CMTM3, Located at the Critical Tumor Suppressor Locus 16q22.1, Is Silenced by CpG Methylation in Carcinomas and Inhibits Tumor Cell Growth through Inducing Apoptosis

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Abstract

Closely located at the tumor suppressor locus 16q22.1, CMTM-like MARVEL transmembrane domain-containing member 3 and 4 (CMTM3 and CMTM4) encode two CMTM family proteins, which link chemokines and the transmembrane-4 superfamily. In contrast to the broad expression of both CMTM3 and CMTM4 in normal human adult tissues, only CMTM3 is silenced or down-regulated in common carcinoma (gastric, breast, nasopharyngeal, esophageal, and colon) cell lines and primary tumors. CMTM3 methylation was not detected in normal epithelial cell lines and tissues, with weak methylation present in only 5 of 35 (14%) gastric cancer adjacent normal tissues. Furthermore, immunohistochemistry showed that CMTM3 protein was absent in 12 of 35 (34%) gastric and 1 of 2 colorectal tumors, which was well correlated with its methylation status. The silencing of CMTM3 is due to aberrant promoter CpG methylation that could be reversed by pharmacologic demethylation. Ectopic expression of CMTM3 strongly suppressed the colony formation of carcinoma cell lines. In addition, CMTM3 inhibited tumor cell growth and induced apoptosis with caspase-3 activation. Thus, CMTM3 exerts tumor-suppressive functions in tumor cells, with frequent epigenetic inactivation by promoter CpG methylation in common carcinomas. [Cancer Res 2009;69(12):5194–201]

Introduction

CMTM-like MARVEL transmembrane domain-containing family (CMTM) is a novel family of proteins linking chemokines and the transmembrane-4 superfamily, consisting of 9 genes, CKLF and CMTM1 to CMTM8. CMTMs play important roles in immune and male reproductive systems as well as tumorigenesis (1–7). Our previous study showed that CMTM5 has tumor-suppressive functions (6). CMTM5 has at least six RNA splicing forms (CMTM5-v1-v6). CMTM5-v1 is evolutionarily conserved and broadly expressed in normal adult and fetal tissues but frequently down-regulated or silenced in tumor cell lines due to promoter methylation. Restoration of CMTM5-v1 in PC-3 and HeLa cells suppresses tumor cell growth and migration (6).

Among the CMTM family, CMTM3 is most closely related to CMTM5-v1, with 42% amino acid identity. CMTM3 is linked to CMTM4, with both located closely on 16q22.1, which is frequently deleted in multiple tumors, such as hepatocellular, breast, and prostate tumors and retinoblastoma, suggesting the presence of critical tumor suppressor genes (TSG) at this locus (8–11).

TSGs can be inactivated by both genetic and epigenetic mechanisms. Genetic deletion and point mutations disrupt TSG functions, whereas epigenetic mechanisms, including promoter CpG methylation and histone modifications, also frequently lead to the loss of TSG functions and involved in tumor development and progression (12, 13). DNA methylation affects various cellular processes including apoptosis, which is a cell “suicide” program required for maintaining the balance between cell proliferation and death (14). Apoptosis can be initiated mainly through two pathways: intrinsic pathway, which originates within the cell involving the mitochondria, and extrinsic pathway, which is mediated by death receptors. Both pathways culminate in the activation of caspases, with caspase-3 as the common important effector caspase (15).

Here, we report that CMTM3 is frequently methylated in multiple carcinoma cell lines and some primary tumors, resulting in loss of its expression at both mRNA and protein levels, whereas CMTM4 is seldom down-regulated in tumor cell lines. Ectopic CMTM3 expression in tumor cells lacking its expression leads to the suppression of cell growth and apoptosis with caspase-3 activation, suggesting that CMTM3 is a novel functional tumor suppressor.

Materials and Methods

Cell lines and tumor samples. Tumor cell lines studied (16) include breast, gastric, nasopharyngeal, hypopharyngeal, esophageal, prostate, colorectal, hepatocellular, lung, cervical, and renal carcinomas, non-Hodgkin’s lymphoma, Hodgkin’s lymphoma, and nasal NK/T cell lymphomas (Supplementary File). Immortalized, nontransformed normal epithelial cell lines (HMEC, HMEpC, HEK293, RHEK1, NP69, Het-1A, NE1, and NE3) were used as normal controls. Cell lines were treated with 10 μmol/L 5-aza-2’-deoxycytidine (Sigma) for 3 days or further treated with 100 nmol/L trichostatin A for additional ~16 h as described previously (16, 17).
DNA samples extracted from tumor samples have been described (16–19). Gastric and colorectal tumor samples were obtained from patients under primary surgery at the Department of General Surgery, General Hospital of People’s Liberation Army, with patients’ consent and institutional ethics approval. H&E-stained frozen sections were prepared for assessing the percentages of tumor cells. Only samples with >50% tumor cells were selected for immunohistochemistry, whereas some other samples were prepared by manual microdissection.

**Reagents and antibodies.** 4,6-Diamidino-2-phenylindole and RNase were purchased from Sigma-Aldrich. FITC-conjugated Annexin V and propidium iodide (PI) were obtained from Beijing Biosea Biotechnology. Monoclonal antibodies anti-β-actin (Sigma-Aldrich) and anti-caspase-3 (Transduction Laboratories), polyclonal anti-pol(ADP-ribose) polymerase (Cell Signaling Technology), and IRDye 800-conjugated secondary antibodies against mouse and rabbit IgG (LI-COR Bioscience) were purchased commercially. Rabbit anti-CMTM3 antibody was prepared and purified in our laboratory (7, 20).

**Semi quantitative reverse transcription-PCR and real-time PCR.** Human multiple tissue RNA samples were purchased from Clontech or Stratagene (16). Reverse transcription using random hexamer and reverse transcription-PCR using Go-Taq (Promega) were done as previously with GAPDH as a control (21). CMTM3 and CMTM4 primers were CMTM3F: 5′-GCTTTGCTGCTGGCAATGAG-3′ and CMTM3R: 5′-TTGGGCTCCTTGTCTCCT-3′, CMTM4F: 5′-CTCAACCTGCACTAGGAT-3′ and CMTM4R: 5′-TGCGGCTGGCTGATGTAGCA-3′, GAPDH: 5′-ATCTCTGCCCTCCTGCTGA-3′, and GAPDHr: 5′-GATGACCTTGGCACCACAGCCT-3′. Reverse transcription-PCR was done for 32 cycles for CMTM3 and CMTM4, but only 23 cycles for GAPDH. Real-time PCR was done as previously described (20), according to the dissolving curve and electrophoresis, the samples with unspecific amplification were regarded as zero, and the expression level of CMTM3 in LNCaP cell set as a baseline.

**DNA bisulfite treatment and methylation analysis.** Bisulfite modification of DNA, methylation-specific PCR (MSP), and bisulfite genomic sequencing (BGS) were carried out as described (22–24). MSP primers detecting methylated or unmethylated alleles of the promoters were CMTM3m3: 5′-CGAGTTTCGGTTTTATCGTC and CMTM3m4: 5′-GGGGCCGGACACAAAGACCGG for methylated and CMTM3u3: 5′-GGTTGAGTTGTTTATGTGT and CMTM3u4: 5′-CCACACAAAACACAAACACA for unmethylated. MSP was done for 40 cycles using AmpliTag Gold and hot start. MSP primers were tested previously for not amplifying any bisulfited DNA and the specificity of MSP was further confirmed by direct sequencing of some PCR products. BGS primers were BGS1: 5′-CRAATATAAACACCCACCTTAAT and BGS2: 5′-TTTGGAGATTAGAGGAAGTT. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen), with 6 to 18 colonies randomly chosen and sequenced.

**Immunohistochemistry.** Paraﬃn tissue sections were dewaxed, rehydrated and placed in 10 mmol/L citrate buffer (pH 6.0), and heated twice in a microwave oven for 5 min each. Sections were incubated with 3% H2O2 for 10 min, washed with PBS, blocked with 10% normal goat serum for 30 min, and then incubated with 4 μg/mL anti-CMTM3 or normal rabbit IgG as a control at 4°C overnight. After washing, the sections were stained with the catalyzed signed amplification system kit (DAKO code K3007) and visualized with a Nikon E800 with photos taken.

**Cell transfection.** CNE2 and T-47D cells were transfected with pcDNA3.1-myc-hisB-CMTM3, pcDNA3.1-myc-hisB-Bax, or vector alone (Invi- troDNA) at a density of 1 × 10^5 per well with G418 selection (400 μg/mL). The selective medium was replaced every 3 days. After 2 weeks, G418-resistant colonies were ﬁxed with 4% paraformaldehyde/PBS and stained with crystal violet. Numbers of colonies (≥50 cells per colony) were counted under a light microscope.

**Soft-agar colony formation assay.** Cells were transfected as described above. At 48 h post-transfection, cells were transfected in RPMI 1640 containing 0.35% agar, 10% fetal bovine serum, and 0.4 mg/mL G418 and layered on RPMI 1640 containing 0.5% agar, 10% fetal bovine serum, and G418 in a 6-well plate. Colonies were photographed in ~2 to 3 weeks post-transfection. All the experiments were done in triplicate wells three times.

**Cell proliferation assay.** Cells transfected with CMTM3, Bax, or vector control plasmids were harvested and plated in 96-well plates at a density of 2,000 per well and then incubated at 37°C. Cell proliferation was analyzed using the Cell Counting Kit-8 (Dojindo). At indicated time points, 10 μL CCK-8 solution was added into each well and incubated for 2 h. Absorbance at 450 nm was measured to calculate the number of viable cells. Results were obtained from three independent experiments in triplicates.

**Cell viability assay.** Cell survival was assessed over a period of 24 h after electroporation using a Vi-Cell Reagent Pak cell viability assay kit (Part No. 383198; Beckman Coulter; refs. 25–27). All of the cell culture solution containing the cells and digestion reagents was placed in a vial, and Beckman Coulter Vi-Cell automated the trypan blue dye exclusion by capturing 100 images to determine cell viability.

4,6-Diamidino-2-phenylindole staining analysis. Twenty-four hours post-transfection, cells were placed into 12-well plates and ﬁxed with 3% paraformaldehyde containing 0.1% Triton X-100 at room temperature for 30 min. Subsequently, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (0.5 μg/mL) and imaged using a CCD camera (Apogee Instruments).

**Flow cytometry analysis of apoptosis.** Phosphatidylserine externalization analysis was done as described previously (28). Briefly, both adherent and nonadherent cells were collected and washed with ice-cold PBS twice and then resuspended in 200 μL binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 1 mmol/L MgCl2, 5 mmol/L KCl, and 2.5 mmol/L CaCl2]. FITC-conjugated Annexin V was added to a ﬁnal concentration of 0.5 μg/mL and incubated for 20 min at room temperature in darkness, and 1 μg/mL PI was incorporated. Samples were immediately analyzed by ﬂuorescence-activated cell sorting. Cells (1 × 10^6) were recorded for apoptosis analysis.

**Evaluation of caspase-3 activity (DEVDase activity analysis).** Transfected cells were harvested in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaHPO4, 130 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L cocktail (Roche Applied Science). Total lysates (5 μg) were added into assay buffer [25 mmol/L HEPES (pH 7.5), 1 mmol/L EDTA, 100 mmol/L NaCl, 0.1% CHAPS, 10 mmol/L DTT] together with the caspase-3 ﬂuorogenic substrate Ac-DEVD-AMC and incubated at 37°C for 90 min. Signals were measured by a FLUO Star ﬂuorometer (BMG Labtechnologies) with a 380 nm excitation filter and a 460 nm emission ﬁlter. Results were obtained from triplicate experiments and presented as relative activities.

**Protein extraction and Western blot analysis.** Cells were harvested and lysed [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl ﬂuoride, and 1% Triton X-100]. Proteins (30 μg) were subjected to Western blotting as described previously (20), with incubation of primary antibodies at 4°C overnight. After washing in TBS-Tween 20 buffer, membranes were incubated for 1 h in darkness with the appropriate IRDye 800-conjugated secondary antibodies in TBS-Tween 20/5% fat-free milk. After washing in TBS-Tween 20 buffer, signals were detected on an Odyssey Infrared Imager (LI-COR Bioscience).

**Statistical analysis.** Data were presented as mean ± SE. Statistical analysis was carried out with Student’s t test. P < 0.05 was considered as statistically significant difference.

**Results.** CMTM3 Methylation in Multiple Cancers

We firstly examined the expression profiles of CMTM3 and CMTM4 in 22 normal adult tissues and 2 adult peripheral blood mononuclear cell samples. Semiquantitative

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Published OnlineFirst June 9, 2009; DOI: 10.1158/0008-5472.CAN-08-3694

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reverse transcription-PCR showed that both CMTM3 and CMTM4 were widely expressed in normal tissues (Fig. 1A; Supplementary Fig. S1). However, CMTM3 was frequently down-regulated or silenced in multiple carcinoma cell lines, including 4 of 9 breast, 1 of 3 prostate, 11 of 16 gastric, 5 of 6 nasopharyngeal, 7 of 18 esophageal, and 1 of 4 colon cell lines (Fig. 1C), as well as occasional other cell lines (Supplementary Fig. S2). In contrast, CMTM4 expression remained high in all cell lines.
examined, except for 4 lymphoma cell lines (Supplementary Fig. S1).

Aberrant promoter CpG methylation is related to gene silencing. As the CMTM3 promoter contains a typical CpG island (Fig. 1B), we analyzed its methylation by MSP. Results showed that cell lines with reduced or silenced expression had methylated promoter, whereas no methylation was detected in immortalized normal epithelial cell lines (Fig. 1C). We also examined the detailed methylation status of individual CpG sites in the promoter by BGS, and results were consistent with those of MSP (Fig. 2B). Real-time PCR also confirmed the relatively high CMTM3 expression in unmethylated cell lines (DU145, HCT116, HT-29, MB231, MCF-7, PC-3, and SW480) and weak expression in methylated cell lines (AGS, C666-1, CNE2, HONE1, Kato III, KYSE510, LNCaP, LoVo, T-47D, and ZR-75-1) (Fig. 1D), supporting the notion that CMTM3 methylation was well correlated with its transcriptional silencing.

We further treated CMTM3-silenced cell lines with a methyltransferase inhibitor 5-aza-2′-deoxycytidine alone or combined with trichostatin A, a histone deacetylase inhibitor. This treatment restored CMTM3 expression along with the demethylation of the promoter (Fig. 2A and B). Thus, these results indicate an epigenetic-mediated mechanism of CMTM3 silencing in tumor cells.

**CMTM3 expression and methylation in primary tumors.** We performed immunohistochemistry for primary gastric and colon tumors. CMTM3 protein was not expressed in 12 of 35 (34%) gastric and 1 of 2 colorectal samples (Fig. 3C; Table 1). Further MSP showed that CMTM3 was methylated in some primary carcinomas, including 28 of 63 (44%) gastric, 3 of 11 (27%) colorectal, 3 of 17 (18%) breast, 2 of 45 (4%) nasopharyngeal, and 1 of 33 (3%) esophageal carcinomas (Fig. 3A) but not in any of the 9 hepatocellular, 17 lung, and 5 prostate tumors (data not shown) as well as 4 normal breast tissues. Only weak methylation was detected in 5 of 35 (14%) gastric cancer adjacent normal tissues (Fig. 3A). A good correlation between CMTM3 protein expression and its methylation status was observed in several randomly chosen cases (Fig. 3C; Table 1). Detailed BGS analyses of some samples also confirmed that the CMTM3 promoter was methylated in primary carcinomas but not in normal epithelial tissue (Fig. 3B).

**Ectopic expression of CMTM3 inhibits tumor cell colony formation and viability.** We investigated the effects of ectopic CMTM3 expression on the growth and viability of CNE2 and T-47D cells with complete methylation and silencing of CMTM3. Results showed that CMTM3 expression significantly suppressed their colony formation abilities, compared with vector-transfected cells (down to 28% and 26%, respectively, Fig. 4A and B), with a well-demonstrated TSG Bax as a positive control. Significant growth inhibition was also detected in KYSE510 and EC109 esophageal carcinoma cells (Supplementary Fig. S3). To further evaluate the effects of CMTM3 on cell proliferation and viability, we performed cell counting assay and results showed that CMTM3 strongly inhibited the proliferation of CNE2 cells (Fig. 4C). Meanwhile, the numbers of viable cells in CMTM3-transfected CNE2 cells were remarkably decreased (Fig. 4D).

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**Figure 2.** A, pharmacologic demethylation with 5-aza-2′-deoxycytidine alone (A) or combined with trichostatin A (A+T) restored CMTM3 expression in methylated and silenced carcinoma cell lines. B, detailed BGS analysis of the CMTM3 promoter. Circles, CpG sites analyzed; row of circles, an individual promoter allele that was cloned, randomly selected, and sequenced; filled circle, methylated CpG site; open circle, unmethylated CpG site.
CMTM3 induced tumor cell apoptosis. We further found that ectopic CMTM3 expression caused obvious cell morphologic changes. Twenty-four hours post-transfection, CMTM3-transfected cells became round and detached, similar to Bax-transfected cells (data not shown). 4’,6-Diamidino-2-phenylindole staining indicated chromatin condensation and rupture in CMTM3-transfected cells, characteristic features of apoptosis (Fig. 5A), whereas no obvious change was seen in vector-transfected cells. We investigated the percentage of apoptotic cells using two-color fluorescence-activated cell sorting analysis in CMTM3-transfected CNE2 cells. Annexin V specifically stains phosphatidylserine at the cell surface, which is a proapoptosis marker, whereas PI assesses the plasma membrane integrity. In CMTM3- and Bax-transfected CNE2 cells, the proportion of Annexin V-positive versus PI-positive cells was increased by >2-fold compared with empty vector-transfected cells (Fig. 5B).

CMTM3 induced apoptosis through caspase-3 activation. To determine the molecular mechanism of CMTM3-induced apoptosis, caspase-3 activity was measured in vector control, CMTM3-transfected, and Bax-transfected cells using a DEVD cleavage assay 36 h after transfection (29). Results indicated that caspase-3 was activated in CMTM3-transfected CNE2 cells (Fig. 5C). We also performed Western blotting analysis and detected an obvious
decrease of pro-caspase-3 and increase of cleaved caspase-3 in CMTM3- or Bax-transfected cells (Fig. 5D). In consistence, cleaved poly(ADP-ribose) polymerase, which is associated with caspase-3 activation (30), was present in CMTM3- or Bax-transfected cells but rarely detected in control cells (Fig. 5D).

**Discussion**

In this report, we found that both CMTM3 and CMTM4 were widely expressed in normal adult tissues. However, in carcinoma cell lines, these two genes showed different expression patterns in which CMTM3 was frequently down-regulated or totally silenced in multiple cancer cell lines, whereas CMTM4 expression was maintained in most cell lines. We found that CMTM3 silencing in cancer cell lines was well correlated with its promoter CpG methylation, which could be restored by pharmacologic demethylation, suggesting a direct epigenetic mechanism. In primary tumors, CMTM3 methylation was closely correlated with decreased CMTM3 expression. Functionally, ectopic expression of CMTM3 in silenced tumor cell lines significantly suppressed their colony formation and growth and induced cell apoptosis via caspase-3 activation. Thus, CMTM3 possesses tumor suppressor functions, and its epigenetic silencing might be important in tumorigenesis.

CMTM3 and CMTM4 are closely located at 16q22.1, an important tumor suppressor locus associated with the pathogenesis of multiple carcinomas (8–11). Nevertheless, only CMTM3 had a significant down-regulation in carcinoma cell lines and tissues, indicating that these two genes play different physiologic roles, especially in tumorigenesis. It has been reported that CMTM4 is also involved in cell division (31) and thus may also be involved in the pathogenesis of some human diseases.

CpG methylation leading to the loss of TSG functions is a major epigenetic alteration in tumor development and progression (13, 32). Our study showed that CMTM3 underwent epigenetic inactivation by CpG methylation in multiple carcinoma cell lines. CMTM3 methylation was also detected in some primary tumors, especially in gastric, colorectal, and breast cancers, further indicating a role of CMTM3 in tumorigenesis. The frequency of CMTM3 methylation in primary tumors is not as high as that in cell lines, indicating that some cancer cell lines might have acquired the methylation during their establishment or maintenance. Similar phenomenon has been reported for some

### Table 1. Correlation of CMTM3 expression with methylation status in primary tumors

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<th>Primary tumor</th>
<th>Immunohistochemistry</th>
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**NOTE:** Only the DNA sample of case #6 was extracted from paraffin-embedded tissue; all the rest of the DNA samples were extracted from frozen tissues.

+, with CMTM3 expression; −, no expression.
other TSGs (16, 21, 33–35). Although we did not detect any homozygous deletion of CMTM3 in cell lines, we still could not exclude the possibility of its hemizygous deletion or microdeletions in tumors.

Restoration of CMTM3 expression in CNE2 cells induced chromatin condensation, phosphatidylserine externalization, and caspase-3 activation. CMTM8, another member of the CMTM family, also induced apoptosis in HeLa and PC-3 cells through the intrinsic pathway (4, 5). On the other hand, caspase-independent apoptotic pathway mediated by mitochondrion-released apoptosis-inducing factor also plays an important role in cell programmed death (36, 37). Further experiments are required to elucidate which pathway CMTM3 is exactly involved in inducing apoptosis.

CMTM is a novel family of proteins, with very limited work conducted thus far for their molecular functions. Our previous study showed CMTM5 methylation in tumor cell lines. Here, we verified that CMTM3 also possesses tumor-suppressive functions and is frequently silenced by promoter methylation in multiple carcinomas. CMTM3 contains a conserved MARVEL domain with CMTM5-v1, which might contribute to their tumor suppressor functions. Thus, our study on CMTM3 extends the current understanding of the role for CMTM family in carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/23/08; revised 4/8/09; accepted 4/10/09; published OnlineFirst 6/9/09.

Grant support: Program for New Century Excellent Talents in University grant NCET-07-0013, China High Tech 863 Program grant 2006AA02A305, and Michael
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We thank Bert Vogelstein, George Tsao, Sun Young Rha, Michael Obster, Paul Murray, (Dolly Huang), Kaitai Yao, Riccardo Dalla-Favera, John S. Rhim, and Shuen-Kuei Liao, (David Y Mason), and Teresa Marafioti for some tumor cell lines and samples and the German Collection of Microorganisms & Cell Cultures for the KYSE cell lines (38).


Published OnlineFirst June 9, 2009; DOI: 10.1158/0008-5472.CAN-08-3694

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