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A new splicing site mutation of the ABCB4 gene in intrahepatic cholestasis of pregnancy with raised serum γ-GT

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ABSTRACT

Background: Intrahepatic cholestasis of pregnancy is a liver disorder with a multifactorial etiology characterized by maternal pruritus, abnormal liver function tests and increased fetal risk. The main biochemical finding is the increase in total serum bile acid concentrations. In a subgroup of women, the serum gamma-glutamyl transpeptidase level is also increased. There is evidence that dysfunction of the ABCB4 gene might play a role in intrahepatic cholestasis of pregnancy development.
Aim: To investigate the role of the ABCB4 gene in Italian women with intrahepatic cholestasis of pregnancy and raised gamma-glutamyl transpeptidase by, analyzing the complete coding sequence and mRNA splicing products.
Methods: Among 299 women with intrahepatic cholestasis of pregnancy, 10 showing raised gamma-glutamyl transpeptidase were enrolled in this study. DNA and RNA were extracted from peripheral blood mononuclear cells using standard procedures. The 27 coding exons and the promoter region were amplified by polymerase chain reaction and analyzed by sequencing. Reverse transcript-polymerase chain reaction analysis of ABCB4 mRNA and cDNA analysis were also performed.
Results: A novel splicing mutation that causes a truncated protein of 249 amino acid was identified in a woman who had the highest serum levels of gamma-glutamyl transpeptidase, alkaline phosphatase, bile acids, and the highest pruritus score. We identified also one already described p.R590Q mutation in a woman who had significantly higher serum levels of alkaline phosphatase, aspartate, and alanine aminotransferase.
Conclusions: Our study demonstrates that splicing mutations in the ABCB4 gene can cause ICP in women with high gamma-glutamyl transpeptidase and thus a complete analysis of coding sequence and cDNA products is required.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a liver disorder that affects approximately 1/125–1/100 (0.8–1%) women in Italy [1]. It is characterized by pruritus in the absence of skin rash and by one or more biochemical laboratory abnormalities that are suggestive of biliary obstruction (i.e., alkaline phosphatase, aspartate/alanine aminotransferase, bilirubin and gamma-glutamyl transpeptidase (γ-GT) levels).

Usually ICP phenotype appears late in pregnancy when levels of female sex hormones are physiologically highest, and therefore predispose to cholestasis. After delivery, with a return to normal levels of these hormones, ICP usually disappears quickly. Maternal prognosis is benign but ICP is associated with increased fetal distress, preterm delivery and stillbirth occurring near term. While fasting serum bile acid values do not change during the course of normal pregnancy, both maternal and fetal values can be markedly raised in ICP. This is thought to be due to abnormal biliary transport across the hepatic canalicular membrane. It has been shown that the risk of fetal death is related to the serum levels of biliary acids [2].

Although some reports describe mutations in more than one gene, wide gaps are still present in our knowledge about this multifactorial disorder [3,4]. Genetic background of ICP is heterogeneous, but there is much evidence corroborating dysfunction in

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Table 1
Patient characteristics.

Patient N°	Parity[a]	γ-GT (U/L)	AST (U/L)	ALT (U/L)	ALP (U/L)	Bile acids (μmol/L)	Tot Bil (mg/dL)	Dir Bil (mg/dL)	Pruritus score
1	1	52	44	72	203	8	0.9	0.3	3
2	1	44	150	185	837	n.d.	1.21	n.d.	1
3	0	62	219	n.d.	190	14.8	0.4	0.1	0
4	1	41	66	147	204	7.2	0.4	0.1	3
5	1	40	43	50	190	10.5	0.9	0.4	2
6	1	61	68	86	157	24	0.4	0.1	1
7	2	45	45	52	86	23.5	0.7	0.5	3
8	1	51	28	58	n.d.	8.7	1	0.3	2
9	1	69	35	54	930	36.9	0.5	0.3	3/4
10	0	42	88	98	486	11.7	0.6	0.2	2

γ-GT: gamma-glutamyl transpeptidase (normal <40 U/L); AST: aspartate aminotransferase (normal <37 U/L); ALT: alanine aminotransferase (normal <40 U/L); ALP: alkaline phosphatase (normal <150 U/L); bile acids (normal <6 μmol/L); Tot bil: total bilirubin (normal <1.9 mg/dL); Dir Bil: direct bilirubin (normal <0.3 mg/dL); n.d.: not determined.
[a] Expressed as the number of children previously born at ICP diagnosis.

the ATP-binding cassette, sub-family B, member 4 (ABCB4) gene (alias *MDR3*), which is associated with the development of ICP with high serum γ-GT activity [5]. ABCB4 protein acts as a floppase at the canalicular level. Its alteration results in lack of phosphatidylcholine protection against the detergent effect of bile acids and damage of the biliary epithelium [6].

The *ABCB4* gene was originally reported to be involved in ICP by de Vree et al. [7] and Jacquemin et al. [8]; moreover, they have showed that the correlation between ICP-phenotype and ABCB4 genotype is not univocal, since cholestasis was not present in every pregnancy of the women with an ABCB4 deficiency. Apart from female sex hormones that could reduce the expression of the normal ABCB4 allele during pregnancy, recently it has been suggested that other environmental factors (e.g. inflammatory factors, selenium deficiency) can contribute to this multifactorial condition [9]. Recent studies have investigated ABCB4 gene sequence variation in women with ICP, supporting a possible involvement of ABCB4 mutations and polymorphisms in the pathogenesis of this condition [5,10,11]. Moreover, ABCB4 disease-alleles are found in ICP women with high but also normal γ-GT values [5,12]. Therefore, it is still under discussion whether ICP in conjunction with raised γ-GT activity can be considered a clinically specific subtype of obstetric cholestasis. The still open question is on the real contribution of the ABCB4 gene mutations in the pathogenesis of ICP with elevated γ-GT levels.

The aim of this study is to investigate whether ICP phenotype with raised γ-GT activity is characterized by ABCB4 mutations or mRNA misprocessing, and therefore, to establish a better correlation between genotype and maternal clinical management to prevent possible stillbirths.

2. Methods

Our study covers a 12-year period (1989–2000), with a total of 30,485 pregnancies followed at the Department of Obstetrics and Gynaecology, University of Milan-Bicocca, S. Gerardo Hospital, Monza, Italy. Among them, 299 women presented with ICP and 21 showed raised γ-GT levels. Only 10 women were available to participate in the present study (Table 1).

The diagnosis of ICP was based on the following criteria: (i) information obtained by clinical examination: pruritus (in the absence of any dermatologic or other systemic medical condition causing pruritus) appearing during the third trimester of the pregnancy, (ii) laboratory testing revealing a cholestatic pattern (serum aspartate and alanine transferase equal or exceeding 40 U/L and/or the concentration of serum total bile acids equal or exceeding 6 μM), (iii) frequent and spontaneous resolution of clinical symptoms, and laboratory findings after delivery, (iv) exclusion of other forms of liver disease and cholestasis, including those supported by a virus (viral hepatitis, cytomegalovirus or Epstein–Barr virus), by autoimmune disease or primary biliary cirrhosis. Furthermore, quantification of γ-GT levels in women with obstetric cholestasis allowed the selection of those with raised values (≥40 U/L). Pruritus was scored by a semiquantitative scale of 1–4, with grade 1 = occasional pruritus, grade 2 = daily intermittent pruritus with preponderance of asymptomatic periods, grade 3 = daily intermittent pruritus with preponderance of symptomatic periods and grade 4 = persistent pruritus, day and night. This system of scoring has already been validated by independent investigators. The management protocol consisted of serial evaluations of serum liver function and semiweekly monitoring of fetal well-being. Patients were delivered at 37–38 weeks.

2.1. Preparation of DNA and RNA

After approval by the Institutional Review Board of the hospital, with written informed consent of participating individuals, blood samples for DNA and RNA extraction were obtained from 10 women with ICP, as well as 43 control women with at least two normal pregnancies. For each woman in the study and 10 control women, a gradient density centrifugation method was used to isolate mononuclear cells (Histopaque-1077; Sigma France, Saint-Quemin, Fallavier, France). DNA and RNA were extracted from 5×10^6 peripheral blood mononuclear cells using a guanidinium thiocyanate-based method (TRIzol; Invitrogen, Gaithersburg, MD USA). The DNA of the remaining control women (33 samples) was extracted using Puregene DNA Isolation Kit (Gentra Systems; Minneapolis), according to the manufacturer's instructions. RNA and DNA concentrations were quantified by spectrophotometry.

2.2. Mutational analysis of the ABCB4 gene

Genomic and isoform B cDNA sequences were derived from GenBank accession numbers CH236949 and NM_018849, respectively. Aminoacid sequence reference was NP_061337. Genomic analysis included 2300 bp of the upstream promoter region. Primer sequences and PCR amplification conditions for the analysis of coding regions were previously reported [13]. Primer sequences and PCR amplification conditions for promoter region are available upon request. The PCR products were sequenced using the Big Dye® Terminator Sequencing kit, and run on 3100 Genetic Analyzer (PE Applied Biosystems); the electropherograms were analyzed using the software Sequencer 4.2 (Gene Codes, Ann Arbor, Michigan, USA).

2.3. Reverse transcript-polymerase chain reaction analysis of ABCB4 splicing variants

Reverse transcription (RT) of 1 μg RNA from each ICP patient was carried out using random hexamers (0.5 μg), 400 units of MMLV-RT, 1.6 mM total dNTPs, 20 units of RNasin, 0.4 mM dithio-