

Original Article

Expression and localization of CMTM2 in human testis and sperm

Xiaowei Zhang^{1*}, Yaojun Dun^{1*}, Zhiping Hu², Yongping Zhao³, Zhengsheng Zhang⁴, Tao Xu¹, Qing Li¹

¹Department of Urology, Peking University People's Hospital, Beijing, China; ²Department of Hepatobiliary, Peking University People's Hospital, Beijing, China; ³Reproductive Medicine Center, Peking University People's Hospital, Beijing, China; ⁴Department of Surgery, Fuping County Hospital, Baoding Area, Hebei Province, China. *Equal contributors.

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Abstract: Purpose: To examine the expression and localization of transmembrane protein CMTM2 in human testis and sperm, and to approach the potential function of the protein in the male reproductive system. Methods: With testicular tissues obtained from obstructive azoospermia patients via biopsy and semen from healthy male volunteers, Western blot and immunohistochemistry were performed on human testis and sperm to explore the expression of CMTM2 on protein level, and immunofluorescence analysis was also used to examine the localization of CMTM2 in human testis and sperm before and after the acrosome reaction. Results: CMTM2 represented high expression in both human testis and sperm. In the testis, CMTM2 immunoreactive particles were observed mainly in the membranes of spermatogenic cells at different stages. In human sperm, CMTM2 was restrictively localized in the posterior head of sperm where sperm-egg fusion occurred, and the localization remained identical before and after sperm acrosome reaction. Conclusion: Expression of CMTM2 in human testis and sperm exhibits cell- and region-specific patterns, which suggests that they may play a pivotal role in spermatogenesis and sperm-egg fusion.

Keywords: CMTM2, western blot, immunohistochemistry, immunofluorescence, sperm

Introduction

Spermatogenesis is a complex and well-regulated process, which is impacted by the spermatogenic cells and a series of cytokines. CKLF-like MARVEL transmembrane domain-containing protein 2 (CMTM2) is one of the members of CMTM family (**Figure 1**). And it is a novel protein family that provides a structural and functional link between chemokines and members of the transmembrane 4 super family (TM4SF) [1, 2]. It has been recognized that CMTM2 exhibits high expression in human testicular tissues [3]. Additionally, the important role of CMTM2 in the process of spermatogenesis is also certified by a close correlation between CMTM2 abnormal expression and a spermatogenesis defect [4]. However, the literature concerning CMTM2 is very limited, and there were only few reports identifying the role of CMTM2 in male reproductive system to date. In the present study, we aimed to study the expression and localization of transmembrane protein CMTM2 in the testis and sperm of adult males and to explore the potential function of the protein in the male reproductive system.

Materials and methods

Materials

The anti-CMTM2 monoclonal antibody was purchased from Abcam (Inc, USA). The horseradish peroxidase (HRP), fluorescein isothiocyanate (FITC), tetramethylrhodamine (TRITC)-conjugated mouse anti-rabbit IgG antibody and 3,3'-diaminobenzidine (DAB) kit were obtained from Beijing Zhongshan Goldenbridge Company (China). FITC-labeled pisum sativum agglutinin (PSA) and A23187 were from Sigma (Inc, USA). And human tubal fluid (HTF) was from In Vitro care (Inc, USA), which was used as medium for *in vitro* fertilization.

Testicular tissue samples were acquired via biopsy from patients with obstructive azoospermia in Peking University People's Hospital. All these patients met the following criteria: 20-45 years old, married and having at least one child during follow up. And some healthy male volunteers treated at reproductive medicine center, Peking University People's Hospital provided semen samples to us, which were all with nor-

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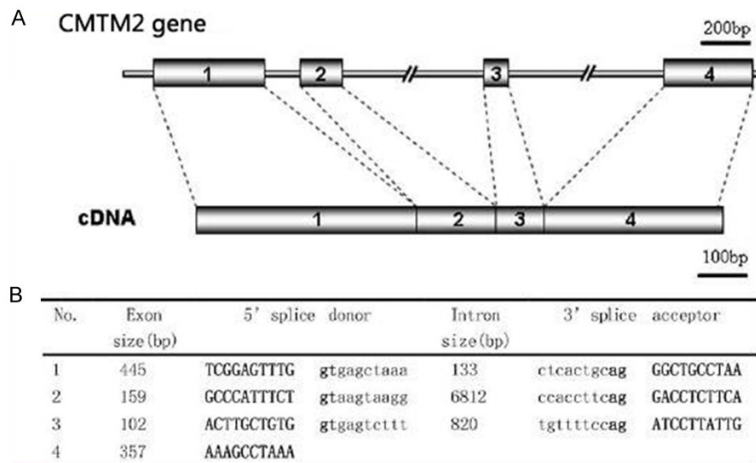


Figure 1. Schematic map of CMTM2.

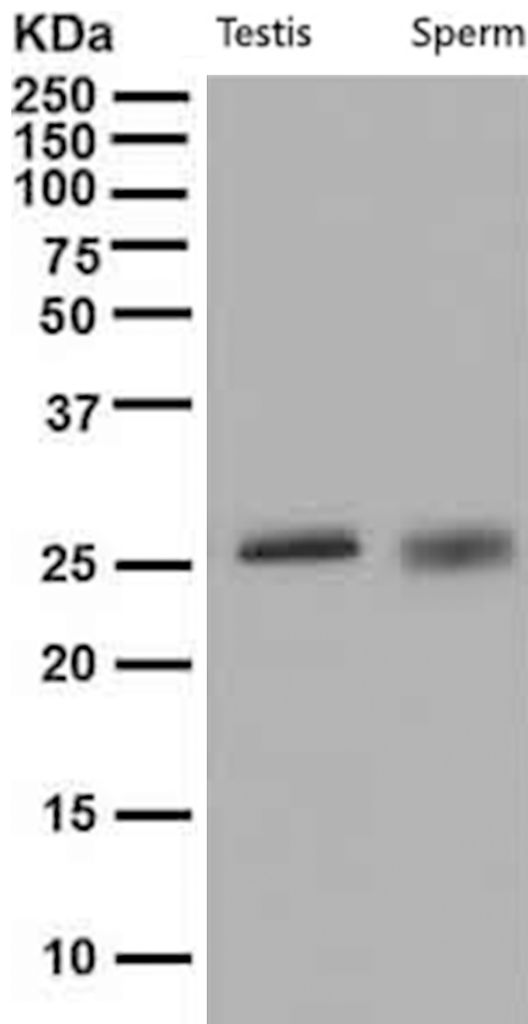


Figure 2. Western blot analysis of CMTM2 in human testis and sperm.

mal semen parameters according to criteria recommended by WHO (5th, 2010). Our study was approved by the ethics committee of Peking University People's Hospital prior to sample collection. And informed consent was signed by all the participants in our study.

Fresh semen was processed in water at 37°C until complete liquefaction of the semen. Then 1 ml liqueficient semen was overlay onto 1 ml PerColl with 60% density which had been preheated in incubator. And sperm cells

were purified from cryoprotectant and somatic cell contamination by centrifugation at ambient temperature for 10 min at 350 g. After the careful removal of the Percoll solution, sperm pellets were washed in HTF for twice.

Western blot

Western blot assays were used to detect the expression of CMTM2 in human testis and sperm. From 15 to 30 ug of total human testis/sperm protein, cytoplasmic protein, or nuclear protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was washed with phosphate buffer saline (PBS). Non-specific sites on the membrane were blocked by incubating the membrane in solution containing 5% non-fat dry milk in PBS for 60 min at room temperature. The membrane was then washed and incubated overnight at 4°C with 200× diluted anti-CMTM2 polyclonal antibody in PBS. Then the membrane was washed again and incubated with 2000× diluted HRP conjugated mouse anti-rabbit IgG antibody in PBS for 1 h at 37°C. After the final washing, the membrane was reacted with enhanced chemiluminescence (ECL) reagent to allow the detection of proteins with a Luminescent Image Analyzer (Fluor Chem 5500, Alpha Innotech, USA).

Immunohistochemistry

Immunohistochemistry experiments were carried out to explore not only expression, but also

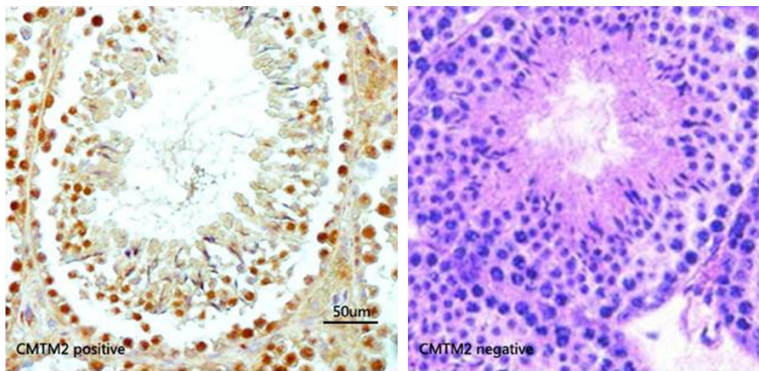


Figure 3. Immunohistochemistry of human testis showing CMTM2 protein expressed in the membrane of different stages of spermatogenic cells (two-step IHC).

distribution of CMTM2 in human testis. After dewaxing and hydrating in descending ethanols, 5-µm paraffin-embedded sections of human testis fixed in Bouin's solution were washed in PBS and were treated with 0.3% H₂O₂ in methanol to inhibit intrinsic peroxidase activity and with 5% normal goat serum for 2 h to prevent nonspecific antibody binding. Subsequently, the sections were then incubated overnight at 4°C with 100× diluted anti-CMTM2 polyclonal antibody in PBS, washed three times in PBS, and again incubated with 1000× diluted HRP conjugated mouse anti-rabbit IgG antibody in PBS for 1 h at room temperature. Sections were washed twice in PBS and the bound antibody was detected using DAB; control sections were stained with pre-immune rabbit serum. Then the prepared slides were coded and examined with an optical microscope (ZEISS Axioskop 2 plus, Germany). And the positive reaction was identified by intensely dark brown staining in cells.

Immunofluorescence

Immunofluorescence analysis was performed to determine the localization of CMTM2 in human testis and sperm. The human testicular tissues were embedded in optimal cutting temperature compound (OCT), then 5-µm sections were made using frozen section machine and placed on glass slides. The slides were first fixed with 4% paraformaldehyde for 30 min at room temperature and incubated with blocking solution for 2 h. The slides were then incubated with 100× diluted anti-CMTM2 polyclonal antibody in PBS at 4°C overnight. Following three times rinse with PBS, The slides were incubated with 100× diluted FITC-conjugated mouse

anti-rabbit antibodies in PBS for 1 h at 37°C. After washing with PBS, the 10 µg/ml Hoechst 33258 staining was performed on slides. Subsequently, the slides were mounted by phosphate buffer solution containing 50% glycerol and fluorescent photomicrographs were immediately taken with microscope.

The purified sperm suspension was placed in 5% CO₂ incubator at 37°C for 5 h to achieve sperm capacitation.

Calcium ionophore (A23187) was added into capacitated sperm suspension with the ultimate concentration at 10 mmol/L. The sperm suspension was then incubated in the same condition for another 30 min to induce acrosome reaction. 20 µL sperm suspension was taken out separately before and after acrosome reaction and placed on glass slides. After natural drying in the air, the slides were fixed with 4% paraformaldehyde for 30 min at room temperature and incubated with blocking solution for 2 h. The slides were then incubated with 100× diluted anti-CMTM2 polyclonal antibody in PBS at 4°C overnight. Following three times rinse with PBS, The slides were incubated with 100× diluted TRITC-conjugated mouse anti-rabbit antibodies in PBS for 1 h at 37°C. The slides were washed with PBS for three times again, then 25 µg/ml FITC-Hoechst staining on slides for 30 min was performed under dark and at room temperature, and the slides were mounted by phosphate buffer solution containing 50% glycerol and examined on microscope. In our evaluation, sperms with the acrosome portion without positive fluorescence signal and only equatorial plate with positive fluorescence signal were regarded as acrosome-reacted, while cells showing FITC-Hoechst positive staining in the anterior part of the head throughout the acrosomal region or almost uniform staining of the whole sperm head were considered as intact, not acrosome-reacted sperm [5].

Results

The expression of CMTM2 in human testis and sperm

As shown in **Figure 2**, both tissue samples (testis and sperm) revealed clear band at 25 kD by

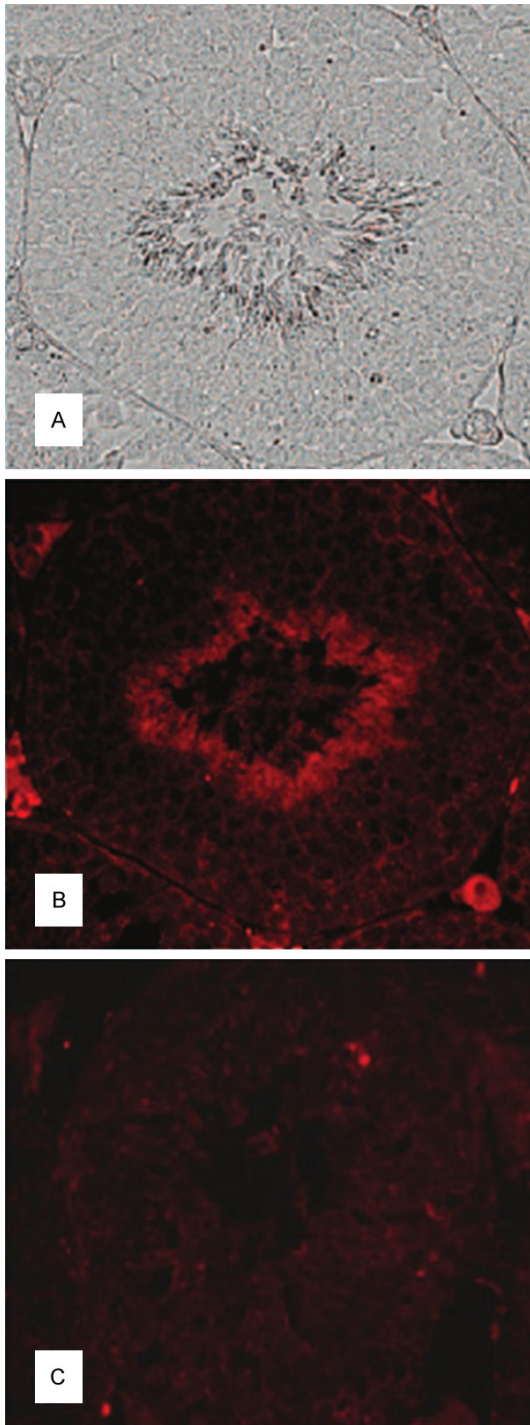


Figure 4. Immunofluorescent localization of CMTM2 in human testis.

western blot, which was comparable to the actual molecular weight of CMTM2. Thus the expression of CMTM2 in human testis and sperm was confirmed. Using immunohistochemistry we further examined the expression of CMTM2 protein in human biopsy testicular

tissues. And we found CMTM2 was highly expressed in human testis.

The localization of CMTM2 in human testis

Moreover, we had also found that CMTM2 was widely expressed in seminiferous tubules of human testis, mainly in cell membranes of spermatogenic cells (**Figure 3**), which is consistent with previous reports [3, 4]. And the immunofluorescence performed on frozen human testis slides showed similar findings with immunohistochemistry (**Figure 4**), which gave weight to the localization of CMTM2 in cell membranes of spermatogenic cells at different stages.

The localization of CMTM2 in human sperm before and after acrosome reaction

TRITC-CMTM2 and FITC-Hoechst double immunofluorescence staining was performed to examine the subcellular localization of CMTM2 in human sperm before and after acrosome reaction. And we found that CMTM2 was localized at the posterior head of sperm before and after acrosome reaction. And the localization and expression of CMTM2 was not affected by sperm acrosome reaction, which was shown in **Figure 5**.

Discussion

Human CMTM2 is a novel protein, and the understanding about transmembrane protein CMTM2 is limited now. Based on previous report, we found that CMTM2 abnormal expression could induce spermatogenesis defect [4]. And with the aggravation of the spermatogenesis defect, the CMTM2-positive cell numbers and mRNA level decreased significantly with no expression in the testes of patients with Sertoli Cell Only Syndrome (SCOS), which is characterized histologically by a complete loss of the germinal epithelium in testicular tubules [4, 6-8]. All the above phenomena provided compelling evidence for a pivotal role of CMTM2 in the process of spermatogenesis. But it remains to be fully elucidated about the definite mechanism.

To the best of our knowledge, it was the first study to systematically examine the expression and localization of CMTM2 in male reproductive system. In the present study, Western blot, immunohistochemistry and immunofluorescence were all performed to explore the expres-

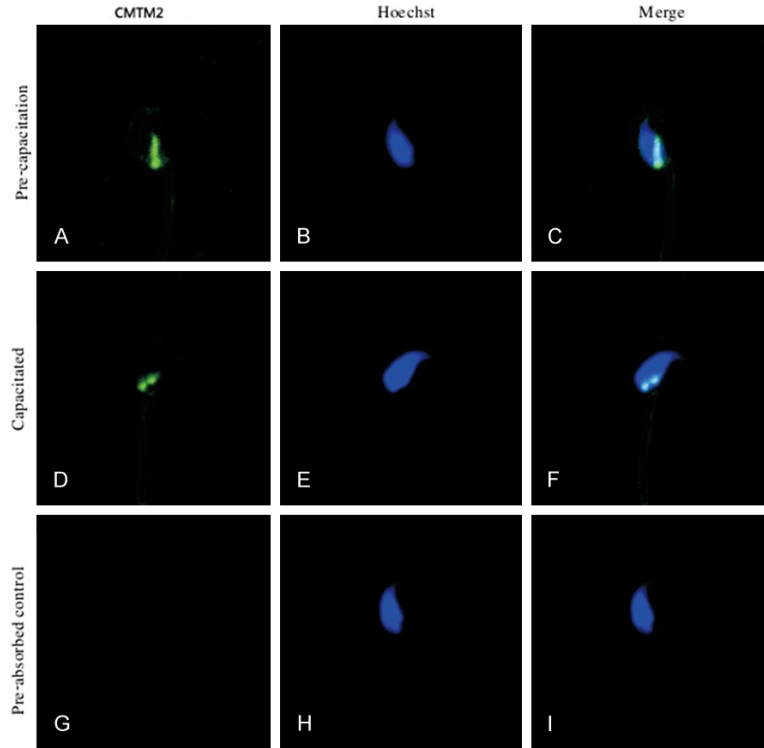


Figure 5. Immunofluorescent localization of CMTM2 in human sperm before and after acrosome reaction.

sion of CMTM2 on protein level. Both western blot analysis and immunohistochemistry had illustrated the high expression of CMTM2 in human testis and sperm. And further study clarified that CMTM2 was localized in cell membranes of spermatogenic cells at different stages, which is in accordance with previous reports [3, 4].

Additionally, CMTM2 protein contains MARVEL, a novel domain with a four transmembrane helix architecture that has been identified in proteins of the myelin and lymphocyte (MAL), physins, gyrins and occluding families [9]. Their function could be related to cholesterol-rich membrane apposition events in a variety of cellular processes, such as biogenesis of vesicular transport carriers [9]. Therefore, CMTM2 was thought to play a crucial role in regulating vesicular transport in cells. And it was also speculated by scholars that CMTM2 may be involved in membrane trafficking, such as intracellular transport, secretion and bullule transport [10]. According to the expression and localization of CMTM2 in spermatogenic cells, we could also conclude that CMTM2 may have a potential role in interaction among spermato-

genic cells such as signal transmission, nutrition support which was provided by Sertoli cells to spermatogenic cells [11]. And through these impacts, CMTM2 could play a crucial role in the process of spermatogenesis.

We had also examined the subcellular localization of CMTM2 in human sperm in this study. According to our findings, CMTM2 was still localized in the posterior head of sperm when sperm-egg fusion occurred after acrosome reaction. And the location was also where sperm-egg fusion occurred [12, 13]. It has been widely recognized that the fertilization in humans was induced by a series of interaction between egg and sperm, including sperm-egg recognition, sperm-egg combination and sperm-egg fusion.

And as the most crucial process in fertilization, sperm-egg fusion was a highly programmed process [14]. It is worthy to note that completing acrosome reaction is an essential prerequisite of sperm-egg fusion.

A series of proteins have been identified to be related with sperm-egg fusion in available literature. In our study, CMTM2 was found to have a similar subcellular localization with NYD-SP8 in sperm [15]. Therefore, we could speculate that CMTM2 was also a fertilization related protein, which may play a role in sperm-egg fusion.

In conclusion, we have examined the expression and localization of CMTM2 in human testis and sperm in this study. The expression of CMTM2 in the male reproductive system of the adult human exhibits cell- and region-specific patterns, which suggests that they may play an important role in spermatogenesis and sperm-egg fusion. However, it still remains to be further elucidated about the definite role of CMTM2 in male reproductive system and the process of spermatogenesis. And *in vitro* fertilization experiments are needed to confirm the role of CMTM2 in fertilization in future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qing Li, Department of Urology, Peking University People's Hospital, 11 Xizhimen South Avenue, Xicheng District, Beijing 100044, China. Tel: +0086-10-88326390; Fax: +0086-10-88326355; E-mail: liqing_1969@sina.com

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