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Research article

In silico investigation of the impact of synonymous variants in *ABCB4* gene on mRNA stability/structure, splicing accuracy and codon usage: Potential contribution to PFIC3 disease

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ABSTRACT

Progressive **F**amilial **I**ntrahepatic **C**holestasis type **3** (PFIC3) is an autosomal-recessive liver disease due to mutations in the *ABCB4* gene encoding for the MDR3 protein. In the present study, we performed molecular and bioinformatic analyses in PFIC3 patients in order to understand the molecular basis of the disease. The three studied patients with PFIC3 were screened by PCR amplification followed by direct sequencing of the 27 coding exons of *ABCB4. In silico* analysis was performed by bioinformatic programs. We revealed three synonymous polymorphisms c.175C > T, c.504C > T, c.711A > T respectively in exon 4, 6, 8 and an intronic c.3487-16T > C variation in intron 26. The computational study of these polymorphic variants using Human Splicing Finder, ex-skip, Mfold and kineFold tools showed the putative impact on the composition of the *cis*-acting regulatory elements of splicing as well as on the mRNA structure and stability. Moreover, the protein level was affected by codon usage changes estimated by the calculation of ΔRSCU and ΔLog Ratio of codon frequencies interfering as consequence with the accurate folding of the MDR3 protein. As the first initiative of the mutational study of *ABCB4* genes in Tunisia, our results are suggestive of a potential downstream molecular effect for the described polymorphisms on the expression pattern of the *ABCB4* underlining the importance of synonymous variants.

1. Introduction

Progressive **F**amilial **I**ntrahepatic **C**holestasis type **3** (PFIC3) is a rare autosomal recessive liver disease with a prevalence 1/100000. It's characterized by early onset of persistent chronic cholestasis leading to cirrhosis and liver failure before adulthood and often requires liver transplantation (Delaunay et al., 2007). Liver histology of PFIC3 patients showed a portal fibrosis and a significant bile duct proliferation with a mixed inflammatory infiltrate (De Vree et al., 1998).

PFIC3 is caused by mutations in *ABCB4* gene; a gene located on 7q21.1 with 27 coding exons from exon 2 to exon 28 (Lincke et al., 1991). This gene encodes a liver-specific canalicular transporter, the **M**ulti-**D**rug **R**esistant **3** (MDR3) that mediates the translocation of phosphatidylcholine through the canalicular membrane. This MDR3 floppase activity provides a protection for cells membranes against the detergent activity of bile salts micelles (Oude Elferink and Paulusma, 2007). Overall, the MDR3 deficiency is related to three distinct hepatobiliary diseases: LPAC, ICP and PFIC3 (Sundaram and Sokol, 2007). The mutational screening of patients presenting typical syndromes of one of these diseases shows the absence of *ABCB4* mutations in a significant proportion suggesting that synonymous SNPs may influence the correct expression of the *ABCB4* gene leading to the pathogenic phenotype (Degiorgio et al., 2007; Rosmorduc et al., 2003). Thereby, according to statistics provided by **N**ational **C**enter for **B**iotechnology **I**nformation (NCBI), nearly 600 polymorphisms were detected in the coding region of *ABCB4* gene including 35% synonymous ones. Those polymorphisms are still unexplored compared to other explored SNPs in genes belonging to the same family of ABC transporter especially the *ABCB11* gene (Davit-Spraul et al., 2014).

Synonymous SNPs may affect the mRNA stability and splice process as well as the protein folding and/or level through codon

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usage change (Cartegni et al., 2002; Komar et al., 1999). Besides, previous functional studies showed that synonymous variants have been identified to be responsible for several diseases such as silent polymorphic variations in *COMT, ACADM* and *CARD15* genes leading respectively to pain sensitivity (Nackley et al., 2006), improved beta-oxidation of medium-chain fatty acids (Bruun et al., 2013) and severe pulmonary sarcoidosis (Sato et al., 2013).

In the present study, which is the first screening of PFIC3 patients in Tunisia, we revealed synonymous polymorphisms in the *ABCB4* gene. Computational analyses provide a strong evidence about the putative effect of these polymorphic variants by their potential interference with the pre-mRNA splicing process and mRNA stability added to their impact on codon usage which could lead to an impaired protein folding.

2. Patients and methods

2.1. Patients

Our study concerned three Tunisian patients from three unrelated consanguineous families. The three patients were examined in the service of pediatric-C.H.U Hedi Chaker-Sfax and were included in the study on the basis of typical clinical features of PFIC3 disease. In fact, they showed symptomatic cholestasis with pruritus and icterus associated with increased values of γ-GT level at presentation compared to the normal values ranging from 7 to 27 U/L. The immunohistochemical staining of patients livers were also performed as a clinical practice using the specific rabbit polyclonal antibody α-REG1, against the N-terminal part of the MDR3 (Marleen et al., 1998). The biological and histochemical data referring to these patients were provided in Table 1.

2.2. Methods

2.2.1. DNA extraction

Blood samples for DNA extraction were obtained from the three patients. Total genomic DNA of peripheral blood leukocytes was extracted using phenol-chloroform procedure according to a previously described protocol (Lewin and Stewart-Haynes, 1992).

2.2.2. Polymerase chain reaction

To amplify the 27 coding exons of the *ABCB4* gene oligonucleotide primers were designed using the primer3 available at http://primer3.ut.ee/. Classic and touchdown PCR reactions were performed in a thermal cycler (Perkin Elmer Gene A PCR System 9700). Reactions were cycled 35 times with denaturation at 95 °C for 35 s, hybridization for 35 s and extension at 72 °C for 45 s. The reaction was carried out in a final volume of 25 μl containing 0.05 μg of genomic DNA, 10 mM dNTP, 25 mM $MgCl_2$, 20 pmol of each primer, 5 μl of 5x buffer, and 0.5 unit of Go Taq DNA polymerase (Invitrogen).

2.2.3. Molecular and in silico analyses of the ABCB4 gene

2.2.3.1. Mutation screening. PCR products of coding exons and all splice junctions of *ABCB4* were purified using an exonuclease before the direct sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM/Biosystems).

2.2.3.2. Bioinformatic analysis. The blast homology searches were performed to compare our sequences with the wild-type one and to identify and locate variations within the *ABCB4* gene.

The use of computational methods is becoming increasingly important for genetic variations found in *ABCB4* to predict and understand their downstream effects on the splicing as well as on the transition process.

The **H**uman **S**plicing **F**inder **HSF** version 3.0 evaluates the putative splicing defects caused by a given substitution (François-Olivier et al., 2009). Moreover, Ex-skip tool takes into account all the sequences **E**xonic **S**plicing **E**nhancers (ESE) and **E**xonic **S**plicing **S**ilencers (ESS) to elaborate a comparison of the total ESS/ESE profile between the normal and the mutated sequence. Based on this comparison, it provides a conclusion about the allele having the highest chance to cause exon skipping (Raponi et al., 2011).

In the other part, the impact of some genetic variants on pre-mRNA structure and mRNA stability was predicted using both Mfold (Zuker, 2003) as a static secondary structure predictor and kineFold as a stochastic secondary structure predictor (Xayaphoummine et al., 2005). Predictions performed by theses software's associate the thermodynamic details ΔG to each predicted structure. A low ΔG value characterizes a stable folding and in each case, the most stable structure was chosen. Further, stability changes induced by exonic variant in the mRNA were assessed by the calculation of $\Delta\Delta G = \Delta G_{variant} - \Delta G_{WT}$. This calculation was performed for different sizes of wild-type *ABCB4* fragments and also for the mutated ones.

For the protein level, changes in codon frequencies were quantified via the calculation of RSCU (**R**elative **S**ynonymous **C**odon **U**sage) and the Log Ratio of codon frequencies followed by a calculation of ΔRSCU and Δ Log Ratio,respectively. All the details concerned the calculations of these two parameters were described in a previous study (Nathan et al., 2012).

To ensure the evaluation of codon frequency changes, the normal frequency of codons through the human *ABCB4* cDNA was determined through the "Sequence Manipulation Suite" site (http://www.bioinformatics.org/sms2/codon_usage.html). Furthermore, the distribution of clusters of frequent and rare codons in this gene was carried out via%MinMax algorithm. Thus, the location of the altered codons within these clusters became possible and provides the best interpretation of results given by the previous calculations (Clarke and Clark, 2008).

Table 1
Biological and histochemical data of the PFIC3 studied patients.

Patients	Sex/Age at onset (years)	First symptoms	Serum concentration of GGT (U/L)	MDR3 detection	outcome to UDCA treatment
P1	M/3	Pruritus, Jaundice	103	+	favorable
P2	F/2,5	Jaundice, hepatomegaly	155	+	favorable
P3	F/4	Pruritus, Jaundice, portal hypertension.	110	+	favorable

GGT: gamma glutamyltransferase.
+: presence of the MDR3 protein in the patient's liver with Immunohistochemical staining.
UDCA: ursodeoxycholic acid.