

CMTM5 Exhibits Tumor Suppressor Activities and Is Frequently Silenced by Methylation in Carcinoma Cell Lines

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Abstract Purpose: *CMTM5* (CKLF-like MARVEL transmembrane domain containing member 5) is located at 14q11.2, a locus associated with multiple cancers. It has six RNA splicing variants with *CMTM5-v1* as the major one. We explored its expression pattern in normal tissues and tumor cell lines, as well as its functions in carcinoma cells.

Experimental Design: We evaluated *CMTM5* expression by semiquantitative reverse transcription-PCR (RT-PCR) in normal tissues and carcinoma cell lines of cervical, breast, nasopharyngeal, lung, hepatocellular, esophageal, gastric, colon, and prostate. We further examined *CMTM5* promoter methylation in these cell lines. We also analyzed *CMTM5* expression after 5-aza-2'-deoxycytidine treatment and genetic demethylation and the functional consequences of restoring *CMTM5* in HeLa and PC-3 cells.

Results: *CMTM5-v1* is broadly expressed in human normal adult and fetal tissues, but undetectable or down-regulated in most carcinoma cell lines. Its promoter methylation was detected in virtually all the silenced or down-regulated cell lines. The silencing of *CMTM5* could be reversed by pharmacologic demethylation or genetic double-knockout of *DNMT1* and *DNMT3B*, indicating methylation-mediated mechanism. Restoration of *CMTM5-v1* suppressed carcinoma cell proliferation, migration, and invasion.

Conclusions: These results indicate that *CMTM5* exhibits tumor suppressor activities, but with frequent epigenetic inactivation in carcinoma cell lines.

CKLFSF is a novel family of proteins linking chemokines and transmembrane 4 super family (TM4SF). In human, nine genes, *CKLF* and *CKLFSF1* to *CKLFSF8*, have been discovered. Most CKLFSF genes have different RNA splicing variants, and the protein product of at least one variant of each member has a MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain. For this reason, in December 2005, *CKLFSF1* to *CKLFSF8* were renamed as *CMTM1* to

CMTM8 (CKLF-like MARVEL transmembrane domain containing member 1 to 8). *CKLF* is the first identified member of this family; with four RNA splicing variants designated CKLF1 to CKLF4. Among them, CKLF1 is more related to chemokine, because it has chemotactic activity on leukocytes with CCR4 as one of its functional receptors (1, 2). *CMTM1* to *CMTM8* were identified based on the cDNA and amino acid sequences of CKLF2 that is the full-length cDNA product of *CKLF*. Of them, *CMTM1* and *CMTM2* are more related to chemokine, whereas *CMTM8* is more related to TM4SF11, with 39% sequence homology at the amino acid level. The characteristics of other CMTM members are intermediate between *CMTM1* and *CMTM8* (3).

Generally, TM4SF includes several types of proteins possessing the four transmembrane-helix structure, such as typical TM4SF (tetraspanin) and MARVEL domain-containing proteins. Tetraspanin-like proteins KAI1/CD82 and CD63 contain a larger second outer loop (EC2) and four to six conserved extracellular cysteine residues. Whereas the MARVEL domain-containing proteins, including MAL, physins, gyrlins, and occludin families, lack the described characteristics of tetraspanins (4). They have essential functions in membrane apposition events. MAL family consists of MAL, MAL-2, BENE, and TM4SF11. The high sequence homology between *CMTM8* and TM4SF11 indicates that *CMTM8* is more related to MAL family, which seems to act as machinery for raft remodeling during differentiation in T cells and for apical transport in epithelial cells during tumorigenesis. MAL and BENE are down-regulated in cervical squamous cell cancers (5). The promoter methylation of MAL occurred in 3 of 13 esophageal cancer cell lines, and

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MAL could suppress the motility, invasion, and tumorigenicity of esophageal cancers (6), indicating that MAL is a candidate tumor suppressor gene (TSG). The methylation of another tetraspanin member KAI1/CD82, which is a functional TSG for multiple tumors, has been well documented (7–9).

Our previous studies showed that CMTM is a novel MARVEL domain-containing protein family; with important roles in many physiologic systems, especially the male reproductive and immune systems. For example, CMTM1, CMTM2, and CMTM3 are highly expressed in testis (10–12), and mouse *Cmtm2a* can directly interact with androgen receptor (ART) *in vivo*, acting as a corepressor of AR-mediated activity (13), whereas *Cmtm2b* is a coactivator of AR (14). CMTM3, CMTM6, and CMTM7 are highly expressed in immune system (12). The functions of CMTM8 in epidermal growth factor receptor endocytosis and apoptosis induction indicate their potential multiple functions during tumorigenesis (15).

CMTM genes form two clusters in the human genome: *CKLF* and *CMTM1* to *CMTM4* as one gene cluster at chromosome 16q22.1 and *CMTM6* to *CMTM8* as another cluster at chromosome 3p23. *CMTM5* is located at 14q11.2, a locus with multiple genes and associated with various carcinomas (16). For instance, the frequent loss of heterozygosity at 14q11.2 in nasopharyngeal carcinoma (NPC) suggests the presence of a functional TSG at this locus (16–18). Another study validated that ~40% of the down-regulated genes in anaplastic meningioma were located on 1p and 14q, whereas a 14q11.2 gene, *NDRG2*, is consistently down-regulated in grade III meningioma (19). Thus, considering the chromosomal location and the protein structure of *CMTM5*, it is possible that *CMTM5* might also be involved in tumorigenesis.

In this report, we identified six RNA splicing variants, CMTM5-v1 to CMTM5-v6, of human *CMTM5*. We also showed that CMTM5-v1 is evolutionarily conserved and broadly expressed in normal adult and fetal tissues but undetectable or decreased in most carcinoma cell lines. Promoter methylation of *CMTM5* was frequently detected in tumor cell lines and could be reversed by pharmacologic demethylation. Restoration of CMTM5-v1 strongly suppressed tumor cell proliferation and migration. These findings indicate that *CMTM5* is a novel gene with putative tumor suppressor functions.

Materials and Methods

Cell lines and tumor samples. A series of carcinoma cell lines were studied (20), including 4 cervical (HeLa, CaSki, C33A, and SiHa), 2 prostate (PC-3 and LNCaP), 6 nasopharyngeal-NPC (C666-1, CNE1, HK1, HNE1, HONE1, and BM1), 1 hypopharyngeal (FaDu), 16 esophageal (EC1, EC18, HKESC1, HKESC2, HKESC3, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE270, KYSE410, KYSE450, KYSE510, KYSE520, and SLMT-1), 11 hepatocellular (Hep3B, huH1, huH6, huH7, Mahlavu, PLC/PRF/5, SNU398, SNU475, SNU387, SNU423, and SNU449), 3 colorectal (HCT116, HT-29, and LoVo), 10 gastric carcinomas (Kato III, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, and YCC16), 4 lung (A549, H-1299, A427, and H292), and 9 breast (BT549, MB231, MB468, MCF-7, T47D, SK-BR-3, YCC-B1, YCC-B3, and ZR-75-1). An immortalized, nontransformed normal breast epithelia cell line (HMEC) was used as a normal control for breast carcinoma (Cambrex, Cat α -2251). Cell lines were treated with 10 μ mol/L of 5-aza-2'-deoxycytidine (Sigma) alone for 3 days and harvested or further treated with 100 nmol/L trichostatin A for additional 24 h, as described previously (21).

Semiquantitative RT-PCR analysis. Reverse transcription was done using the ThermoScript First-Strand Synthesis kit (Invitrogen Technologies). Human multiple tissue cDNA panel was purchased from Clontech. Nested PCR for mouse *Cmtm5* amplification was done using the following primers: forward primer, 5'-CAAGCCTCCCTGGTACTAC; reverse primer, 5'-CTTCTCAAGCGCACATCTCTG; nested forward primer, 5'-GTGAGCCTCAGAGATGTTTC; nested reverse primer, 5'-GTAACCCTGGAATCAGCTGCTG. For the first round of amplification, the annealing temperature was 59°C for 35 cycles. The nested amplification was carried out at the same annealing temperature for 38 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

For screening *CMTM5* expression in a panel of human normal tissues, primers used were CMTM5-v1F: 5'-CTTCCTCACCTCCCACAAG (on exon 1) and CMTM5-v1R: 5'-AGATGGAAACCAGGATGATG (on exon 5). Reverse transcription-PCR (RT-PCR) was done for 37 cycles with hot start, using AmpliTaq Gold (Applied Biosystems; ref. 22).

DNA bisulfite treatment and promoter methylation analysis. Bisulfite modification of DNA, methylation-specific PCR (MSP), and bisulfite genomic sequencing (BGS) were carried out as described previously (22–24). The bisulfite-treated DNA was amplified with the methylation-specific primer set CMTM5m3: 5'-CGTGGTGTGTTGAATATTCGC, CMTM5m2: 5'-TCCAACATACAAAAACGCG or the unmethylation-specific primer set CMTM5u3: 5'-ATTTGTGGTGTGTTGAATATTTGT, CMTM5u2: 5'-TCTCCAACATACAAAAACACA. MSP was done for 40 cycles using AmpliTaq Gold and hot start. MSP primers were tested previously for not amplifying any unbisulfited DNA, and the MSP products of several cell lines were confirmed by direct sequencing, indicating that the MSP system is specific. For BGS, bisulfite-treated DNA was amplified using primers BGS1: 5'-AATGGTTAATTTG-TATTGGTATT and BGS2: 5'-CTCTAACCTTAACCTCTTATTAA. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen) with 8 to 10 colonies randomly chosen and sequenced.

Cell transfection. Transfection of PC-3 and HeLa cells was done by electroporation (25). pCDNA3.1-myc-hisB (Invitrogen) and pCDNA3.1-myc-hisB-CMTM5-v1 plasmids were used. Cells (2×10^6) were mixed with 10 μ g DNA in 400- μ L serum-free medium. The DNA-cell mixture was electroporated with a 140 V/20 ms pulse using a BTX T820 square wave electroporator in a 2-mm cuvette (BTX Inc.). Transfection efficiency was monitored by pEGFP-N1 plasmid (CLONTECH). Cells with >75% transfection efficiency were used for further experiments. For stable transfection, plasmids were linearized by *Bsp*HI prior to transfection, and 36 h posttransfection, 800 μ g/mL G418 were added to cultures. Twelve cell clones of PC-3 stably transfected with CMTM5-v1 expression vector were picked and designated as PC-3/CMTM5-v1 5A1 to 5A12. Ten cell clones stably transfected with empty vector were designated as PC-3/Mock 5B1 to 5B10.

Western blot. Lysates of stably transfected cells were subjected to 12.5% SDS-PAGE, then electrophoretically transferred to nitrocellulose membranes. The membranes were reacted with anti-CMTM5 antiserum and then with IRDye 800-conjugated anti-IgY. The signals were detected using the Odyssey Imaging System (LI-COR Bioscience, Inc.).

Colony formation assays. The assay was done as described previously (26). In brief, PC-3 and HeLa cells transfected with CMTM5-v1 expression vector or empty vector were plated at 200 cells per 35-mm dish. At 48 h after transfection, 800 μ g/mL G418 were added, and the medium was changed every 3 days. On day 15, G418-resistant colonies were fixed with 2% PFA/PBS. After crystal violet staining, colonies with ≥ 50 cells were counted.

Proliferation assays. For the assessment of DNA synthesis, [3 H]thymidine was added (1 μ Ci/mL) at the indicated time points, samples were incubated for 6 h, and cells were harvested by a TOMTEC Harvester 96R onto a 96-well Filtermat (Perkin-Elmer). The incorporation of [3 H]thymidine was determined with the MicroBeta Windows Workstation software (Perkin-Elmer).

Cell migration assay. The migration assay was done in a 48-well Chemotaxis chamber (Neuroprobe, Inc.), according to the manufacturer's instruction, as previously described with slight modification

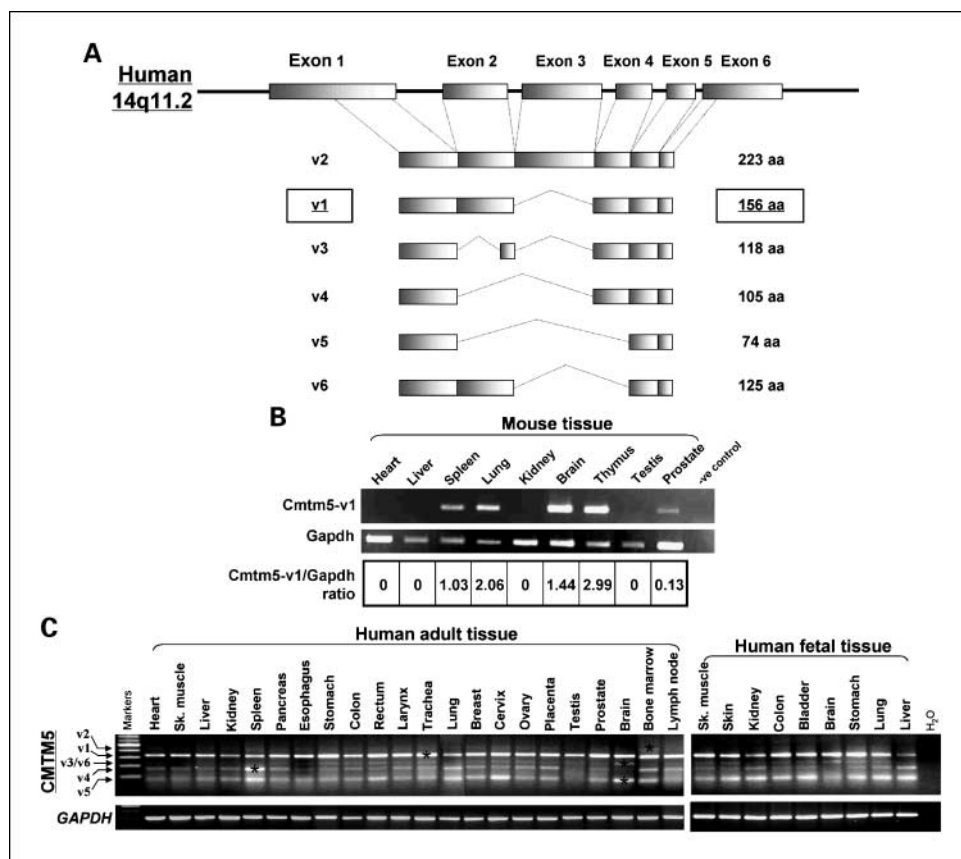


Fig. 1. The genomic organization, splicing variants, and expression profiles of *CMTM5*. **A**, *CMTM5* is located at human 14q11.2 and has six exons and six RNA splicing variants (CMTM5-v1 – CMTM5-v6), wherein CMTM5-v1 is the major form. **B**, only Cmtm5-v1 was identified in mouse tissues. **Bottom**, relative expression ratio of Cmtm5-v1 compared with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). **C**, CMTM5-v1 is the major form expressed in human normal adult and fetal tissues. *Sk*, skeleton.

(27). Briefly, conditioned medium prepared by growing NIH 3T3 cells in serum-free medium containing 0.5 $\mu\text{g}/\text{mL}$ bovine serum albumin for 24 h was added to the bottom part of the chamber. A polycarbonate filter (8- μm pore size) was used. The cells were trypsinized and washed twice, and 50 μL of the single-cell suspension (3×10^5 cells/mL in 0.1% bovine serum albumin/RPMI 1640) was added to the upper well of the chamber. The cells were incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere. After incubation, nonmigrated cells in the upper chamber were scraped off and the migrated cells on the bottom part of the filter were identified through fixing with methanol and staining with H&E. In each individual experiment, cells that migrated through the filters were counted from at least three randomly selected fields. Results were obtained from at least three individual experiments and represented as the cell migration index, which was the number of cells per high power field.

Wound healing assay. Cell mobility was assessed using a scratch wound assay. Stably transfected cells were cultured in a 24-well plate until confluent. The cell layer was carefully wounded using sterile tips and washed twice with fresh medium. After incubation for 12, 24, or 36 h, the cells were photographed at low magnification (4 \times objective). The experiments were done in triplicate.

Statistical analysis. The results are expressed as values of mean \pm SE. Statistical analysis was carried out with Student's *t* test; $P < 0.05$ was considered as statistically significant difference.

Results

CMTM5 has at least six RNA splicing variants (CMTM5-v1 – CMTM5-v6) with CMTM5-v1 as the main form expressed in multiple normal tissues. Bioinformatics analysis indicates that *CMTM5* is broadly expressed. To examine the expression profile of *CMTM5*, we obtained its cDNA sequences with cDNA

libraries of leukocyte, brain and liver, using nested RT-PCR method. The results indicate that *CMTM5* has multiple RNA splicing variants. After cloning and sequencing, we identified six variants CMTM5-v1 to CMTM5-v6 (Genbank accession numbers: AF479262, AF527413, AF527414, AF527949, AF527948, and AY820135). Among these, CMTM5-v2 is the longest cDNA sequence with six exons encoding 223 amino acids. Exons 1, 5, and 6 are commonly used by CMTM5-v1 to CMTM5-v6, whereas exons 2, 3, and 4 are selectively used (Fig. 1A).

We conducted RT-PCR analysis with mouse normal tissues and found that Cmtm5-v1 (NM026066) is the main form of mouse *Cmtm5*, with higher expression level in thymus, lung, and brain (Fig. 1B). We further examined the expression profile of human *CMTM5* in a panel of 31 normal adult and fetal tissues using semiquantitative RT-PCR. The results showed that CMTM5-v1 is also the major form expressed in all human normal tissues except for spleen (Fig. 1C). The sequence similarity of CMTM5-v1 between human and mouse is 90% on the overall amino acid level, indicating that CMTM5-v1 is highly conserved.

CMTM5 is silenced by methylation in carcinoma cell lines and could be activated by pharmacologic and genetic demethylation. As *CMTM5* is widely expressed in normal tissues, it is of interest to examine its expression levels in tumor cell lines. Using semiquantitative RT-PCR, we found that CMTM5-v1 was reduced or silenced in most of the carcinoma cell lines, including 6 of 6 nasopharyngeal, 1 of 1 hypopharyngeal, 12 of 16 esophageal, 10 of 11 hepatocellular, 9 of 9 breast, 3 of 4 lung, 2 of 3 colon, 8 of 10 gastric, 3 of 4 cervical, and 2 of 2

prostate cancer cell lines (Fig. 2). Except for rare cell lines, the expression of other *CMTM5* variants was not detected in these cell lines. Thus, *CMTM5* down-regulation was commonly detected in carcinoma cell lines.

As promoter methylation is the major mechanism of gene silencing, we further explored whether there was any epigenetic inactivation of this gene. *CMTM5* promoter methylation status was analyzed by MSP (28). Virtually all the cell lines with reduced or silenced expression were methylated, whereas no methylation was seen in *CMTM5*-v1-expressing cell lines, including Kato III, and the normal breast epithelial cell line HMEC (Fig. 2). We also examined the detailed methylation status of individual CpG sites in the *CMTM5* promoter by BGS (Fig. 3A and B). BGS analysis in several cell lines confirmed the MSP results (Fig. 3B). Thus, a correlation between transcriptional silencing and promoter methylation of *CMTM5* was seen in tumor cells.

To further assess the link between *CMTM5* methylation and its silencing, we treated several silenced cell lines with a methyltransferase inhibitor Aza, alone or combined with trichostatin A (TSA), a histone deacetylase inhibitor. The treatment restored *CMTM5*-v1 expression along with the demethylation of the promoter (Fig. 3C and D). Moreover, in the colorectal cancer cell line HCT116, which is methylated for *CMTM5* (with only trace of expression), *CMTM5* could be activated by genetic demethylation through double knockout of the two DNA methyltransferase genes *DNMT1* and *DNMT3B* (DKO cell line; Fig. 3D). Concomitantly, unmethylated *CMTM5* promoter alleles were induced in DKO cells, similar to the situation of other tumor suppressor genes we examined before, such as *PCDH10*, *WIF1*, and *DLC1* (29–31). Genetic demethylation was even more dramatic than the pharmacologic demethylation with Aza, with or without TSA (Fig. 3D). *DNMT1* and *DNMT3B* are two major DNA methyltransferases responsible for maintenance and *de novo* CpG methylation, and disruption of these two genes results in >95% loss of overall genomic methylation and CpG island demethylation in HCT116 cells (32). Taken together, these results suggest that methylation mediates the silencing of *CMTM5* in tumor cells.

Overexpression of *CMTM5*-v1 has tumor inhibitory effect on the colony formation of tumor cells. The frequent silencing of *CMTM5* in multiple carcinoma cell lines, compared with its broad expression in normal tissues, suggests that *CMTM5* might

have tumor suppressor function. We investigated whether restoration of *CMTM5*-v1 could suppress the clonogenicity of carcinoma cell lines PC-3 and HeLa, which are methylated and silenced for *CMTM5*-v1. As shown in Fig. 4A and B, 2 weeks after transfection and subsequent selection of drug resistant colonies (33, 34), the numbers of colonies produced by *CMTM5*-v1-transfected cells was significantly less than that by empty vector-transfected cells, suggesting that *CMTM5*-v1 does suppress the colony formation of tumor cells.

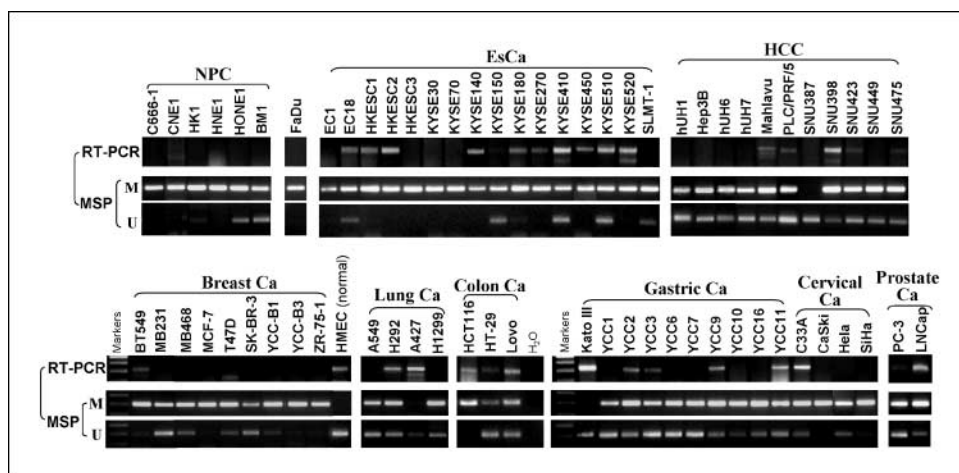
Stable overexpression of *CMTM5*-v1 inhibits the proliferation of PC-3 cells. To further test the effect of *CMTM5*-v1 on cell proliferation, we generated stably transfected PC-3/*CMTM5*-v1 cells. Three *CMTM5*-v1-transfectants, 5A2, 5A4, and 5A10, expressing *CMTM5*-v1 protein as shown by RT-PCR and Western blot, were successfully obtained (Fig. 4C). Ten vector transfectants were also successfully obtained, with only two, 5B1 and 5B2, randomly chosen and used in further experiments.

Subsequently, we used [³H]thymidine incorporation assay to measure tumor cell proliferation in response to *CMTM5*-v1 restoration. The PC-3 cells overexpressing *CMTM5*-v1 (5A2, 5A4, and 5A10) exhibited reduced incorporation compared with vector controls (Fig. 4D). We further investigated the growth of these transfectants in serum-deprived medium. At the indicated time points, the cells were starved for 24 h before the addition of [³H]thymidine reagents. As shown in Fig. 4E, the DNA synthesis of serum-starved *CMTM5*-v1-transfected PC-3 cells was also inhibited.

Stable overexpression of *CMTM5*-v1 inhibits the invasion and migration of PC-3 cells. As other MARVEL domain-containing proteins, like MAL, are methylated in carcinoma cell lines and suppress the motility, invasion, and tumorigenicity of tumor cells, we also assessed the effects of *CMTM5*-v1 expression on the invasion and migration of PC-3 cells. We examined the motility of PC-3 transfectants using a Boyden chamber migration assay. As shown in Fig. 5A and B, the migration of *CMTM5*-v1-transfected cells was remarkably reduced compared with that of vector-transfected cells, indicating that restoration of *CMTM5*-v1 strongly inhibits the invasion of PC-3 cells.

Furthermore, the effect of *CMTM5*-v1 expression on PC-3 cell motility was assessed by scratch wound healing assay. As shown in Fig. 6, *CMTM5*-v1-expressing cells spread along the wound edges significantly slower than the vector-transfected cells at 24 or 36 h, indicating that *CMTM5*-v1 inhibits tumor cell migration.

Fig. 2. *CMTM5*-v1 is silenced and methylated in most carcinoma cell lines. *Ca*, carcinoma; *NPC*, nasopharyngeal carcinoma; *EsCa*, esophageal carcinoma; *HCC*, hepatocellular carcinoma; *M*, methylated; *U*, unmethylated.



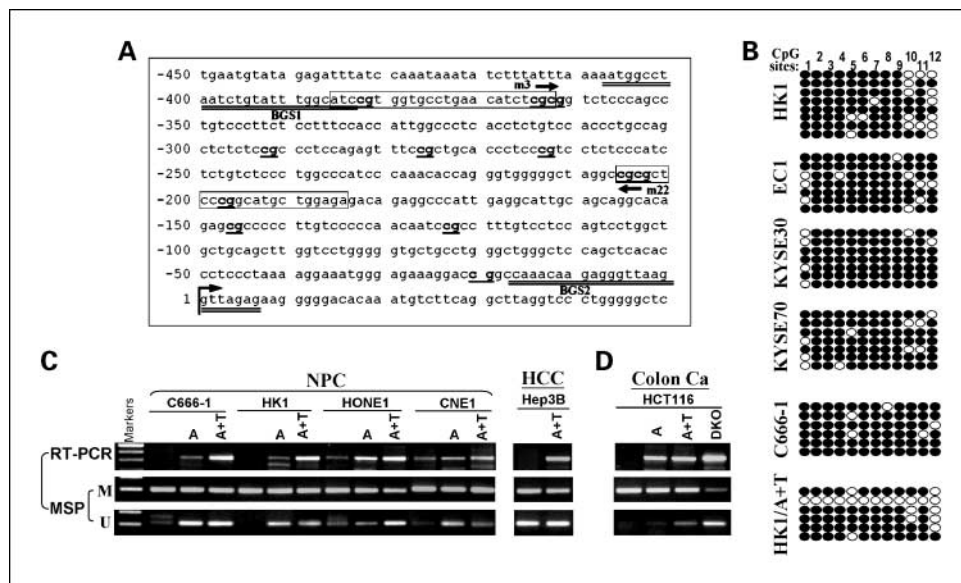


Fig. 3. CMTM5-v1 is silenced and methylated in carcinoma cell lines. *A*, sequence of the CMTM5 promoter, CpG sites, and primer positions for methylation analyses; MSP primers (*m3*, *m22*) and BGS primers (*BGS1*, *BGS2*) are indicated. The transcription start site is indicated by a curved arrow. *B*, bisulfite genomic sequencing of the CMTM5 promoter in some cell lines. Each row represents an individual allele that was randomly cloned and sequenced. Circles represent CpG sites (12 sites) analyzed: *black circle*, methylated CpG site; *white circle*, unmethylated CpG site. Demethylation in HK1 with Aza (*A*) combined with TSA (*T*). *C* and *D*, pharmacologic demethylation with Aza alone or combined with TSA (*A + T*) – induced CMTM5-v1 expression in methylated and silenced carcinoma cell lines. *D*, CMTM5 could be activated by pharmacologic demethylation with Aza along or combined with TSA and also by genetic demethylation through double knockout of two DNA methyltransferase genes *DNMT1* and *DNMT3B* (DKO cell line) in colon cancer cell line HCT116.

Discussion

CMTM5 is located at 14q11.2, an important locus associated with the pathogenesis of multiple carcinomas. We observed broad expression of CMTM5 in normal adult and fetal tissues but reduced or silenced expression in most carcinoma cell lines.

We also identified the frequent methylation of CMTM5 promoter in these cell lines, which correlates with its loss of expression. Treatment with Aza and trichostatin A demethylated the promoter and led to transcriptional reactivation of CMTM5 in silenced cell lines. We further showed that restoration of CMTM5-v1 in silenced carcinoma cell lines

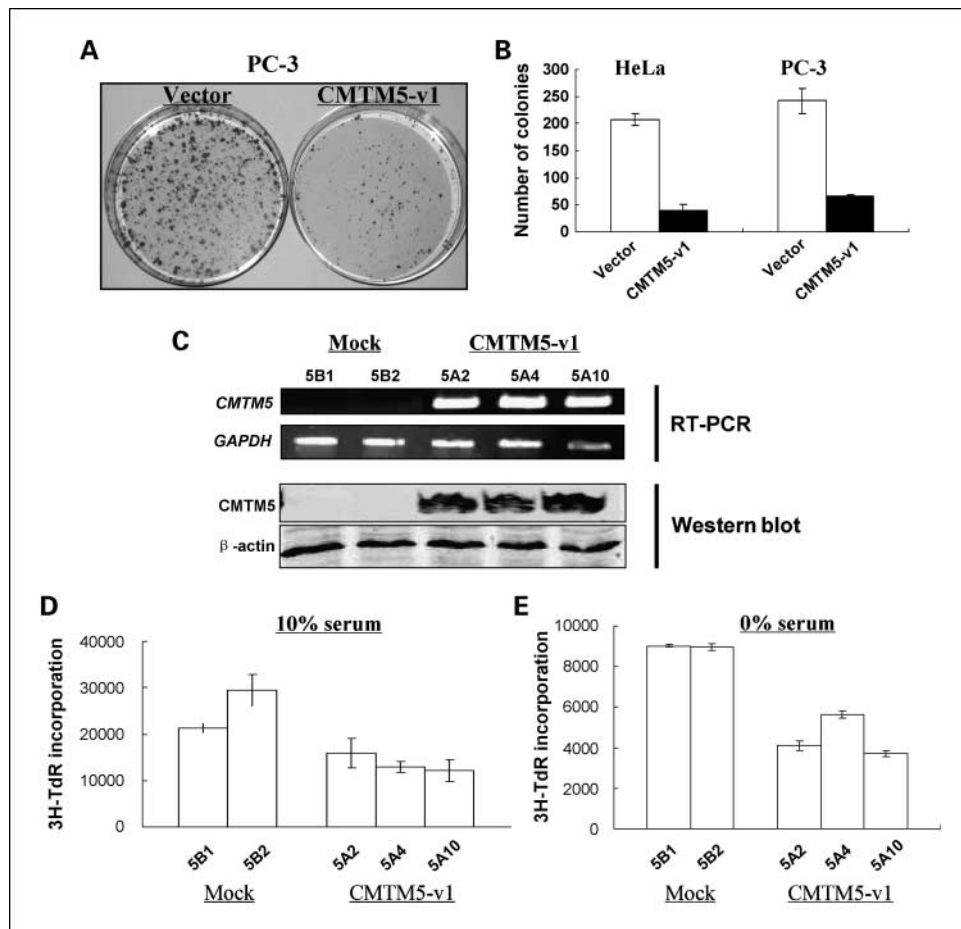


Fig. 4. *A*, restoration of CMTM5-v1 inhibits the clonogenicity of carcinoma cells. Representative inhibition of colony formation by CMTM5-v1 on PC-3 cells. *B*, quantitative analysis of colony numbers. *Columns*, mean of three separate experiments; *bars*, SE. $P < 0.005$ was found between the mock and CMTM5-v1 transfectants. *C*, CMTM5-v1 expression in PC-3 stable transfectants by RT-PCR and Western blot. Total RNA was isolated, and equal amount of total RNA was applied for reverse transcription. GAPDH was used as an internal standard. Equal amount of cell lysate was loaded, with actin as a control. The proliferation of stable transfectants under normal culturing (*D*) and serum deprivation (*E*) was measured by [³H]thymidine incorporation assay. The differences between the three CMTM5-v1 stable transfectants and mock PC-3 cells were all of great significance ($P < 0.05$).

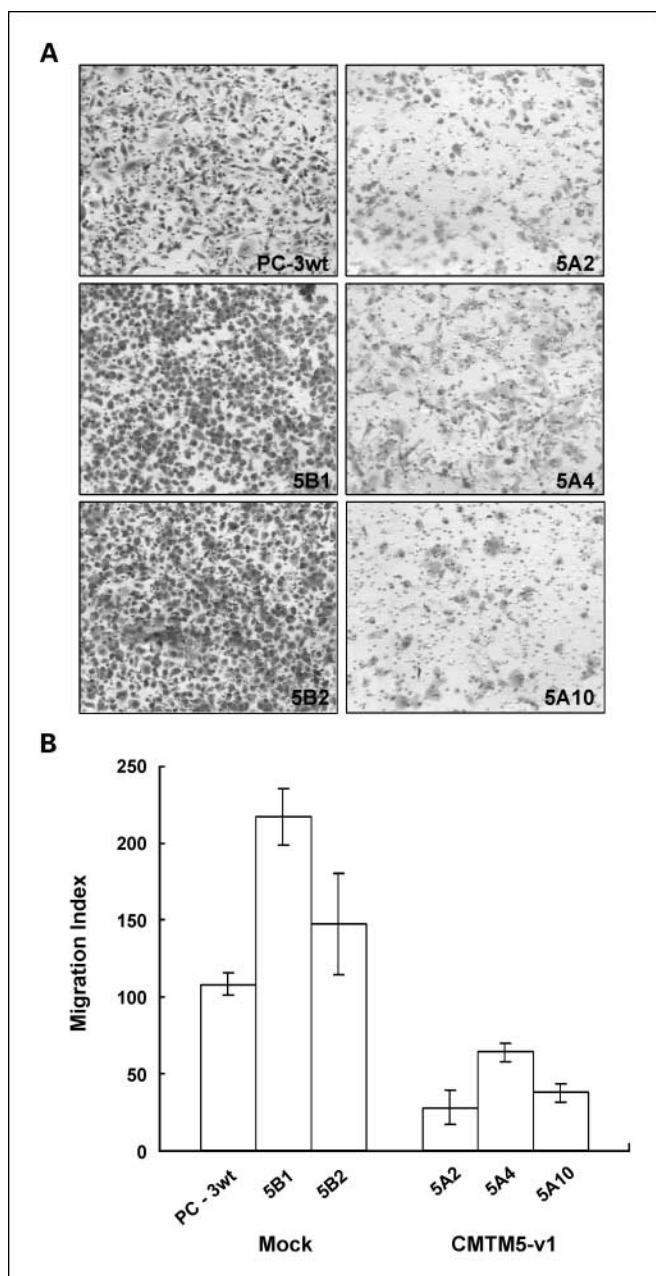


Fig. 5. Ectopic CMTM5-v1 expression suppresses the invasion of PC-3 cells. Columns, means with $P < 0.001$ found between the mock and CMTM5-v1 transfectants; bars, SE. 5A2, 5A4, and 5A10 are CMTM5-v1 stable transfectants expressing the CMTM5-v1 protein. 5B1 and 5B2 are two vector stable transfectants.

dramatically reduces not only tumor cell proliferation and colony formation, but also their invasion and migration abilities. These findings indicate that CMTM5 possesses tumor suppressor functions, and its epigenetic silencing is probably involved in tumor pathogenesis.

Human CMTM5 has at least six RNA splicing variants, CMTM5-v1 to CMTM5-v6, whereas in mouse tissues, we only detected Cmtm5-v1. CMTM5-v1 is also the major expressed variant in human, in both normal tissues and tumor cell lines. Thus, we selected only CMTM5-v1 for functional studies. Further study is needed to elucidate the possible functions of other CMTM5 isoforms.

CpG methylation leading to the loss of TSG functions is a major epigenetic alteration in tumor development and progression (35, 36). Our observation that CMTM5 undergoes epigenetic inactivation by CpG methylation in various carcinoma cell lines provides further insight into the contribution of CMTM5 to tumorigenesis, probably at the metastasis and invasion stages.

As the CMTM family was just recently discovered in 2003 by our group, the functions of most CMTM members have not been well defined, especially with respect to tumorigenesis. Our study is the first report that a CMTM gene possesses tumor suppressor functions when inactivated epigenetically in tumor cell lines. Although we did not detect any homozygous deletion of CMTM5 in cell lines, more work is required to address whether other genetic inactivation, including point mutations, is involved. The molecular mechanism of how CMTM5-v1 inhibits tumor cell growth and migration is also unclear. CMTM5 is a MARVEL domain-containing protein. The mechanism how MARVEL domain-containing proteins affect tumor cell growth and migration is poorly characterized, most probably through cell-cell contact inhibition, similar to KAI1/CD82 (7–9). The tumor suppressor function of MAL in

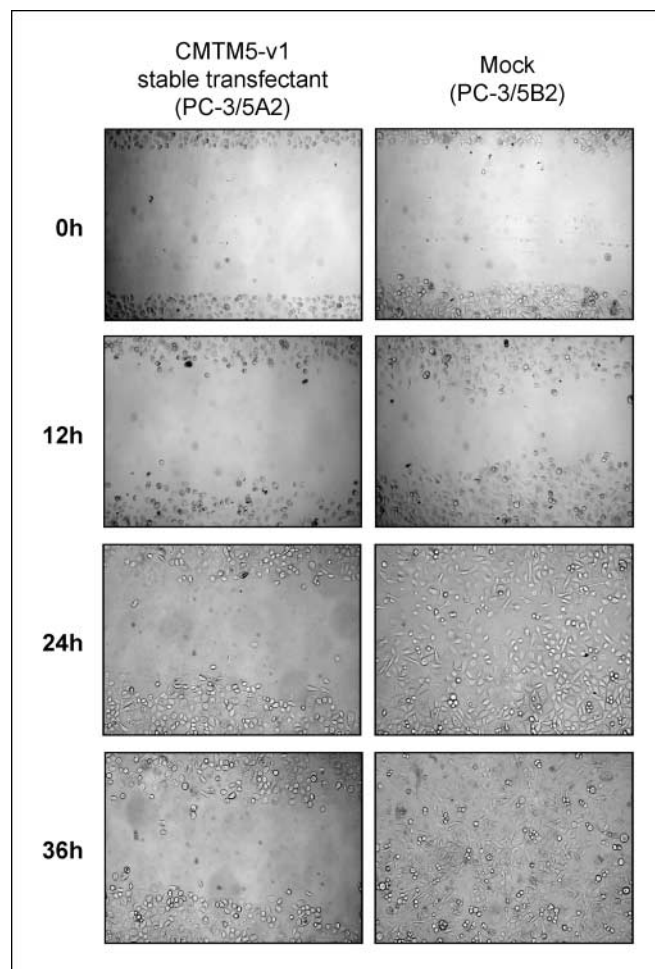


Fig. 6. Ectopic CMTM5-v1 expression inhibits the migration of PC-3 cells. Representative scratch wound healing assay result. Photos were taken at different time points after the scratch.

esophageal cancer is through G1-S block and apoptosis induction via the Fas signaling pathway (5). Thus, more mechanism studies on CMTM5 or other MARVEL domain-containing proteins will uncover their roles in tumorigenesis and may also lead to potential applications in tumor therapies.

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