

A Novel Human Lipid Binding Protein Coding Gene: PERF15, Sequence and Cloning

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Abstract

Introduction: PERF15 is a testicular germ-cell specific fatty-acid binding protein (FABP) isolated from mammals, originally from rats. It encodes one of the most abundant proteins of rat spermatozoa localized in the perinuclear theca. Northern blot analysis has demonstrated that rat PERF15 mRNA is exclusively transcribed during meiosis and post-meiosis. In this study, we cloned and sequenced human PERF15 gene.

Materials and Methods: According to the open reading frame of automated computational analysis of Homo sapiens similar to testis fatty acid binding protein nine, two specific Primers were designed to amplify human PERF15 gene. To confirm the identity of the amplified gene, PCR products of PERF15 were cloned into appropriate plasmid vectors followed by sequencing of the inserts.

Results: A unique band of ~3kb was obtained after PCR amplification. Restriction enzyme digestion using PvuII confirmed that the fragment was related to PERF15. Gene alignment, direct sequencing and application of specific primers to the gene showed 100% similarity between this gene and the computational data by gel extraction of the ~3 kb band. The human PERF15 gene contained four exons and three introns. Exons one, two, three and four, respectively, coded for 24, 57, 34 and 17 amino acids. The existing three introns were composed of 2113, 461, and 168 nucleotides.

Conclusion: In spite of the homology between exonic regions and exon-intron boundaries of human PERF15 gene and that of animals, human PERF15 gene is different in size and sequence from corresponding introns in rat and murine PERF15.

Keywords: Cloning, Fatty acid binding protein, Fertilization, PERF15, Testis genes.

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Introduction

Sperm quality control is critical for efficient reproduction. In germ cells, apoptosis involves different processes and whether it triggers the induction of cell death in germ cells is still unclear. Sperm fatty-acid composition is involved in its fertilizing ability. Fatty-acids com-

position of germ cells changes dynamically during spermatogenesis and fatty acid-binding protein (FABP) may be involved in these processes (1).

Mammalian spermatozoon is made of several unique cytoskeletal elements such as perinuclear theca (PT), the outer dense fibers and the fibrous

sheath (2). Perinuclear theca (PT) is a prominent cytoskeletal element that covers sperm nucleus. PT is composed of two parts. The subacrosomal part is sandwiched between the inner acrosome membrane and the nuclear envelope. The post-acrosomal part is a sheath that lies between the plasma membrane and the outer nuclear membrane (3). PERF15 is a major protein of rat PT found between the inner acrosome and the outer face of the mammalian sperm nuclear envelope. PERF15 belongs to a family of fatty-acid binding proteins (FABP). It is very similar to myelinP2, adipocyte lipid binding protein (ALBP) and heart-type fatty-acid binding proteins (3). PERF15 expression changes during meiosis, therefore, its immunoreactivity has been weak in pachytene spermatocytes and strong in spermatids, especially in early elongated spermatids (4).

FABPs are members of a family of conserved intracellular lipid binding proteins whose major function is thought to be intracellular fatty-acids binding. Different roles have been proposed for FABPs. FABPs may modulate the effect of fatty acids on various enzymes and receptors, as well as cellular processes such as signal transduction and gene expression (5). PERF15 protein is strongly expressed in apoptotic spermatocytes (6) as the fatty acid composition of germ cells changes dynamically during germ cell development (7). PERF15 could play an important role in this developmental system (8). It is likely that PERF15 would protect sperm fatty acids from oxidation to maintain fertilizing ability of spermatozoa (8).

Our goal for carrying out the present study was cloning and sequencing the entire human PERF15 gene and comparing it to other FABP gene families.

Materials and Methods

This research was approved by the ethics committee of Shaheed Beheshti University of Medical Sciences.

RNA Analysis: Total RNA was isolated from 50mg of fresh adult human testis tissue according to guanidine/ thiocyanate/ phenol/ chloroform extraction method (9). For this purpose, fresh testis tissue was sliced into small pieces and transferred into a 1.5ml Eppendorf tube, 300 μ l RNA Bee solution (Biosite, CytoVision Molecular Diagnosis, Germany) was added and homogenized by Pellet Pestle (Sigma, Germany). Then, it

was laid on ice and 30 μ l (0.1 tube volume) of chloroform was added, mixed and centrifuged at 12000rpm, at 4°C for 10 minutes. For RNA precipitation, the upper phase was transferred to a clean micro-tube, where isopropanol (v/v) was added and it was incubated at -20°C for 1.5 hours and centrifuged at 12000rpm, at 4°C for 12 minutes. The pellet was kept and 700 μ l of 75% ethanol was added and centrifuged at 12000rpm at 4°C for 15 minutes. Then, the pellet was kept at room temperature to dry. The dried sediment was finally resolved in DDW. The collected RNA was spectroscopically quantified at 260nm by Ultraspec 3100 Pro (Biochrom Ltd., Cambridge, UK). The purity of RNA was verified by optical density (OD) absorption ratio OD_{260nm}/OD_{280nm} determination (1.80 – 2.06; mean=2.0).

First strand cDNA synthesis: The extracted RNA was converted to cDNA by to RT-PCR method (10). Briefly, 1 μ g of the total RNA was heated at 90°C for 2.5-5 minutes, to release any existing secondary structure in RNA strands, and then it was immediately cooled on ice. Then, the RNA was enzymatically reverse-transcribed with 1 μ l of 20U/ μ l of M-MLV (1U/ final concentration, Fermentas, Cinna Gen Inc, Iran), 4 μ l of 5X RT buffer (1 \times final concentration, Fermentas), 2 μ l of 5mM dNTP (500 μ M, Roche, Diagnostics, Mannheim, Germany), 1 μ l of 10 picomole of Random Hexamer, N6, primer (Pharmacia, Sweden), and double distilled water was added upto a final volume of 20 μ l. The reaction was then transferred to a thermo cycler for a three-step heating at 25°C for 10 minutes, 42°C for 60 minutes and 70°C for 10 minutes, and later it was transferred onto ice and it was immediately cooled down to -20°C.

Polymerase chain reaction (PCR) amplification of cDNA: Two pairs of primers were designed to amplify human PERF15 cDNA based on the automated computational analysis of Homo sapiens similar to testis fatty-acid binding protein 9 (Gene bank, accession number XM_378035). Forward primer hP1 (5' ATG GTT GAG CCC TTC TTG GGA AC 3') and reverse primer hP6 (5' TCA CAC CTT TTC GTA GAT TCT GGT G 3') were synthesized (VWR, Stockholm, Sweden). The PCR was done using the master mix containing 2.5 μ l of PCR buffer (10 \times), 2 μ l of 25mM MgCl₂, 1 μ l of 5mM dNTP, 2 μ l of 5 μ M forward primer, 2 μ l of 5 μ M reverse primer, 0.25 μ l

of Taq DNA polymerase (Roche, Diagnostics), 25ng of cDNA and double distilled water added upto a total volume of 25 μ l. The mixture was amplified in a thermocycler (Eppendorf, Germany) following an initial denaturation at 94°C for 4 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 63.5°C for 30 seconds, elongation at 72°C for 1 minute and a final extension at 72°C for 10 minutes.

DNA extraction: Human blood was used for the preparation of genomic DNA using a DNA extraction kit (Qiaquick kit, VWR Stockholm, Sweden) and salting out method (11).

Ten milliliters of peripheral blood was obtained from a 47-year old fertile man. Subsequently, 900 μ l of lysis buffer was added to 300 μ l of the blood sample and incubated at room temperature for 10 minutes. The hemolysate was centrifuged at 3000rpm for 5 minutes. The supernatant was discarded and the remainder was washed one more time by the lysis buffer. The washing step was repeated three times until the precipitated cells turned pink. Six-hundred microliters of the lysis buffer and 15 μ l of proteinase K (20mg/ml) were added, mixed and incubated at 37°C overnight. Two hundred microliters of potassium acetate solution (5M) was then, added and incubated at 4°C for 10 minutes and centrifuged at 12000rpm for 10 minutes. The supernatant was transferred into a clean tube. Then, 600 μ l of isopropanol was added to the tube and it was gently mixed to isolate the DNA. The mixture was centrifuged at 12000rpm at 4°C for 10 minutes. The precipitated DNA was washed by 600 μ l of 70% Ethanol, dried at room temperature and dissolved in 50-100 μ l of double-distilled water.

PCR Amplification of Genomic DNA: PCR was applied to amplify human PERF15 DNA. The primer pairs hP1 (5' ATG GTT GAG CCC TTC TTG GGA AC 3') and hP6 (5' TCA CAC CTT TTC GTA GAT TCT GGT G 3') were used to amplify the entire PERF15 gene segment. For this purpose, 2.5 μ l of 10 \times PCR buffer containing MgCl₂, 1 μ l of 5mM dNTP mixture (Roche, Diagnostics), 3 μ l of 5 μ M of each forward and reverse primers, 0.2 μ l of expand long DNA polymerase (Roche, Diagnostics) and 3ng/ μ l of DNA template were mixed in a 200-microliter tube (Treff) reaching a total volume of 25 μ l by adding double-distilled water (DDW). Each DNA sample was amplified in a thermo-cycler

(Eppendorf, Germany) through one denaturizing step at 94°C for four seconds, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 sec. and a final extension step at 72°C for 7 minutes.

Extraction and purification of the PCR products: The PCR product (30–40 μ l of each) was electrophoresed on 1.5% agarose gel at a voltage of 70mv for 1-1.5 hours. Observed bands were cut off and isolated using a Qiaquick kit (VWR, Stockholm, Sweden) according to the manufacturer's instructions.

Enzymatic digestion: A WebCutter DNA database was used to find a unique restriction site to be able to cut the human PERF15 gene into specific fragments. Then, the PvuII enzyme was selected to digest the 3kb PCR product which yielded two 1159 and 1924bp fragments. The digestion reaction contained 3 μ l of 10 \times buffer (New England Biolabs, UK), 2IU/ μ g of PvuII, and 5-10IU/ μ l of the purified PCR product. Then, double-distilled water was added up to 30 μ l to digest the human PERF15 gene. The restriction enzyme cut the gene structure at nucleotide 1159 of the PCR product and produced a.

Sequencing: To sequence the 3kb PCR product on both strands, the purified fragment was directly sequenced using Automatic sequencer (version 3130XL Genetic Analyzer, ABI Applied Biosystems). Primer pairs hP1 and hP2 were used for sequencing using the dideoxy sequencing method. Then, the obtained sequence was compared to the computational analysis of Homo sapiens, similar to fatty-acid binding protein gene (NCBI – accession #XM_378035).

Sub-cloning and Amplification of the PCR Product: Sub-cloning of the PCR product was performed according to the previously mentioned method (12). For this aim, the three purified PCR fragments were inserted into a plasmid (P^{BlueScript} Cloning Vector System; Molecular Biology Research Center, Iran). The generated recombinant DNA was transformed into E. coli XL1 blue (Molecular Biology Research Center, Iran) and incubated for 24 hours on selective agar plates. Several white colonies were selected and amplified using universal primers recognizing T7 and T3 plasmid promoters. Positive colonies were selected and plasmids were isolated and digested by NdeI restriction enzyme.

DNA inserts were sequenced using Sanger's

method. A DNA comparison program was used to compare the nucleotide sequences of the gene fragments and the previously registered computational human gene sequence at NCBI with an accession number of XM_378035.

Results

RT-PCR of human RNA, through the use of hP1 and hP6 primers, allowed us to amplify the human cDNA as it is shown in figure 1.

This fragment was purified, sequenced according to Sanger's method (Cinagene, Iran) and compared to rat cDNA sequence. The comparison showed 87% homology. Application of PCR on human genomic DNA, by using primer pairs hP1/hP6, created a fragment of ~3000bp length. On using web-cutter DNA database for finding a unique restriction site to be able to cut the human PERF15 gene into specific fragments, we found that PvuII restriction endonuclease digests PERF15 gene into two fragments. PvuII restriction endonuclease digestion confirmed that the 3kb PCR product belonged to human PERF15 gene. The enzyme recognized a CAGCTG sequence in the 3kb PCR product and cut the fragment into two expected 1159 and 1934bp fragments (Figure 2).

The sub-cloning of the 3 kb fragment into an appropriate vector, and its propagation in host cells, allowed the selection of several positive colonies which were later confirmed by PvuII

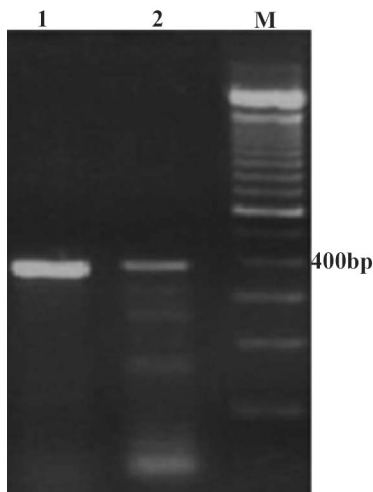


Figure 1. The result of RT-PCR amplification of the human and rat testis cDNA. Lane 1: rat RT-PCR product using rat specific primer pairs P1: 5'ATGATGAACCCTTCTTGGGG3' and P6: 5'CACCCTTTCGTAGGTCCTAGT 3', Lane 2: human RT-PCR product with hP1-hP6 primers, M: molecular weight marker.

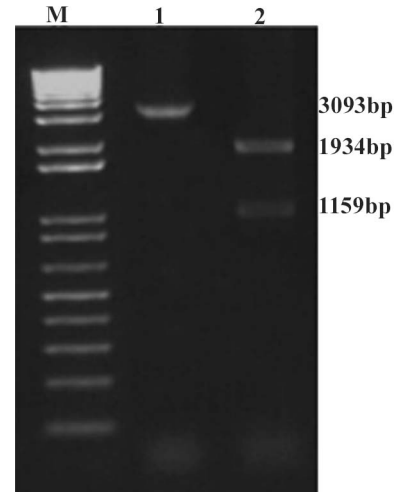


Figure 2. PvuII digestion of the human ~3kb PCR product. This enzyme recognizes a CAG CTG sequence in human PERF15 gene and cuts this gene into 2 fragments of 1159bp and 1934bp. Lane 1: undigested human PCR product amplified with hP1/hP2. Lane 2) the same human PCR product after digestion with PvuII. M) Molecular size marker.

restriction enzyme (Figure 3 and 4).

The DNA comparison program for comparing the nucleotide sequences of the 3kb gene fragment and the previously registered computational human gene sequence at NCBI with an accession number of XM_378035 confirmed the 3kb PCR product to be the intended gene. Human PERF15 gene, like rat PERF15 gene, has four exon and three intron regions. The length of the gene is three kilobases from the translating start codon. The sequences of the exons showed a 20% homology, but no significant homologies were

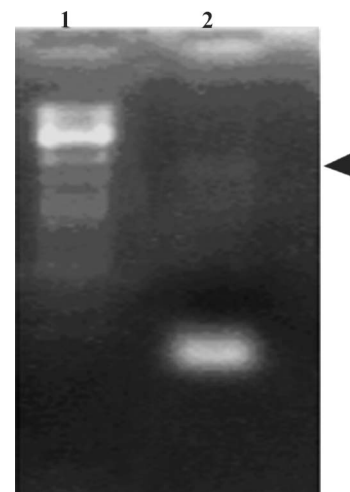


Figure 3. Amplification of PERF15 gene fragment. Samples of 50µl containing standard buffer, 200µM of each dNTP, 40pM of each primer, 100ng genomic DNA, 1.25unit of Taq DNA polymerase to 30 cycles of PCR amplification. Each sample was resolved on a 1% agarose gel containing ethidium bromide. Lane 1(100bp DNA marker), Lane 2 (PCR product).

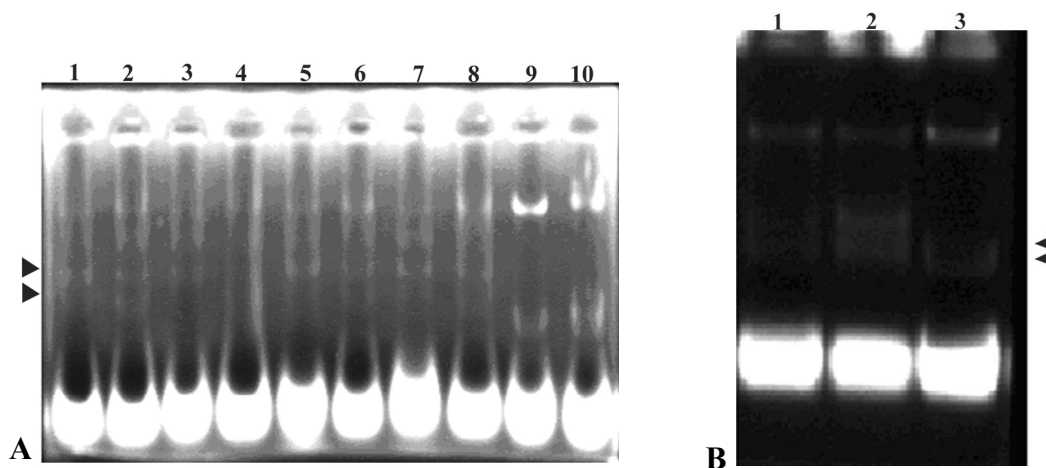


Figure 4. Restriction digest of amplified vector after the sub cloning of 3kb insert. A: Each plasmid was digested with NdeI restriction enzyme (lanes 1-8), undigested recombinant vector (lanes 9& 10), and the very upper band of each lane demonstrates open ring plasmid. B: lanes 1 and 3 show undigested plasmid, lane 2 shows digested recombinant plasmid.

found in the corresponding intron regions of rats (Figure 5).

Discussion

Body fatty acid composition changes during spermatogenesis (7) and sperm abnormal fatty acid composition has been linked to infertility (13).

PERF15, a FABP, is expressed only during spermatogenesis (1), in mammalian testes (14), and in spermatozoa of rats (15, 2), Bulls (15), and mice (16). The protein could be influenced by changes in the composition of fatty acids. Due to PERF15 localization in spermatozoon that lies between the inner acrosomal and the outer nucleus membranes (14), and its high homology with other FABPs, it is presumed that this protein is required for sperm development (14). This protein shares physicochemical properties with anti-cellular retinoic acid-binding protein (CRABP), (17). Nucleotide comparison between rat and mouse PERF15 shows that both gene nucleotide sequences are identical except in nucleotide 183, which results in Lysine/Glutamic acid substitution in the protein chain. Additionally PERF15 is involved in spermatogenesis and programmed cell death in germ cells and degeneration is a necessary process for normal sperm maturation (18). This evidence suggests the involvement of PERF15 in spermatogenesis. Since human PERF15 is made of four exons and three introns and it shows a significant sequence similarity to other members of the gene family – such as rat PERF15 (66%), to ALBP, a lipid carrier molecule in adipocyte tissue (65%), and a 50% homology to

cardiac FABP (picture) – the same function is presumed for all members of the supper gene family. Although, we did not arrange a hoisto-cytochemical experiment for detection the exact cell location of the human protein, the rate of gene similarities confirms the idea that human PERF15 has a lipid attachment potential like other proteins of the gene family. PERF15 binding affinity for phospholipids of the opposite membranes of sperm cytoplasmic organelles, i.e. acrosome and nucleus, causes mediated attachment of these membranes, which is essential for species-specific head formation and fertilization. Due to the similarities to other pointed mammalian PERF15, the same membrane attachment function is assumed for the human protein. Therefore, PERF15 seems to have a basic role in a successful fertilization (15, 19-20).

Previous investigations on human sperm pathology have revealed that in acrosomeless sperm from infertile men, perinuclear theca is absent (21-23). Considering the mentioned experiments, we assume that this human gene is also expressed in sperm cells during spermatogenesis and its function, due to its high homology in structure to other mammalian PERF15 like rat protein, might be to maintain a normal sperm morphology. Future studies using the cloned PERF15, may well advance our understanding of its involvement in the male reproductive system and its association with some male infertilities.

Conclusion

The perforatorium lipid binding protein (PERF15), a member of intracellular fatty-acid

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