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Characterization and Expression Profile of CMTM3/CKLFSF3

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CMTM/CKLFSF is a novel family of proteins linking chemokines and TM4SF. In humans, these proteins are encoded by nine genes, *CKLF* and *CMTM1-8/CKLFSF1-8*. Here we report the characteristics and expression profile of *CMTM3/CKLFSF3*. Human CMTM3/CKLFSF3 has a high sequence identity among various species and similar characteristics as its mouse and rat homologues. Established by results both of RT-PCR and Quantitative Real-time PCR, the gene is highly transcribed in testis, leukocytes and spleen. For further verification, we generated a polyclonal antibody against human CMTM3/CKLFSF3 and found that the protein is highly expressed in the testis and some cells of PBMCs. Therefore, *CMTM3/CKLFSF3* is an evolutionarily conserved gene that may have important roles in the male reproductive system and immune system. Further studies are necessary to validate its functions in the two systems.

Keywords: CKLFSF, CMTM3, Marvel, PBMC, Testis

Introduction

CKLFSF is a novel family of proteins linking chemokines and Transmembrane 4 Super Family (TM4SF), which are encoded by nine genes, *CKLF* and *CKLFSF1-8*, in humans. *CKLF*, *CKLFSF1*, and *CKLFSF2* are active during evolution, and *CKLFSF2* has two orthologues in rat and mouse designated *Cklfsf2a* and *Cklfsf2b*. *CKLFSF3-8* are evolutionarily conserved. *CKLFSF* members form two gene clusters: *CKLF* and *CKLFSF1-4* at chromosome 16q22.1, and *CKLFSF6-8* at chromosome 3p23. Most CKLFSFs have different spliced forms, and the protein product of at least one spliced form of each member has a MAL and related proteins for vesicle trafficking and membrane link (MARVEL) domain (Han *et al*., 2003). For this reason, *CKLFSF1-8* were renamed *CMTM1-8*, or CKLF-like MARVEL transmembrane domain containing 1-8. In this superfamily, the protein products of *CKLF*, *CMTM1/CKLFSF1*, and *CMTM2/CKLFSF2* are similar in structure and function to chemokines, while the protein products of the other members are more closely related to TM4SF.

CKLF is the first discovered member of this family, with four different spliced forms designated CKLF1, 2, 3, and 4. CKLF1 has chemotactic activity towards leukocytes, and overexpression of human CKLF1 in mouse can induce asthma-like changes (Han *et al*., 2001). When T cells were stimulated with Phytohemagglutinine (PHA), the expression of CKLF1 was up-regulated (Li *et al*., accepted in 2006). Like human CKLF1, rat Cklf1 also has chemotactic effects towards leukocytes and was up-regulated in the rat arthritis model (Lou *et al*., 2003). These observations suggest that CKLF1 plays significant roles in immune responses and may be involved in autoimmune diseases. Our previous study showed that CMTM3/CKLFSF3, CMTM6/CKLFSF6, and CMTM7/CKLFSF7 are highly expressed in peripheral blood mononuclear cells (PBMCs), suggesting that they have functions in the immune system (Han *et al*., 2003).

In addition, previous studies also indicated that several members in this superfamily may play a role in the male

Abbreviations: CKLFSF, Chemokine-like factor super family; CKLF, Chemokine-like factor; HGNC, HUGO Gene Nomenclature Center; CMTM3, CKLF-like MARVEL transmembrane domain containing 3; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ORF, open reading frame; PCR, Polymerase Chain Reaction; RT-PCR, reverse transcription PCR; TM4SF, Transmembrane 4 Super Family; EST, expressed sequence tag; UTR, untranslated region; MARVEL, MAL and related proteins for vesicle trafficking and membrane link; AR, androgen receptor; ARR19, Androgen Receptor Corepressor-19 kDa; NCBI: National Center for Biotechnology Information; PBMC: peripheral blood mononuclear cells; SH3: Src Homology 3; BASH: B-cell specific adaptor protein; ERK: extracellular signal-regulated kinase; BNAS2: BASH N terminus associated protein 2.

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reproductive system. For example, rat and mouse Cklfs are predominantly expressed in testis (Lou *et al*., 2003; Rui *et al*., 2003). Human CMTM1/CKLFSF1, with at least 23 isoforms, is predominantly expressed in human testis and is most abundant in spermatocytes (primary and secondary), as well as in the tissue fluid surrounding spermatogonia and spermatocytes (Wang *et al*., 2004). Human CMTM2/CKLFSF2 was also found to be highly expressed in testis (Shi *et al*., 2005). In addition, its mouse homolog Cmtm2a/Cklfsf2a interacts directly with androgen receptor (AR) in vivo and acts as a corepressor of AR-mediated activity (Jeong *et al*., 2004), while human CMTM2/CKLFSF2 and Cmtm2b/Cklfsf2b were also reported to be coactivators of AR (Li *et al*., 2006). The function of CMTM8/CKLFSF8 in EGFR endocytosis and degradation suggests the multiple potential functions of this superfamily (Jin *et al*., 2005).

We previously reported the molecule cloning of CMTM3/CKLFSF3. Here, we report its evolutionary conservation based on sequence identity, structural characterization, and genomic structure. We also found that CMTM3/CKLFSF3 is highly expressed at the mRNA and protein levels in the male reproductive system and the immune system. Together with previous studies of the other members of this superfamily, we propose that human CMTM3/CKLFSF3 may play important roles in the male reproductive and immune systems. Further study is required to clarify the exact role of CMTM3/CKLFSF3 both in vitro and in vivo.

Materials and Methods

Bioinformatics. Sequence identity and open reading frame (ORF) prediction were performed using software available from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The exon/intron structure of *CMTM3/CKLFSF3* was identified by comparing the cDNA to the genomic sequence using BLAT Search Genome (http://genome.ucsc.edu/cgi-bin/hgBlat). The exact exon/intron boundaries were determined using SIM4 software (Florea, Hartzell, Zhang, Rubin, & Miller, 1998). The Gene Atlas database (http://symatlas.gnf.org) and the GeneCard database (http://bioinfor.weizmann.ac.il/cards) were used to analyze the expression profile, while protein motifs were predicted using PFSCAN software (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Falquet L, et al, 2002). In addition, the transmembrane region of the putative protein was predicted with the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). Subcellular location and signal peptides were predicted by PSORT II (http://psort.nibb.ac.jp/form2.html) (Nakai, 2000). DNAstar software was used for multiple sequence alignments and protein characteristics.

RT-PCR

Non-quantitative RT-PCR. The expression pattern of human CMTM3/CKLFSF3 was detected using a human multiple tissue cDNA panel and a human blood fractions cDNA panel (BD Biosciences Clontech, San Jose, CA USA) with specific primers for CMTM3/CKLFSF3 and GAPDH (Table 1) according to the manufacturer's instructions. Two microliters of each cDNA template were used in a 25 μl PCR amplification system of LA Taq™ (Takara Bio Inc. Japan). DNA was denatured at 94°C for 5 min followed by 34 cycles of 'Touchdown' PCR and a final extension for 7 min at 72°C (Don *et al*., 1991). In each cycle of the 'Touchdown' PCR, the denaturing temperature was at 94°C for 30 s, the extension temperature was at 72°C for 30 s, and the annealing temperature was decreased by 2°C for 30 s every second cycle from 57°C to a 'touchdown' at 45°C, at which temperature 24 cycles were carried out. Five microliters of the PCR product were analyzed on a 1.0% agarose gel.

Table 1. Primer pairs for both CMTM3/CKLFSF3 and GAPDH

	Upstream primer	Downstream primer
CKLFSF3	5'-ccaccatgtggcccccagacc -3'	5'-aggccttcagtcagagtcc -3'
Cklfsf3	5'-accgcggccctcatctactt -3'	5'-tcttgtgggctgtggtctca-3'
GAPDH	5'-tgaaggtcggagtcaacggatttggt-3'	5'-catgtgggccatgaggtccaccac-3'

Table 2. Peptides used for the preparation of the polyclonal anti-CMTM3/CKLFSF3 antibody. All the peptides were listed from the N-terminal to the C-terminal. *At the C-terminal of the sequence, the extra addition of C (Cysteine) was aimed to facilitate its linkage with KLH

	Location	Sequence	AA number
Peptide 1	N- terminal	MWPPDPDPDPDPEPAGGSRPGPAVC*	25
Peptide 2	Middle	MQLNDKWQGLC	11
Peptide 3	C- terminal	KFLKQGDSADETTAHKTEEENSDSDSDC*	28

Table 3. Primers for the amplification for the CMTM3/CKLFSF3 and β-actin for the Real-time PCR

	Upstream primer	Downstream primer
CMTM3	5'- accgcggccctcatctact -3'	5'- aggccttcagtcagagtcc -3'
β-actin	5'-cagcacaatgaagatcaagatca-3'	5'- cggactcgtcatactcctgc -3'

To detect mouse Cmtm3/Cklfsf3, total RNA for RT-PCR was extracted from Balb/c mouse tissues using Trizol (Life Technologies Inc., USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed according to standard protocols using the ThermoSCRIPT™ cDNA synthesis system (Invitrogen, USA). One microliter of the cDNA solution was used as the template to amplify mouse Cmtm3/Cklfsf3 using specific primer pairs (Table 2). The DNA was amplified by 'Touchdown' PCR and analyzed as described above.

The RT-PCR products of the two genes were cloned into the pGEM-T-easy vector (Promega, Madison, WI USA), and plasmids were verified by sequencing.

Real-time quantitative PCR. To further quantify the expression level of human CMTM3/CKLFSF3 in various tissues, the cDNA libraries of human ovary, testis, spleen, thymus and leukocyte from the previous multiple tissue cDNA panel(BD Biosciences Clontech, USA) were used with specific primers for CMTM3/CKLFSF3 and β-actin (Table 3). Quantitative Real-time PCR assay was carried out in an ABI Sequence Detection System (Applied Biosystems, USA). PCR reactions were performed according to the user manual.

Preliminary reactions were run to optimize the concentration and ratio of each primer set. All the cDNA templates were diluted 100 times and eight microliters of each diluted cDNA template were used in a 20 μl Real-time PCR amplification system of SYBR Green PCR Master Mix Kit as the manufacturer directed. DNA was denatured at 95°C for 10 min followed by 35 cycles of 15 seconds at 95°C together with 1 min at 60°C. The expression level of CMTM3/CKLFSF3 in the ovary was treated as the baseline. The experiments were repeated twice with consistent results.

Antibody preparation and characterization. Three peptides from human CMTM3/CKLFSF3 were synthesized and polyclonal antibodies were generated by immunizing rabbits four times with all three peptides together at 100 μg per peptide. Rabbit serum was collected before immunization as a negative control. An ELISA assay showed that serum titers against CMTM3/CKLFSF3 were as high as $1:10^6$ and could specifically react with the mixed polypeptides, which was further confirmed by Western Blot analysis.

The plasmid for mammalian expression of CMTM3/CKLFSF3 was constructed by cloning the gene into BglII (Takara Bio Inc. Japan) sites that had been introduced into the adenovirus-mediated expression vector pDC315 (Vector Gene Technology Company Ltd., China) to produce Ad-CMTM3/CKLFSF3. PC-3 cells were grown in RPMI 1640 medium (Life Technologies Inc., Rockville, MD USA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT USA). Cells were replated 24 hrs prior to the experiment and fed with fresh culture medium. After 20-22 hours, cells were infected with either empty vector Ad-Null or with Ad-CMTM3/CKLFSF3 at MOI = 120 (Davies *et al*., 2002). Cells were collected 48 hours after infection and whole cell lysates were prepared was separated on a 12.5% SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose membranes and incubated with antiserum against CMTM3/CKLFSF3 at a titer of 1 : 1000 followed by IRDye™ 800 anti-rabbit secondary antibody. Signals were visualized with the Odyssey® Imaging System (LI-COR Biosience Inc.)

Immunohistochemistry and immunocytochemistry. Human testis tissue was fixed in formalin and embedded in paraffin. Tissue sections were rehydrated through graded ethanol washes, placed in 10 mM citrate buffer (pH 6.0), and then heated twice in a microwave oven for 5 min each. The slides were then incubated with 0.3% hydrogen peroxide for 5 min, washed with PBS, and blocked in PBS plus 10% normal goat serum for 10 min (Shi *et al*., 1992). After removing excess blocking buffer, rabbit polyclonal anti-human CMTM3/CKLFSF3 (1:500) was added and the slides were incubated for 45 min at 37°C in a humidified chamber. After thorough washing, the appropriate IRDye™ 800-conjugated secondary antibodies were added (LI-COR BIOSCIENCE INC., USA) and staining was visualized using the Odyssey® Imaging System (LI-COR Biosience Inc., USA). Human PBMCs were isolated from healthy donors with Percoll gradients (Amersham Pharmacia, UK), and cells were centrifuged at 2,000 × g for 5 min and fixed with acetone. Immunocytochemistry was performed similarly.

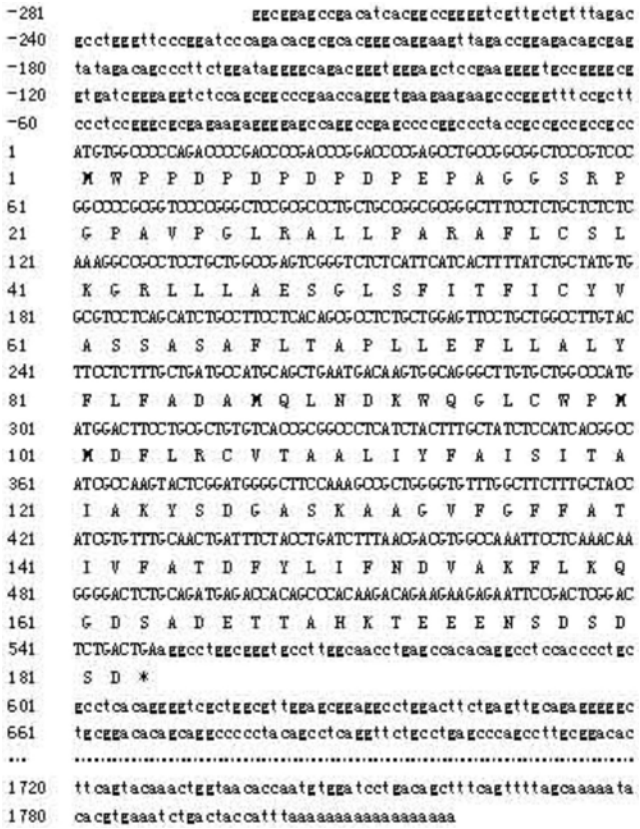


Fig. 1. The nucleotide sequence of the message strand is numbered from 5' to 3'. Coding bases are shown in capital letters; bases in the UTR are shown in small letters. Numbering is based on +1 being the first base of the ORF, and an asterisk indicates the stop codon. These sequences have been deposited in GenBank under accession number AF479813.

Results

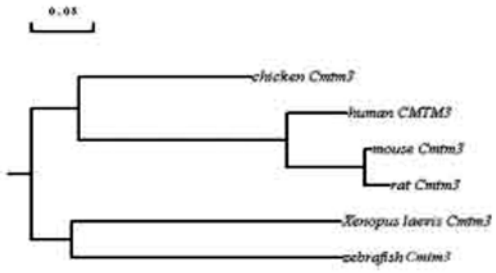
The cDNA and deduced amino acid sequences of human CMTM3/CKLFSF3. In our previous study, we reported the cloning of human CMTM3/CKLFSF3 gene (Han *et al*., 2003). In this paper, we report the cDNA and deduced amino acid sequences of CMTM3/CKLFSF3. The first methionine codon in the longest open-reading frame is preceded by an in-frame stop codon 198 bp upstream and flanked by a Kozak non-consensus site (GCCATGT) (Kozak, 1991). The cDNA sequence encodes a complete ORF of 182 amino acids and has a poly (A) tract at 1804 bp. No typical polyadenylation signal (AATAAA or ATTAAA) (Swimmer *et al*., 1985) was found in the 3' untranslated region (UTR) (Fig. 1).

CMTM3/CKLFSF3 is conserved in vertebrate animals. With human CMTM3/CKLFSF3 as the query sequence, we performed BLASTP searches against the public database of genomes on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). CMTM3/CKLFSF3 homologs were conserved in

(A)

	human CMTM3	mouse Cmtm3	rat Cmtm3	zebrafish Cmtm3	chicken Cmtm3	Xenopus laevis Cmtm3
human CMTM3	100%					
mouse Cmtm3	89%	100%				
rat Cmtm3	86%	97%	100%			
zebrafish Cmtm3	49%	47%	47%	100%		
chicken Cmtm3	66%	62%	60%	57%	100%	
Xenopus laevis Cmtm3	47%	48%	47%	57%	58%	100%

(B)



(C)

Fig. 2. Sequence alignment of human CMTM3/CKLFSF3 and its homologs (A) Amino acid sequence alignment of human CMTM3/CKLFSF3 with mouse, rat, chicken, zebrafish and Xenopus laevis homologs. Sequence alignment was obtained using the DNAstar software. The boxed areas indicate matching residues. (B) Sequence comparison of human CMTM3/CKLFSF3 with the above five homologs. The numbers represent the percentage of sequence identity determined by the DNAstar software. (C) Phylogenetic analysis of human CMTM3/CKLFSF3 and its five homologs. The inferred phylogenetic tree was generated based on the degree of amino acid sequence identity shown in B.

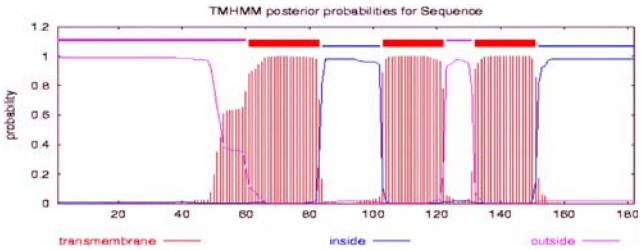


Fig. 3. Putative transmembrane regions analysis of human CMTM3/CKLFSF3.

most vertebrate animals, including zebrafish, Xenopus laevis, chicken,mouse and rat homologs (under accession number AAH77159, AAH87295, XP_424225, AAP33492 and XP_226200, respectively). As shown in Fig. 2, mouse and rat homologs shared the highest similarity with human CMTM3/CKLFSF3 (86-89%), while zebrafish, *Xenopus laevis*, and chicken homologs shared a lower similarity (47-66%) with the human gene. Compared with CKLF, CMTM1/CKLFSF1 and CMTM2/CKLFSF2 in the CMTM superfamily, CMTM3/CKLFSF3 showed higher conservation during evolution. However, no homologs were found in the invertebrate animals, therefore CMTM3 is only conserved in vertebrate animals.

All members of this superfamily contain four putative transmembrane regions except CMTM3/CKLFSF3, which contains only three putative transmembrane regions. Bioinformatics analysis indicated that human CMTM3/CKLFSF3 was a highly hydrophobic protein, with the N-terminus and one loop outside the cell membrane and the C-terminus and the other loop inside the cell membrane (Fig. 3) (Krogh *et al*., 2001). The protein also contains a MARVEL domain (Sanchez-Pulido *et al*., 2002), similar to the other members of the CMTM/CKLFSF superfamily.

	Transmembrane regions			MARVEL domain	Signal peptide	PI	Amino acids
	1st	2nd	3rd				
hCMTM3	61-83	103-122	132-151	36-155	No	4.15	182
mCmtm3	61-83	103-122	132-151	36-155	No	4.46	184
rCmtm3	61-83	103-122	132-151	36-155	No	4.59	184

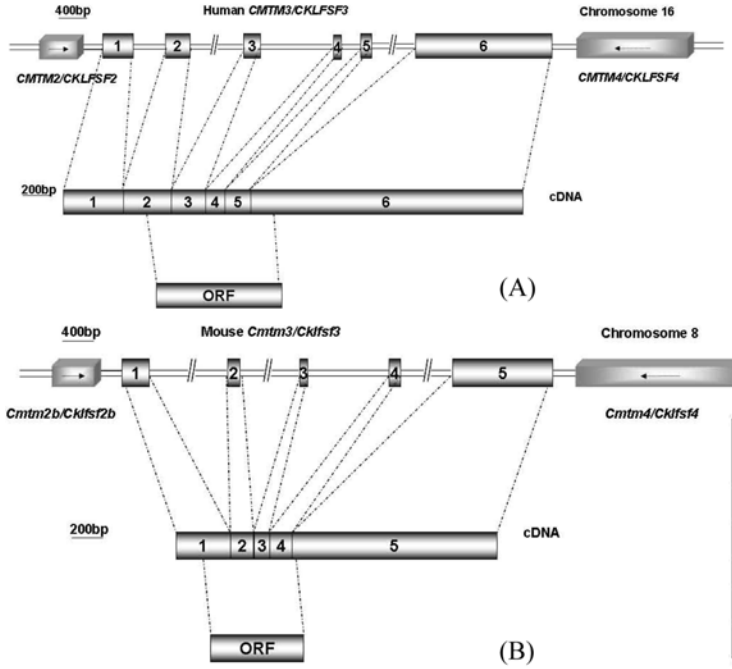
Fig. 4. The comparison of the characterizations on the protein levels among three species.

To further investigate the conservation of human CMTM3/CKLFSF3, we put the mouse and rat homologs through the same analyses and found that both homologs have three putative transmembrane regions, a MARVEL domain, and no signal peptides, similar to human CMTM3/CKLFSF3. Notably, their transmembrane compositions and MARVEL domain are almost identical to human CMTM3/CKLFSF3, including the initial and final amino acid numbers in each transmembrane region. Their other characteristics are also quite similar (Fig. 4). Together, these data show that CMTM3/CKLFSF3 is highly conserved.

Chromosome localization and genomic organization of human CMTM3/CKLFSF3 and mouse Cmtm3/Cklfsf3. Human *CMTM3/CKLFSF3* was localized to chromosome 16q22.1, forming a gene cluster with *CMTM4/CKLFSF4* downstream and with *CMTM2/CKLFSF2* upstream. Mouse Cmtm3/Cklfsf3 was located on chromosome 8, also forming a gene cluster with the above-mentioned members of this superfamily, with *Cmtm2b/Ckflsf2b* found in the same location as *CMTM2/CKLFSF2* in human.

Comparison the cDNA sequence of *CMTM3/CKLFSF3* with the human genome using BLAT Search Genome software revealed that human *CMTM3/CKLFSF3* occupied about 9.9 kb of chromosome 16 and contained 6 exons and 5 introns (Fig. 5A). Each of the 5'-donor and 3'-acceptor splice sites conformed to the expected consensus sequence of eukaryotic splice junctions, with invariable GT/AG dinucleotides present at each of the immediate exon/intron boundaries (Fig. 5C) (Mount, 1982). Mouse *Cmtm3/Cklfsf3* was mapped to mouse Chromosome 8 and contained 5 exons and 4 introns(Fig. 5B). Its exon/intron boundaries also complied with the "GT-AG rule" (Fig. 5D). Interestingly, the third, fourth and fifth exons of human *CMTM3/CKLFSF3* are the same with the second, third, and fourth exons, respectively, of its mouse homolog.

Human CMTM3/CKLFSF3 and mouse Cmtm3/Cklfsf3 are highly expressed in the immune and male reproduction systems at the mRNA level. Analysis with Gene Atlas and GeneCards suggested that CMTM3/CKLFSF3 was highly expressed in the male reproductive system and the immune system. Northern blotting from our previous study is consistent with this result (Han *et al*., 2003). We further studied the



No.	Exon Size (bp)	5' splicer donor	Intron Size (bp)	3' splicer acceptor
1	246	GAAGAAGAAG gtggagtctg	176	ttccgcacag CCCGGGTTTC
2	222	GGCCGAGTCG gtgagtgcgg	3357	ttccccgcag GGTCTCTCAT
3	156	GCCCATGATG gtgaggacag	971	ctctccccag GACTTCCTGC
4	96	AGCCGCTGGG gtgagcagcc	353	ctgtccacag GTGTTTGGCT
5	121	AAGACAGAAG gtaagcggct	2602	ttcctttcag AAGAGAATTC
6	1284	TACCATTTAA		

(C)

No.	Exon Size (bp)	5' splicer donor	Intron Size (bp)	3' splicer acceptor
1	296	GGCCGAGTCG gtgagtgcgt	2862	tcttccgcag GGTCTTTCAT
2	156	GCCCATGATG gtaaggatcg	863	ctctcctcag GACTTCCTGC
3	96	AGCAGCTGGA gtgagtggct	399	tatcccacag GTTTTTGGCT
4	121	AGGACAGAAG gtgagtggcc	1339	ttcctttcag AAGAGAACTC
5	941	ACTGACTACA		

(D)

Fig. 5. Chromosome localizations and genomic structures of the human *CMTM3/CKLFSF3* and the mouse *Cmtm3/Cklfsf3*. (A) and (B) are the schematic maps of the human *CMTM3/CKLFSF3* and the mouse *Cmtm3/Cklfsf3*, respectively, with the genes nearby also outlined. (C) and (D) are the sizes of exons and introns, and splice junction sequences of the human *CMTM3/CKLFSF3* and the mouse *Cmtm3/Cklfsf3*. The characters in red are aimed to emphasis the invariable GT/AG dinucleotides presence at each of the immediate exon/intron boundaries.

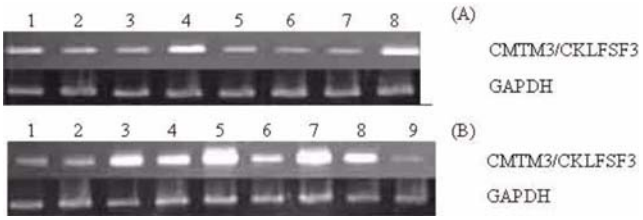


Fig. 6. Expression profiles of human CMTM3/CKLFSF3. (A) its expression profile in multiple tissues. 1. spleen; 2. thymus; 3. prostate; 4. testis; 5. ovary; 6. small intestine; 7. colon; 8. leukocyte. (B) its expression profile in blood cell fractions. 1. rested PBMC; 2. rested $CD8^+$; 3. rested $CD4^+$; 4. rested $CD14^+$; 5. rested $CD19^+$; 6. activated $CD19^+$; 7. activated PBMC 8. activated $CD4^+$; 9. activated $CD8^+$.

Fig. 7. Expression level of mouse *CMTM3/CKLFSF3* in different mouse tissues. 1. heart; 2. liver; 3. spleen; 4. lung; 5. kidney; 6. brain; 7. testis; 8. thymus; 9. prostate.

distribution of human CMTM3/CKLFSF3 mRNA across multiple tissues by RT-PCR (Fig. 6A). Consistent with the bioinformatics and Northern blot results, CMTM3/CKLFSF3 was present in most tissues, with higher levels in the testis, leukocyte and spleen. Considering the high heterogeneity of the leukocyte, we further detected the expression levels of CMTM3/CKLFSF3 in each blood cell fraction, with the highest expression in resting $CD19^+$ cells and decreasing sharply upon activation (Fig. 6B). Given the high similarity between mouse cmtm3/cklfsf3 and human CMTM3/CKLFSF3, we also detected mouse cmtm3/cklfsf3 in nine different tissues, with the highest expression in testis and spleen (Fig. 7).

The above results elucidated that human CMTM3/CKLFSF3 and its mouse homolog were both highly expressed in the testis and spleen In order to further ascertain expression levels of human CMTM3/CKLFSF3 in those tissues, we selected the cDNA libraries of five tissues including thymus, spleen, testis, ovary and leukocyte and performed Real-time PCR. The result validated that compared with that in ovary,

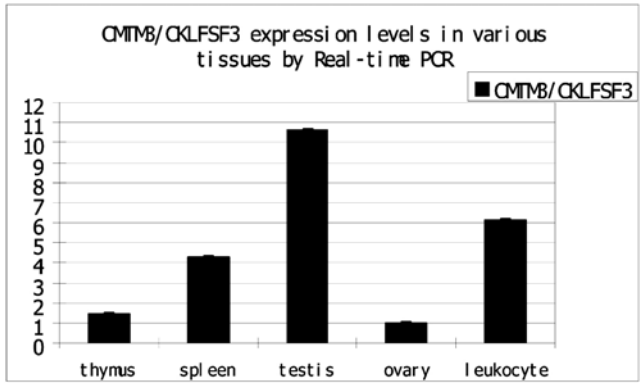


Fig. 8. The expression levels of human CMTM3/CKLFSF3 detected by Real-time PCR in several tissues. The expression level in the ovary was treated as 1.

CMTM3/CKLFSF3 was predominantly expressed in testis and also highly expressed in leukocyte and spleen. The expression level in thymus was also higher than that in ovary (Fig. 8).

Preparation and identification the polyclonal antibody against human CMTM3/CKLFSF3. To further verify the high expression of human CMTM3/CKLFSF3 in PBMCs and testis tissue, we generated polyclonal antibodies. Based on the bioinformatics analysis of human CMTM3/CKLFSF3 (Fig. 9), we selected three segments as shown in Table 2 and had these polypeptides synthesized.

PC-3 cells overexpressing CMTM3/CKLFSF3 protein via an adenovirus-mediated expression vector were used to test antibody specificity (Fig. 10). Compared with preimmune serum, the CMTM3/CKLFSF3 polyclonal antibody was able to recognize CMTM3/CKLFSF3 overexpressed in PC-3 cells. The antibody detected no CMTM3/CKLFSF3 expression in cells transfected with Ad-null or in wild type PC-3 cells, which might have resulted from the low level of CMTM3/CKLFSF3 in this cell line.

CMTM3/CKLFSF3 is highly expressed in testis and some cells of PBMCs at the protein level. Our results for human CMTM3/CKLFSF3 expression at the mRNA level suggested

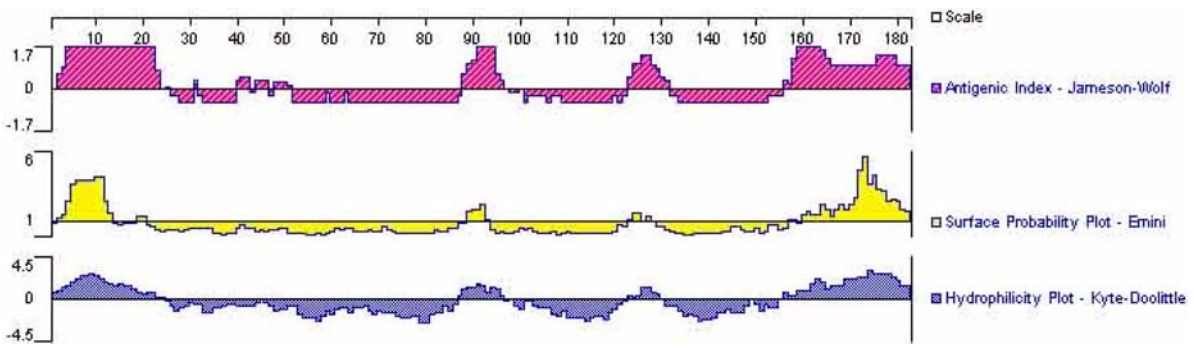


Fig. 9. Bioinformatics analysis of human CMTM3/CKLFSF3 on its antigenic index, surface probability and hydrophilicity.

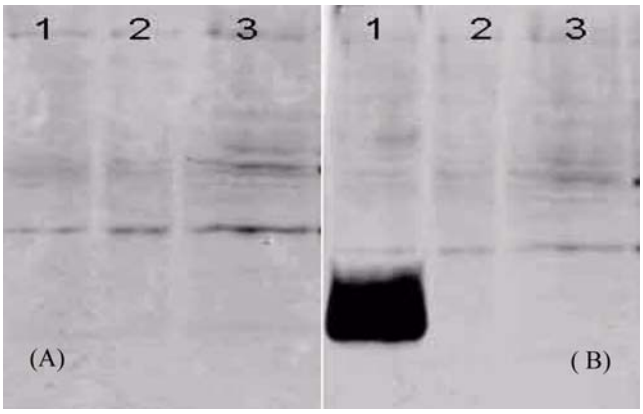


Fig. 10. Human CMTM3/CKLFSF3 polyclonal antibody verification. (A) was the serum before immunization while (B) was the serum after immunization. 1. Ad-CMTM3/CKLFSF3; 2. Ad-null; 3. PC-3 wild type.

that it was predominantly highly expressed in human testis and leukocyte. To examine CMTM3/CKLFSF3 expression at the protein level, we performed immunohistochemistry and immunocytochemistry using our polyclonal antibody. The strong positive signals in testis tissue confirmed the high expression of human CMTM3/CKLFSF3 in the testis (Fig. 11A). Positive signals were also detected in human PBMCs, although the signal staining varied in intensity, implying different expression levels (Fig. 11B). This might be due to the heterogeneity of PBMCs, which was consistent with our RT-PCR result (Fig. 6B). Further confirmation needs to be done by isolating the corresponding blood fraction cells.

Discussion

Here we reported the characteristics and expression profile of *CMTM3/CKLFSF3*, a member of the novel gene family CMTM/CKLFSF. Human CMTM3/CKLFSF3 has a high sequence identity and similar expression profile as mouse Cmtm3/Cklfsf3. The gene is conserved during evolution, suggesting it may possess important functions.

Human CMTM3/CKLFSF3 was predominantly expressed in leukocytes and spleen in the multiple tissue cDNA library and highly expressed in resting B lymphocytes in the peripheral blood fraction cDNA library, while mouse Cmtm3/Cklfsf3 was highly expressed in spleen and testis. The spleen is rich in B lymphocytes and is one of the main sites of humoral immunity. Therefore, we propose that CMTM3/CKLFSF3 may play a significant role in the humoral immune response. Yasuhiro Imamura *et al*. reported that the chicken homolog of human CMTM3/CKLFSF3, BNAS2, could bind to the N-terminal of BASH as well as ERK and Btk, thus implying that BNAS2 may have an important role in ERK activation upon B-cell receptor (BCR) ligation in B cells

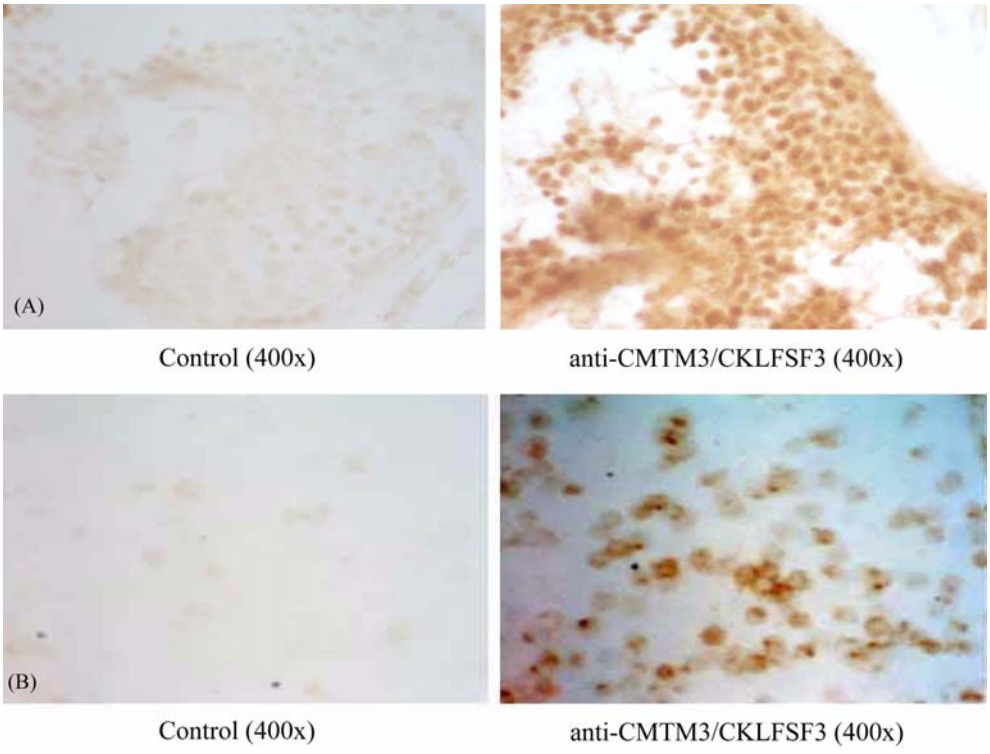


Fig. 11. CMTM3/CKLFSF3 has a high expression level in testis and some cells of PBMC. (A) testis tissue slides. Strong positive signals were detected in the cells. (B) PBMC slides. Mottled positive signals were detected.

(Yasuhiro *et al*., 2004.). Activation of the BCR-mediated ERK pathway was reported to correlate with cell-cycle entry, pre-B cell development, and even the activation of B cells (Tordai *et al*., 1994). Therefore, it may be useful to study the function of human CMTM3/CKLFSF3 during B cell development and humoral immune responses. Indeed, the high expression of CMTM3/CKLFSF3 in $CD4^+$ T lymphocytes and monocytes indicated its potential regulatory role in the immune system. Future work in this area could be of much significance.

Previous data from Northern Blot verified the high expression of human CMTM3/CKLFSF3 in testis. Here the result of Real-time PCR elucidated that human CMTM3/CKLFSF3 was predominantly expressed in human testis tissue, about ten times higher than that in ovary. In addition, mouse Cmtm3/Cklfsf3 was also highly expressed in testis. Therefore, this gene may play an important role in the male reproductive system in addition to its functions in the immune system. Published data established the high expression in testis of not only human CMTM1/CKLFSF1 and CMTM2/CKLFSF2, but also rat and mouse Cklfs and mouse Cmtm2a/Cklfsf2a and Cmtm2b/Cklfsf2b. In addition, human CMTM2/CKLFSF2 and mouse Cmtm2b/Cklfsf2b were AR coactivators, while mouse Cmtm2a/Cklfsf2a was an AR corepressor. ARR19, a novel AR corepressor reported by Jeong et al., was in fact the mouse Cmtm2a/Cklfsf2a. Motif analysis revealed that human CMTM2/CKLFSF2 possesses a leucine zipper, as well as "LXXLL" (where L represents leucine and X represents any amino acid) motifs. These are often present in the transcription factors and steroid receptor (including AR and ER) binding proteins and are responsible for translocation into the nucleus (Heery *et al*., 1997, Torchia *et al*., 1997). CMTM3/CKLFSF3 also has leucine zipper and "LXXLL" motifs. The structural characterization of CMTM3/CKLFSF3 combined with its high conservation during evolution and high expression in the male reproductive system strongly implies its possible importance in regulating AR activity.

In summary, we report that *CMTM3/CKLFSF3* is highly conserved and highly expressed in both the immune system and the male productive system. Based on the current data and previous published data, we strongly believe that human CMTM3/CKLFSF3 may play an important role in these two systems. Future investigation into CMTM3/CKLFSF3 in these systems would not only help elucidate its exact functions, but also help unravel the molecular mechanisms involved in male reproduction. Moreover, this study may also be of considerable benefit in deciphering the functions of other members in this family.

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