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cDNA cloning, gene organization and expression analysis of human peptidylarginine deiminase type VI✪⋆

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Peptidylarginine deiminase (PAD) catalyzes the post-translational modification of protein through the conversion of arginine to citrulline in the presence of calcium ions. Human, similar to rodents, has four isoforms of PAD (type I, II, III and IV/V), each of which is distinct in substrate specificity and tissue specific expression. In our large-scale sequencing project, we identified a new human PAD cDNA from a human fetal brain cDNA library. The putative protein encoded by this cDNA is designated hPADVI. Expression analysis of hPADVI showed that it is mainly expressed in adult human ovary and peripheral blood leukocytes. We conclude that hPADVI may be orthologous to mouse ePAD, basing on sequence comparison, chromosome localization and exon-intron structure analysis. PAD-mediated deimination of epithelial cell keratin resulting in cytoskeletal remodeling suggests a possible role for hPADVI in cytoskeletal reorganization in the egg and in early embryo development. This study describes a new important member of the human PAD family.

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⋆The nucleotide sequence reported in this paper has been submitted to GenBank under accession number AY443100.

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Abbreviation: PAD, peptidylarginine deiminase.

Peptidylarginine deiminases (protein-L-arginine iminohydrolase, EC 3.5.5.15, PAD) are a group of enzymes that convert peptide bound arginyl residues to citrullinyl residues in proteins (Rothnagel & Rogers, 1984). Enzymatic deimination abolishes positive charges of native protein molecules, inevitably causing significant alteration in their structure and function (Lamensa & Moscarello, 1993; Imparl *et al.*, 1995; Tarcsa *et al.*, 1996). All the enzymes known to date show absolute requirement for calcium ion (Nakashima *et al.*, 1999). Deimination of arginine residues of vimentin, desmin and glial fibrillary acidic protein (GFAP) by PAD interferes with the ability of these proteins to polymerize (Inagaki *et al.*, 1989). Deimination of trichohyalin results in loss of secondary structure and such modified protein is then more easily cross-linked by a transglutaminase (Tarcsa *et al.*, 1996; 1997). Early research described four isoforms of PADs in rodents (Ishigami *et al.*, 1998). These isoforms displayed nearly identical amino -acid sequences, but different tissue-specific expression (Ishigami *et al.*, 2001). Recently, oocyte and early embryo abundant peptidylarginine deiminase-like protein, ePAD, has been reported in mouse (Wright *et al.*, 2003). Concerning human tissues, four types of PAD have been cloned, i.e., PAD type I (Guerrin *et al.*, 2003), PADII (Ishigami *et al.*, 2002), PADIII (Kanno *et al.*, 2000), and PADIV/V (Guerrin *et al.*, 2003). Human PADI mRNAs were detected by reverse transcriptase-PCR in various organs, including epidermis, testis, placenta, spleen and thymus (Guerrin *et al.*, 2003). Human PADII mRNA was detected in the epidermis, the type II enzyme was expressed in all the living epidermal layers, suggesting that PADII is functionally important during terminal differentiation of epidermal keratinocytes. Human PADIII is the predominant isoform in hair follicles and may function as a modulator of hair structural proteins, including trichohyalin during hair and hair follicle formation (Kanno *et al.*, 2000). Human PADIV/V is present in human myeloid leukemia HL-60 cells induced to differentiate into granulocytes by retinoic acid and later in peripheral blood granulocytes (Nakashima *et al.*, 1999).

Here we report a new gene, which encodes PAD, whose transcript is detected mainly in the ovary and peripheral blood leukocytes. A bioinformatic analysis suggests that it is an orhtologous gene to mouse ePAD.

MATERIAL AND METHODS

cDNA library construction. A cDNA library was constructed in a modified pBluescript II SK (+) vector (Stratagene). The modified vector was constructed by introducing two *Sfi*I recognition sites, i.e. *Sfi*IA (5′ggccattatggcc 3′) and *Sfi*IB (5′ggccgcctcggcc 3′) between the *Eco*RI and *Not*I sites of pBluescript II SK (+). Fetal brain mRNA was purchased from Clontech. Double-stranded cDNA was synthesized and inserted into pBS vector between the above sites using SMART^{TM} cDNA Library Construction Kit (Clontech) following manufacturer's instructions. The cDNA inserts were sequenced on an ABIPRISM^{TM} 377 DNA sequencer (Perkin-Elmer) using the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) with –21M13 primer, M13Rev primer and synthetic internal-walking primers designed according to the obtained cDNA sequence fragments. Each part of the insert was sequenced at least three times bidirectionally. Subsequent editing and assembly of all the sequences from one clone were performed using Acembly (Sanger Center).

Bioinformatic analysis. To verify the new full length cDNAs, a database search was performed with the basic local alignment search tools (BLAST) network service at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Profile scan and alignment were done at http://www.expasy.org/pfscan. Other sequence analysis was performed online.

RT-PCR. To investigate the expression pattern of hPADVI in different tissues, a multiple tissue cDNA (MTC, Clontech) based RT-PCR was employed. Panel I/II and Advantage 2 Kit (Clontech) were used in the reaction. The hPADVI specific primer pairs (hPADVIF: 5′cagcagcttttaccccagtgcagaggg 3′ and hPADVIR: 5′tcttgcccatcacaatcatccgcaacag 3′) were designed to amplify a 500 bp fragment. A glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control primer pair included in the panels was used to verify the normalization of the MTC panel. The sequences of the primers for amplifying G3PDH were 5′tgaaggtcggagtcaacggatttggt 3′ (G3PDHF) and 5′catgtgggccatgaggtccaccac 3′ (G3PDHR). A total of 35 cycles of amplification was performed in a total volume of 50 μl. The cycling conditions were as follows: 5 min at 94°C, followed by 35 cycles of 95°C for 30 s, 68°C for 60 s, 72°C for 5 min. HPADVI and G3PDH cDNAs were amplified in a parallel RT-PCR reaction. Five microlitters of each product was later resolved on 1.5% agarose gels.

RESULTS

Sequence characterization

The nucleotide sequence and deduced amino-acid sequence of this gene are shown in Fig. 1. An open reading frame encodes a protein of 694 amino acids. The molecular mass and isoelectric point of the predicted translation product are calculated to be 77.4 kDa and 5.02, respectively. Comparison of the se-

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          agggcgtctgaggctgctgtgctgagtgagggctgcggtgcaggcctgagg
atggtcagcgtggagggccgagccatgtccttccagagtatcatccacctgtccctggac
M  V  S  V  E  G  R  A  M  S  F  Q  S  I  I  H  L  S  L  D
agccctgtccatgccgtttgtgtgttgggcacagaaatctgcttggatctcagcgggtgt
S  P  V  H  A  V  C  V  L  G  T  E  I  C  L  D  L  S  G  C
gccccccagaagtgccagtgcttcaccatccatggctctgggagggtcttgatcgatgtg
A  P  Q  K  C  Q  C  F  T  I  H  G  S  G  R  V  L  I  D  V
gccaacacggtgatttctgagaaggaggacgcccaccatctggtggcccctgtctgatccc
A  N  T  V  I  S  E  K  E  D  A  T  I  W  W  P  L  S  D  P
cccctgtctgatcccacgtacgccacagtgaagatgacatcgcccagcccttccgtggat
P  L  S  D  P  T  Y  A  T  V  K  M  T  S  P  S  P  S  V  D
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A  D  K  V  S  V  T  Y  Y  G  P  N  E  D  A  P  V  G  T  A
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K  T  K  K  V  I  F  S  E  E  I  T  N  L  S  Q  M  T  L  N

gtccaaggccccagctgtatcttaaagaaatatcggctagtcctccatacctccaaggaa
V  Q  G  P  S  C  I  L  K  K  Y  R  L  V  L  H  T  S  K  E
gagtcgaagaaggcgagagtctactggccccaaaaagacaactccagtacctttgagttg
E  S  K  K  A  R  V  Y  W  P  Q  K  D  N  S  S  T  F  E  L
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T  F  Y  V  E  A  I  A  F  P  S  A  E  F  S  G  L  I  S  Y
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W  L  Q  D  E  M  A  F  C  Y  T  Q  A  P  H  K  T  T  S  L
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I  L  D  T  P  Q  A  A  D  L  D  E  F  P  M  K  Y  S  L  S
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P  G  I  G  Y  M  I  Q  D  T  E  D  H  K  V  A  S  M  D  S
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R  V  L  I  G  S  S  F  Y  P  S  A  E  G  R  A  M  S  K  T
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G  K  K  G  F  L  L  L  L  A  S  P  S  A  C  Y  K  L  F  R
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E  N  Q  K  E  G  Y  G  D  A  L  L  F  D  E  L  R  A  D  Q
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L  T  N  I  P  S  D  Q  Q  P  K  R  S  F  A  R  P  Y  F  P
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D  L  L  R  M  I  V  M  G  K  N  L  G  I  P  K  P  F  G  P
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Q  I  K  G  T  C  C  L  E  E  K  I  C  C  L  L  E  P  L  G
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ccctatggggaaaagatgcaaaagtgttcagccaagtgacgtttactaaatagccaataa
agggctggtgggtgtgaatgc
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Figure 1. Nucleotide and deduced amino-acid sequence of human hPADVI (GenBank Accession No. AY443100).

The nucleotide sequence is shown in the top lines, and the deduced amino-acid sequence below in the singer-letter code. The ORF extends from nucleotide 52 to 2136 and encodes a protein of 694 amino acids. An asterisk represents the stop codon; at the 3′ end the possible polyadenylation signal (AATAAA) is boxed.

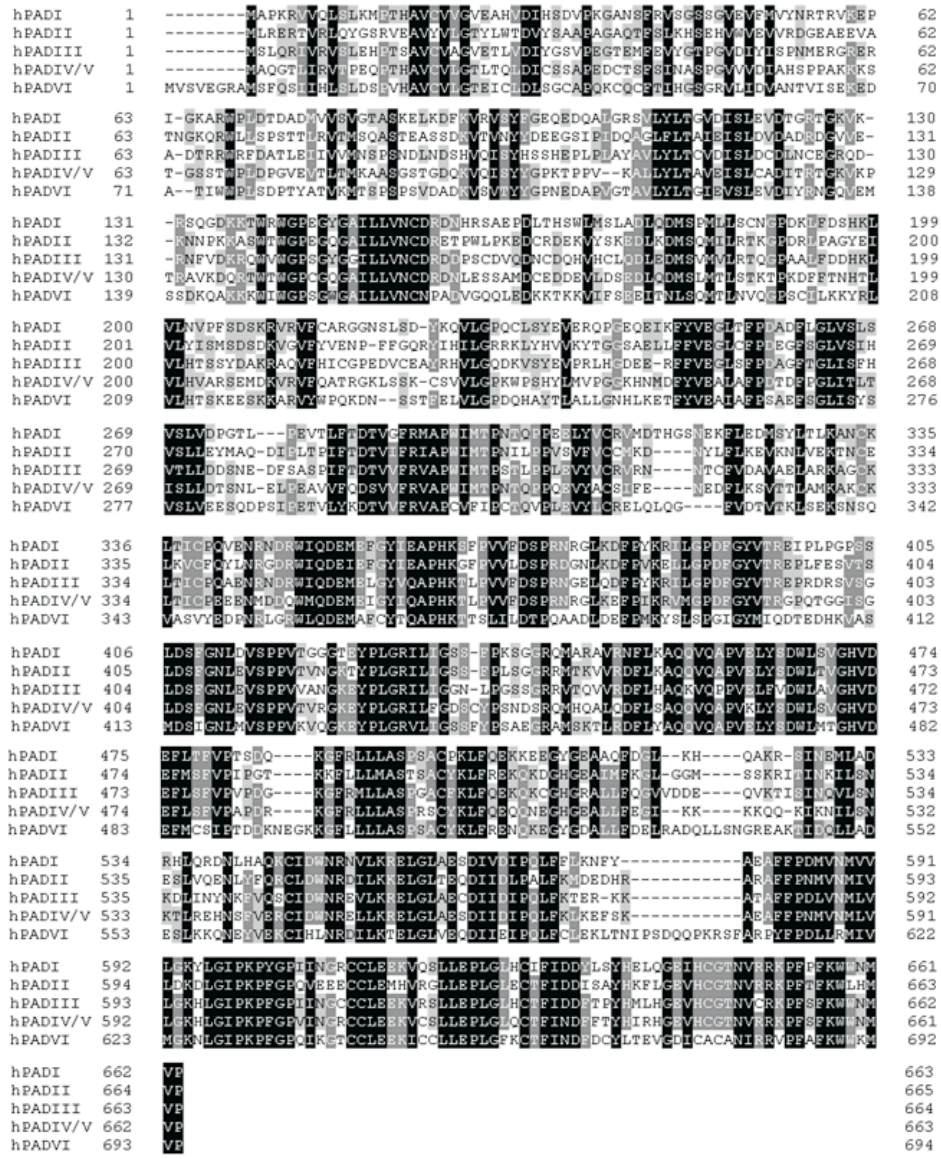

Figure 2. A. Alignment of human PAD types I, II, III, IV/V, and VI.

The alignment was performed by the Align X program of vector NTI suite 5.5, and amino acids are shaded according to the degree of conservation using GeneDoc (http://www.cris.com/~Ketchup/genedoc.shtml): black (100% similarity); gray (80–90% similarity); light gray (60–70% similarity). The accession numbers of the sequence data cited for comparison have the following designations: PADI, AB033768; PADII, AB030176; PADIII, AB026831; PADIV/V, AB017919; PADVI, AY443100. (continued on next page)

quence against the NCBI nonredundant database using the BLAST algorithm found that the sequence was 89% identical to a recently submitted putative peptidylarginine deiminase protein sequence (XP_372767), which was predicted by the NCBI's automated annotation tool GNOMON. The protein has 42%, 43%, 41% and 42% homology to human hPADI, hPADII, hPADIII, and hPADIV/V, respectively (Fig. 2A). It shares a higher homology (65% identity) with mouse ePAD (Fig. 2B). We term this gene hPADVI following the HUGO Nomenclature Committee (http://www.gene.vcl.ac.vk/nomenclature).

Chromosomal localization

Using the international human genome database on NCBI, we found that hPADVI localizes on 1p36.13. The gene spans 28.8 kbp and con-

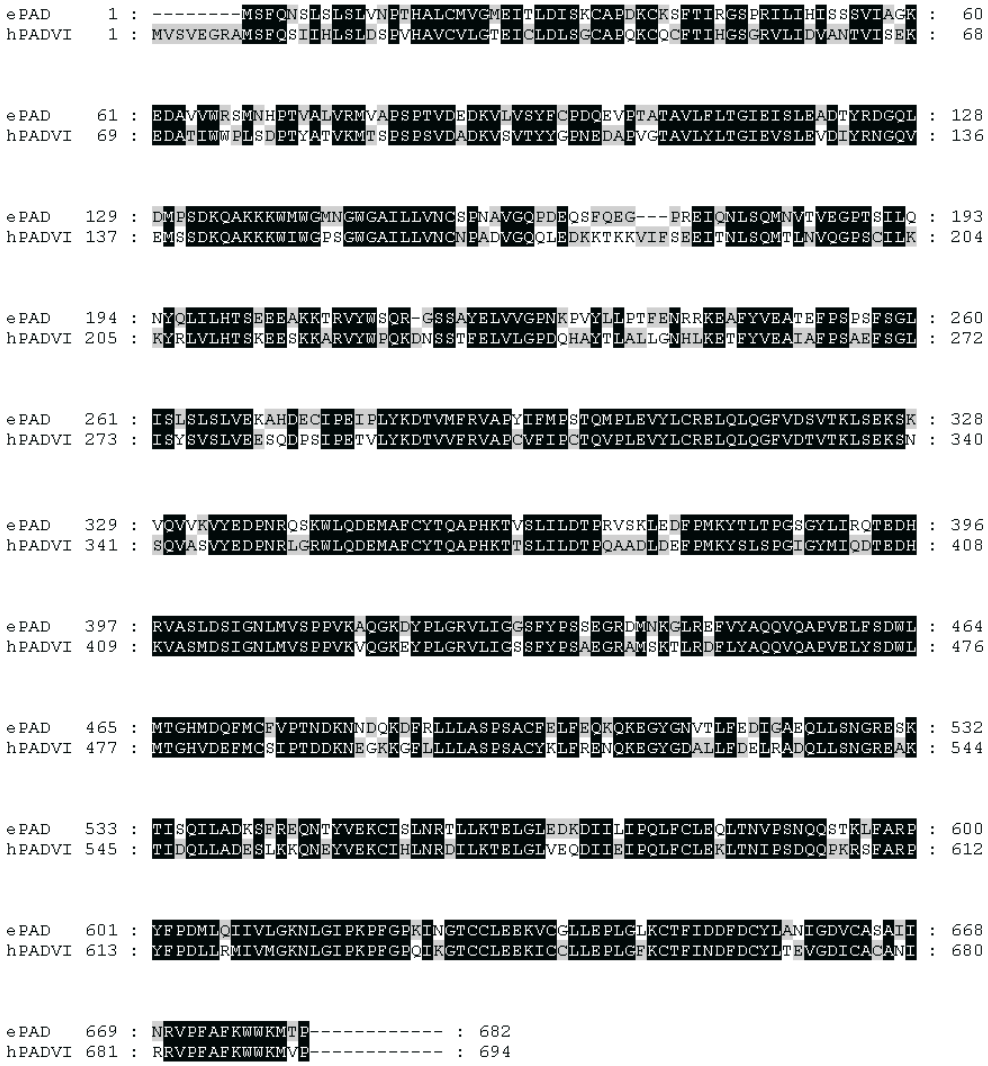

Figure 2. B. Alignment of human PADVI and mouse ePAD.

sists of 16 exons. All sequences of the exon-intron junctions are consistent with the AG-GT rule (Table 1). hPADII, hPADI, hPADIII and hPADIV/V link with hPADVI in tandem, and mouse PADII, PADI, PADIII, PADIV and ePAD were linked in the same order (Fig. 3A). The lengths of the corresponding exons of hPADVI and ePAD were equal (Fig. 3B).

Expression pattern of hPADVI

The tissue distribution of hPADVI mRNA was determined by RT-PCR. The result showed that hPADVI was expressed mainly in the ovary and peripheral blood leukocytes, and slightly expressed in the liver, thymus, testis, lung and spleen of the 16 tissues examined (Fig. 4).

DISCUSSION

In a large-scale cDNA sequence study, we isolated a 2397 bp cDNA that encodes human peptidylarginine deiminase type VI gene. The cDNA containing an ORF from 52 to 2136 bp encodes a protein of 694 residues. The putative initiation ATG codon at 52 bp (CTG-AGGATGG) conformed to the Kozak consensus sequence (A/GXXATGG) apparently controlling the translational efficiency of mammalian mRNAs (Kozak, 1987). The poly-

Table 1. Exon-intron structure of human hPADVI gene

	Exon	Size (bp)	5′-splice donor	Intron	Size (bp)
	1		TCTCAGCGGG**gt**gagatgctgg	1	690
cgggcaaacc**ag**GTGTGCCCCC	2	178	TGCGGATAAG**gt**aagcctcagg	2	2.189
ctgtctccac**ag**GTCTCGGTCA	3	73	ACTGGCATTG**gt**gagtgttgct	3	4.416
cttctgtttc**ag**AGGTCTCTCT	4	68	ACAGGCTAAG**gt**gagtctgcca	4	1.055
tctcatttgc**ag**AAAAAATGGA	5	118	TTTTCAGAGG**gt**aggacctcag	5	800
tcttttgccc**ag**AAATAACGAA	6	126	TGGCCCCAAA**gt**gagtgttctt	6	6.284
tttctctcct**ag**AAGACAACTC	7	179	TCAAGACCCG**gt**atgtccccat	7	213
gtcttgttgc**ag**TCAATTCCAG	8	104	ACCTGTGCAG**gt**gagagaccat	8	3.229
ctcccatggc**ag**GGAGCTGCAG	9	112	GTGGCTCCAG**gt**aacaccccac	9	1.745
tctccattcc**ag**GATGAGATGG	10	108	ACTCACTGGT**gt**ggaacttggt	10	213
tctctccccc**ag**AGCCCTGGTA	11	155	TTTACCCCAG**gt**gagccacaaa	11	492
tcttccttct**ag**CGCAGAGGGC	12	157	GGGCAAAAAG**gt**ctgctttggg	12	428
tctgtttccc**ag**GGCTTCCTGC	13	124	CTGTCTAATG**gt**aagggaactc	13	1.403
ttcttcctac**ag**GAAGGGAAGC	14	71	ATACGTGGAG**gt**aggaccagtg	14	1.540
acccacccac**ag**AAGTGCATTC	15	162	CCCTGACCTG**gt**gaggggcgac	15	2.353
tctttctaac**ag**TTGCGGATGA	16			16	

Intron and exon junction nucleotide sequences are shown in lowercase and uppercase letters, respectively. Bold letters stand for donor and acceptor splice site.

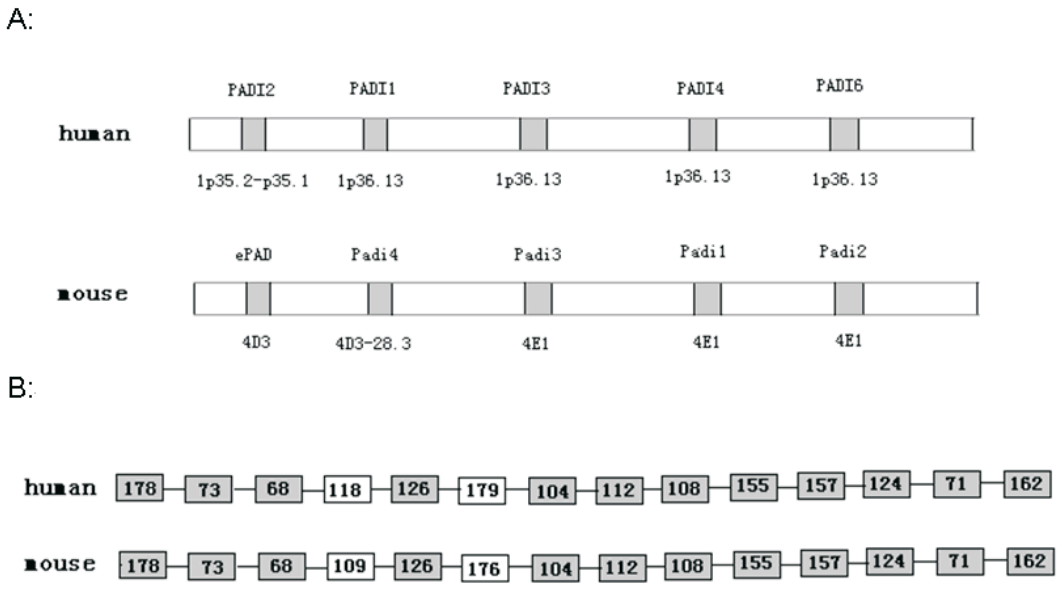

Figure 3. A. Chromosomal localization of the human and mouse PAD families. B. Exonic organizations of human hPADVI and mouse ePAD.

Exons are represented as boxes with lengths in nucleotides. Introns are shown by lines. The exons of equal lengths in the two genes are indicated by gray boxes (the Figure shows the second through fifteenth exons).

adenylation signature (AATAAA) is located at 2372 bp.

The putative protein shows 42%, 43%, 41% and 42% identify to human hPADI, hPADII, hPADIII, and hPADIV/V, respectively. Moreover, it shows a higher identity (65%) to mouse ePAD. The domain that is conserved between the known PADs is also conserved in this protein, suggesting that the putative protein represents a new member of the PAD enzyme family. We have named this protein hPADVI, in agreement with HUGO Nomenclature Committee (http://www.gene.vcl.ac.vk/nomenclature).

Alignment of hPADVI cDNA against NCBI database revealed that the cDNA sequence covered 28.8 kb. The hPADVI gene consists of 16 exons along the human chromosome 1P36.13, where the human PAD gene family is located as a cluster. Interestingly, mouse ePAD gene also consists of 16 exons along mouse chromosome 4D3, where the mouse PAD gene family presents the same pattern (Fig. 3A). We also found that hPADVI and ePAD have the same number of amino-acid residues coded for corresponding exons except for the fifth and seventh exons, and these two exons do not change the open reading frame (Fig. 3B). The similar gene organization of hPADVI and ePAD suggests that hPADVI is the counterpart of ePAD.

Peptidylarginine deiminases (PADs) are posttranslational modification enzymes that convert protein arginine to citrulline residues in a calcium-dependent ion manner. In rodents and human, different isoforms of PAD are distinct in substrate specificity and tissue specific expression. The relatively high sequence conservation in the C-terminal region suggests that this enzyme is involved in such common physiological functions as catalysis and calcium binding. The N-terminal region might be involved in selective recognition of target proteins in relevant tissues (Fig. 2). The RT-PCR showed that hPADVI is mainly expressed in the ovary and peripheral blood leukocytes. Mouse ePAD localizes to egg cytoplasmic sheets, a unique keratin-containing intermediate filament structure found only in mammalian oocytes and in early embryos, and known to undergo reorganization at critical stages of development. The specific localization of ePAD to oocytes in ovarian sections

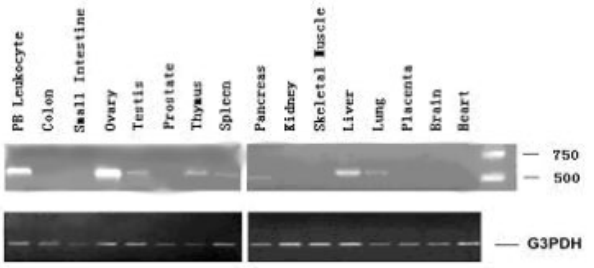

Figure 4. Multiple tissue cDNA based RT-PCR expression pattern of hPADVI.

Twenty-six cycles (for G3PDH) and 35 cycles (for hPADVI) were performed with Advantage 2 Kit (Clontech).

and its homology to a well-characterized enzyme family that has known *in vitro* and *in vivo* substrates supports further development of small molecule inhibitors of new potential contraceptive targets (Wright *et al.*, 2003). hPADVI, the counterpart gene of mouse ePAD might have a similar role in cytoskeletal reorganization in the egg and early embryo. Further study should be made to clarify the precise role of hPADVI.

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