Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2) in Children with ABCB11 Gene Mutations and Bile Salt Export Pump Dysfunction

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Abstract

Background: Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2) is a rare inherited disease due to mutations in ABCB11 encoding the canalicular Bile Salt Export Pump (BSEP) of hepatocyte which is the major transporter responsible for bile acid excretion. Impaired biliary excretion leads to bile salt accumulation in hepatocytes, ongoing hepatocellular damage and increased risk of hepatocellular carcinoma.

Aim of Work: To detect mutations in Exon 8 of ABCB 11 gene in patients with suspected PFIC2 among the selected group of Egyptian population to confirm diagnosis of PFIC2 and to detect mutations that impair BSEP protein function and bile acid excretion.

Patients and Methods: Thirty three subjects were enrolled in this study including 11 suspected PFIC2 patients and 22 healthy control subjects. ABCB 11 genotyping was performed by DNA extraction followed by PCR amplification, purification then sequencing of Exon 8 of the gene.

Results: No mutations or variations in sequence results involving Exon 8 of the ABCB 11 gene of studied Egyptian populations were detected.

Conclusion: This work detected no mutations or variations involving Exon 8 of ABCB 11 gene in studied Egyptian populations were detected.


Introduction

PROGRESSIVE Familial Intrahepatic Cholestasis type 2 (PFIC2) is a rare inherited disorder characterized by progressive liver disease that typically manifest in childhood as impaired bile flow in the absence of hepatobiliary structural abnormality [1].

Progressive familial intrahepatic cholestasis type 2 is caused by mutations in the ABCB 11 gene. The ABCB 11 gene encodes the ATP-dependent canalicular bile salt export pump of human liver and is located on human chromosome 2 [2].

The primary transporter responsible for bile salt secretion is the bile salt export pump, a member of ABC superfamily, which is located at the bile canalicular apical domain of hepatocytes [3].

Bile salt export pump consists of 1321 amino acids with a molecular mass of approximately 160kDa. Like other full-length transporters of the ABC superfamily, BSEP is predicted to be a duplicated structure with each half of the molecule consisting of six predicted TMD and a large cytoplasmic NBD in a TMD-NBD-TMD-NBD organization [4].

Mutations in ABCB 11 is responsible for the decreased biliary bile salt secretion described in affected patients, leading to decreased bile flow and accumulation of bile salts inside the hepatocyte with ongoing severe hepatocellular damage [5].

Severe phenotypes are often associated with mutations leading to premature protein truncation or failure of protein production. Insertion, deletion, non sense and splicing mutations result in damaging effects and patients exhibited little or no detectable BSEP at the hepatocyte canaliculus. Missense mutations are also common defects that either affect protein processing and trafficking or disrupt functional domains and protein structure [5].
Patients with PFIC2 typically present with severe jaundice, hepatomegaly, failure to thrive, and pruritus. Laboratory work up show direct hyperbilirubinemia, elevated serum aminotransferases, and paradoxically normal serum gamma glutamyl transpeptidase and cholesterol concentrations. Biliary bile acid concentrations are less than 1% of the normal. There is a rapid progressive course to cirrhosis, liver failure and hepatocellular carcinoma, which can only be cured by liver transplantation [7].

The identification of ABCB 11 mutations is the only way to confirm diagnosis of PFIC2 in suspected patients by clinical, laboratory, radiological and histological findings and to differentiate also between PFIC2 and PFIC1 patients who show similar clinical and laboratory findings [8].

All these help for close follow-up of these patients that usually show rapid progression to liver cirrhosis and hepatocellular carcinoma [9].

**Patients and Methods**

The current study was approved by the Ethical Committee of Faculty of Medicine, Cairo University. It was done over the years 2011 to 2013 on 33 subjects, eleven suspected PFIC2 patients who were attending the Pediatric Hepatology Unit in Cairo University Pediatric Hospital, and 22 control subjects. The objective of the study was clearly explained to all the patients participating in the study and parents of patients signed an informed consent before participating in the study.

Diagnosis was based on history chronic cholestasis in the form of recurrent episodes of jaundice from early months of life that become permanent later in the course of the disease. Severe pruritis is commonly observed. Clinical manifestations together with normal (GGT) activity, very high serum bile acid concentration, radiological and histological approaches suggested the diagnosis especially after exclusion of other main causes of cholestasis in children.

*All patients were subjected to:*

- Full history taking including: Age at presentation, familial pedigree, familial illness, current age. Clinical features including: Continuous jaundice or intermittent, pruritis and its severity, hepatomegaly, splenomegaly, diarrhea, hearing loss, manifestation of vitamins (A, D, E, K) deficiency. Laboratory investigation: GGT, AST, ALT, Bilirubin, serum bile acids. Radiological examination including: Abdominal U/S. Histological examination in the form of: Liver biopsy. Finally molecular investigation included DNA sequencing of ABCB 11 gene (Exon 8) for patients and controls.

Three ml of venous blood were collected from each subject in a sterile EDTA vacutainer for the genotyping technique. DNA was extracted from fresh samples to be used for performing the PCR for ABCB 11 gene study. Sequencing of Exon 8 of ABCB 11 gene required the following steps: DNA extraction, amplification using the polymerase chain reaction, detection of PCR amplification products, DNA purification, cycle sequencing, long read capillary electrophoresis and finally analysis of data.

**Amplification of Exon 8 of ABCB11 gene by Polymerase Chain Reaction (PCR):**

Genomic DNA was extracted from peripheral blood EDTA samples by QIAamp DNA extraction kit (QIAGEN GmbH, Germany) according to manufacturer’s instructions. Amplification of the extracted DNA was done according to the protocol proposed by Thompson et al., [10] followed by detection of PCR amplification products using 1.5% agarose gel electrophoresis containing ethidium bromide and ultraviolet light transillumination. Using HOT FIREPol (10X) Master Mix (Solis BioDyne, Tartu.Estonia) which contain: Ready to use HotStart Taq DNA polymerase (recombinant) mixture, optimized HotStart PCR buffer, MgCl2 and dNTPs. Forward and reverse primers (supplied by Bioscience GmbH, Jena, Germany) and annealing temperature for Exon 8 of ABCB 11 gene are described in (Table 1).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Exon 8</th>
</tr>
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<tbody>
<tr>
<td>Forward primer</td>
<td>5'-AGGGTGATAGGGAGAGAGAGAG-3'</td>
</tr>
<tr>
<td>Reversed primer</td>
<td>5'-GCTAACTGTACTCAGAAAAAGGGACTC-3'</td>
</tr>
<tr>
<td>Amplification protocol</td>
<td>Initial activation at 95°C for 15 minutes.</td>
</tr>
<tr>
<td></td>
<td>- 34 cycles of:</td>
</tr>
<tr>
<td></td>
<td>- 94°C for 45 seconds.</td>
</tr>
<tr>
<td></td>
<td>- 62°C for 45 seconds.</td>
</tr>
<tr>
<td></td>
<td>- 72°C for 1 minute.</td>
</tr>
<tr>
<td></td>
<td>- Final elongation at 72°C for 1 minute.</td>
</tr>
</tbody>
</table>

Reactions were performed in a total volume of 25μL containing: 2μl PCR HotStart Master Mix (10X), 1μl forward primer (100pmol/μl), 1μl reverse primer, 14μl nuclease-free water and 7μl purified DNA solution. Gradient thermal cycler (Professional Thermocycler, Biometra; Applied Biosystems, Foster City, CA, USA) was programmed according to the following conditions:
Initial activation at 95°C for 15 minutes, then 34 cycles of denaturation at 94°C for 45 seconds, annealing at 62°C for exon 9 for 45 seconds, extension at 72°C for 1 minute. Final elongation was done at 72°C for 1 minute. The DNA quality was examined using 1.5% agarose gel electrophoresis, the amplified DNA ran as a single bright band on an agarose gel which indicated successful amplification.

**Cycle sequencing and capillary electrophoresis (on the ABI 3500 genetic analyzer):**

The amplified DNA was purified using QIAquick PCR purification kit supplied by Qiagen, Germany. Then Frederick Sanger’s enzymatic dideoxy DNA sequencing technique was applied which was based on the chain-terminating dideoxynucleotide analogues [11] using ABI PRISM Big Dye Terminator cycle Sequencing Ready Reaction Kit v 3.1. (Contain dideoxynucleotides) and BigDye 5X dilution buffer supplied by Applied biosystem (Life Technologies Corporation, California, USA).

For each reaction the following reagents were added to a separate tube: 2.0 μl of BigDye Terminator, 1.0 μl of 5X Dilution buffer, 2.0 μl of the forward Primer used for PCR reactions, 2.0 μl of template DNA, and 3.0 μl Nuclease free water to complete the total volume to 10 μl.

The thermal cycler was adjusted to the following conditions: Initial denaturation at 96°C for 1 minute followed by 25 cycles of: Denaturation at 95°C for 10 seconds, annealing temperature 62°C for Exon 8 for 5 seconds, and extension at 72°C for 4 minutes. Then purification step was performed using BigDye X terminator purification kit supplied by Applied Biosystem to sequester unincorporated dye terminators and dNTPs to prevent their co-injection with dye-labeled extension products. The “cleaned up” sequencing reactions were resuspended in 15 μl of Hi Di formamide for denaturing DNA before injection, then it was injected in Applied Biosystems 3500 Genetic Analyzers Fig. (1).

![Fig. (1): The sequence results of PCR product of Exon 8 of suspected PFIC2 patient no (10).](image-url)
**Analysis of data:**

Sequences were compared to the published sequence (NM-003742.2). According to NM-003742.2 Exon 8 is from 738 to 909 nucleotide base. According to Homo sapiens ATP-binding cassette, sub-family B (MDR/TAP), member 11 (ABCB 11), mRNA by NCBI (National Centre of Biotechnology Information) [12]. Reference Sequence: NM-003742.2 which is 4775bp mRNA and its translated protein (bile salt export pump) NP-003733.2 which is 1321 amino acid analysis was done by BLAST [Basic Local Alignment Search Tool] [12] (www.ncbi.nlm.nih.gov) and the CLC-BIO sequence viewer 6 program (www.clcbio.com).

**Statistical methods:**

Data obtained from the study was coded and entered using the software SPSS (Statistical package for social science) version 17. (SPSS, Inc. Chicago, IL, USA) parametric data were summarized using mean and standard deviation, while non-parametric data were summarized as median and percentiles for quantitative variables. Frequency and percentages were used for qualitative variables. Comparison between groups was done using Chi square and Fischer exact test for qualitative variable, t-test and non-parametric MannWhitney test were used to compare two groups. p-value was considered significant if <0.05.

**Results**

**Clinical data:**

This cross-sectional study was performed on 11 suspected PFIC2 patients with chronic intrahepatic cholestasis presented to Pediatric Hepatology Unit at Cairo University Pediatric Hospital with unexplained cholestasis and normal GGT. Suspected PFIC2 patients their age ranged (1-6) years. They were suspected to be diagnosed as PFIC2 before the age of 3 years. Ten cases (90.9%) were males and 1/11 (9.1%) was a female. Eight of cases were positively consanguinous (72.7%), one of these families there was one sibling died at 6 month and he was jaundiced. The (control group) consisted of 22 subjects, 11 males (50%) and 11 (50%) females. Biochemically, all patients had high bilirubin levels (total and direct), normal GGT, and elevated transaminases (AST, ALT) with characteristic increased level of serum bile acid concentration (Table 2).

Radiologically, two patients (18.2%) had average size liver and spleen, seven of them (63.6%) had hepatomegaly, and two had hepatosplenomegaly (18.2%) detected by abdominal ultrasound. Pathologically, one of the patients (number 10 among subjects) showed developed chronic hepatitis with impending fibrosis. Two of patients 2/11 (18.2%) showed Paucity of Intralobular Bile Ducts (PIBD). Six of cases 6/11 (54.54%) had blurred lobular architecture, intracellular and canaliculial cholestasis together with diffuse ballooning degeneration of hepatocytes (Table 3).

**DNA sequencing:** The DNA sequence analysis of Exon 8 of the ABCB 11 gene revealed no mutations or variations in sequence results involving phenotypically normal control subjects and suspected PFIC2 patients as shown in (Table 4).

<table>
<thead>
<tr>
<th>Data</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at presentation (months)</td>
<td>6.5±10.2</td>
<td>36</td>
</tr>
<tr>
<td>Current age (years)</td>
<td>3.4±1.8</td>
<td>1-6</td>
</tr>
<tr>
<td>Alanine Transaminase (ALT)</td>
<td>100.3±88.1</td>
<td>47-350</td>
</tr>
<tr>
<td>Aspartate Transaminase (AST)</td>
<td>129.7±143.5</td>
<td>55-512</td>
</tr>
<tr>
<td>Bilirubin (total and direct)</td>
<td>T: 8.0±3.99</td>
<td>3.5-14.6</td>
</tr>
<tr>
<td></td>
<td>D: 5.2±2.4</td>
<td>2.7-10.0</td>
</tr>
<tr>
<td>Serum bile acids</td>
<td>223.7±177.26</td>
<td>19-522</td>
</tr>
</tbody>
</table>

**Table (3): Ultrasound and liver biopsy results among patients.**

<table>
<thead>
<tr>
<th>Data</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/S findings:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>7/11</td>
<td>63.6</td>
</tr>
<tr>
<td>Hepatosplenomegaly</td>
<td>2/11</td>
<td>18.18</td>
</tr>
<tr>
<td>Average size liver &amp; spleen</td>
<td>2/11</td>
<td>18.18</td>
</tr>
<tr>
<td>Liver biopsy result:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal hepatitis</td>
<td>6/11</td>
<td>54.54</td>
</tr>
<tr>
<td>PIBD</td>
<td>2/11</td>
<td>18.18</td>
</tr>
<tr>
<td>Not available</td>
<td>3/11</td>
<td>27.27</td>
</tr>
</tbody>
</table>

**Discussion**

Progressive familial intrahepatic cholestasis type 2 is an autosomal recessive condition that disrupt bile transport. BSEP deficiency (PFIC2) is among disorders with low serum concentrations of γ-glutamyltransferase activity despite conjugated hyperbilirubinemia, as in familial intrahepatic cholestasis 1 deficiency caused by mutations in ATP8B1 [13].
It was explained that young patients with severe BSEP deficiency syndrome are at risk to subsequently develop Hepatocellular Carcinoma (HCC) and cholangiocarcinoma, with the reported cases of HCC being much greater than cholangiocarcinoma. These patients frequently present with splice site changes, deletion, insertions, and nonsense mutations that result in the absence of functional protein. This absence results in elevated levels of intracellular bile salts that have been shown to influence many aspects of cell function, including mitochondrial function, cell cycle, DNA repair, homeobox gene activation, cell polarization and differentiation [14].

Many polymorphisms in ABCB 11 have been described in a population with different ethnic distribution all over the world with respect to allele number, frequency of common and population-specific sites. These polymorphisms are located in exons and introns, as well as in 5′-flanking regions, but no effect on the mRNA or protein has been determined. However, population genetic analysis suggested some selective pressure against changes in the protein, supporting the important endogenous role of these transporters [15].

Many different ABCB 11 disease causing variants have been detected worldwide, most of them are mutations grouped as missense, nonsense, deletions and insertions, and splice-site mutations. The results of these gene mutations is the decrease or total loss of expression of the BSEP protein on the hepatic canalicular membrane [4].

In this study, 33 Egyptian subjects were investigated; twenty two control subject (22/33), eleven subjects (50%) were males and 11/22 (50%) were females, and eleven suspected PFIC2 patients (11/33), their age ranged from 1 to 6 years. All patients were subjected to full history taking including: Age at presentation, familial pedigree, familial illness, current age, clinical features including: Continuous jaundice or intermittent, pruritus and its severity, hepatomegaly, spleenomegaly, diarrhoea, hearing loss, manifestation of vitamins (A, D, E, K) deficiency, results of GGT, AST, ALT, Bilirubin, serum bile acids, radiological, histological examination and laboratory investigation included DNA sequencing of ABCB 11 gene (Exon 8) for patients and controls.

We investigated the possible association of mutations in Exons 8 which is considered site for common mutations of ABCB 11 gene as a cause of ABCB 11 transporter functional deficiency that leads to Progressive Familial Intrahepatic Cholestasis type 2 (PFIC2) among studied Egyptian children and our study revealed no mutations or variations in the sequencing results involving Exons 8 of the ABCB 11 gene of studied PFIC2 patients and phenotypically healthy subjects.

The clinical phenotype of Exon 8 of ABCB 11 gene encodes for TMD4 in the BSEP protein [4]. Our results were similar to the results of Lang et al., [15] who studied genetic variability and haplotype structures of ABCB 11 in 292 healthy populations of different ethnic backgrounds in which they detected 28 genetic variants in 27 coding Exons and 1 noncoding exon; among these were 10 missense mutations, 17 silent mutations and 1 mutation in the untranslated exon, with the two Casasian-specific variants in two exons not including exon 8. The two Casasian-specific variants were in exon 13 (c.1331T>C; 59.4%) and exon 17 (c.2029A>G; 4.2%) coded for amino acid substitutions p.V444A and p.M677V respectively. All amino acid polymorphisms of ABCB 11 were predicted to be located in the extracellular region.

On the contrary, many collaborative studies have identified mutations in Exon 8 of ABCB 11 producing marked BSEP protein deficiency [4,6,16].

Lam et al., [4] made a molecular model of BSEP illustrating some of the most common ABCB gene mutations that may result in a clinical phenotype of PFIC2 with marked protein deficiency; p.G238V (located in TMD4 and encoded by Exon 8), p.E297G (located in intracellular span between TMD4 and TMD5 and encoded by Exon 9), p.D482G (located in NBD1 and encoded by Exon 14), p.G982R (located at TMD 11 and encoded by Exon 23), and p.R1268Q (located in NBD2 at conserved signature C motif and encoded by Exon 28).

Strautnieks et al., [6] identified Eighty-two different mutations (52 novel) (9 nonsense mutations, 10 small insertions and deletions, 15 splice-site changes, 3 whole-gene deletions, 45 missense changes); p.G23 8V (located in TMD4 and encoded by Exon 8) were included in missense mutation results.

In addition, Byrne et al., [16] classified the majority (63) of known ABCB 11 mis-sense mutations and 21 Single-Nucleotide Polymorphisms (SNPs), e.g. (p.G260D, p.E297K, p.D482G R832C, p.A1028A, p.S1144R, and p.R1153H) and they found reduced wild-type splicing for 20 mutations/SNPs, with normal mRNA levels reduced to 5% or less. The common ABCB 11 missense mutation encoding p.D482G enhanced aberrant splicing.
whereas the common SNP p.A1028A promoted exon skipping. Addition of exogenous splicing factors modulated several splicing defects of the mutants expressed in vitro, most appeared to be retained in the endoplasmic reticulum and degraded. All these mutations can result in truncated or misfolded BSEP proteins. Thus, primary defects at either the protein or the mRNA level (or both) contribute significantly to BSEP deficiency. These results will help to develop mutation-specific therapies for children and adults suffering from intrahepatic cholestasis due to BSEP deficiency.

Conclusion:
In conclusion, this work detected no mutations or variations involving Exon 8 of ABCB11 gene in studied Egyptian patients with PFIC2 as a cause of severe BSEP deficiency, so further investigation of other exons of the gene are necessary to confirm diagnosis of PFIC 2 and to prove that inspite of considering exon 8 mutations are common worldwide, but these mutations are not that common among Egyptian ethnic population, however larger sample size is needed.

References
الخلاصة: يعد مرضى احتباس العصارة الصفراوية الوراثي المتضاد الكبد من النوع الثاني (PFIC2) مرضًاً وراثيًاً نادراً يتميز بكثرة ناجمة عن مرض بالكبد تظهر أعراضه بشكل نموذجي أثناء فترة الطفولة على هيئة خلل في تنقية العصارة الصفراوية. يحدث هذا المرض بسبب طفرة في الجين (ABC11) (PFIC2) في الكبد البشري وتفرز هذه المضخة البروتين الخاص بها عند الفشل الشامل ما بين خلايا الكبد والأنبوات المرارية. حدوث الطفرة في هذه المضخة هو المسؤول الأول عن نقص إفراز أملاح العصارة الصفراوية والذي يحدث بشكل واضح في المرضى الذين يتأثرون بهذا المرض، والذي يؤدي بدوره إلى توقف تنقية العصارة الصفراوية وتراكم الأملاح الصفراوية داخل خلايا الكبد مع حدوث تلف شديد في خلايا الكبد مما يؤدي إلى اورام بالكبد.

الهدف من البحث: وعندت هذه الدراسة إلى الكشف عن الطفرات التي تحدث في (Exon 8) للجين (ABC11) في مرضى (PFIC2) من النوع الثاني بين المصريين الذين تسببت هذه الطفرات في خلل في المضخة الصفراوية وتفاقم الاملاح الصفراوية من الكبد.

طريقة البحث: وضعت حوالي (32) مصرى تلك الدراسة منهم (22) كمجموعة تحكم و (11) من مرضى (PFIC2) والفحوصات الفعالة، بما فيها تتابع الحمض النووي DNA لكل من المرضى ومجموعة التحكم.

(Exon 8 of the ABC11 gene) نتائج البحث: لم تظهر الدراسة أي طفرات أو اختلافات في النتائج: الجين للمرضى المصريين والأنبوات الخارجية للأفراد الأصحاء.