Biochemical and Biophysical Research Communications 387 (2009) 139–142

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CMTM5 induces apoptosis of pancreatic cancer cells and has synergistic effects with TNF-α

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ARTICLE INFO

Article history:
Received 25 June 2009
Available online 3 July 2009

Keywords:
CMTM5
Pancreatic cancer
Apoptosis
TNF-α

ABSTRACT

Our previous data show that CKLF-like MARVEL transmembrane domain-containing member 5 (*CMTM5*) is a potential tumor suppressor gene, but its function in pancreatic cancer is unknown. Herein we first report that *CMTM5* is also absent in pancreatic cancer cell lines with promoter methylation. Compared with normal pancreatic tissues, *CMTM5* is significantly decreased in cancer tissues. Restoration of CMTM5-v1 not only induces MIA PaCa-2 cell apoptosis with activation of caspase 3, 8 and 9, but also has synergistic effects with TNF-α. Thus, *CMTM5* may play a role in the pancreatic cancer.

Introduction

CMTM is a novel family of proteins linking chemokines and TM4SF. In humans, CMTM consists of nine genes (*CKLF* and *CMTM1–8*) [1,2]. *CMTM5* has at least six RNA splicing forms, CMTM5-v1–v6, with CMTM5-v1 as the main form. CMTM5-v1 is broadly expressed in normal adult and fetal tissues, while CMTM5-v1 is either decreased or undetectable in most tumor cell lines due to promoter methylation. Restoration of CMTM5-v1 can strongly suppress tumor cell growth and migration [3] and induces apoptosis of cervical cancer cells [4], but the expression and function of CMTM5-v1 in pancreatic cancer is unknown. Pancreatic cancer is lethal, with 5-year survival rates <5% [5]. Thus, it is necessary to identify novel genes involved in pancreatic carcinogenesis.

Apoptosis is a cell suicide program required for maintaining the balance between cell proliferation and death. There are two main pathways involved in the induction of apoptosis: a death-receptor-initiated caspase 8-mediated pathway and a mitochondria-initiated caspase 9-mediated pathway. Caspase 8 can be activated by several ligands of the different death receptors, including TNF and CD95L. The mitochondrial pathway can be activated by a diverse range of stimuli, including reactive oxygen species, radiation and chemotherapeutic agents. Either caspase 8 or caspase 9 can activate caspase 3. Caspase 3 functions as the main effector or "executioner" caspase, cleaving various substrates, including PARP, that ultimately causes morphologic and biochemical changes in apoptotic cells [6].

Herein we report for the first time that *CMTM5* is undetectable in pancreatic cancer cell lines with promoter methylation and decreased in pancreatic cancer tissues. Restoration of CMTM5-v1 induces apoptosis of pancreatic cancer cells with activation of caspase 3, 8 and 9. Furthermore, CMTM5-v1 has synergistic effects with TNF-α.

Materials and methods

Cell lines and tissue samples. The human pancreatic cancer cell lines, AsPC-1, BxPC-3, PANC-1, and MIA PaCa-2, were purchased from the American Type Culture Collection (Rockville, MD, USA). AsPC-1 and BxPC-3 cells were grown in RPMI-1640 supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany). MIA PaCa-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Life Technologies, Grand island, NY, USA) supplemented with 4 mM L-glutamine, 10% FBS, and 2.5% horse serum. PANC-1 cells were maintained in DMEM medium supplemented with 4 mM L-glutamine and 10% FBS.

The five pancreatic cancer and normal pancreas tissues for Western blotting were obtained from the Tissue Bank of Peking University School of Oncology with the patients' consent and Institutional Ethics approval. The pancreatic cancer tissue chips were purchased from Chaoying Biotechnology Co. (Xian, China).

Reagents: Monoclonal antibody against β-actin and 6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Propidium iodide (PI) was obtained from

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doi:10.1016/j.bbrc.2009.06.148

the Beijing Biosea Biotechnology Co. (Beijing, China). Ac-DEVD-AMC was purchased from ALEXIS Biochemicals (Lausen, Switzerland). Monoclonal antibody against caspase 3 was obtained from Transduction Laboratories (Lexington, KY, USA). Recombinant human TNF-α and monoclonal antibody against caspase 8 were obtained from R&D systems, Inc. (Minneapolis, USA). Monoclonal antibodies recognizing caspase 9 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). IRDye™ 800-conjugated secondary antibodies against mouse and rabbit IgG were from LICOR Bioscience (Lincoln, NE, USA). Polyclonal antibody against *CMTM5* was prepared as previously described [4].

Semi-quantitative RT-PCR analysis: Reverse transcription was done using the ThermoScript First-Strand Synthesis kit (Invitrogen Technologies, Carlsbad, California, USA). The normal pancreas cDNA library was purchased from Clontech (Mountain View, California, USA). *CMTM5* was amplified with the following primers: forward primer, 5′-ATCTGCTTCACGGCCTCC-3′; reverse primer, 5′-GTGCCATCTCAGTCCGGTAG-3′. The annealing temperature was 60 °C for 35 cycles.

MSP and BGS analysis: Bisulfite modification of DNA, methylation-specific PCR (MSP), and bisulfite genomic sequencing (BGS) were carried out as previously described [3]. For BGS, the PCR products were cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) with 6–10 clones randomly chosen and sequenced.

Immunohistochemistry: Immunohistochemistry was done as previously described [7] and the antibody concentration was 10 mg/L.

Cell infection and TNF-α treatment: Cells were infected with CMTM5-v1 adenovirus (ad-CMTM5-v1) and ad-null (vector-containing adenovirus, defined as MOCK) at a multiplicity of infection (MOI) of 30, as previously described [4].

For TNF-α treatment, MIA PaCa-2 cells were infected at a MOI of 15, and TNF-α was added 6 h after infection at a concentration of 5 ng/mL.

DAPI staining and DNA fragmentation analysis: The cells were fixed with 3% paraformaldehyde containing 0.1% Triton X-100 36 h post-infection at room temperature for 30 min. Subsequently, the cell nuclei were stained with DAPI (0.5 μg/mL) and imaged using a CCD camera (Apogee Instruments, Roseville, California, USA).

The measurement of the DNA fragment was followed as previously described [8]. One × 10^4 cells were collected and recorded by a FACS instrument.

Evaluation of caspase 3 activity (DEVDase activity analysis): DEVDase activity was done as previously described [4]. Infected cells were harvested in lysis buffer. Five micrograms of total lysate was added into the assay buffer 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, Offenburg, Germany) with a 380 nm excitation filter and a 460 nm emission filter.

Protein extraction and Western blot analysis: Cells were harvested, lysed, and proteins (30 μg) were subjected to Western blot as previously described [4].

Statistical analysis: Data were presented as the mean ± SE. Statistical analysis was carried out with Student's *t*-test. A $P < 0.05$ was considered to be a statistically significant difference.

Results

CMTM5 is silenced in pancreatic cancer cell lines with promoter methlyation

Our previous study [3] illustrated that *CMTM5* is silenced in many cancer cell lines, including breast, nasopharyngeal, esophageal, and gastric cancers. Herein we examined *CMTM5* at the mRNA level in normal pancreatic tissue and four pancreatic cancer cell lines (AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1). RT-PCR analysis showed that compared to the moderate level of expression in normal pancreatic tissue, CMTM5-v1 was undetectable in pancreatic cancer cell lines (Fig. 1A).

The silence of *CMTM5* in cancer cell lines is due to promoter methylation, so we also detected *CMTM5* in pancreatic cancer cell lines. MSP showed that all the cell lines were methylated (Fig. 1B). The detailed methylation status of each individual CpG site was examined by BGS. The results were consistent with those of MSP (Fig. 1C).

CMTM5 is decreased in pancreatic cancer tissues

Using a pancreatic cancer tissue microarray, we evaluated the expression of *CMTM5* protein in samples of normal pancreas ($n = 22$) and pancreatic adenocarcinoma ($n = 57$). The level of expression of *CMTM5* in individual samples was scored as negative, weakly positive, and positive by a blinded pathologist. The expression of *CMTM5* was observed primarily in the cytoplasm (Fig. 2A). All the normal tissue samples were graded as positive. However, most pancreatic cancer samples were graded as negative (40 of 57 [70%]), whereas 25% were weakly positive (Fig. 2B).

To determine if the expression of *CMTM5* correlated with the differentiation status of pancreatic adenocarcinoma, cancer sam-

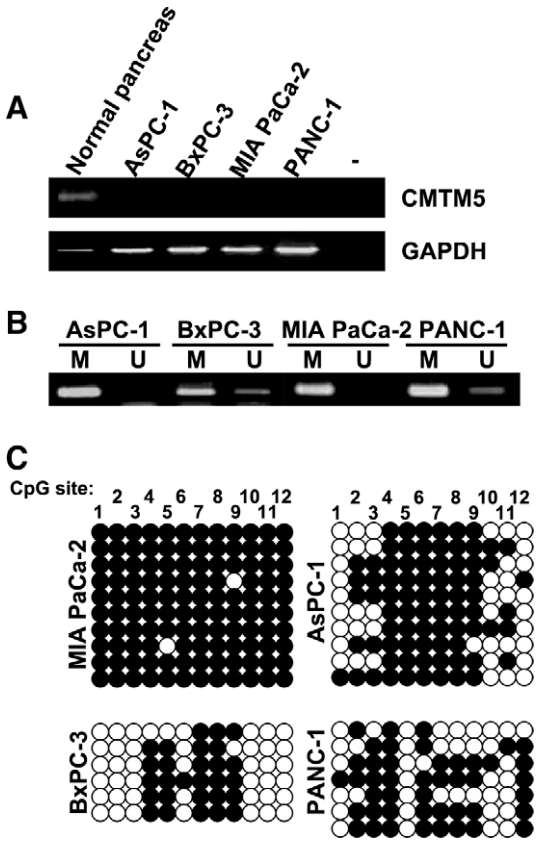

Fig. 1. *CMTM5* is silenced and methylated in pancreatic cancer cell lines. (A) RT-PCR analysis of *CMTM5* expression in normal pancreatic tissue and pancreatic cancer cell lines. GAPDH was used as a reference gene. (B) MSP analysis of *CMTM5* in pancreatic cancer cell lines. M, methylated; U, unmethylated. (C) bisulfite genomic sequencing of the *CMTM5* promoter in pancreatic cancer cell lines. Each row represents an individual allele that was randomly cloned and sequenced. Circles represented CpG sites (12 sites) analyzed: black circle, methylated CpG site; white circle, unmethylated CpG site.

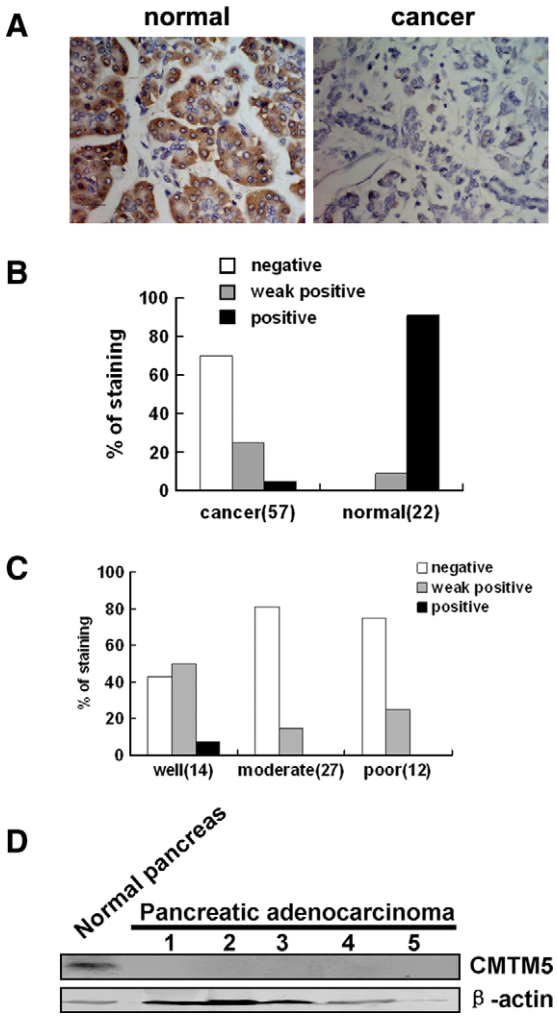

Fig. 2. *CMTM5* is decreased in pancreatic cancer tissues and its expression level correlates with pancreatic cancer differentiation status. (A) Immunostaining of *CMTM5* in normal pancreas and pancreatic adenocarcinoma tissues. Left, normal pancreas; right, adenocarcinoma. (B) Analysis of *CMTM5* expression in the pancreatic cancer tissue array. (C) Correlation between *CMTM5* expression and the differentiation status of cancers. Columns, percentage of total sample type. (D) Western blot analysis of *CMTM5* in normal pancreatic tissue and pancreatic adenocarcinoma.

ples on the tissue microarray were graded as well, moderately, and poorly-differentiated. We found a direct correlation between *CMTM5* expression and the differentiation status of tumors. Greater than 75% (9 of 12) of poorly-differentiated cancers lost *CMTM5* expression compared with 43% (6 of 13) of well-differentiated cancers (Fig. 2C). We did not observe a correlation between the level of *CMTM5* expression and patient gender or age (data not shown).

We also examined the expression of *CMTM5* in normal pancreatic and pancreatic adenocarcinoma by Western blot. In agreement with the immunohistochemistry findings, *CMTM5* was expressed in normal pancreas, but undetectable in pancreatic adenocarcinoma samples (Fig. 2D).

Restoration of CMTM5-v1 induces apoptosis of pancreatic cancer cells

We investigated the effects of ectopic *CMTM5* expression by the Ad5 system on MIA PaCa-2 cells, in which *CMTM5* was completely methylated and silencing. Thirty-six hours after infection, compared with MOCK, ad-CMTM5-v1-infected cells were round, shrunken with blebbing, and detached from the dish (Fig. 3A). DAPI

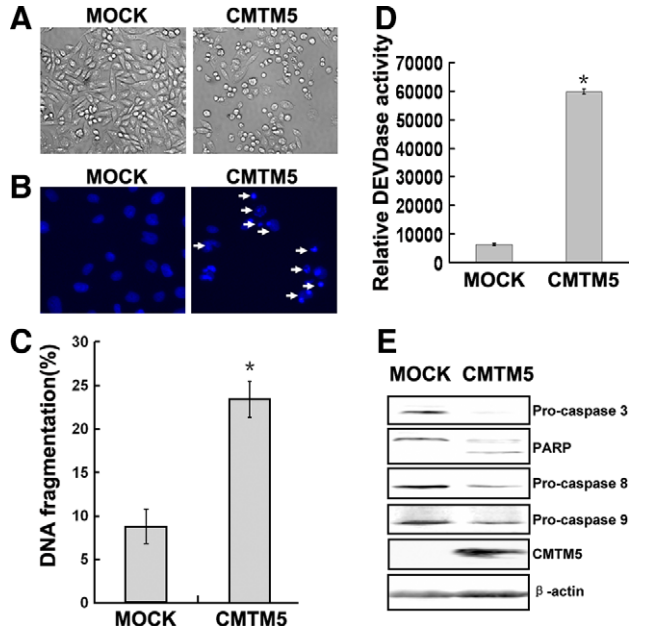

Fig. 3. Restoration of CMTM5-v1 induces apoptosis of MIA PaCa-2 cells. (A) Morphologic changes induced by CMTM5-v1. (B) DAPI staining assay. White arrows, apoptosis-associated changes in nuclei. (C,D) DNA fragmentation assay and caspase 3 activity assay. Mean ± SD of three independent experiments. $^{*}P < 0.05$, compared with MOCK. (E) Western blot showed decreased pro-caspase 3, 8, and 9 and cleavage of PARP. β-Actin was used as a protein loading control.

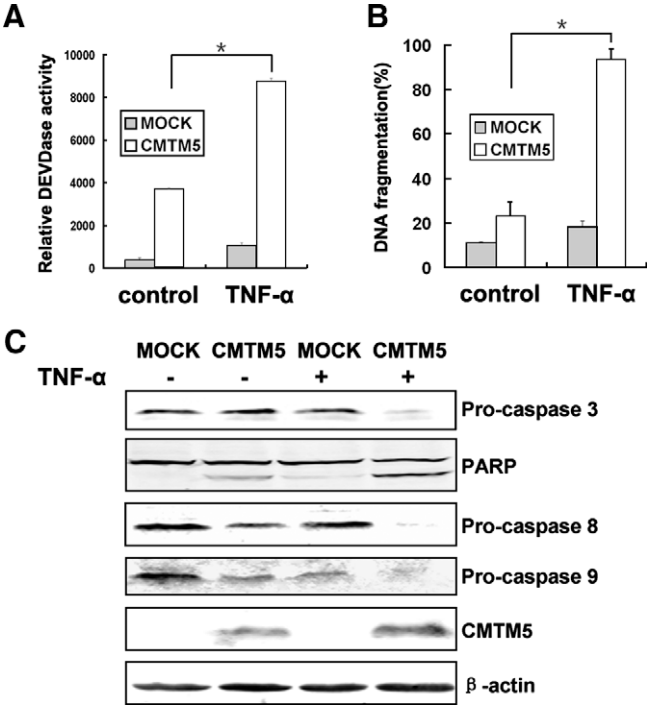

Fig. 4. CMTM5-v1 has synergetic effects with TNF-α. (A,B) Caspase 3 activity and DNA fragmentation assay. Mean ± SD of three independent experiments. $^{*}P < 0.05$, compared with control. (C) Western blot analysis of pro-caspase 3, 8, and 9 and PARP.

staining showed chromatin condensation and rupture in ad-CMTM5-v1-infected cells, which are characteristic features of apoptosis (Fig. 3B). Similar changes were also detected on PANC-1 cells (data not shown). The DNA fragmentation in ad-CMTM5-

v1-infected cells was increased 3-fold compared with the control (Fig. 3C).

Caspase 3 is the most common important effector caspase during apoptosis. Above all, we measured caspase 3 activity using a DEVD cleavage assay. CMTM5-v1 initiated the activity 10 times higher than MOCK (Fig. 3D). The subsequent Western blot analysis detected an obvious decrease of pro-caspase 3 in CMTM5-v1-infected cells. Cleaved PARP associated with caspase 3 activation was present in CMTM5-v1-infected cells, but rarely detected in control cells (Fig. 3E). Western blot also showed both pro-caspase 8 and 9 decreased in ad-CMTM5-v1-infected cells (Fig. 3E).

CMTM5-v1 has synergistic effects with TNF-α

Since TNF-α also induced apoptosis of MIA PaCa-2 cells, we tested whether CMTM5-v1 had synergistic effects with TNF-α. The caspase 3 activity and DNA fragmentation assay indicated that ad-CMTM5-v1-infected cells plus TNF-α was significantly higher than ad-CMTM5-v1-infected cells alone or cells treated with TNF-α alone (Fig. 4A and B). The subsequent Western blot showed a more remarkable decrease in pro-caspase 3, 8, and 9 and an increase in cleaved PARP in ad-CMTM5-v1-infected cells combined with TNF-α (Fig. 4C).

Discussion

Tumor suppressor genes (TSG) can be inactivated in tumors by several mechanisms, including deletion and mutation in the genomic sequence, or by epigenetic mechanisms, such as promoter DNA methylation. In cancer, DNA methylation is a powerful way of inactivating tumor suppressor and DNA repair genes. *CMTM5* is a candidate tumor suppressor gene. We have shown that *CMTM5* is either decreased or undetectable in most tumor cell lines due to promoter methylation and restoration of CMTM5-v1 can strongly suppress tumor cell growth and migration [3]. Herein we investigated for the first time the tumor suppressor effects of CMTM5-v1 in pancreatic cancer. Like other tumor cell lines, *CMTM5* was undetectable in AsPC-1, BxPC-3, MIA PaCa-2, and PANC-1 cells and the promoter was also methylated in these four cell lines. *CMTM5* is significantly decreased in pancreatic adenocarcinoma tissues and its level of expression is correlated with their differentiation status.

The MIA PaCa-2 cell line is a common cell model for pancreatic cancer research, which has functional TNF and TRAIL receptors, and TNF-α exposure causes concentration dependent apoptosis (0.5–25 ng/mL) [9]. Restoration of CMTM5-v1-induced apoptosis of MIA PaCa-2 cells with caspase 3 activation. The cleavage of procaspase 8 and 9 verified that both of them were activated and participated in CMTM5-v1 induced apoptosis.

TNFR is one of the most important members of the death receptor family [10]. The binding of TNF-α to TNFR1 results in the recruitment of TRADD, FADD, RIP, and TRAF2, leading to the activation of the NF-κB and JNK pathways, and then breaks the balance between cell death and survival [11]. Since CMTM5-v1 and TNF-α has synergistic effects, we first detected the influence of CMTM5-v1 on TNFR1 expression. At the mRNA and protein levels, TNFR1 had no difference between MOCK and Ad-CMTM5-v1, including total protein, protein on the cell membrane, and protein on lipid rafts (data not shown). Restoration of CMTM5-v1 also activated the JNK pathway and inhibited NF-κB activation caused by TNF-α (data not shown), but the detailed mechanism remains to be determined.

CMTM5 is a member of the CMTM family, which belongs to TM4SF. Several TM4SF members are related to pancreatic cancer progression and metastasis, such as CD151, CO-029, and Claudin-4 [7,12]. *CMTM5* is undetectable in pancreatic cancer cell lines and downregulated in pancreatic cancer tissues; restoration of *CMTM5* induces apoptosis of pancreatic cancer cells and has synergistic effects with TNF-α, which will provide new clues for further studies of other MARVEL domain-containing proteins and be helpful for pancreatic cancer research.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 30571691), the National High Technology Research and Development Program of China (863 Program; No. 2006AA02A305), and the Program for New Century Excellent Talents in University (No. NCET-07-0013). The manuscript language was refined by language company-International Science Editing (Ireland).

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