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Original Article

Cloning, Expression and Characterization of Zebra Fish Ferroportin in Hek 293T Cell Line

*A Rafiee [1], SMR Fatemi [1], S Jamili [1], S Ajdari [2], F Riazi-rad [2], A Memarnejadian [3], *MH Alimohammadian[2]*

[1]Dept. of Marine Biology, Sciences & Research Branch, Islamic Azad university, Hesarak, Tehran, Iran
[2]Dept. of Immunology, Pasteur institute of Iran, Tehran, Iran
[3]Dept. of Hepatitis and Aids, Pasteur institute of Iran, Tehran, Iran

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Abstract

Background: Ferroportin (Fpn), a regulator of iron homeostasis is a conserved membrane protein that exports iron across the enterocytes, macrophages and hepatocytes into the blood circulation. Fpn has also critical influence on survival of microorganisms whose growth is dependent upon iron, thus preparation of Fpn is needed to study the role of iron in immunity and pathogenesis of micoorganisms.

Methods: To prepare and characterize a recombinant ferroportin, total RNA was extracted from Indian zebrafish duodenum, and used to synthesize cDNA by RT-PCR. PCR product was first cloned in Topo TA vector and then subcloned into the GFP expression vector pEGFP–N1. The final resulted plasmid (pEGFP-ZFpn) was used for expression of Fpn-EGFP protein in Hek 293T cells.

Results: The expression was confirmed by appearance of fluorescence in Hek 293 T cells. Recombinant Fpn was further characterized by submission of its predicted amino acid sequences to the TMHMM V2.0 prediction server (hidden Markov model), NetOGlyc 3.1 and NetNGlyc 3.1 servers. The obtained Fpn from indian zebrafish also contained eight transmembrane domains with N- and C-termini inside the cytoplasm and harboured 78 O-glycosylated amino acids.

Conclusion: The recombinant Fpn from Indian zebra fish was successfully expressed in Hek 293 cell line. Although the discrepancy in two amino acids was observed in our produced Fpn and resulted in an additional O-glycosylation site, but had no effect on the topology of the protein compared to other Fpn described by other researchers. Therefore this construct can be used in future iron studies.

Keywords: Iron homeostasis, Ferroportin, Cloning, Expression, Topology

Introduction

Iron is an abundant material in the earth and an essential element for human and other living organisms (1). In human, iron is absorbed in intestine, circulates in the blood, and is stored in the macrophages and hepatocytes. Cellular absorption of iron is mediated by molecules such as transferrin receptor and divalent metal transporter 1 (DMT-1) (2). Moreover, macrophages express natural resistance-associated macrophage protein 1 (Nramp-1) in late phagolysosomes, which is able to transport iron across the membranes (3). Ferroportin (Fpn) is the only molecule that is involved in the release of iron from cells to the plasma (4). Homeostasis of iron, in fact, depends on maintenance of iron-transferrin concentration in plasma and

***Corresponding Author:** Fax: +9821 66968857, E-mail address: mhalimohammadian@yahoo.com

extracellular fluids. Three major pathways are required to support this status: a) absorption of iron from intestine (dietary iron); b) release of stored iron from liver; and c) recycling of iron from senescent red blood cells by macrophages in spleen (4, 5). These pathways are controlled by influence of hepcidin on *Fpn* expression. By ligation of hepcidin, Fpn is internalized and degraded in iron-exporting cells (6, 7) hence the export of cellular iron into the plasma is diminished (8).

Fpn (also called Ireg1, MTP1or SLC40AI) is a membrane iron exporter that is expressed in all tissues involved in regulation of iron flow, including duodenal enterocytes and macrophages. Inactivation of the *Fpn* gene leads to iron accumulation in these cells (5). It acts as the ligand of hepcidin, which is a liver-produced iron regulatory hormone (6). Previous reports have shown that Fpn of zebrafish is a protein with 10 putative trans-membrane domains that consists of 562 amino acids (9). This protein has been reported as a conserved vertebrate iron exporter and regulatory protein (9-12). Available evidence also suggests the presence of high similarities among Fpn of zebrafish, mouse and human. Zebrafish contains a known genome and is a perfect system for studying of genes that involve in iron metabolism (13).

In the present study, zebrafish *Fpn* gene was used to produce a recombinant Fpn. This protein is applicable in future studies to investigate its influence on iron homeostasis and iron disorders. It can also use to evaluate its correlation with other iron modulator proteins and to study its role in treatment of infections caused by intracellular microorganisms because of their requirements to iron for survival. To attain this purpose, *Fpn* was expressed and its topology was determined. Therefore, the total RNA was extracted from zebrafish intestine tissue and used for cDNA synthesis. Amplified DNA was then cloned in *E. coli* and expressed in Hek 293T cell line. Its topology including transmembrane domains and glycosylation sites were also analyzed and compared with other reports.

Materials and Methods

Extraction of ferroportin RNA

Total RNA was extracted from intestinal tissue of Indian zebrafish by RNX^{TM} reagent (Cinnagen, Iran) according to the procedure supplied by the manufacturers.

cDNA synthesis and cloning

One µg of extracted RNA was used for cDNA synthesis by High Fidelity Prime Script^{TM} RT-PCR kit (TAKARA, Japan) according to the manufacturer's protocol. A pair of primer was designed based on *Fpn* mRNA sequence (Gene Bank accession number NM_131629): *Xho*I-tailed forward (5`- TCG CTC GAG AAA ACG CAC AAT GGA CAG CCC TG -3') and *Sma*I-tailed reverse (5`- TTA AGG GCC CAT ACA GAG TTT GGA AGT GAG GG -3`) primers (restriction sites are underlined) and named as Fpn-F and Fpn-R, respectively. PCR mixture was prepared in a total volume of 20 µl containing 2 µl of cDNA, 5 pmol of each primer, 1.8 mM $MgCl_2$, 0.5 mM dNTPs, 1X PCR buffer, 1 unit of *Pfu* DNA polymerase (Fermentas, Lithuania) and dH_2O up to 20 µl. PCR program was started at 94^{o} C for 5 min, followed by 30 cycles of 94^{o} C for 1 min, 59^{o} C for 1 min, 72^{o} C for 5 min and completed with a final extension of 72^{o} C for 10 min.

PCR product (1714 bp) was initially treated with *Taq* DNA polymerase at 72 ^{o} C for 10 min and then cloned into the TOPO TA Cloning plasmid (invitrogen). Following the extraction of recombinant plasmid from transformed TOP10 *E. coli* cells by *AccuPrep*® Plasmid Mini Extraction Kit (Bioneer), the cloned gene was analysed by restriction enzymes of EcoR1 and Xmn1 (data not shown). Plasmid containing *Fpn* gene was digested with *Xho*I/*Sma*I (Fermentas) and subcloned in the pEGFP-N1 expression vector (Clontech) to construct the

final recombinant plasmid of pEGFP-ZFpn, which was sequence-confirmed by SeqLab laboratories (Germany).

Cell line, transfection and expression detection

HEK 293T cell line (Human Embryonic kidney 293 cell) which is a cell line originated from human embryonic kidney cells and carries the SV40 T antigen was used for pEGFP-Z*Fpn* plasmid propagation and expression. These cells were maintained and cultivated in RPMI1640 medium (Sigma) supplemented with 2.0 mM L-gluthamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (Biosera, South Korea) at 37 °C, 5% CO2. Cells were transiently transfected with the DNA construct by PolyFect transfection reagent (Qiagen, Germany). In brief, according to the manufacturer's instruction the transfection-complex was prepared based on the optimized amounts of plasmid and PolyFect reagent (4 µg and 40 µl, respectively) and transferred to the 40-80% confluent HEK cells. At 48 h post-transfection, cells were washed with PBS buffer and tested. Expression of enhanced green fluorescence protein (EGFP) was first visualized by fluorescent microscopy (Leitz Germany) and then the percentage of fluorescent emitting cells was determined by flow cytometry (BD, FACScan).

Bioinformatics analyses

Membrane topology and glycosylation sites of the recombinant protein was investigated using the TMHMM V2.0 (http://cbs.dtu.dk/services/TMHMM), NetNGlyc 3,1 (http://cbs.dtu.dk/services/NetNGlyc) and NetOGlyc 3.1 (http://cbs.dtu.dk/services/NetOGlyc) prediction servers, respectively.

Results

Construction of pEGFP-ZFpn expression plasmid

Constructed cDNA corresponding to *Fpn* was initially PCR-amplified. Restriction analysis of *Fpn* PCR product (1714 bp) with *Bam*HI and *Xmn*I enzymes resulted in the isolation of 1083 + 631 bp and 1070 + 644 bp fragments, respectively (Fig.1). Then *Fpn* PCR product was cloned into a TOPO TA vector, which led to the construction of TOPO-*Fpn* intermediate plasmid. Double digestion of TOPO-*Fpn* with *Xho*I/*Sma*I enzymes provided the *Fpn* gene with suitable ends for cloning in pEGFP-N1 plasmid, which contained the SV40 origin and hence was capable of replicating in mammalian cells expressing the SV40 T antigen. The final resulted plasmid (pEGFP-ZFpn) afforded the expression of *Fpn* and EGFP reporter under the direction of a CMV promoter in a single open reading frame (Fig. 2), which led to the production of Fpn-EGFP as a C-terminal fusion protein. Restriction enzyme analysis of pEGFP-Z*Fpn* with *Xho*I/*Sma*I enzymes produced the cloned 1705 bp PCR product (Fig. 3) and finally sequencing reactions confirmed the authenticity of pEGFP-Z*Fpn* fusion fragment (data not shown).

Expression and characterization of ZFpn protein

To confirm the expression of Fpn protein, pEGFP-Z*Fpn* plasmid was transfected into the HEK 293T cell line. Forty eight hours post-transfection the cells emitted the green fluorescence (Fig. 4a), which was distinct from the non-transfected control cells (Fig. 4b), under the immunofluorescence microscopy. Additionally, flow cytometric analysis of the transfected cells indicated that 68% of the cells express GFP.

Bioinformatic analysis of Fpn protein sequences

To further characterize the cloned *Fpn* gene (submitted to the GenBank with the accession No. of HM068067), its amino acid residues were determined for the corresponding nucleotide sequence using the Gene Runner v3.05 software. The predicted amino acid sequences of our expressed Fpn protein was also analyzed (Fig. 5). Furthermore, analysis of the protein sequences in NetN- and NetOGlyc server displayed the presence of 4 N-glycosylation and 78 O-glycosylation sites, respectively (data not shown).

To determine the topology of the recombinant protein, the obtained amino acid sequences were submitted to the TMHMM v2.0 prediction server (14). Data displayed that N- and C-terminal ends of Fpn were most likely cytosolic and the protein contains 8 transmembrane domains, as shown in Fig. 6.

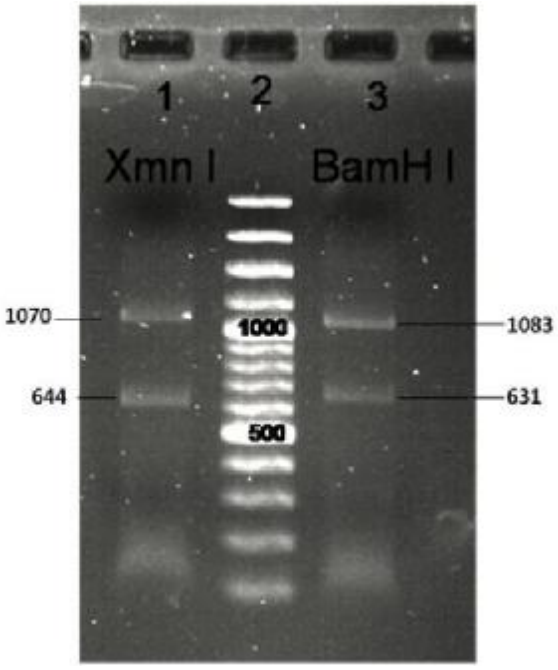

Fig. 1: Restriction enzyme pattern of TOPO-Fpn plasmid in 1.2% agarose gel.
Lane 1: digested plasmid with *Xmn*I (644 and 1070 bp products), Lane 2: 1-kb ladder, Lane 3: digested plasmid with *Bam*HI (631 and 1083 bp products)

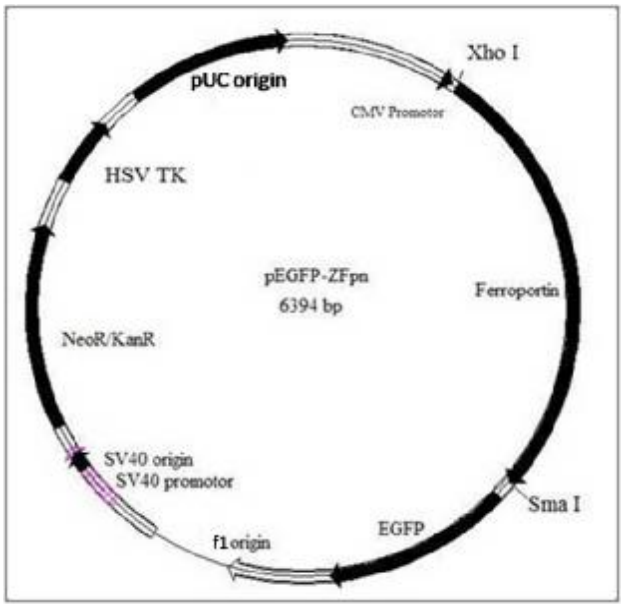

Fig. 2: pEGFP-ZFpn plasmid map. *Fpn* is cloned into the multiple cloning site of pEGFP-N1 vector by *Xho*I/*Sma*I double digestion. Ferroportin-EGFP fusion protein is expressed under the control of CMV promoter. Plasmid carries the f1 for single stranded DNA production and pUC origin of replication for propagation in prokaryote cells. SV40 origin also provides its efficient propagation in T antigen varying cell lines. HSV TK and NeoR/KanR denote to Herpes simplex virus thymidine kinase, neomycin and kanamycin resistance genes, respectively

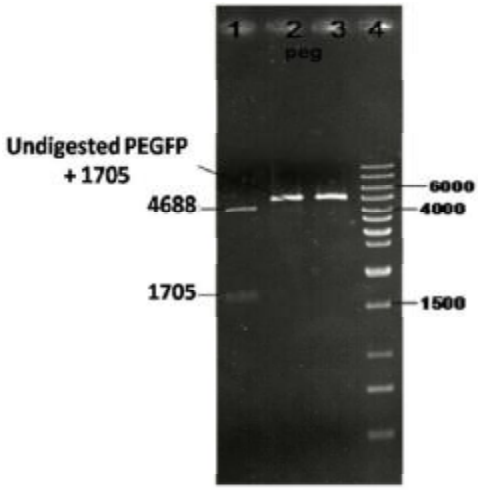

Fig. 3: Restriction enzyme pattern of pEGFP-ZFpn plasmid in 1.2% agarose gel. Lanes1: *Xho*I/*Sma*I double digested vector, Lanes 2 and 3 undigested vector, Lane 4: 1-kb ladder

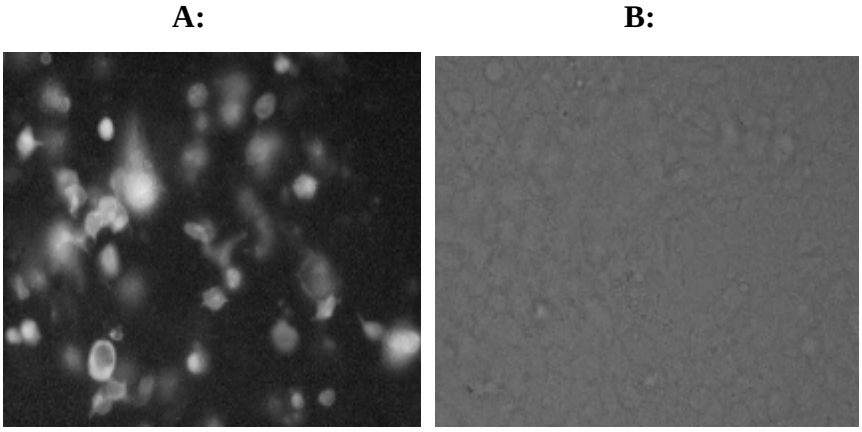

Fig. 4: Detection of pEGFP-ZFpn plasmid expression under immunofluorescence microscope. While the transfected HEK 293T cells showed the GFP emission (A), non-transfected control cells indicated no positive signals (B). Pictures are taken with the magnification of 1000×

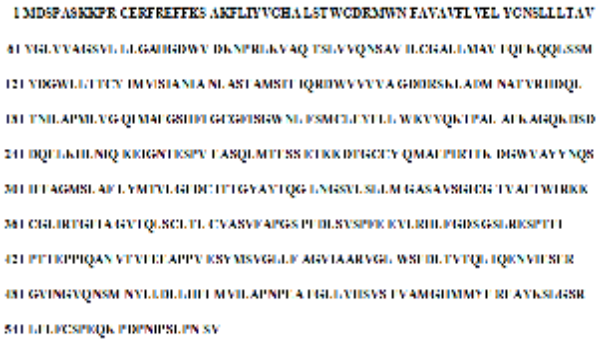

Fig. 5: Predicted amino acid sequences of zebrafish ferroportin, using the Gene runner software

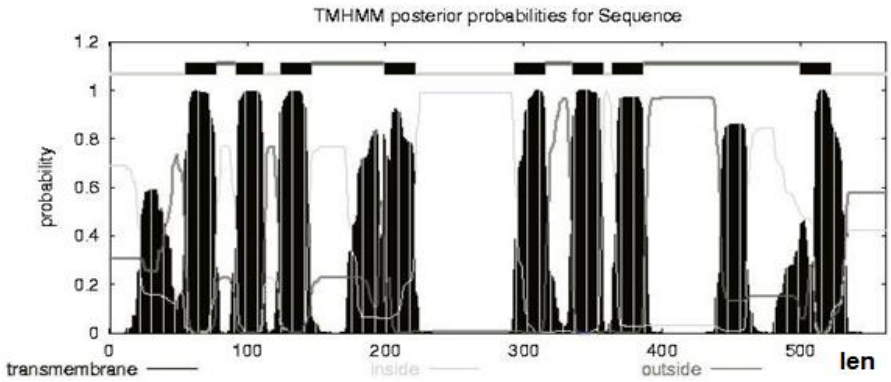

Fig. 6: Membrane topology of ferroportin. The plot was predicted with TMHMM v2.0 server shows the posterior probabilities of inside/outside/TM helices. The prediction gives the most probable location and orientation of transmembrane helices in the sequence at the top of the plot (between 1 and 1.2). The black lines indicate the number of predicted transmembrane helices. (len: the length of the protein sequence) which contains the 55-77, 92-111, 124-146, 199-221, 293-315, 335-357, 364-386, 500-522 aa and the number of predicted TMHs are 8. The light grey lines indicate the inside region of FPN which contains the 1-54, 112-123, 222-292, 358-363, 523-562 aa and the dark grey lines indicate the outside region of FPN which contains the 78-91, 147-198, 316-334, 387-499 aa

Discussion

Iron is needed for survival and growth of all living cells and most microorganisms. Also there is strong evidence that iron is one of the important elements involved in innate immunity. In response to immunity the host has evolved countermeasures to limit the availability of iron to the pathogen (1). Regulation of iron homeostasis in the body is mediated by different proteins including ferroportin (Fpn). Evidence shows that Fpn is predominantly expressed in enterocytes, spleen macrophages and liver kupffer cells. Fpn has been suggested to play an important role in uptake of new iron via enterocytes (4). Other reports suggest the influences of Fpn in the growth of some intracellular pathogens like *Salmonell enterica* (15). Preparation of a recombinant Fpn protein could be useful to study critical role of this protein in the survival and growth of intracellular pathogens.

In this study, we have used zebrafish gene to identify the multiple-transmembrane domain protein Fpn. Total RNA was extracted from duodenal cells of Indian zebrafish and cDNA was synthesized by RT-PCR using specific primers to obtain *Fpn* gene. Similar to other reports (7) and to simplify the detection of Fpn, the isolated gene was cloned into the eukaryotic GFP encoding vector for expression and GFP-based detection in HEK 293T cell line. The Fpn-GFP fusion protein was detected in transfected HEK cells which showed clear indication for *Fpn* expression. Considering that Fpn undergoes post-translational modifications (16) and is a large (>500 amino acids) cell-surface protein (17), we selected eukaryotic HEK 293T cell line for its expression. In fact mammalian cells would seem to be the safest choice for expressing a therapeutic protein in order to maintain the correct glycosylation, folding, and other post-translational modifications (18).

The amino acid sequence of the Fpn protein was deduced from its cDNA sequence using the Gene runner software and the result was compared with other reports. This gene, *ferroportin1*, has a predicted open reading frame of 562 amino acids. Although, our protein showed 100 % homology with data reported by Wood et al (Genebank accession No. CAQ15734.1), however two obvious differences in amino acid sequences was displayed when compared with data reported by Donovan et al (9). The first difference was the substitution of threonine instead of proline at position 272, and the second one was replacement of glutamine with lysine at position 400. In addition, the first (threonine) and the second (glutamine) amino acids were located inside and outside of the membrane respectively. Moreover, the predicted glycosylation sites of zebrafish Fpn presented by Denovan et al. (9) was compared with our Fpn sequences using Net NGlyc 3.1 and Net OGlyc 3.1 servers, and the results showed no differences in N-glycosylation sites, however, our Fpn sequences showed an additional O-glycosylation site at the residue of 272 which probably was due to replacement of threonine with proline. Our data showed no difference in N-glycosylation and O-glycosylation sites when compared with data of Wood et al in Gene Bank.

Considering the two mutations, membrane topology of Fpn and the number of transmembrane (TM) domains had to be determined, so we submitted the full length zebra fish Fpn protein sequence for transmembrane prediction using Hidden Markov Model (TMHMM v2.0) prediction server (13). The results reported by TMHMM showed 8 transmembrane domains for our zebrafish Fpn which differed from the reports of both Denovan et al (9), who calculated 10 TM domains and Rice et al. that had predicted 12 TM domains for the Fpn sequences (16). However, when the amino acid sequences of our zebrafish Fpn along with amino acid sequences of Fpn reported by Donovan el. submitted into TMHMM server, both Fpns showed 8 transmembrane domains with no differences in their topologies. Although,

Rice et al. had predicted 12 TM for zebrafish Fpn by using all probabilities for being a TM, but the TMHMM server had been marked 8 TMs for their zebrafish Fpn when they submitted the protein sequences to server (15). Therefore it seems that the replacement of two amino acids in our produced Fpn has no effect on the topology of the protein. Also using the prediction software TMHMM, we predicted that the N- and C-terminus of Fpn are cytosolic, not differing from prior predictions. Our model also places the 55-77, 92-111, 124-146, 199-221, 293-315, 335-357, 364-386, 500-522 aa in transmembrane helices, 1-54, 112-123, 222-292, 358-363, 523-562 aa as the inside region of Fpn and the 78-91, 147-198, 316-334, 387-499 aa as the outside region of Fpn.

In conclusion, this study described the cloning, successful eukaryotic expression and characterization of Indian zebrafish ferroportin. Our in silico analysis also predicted two new mutations within the amino acid sequence of expressed Fpn with no differences in topology of this protein. Following this study, the constructed pEGFP-Z*Fpn* plasmid will be used to investigate the influence of recombinant Fpn on the survival and growth of intracellular parasites in experimental model.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgement

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