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A novel exon generates ubiquitously expressed alternatively spliced new transcript of mouse *Abcc4* gene

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ABSTRACT

Abcc4 gene codes for a protein (ABCC4) involved in the transportation of different classes of drugs outside the cells. Various important drugs transported by ABCC4 include antiviral and anticancer drugs as well as endogenous molecules such as bile acids, cyclic nucleotides, folates, prostaglandins and steroids. Alternative splicing generates multiple mRNAs that encode protein isoforms having diverse functions. In this study, we have identified a novel transcript of mouse *Abcc4* gene using a combination of bioinformatics and molecular biology techniques. This transcript was found to be different from the reported transcript in having a different first exon that was found to be located on previously identified first intron. Newly identified transcript was found to be expressed across different tissues we studied and in different developmental stages. Expression level of novel and reported transcripts was studied using quantitative real-time PCR. After conceptually translating the novel transcript, various post-translational modifications were studied. Translation efficiency and predicted half life of encoded protein isoforms were analysed *in silico*. Molecular modelling was performed to compare the structural differences in both isoforms. The diversity at N-termini in these protein isoforms explains the diverse function of ABCC4 in mouse.

1. Introduction

Multiple drug resistance protein 4 (MRP4/ABCC4) is an ATP-binding cassette (ABC) transporter included in the subfamily C of the ABC transporter superfamily. MRP4 is encoded by *Abcc4* gene, which is involved in the transport of diverse sets of drugs outside the cell (Zhao et al., 2014). Various anticancer drugs that are transported by ABCC4 include 6-mercaptopurine, 6-thioguanine, camptothecins and others (Lin et al., 2013; Tian et al., 2005; Leggas et al., 2004). ABCC4 is reported to be overexpressed in retinoblastomas, neuroblastomas, gliomas, melanomas, colon cancer and colorectal cancer cells (Hendig et al., 2009; Holla et al., 2008). Moreover, ABCC4 expression is associated with drug resistance in leukaemia and ovarian cancer cells (Beretta et al., 2010). Recently, a study showed that downregulation of ABCC4 increased apoptosis in drug-resistant human gastric cancer cells, thereby restoring the sensitivity of the drug-resistant cancer cells to 5-FU (Zhang et al., 2015) while another study showed protective effect of ABCC4 against cytarabine-mediated damage in leukemic and host myeloid cells (Drenberg et al., 2016). ABCC4 is also reported to play an important role in cAMP homeostasis and related pathways (Belleville-Rolland et al., 2016). Such a diverse role of ABCC4 makes it an interesting target for research.

ABC transporters can be classified by presence of three sequence motifs located in their cytoplasmic ATP-binding domain or nucleoside binding domains (NBDs). These are Walker A motifs, Walker B motifs, and ABC signature motif (Haimeur et al., 2004). The core functional structure of most ABC transporters include two NBDs, nucleoside binding domains and two sets of membrane spanning domains (MSDs) typically containing six membrane-spanning alpha helices (Haimeur et al., 2004).

Alternative splicing plays an important role in creating diversity and regulating cellular functions (Kelemen et al., 2013; Nilsen and Graveley, 2010; Stamm et al., 2005). Recently, alternative splicing was reported to play an important role in creating functional diversity in ABCC1 (MRP1) of sea urchin (Gökirmak et al., 2016). In case of ABCC4, there is no study reporting the alternatively spliced isoforms in recent years. In this study, we have analysed the alternative splicing of the mouse *Abcc4* gene due to its important and diverse roles inside the cell. Mouse *Abcc4* gene is located on chromosome 14 and contains 31 exons that code for 1325 amino acids protein. According to consensus CDS project, two CCDS are reported, CCDS27335.1 and CCDS49565.1, that differ in exclusion of exon 4 in one of them. To study the alternative splicing of an *Abcc4* gene, we followed a novel methodology that combines the use of various bioinformatic tools and molecular biology techniques to predict and then confirm the existence of novel isoforms as described earlier (Banday et al., 2012). Using such methodology, we predicted and

Abbreviations: RT-PCR, Reverse transcriptase polymerase chain reaction; EST, Expressed sequence tags; PN, Postnatal; SMS, sequence manipulation suite; qRT-PCR, quantitative real-time PCR.

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confirmed one exon located in the intronic region between exon 1 and exon 2 of *Abcc4* gene that could participate in alternative splicing with other internal exons. With the help of various molecular biology techniques, we confirmed the expression of one new transcript in different tissues of mouse. Further, using various bioinformatics tools, prediction of several post-translational modifications of the novel isoform was performed.

2. Materials and methods

2.1. Computational analysis and prediction of novel exons

Our novel methodology involves the extensive use of various databases and gene/exon finding tools. *Abcc4* gene of mouse was studied for predicting exons that could participate in alternative splicing with existing exons leading to generation of novel isoforms. Genomic DNA, cDNA and protein sequences of Abcc4 were downloaded from Mouse Genome Informatics (MGI). Published exons and introns were first located on the genomic sequence. Upstream region of the gene was also analysed for putative novel exons using gene and exon finding tools like GENSCAN (Burge and Karlin, 1997), FGENESH (Solovyev et al., 2006) and FEX (Solovyev et al., 1994). These tools were able to identify the published exons with high score and served as a positive control. Several false positives arising in the prediction process were filtered out using the knowledge of comparative genomics and alternate splicing. The removal of redundant data was done carefully by manual curation. The new exons identified were then subjected to test code analysis available at sequence manipulation suite (SMS). Test code recognises the protein coding nature of the DNA sequences and is based on statistical correlation of nucleotide sequences to be coding or non-coding in nature (Fickett, 1982). Based on the results obtained from test code analysis, exons were chosen for confirmation by wet lab experiments involving RT-PCR and sequencing. Different online tools used in our study are GENSCAN (http://genes.mit.edu/GENSCAN.html), FGENESH (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind), FEX (http://linux1.softberry.com/berry.phtml?topic=fex&group=programs&subgroup=gfind), ClustalOmega (http://www.ebi.ac.uk/Tools/msa/clustalo/), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Sequence Manipulation Suite (http://www.bioinformatics.org/sms/), ExPASy translate tools (http://web.expasy.org/translate), ExPASy (http://www.expasy.org/proteomics), TermiNator (http://www.isv.cnrs-gif.fr/terminator3/index.html), TFBIND (http://tfbind.hgc.jp/). Exon specific primers were designed and synthesised (Sigma Aldrich, Bangalore, India) and were as follows.

Primer	Sequence (5′-3′)	Direction	Primer location
FC	CTG GTC ATA AGC GGA GAC TGG AA	Forward	Exon E2
FE1A	GTG TTT TCA CCA ACG TCC AGG AAG	Forward	Exon E1A
FE1B	ATG CTG CCG AGT GAG GTG GTG AA	Forward	Exon E1B
R1	GCA GAG GCA GAA GAA TAA CCA GAA C	Reverse	Exon E6
R2	CTG CCC ACA GAA AGT GCA AGA AG	Reverse	Exon E6

2.2. Preparation of RNA from different tissues of mouse

Total cellular RNA was isolated from different mouse tissues using RNA extraction kit (Intron Biotechnology, Gyeonggi-do, Korea) according to manufacturer's instructions. Isolated RNA was dissolved in diethyl pyrocarbonate treated water and quantitated spectrophotometrically. Integrity of RNA was confirmed by denaturing agarose gel electrophoresis. RNA prepared was either used immediately or stored at −80 °C. Animal experimentations were permitted by the Ministry of Environment and Forests, Government of India under registration no. 714/02/a/CPCSEA. It was issued by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) dated 25th October,

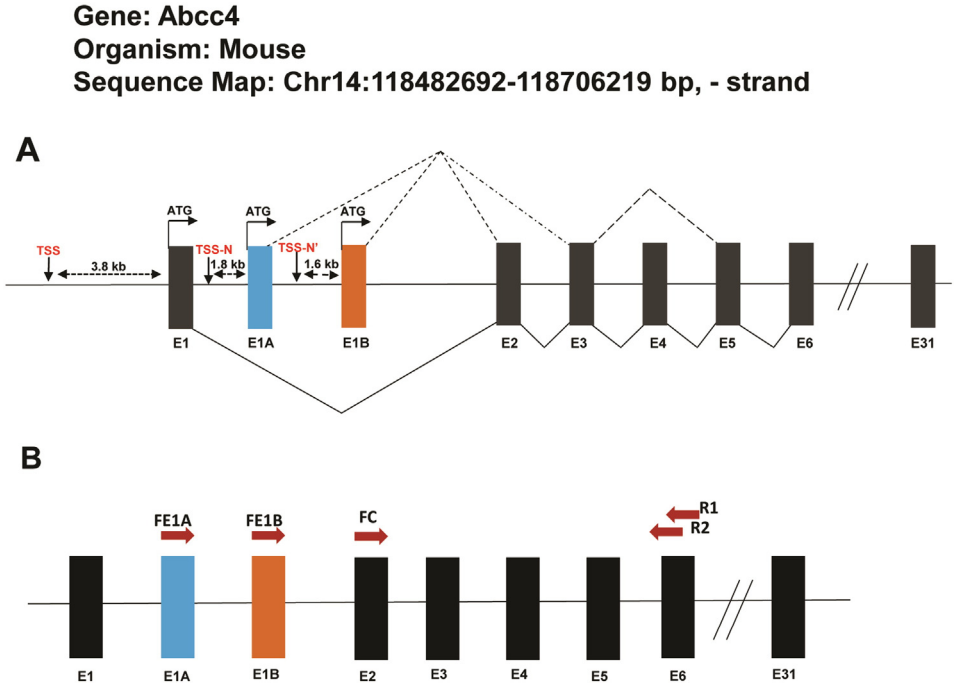

Fig. 1. Exon-intron organisation and alternative splicing pattern of published and newly predicted Abcc4 transcripts of mouse. (A) Exons are represented with rectangular boxes and inter connecting lines represent introns. Known exons (E1 to E31) are shown in black boxes whereas coloured boxes represent newly predicted exons. Sizes of at the exons/introns are not to scale. Dashed lines show the splicing pattern, predicted to generate two new transcripts having either exon E1A or exon E1B as the 1st exon at the 5′ end along with a known transcript having exon E1 as first exon at the 5′ end. TSS is putative transcription start site for known variant containing E1 while TSS-N and TSS-N' is putative transcription start site for novel variants containing E1A and E1B respectively. (B) Designing of forward and reverse primers. Control forward primer (FC) was designed from published exon 2 region. Reverse primer R1 was from exon 6 while reverse primer R2 was located internally (from exon 6) to R1. R2 primer was designed for semi-nested PCR. Forward primers from newly predicted exons, FE1A and FE1B were designed from newly predicted exons E1A and E1B respectively.