Preliminarily functional analysis of a cloned novel human gene *ADAM29*

WANG Fang (王 昉)*, XU Rener (徐人尔)*, ZHU Pengcheng (朱鹏程), HU Junjie (胡俊杰), YING Beibei (应蓓蓓), ZHAO Shouyuan (赵寿元) & LI Changben (李昌本)

School of Life Science, Institute of Genetics, State Key Laboratory of Genetic Engineering, Fudan University, Shanghai 200433, China
Correspondence should be addressed to Li Changben (email: cbli@fudan.ac.edu.cn)

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Abstract ADAM is a family of type I integral membrane proteins which are characterized by sharing a disintegrin and metalloprotease domain and involved in many important physiological processes such as fertilization, neurogenesis and inflammatory response. A novel human ADAM gene ——*ADAM29*, which was cloned in our laboratory, is exclusively expressed in human testis and contains a potential fusion domain. A full-length cDNA of *ADAM29* was obtained by using multiple-step PCR. Phylogenetic tree of known mammalian *ADAMs* specifically expressed in testis was reconstructed. Polyclonal antiserum was raised by immunizing the rabbits with sub-peptide of ADAM29 (Leu268—Asp374) as immunogen. The result of immunohistochemical test on human testis showed that *ADAM29* is expressed in different stages of spermatogenesis and in interstitial cells. ADAM29 may play a certain role in the signal transduction during the maturation of testis-associated cells.

Keywords: ADAM family, ADAM29, phylogenetic tree, immunohistochemistry, testis, interstitial cell.

ADAM (A Disintegrin And Metalloprotease) is a family of cell surface proteins with a multi-domain including the pro-domain, metalloprotease domain, disintegrin domain, cysteine-rich domain, epidermal growth factor (EGF)-like domain, transmembrane domain and cytoplasmic tail domain. Approximately 31 members of ADAM family have been identified in several animal species[12]. Although emerging data show that ADAMs are playing important roles in many physiological processes, such as fertilization, neurogenesis, myogenesis, TNF and TGF-α release and inflammatory response, for most of ADAM members, their precise functions remain to be determined. Recently, a novel human ADAM gene——*ADAM29* has been cloned in our laboratory and it was found that *ADAM29* is exclusively expressed in human testis[1]. Here, cloning of the full-length cDNA of *ADAM29* was reported. After computer prediction of its antigen distribution and homology analysis between the related ADAM molecules (data not shown), a peptide (Leu268—Asp374 of ADAM29) was selected to be produced in engineered *E.coli* as a fusion protein. The purified fusion protein was used as an antigen to immunize the rabbits. Polyclonal

* These authors contribute equally to this work.

antisera were prepared and used to detect the distribution of ADAM29 in human testis. The relationship between domain structure and function of testis-predominant ADAMs was discussed. The results suggested those ADAMs may play a special role in the process of spermatogenesis and fertilization.

1 Materials and methods

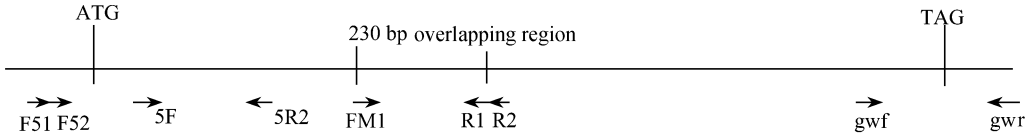
1.1 Cloning of full-length cDNA of *ADAM29*

1st PCR: A human testis cDNA library (Clontech) was used as template, and a 1.2 kb DNA fragment (product 1) was obtained by nested PCR with primers F51, R2 and F52,R1. In the same way, another DNA fragment, with a length of 1.6 kb (product 2) was amplified with primers FM1 and gwr. There is a 230 bp overlapping region between the two products.

2nd PCR: Products 1 and 2 in equal moles were mixed well, denatured at 95℃ for 5 min, annealed at 60 ℃ for 3 min, and cooled to 4℃. Then the resultant DNAs were used as templates for 2nd PCR with primers F52 and gwr.

3rd PCR: 2.5 kb DNA (product 3) recovered from 2nd PCR was used as the template and PCR was carried out with the primers the same as the 2nd PCR. The 2.5 kb DNA fragment (product 4) amplified from the 3rd PCR was ligated into vector pGEM-T (Promega), and sequenced.

All PCRs were hot-started with the LA Taq polymerase (TaKaRa). PCR primers used in this study are listed as follows:



Name	Sequence	Orientation	Position
F51	5′-ATCGTATAACCATCAGCAAGAA-3′	forward	104—125
F52	5′-CCATAACAGGGACTTCAAAATCACT-3′	forward	332—356
5F	5′-GAGGCCAGAAACACATTATC-3′	forward	554—573
FM1	5′-ATAGGAGCTTTTAGAGGAATG-3′	forward	1276—1296
gwf	5′-CACCCCCTAAGAGAAAGAAG-3′	forward	2375—2394
5R1	5′-TGAAAACCCCCAAAACAG-3′	backward	749—732
5R2	5′-ATGCTAGGGGCTTGATTT-3′	backward	799—782
R1	5′-TTCCCAAAATCACCCATAACTAC-3′	backward	1506—1484
R2	5′-ATTCCCACAGCGCTTCACATTA-3′	backward	1584—1563
gwr	5′-CGGTAAGGTGTCACATGAG-3′	backward	2948—2930

1.2 Immunohistochemistry analysis of human testis slices

The cDNA fragment encoding the ADAM29 peptide Leu268—Asp374 was amplified with primers 5′ CTCGGATCCCTGTATTGCAAGTGGAAGTC 3′ and 5′ GTCGAATTCTCAAT-

CACCATAACTACAATTGCT 3′, and cloned into expression vector pRSET-A (Invitrogen) and finally transformed into *E.coli* DE3/LysE strain (Promega). His-tagged fusion protein (named TAN) was produced and then purified according to the Invitrogen pRSET system manual.

Polyclonal antiserum (rabbit anti-human TAN, named Ab-TAN) was prepared and purified by Prof. Liu Quanhai (Institute of Medical Industry, Shanghai)[4].

Western blotting assay was carried out as described in ref. [3]. Primary antibody, Ab-TAN was diluted by 1∶1000; secondary antibody, goat anti-rabbit IgG-AP(Huamei) was diluted by 1∶500.

Using Envision kit and its protocol (Daka), immunoperoxidase staining of formalin-fixed, paraffin-embedded human testis tissue (kindly provided by Prof. Zhou Guomin, Shanghai Medical University) was performed. Primary antibody is Ab-TAN and secondary antibody, horseradish peroxidase conjugated Envision antibody.

1.3 Sequence comparison

Using program PILEUP (GCG software, Winscosin Package, Genetic Computer Group), the protein sequence properties of eleven ADAM members (ADAM2, 3, 5, 18, 20, 21, 24—26, 29, 30, which exclusively expressed in mammalian testis[2]) were analyzed. Gap-open penalty and gap-extend penalty are 30 and 1, respectively.

2 Result

2.1 Cloning of full-length cDNA of *ADAM29*

Multiple-step PCR was applied to obtain the 2.5 kb full-length cDNA of *ADAM29* (fig. 1). Five pairs of different PCR primers covering *ADAM29* gene were designed and amplified products were in the size as expected. Finally, the sequence of an amplified DNA convinced that it contained a full-length cDNA of *ADAM29*.

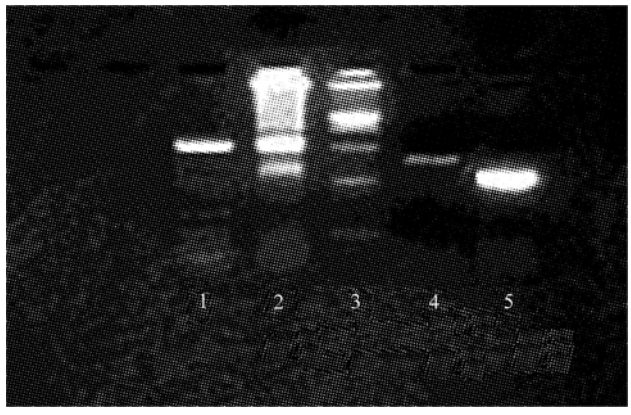


Fig. 1. Cloning of full-length cDNA of *ADAM29* by recombinant PCR. 1, 3rd PCR : product 4 (2.5 kb); 2, 2nd PCR: product 3; 3, DL-15000 marker; 4, 1st PCR: product 2 (1.6 kb); 5, 1st PCR: product 1 (1.2kb).

2.2 Fusion protein (antigen) purification

The cDNA encoding a peptide Leu268—Asp374 was inserted into vector pRSET-A correctly in-frame with the His-tag. Fusion protein was produced mostly in the form of inclusion body (fig. 2). Faint band with double molecular size could be seen in SDS-PAGE gel. It indicates that the fusion protein may form a dimer even in denatured condition (dimer formation is consistent with the existence of several cysteine residues in the peptide).

2.3 Western blotting

As shown in fig.3, polyclonal antiserum was specifically binding to the fusion protein (antigen). A band corresponding to the twice molecular weight was detected by Western blotting as well, confirming the formation of dimers.

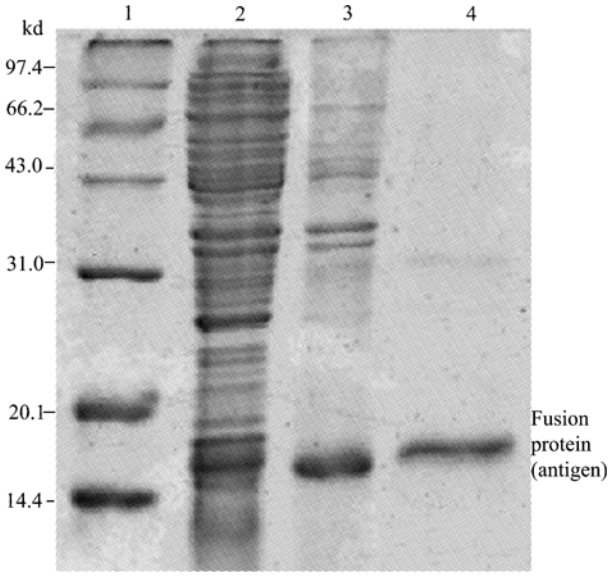


Fig. 2. SDS-PAGE gel of ADAM29 fusion protein (antigen). 1, Protein marker; 2, total proteins of expression strain; 3, pellet after sonication; 4, fusion protein after His-bond column purification

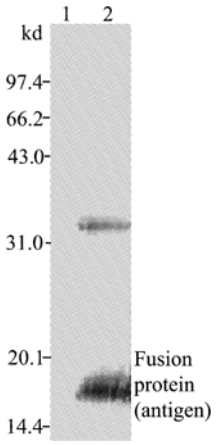


Fig. 3. Western blotting analysis. 1, Control protein (RNase A); 2, total protein of expression strain.

2.4 Immunohistochemical test

In the cross section of human testis, two types of cells, Sertoli cells and spermatogenic cells can be seen in seminiferous tubules. The latter are lying in the order of maturation stages from periphery to lumen of the tubule. The interstitial cells are located in the connective tissue between seminiferous tubules. Immunoperoxidase staining found that ADAM29 protein (brown spot) existed in various types of cells in human testis, however, the interstitial cells would appear to be the major source of this protein (fig. 4).

2.5 Sequence comparison

The sequence homology of eleven mammalian *ADAM* members expressed specifically in testis were compared each other using computer program PILEUP, and a phylogenetic tree is recon-

structed (fig.5). ADAM20 and 21 were the closest members to ADAM29. The consensus amino acid within different domains of these ADAMs are listed in fig. 6.

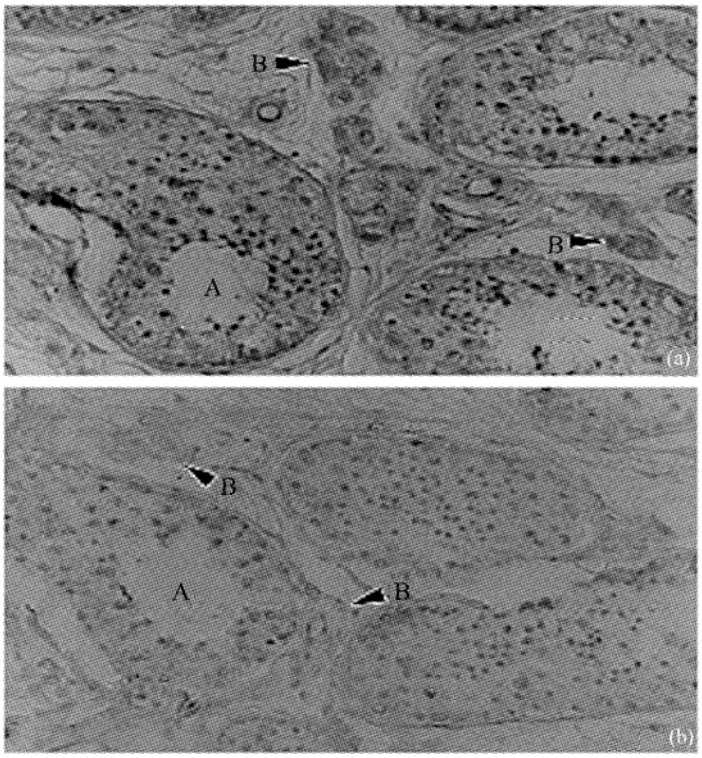


Fig. 4. Immunoperoxidase staining of human testis section. (a) Primary antibody (Ab-TAN) diluted by 1:1000; (b) no addition of primary antibody (as control). A, Seminiferous tubule; B, interstitial cells.

3 Discussion

Along with the identification of new *ADAM* members, people begin to pay much more attention to their functions, which mainly stick to two aspects, i.e. the function of the molecules with metalloprotease activity and the function of the molecules with cell adhesion activity. Results of the present research would provide helpful clues to further investigation of their functions.

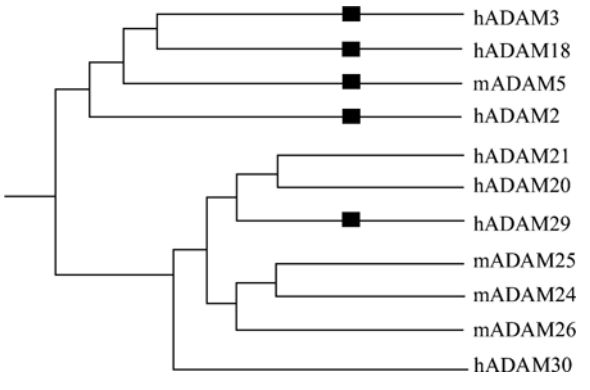


Fig. 5. Phylogenetic tree of eleven testis-specific expressed mammalian *ADAMs*. m, *Mus musculus*; h, *Homo sapiens*. ■, ADAMs with no potential protease activity.

It is reported that 17 ADAMs (1, 8—10, 12, 13, 15—17, 19—21, 24—26, 28, 30) have protease activity[2], which can cleave membrane-anchored cyto-

kines, growth factors and receptors, and then release them to cell matrix. Therefore, the ADAMs are also named sheddase. Three best-studied cases are ADAM17/TACE, ADAM10/Kuzbanian and ADAM9/MDC9. TACE can release an active soluble extracellular domain from membrane-anchored TNF-α. The latest research indicates that TACE and ADAM10 are related with Alzheimer[11]. There is a conserved sequence in all ADAMs with protease activity, i.e. HEXXHXXGXXH. Three histidines (H) bind Zn^{2+} and helix turn point occurs at glycine (G); glutamic acid (E) performs as an active site. ADAM29 is supposed to be out of protease activity due to the replacement of E by H in the sequence (fig. 6).

–	hADAM3	SQLLGINLGLAY	PEQCDCG
–	hADAM18	AQLLGLNVGLTY	NEECDCG
–	mADAM5	LQLLSIGMGLTY	NEQCDCG
–	hADAM2	AQLLSLSMGTTY	GEECDCG
+	hADAM21	HELGHTLGMQHD	EEQCDCG
+	hADAM20	HELGHNLGMQHD	GEECDCG
–	hADAM29	HHLGHNLGMNHD	GEECDCG
+	mADAM25	HEMGHNLGMEHD	GEQCDCG
+	mADAM24	HEIGHNLGMSHD	GEQCDCG
+	mADAM26	HEMGHNFGMKHD	GEQCDCG
+	hADAM30	HELGHAVGMSHD	NEECDCG

Fig. 6. Sequence comparison of eleven testis-specific expressed mammalian ADAMs. Zn^{2+} -binding consensus site: HEXXHXXGXXH; consensus site in disintegrin domain: GE (E/Q) CDCG. "+" and "–" stand for metalloprotease activity.

Generally, we can explore the cell adhesion activity of ADAMs from the disintegrin domain that can bind to integrin. Unfortunately, we still cannot make sure the active site of disintegrin domain which is responsible for cell adhesion activity. But research results indicate disintegrin loop and its conserved sequence GE (E/Q) CDCG, especially D residue, are critical to this activity [5,6]. It is very likely for ADAM29 to interact with integrin and other molecules because of the existence of this structure and conserved sequence of GEECDCG.

More than ten ADAMs are expressed specifically or very predominantly in the testis. ADAM 29 is one of them. Most researches in this field are related to the process of spermatogenesis and fertilization. By using polyclonal antisera, it has been found that several ADAM family members (ADAM1/fertilin-alpha, ADAM2/fertilin-beta, ADAM3/cyritestin/tMDCI, ADAM5/ tMDCII, and ADAM18/tMDCIII)[7] expressed in spermatogenic cells. However, only ADAM1 has protease activity. Two molecules (Fertilin-beta and cyritestin) may be involved in sperm-egg recognition. Male mice lacking fertilin-beta or cyritestin are sterile. Fertilin-beta$^{-/-}$ sperm is deficient in sperm-egg membrane adhesion, sperm-egg fusion, migration from uterus into oviduct and binding to the egg zona pellucida, while cyritestin$^{-/-}$ sperm is only unable to bind to the zona pellucida[8,9]. Then, what is the function of ADAM29?

After analyzing the amino acid sequence of testis-specific ADAMs in mammals, we found (figs. 5 and 6): (i) Generally, all these ADAM family members have a conserved sequence GE (E/Q) CDCG in disintegrin domain, especially CDCG, which indicates that the function of disintegrin domain is extremely important for these members. (ii) All the positions of cysteine (C) are very conserved in these ADAMs (data not shown), which reveals cysteine's significance to the function of proteins. Cysteine is apt to form disulfide bond, therefore probably being critical in the formation of protein structure of ADAMs. Also it may facilitate ADAMs to form homo-polymer or hetero-polymer. It is still unknown whether the active ADAMs are polymer or not. (iii) ADAM29 and ADAM20, 21, 24—26 have a high similarity. However, till now there have been no reports concerning the functions of these 6 molecules. From the reconstructed phylogenetic tree we inferred that distinct differentiation within ADAM family members may occur in their early evolutionary stage and then diverge into two major groups. One group is characterized by metallopro- tease activity within most members, while the other one has no such activity. Although ADAM29 belongs to the former group, a replacement of residue (E replaced by H) on a key point (active site) resulted in a loss-of-function mutation leading to losing the protease activity.

Moreover, it has been proved by immunohistochemical analysis that *ADAM29* was mainly expressed in interstitial cells. Interstitial cells produce male hormone and will be activated or inactivated by all kinds of signal factors during the development in order to control the secretion of male hormone. ADAM29 may participate in the process of signal transduction on cell surface, especially its relatively long cytoplasmic tail, which contains more than one hundred residues. It is very likely for it to interact with the downstream molecules in the cytoplasm thus to transduce the signals. There are 6 repeated sequences of PSQSQP and 3 similar sequences, PPQSQP, PSQSHP and PSQRQP. So many repeated sequences in the cytoplasmic tail might also relate to signal transduction. It was reported lately that there are 3 different splicing sites in cytoplasmic tail, coding for 3 proteins with different sizes[10]. This also implies that the function of ADAM29's cytoplasmic tail is very much likely to be involved in signal transduction. Therefore, our results provide useful clues for further systematical analysis on the roles of ADAM family in the signal transduction.

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