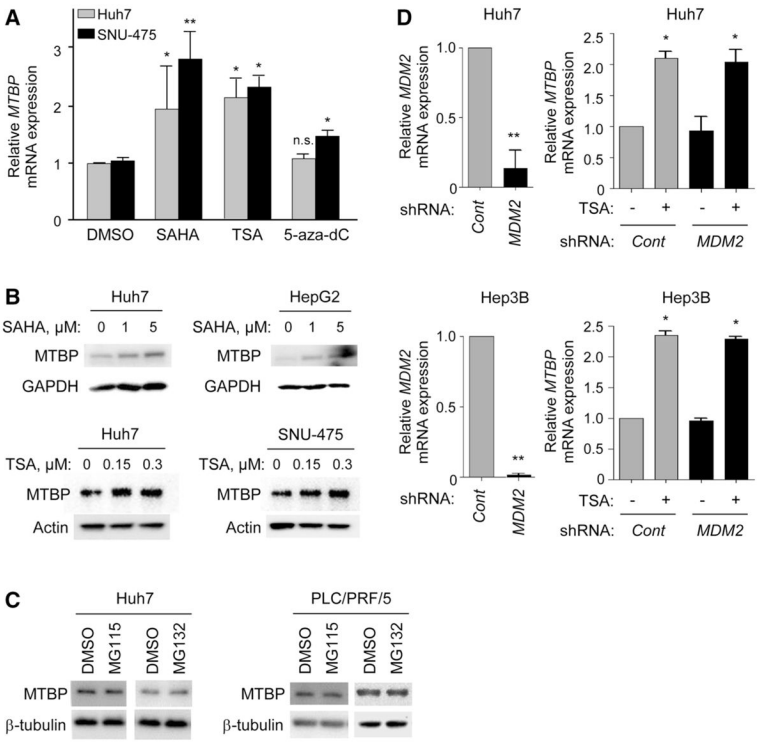

Fig. 5.

MTBP expression can be transcriptionally silenced in HCC. **a** Results of qRT-PCR 24 h after treatment of Huh7 (*grey*) and SNU-475 (*black*) cell lines with either 5 μM of SAHA, 0.3 μM of TSA, or 10 μM of 5-aza-dC. Data are shown as relative *MTBP* mRNA expression to that of *GAPDH*. **b** Western blotting for MTBP and GAPDH or actin at 24 h after treatment of indicated HCC cells with SAHA (*top*) or TSA (*bottom*). **c** Western blotting for MTBP and β-tubulin (control) following treatment of indicated cells with proteasome inhibitors MG132 and MG115 at 30 μM for 4 h. Equivalent dilutions of dimethyl sulfoxide (DMSO) were used as controls. **d** Results of qRT-PCR for *MDM2* (*left*) and *MTBP* (*right*) using Huh7 (p53Y220C) and Hep3B (p53-null) cells (*grey*) and those down-regulated for MDM2 (*black*) with or without 0.3 μM of TSA treatment for 24 h. Cont: Control. Data are shown as relative *MDM2* or *MTBP* mRNA expression to that of *GAPDH*. Error bars: mean ± S.D. from three independent experiments. *P <0.05 and **P < 0.01; Student's *t* test. n.s., not significant

Table 1

MTBP expression and clinicopathological parameter

Characteristics	Case# tested	MTBP intensity		P value
		0–1[a]	2–3[a]	
Age				
> 52	50	13	37	n.s.
≤52	52	9	43	
Gender				
Male	72	15	57	n.s.
Female	30	7	23	
Tumor size				
> 5 cm	43	7	36	n.s.
≤5 cm	59	15	44	
Edmondson-Steiner grade				
G1, 2	77	16	61	n.s.
G3, 4	25	6	19	
Capsular/vascular invasion				
Negative	77	11	66	< 0.001
Positive	25	11	14	
Lymph node metastasis				
Negative	68	10	58	< 0.05
Positive	34	12	22	

[a]MTBP staining 0–1 (none to low), 2–3 (middle to high)

n.s. not significant