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New Phenomenon

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# CMTM3 presents a secreted form released via exosomes

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CKLF-like MARVEL transmembrane domain-containing family (CMTM) is a family of proteins linking classical chemokines and the transmembrane-4 superfamily (TM4SF). In humans, they are encoded by nine genes, including chemokine-like factor (*CKLF*) and *CMTM1* to *CMTM8* [1]. Of these, CKLF1, CMTM1, and CMTM2 are related to chemokines, whereas CMTM8 is related to TM4SF11. The characteristics of other CMTM members are intermediate between those of CMTM1 and CMTM8. MARVEL domain-containing proteins usually have no signal peptide, but several reports have shown that MARVEL proteins, like MAL and CMTM5, are secreted via a vesicle-mediated secretory pathway [2,3]. CMTM3 is the most closely related to CMTM5-v1 in the CMTM family, with 42% amino acid identity. However, by far, CMTM3 is known as a membrane-associated tumor suppressor protein [4]. Thus, it is necessary to know if CMTM3 can be secreted in vesicles.

As CMTM3 is highly expressed in the male reproductive system and inhibits prostate-specific antigen expression, we examined whether CMTM3 protein can be released in human normal prostate and prostate hyperplasia tissues by immunohistochemistry assay. Of the 49 normal prostate and prostate hyperplasia tissues, 38 cases (77.6%) have the secreted form of CMTM3 in the prostate lumen (**Fig. 1A**). To verify this result, we first infected PC-3 cells with adenovirus and confirmed the expression of CMTM3 (**Fig. 1B**). Then, the supernatants were collected with sequential centrifugation for western blot analysis. CMTM3 can be sedimented through centrifugation (100,000 *g*) from the conditioned medium (**Fig. 1B**), indicating that it is secreted in vesicles.

To further confirm the type of secreted form of CMTM3, vesicles isolated by centrifugation at 100,000 *g* were subject to sucrose gradient ultracentrifugation, and then western blot analysis was used to detect CMTM3 in the fractions. The secreted form of CMTM3 was found to float at the density of 1.19 g/ml on a sucrose gradient (**Fig. 1C**), corresponding to the known density of exosomes. The vesicles isolated by centrifugation at 100,000 *g* were also examined by electron microscopy. As shown in **Fig. 1D**, the secreted CMTM3 was located in small vesicles (<100 nm in diameter). Furthermore, CMTM3-positive vesicles can occasionally be detected in PC-3 cells infected with vector-containing adenovirus (MOCK), suggesting the existence of endogenous CMTM3 in vesicles.

To investigate the mechanism underlying CMTM3 secretion, both the classical and non-classical vesicle-mediated secretory pathways were investigated. Cells were treated with 2.5 μg/ml of brefeldin A (BFA) or 50 μM of LY294002. BFA inhibits the classical protein transport pathway [5], whereas LY294002 is a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) which can inhibit internal vesicle formation within the intracellular multivesicular bodies (MVBs) and suppress the secretion of exosomes [6]. Results showed that BFA had no effect on CMTM3 release into the media (**Fig. 1E**). In contrast, LY294002 greatly reduced the release of CMTM3 (**Fig. 1E**), suggesting that CMTM3 is released via an exosome route.

The above finding that CMTM3 secretion is via an exosome route led us to examine whether CMTM3 is co-localized with exosomal marker. The co-localization of CD63 and overexpressed CMTM3 were detected. CMTM3 had partial co-localization with CD63 (**Fig. 1F**). Further subcellular localization analysis indicated that CMTM3 had no obvious co-localization with endoplasmic reticulum or Golgi (data not shown).

Characteristics of the secreted form of CMTM3 are consistent with the criteria of exosomes. Among the various kinds of secreted membrane vesicles, exosomes have received much attention over the past several years. It has been revealed that exosomes, with a size between 30 and 100 nm, are formed as intraluminal vesicles in MVBs and released when MVBs fused with the plasma membrane [7]. Recently, some tumor suppressor molecules have been reported to be secreted via exosomes, which further exert tumor-suppressive functions [8,9]. For example, PTEN, a tumor suppressor protein normally localized in the cytoplasm and nucleus, can be secreted

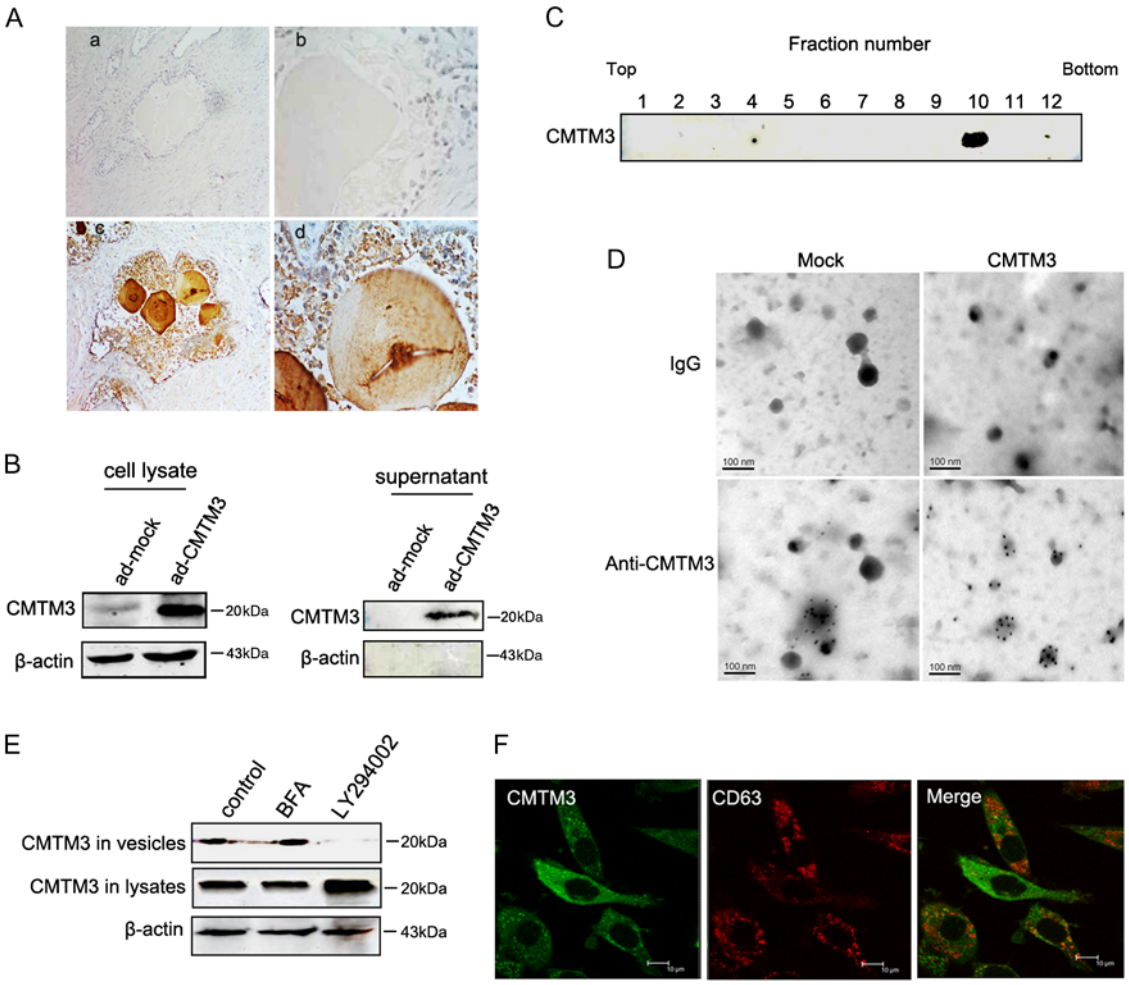


**Figure 1. Identification of the secreted form of CMTM3** (A) CMTM3 occurs as the secreted form in the prostate lumen. Human prostate hyperplasia tissues were stained with normal rabbit IgG as control (a and b) or with polyclonal antibody specific against CMTM3 (c and d). Magnification: (a) and (c), ×100; (b) and (d), ×400. (B) The cell lysates of PC-3 cells infected with ad-null (vector-containing adenovirus, defined as Mock) or ad-CMTM3 were detected using anti-CMTM3 pAb. Vesicles isolated from conditioned medium were analyzed by western blot analysis. β-actin served as a cell lysate protein loading control and as the negative control for supernatant proteins. (C) Vesicles containing CMTM3 floated on a continuous sucrose density gradient. Aliquots from each of the first 12 fractions were detected by western blot analysis. (D) Vesicles isolated from conditioned medium of PC-3 cells infected with Mock or ad-CMTM3 were applied to Formvar carbon-coated electron microscope grids, and immunostained with anti-CMTM3 antibody. Scale bars represent 100 nm. (E) PC-3 cells transiently expressed CMTM3 were incubated with 2.5 μg/ml of BFA or 50 μM LY294002 in conditioned medium for 24 h. Vesicles from conditioned medium and cell lysates were analyzed by western blot analysis using specific antibody against CMTM3. β-actin served as a cell lysate protein loading control. (F) PC-3 cells transiently expressed CMTM3 were fixed and doubly labeled with antibodies against CMTM3 (green) and CD63 (red). Scale bars represent 10 μm.

in exosomes. Secreted PTEN reduces recipient cell proliferation and phosphorylation of the serine and threonine kinase Akt. Therefore, it is reasonable to infer that CMTM3-containing vesicles might also play an important role in pathophysiological processes.

The MARVEL domain has been identified in proteins of the myelin and lymphocyte (MAL), physins, CMTM, gyrins, and occludin families. Most MARVEL domain proteins are involved in membrane apposition and vesicle-trafficking events. MAL was reported to have a form secreted as exosome-like vesicles, except that its size is a little bigger than that of typical exosomes [2]. However, the size and density of CMTM3-containing vesicles are the same as those of typical exosomes. Interestingly, although human CMTM3 is the most closely related to CMTM5-v1 among the CMTM family, they also share different secretory characteristics. Unlike CMTM3, CMTM5-v1 had no obvious co-localization with the exosomal marker CD63, and its secretion was inhibited by BFA, but not by wortmannin, an inhibitor of the PI3K. These results suggest that different MARVEL proteins might be involved in different secretory pathways.

In summary, we identified CMTM3 as a novel secretory protein released via exosomes. It will be important to investigate the source of secreted CMTM3, its tumor-suppressive effect, and signaling pathway. As exosomes secreted from prostate could be detected in urine, it will be interesting to analyze the expression level of exosomal CMTM3 in urine samples from prostate cancer and benign prostatic hyperplasia, to explore the potential of secreted CMTM3 as a diagnostic marker for prostate cancer.

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