MTBP Is Overexpressed in Triple-Negative Breast Cancer and Contributes to Its Growth and Survival

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Abstract

Triple-negative breast cancer (TNBC) is a clinically aggressive subtype of breast cancer commonly resistant to therapeutics that have been successful in increasing survival in patients with estrogen receptor–positive (ER+) and HER2+ breast cancer. As such, identifying factors that contribute to poor patient outcomes and mediate the growth and survival of TNBC cells remain important areas of investigation. MTBP (MDM2-binding protein), a gene linked to cellular proliferation and a transcriptional target of the MYC oncogene, is overexpressed in human malignancies, yet its contribution to cancer remains unresolved. Evaluation of mRNA expression and copy number variation data from The Cancer Genome Atlas (TCGA) revealed that MTBP is commonly overexpressed in breast cancer and 19% show amplification of MTBP. Increased transcript or gene amplification of MTBP significantly correlated with reduced breast cancer patient survival. Further analysis revealed that while MTBP mRNA is overexpressed in both ER+ and HER2+ breast cancers, its expression is highest in TNBC. MTBP mRNA and protein levels were also significantly elevated in a panel of human TNBC cell lines. Knockdown of MTBP in TNBC cells induced apoptosis and significantly reduced TNBC cell growth and soft agar colony formation, which was rescued by expression of shRNA-resistant Mtbp. Notably, inducible knockdown of MTBP expression significantly impaired TNBC tumor growth, in vivo, including in established tumors. Thus, these data emphasize that MTBP is important for the growth and survival of TNBC and warrants further investigation as a potential novel therapeutic target.

Implications: MTBP significantly contributes to breast cancer survival and is a potential novel therapeutic target in TNBC. Mol Cancer Res; 12(9); 1216–24. ©2014 AACR.

Introduction

The use of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) as biomarkers is standard practice in the clinical management of breast cancer. Their expression directs the use of targeted therapeutics, such as tamoxifen and trastuzumab, that have dramatically improved patient survival. Unfortunately, such improvements in clinical outcomes have not been realized in the management of triple-negative breast cancer (TNBC), a subset of breast cancers lacking HER2 amplification and expression of ER and PR (1). TNBC comprises 10% to 20% of breast cancer cases and is more commonly identified in younger women and those with African-American or Hispanic heritage (2). It is clinically aggressive, correlating with an increased risk of distant recurrence within 3 years following treatment and a significant decrease in overall patient survival, compared with receptor-positive cases (3, 4). While there has been some success in exploiting novel molecular targets, such as PARP inhibitors in BRCA1-mutant tumors with errors in DNA break repair (5, 6), these cases are isolated and applicable to only select TNBCs. Other targets such as mTOR, Src, and HER1 tested in phase II clinical trials have shown only minimal success (7–9). Thus, there is a need to identify and test the therapeutic efficacy of novel molecular targets in TNBC.

The Mdm2 (Two)-binding protein (MTBP) was first identified as a potential tumor suppressor that binds Mdm2, a negative regulator of p53 (10). However, subsequent genetic studies indicated it functions independent of Mdm2 and, instead, contributes to tumor development induced by the Myc oncogene (11–13). Recently, MTBP has been implicated in regulating proliferation and cell-cycle progression (12, 14, 15). MTBP is a transcriptional target of MYC, and its protein expression increased in response to proproliferative signals and decreased upon growth factor withdrawal (12). In mouse models, Mtbp heterozygosity led to reduced levels of Mtbp protein and this inhibited Myc-induced B-cell proliferation, resulting in a significant delay in...
lymphoma development (12). Furthermore, siRNA-mediated knockdown of MTBP was reported to delay cell-cycle progression through the S and G2–M phases of the cell cycle (14, 15). Therefore, MTBP appears to contribute to the development and possibly the maintenance of tumors through regulation of proliferation, but further investigation is needed.

Here, we report that MTBP is overexpressed and amplified in breast cancer, correlating with decreased patient survival. Notably, MTBP mRNA expression was highest in TNBC. shRNA-mediated knockdown of MTBP in human TNBC cell lines inhibited their expansion and induced apoptosis, in vitro, as well as significantly reduced tumor growth, in vivo. Our data reveal that MTBP significantly contributes to breast cancer and is a potential novel therapeutic target in the treatment of TNBC.

Materials and Methods

Patient data

Patient survival and gene expression data for 844 breast cancers was accessed from The Cancer Genome Atlas (TCGA) public data portal (https://tcga-data.nci.nih.gov/tcga/) January–April 2013. For Kaplan–Meier survival curves, normalized RNA-Seq data (version 2, level 3) was used as gene expression values and the median was used to classify samples into high and low expression groups. Log-rank tests were used to compare survival between groups. Box and whisker plots (box represents first and third quartiles, thick band is median value, and bars extend to ±1.58 the interquartile range divided by the square root of the number of samples) were applied to describe MTBP gene expression values. Groups were compared using a Wilcoxon rank-sum test. Gene copy number alteration (CNA) and survival data for 913 breast cancers was obtained from the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) May 2013.

Cell culture, vectors, transfection, and infection

The human cell lines MDA-MB-231, HCC1806, and HCC1937 were cultured as described by the American Type Culture Collection and were provided by Dr. Jennifer Pietenpol. Cells were transfected with Effectene (Qiagen)–Pietenpol. Cells were transfected with Effectene (Qiagen) or were infected with retroviruses, as previously described (16). Cells were plated in triplicate and MTT assays were performed as per manufacture’s protocol (Sigma). Cell cycle (Dean–Jett–Fox analysis) and apoptosis (sub-G1 DNA content) were evaluated with FlowJo software (TreeStar Inc.) following DNA staining with propidium iodide and flow cytometry. Apoptosis was also evaluated by flow cytometric analysis of Annexin V–APC binding (Life Technologies) and caspase-3 cleavage by Western blotting (see below). Cell viability was assessed by Trypan Blue Dye exclusion. Soft agar assays were performed as previously described (18). For doxycycline-inducible shRNA experiments, 0.5 to 1 μg/mL of doxycycline was added to the cultures.

Mice

Female athymic nude mice (5–6 week old; Harlan) were injected subcutaneously in the flank with 3 × 10^6 HCC1806 cells. Mice were housed with drinking water supplemented with 5% sucrose with or without 2 g/L of doxycycline (Research Products International Corp.) that was changed every 48 hours beginning on the day of injection or 10 days later. Tumor volume was calculated from measurements with electronic calipers. At time of sacrifice, mice were photographed, and tumors were extracted, photographed, and weighed. A piece of each tumor was frozen for Western blot analysis. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee and followed all federal and state rules and regulations.

Western blotting and quantitative real-time PCR analysis

For Western blotting, cells or tumors that were infected or transiently transfected (see above) were harvested after 48 hours or at indicated times and were lysed as previously reported (16, 18). Equal amounts of protein were resolved by SDS-PAGE and Western blotted using antibodies specific for MTBP (B5, Santa Cruz Biotechnology), cleaved caspase-3 (D175, Cell Signaling Technology), MYC (C33, Santa Cruz Biotechnology), and β-ACTIN (AC15, Sigma). To evaluate mRNA expression, total RNA was isolated, cDNA was generated, and qRT-PCR for MTBP and β-ACTIN levels was performed as previously described (12). mRNA data are relative to β-ACTIN levels.

Statistical evaluation

Wilcoxon rank-sum test (Figs. 1A and 2A), log-rank tests (Fig. 1B and C), the Student t-test (Figs. 2–6), and Cox regression analysis were used to compare data. Error bars represent SD (Figs. 2–5) or SEM (Fig. 6).

Results

MTBP is overexpressed in human breast cancer and correlates with decreased patient survival and triple-negative status

We previously detected MTBP/Mtbp overexpression in human lymphoma cell lines and primary murine lymphomas (12). MTBP was also reported amplified in colorectal carcinoma and multiple myeloma (19, 20), as well as several human cancer cell lines (21), suggesting that its overexpression...
contributes to human cancers. To specifically evaluate MTBP expression in human breast cancer, mRNA expression and patient survival data for 844 breast cancers from TCGA were assessed. MTBP was significantly elevated in breast cancer samples compared with normal breast tissue ($P = 2.2 \times 10^{-16}$; Fig. 1A). When cancers were separated by their MTBP expression, those patients whose breast cancers had elevated MTBP expression exhibited reduced overall survival compared with patients whose breast cancers had lower levels of MTBP ($P = 0.0337$; Fig. 1B). A Cox regression analysis also showed that increased MTBP levels are significantly linked with worse patient survival ($P = 0.033$). Moreover, MTBP was amplified in 19% of breast cancers, and this amplification decreased overall patient survival compared with tumors without amplified MTBP ($P = 0.01955$; Fig. 1C; refs. 22, 23). These data indicate that MTBP overexpression is common and thus, likely selected for during breast cancer formation and/or progression. The results also show that elevated levels of MTBP correlate with reduced breast cancer patient survival.

Figure 1. MTBP overexpression in breast cancer decreases survival. A and B, RNA-Seq mRNA expression data for normal and breast cancer tissue from TCGA database. A, box and whisker plot of relative MTBP mRNA expression in normal and cancerous breast tissue with n indicating the number of samples. B, Kaplan–Meier survival curves for patients with breast cancer divided by the median value into low and high MTBP mRNA expression (n = 421 per group). C, Kaplan–Meier survival curves of MTBP gene copy number in breast cancer samples with (n = 171) and without (n = 742) amplified MTBP from the cBio Portal for Cancer Genomics (22, 23). $P$ values calculated using a Wilcoxon rank-sum test for A and a log-rank test for B and C.

Figure 2. MTBP is overexpressed in TNBC. A, box and whisker plot of relative MTBP mRNA expression from TCGA RNA-Seq mRNA expression data, representing normal breast tissue and breast cancers divided into ER$^+$, HER2$^+$, or triple-negative (TN; ER$^-$, PR$^-$, HER2$^-$). The n indicates the number of samples. B, expression of MTBP mRNA measured by qRT-PCR in TNBC cell lines and normal HMECs ($P < 0.0001$, $P < 0.001$). C, Western blot analyses of whole-cell lysates of TNBC cell lines and HMECs for the proteins indicated.

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MTBP expression with 2 different MTBP shRNAs in the MDA-MB-231 and HCC1806 TNBC cell lines. While both cell lines overexpress MTBP (Fig. 2B), they represent distinct subtypes of TNBC (mesenchymal-like and basal-like, respectively), and HCC1806 cells have an MTBP amplification (21, 25). In both cell lines, reduced MTBP expression resulted in a decrease in proliferation that correlated with the amount of MTBP protein present, where MTBP shRNA1 was more effective at reducing MTBP protein levels than MTBP shRNA2 (Fig. 3A). Similarly, anchorage-independent growth in soft agar was significantly reduced for both the MDA-MB-231 and the HCC1806 cells when MTBP was knocked down (Fig. 3B). To ensure the observed effects were due to reduced MTBP expression, shRNA resistant murine Mtbp was co-expressed with MTBP shRNA1. The murine Mtbp rescued the ability of MDA-MB-231 cells to form colonies in soft agar in the presence of MTBP shRNA1, whereas cells cotransfected with vector control and MTBP shRNA1 still showed decreased colony formation (Fig. 3C). These results indicate that knockdown of MTBP inhibits TNBC expansion and anchorage-independently.

To further evaluate the antiproliferative effects of MTBP knockdown in TNBC cells, MTBP shRNA1 was adapted to a lentiviral doxycycline-inducible system (17). Using the 3 TNBC cell lines MDA-MB-231, HCC1806, and the basal-like HCC1937 cells that also overexpress MTBP (Fig. 2C), we observed that doxycycline-induced MTBP shRNA1 resulted in reduced MTBP protein expression within 24 hours (Fig. 4A) and significantly
decreased (37%–40% reduced) proliferation within 72 hours (Fig. 4B) in all three lines. The steady-state levels of MYC remained unchanged (Fig. 4A). Expression of the shRNA-resistant murine Mtbp allowed MTBP shRNA1-expressing MDA-MB-231 cells to continue growing at rates analogous to that of cells treated with vehicle control (Fig. 4C). Therefore, disruption of MTBP expression with constitutive or inducible shRNA caused a reduction in the ability of TNBC cells to form colonies in soft agar and to proliferate.

MTBP knockdown induces apoptosis in TNBC cells
To investigate the biologic reason that MTBP knockdown inhibited the expansion of TNBC cells, HCC1806 cells expressing the doxycycline-inducible MTBP shRNA1 or the nontargeting shRNA control were cultured with or without doxycycline for 72 hours. At this time, there were visibly fewer adherent and more floating doxycycline-treated MTBP shRNA1-expressing cells compared with vehicle control-treated cells and nontargeting shRNA-expressing cells cultured with or without doxycycline (Fig. 5A). Evaluation of the cell cycle revealed no significant difference in G1, S, or G2–M distribution of the cells where MTBP shRNA1 had been induced with doxycycline compared with vehicle control (Fig. 5B). In contrast, doxycycline-treated MTBP shRNA1-expressing HCC1806 cells had an increase in the percentage of cells with sub-G1 DNA content (Fig. 5B). There was also a significant decrease in viability (Fig. 5C) and an increase in Annexin V–positive (Fig. 5D) MTBP shRNA1-containing cells. Moreover, in HCC1806 cells with MTBP shRNA1, cleaved caspase-3 was visible after the addition of doxycycline compared with vehicle control or with those cells with the nontargeting shRNA control (Fig. 5E). Thus, shRNA-mediated knockdown of MTBP in TNBC cells induced apoptosis, without detectable alterations in phases of the cell cycle.

MTBP loss inhibits TNBC growth, in vivo
To evaluate whether MTBP knockdown would alter TNBC growth in vivo, HCC1806 cells expressing doxycycline-inducible MTBP shRNA1 were subcutaneously injected into the flanks of athymic nude mice. Their drinking water was supplemented with or without doxycycline. By day 7, tumors in mice receiving doxycycline to induce MTBP shRNA1 showed a statistically significant decrease in volume compared with tumors in control mice not receiving doxycycline (24 vs. 43 mm3; P < 0.0001), and this difference continued to increase through the duration of the experiment (Fig. 6A). At the time of sacrifice (day 21), tumors that expressed MTBP shRNA1 due to doxycycline...
exposure were smaller in volume and weighed significantly less than the tumors from control mice (Fig. 6A–C). There was a 70% reduction in tumor volume in the mice expressing MTBP shRNA1 compared with controls. These tumors showed reduced levels of MTBP protein, verifying MTBP shRNA1 expression persisted over the course of the experiment (Fig. 6C). MYC protein levels were similar in all tumors (Fig. 6C).

We also tested whether established TNBC tumors would be affected by knockdown of MTBP. Specifically, HCC1806 cells expressing the doxycycline-inducible MTBP shRNA1 were injected into the flanks of nude mice at the same time as cells for the experiment described above and were allowed to grow. After 10 days when the tumors averaged 100 to 150 mm³, these mice were given doxycycline to induce MTBP shRNA1 expression. Within 72 hours these tumors were significantly smaller than tumors that were not exposed to doxycycline (188 vs. 350 mm³; \( P = 0.0057 \)), yet tumors did not completely disappear (Fig. 6A). Instead, after the initial decrease in tumor size, a significant reduction in the rate of tumor growth was observed that was analogous to the rate of tumor growth for the mice that received doxycycline on day one. At sacrifice (day 21), the tumors from the mice that received doxycycline to induce MTBP shRNA1 after tumors had established were smaller and weighed significantly less than the tumors from mice that never received doxycycline (Fig. 6B and C). The tumors were similar in size and weight to the tumors from mice that been exposed to doxycycline for the entire experiment. These data collectively indicate that targeting MTBP, reducing its expression, significantly limits the growth of TNBC cells in vivo, including established TNBC tumors.

**Discussion**

Studies have linked MTBP to cancer (11, 12, 19, 20, 26), but little was known about MTBP in established cancer cells, particularly how it influenced proliferation, cellular survival, and patient outcomes. Here, we show MTBP is overexpressed in human breast cancer, and this correlated with significantly decreased patient survival. Notably, among the
cells were also observed being critical for the growth and survival of TNBC cells. The cancer patient prognosis and triple-negative status as well as significantly contributes to tumor development (12, 13, 15). Here we show that MTBP overexpression is an important indicator of poor breast cancer patient prognosis and triple-negative status as well as being critical for the growth and survival of TNBC cells. The results of this study support further investigation into MTBP as a novel therapeutic target in TNBC.

This study reveals that MTBP overexpression contributes in a significant way to human breast cancer and increases understanding of MTBP in cancer. Specifically, we previously reported that MTBP is overexpressed in human and murine B-cell lymphomas (12). Others have shown that the region of the genome encoding MTBP is amplified in colorectal cancer and multiple myeloma (19, 20). Similarly, evaluation of copy number variation data from TCGA indicates that MTBP is amplified in many types of human cancer (13, 22, 23). For breast cancer, we determined MTBP amplification occurred in 19% of the tumors, and this significantly correlated with decreased patient survival.

Because most breast cancer deaths are associated with metastasis, the current analysis suggests that MTBP overexpression is a potential novel indicator of aggressive breast cancers with increased metastatic potential that are more likely to result in patient death. However, this concept conflicts with experimental data that indicate decreased MTBP expression increases cell migration, invasion, and metastasis (11, 26). It is possible that both are correct if, as has been shown for MYC (27), MTBP is temporarily downregulated when cancer cells move and is then upregulated after cancer cells seed metastatic sites and begin to proliferate again. However, there is also one report showing that decreased MTBP expression was highest in the TNBC subtype, which lacks oncogene expression (MYC and E2F1) or growth factor exposure, and MTBP could have a tissue-specific or cancer cell mutation–specific function, much of the data suggest that in multiple hematopoietic and nonhematopoietic human cancers, including breast cancer, MTBP overexpression is selected for and contributes to cancer development and progression. The current study links MTBP overexpression in breast cancer to advanced disease and poor patient prognosis.

Data from multiple groups, including our own, suggest that MTBP has a critical function in proliferation, and that this significantly contributes to tumor development (12, 13, 15). Specifically, we previously determined that an Mtbp haploinsufficiency suppressed proliferation mediated by the Myc oncogene, significantly inhibiting the ability of Myc to induce B-cell lymphoma development (12). Moreover, MTBP mRNA and protein expression increased in response to oncogene expression (MYC and E2F1) or growth factor exposure, and MTBP was shown to be a transcriptional target of MYC (12). Others have indicated that MTBP contributes to cell-cycle progression by linking MTBP to DNA replication origins and mitotic progression (14, 15). In addition, we recently determined that elevated levels of MTBP resulted in enhanced cellular proliferation and transformation, in vitro and in vivo (13). Here we show that MTBP is overexpressed in breast cancers and its expression is the highest in the TNBC subtype. TNBCs are reported to have a higher proliferative index than receptor-positive high-grade invasive carcinomas (29). In addition, elevated MYC transcriptional activity, which is correlated with decreased breast cancer patient survival, is linked to increased proliferation in different breast cancer subtypes, we determined that MTBP expression was highest in the TNBC subtype, which lacks targeted therapies and is known for being clinically aggressive (1, 3, 4). Experiments also revealed that reducing MTBP expression in human TNBC cell lines with shRNA significantly inhibited cell expansion by inducing apoptosis. The growth-inhibitory effects of MTBP knockdown in TNBC cells were also observed in vivo, in xenografts, and, importantly, in established TNBC tumors. Therefore, this study identifies MTBP as an important indicator of poor breast cancer patient prognosis and triple-negative status as well as being critical for the growth and survival of TNBC cells. The
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breast cancer, and the TNBC subtype has the highest MYC transcriptional activity (24). We recently determined that patients with breast cancers who express high levels of both MYC and MTBP have a worse prognosis than those with just high MYC expression (13), suggesting cooperation between MYC and MTBP overexpression in breast cancer. Therefore, MTBP appears to be a proproliferative factor where its overexpression supports the increased proliferative capacity of cancer cells, which is associated with poor patient survival in many human cancers (29–35).

Deletion of Mtbp is embryonic lethal in mice, indicating it has an indispensable function in development (11). Here we show that MTBP also has an essential function in breast cancer cell survival. Knockdown of MTBP in human TNBC cell lines using constitutive or inducible MTBP shRNA severely limited TNBC growth, in vitro and in vivo, due to induction of apoptosis. This is similar to the oncogene addiction–mediated cell death observed when an oncogene, such as MYC, is knocked down in cancer cells (36–38), suggesting that TNBC cancer cells can become reliant on MTBP for their continued growth and survival. Support of this concept was reported when knockdown of MTBP with siRNA in HeLa cells delayed DNA replication or mitosis and also led to cell death (14, 15). Although we did not detect cell-cycle changes with MTBP knockdown in TNBC cells, as was reported for HeLa cells (14, 15), we did observe significant apoptosis resulting in reduced TNBC cell survival. The apoptosis that occurred upon MTBP knockdown resulted in a reduction in the ability of the TNBC cells to grow in soft agar and in vitro in mice. Notably, using inducible MTBP shRNA revealed that, in vivo, established breast cancers rely on MTBP for their continued growth. Therefore, our data identify MTBP as a protein TNBC cells need to survive and grow. Thus, MTBP is a potential novel therapeutic target in TNBC warranting further investigation. Moreover, additional studies are needed to examine whether MTBP has a similar essential function in other human malignancies, including receptor-positive subtypes of breast cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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