Tumor and Stem Cell Biology

Genetic Ablation of Metadherin Inhibits Autochthonous Prostate Cancer Progression and Metastasis

Liling Wan1, Guohong Hu1,2, Yong Wei1, Min Yuan1, Roderick T. Bronson3, Qifeng Yang4, Javed Siddiqui5, Kenneth J. Plinta6, and Yibin Kang1,7

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Phone: 609-258-8834; Fax: 609-258-2340; E-mail: ykang@princeton.edu

Washington Road, LTL 255, Princeton University, Princeton, NJ 08544.

Yibin Kang, Department of Molecular Biology, Corresponding Author:

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Introduction

Prostate cancer is the most common type of cancer among men in the United States. It accounts for 28% of the total cancer incidences and 10% of the total cancer deaths of American men (1). Prostate cancer–related mortality is mainly caused by advanced cancers that have disseminated and metastasized to distant organs such as the bone, lung, and liver (2). Once cancer cells establish secondary tumors in these vital organs, treatment usually becomes more complicated with worse outcome. Therefore, defining and understanding novel molecular targets that drive prostate cancer progression and metastasis is crucial for developing effective therapeutic approaches for prostate cancer.

We previously identified metadherin (MTDH; also known as AEG-1, 3D3/LYRIC) as a prometastasis gene that resides in 8q22, a frequently amplified genomic locus linked to poor relapse-free survival of breast cancer (3). In recent years, elevated levels of MTDH have been reported in more than 20 cancer types (4), suggesting a potentially crucial and broad functionality of this gene in human cancer. Depending on the cancer type tested, recent studies using mainly cell culture systems have implicated MTDH in many cancer-related processes, including cellular proliferation, stress-induced cell death, invasion, chemoresistance, and metastasis (5, 6).

These pleiotropic tumor-promoting roles of MTDH may stem from the complex nature of this protein, as revealed by its initial identification. MTDH was originally reported as an HIV-induced gene in astrocytes (7), a cell-surface molecule mediating the homing of mammary tumor cells to the lung endothelium (8), a lysine-rich CEACAM1 co-isolated (LYRIC) protein associated with tight junctions in prostate epithelial cells (9), and as a novel transmembrane protein present in the different subcellular compartments (10). At the molecular level, the human MTDH encodes a 582-amino acid protein with no recognizable domains that could indicate its biologic function, except for a putative transmembrane domain and 3 lysine-rich nuclear localization signals (11). MTDH has recently been reported to interact with multiple proteins. In the nucleus, MTDH was shown to interact with PLZF (11), BCCIPα (12), and NF-κB subunit p65 (13, 14). In cytoplasm, MTDH was reported to interact with staphylococcal nuclease domain-containing protein 1 (SND1; refs. 15–17). MTDH has also been linked to multiple classical oncogenic signaling pathways such as PI3K/AKT and Wnt signaling (5) in a cancer cell-type–dependent manner. However, whether MTDH exerts its function through interacting with its binding partners, and how
MTDH modulates abovementioned oncogenic pathways remain largely unknown. A few studies so far suggest that MTDH may play a role in prostate cancer. MTDH was shown to be overexpressed in prostate tumor tissues and tumorigenic cell lines compared with benign hyperplastic tissues and normal epithelial cells (18, 19). Knockdown of MTDH in cultured prostate cancer cells enhanced apoptosis and inhibited Matrigel invasion (18, 19). However, the expression profiles of MTDH at different stages of prostate cancer progression, the physiologic roles of MTDH in prostate tissue development, and the in vivo functional involvement of MTDH in prostate cancer development remain unaddressed. In this study, we investigate the clinical relevance and functional importance of MTDH in prostate cancer.

Materials and Methods
Detailed descriptions of the materials and the methods are presented in the Supplementary Materials.

Mice
All experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee of Princeton University. Mtdh-/- mice were generated by injecting ES cell line XB780 (Bay Genomics) containing a gene-trapped allele of Mtdh into C57BL/6 blastocysts followed by confirmation of germline transmission by PCR. All Mtdh-/- mice were backcrossed to C57BL/6 background for >6 generations before breeding with C57BL/6 transgenic adenocarcinoma of mouse prostate (TRAMP) transgenic mice (Jackson Laboratory; ref. 20). Female TRAMP mice were bred to Mtdh-/- male mice to obtain female TRAMP/Mtdh +/- and male Mtdh-/- founder mice. Subsequently, these founder mice were bred to generate TRAMP/Mtdh +/-, TRAMP/Mtdh +/+, and TRAMP/Mtdh -/- male mice. This breeding strategy ensured that all experimental mice were heterozygous to the SV40 transgene. DNA was extracted from the tail biopsy and PCR was performed for recombination of MTDH in prostate cancer.

Transplantation
Athymic nude male mice were injected subcutaneously with 5 \times 10^5 TRAMP-C1 cells, and tumors were measured by calipers twice a week for calculation of tumor volumes (\(\pi \times \text{length} \times \text{width}^2/6\)).

Cell culture
TRAMP-C1 cell line was obtained from ATCC and authenticated using short tandem repeat (STR) profiling method. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 0.005 mg/mL bovine insulin, 10 nmol/L dehydroisoandrosterone, 5% fetal bovine serum, and 5% Nu-Serum IV.

Tissue examination and tumor grading
Tissues were fixed, embedded, and sectioned. The hematoxylin and eosin (H&E)-stained prostate sections were each blindly scored on a scale of 1 to 6 (21-23) and each section was designated with a highest grade for the area of most severe pathology.

Human samples
Tumor specimens were obtained from the Comprehensive Cancer Center at the University of Michigan with informed consent from all subjects in accordance with the Institutional Review Board of the University of Michigan. Two prostate tissue microarrays composed of 62 normal prostate tissues, 10 benign prostatic hyperplasia (BPH), 10 prostate atrophy or prostate inflammatory atrophy (PIA), 10 prostatic intraepithelial neoplasia (PIN), 72 prostate tumors, and 10 distant metastases were used in our clinical study. At the time of surgery, the ages of the patients with available information were 56 to 90 years (median = 76 years, SD = 7.6 years). All the patients were not treatment with hormone or radiation therapy.

Western blot analysis
Total cell or tissue lysates were generated using RIPA buffer. Western blots of lysates resolved by SDS-PAGE were probed with the antibodies listed in the Supplementary Table S2.

qRT-PCR analyses
RNA isolation and real-time RT-PCR were performed following standard procedures. The mRNA levels were normalized based on GAPDH. Primer sequences are listed in Supplementary Table S1.

Fluorescence in situ hybridization
Tissue FISH was performed in the Dana-Farber Cancer Institute Cytogenetic Core Facility. Probes were labeled using the Nick Translation Kit, SpectrumOrange dUTP (Vysis), and SpectrumGreen (Vysis). Hybridization signals were viewed on fluorescence microscope and analyzed.

Immunohistochemistry
Immunohistochemistry staining was performed following standard protocols. Briefly, antigen heat retrieval with citric buffer was used on paraffin-embedded sections. Sections were then incubated with primary and secondary antibodies listed in the Supplementary Table S2, followed by incubation with ABC reagents and 3,3-diaminobenzidein substrate.

Statistical analysis
All results wherever necessary were subjected to statistical analysis. A log-rank test, a nonparametric Mann–Whitney test, \(\chi^2\) test, and unpaired, 2-sided, independent Student t test with equal variance assumption were used for most studies as indicated in figure legends. For all statistics test, \\
\(\text{","} P < 0.05;
\text{"";} P < 0.01; \text{and } ^{***} P < 0.001.

Results
MTDH is associated with tumor progression and metastasis in human prostate cancer
First, we investigated the clinical relevance of MTDH in human prostate cancer samples. Two prostate tissue microarrays, consisting samples of normal prostates BPH, PIN, prostate primary tumors, and distant metastasis were analyzed.
by immunohistochemistry, and the MTDH protein levels were compared between samples of different clinical stages (Fig. 1A and Supplementary Table S3). MTDH protein was undetectable or expressed at very low levels in all normal or BPH prostate tissues. The protein levels of MTDH gradually increased in premalignant tissues (PIN), primary tumors, and metastasis, with the percentages of samples expressing medium or high amount of MTDH as 10%, 47.2%, and 80.0%, respectively, revealing a tight correlation of MTDH levels with clinical progression of prostate disease (Fig. 1A, left; \( \chi^2 \) test; \( P < 0.001 \)). A difference in MTDH protein levels was already apparent when we compared BPH and PIN, two types of noncancerous tissues (Fig. 1A, right; \( \chi^2 \) test; \( P = 0.023 \)), indicating an increase of MTDH expression during the transition from benign to premalignant stage. MTDH levels were also correlated with Gleason scores of primary tumors, as evidenced by higher average staining intensity of MTDH (Fig. 1C, blue curve) or a greater percentage of samples with at least medium levels of MTDH (Fig. 1C, red curve) in late-stage tumors. Importantly, the prostate-specific antigen (PSA)–based recurrence rate in patients with medium or high levels of MTDH was significantly higher than that in patients with low levels of MTDH in their prostate tumors (Fig. 1D). Together with the fact that MTDH protein levels in prostate cancer distant metastasis are markedly higher than those in primary tumors (Fig. 1A and 1B), these data document a strong positive correlation of MTDH levels with prostate cancer recurrence and metastasis.

**MTDH genomic gain is associated with MTDH overexpression and clinical progression in prostate cancer**

We previously mapped MTDH at the center of a common 8q22 gain that is associated with poor prognosis of breast cancer (3). To test whether there is also increased genomic copy number of MTDH in prostate tumors, we analyzed a prostate tissue microarray with interphase FISH using probes against either the MTDH genomic locus or the chromosome 8 centromere region (as control). FISH analysis revealed a significant portion of prostate cancer samples harbored extra
genomic copies of the MTDH gene (Fig. 2A). Importantly, MTDH DNA gain was significantly associated with higher levels of protein in the cells (Fig. 2B), indicating that DNA gain is a mechanism for MTDH overexpression in prostate cancer. Furthermore, MTDH DNA gain was tightly correlated with clinical stages and prognosis, such that of samples before or at the premalignant stages (normal, BPH, or PIN), local tumors, and distant metastasis, the portions with DNA gain were 0%, 24%, and 50%, respectively (Fig. 2C). In addition, patients with increased MTDH copies suffered from earlier recurrence than those without MTDH genomic gain (Fig. 2D). These clinical data demonstrate the prognosis values of DNA and protein status of MTDH, and suggest a potentially crucial role of MTDH in prostate cancer progression and metastasis.

**Generation and characterization of Mtdh-deleted TRAMP mice**

To investigate the roles of Mtdh in normal development and cancer, we first created whole-organism Mtdh-knockout (KO) mice using ESC line XB780 from Bay Genomics gene trap database (24). The mutant allele contains a LacZ transgene insertion into the second intron of Mtdh, which results in premature termination of transcription. Mtdh-KO (Mtdh<sup>-/-</sup>) mice were viable and fertile, and no overt abnormality in any organs were seen at autopsy. Specifically, the gross morphology (Supplementary Fig. S1A) and relative weights (Supplementary Fig. S1B) of the lower genitourinary tract that includes both the prostate and seminal vesicles were comparable between wild-type mice and the Mtdh<sup>-/-</sup> mice (Supplementary Fig. S1C). Morphological abnormality in the prostate epithelium of Mtdh<sup>-/-</sup> mice was observed more abundant expression of Probasin (Fig. 3C). Consistently, a significantly increased mRNA was detected in TRAMP/Mtdh<sup>+/−</sup> prostates compared with normal controls (Fig. 3D). These data indicate that Mtdh is upregulated during prostate tumorigenesis in mice and suggest that higher levels of Mtdh may confer a competitive advantage for tumor cells.

To test whether Mtdh loss affects the expression of the PB-Tag transgene, we immunostained SV40 T antigen in prostates from TRAMP mice. The T antigen was detected in prostate tumorigenesis, we isolated prostate tissues from normal and TRAMP mice and examined the mRNA (Fig. 3C) and protein (Fig. 3D) levels of Mtdh. As expected, no Mtdh mRNA was detected in Mtdh<sup>-/-</sup> prostate tissues (Fig. 3C), confirming that the gene-trapped method completely abolished Mtdh expression. Furthermore, we observed more abundant Mtdh mRNA in prostate tissues from TRAMP/Mtdh<sup>+/−</sup> and TRAMP/Mtdh<sup>-/-</sup> mice compared with matched control mice that do not express SV40 T antigen (Fig. 3C). Consistently, a significant increase in protein levels of Mtdh was detected in TRAMP/Mtdh<sup>+/−</sup> prostates compared with normal controls (Fig. 3D). These data indicate that Mtdh is upregulated during prostate tumorigenesis in mice and suggest that higher levels of Mtdh may confer a competitive advantage for tumor cells.

Fig. 2. MTDH genomic gain is associated with MTDH protein levels and clinical progression in prostate cancer. A, a prostate tumor tissue microarray was analyzed for MTDH genomic copy number by FISH. Shown are examples of tumors without (left) or with (right) MTDH genomic gains. SpectrumGreen (green) and SpectrumOrange (pale orange) probes detect chromosome 8 centromere and the 8q22 region, respectively. Scale bar, 1 μm. B, MTDH genomic gain is correlated with MTDH protein levels. Samples with MTDH gain, n = 11; samples without MTDH gain, n = 64. P = 0.0018 by the χ² test. C, frequency of MTDH genomic gain in prostate tissues with benign or premalignant tumors, n = 38; primary tumors, n = 29; distant metastasis, n = 28. P values by the χ² test are shown. D, the Kaplan–Meier analysis of recurrence-free survival of patients with prostate cancer with and without MTDH genomic gain in their tumors. Cox proportional hazard ratio (HR) is shown.
epithelial cells as early as 8 weeks of age (Fig. 3E) and continuously expressed in the prostatic epithelium and tumor cells at later stages (Fig. 3F). There was no noticeable difference in the T antigen immunoreactivity in individual prostatic epithelial cells between TRAMP/Mtdh+/+ and TRAMP/Mtdh+/– mice at either early or late stages of cancer progression. Furthermore, SV40 t and T mRNA expression were comparable in TRAMP mice with different Mtdh status (Supplementary Fig. S2A and S2B). As expected for the negative control, SV40 antigens were undetectable in prostates from normal mice (Supplementary Fig. S2A and S2B). These results clearly demonstrate that Mtdh deletion does not affect the PB-Tag transgene expression in prostate epithelial cells.

Loss of Mtdh suppresses prostate tumor formation and increases survival rate

To investigate the overall effect of Mtdh on oncogene-induced prostate tumorigenesis, we studied cohorts of TRAMP mice (n > 100) with different Mtdh status for their prostate tumor development and cancer-related mortality.
MTDH Promotes Prostate Cancer Progression and Metastasis

First, as genitourinary weight is a reliable indicator of tumor burden in TRAMP mice (22), we dissected and measured the urogenital apparatus from TRAMP mice at different ages to monitor tumor formation at a gross level. As Mtdh expression was comparable between Mtdh+/+ and Mtdh+/− prostate tissues (Fig. 3C), we grouped these mice as Mtdh-positive group (Mtdh+) as opposed to Mtdh-negative group (Mtdh−). The SV40 T antigens induced a drastic expansion of the urogenital mass in TRAMP/Mtdh+ mice as a function of age (Fig. 4A, red), such that many of these mice displayed severely enlarged abdomen at 36 weeks of age. In contrast, the wet weights of genitourinary tissues (Fig. 3C), we grouped these mice as P, prostate; SV, seminal vesicle) excised from 36-week-old male mice with indicated genotypes. Scale bar, 1 cm. D, tumor incidence scored by examining histologic sections of prostate glands from cohorts of TRAMP mice with indicated Mtdh genotype at different ages. The numbers on top of each bar graph indicates the number of mice with prostate cancer versus total number of mice examined in a given group. **, P < 0.01 and ***, P < 0.001 based on the χ2 test. E, mortality rate of TRAMP mice with indicated Mtdh genotypes by 1 year of age. ****, P < 0.001 based on the χ2 test.

To further assess the occurrence of prostate cancer at histologic level, we sectioned prostate tissues from TRAMP mice and performed H&E staining. About 50% of 23- or 28-week-old TRAMP/Mtdh− mice developed prostate cancer lesions, and by 36 weeks of age, almost all the mice in this group developed multifocal prostate tumors (Fig. 4D, red). In contrast, no prostate cancer was detected in 28-week-old TRAMP/Mtdh+/− mice, and prostate cancer lesions were detected in only 50% of 36-week-old TRAMP/Mtdh+/− mice at very limited regions of the prostate epithelium. These histopathologic results are consistent with the morphologic observations showing that most TRAMP/Mtdh+/− mice did not display visible signs for prostate tumor formations by 36 weeks of age (Fig. 4C). Of note, tumors formed in TRAMP mice exhibit diverse histologic features that are characteristics of adenocarcinomas (Supplementary Fig. S3A, top), phyllode-like tumors (Supplementary Fig. S3B, top), and neuroendocrine tumors (Supplementary Fig. S3C, top), and it has been suggested that these morphologically distinct tumors may originate from different cell lineages within the prostate or develop independently from common early progenitors (25). Interestingly, TRAMP/Mtdh+/− mice showed a delay in the occurrence of all these different tumor subtypes (Supplementary Fig. S3),
suggesting a possible lineage-independent role of Mtdh in prostate cancer.

As a consequence of malignant prostate cancer development, a significant percentage (~50%) of TRAMP/Mtdh+ died before reaching 1 year of age. In contrast, only 1 of 14 TRAMP/Mtdh−/− examined died from cancer-related disease (Fig. 4E, P < 0.001). These data together demonstrate that Mtdh deletion in mice inhibits oncogene-driven formation of prostate cancer and extends the life span of TRAMP mice.

Inactivation of Mtdh impedes prostate cancer progression

Prostate cancer progression consists of multiple stages starting from premalignant lesions such as PIN to well, moderately, and poorly differentiated carcinoma. To investigate how Mtdh loss affects the initiation and progression of prostate cancer, we performed histologic examination on dorsal and lateral lobes of each prostate gland, the most frequent and severe sites subjected to tumorigenesis in TRAMP mice (Fig. 5A; ref. 21). On the basis of previously described grading systems for TRAMP tumors (21, 22), each prostate was assigned a highest score ranging from a score of 1 for normal prostate and 6 for poorly differentiated carcinoma including neuroendocrine tumors. PIN lesions were readily detected in both TRAMP/Mtdh+ and TRAMP/Mtdh− prostates from 8 to 12 weeks of age (Fig. 5B, first panel), as evidenced by epithelial hyperplasia and the presence of an intact basal cell layer (Fig. 5A, T8 and T12). This was consistent with comparable proliferation (Supplementary Fig. 5A) and apoptosis (Supplementary Fig. 4B) indices in prostate epithelium from 10-week-old TRAMP/Mtdh+ and TRAMP/Mtdh− mice. A substantial fraction of TRAMP/Mtdh− mice developed well or moderately differentiated adenocarcinoma with invasive lesions at 23 and 28 weeks, respectively, and by 36 weeks, 84% of TRAMP/Mtdh+ mice only developed PIN or early-stage, well-differentiated adenocarcinomas or phyllode-like tumors by 36 weeks. Consistently, the expression of E-cadherin was gradually reduced or even became absent as prostate tumors progressed to less-differentiated stages in TRAMP/Mtdh+ mice (Fig. 5C, top), whereas abundant E-cadherin was detected in prostate tissues in TRAMP/Mtdh−/− mice across different ages examined (Fig. 5C, bottom). In addition, malignant progression of Mtdh+ tumors was accompanied by a significantly higher percentage of proliferating cells (Fig. 5D, P < 0.01), whereas the percentage of apoptotic cells were similar in Mtdh− and Mtdh− tumors at this stage (Supplementary Fig. 4C). Taken together, these findings suggest that inactivation of Mtdh arrests prostate cancer progression at early stages of tumorigenesis and prevents premalignant lesions to expand and progress into advanced stages.

Ablation of Mtdh reduces systemic metastasis of prostate tumor cells

We examined metastatic disease in cohorts of TRAMP mice at around 50 weeks of age, a time point when most of TRAMP/Mtdh+ and Mtdh−/− mice have developed prostate cancer lesions. We dissected livers, lungs and periaortic lymph nodes, frequent sites of metastasis in the TRAMP model (26), and examined the appearance of metastatic lesions (Fig. 6A and B) at both macroscopic (Fig. 6C) and histology levels (Fig. 6D). Multiple large metastatic nodules were observed in livers from 34% of TRAMP/Mtdh+ mice, resulting in markedly increased liver size. Conversely, none of TRAMP/Mtdh−/− mice examined developed visible metastases in the liver. Similarly, multiple metastatic deposits in the lung were found in 39% of TRAMP/Mtdh+ mice, but only 2 of 17 TRAMP/Mtdh−/− mice had 1 or 2 metastatic lesions detected in their lungs. Enlargement of lymph node were frequently identified in TRAMP/Mtdh+ mice (9/20) and this incidence decreased to 30% in TRAMP/Mtdh−/− mice. To demonstrate that the metastatic cells were of prostatic origin, immunohistochemical analysis was performed on serial sections of the liver, lung, and lymph node to detect the T-antigen oncoprotein. We observed a uniform expression of the T-antigen oncoprotein confined to the metastatic deposits but not surrounding normal cells (Fig. 6E), in agreement with the tissue-specific pattern of PB-directed transgene expression (26). These data together clearly show that Mtdh deletion in mice significantly reduced systematic metastasis in the TRAMP model. Of note, because of the fact that close to 50% of TRAMP/Mtdh+ mice had died from large primary and likely metastatic tumors before reaching one year of age (Fig. 4E), the observed difference in metastasis between Mtdh+ and Mtdh− groups was very likely to be underestimated.

Knockdown of Mtdh in prostate cancer cells reduces proliferation in vitro and tumor formation in vivo

As Mtdh is widely expressed in mice (data not shown), the tumorigenesis defects in whole-organism Mtdh-KO mice could result from either loss of Mtdh in prostate epithelial cells or other cell/tissue types. To distinguish between these two possibilities, we examined the necessity of Mtdh in tumorigenic ability of TRAMP-C1 cell line, which is characteristic of an advanced prostate tumor in the TRAMP model (27). We knocked down (KD) Mtdh (Fig. 7A and 7B) and found that Mtdh-KD decreased proliferation of TRAMP-C1 cells in vitro (Fig. 7C). To examine the consequence of Mtdh-KD in tumor formation in vivo, we injected control and Mtdh-KD cells into the flanks of recipient mice and monitored tumor occurrence over time. The occurrence of palpable tumors was significantly delayed in the KD groups (Fig. 7D), and tumors dissected from the KD groups were much smaller than those from the control group (Fig. 7E and 7F). Similar results were obtained in another 2 independent of experiments (Supplementary Fig. S5). Interestingly, tumors that eventually formed in the Mtdh-KD groups expressed similar levels of Mtdh as tumors in the control group (Fig. 7G), which could result from either the clonal expansion of the cells without Mtdh inhibition or from re-expression of Mtdh in Mtdh-KD cells.

Discussion

Human prostate cancer is characterized by rampant recurrent amplifications and deletions, suggesting that along with a...
Figure 5. Loss of Mtdh inhibits malignant progression of prostate cancer. A, H&E-stained histologic sections of prostates dissected from TRAMP/Mtdh+ and TRAMP/Mtdh– mice at indicated ages. Scale bar, 200 μm. B, each prostate from mice with indicated genotypes and ages was assigned a single highest grade. + and – indicate TRAMP/Mtdh+ and TRAMP/Mtdh– mice, respectively. Grade scores: 1, normal; 2, low grade PIN; 3, high-grade PIN; 4, well-differentiated adenocarcinoma and phyllode tumor; 5, moderately differentiated adenocarcinoma; 6, poorly differentiated adenocarcinoma and neuroendocrine tumors. The grading scheme followed standard protocol as previously described (23). The numbers at the bottom of each column indicate total number of prostate glands evaluated in each group. P < 0.05 by the χ2 test in T36 group. C, analysis of E-cadherin during prostate tumor progression. Sections from dorsal–lateral lobes were immunostained with an antibody against E-cadherin and images shown are representative of n > 3 animals per group. Scale bar, 25 μm. D, left, representative images of Ki67-stained sections of prostate tumors from 36-week-old TRAMP/Mtdh+ (n = 6) and TRAMP/Mtdh– (n = 3) mice. Scale bars, 25 μm. Right, quantification of Ki67-positive epithelial cells. Data represent mean ± SEM. **, P < 0.01 based on the Student t test.
few well-known genetic lesions, such as loss of tumor suppressors PTEN and p53, there remain many uncharacterized genes governing the genesis and progression of this malignancy (28). In this study, we demonstrate that *MTDH* is frequently overexpressed and amplified in human prostate cancers and its expression levels strongly correlate with disease progression and poor survival outcome. In addition, our genetic studies utilizing *Mtdh*-KO mice provide the first *in vivo* evidence that *Mtdh* plays a critical role in spontaneous prostate cancer progression and metastasis without affecting normal development.

The TRAMP model closely resembles human prostate cancer as it spontaneously develops progressive prostate cancer that can metastasize to multiple different organs. Importantly, we show *Mtdh* levels are elevated in both human and SV40-driven murine prostate tumors compared with normal prostate tissues. Interestingly, *Mtdh* was recently shown to be overexpressed through genomic amplification in a p53/Pten-deficient metastatic prostate cancer mouse model, in which telomerase activity was reactivated following telomere dysfunction (29), suggesting that *Mtdh* can be activated in mice through genomic amplification mechanism similar to what we found in human prostate cancer. Future studies are needed to investigate the molecular basis of *Mtdh* overexpression in SV40-transformed tumors.

We show that *Mtdh* knockout in the TRAMP mice significantly impaired tumor formation and decreased cancer-related mortality. The findings that silencing of *Mtdh* in TRAMP-C1 cancer cells prolonged tumor-free survival and reduced tumor growth *in vivo* lend further support for a prostate tumor cell-intrinsic role of *Mtdh* in prostate cancer. Of note, tumors eventually formed by TRAMP-C1 cancer cells that presumably carried *Mtdh*-targeting shRNA regained the expression of *Mtdh*, reinforcing the notion that *Mtdh* is essential for prostate cancer formation *in vivo*. We observed a blockage of prostate cancer progression at benign or well-differentiated stages in TRAMP/*Mtdh*+/− mice, a notion that is further supported by constitutively high levels of epithelial marker E-cadherin in TRAMP/*Mtdh*+/− prostate tissues. Consistently, although TRAMP/*Mtdh*+/− mice displayed enlarged prostate glands and obstructed seminal vesicles, a phenomenon often resulted from invasive and malignant growth of prostate tumor cells into the urethra and seminal vesicles, urogenital track from TRAMP/*Mtdh*+/− mice seemed largely normal. In line with our findings from mouse models, MTDH exhibits a progression-correlated expression pattern in human prostate cancer. These results may suggest the potential use of MTDH as a biomarker for prostate cancer progression and a possible therapeutic target to halt malignant progression of human prostate cancer.

The fact that a small percentage of TRAMP/*Mtdh*+/− mice still developed invasive prostate cancer after long latency and...
Figure 7. Silencing of Mtdh in TRAMP-C1 prostate cancer cells decreases proliferation in vitro and tumor formation in vivo. A and B, Mtdh was KD by two independent shRNA, as quantified by qPCR (A) and Western blot analysis (B). C, proliferation rate of control and Mtdh-KD TRAMP-C1 cancer cells after 48 hours. D, kinetics of tumor formation in D. G, Mtdh mRNA levels in tumors formed in control and KD groups. Note the tumors that eventually grew in the KD groups expressed similar levels of Mtdh as the controls. A, C, and G, data represent mean ± SEM and P values based on the Student’s t test. **, P < 0.01; ***, P < 0.001; n.s., not significant.

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died from the disease (1 of 14) suggests that some TRAMP/Mtdh−/− tumors have overcome the deficiency of Mtdh and utilized Mtdh-independent pathways to support their malignant growth and progression. Indeed, the induction of SV40 oncogene occurs uniformly in prostate epithelial cells as early as puberty; however, the development of invasive prostate cancer and metastasis occurs much later and seems to be highly heterogeneous, suggesting that individual tumor may acquire different genetic and epigenetic alterations to fully transform SV40 antigen-positive cells. Although inactivation of Mtdh inhibits Mtdh-dependent signaling and prostate cancer progression as observed in the majority of TRAMP/Mtdh−/− mice, it would not block tumor growth that is supported by Mtdh-independent mechanisms. This is also in line with the observation that MTDH is highly expressed in a large percentage but not all human prostate cancer. Nevertheless, cancer cells that have depended on high levels of MTDH for their progression to a malignant stage would be sensitive to MTDH-targeting therapeutics, as supported by the finding that inhibition of Mtdh in Mtdh-positive TRAMP-C1 cells significantly impairs the tumorigenic potential of these cells.

Metastasis to distant organs is responsible for the majority of cancer-related death in patients with solid cancer (30). We previously demonstrated that MTDH is amplified and/or overexpressed in human breast cancer with a higher risk of metastasis (3). In this study, we found that a substantial fraction of distant metastases of human prostate cancer exhibited high levels of MTDH and harbored amplification of the 8q22 genomic loci. Functionally, Mtdh ablation significantly decreased the incidence and burden of metastases in distant organs in the TRAMP model. In concordance with our findings, a recent study shows that telomerase reactivation following telomere dysfunction in a prostate cancer–prone mouse model driven by Pten and p53 loss facilitates the selection of copy number alterations at cancer-relevant loci, and Mtdh was found to be at the top of a selective list of genes whose genetic amplification is strongly associated with cancer progression and metastasis in this mouse model (29). This suggests a possibly crucial role of Mtdh in prostate cancer driven by genetic events in addition to SV40 oncogenes. Finally, it should be noted that the metastasis-promoting function of MTDH is likely to exist in other cancer types, beyond breast and prostate cancers. Several recent studies indeed showed abnormal MTDH expression was associated with lymph node metastasis and a worse prognosis in additional cancer types including laryngeal squamous cell carcinoma (31), squamous cell carcinoma of the head and neck (32), and hepatocellular carcinoma (33), and overexpression of MTDH increases experimental metastasis of hepatocellular carcinoma cells (34).

In summary, our study reports that MTDH is overexpressed in both mouse and human prostate cancer and this elevated level of MTDH is critical for spontaneous prostate cancer
progression and metastasis in vivo. Given the broad over-expression of MTDH in diverse cancer types, the tumor-promoting roles of MTDH may not be restricted to the models used in the study. Indeed, ongoing studies in our laboratory have also found that deletion of Mtdh drastically inhibits spontaneous mammary tumor formation and metastasis (unpublished data). Future studies are needed to understand the underlying mechanisms and signaling pathways that responsible for the tumor-promoting function of MTDH and to facilitate the development of MTDH-targeting therapeutics to control human cancer.

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

Authors’ Contributions
Conception and design: L. Wan, Y. Kang
Development of methodology: L. Wan, Qi. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Wan, G. Hu, M. Yuan, R.T. Bronson, Q. Yang, J. Siddiqui, K. J. Pienta
Analysis and interpretation of data (e.g., statistical analysis, bioscience, computational analysis): L. Wan, G. Hu, J. Siddiqui

References

Writing, review, and/or revision of the manuscript: L. Wan, J. Siddiqui, K. J. Pienta, Y. Kang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wan, M. Yuan, Qi. Yang, J. Siddiqui

Study supervision: L. Wan, Y. Wei, Y. Kang

Other (did the pathology for this paper): R.T. Bronson

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