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Metallothionein 1 h tumour suppressor activity in prostate cancer is mediated by euchromatin methyltransferase 1

Yu-Chen Han^{1,†}, Zhong-Liang Zheng^{1,†}, Ze-Hua Zuo¹, Yan P Yu¹, Rui Chen², George C Tseng², Joel B Nelson³, and Jian-Hua Luo^{1,*}

¹Department of Pathology, 3550 Terrace Street, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

²Department of Biostatistics, 3550 Terrace Street, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

³Department of Urology, 3550 Terrace Street, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

Abstract

Metallothioneins (MTs) are a group of metal binding proteins thought to play a role in the detoxification of heavy metals. Here we showed by microarray and validation analyses that MT1h, a member of MT, is down-regulated in many human malignancies. Low expression of MT1h was associated with poor clinical outcomes in both prostate and liver cancer. We found that the promoter region of MT1h was hypermethylated in cancer and that demethylation of the MT1h promoter reversed the suppression of MT1h expression. Forced expression of MT1h induced cell growth arrest, suppressed colony formation, retarded migration, and reduced invasion. SCID mice with tumour xenografts with inducible MT1h expression had lower tumour volumes as well as fewer metastases and deaths than uninduced controls. MT1h was found to interact with euchromatin histone methyltransferase 1 (EHMT1) and enhanced its methyltransferase activity on histone 3. Knocking down of EHMT1 or a mutation in MT1h that abrogates its interaction with EHMT1 abrogated MT1h tumour suppressor activity. This demonstrates tumour suppressor activity in a heavy metal binding protein that is dependent on activation of histone methylation.

Keywords

prostate cancer; tumour; suppression; metallothionein; histone methylation

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*Correspondence to: Jian-Hua Luo, Scaife S-760, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA. luoj@msx.upmc.edu.

[†]These authors contributed equally to this work.

Author contribution statement

JHL and JBN conceived and designed the experiments. YPY and GCT supervised the experiments and analyses. YCH, ZLZ, ZHZ, and RC performed the experiments and analysis.

SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Table S1. siRNA specific MTs and EHMT1.

Table S2. Primer sequences for MTs' RT-PCR.

Introduction

Metallothioneins (MTs) are a group of evolutionarily conserved cysteine-rich metal binding proteins thought to be involved in detoxification of heavy metals [1,2]. MTs are classified into multiple classes based on cysteine distribution and spacing. MT1 has the characteristic sequence of K-x(1,2)-C-C-x-C-C-P-x(2)-C and is mostly present in vertebrates, including man [3]. Cysteine residues from MTs serve to neutralize harmful oxidant radicals, such as the superoxide and hydroxyl radicals, by oxidizing itself into cystine [4,5]. This reduces the oxidized heavy metals and prevents them from damaging cellular proteins, lipids, and nucleic acids. However, the broader biological function of MTs remains unclear.

Previous studies suggest that multiple MT proteins are abnormally expressed in many human malignancies [6,7]. The close similarity of MT species has made in-depth studies in human cancer and other diseases difficult. In this study, we analysed the expression of one of the MT species, MT1h, in liver and prostate cancer. We found that MT1h is widely down-regulated in liver and prostate cancers. Its promoter is heavily methylated. MT1h carries out its tumour suppressor activity through activation of euchromatin histone methyltransferase 1 (EHMT1), which induces histone methylation and possibly gene expression suppression [8].

Materials and methods

Primers, tissues, cell lines, and cell culture

Prostate cancer cell lines, DU145 and PC3, and an immortalized prostate epithelial cell line, RWPE-1, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown using standard condition (see Supplementary materials and methods).

Cloning, PCR, and other molecular biological methods

These methods were performed by standard techniques, with details to be found in the Supplementary materials and methods, and Supplemental Table 1 and 2.

Screening and validation of protein–protein interactions in yeast

Yeast two-hybrid screening was performed as previously detailed [9]. Briefly, plasmid DNA from positive clones was isolated from yeast and sequenced. For validation, an isolated clone of pACT2-EHMT1 was co-transformed with pBD-MT1h into AH109 yeast cells and plated on SD-Ade/-His/-Leu/-Trp high stringency plates. Co-transformation of pGADT7T and pGBKT7-53 was used as a positive control for protein–protein interactions, while co-transformation of pGADT7T and pGBKT7-Lam was used as a negative control. Validation was performed by immunoprecipitation and pull-down experiments (see Supplementary materials and methods for details).

H3K9 methyltransferase assays

Nuclear extracts from MT1h#4, MT1h#6, DMT1h#8, and DMT1h#9 cells induced with or without tetracycline were immunoprecipitated using an anti-EHMT1 antibody. H3K9 methyltransferase assays were subsequently performed using substrates obtained from

Epigentek, Inc (Farmingdale, NY, USA), according to the manufacturer's instructions. Negative controls were immunoprecipitates that did not include anti-EHMT1 antibodies or that included UNC0638, an EHMT1 inhibitor.

Colony formation assays, tumour growth, and spontaneous metastasis assays

Colony formation assays were performed as previously described [9,10]. For tumour growth and spontaneous metastasis assays, tumour xenograft models were established in severe combined immunodeficiency disease (SCID) mice as previously described [9–13] (see Supporting information for details). All studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Results

To investigate whether MT1h is down-regulated in human cancers, we analysed MT1h expression from publicly available microarray data [6]. We found MT1h to be down-regulated in 30 sets of microarray data of human malignancies, including, but not limited to, prostate cancer, breast cancer, colon cancer, bladder cancer, leukaemia, B-cell lymphoma, neuroblastoma, small cell lung cancer, and melanoma. There is a consistent down-regulation of MT1h in these tumours compared with normal tissues (Table 1). Analysis of prostate and liver cancer microarray data from the University of Pittsburgh [14,15] (Figure 1A) suggested that reduced expression of MT1h RNA occurs not only in tumour samples, but also in benign liver or prostate tissues adjacent to the tumours. However, the magnitude of the down-regulation of MT1h was significantly larger in cancer samples. To validate the microarray data, semi-quantitative RT-PCR was performed on the prostate cancer cell lines DU145, PC3, and LNCaP. There is no detectable expression of MT1h in these cell lines. In contrast, a high level of MT1h expression was found in prostate and liver organ donors. A 10- to 100-fold decrease of MT1h expression was found in both liver cancer and prostate cancer (Figure 1B). MT1h shares 87–92% amino acid homology with seven other MT1 members, but only 63–76% nucleotide homology.

To detect the specific expression of MT1h, we selected three regions of MT1h that do not contain homology with other MT1 members as target regions for *in situ* hybridization (see Supporting information). These probes were then hybridized to formalin-fixed, paraffin-embedded tissue array slides containing 305 normal prostate tissues, 220 prostate cancer, 89 normal liver, and 162 liver cancer samples. The results of the MT1h *in situ* hybridization were then graded as strong (3), moderate (2), weak (1), focal (0.5), or negative (0). As shown in Figures 1C and 1D, moderate expression of MT1h was found in both normal liver (1.91) and prostate glands (1.89). The expression of MT1h in prostate cancer (0.22, $p < 0.001$) and liver cancer (0.23, $p < 0.001$) was significantly decreased. These results confirm and substantiate the validity of previous microarray results.

Prostate cancer samples were then divided into two groups: one group containing samples from patients who did not experience chemical relapse for at least 5 years, and one containing samples from patients that experienced relapse within 5 years of radical prostatectomy. The average score for the levels of MT1h expression detected for the relapse group (0.18) was lower than that for the patients that did not experience relapse (0.34) ($p =$

0.024). Similarly, when liver cancer samples were divided according to patients who died within 5 years of diagnosis versus patients who survived longer than 5 years, the average score of the former group (0.08) was lower than that of the latter group (0.59) ($p < 0.001$). Taken together, these results suggest that low levels of MT1h expression are associated with a poor prognosis for both prostate and liver cancer patients.

When MT1h expression was separated into a group with at least focal expression versus a group with negative expression, 53% (46/86) of patients with negative expression of MT1h survived 5 years prostate cancer-free, while 77% (34/44, $p = 0.012$) of patients with at least focal expression of MT1h did the same (Figure 2). For liver cancer samples, a similar separation yielded 38% 5-year survival for samples from patients with negative MT1h expression. This was significantly lower than the 79% 5-year survival for samples from patients with at least focal expression of MT1h (Figure 2; $p < 0.001$). These analyses suggest that MT1h expression plays an important role in tumour progression and tumour-related mortality.

To explore the mechanism of down-regulation of MT1h, Q-PCR was performed on DNA from normal and neoplastic prostate tissues. Only two samples appeared to have deletion of MT1h. Analysis of an Affymetrix SNP 6.0 chip of 104 prostate cancer samples [16] revealed less than 9% (9/104) of prostate cancer samples containing hemizygous deletion of MT1h and zero cases with MT1h deletion in 98 liver cancer samples [17]. These results indicate that genome copy deletion cannot account for the widespread down-regulation of MT1h in prostate and liver cancers. However, a CpG island was identified in the promoter/exon 1 regions of MT1h, spanning -345 to +190 of the MT1h genome (Figure 3A). To investigate whether the promoter region of MT1h is methylated, a pair of methylation-specific primers was designed to cover the MT1h promoter region (-317 to -91). As shown in Figure 3B, 41 of 43 (95%) primary prostate cancer samples were methylated. The MT1h promoter in the prostate cancer cell lines PC3, DU145, and LNCaP was also methylated. All ten liver cancer samples showed methylation of the MT1h promoter. The methylation PCR products of six prostate cancer and two liver cancer samples were ligated into the TA cloning vector and 5–6 clones of each PCR product were sequenced. The sequencing confirmed hypermethylation of CpG in this region of the MT1h promoter (Figure 3C). To investigate the impact of methylation on MT1h expression, PC3 and DU145 cells were treated with aza-cytidine, which causes demethylation of genomes in progeny cells. As shown in Figure 3D, treatment of PC3 and DU145 cells with aza-cytidine caused demethylation of the MT1h promoter in these cells and reversed the expression of MT1h from negative to strongly positive, suggesting a critical role of promoter methylation for MT1h expression suppression.

To test the notion that MT1h contains tumour suppressor activity, MT1h cDNA was ligated into pCDNA4-FLAG in frame to produce an MT1h-FLAG fusion protein. The pCDNA4-MT1h-FLAG/pCDNA6 constructs were then transfected into PC3 cells. Multiple transfectants with tetracycline-inducible MT1h-FLAG were identified and three colonies were selected for further studies. As shown in Figure 4A, induced expression of MT1h-FLAG reduced colony formation by 40–55% ($p < 0.001$). Induced MT1h expression also reduced entry of the S and M phases of cells and increased the G0/G1 phase: S phase

decreased from 33% to 20% ($p < 0.01$), M phase from 41% to 28% ($p < 0.01$), and G0/G1 phase increased from 24% to 53% ($p < 0.001$) for clone 4; for clone 6, S phase decreased from 37% to 24% ($p < 0.05$), M phase from 34% to 27% ($p < 0.05$), and G0/G1 phase increased from 29% to 51% ($p < 0.001$); for clone 15, S phase decreased from 18% to 12% ($p < 0.05$), M phase from 65% to 51% ($p < 0.05$), and G0/G1 phase increased from 19% to 34% ($p < 0.01$). These results indicate that MT1h suppresses cell growth by reducing cell entry into the S and M phases.

We then investigated whether expression of MT1h suppresses tumour cell migration and invasiveness; wound healing assays were performed on MT1h-FLAG inducible clones. As shown in Figure 4C, expression of MT1h in clones 4, 6, and 15 reduced migration by 34% ($p < 0.05$), 32% ($p < 0.05$), and 35% ($p < 0.05$), respectively. Matrigel traverse analyses were performed to investigate whether expression of MT1h suppresses the invasiveness of tumour cells. As shown in Figure 4D, when MT1h-FLAG was induced with tetracycline, PC3 cell invasion into the Matrigel was reduced by 42% ($p < 0.01$) for clone 4, by 41% ($p < 0.01$) for clone 6, and by 54% ($p < 0.01$) for clone 15. These experiments suggest that MT1h suppresses the invasiveness of prostate cancer.

Clinical data analyses suggest that down-regulation of MT1h is associated with aggressive behaviour of prostate and liver cancers. To examine whether reversing the down-regulation of MT1h in PC3 cells suppresses tumour growth and aggressiveness *in vivo*, clones 4 and 6 were subcutaneously implanted into SCID mice as a xenograft. These mice were treated with tetracycline (10 $\mu\text{g/ml}$) in the water daily. Mice treated with tetracycline had smaller tumour volumes: 0.22 cm^3 versus 0.61 cm^3 of the untreated controls ($p < 0.01$) for clone 4, and 0.31 cm^3 versus 1.24 cm^3 for clone 6. There was no incidence of metastasis in any of the tetracycline-treated groups, while metastases occurred in 25% (2/8) of mice with untreated clone 4 and 37.5% (3/8) with untreated clone 6 (Figure 5). Expression of MT1h also reduced the mortality rate from 56.3% to 12.5% in comparison with control groups ($p = 0.0012$). These results indicate that MT1h contains tumour suppressor activity *in vivo*. The SCID mice xenograft tumour model is similar to the clinical finding that expression of MT1h is associated with higher cancer-free survival rates.

To better understand the tumour suppressor activity of MT1h, we ligated MT1h cDNA into the pGBKT7 vector to create pBD-MT1h to create GAL4 bait domain-MT1h fusion protein. We subsequently performed a yeast two-hybrid screening to identify proteins that interact with MT1h using a pAD-cDNA library from human prostate tissues. Using pBD-MT1h, we identified 17 positive colonies after three rounds of metabolic screening. These colonies were subsequently isolated. After several restriction enzyme digestions, several redundant clones were eliminated. Nine unique clones were identified and sequenced. One of these clones contains a cDNA encoding euchromatin histone-lysine-*N*-methyltransferase 1 (EHMT1).

To validate the yeast two-hybrid screening results, pAD-EHMT1 and pBD-MT1H were co-transfected into yeast AH109 cells, grown in high stringency medium, and tested for α -galactosidase activity. Both pBD-MT1h (full-length) and positive control (pBD-T antigen and pAD-p53) showed positive galactosidase activity, while pGBKT7 and pAD-EHMT1

were negative, suggesting that the EHMT1 binding activity was specific for MT1H (Figure 6A). Among the prostate cell lines, EHMT1 was abundantly expressed in RWPE-1 cells (immortalized prostate epithelial cells) as well as PC3 and DU145 cells (prostate cancer cells). To verify the interaction, an *in vivo* MT1H-EHMT1 binding analysis was performed in protein extracts of PC3 cells transformed with pCDNA4-MT1h-FLAG/pCDNA6. As shown in Figure 6B, co-immunoprecipitation of MT1H-FLAG and EHMT1 was readily apparent. To visualize whether MT1H and EHMT1 co-localize in the nucleus, double immunofluorescence staining using antibodies against the FLAG tag and EHMT1 was performed in PC3 cells transformed with pCDNA4-MT1h-FLAG/pCDNA6. As demonstrated in Figure 6C, MT1H and a substantial amount of EHMT1 co-localized in the nuclei of RWPE-1 cells. To validate the interaction between MT1h and EHMT1 *in vitro*, full-length MT1H was constructed into pGEX-5 T to create a GST-MT1h fusion protein. To rule out potential bridging proteins between EHMT1 and MT1H interactions, EHMT1 cDNA was ligated into the pET-28 vector to express a His-EHMT1 fusion protein in bacteria. The binding analysis with the recombinant His-EHMT1 and GST-MT1H (2–61 amino acids) showed that EHMT1 binds with the MT1H N-terminus directly (Figure 6D), suggesting that the interaction between MT1h and EHMT1 is direct and does not require a ‘bridge protein’ for the interaction. A series of deletion mutants of GST-MT1h was constructed to identify the motifs that are required to interact with EHMT1. A stretch of 19 amino acids located at positions 2–20 of MT1h was found to be crucial for MT1h binding with EHMT1, because fusion proteins lacking this sequence did not bind with EHMT1, while all proteins containing this sequence bound with EHMT1 (Figure 6D).

EHMT1 is a critical molecule responsible for histone methylation, which represses transcription [18,19]. Its main target is the lysine residue at the ninth position of histone 3. To examine the impact of the MT1h–EHMT1 interaction on methylation of histone 3, clones 4 and 6 were induced to express MT1h-FLAG. Histone 3 methylation was then examined using antibodies specific for tri-methyl-histone 3-lysine 9 (trimethyl-H3K9). As shown in Figure 7A, induction of MT1h-FLAG expression markedly enhanced the methyltransferase activity on H3K9. Knockdown of MT1h in RWPE-1 cells dramatically decreased the methylation of H3K9, while knockdown of other family members of MTs, including A, B, E, G, M, and X, had no appreciable effect, suggesting MT1h specificity on activation of methyltransferase activity for histone 3. The knockdown of EHMT1 using siRNA specific for EHMT1 largely abrogated the methylation of H3K9, indicating that EHMT1 is essential for MT1h-induced methyltransferase activity on histone 3 (Figure 7B). To investigate whether interaction between MT1h and EHMT1 is required for methyl-transferase activity of histone 3, a mutant MT1h that is defective in binding with EHMT1 was generated. The cDNA of this mutant was ligated into pCND4 to generate pCDNA4- MT1h, where amino acids 2–19 of MT1h were deleted. Induction of mutant MT1h led to no discernible increase of methyltransferase activity (Figure 7A). Subsequently, the methyltransferase activities of EHMT1 were quantified *in vitro* to investigate the impact of MT1h. As shown in Figure 7C, induction of MT1h enhanced the methyltransferase activity of EHMT1 by 109% ($p < 0.001$) for clone 4 and by 85% ($p < 0.001$) for clone 6. In contrast, induction of mutant MT1h had no appreciable impact on the methyltransferase activity of EHMT1. In parallel with the methyltransferase activity experiments, we found that knockdown of EHMT1 completely

eliminated the tumour colony suppression activity of MT1h. Mutant MT1h that lost the ability to interact with EHMT1 did not possess tumour suppressor activity (Figure 7D). Taken together, these experiments indicate that the tumour suppressor activity of MT1h is dependent on its ability to interact and to enhance the histone methyltransferase activity of EHMT1.

Discussion

We have shown that a member of a heavy metal binding protein family contains tumour suppressor activity. There are several lines of evidence supporting this idea: (1) widespread methylation of the *MT1h* gene in both liver and prostate cancer; (2) down-regulation of MT1h in 28 different malignant tumours; (3) suppression of prostate cancer growth and invasion *in vivo* and *in vitro* after expression of MT1h was restored; and (4) a credible mechanism of the MT1h–EHMT1 pathway leading to tumour suppression. Our analysis links MT1h expression with the activation of EHMT1 and suggests that MT1h is an important regulator of E2F6 activity. We hypothesize that MT1h is a co-factor of EHMT1 methyltransferase. Since the MT1h promoter is highly methylated in both liver and prostate cancers, there is little expression of MT1h in these tumours. As a result, there is very limited methyltransferase activity of EHMT1 in these cells. The restoration of MT1h by forced expression reverses the inactivation of EHMT1, which leads to inhibition of cell growth due to cell cycle arrest.

Few biological studies have been conducted on specific isoforms of MT, due to high sequence homology. However, it is believed that MTs play an important role in facilitating the signal transduction of the insulin receptor by decreasing its dephosphorylation [20,21]. Zinc ion is a co-factor for MTs, and both zinc ion and MTs are potent stimulators of insulin-induced lipogenesis and glucose uptake [21]. Deficiency of zinc has been associated with an increased risk of diabetes in some human populations [22,23]. In this study, however, MT1h, which is one of the family members of MT1, plays a counterbalancing role by activating EHMT1 and suppressing cell growth. In contrast to some MT isoforms, the down-regulation of MT1h was found in a large number of tumours, revealing a complex nature of the function of MT family members. It is likely that MT members have diverse targets and functions in regulating cell proliferation and death. Characterization of the biological function of each MT isoform will shed light on our understanding of these diverse and important molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Down-regulation of MT1h in liver and prostate cancers. (A) Microarray analysis of hepatocellular carcinoma and prostate adenocarcinoma of MT1h expression. Normal organ donor liver and prostate specimens were used as references. AT denotes benign tissues adjacent to cancer. T denotes tumour tissues. (B) Semi-quantitative RT-PCR on primary prostate cancer (T65 and T49), liver cancer (HC14 and HC13), normal prostate donor (PD01, PD04, and PD12), normal liver donor (NL103 and NL13), and prostate cancer cell line (DU145, PC3, and LNCaP) samples. cDNA synthesized from 1 µg of total RNA was diluted into 1 : 10, 1 : 100, 1 : 1000, and 1 : 10 000 solutions. PCR was then performed using primers specific for MT1h and β-actin. (C) Representative images of *in situ* hybridization of MT1h using a cocktail of three biotin-labelled oligo probes complementary to *MT1h* mRNA. (D) Expression of MT1h is down-regulated in prostate and liver cancers. Left panel: mean expression scores and standard errors of MT1h in benign prostate tissues (N), prostate cancer (PC), prostate cancer that did not relapse for more than 5 years (PCnone), and tumours that relapsed within 5 years of prostatectomy (PCrelapse). Right

panel: mean expression scores and standard errors of MT1h in benign liver tissues (N), hepatocellular carcinoma (HCC), HCCs from patients who survived more than 5 years (HCCalive), and HCCs from patients who died within 5 years of diagnosis (HCCdeath).

Figure 2.

Low-level expression of MT1h is associated with poor clinical outcomes of prostate liver cancer patients. (A) Kaplan–Meier analysis of survival of liver cancer patients with at least focal expression of MT1h versus negative expression in liver cancer samples. (B) Kaplan–Meier analysis of disease-free survival of prostate cancer patients with at least focal expression of MT1h versus negative expression in prostate cancer samples.

Figure 3.
The MT1h promoter/exon 1 region is methylated. (A) Schematic diagram of the CpG islands of the MT1h promoter/exon 1 regions. Arrow indicates mRNA start site. Blue denotes CpG island. Each CpG is represented by a vertical stroke. (B) Methylation-specific PCR of the promoter region of MT1h. Lanes 1–4; primer specificity of MT1h methylation and unmethylation primers. Unmethylated DNA templates were treated with (lanes 1, 2) or without (lanes 3, 4) SSSI methylase. The samples were subsequently treated with sodium bisulphite. PCR was performed with methylation primers (lanes 1 and 3) or unmethylation

primers (lanes 2 and 4). Lanes 5–104: methylation-specific PCR on 46 prostate cancer samples and cell lines. Four normal prostate samples were included (N2, N4, N7, and N8). Lanes 105–128: methylation-specific PCR on ten HCC and two normal liver samples (LD1 and LD3). M denotes PCR using methylation-specific primers; U denotes unmethylation-specific primers. (C) Representation of methylation sequencing of selected samples. The PCR product (–91 to –317) contains 15 CpGs excluding those in the primer region and a 182 bp non-primer sequence (226 bp if primers are included). Preservation of cytosine nucleotide after bisulphite treatment in CpG is indicated as CG. Conversion to thymidine after treatment is indicated as TG. Sequencing was performed on templates from clones of the TA vector ligated with the PCR products. (D) Treatment with 5-aza-2'-deoxycytidine reverses the suppression of MT1h expression. Upper panel: PC3 (lanes 1–4) and DU145 cells (lanes 5–8) were treated with or without Aza-C as indicated. Methylation-specific PCR was performed to detect the methylation status of MT1h promoter as C. Lower panel: semi-quantitative RT-PCR of *MT1h* mRNA. cDNA synthesized from 1 µg of total RNA was diluted into 1 : 10, 1 : 100, 1 : 1000, and 1 : 10 000 solutions. PCR was then performed using primers specific for MT1h and β-actin.

Figure 4.

MT1h suppresses tumour growth and invasiveness of prostate cancer. (A) Expression of MT1h-FLAG in pCDNA4-MT1h-FLAG/pCDNA6 in PC3 cells. Protein extracts from stable clones 4, 6, and 15 treated with or without tetracycline were immunoblotted with antibodies specific for FLAG and β -actin. (B) Colony formation assays of replica of A. (C) BrdU labelling and cell cycle analysis of replica of A. (D) Wound healing analyses of replica of A. (E) Matrigel traverse analysis of replica of A.

Figure 5.

MT1h expression decreases the growth, metastasis, and mortality of PC3 xenograft tumours.

(A) MT1h expression reduced the tumour volume of PC3 cell xenografts in SCID mice.

Left: weekly mean of tumour volumes of clone 4 of pCDNA4-MT1h-FLAG/pCND6 transformed PC3 cells treated with or without tetracycline. Right: weekly mean of tumour volumes of clone 6 of pCDNA4-MT1h-FLAG/pCND6 transformed PC3 cells treated with or without tetracycline. (B) MT1h expression reduced the incidence of metastasis. (C) MT1h expression reduced the mortality of xenograft SCID mice.

Figure 6.

MT1h interacts with EHMT1. (A) MT1h bound EHMT1 in a yeast two-hybrid co-transfection. Growth and α -galactosidase activity of yeast AH109 cells co-transfected with the indicated vectors in SD-4 (-Leu/-Trp/-Ade/-His) medium transformed with pBD-MT1h and pAD-EHMT1. pBD-T antigen/pAD-p53 represents the positive control, while pBD/pAD-EHMT represents the negative control. (B) Co-immunoprecipitation of MT1h-FLAG and EHMT1 from pCDNA4-MT1h-FLAG/pCDNA6 transformed PC3 cells. Protein extracts from clone 4 of pCDNA4-MT1h-FLAG/pCDNA6 transformed PC3 cells treated with 5 μ g/ml tetracycline were immunoprecipitated with the indicated antibodies, resolved by 8% SDS-PAGE, and immunoblotted with either anti-EHMT1 (upper panel) or anti-FLAG antibodies (lower panel). (C) Co-localization of MT1h-FLAG and EHMT1 in pCDNA4-

MT1h-FLAG/pCDNA6 transformed PC3 cells. The transformed PC3 cells were immunostained with antibodies specific for FLAG (mouse) and EHMT1 (goat). Immunofluorescence staining was then performed using FITC-conjugated antibodies against mouse (FLAG) or rhodamine-conjugated antibodies against goat (EHMT1). (D) *In vitro* binding analysis of GST-MT1h fusion proteins with His-EHMT1 purified from *E. coli* transformed with pET28-EHMT1. Top: schematic diagram of GST-MT1h fusion protein and deletion constructs. Bottom: upper panel: immunoblots of HisTAG-EHMT1 from a pull-down assay with GST-MT1h and its mutant fusion proteins. Lower panel: Coomassie staining of GST-MT1h and its mutant fusion proteins.

Figure 7.

MT1h tumour suppressor activity is mediated by MT1h/EHMT1 interaction. (A) MT1h/EHMT1 interaction enhances the methylation of histone 3 lysine 9. Left: clones 4 and 6 of pCDNA4-MT1h-FLAG/pCDNA6 transformed PC3 cells were treated with or without tetracycline. Protein extracts were immunoblotted with antibodies specific for FLAG, β -actin, tri-methyl-H3K9, and histone 3. Middle: cells of clone 4 were transfected with siRNA specific for EHMT1 (sE1) or scramble control (Scr). The cells were then induced with tetracycline and immunoblotted by antibodies specific for FLAG, EHMT1, β -actin, tri-methyl-H3K9, and histone 3. Right: clones 8 and 9 of pCDNA4-MT1h-FLAG/pCDNA6 transformed PC3 cells were treated with or without tetracycline and immunoblotted as in the left panel. (B) MT1h-specific methylation of H3K9. siRNAs specific for MT1a (sM1a), MT1b (sM1b), MT1e (sM1e), MT1f (sM1f), MT1g (sM1g), MT1h (sM1h), MT1m (sM1m), MT1x (sM1x), and scramble control (Scr) were transfected into RWPE-1 cells. RT-PCRs were performed to examine the knockdown of these MTs. Immunoblots were performed

using antibodies specific for tri-methyl-H3K9 and histone 3. (C) MT1h enhances EHMT1 methyltransferase activity *in vitro*. Clones 4 and 6 of pCDNA4-MT1h-FLAG/pCDNA6 transformed PC3 cells and clones 8 and 9 of pCDNA4- MT1h-FLAG/pCDNA6 transformed PC3 cells were treated with or without tetracycline. Methyltransferase assays were performed on immunoprecipitates of EHMT1 using an EHMT1-specific substrate. (D) Colony formation of replica of A.

Table 1

Human malignancies with MT1h down-regulation

Malignancy	Fold change	<i>p</i> value
B-cell lymphoma	-16	$< 1 \times 10^{-10}$
Bladder cancer	-12	$< 1 \times 10^{-10}$
Breast cancer	-6.3	3.75×10^{-9}
Colorectal cancer	-6.2	11×10^{-8}
Embryonal rhabdomyosarcoma	-6.4	2.47×10^{-9}
Ewing's sarcoma	-12	$< 1 \times 10^{-10}$
Ganglioneuroblastoma	-4.6	3.19×10^{-5}
Malignant peripheral nerve sheath tumour	-3.2	0.007
Monophasic synovial sarcoma	-6.2	2.2×10^{-8}
Multiple myeloma	-3.3	0.004
Myelogenous leukaemia	-13	$< 1 \times 10^{-10}$
Neuroblastoma	-12	$< 1 \times 10^{-10}$
Neuroblastoma – differentiating	-3.8	7.23×10^{-4}
Neuroblastoma – poorly differentiated	-8.1	$< 1 \times 10^{-10}$
Neuroblastoma – undifferentiated	-2.5	0.037
Plasma-cell leukaemia	-4.5	3.9×10^{-5}
Precursor T lymphoblastic leukaemia	-15	$< 1 \times 10^{-10}$
Lung small cell cancer	-2.6	0.03
T-cell acute lymphoblastic leukaemia	-8.6	$< 1 \times 10^{-10}$
Acute lymphoblastic leukaemia	-15	$< 1 \times 10^{-10}$
Acute myeloid leukaemia	-26	$< 1 \times 10^{-10}$
Alveolar rhabdomyosarcoma	-4.4	6.29×10^{-5}
Choriocarcinoma	-4.8	1.01×10^{-5}
Chronic myelogenous leukaemia	-8.6	$< 1 \times 10^{-10}$
Chronic myeloid leukaemia	-9.1	$< 1 \times 10^{-10}$
Colon adenocarcinoma	-6.8	1.33×10^{-10}
Diffuse large B-cell lymphoma	-7.7	$< 1 \times 10^{-10}$
Melanoma	-8.2	$< 1 \times 10^{-10}$
Prostate carcinoma	-6.9	$< 1 \times 10^{-10}$
Smoldering myeloma	-4.3	1.02×10^{-4}