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Molecules and Cells



# Identification and Characterization of *CMTM4*, a Novel Gene with Inhibitory Effects on HeLa Cell Growth through Inducing G2/M Phase Accumulation

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**Human *CMTM* is a novel gene family consisting of *CKLF* and *CMTM1-8*. *CMTM4* is the most conserved gene and has three RNA splicing forms designated as CMTM4-v1, -v2 and -v3, but in many types of tissue and cell lines, only CMTM4-v1 and -v2 could be detected. CMTM4-v2 is the full length cDNA product, which has been highly conserved during evolution. CMTM4-v1 and -v2 are broadly expressed in normal types of tissue. They are distributed on the cell membrane and across the cytoplasm in a speckled pattern. Overexpression of CMTM4-v1 and -v2 can inhibit HeLa cell growth *via* G2/M phase accumulation without inducing apoptosis. Therefore, *CMTM4* might be an important gene involved in cell growth and cell cycle regulation.**

## INTRODUCTION

*CMTM* is a gene family consisting of *CKLF* and *CMTM1-8.* The characteristics of their encoding products are between classical chemokines and TM4SF. They have important roles in the immune and male reproductive systems and participate in tumorigenesis. *CKLF* was identified first with four alternative splicing forms designated as CKLF1-4. CKLF1 has chemotactic activity towards leukocytes and CCR4 is one of its functional receptors (Han et al., 2001; Wang et al., 2006). *CMTM1*, *-2*, and *-3* are highly expressed in testis and have regulatory effects on androgen receptor transactivation (Shi et al., 2005; Wang et al., 2004; 2008; Zhong et al., 2006). *CMTM3* and *CMTM5* have recently been identified as tumor suppressor candidates (Shao et al., 2007). Moreover, *CMTM8* accelerates the ligand-induced clearance of epidermal growth factor receptor (EGFR) from the cell surface leading to the absence of EGFR mediated signaling and induces cells to undergo apoptosis via caspase-dependent and -independent pathways (Jin et al., 2005).

*CMTM4* is the most conserved member of this family. Using an esiRNA technique, Kittler et al. (2004) indicated *CMTM4* might have essential functions in HeLa cell division since its knockdown resulted in a cell cleavage defect with malformation of the cytoskeleton apparatus leading to binucleated cells after mitosis. They used 5,305 endonuclease-prepared siRNAs from a library representing 15,497 human genes to screen for genes required for cell division in HeLa cells. *CMTM4* is one of 37 genes that have been identified by this method.

In order to study the characteristics and function of *CMTM4* further, we analyzed its conservation, expression profile, subcellular localization and effects on HeLa cell growth. We found that CMTM4-v2 is the full length cDNA product and it has been highly conserved during evolution. CMTM4-v1 and -v2 are broadly expressed in normal tissues, and they are distributed on the cell membrane and across the cytoplasm in a speckled pattern. CMTM4-v1 and -v2 inhibit HeLa cell growth by causing G2/M phase accumulation without inducing apoptosis. Therefore, *CMTM4* might be an important gene involved in cell growth and cell cycle regulation.

## MATERIALS AND METHODS

### Bioinformatics

Software provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was applied to perform sequence analysis, open reading frame prediction, and determination of the deduced amino acid chains. Sequence identity and conservation analyses were carried out by using DNAstar software. The structures of exons and introns in *CMTM4* were obtained by comparing the cDNA to the genomic sequence using BLAT Search Genome (http://genome.ucsc.edu/cgi-bin/hgBlat).

### RNA extraction and RT-PCR

Two tissue cDNA panels were purchased from BD Biosciences Clontech (USA). The total RNA was extracted from HeLa cells using TRIZOL®Reagent (Life Technologies, USA) according to the manufacturer's instructions. A Thermoscript™ RT-PCR System (Invitrogen life technologies, USA) was used to perform reverse transcription for cDNA synthesis and PCR. The following primers were used: CMTM4-v1 upstream 5′-ATGCGGAGCGGCGAG-3′, downstream 5′-CTAGACACCTGGGGTGGCGTG-3′; CMTM4-v2 upstream 5′-GGGCGGCAGCATGCGG-3′, downstream 5′-CTGGACACGTGAGGACCTGCC-3′; GAPDH upstream 5′-TGAAGGTCGGAGTCAACGGATTGGT-3′, down-

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stream 5′-CATGTGGGCCATGAGGTCCACCAC-3′. Each reaction vial contained 1 μl template cDNA in a total reaction volume of 20 μl. DNA was first denaturated at 95°C for 4 min followed by 39 cycles of touchdown PCR to amplify CMTM4-v1. In each cycle, the denaturating temperature was 95°C for 30 s, the extension temperature was set to 72°C for 1 min, and annealing temperatures were successively decreased by 3°C starting from 65°C (3 cycles) to a touchdown temperature of 54°C (2 cycles for each touchdown step) and finally reaching 52°C for 28 cycles. To amplify CMTM4-v2, DNA was denatured at 94°C for 5 min followed by 36 cycles of denaturation at 94°C, primer annealing at 65°C and extension at 72°C each for 30 s. Final extension temperatures for both CMTM4-v1 and -v2 were 72°C for 7 min. GAPDH was amplified as an internal standard with annealing at 58°C. All PCR reactions were analyzed by agarose gel electrophoresis.

**Real-time quantitative PCR**

For expression analysis in normal human tissues, a multiple tissue cDNA panel (BD Biosciences Clontech, USA) was used and CMTM4-v1 and -v2 were amplified using the appropriate specific primers as following: CMTM4-v1 upstream 5′-GATCCAGCGCCTGGACACT-3′, downstream 5′-TCAGCAGGTTTGTGGGTGATT-3′; CMTM4-v2 upstream 5′-TAAACCATAGAGCCGGAGCAG-3′, downstream 5′-GAGGAGGATCCAGGCAGGTC-3′. Quantitative real-time PCR was carried out in an ABI Sequence Detection System (Applied Biosystems, USA) and reactions were set up according to the manufacturer's instructions. Preliminary reactions were run to optimize the concentration and ratio of each primer set. All cDNA templates were diluted 100 times and 8 μl of each diluted template was used in a 20 μl real-time PCR amplification system of a SYBR Green PCR Master Mix Kit according to the manufacturer's manual. DNA denaturation was performed at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The expression level of CMTM4 in the skeletal muscle was treated as the baseline.

**Cell culture**

The cervical carcinoma cell line HeLa was purchased from ATCC and cultures were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified 5% $CO_2$ atmosphere and used for studies during exponential growth phases.

**Plasmids and transient cell transfection**

Plasmids for eukaryotic expression of CMTM4-v1 and -v2 were constructed using pcDNA3.1-myc-his b (-) (Invitrogen Technologies, USA) and pEGFP-N1 (Clontech, USA) expression vectors. Transient transfection of HeLa cells was performed by electroporation at 120 V, 20 ms in 2 mm gap cuvettes using a BTX T820 square wave electroporator (BTX Inc., USA). Transfection efficiency was monitored by pEGFP-N1 plasmid and cells with at least 70% transfection efficiency were used for further experiments.

**Confocal microscopy**

The plasmids of pEGFP-N1-CMTM4-v1 and pEGFP-N1-CMTM4-v2 were transfected into HeLa cells, alone or together with plasmids that expressed DsRed-tagged Golgi or ER trackers. Forty-eight hours after transfection, LysoTracker® Red DND-99 (50 nM, Invitrogen) or MitoTracker Red CMXRos (100 nM, Molecular Probes) was added to the media of the cells that only overexpressed the GFP fusion proteins of CMTM4-v1 or -v2. After 30 min incubation, cells were analyzed by confocal microscopy as described previously (Jin et al., 2005) to see if there were co-localizations between CMTM4-v1 or -v2 and these organelles.

**Preparation of cell lysates and Western blot analysis**

Cells were harvested, washed twice in ice-cold PBS, pH 7.2, and pelleted by centrifugation at 800 × *g*. All experimental steps were carried out on ice or at 4°C. Cell pellets were resuspended in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 1% Triton-X100, placed on ice for 30-60 min and subsequently treated with 10 strokes of ultrasonication. Triton insoluble material was then removed by centrifugation at 18,000 × *g* for 20 min and the supernatants were harvested. Protein concentrations were determined by using a BCA protein assay reagent (Pierce, USA) according to the manufacturer's instructions. Equal amounts of proteins were separated on 12.5% SDS-PAGE and subsequently electroblotted at 100 V for 2 h onto a nitrocellulose membrane (Amersham Pharmacia, UK). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat milk for 1 h and incubated with rabbit antibodies against CMTM4-v1 and -v2 (Li et al., 2008) at 1:50 dilutions in 5% nonfat milk in TBST at 4°C over night. Membranes were then extensively washed in TBST and incubated for 1 h with the appropriate IRDye™ 800 conjugated secondary antibody (LICOR Bioscience, USA) in the dark and again extensively washed in TBST. Signals were visualized with an Odyssey Infrared Imager (LI-COR Bioscience, USA). β-actin was detected using the antibody (Sigma, USA; 1:3000) as an internal standard.

**Colony formation assay**

The assay was done as described previously (Srikantan et al., 2002). In brief, HeLa cells transfected with CMTM4-v1 and -v2 expression vectors or empty vectors were seeded into six well culture plates at a density of 2,000 cells/well. Forty-eight hours after transfection, G418 (Roche) was added to a final concentration of 800 μg/ml. The medium was changed every third day and each time G418 was added. Twenty-one days later, G418 resistant colonies were fixed with 2% polyformaldehyde/PBS and stained with crystal violet. Colonies containing at least 50 cells were counted and statistically analyzed.

**Cell counting assay**

The cell counting assay was carried out using a Vi-CELL TM_XR Cell Viability Analyzer (Beckman Coulter, USA). Trypan blue positive cells were considered dead, and the number of viable cells were calculated by the analyzer according to the manufacturer's instructions.

**Annexin V and PI-staining**

Cells were collected by brief trypsinization 72 h after transfection, washed twice in PBS and were resuspended in 200 μl binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2.5 mM $CaCl_2$). FITC conjugated Annexin V was added to a final concentration of 0.5 μg/ml and samples were incubated 20 min in the dark. After brief filtration, PI was added at 1 μg/ml and samples were subsequently analyzed by a FACSCalibur flow cytometer (Becton Dickinson, USA). The 1 × $10^4$ cells were collected and apoptotic cells were detected.

**Cell cycle analysis**

At the indicated time points after transfection or after cell cycle synchronization, the medium was removed and cells were

**Table. 1.** Sizes and boundaries of exons and introns of *CMTM4* with invariable GT/AG nucleotides present at each of the immediate exon/introns boundary (in bold letters).

| No. | Exon size (bp) | 5′ splicer donor | Intron size (kb) | 3′ splicer acceptor |
|---|---|---|---|---|
| 1 | 368 | CGCCCAAGTG**gt**aggtgccg | 59.7 | ttcttcct**ag**ATCTTGGCCC |
| 2 | 177 | GAATCTGACA**gt**gagtacaa | 12.9 | ctcccttt**ag**GATTTGGTCA |
| 3 | 99 | TGCTGCCGTG**gt**agcaaac | 1.2 | gtgttttt**ag**ATATTTGGCT |
| 4 | 7473 | | | |

washed in PBS, trypsinized, collected, washed again in PBS, and fixed in 70% ethanol at -20°C over night. The fixed cells were then washed again two times in PBS and incubated in 0.1% RNase/PBS solution at 37°C for 30 min. To assess DNA content, 10 μg/ml of propidium iodide was added to each sample and cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, USA). The obtained data were then analyzed using ModFit LT software (Verity Software House, USA).

For cell cycle synchronization, cells were treated with 2 mM thymidine 12 h after transfection and the medium was changed 24 h later after washing the cell layers three times with PBS pH 7.2 to remove all thymidine traces. Twelve hours later the medium was changed again and replaced by thymidine containing (2 mM) fresh medium. Finally, 12 h later cells were released from the thymidine treatment. Fresh medium was added; cells were collected at the indicated time points and prepared for cell cycle analysis as described above.

## RESULTS

### Amino acid sequences, conservation analysis and genomic organization of CMTM4-v1 and -v2

*CMTM4* has three RNA splicing forms designated as CMTM4-v1, -v2 and -v3, but CMTM4-v1 (NM_178818) and -v2 (NM_181521) are the main forms for which molecular sizes are consistent with Northern blot analysis (Han et al., 2003). The cDNA sequence of CMTM4-v1 is much shorter than that of CMTM4-v2, but its amino acid sequence is longer. As illustrated in Fig. 1A, only the last 26 amino acids located at the C-terminus are CMTM4-v1 specific, and the others are completely identical with CMTM4-v2. Both CMTM4-v1 and -v2 possess a leucine zipper motif. CMTM4-v2 is highly conserved in most vertebrate animals (Fig. 1B), and the chimpanzee homologue is identical with human CMTM4-v2. Mouse and cattle share 95.7% of the sequence.

Combined analysis of CMTM4-v1 and -v2 indicates *CMTM4* contains four exons and three introns and the last exon can be divided into three parts; i.e., A, B and C. CMTM4-v2 contains all of the exons, whereas CMTM4-v1 contains exons 1, 2, 3 and part A and part C of exon 4 (Fig. 1C). Each of the 5′-donor and 3′-acceptor splice sites conformed to the expected consensus sequence of eukaryotic splice junctions (Mount et al., 1982), with invariable GT/AG dinucleotides located at each of the exon/intron boundaries (Table 1).

### CMTM4-v1 and -v2 are broadly expressed in normal human tissues on the mRNA level

To further study the expression profile of CMTM4-v1 and -v2, RT-PCR and Real Time PCR were carried out. Both isoforms show a broad expression pattern across the 16 examined human tissues, however, their expression patterns were different from each other (Figs. 2A and 2B). CMTM4-v1 shows the highest expression level in testis and next in prostate, while CMTM4-v2 has the highest expression level in pancreas and second highest in testis.

### CMTM4-v1 and -v2 are membrane-associated proteins

In order to characterize the subcellular localization of CMTM4, we transfected HeLa cells with pEGFP-N1-CMTM4-v1 and pEGFP-N1-CMTM4-v2 and assessed whether there were co-localizations with the endoplasmic reticulum (ER), the Golgi-System, the lysosomal compartment or with mitochondria. Figures 3A and 3B show that overexpressed CMTM4-v1 and -v2 are both located on the cell membrane and show a speckled distribution pattern across the cytoplasm with no obvious co-localization to the ER, lysosomes and mitochondria. However, a few cells have co-localization of CMTM4 with the Golgi-System.

### CMTM4-v1 and -v2 can inhibit the colony formation ability of HeLa cells

As it was reported that CMTM4 might have essential functions in HeLa cell division since its knockdown resulted in a cell cleavage defect with malformation of the cytoskeleton apparatus leading to binucleated cells after mitosis (Kittler et al., 2004), we focused on the effects of CMTM4-v1 and -v2 on HeLa cell growth. We first assessed their endogenous expression in HeLa cells, which showed that both CMTM4-v1 and -v2 were moderately expressed in HeLa cells on a mRNA level (data not shown). Then, we constructed the eukaryotic expression vector of CMTM4-v1 and CMTM4-v2, respectively, for functional studies. As illustrated in Fig. 4A, CMTM4-v1 and CMTM4-v2 were undetectable on a protein level in HeLa cells, but they could be successfully overexpressed. Subsquently, we observed their long term effects on HeLa cell growth through a colony formation assay. Transfected cells were plated out and the growth of G418-resistant colonies was observed for 21 days. Both CMTM4-v1 and -v2 led to a marked decrease of colony numbers compared with cell colonies carrying the empty vector (Figs. 4B and 4C).

### CMTM4-v1 and -v2 inhibit HeLa growth without inducing apoptosis

To find the way that CMTM4 influenced the growth of HeLa cells, we conducted a cell counting assay 72 h after transfection. As shown in Fig. 5A, both total and viable cell numbers were decreased after CMTM4-v1 and -v2 overexpression compared with the empty vector. To address the question whether the observed inhibitory effects of CMTM4-v1 and -v2 on HeLa cell growth was caused by apoptosis, we performed Annexin V- and PI-staining. CMTM4-v1 and -v2 did not induce apoptosis 72 h after transfection (Fig. 5B), but Bax induced significant apoptosis of HeLa cells.

### CMTM4-v1 and -v2 induces G2/M phase accumulation both in non-synchronized and synchronized HeLa cells

Regular cell cycle progression is a key factor for cell proliferation, and alterations of the cell cycle may influence cell growth. There-

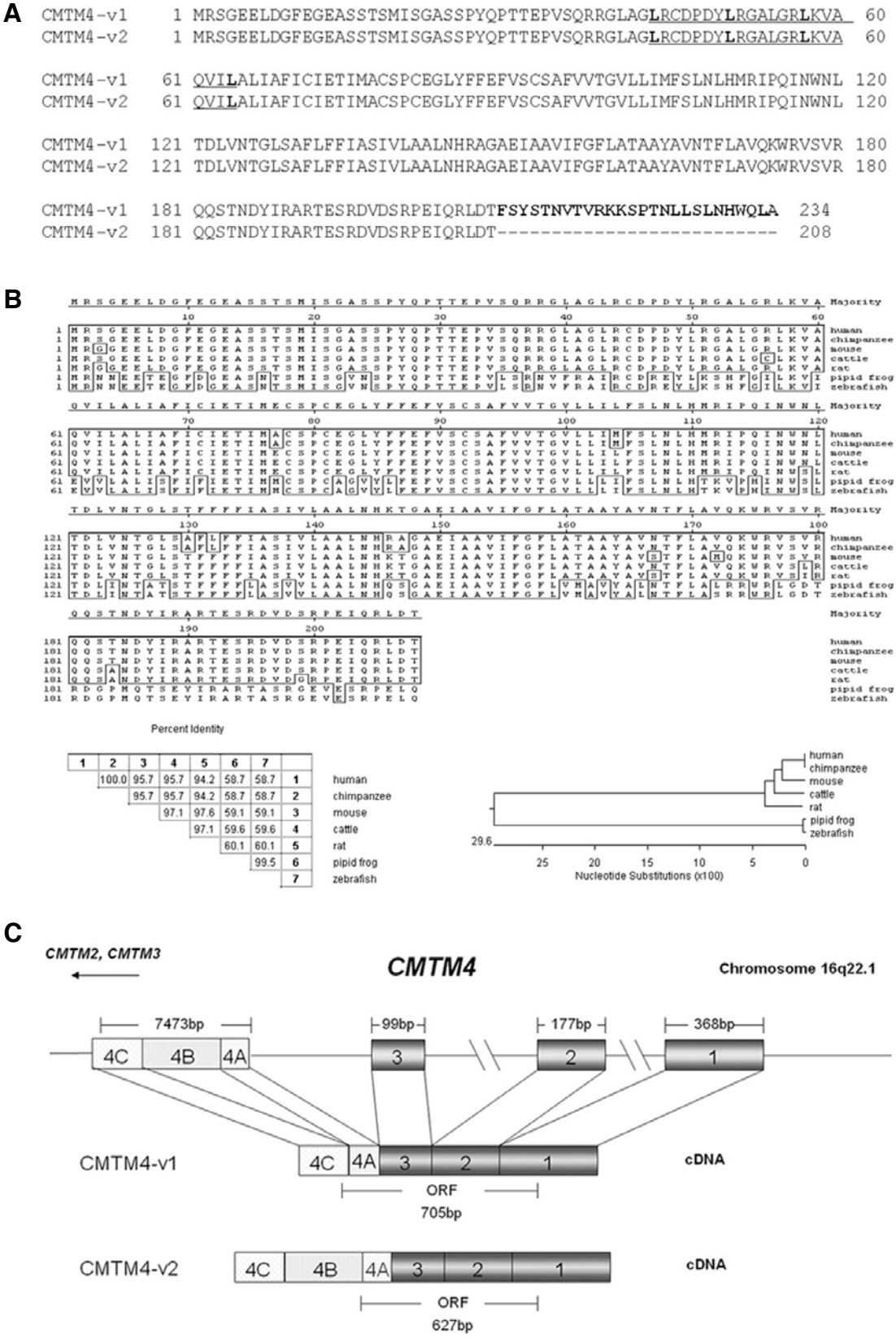


**Fig. 1.** (A) Comparison of the amino acid sequences of CMTM4-v1 and CMTM4-v2. Specific amino acids of CMTM4-v1 are in bold letters, the leucine zipper motif is underlined. (B) Sequence alignment of human CMTM4-v2 and its homologues, percentage of sequence identities, and phylogenetic tree representing nucleotide substitution among species. (C) Genomic organization of *CMTM4*.

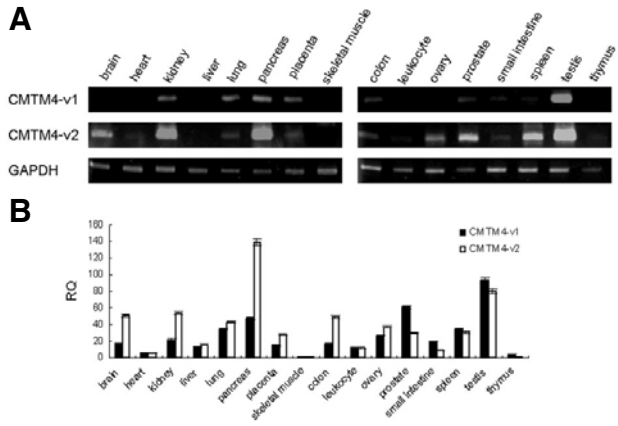


**Fig. 2.** Expression profiles of CMTM4-v1 and -v2 in normal human tissues. (A) PCR products amplified from cDNA panels. (B) Real time PCR, with the expression of CMTM4-v1 and -v2 in skeletal muscle treated as the baseline. "RQ" means the Relative mRNA levels.

fore, we investigated the effect of CMTM4-v1 and -v2 on the cell cycle. First, we analyzed the effects in non-synchronized cells. As illustrated in Figs. 6A and 6B, G2/M phase cell populations increased after CMTM4-v1 or -v2 overexpression: 20.8% of CMTM4-v1 transfected cells and 21.8% of CMTM4-v2 transfected cells were recovered in the G2/M phase compared with 14.5% of cells transfected with the empty vector 48 h after transfection.

In order to verify the effects further, we performed cell cycle analysis using synchronized HeLa cells. As illustrated in Fig. 6C, HeLa cells were successfully synchronized by double-thymidine block (DTB) in G1 phase; and 6 h after release, about 80% of cells moved forward to S phase. Consistent with the demonstrated results, cell populations overexpressing CMTM4-v1 or -v2 were increased in the G2/M phase, most obviously 48 h after the cells were released from the thymidine block, with 19.5% of CMTM4-v1, and 23.0% of CMTM4-v2 overexpressed cells compared with 12.5% of empty vector carriers recovered in this cell cycle stage (Figs. 6D and 6E).

## DISCUSSION

Human CMTM is a novel gene family consisting of *CKLF* and *CMTM1-8*. In this report we introduce the characteristics of *CMTM4,* the most conserved member of this gene family. Kittler et al. (2004) identified *CMTM4* as an essential gene for division of HeLa cells. Knockdown of *CMTM4* can lead to cell cleavage defects and binucleated cells after mitosis (Kittler et

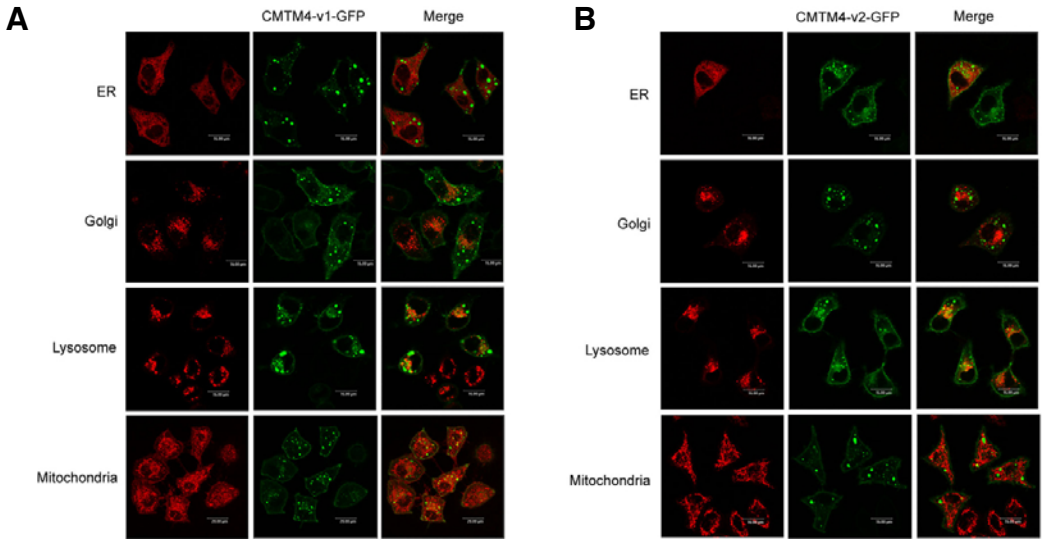


**Fig. 3.** Confocal microscopy of overexpressed CMTM4-v1 (A) and -v2 (B) with ER, Golgi-System, lysosomes and mitochondria (red). CMTM4-v1 and -v2 are distributed on the cell membrane and across the cytoplasm in a speckled pattern and show no co-localization with these organelles except in a few cells they were partially co-localized with the Golgi-System.

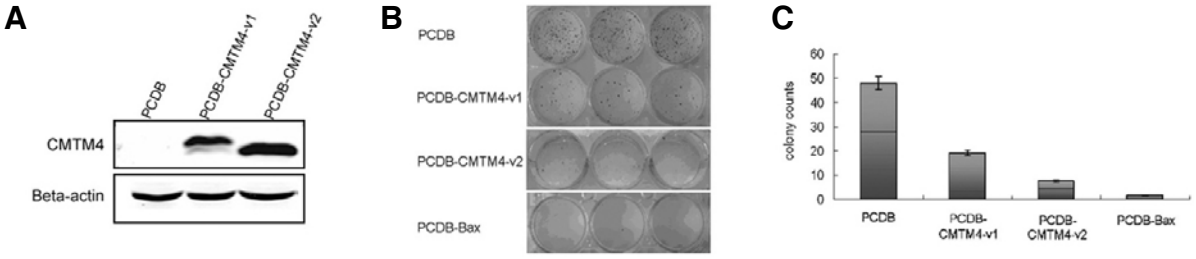


**Fig. 4.** Both CMTM4-v1 and -v2 can inhibit the colony formation of HeLa cells. (A) Verification of CMTM4-v1 and -v2 overexpression in HeLa cells by Western blot 72 h after transfection. (B) Crystal violet stained colonies 21 days after cell transfection. (C) Colonies contained more than 50 cells were counted. Bax was used as a positive control. The data present the mean of three independent experiments.

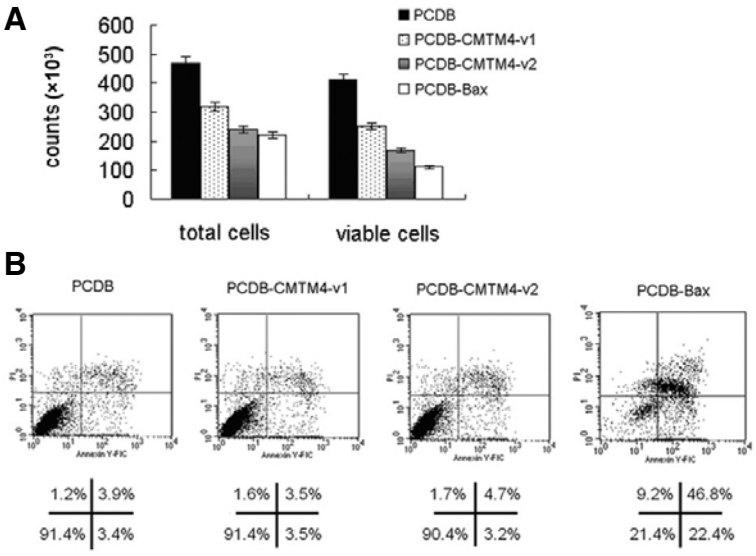


**Fig. 5.** CMTM4-v1 and -v2 inhibit HeLa cell growth without inducing apoptosis. (A) The number of total and viable cells decreased in CMTM 4-v1 or -v2 overexpressed HeLa cells 72 h after transfection. (B) AnnexinV/PI staining showed no apoptosis after CMTM4 overexpression. Bax was used as a positive control.

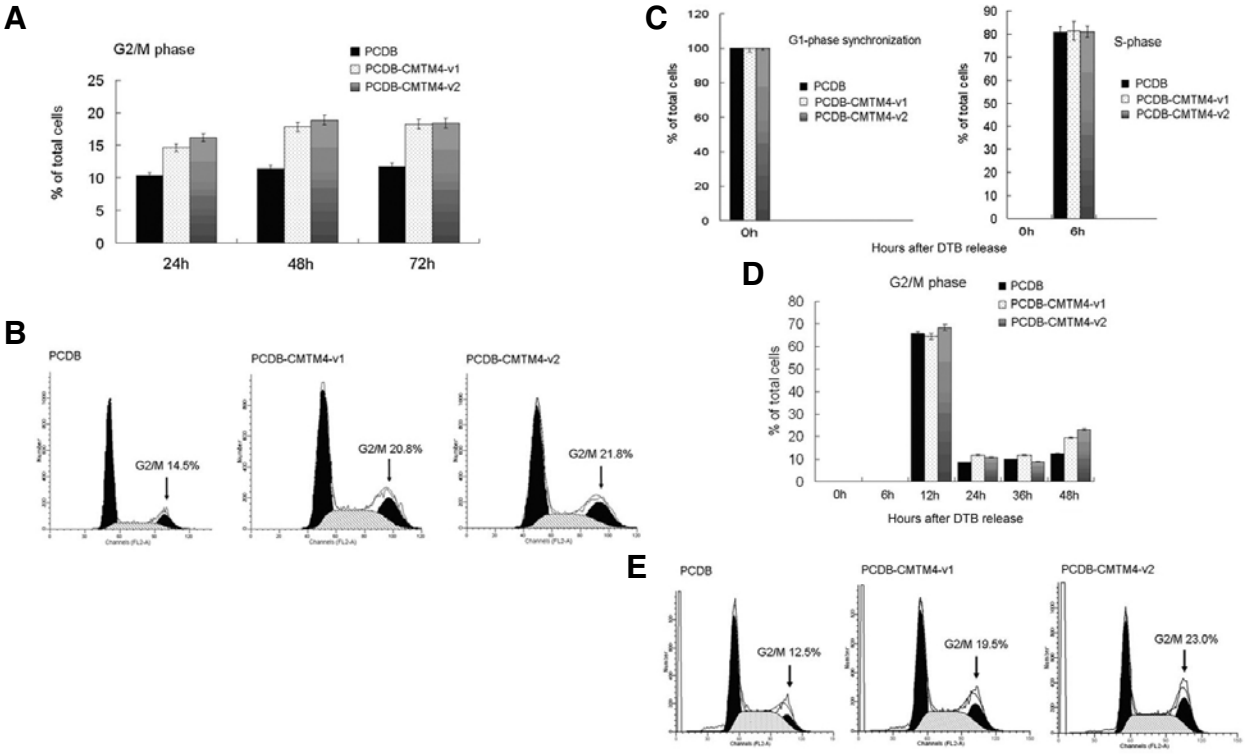


**Fig. 6.** CMTM4-v1 and -v2 cause G2/M phase accumulation both in non-synchronized and synchronized HeLa cells. (A) Overexpression of CMTM4-v1 and -v2 both can induce cell cycle accumulation in non-synchronized HeLa cells at G2/M stage. (B) Cell-cycle distribution of non-synchronized HeLa cells 48 h after transfection. The plot shown is a representative of three different experiments. (C) HeLa cells were synchronized in G1 phase after double thymidine block (DTB), and 6 h after release from G1 block, about 80% of cells moved forward to S phase following regular cell cycle progression. (D) Cell populations overexpressing CMTM4-v1 or -v2 were increased in the G2/M phase, most obviously 48 h after the cells were released from DTB. (E) Cell-cycle distribution of synchronized HeLa cells 48 h after transfection. The plot shown is a representative of three different experiments.

al., 2004). Based on this observation, we further investigated *CMTM4* and found CMTM4-v1 and -v2 are the main forms. Overexpression of CMTM4-v1 and -v2 inhibit HeLa cell growth by inducing G2/M phase accumulation in non-synchronized as well as in synchronized cells. Our results accompanied with the previous findings by Kittler et al. (2004) demonstrate that the changes in *CMTM4* expression may affect HeLa cell cycle and division. Regular cell proliferation and tissue differentiation are

the results of a tightly controlled cell cycle progression with a balance between inhibitors and activators, while disturbance of this system can lead to uncontrolled cell proliferation, which is a hallmark of tumor growth (Vermeulen et al., 2003). The present study suggests that *CMTM4* might have crucial functions in the cell cycle and might be an important novel regulator of cell growth.

*CMTM4* is tightly linked with *CMTM1-3*. Our previous studies verified that CMTM1-v17, CMTM2 and CMTM3 have highest expression levels in testis with regulatory effects on AR transactivation. The amino acid sequences of both CMTM4-v1 and -v2 possess a leucine zipper motif, which usually plays an important role in mediating the binding process of proteins to target nuclear receptors (Heery et al., 2001). CMTM4-v1 and -v2 show higher expression levels in testis on a mRNA level, which is consistent with an immunohistochemistry assay (Li et al., 2008). This expression characteristic and the leucine zipper motif suggest they might also play important roles in AR transactivation such as CMTM1, -2 and -3. Further experiments are necessary to uncover their functions in this aspect.

*CKLF* and *CMTM1-4* form a gene cluster on chromosome 16q22.1, a locus frequently deleted in multiple tumors that might harbor novel tumor suppressor genes (Dorion et al., 1995). Therefore, it will be interesting to observe if *CMTM4* is down regulated or deleted in tumors, which will provide further insights into the contribution of *CMTM4* to tumor genesis *CMTM3* and *CMTM5* are novel tumor suppressor candidates with promoter methylation in many tumor cell lines and tumor. tissues, which has inhibitory effects on cell growth. Extensive studies on *CMTM4* will be helpful to uncover the function of CMTM and other MARVEL-domain containing proteins and may have potential applications in tumor therapy.

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