LAB RESEARCH

Clinical profile and screening of exon 6 and 14 of *ABCB4* gene in obstetric cholestasis patients at a tertiary care hospital in Rawalpindi, Pakistan

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Abstract

Objective: Intrahepatic Cholestasis of Pregnancy (ICP) is a rare pregnancy specific disorder. Genetic variants of *ABCB4* gene increase ICP risk. This study was conducted to determine frequency of ICP cases presented at a tertiary care hospital in Rawalpindi, Pakistan and to screen for genetic variants of exon 6 and 14 of *ABCB4* gene in ICP cases.

Methods: This analytical study included ICP patients presenting at Department of Gynaecology and Obstetrics, Holy Family Hospital Rawalpindi, from February 2017 to May 2017. Sanger's sequencing was performed using genomic DNA extracted from blood samples of patients and controls.

Results: Twenty pregnant women out of 1150 (1.74%) had ICP and were enrolled during study period. Overall (19/20) 95% patients had pruritus and among them (8/20) 40%, (4/20) 20% and (2/20) 10% had a history of miscarriages, stillbirths and familial ICP respectively. Genetic analysis revealed an already reported variant i.e., c.504C>T in exon 6 in thirteen patients and a novel variant i.e., c.1686A>G in exon 14 in five patients. Both variants were not present in controls. In silico analysis suggested that both variants might affect pre-mRNA splicing of *ABCB4* transcript.

Conclusion: ICP had a frequency of 1.74% among pregnant women. Identification of a novel heterozygous variant in five patients and an already reported variant in thirteen patients reaffirms genetic heterogeneity and role of *ABCB4* in ICP etiology.

Keywords: intrahepatic cholestasis of pregnancy, *ABCB4* gene, Single Nucleotide Polymorphism (SNP), DNA sequencing.

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Introduction

Intrahepatic Cholestasis of Pregnancy (ICP), also known as obstetric cholestasis is a pregnancy-specific hepatic disorder. Familial as well as sporadic cases of ICP have been reported[1-3]. Usually, onset of disease is during third trimester of pregnancy; however, there are few reports regarding early onset of disease. Prevalence of ICP varies between 0.2%-2%.[4] An incidence rate of 3.1% for ICP was reported in a study from Pakistan.[5] However, to date highest incidence of 16% was detected in Chile.[6] Seasonal variation in incidence of ICP has also been noted with higher incidence during winters, which also correlates with poor selenium intake[7] and deficiency of vitamin D.[8]

ICP is diagnosed based on pruritus, abnormal liver function tests (LFTs) and elevated serum bile acids.[2,5] These symptoms are attributed to disrupted transport of bile salts from liver to gallbladder and their compensatory transport from hepatocytes into the blood.[9] ICP is a multifactorial disease that occurs due to genetic predisposing factors as well as non-genetic risk factors.[10] Among non-genetic causes, overload of female sex hormones during pregnancy contributes to decreased efficiency of hepatocyte transporters and accumulation of bile acids.[2] The risk of foetal complication such as foetal distress, intrauterine death and premature birth is associated with increased amount of serum bile acids. During normal pregnancy, there is trans-placental gradient that facilitates transport of bile acid and toxin across it. However, in ICP this gradient is reversed and increased transfer of bile acid from mother to foetus across the placenta, results in accumulation of bile acid in foetal compartment, risking the well-being of the foetus.[11] As pathophysiology of ICP is still not certain there is no approved specific treatment, however, ursodeoxycholic acid – a tertiary bile acid present in human serum (3-8%) is normally used to treat maternal symptoms and it has effectively reduced pruritus in recent studies.[9,10]

Among known genetic contributors mutations in *ABCB4* (Multidrug resistant protein 3, MDR3)[1], ABCB11 (ATP binding cassette subfamily B member 11),[12] ATP8B1 (ATPase class 8B member 1)[13] and ABCC2 (ATP binding cassette subfamily C member 2) have been identified to cause ICP 2.

ABCB4 has 27 coding exons encoding a phosphotidylcholinefloppase MDR3. Homozygous mutations in *ABCB4* are reported to cause progressive familial intrahepatic cholestasis type 3 (PFIC3), whereas heterozygous mutations of *ABCB4* have been identified in familial as well as sporadic cases of ICP.[2,3] According to

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Dixon and Williamson, 10% of ICP affected women harbour an *ABCB4* mutation usually a missense variation.[14] *ABCB4* mutations also underline drug-induced cholestasis as well as low phospholipid linked cholelithiasis.[15,16] In the present study, we aimed to screen exon 6 and 14 of *ABCB4* gene to identify genetic variants in Pakistani ICP cases.

Patients and Methods

This case control study enrolled twenty pregnant females diagnosed with ICP through the gynaecologist at Department of Gynaecology and Obstetrics, Holy Family Hospital Rawalpindi during February 2017- May 2017.Ethical approval for execution of this study was obtained from institutional research forum RMC, Rawalpindi in accordance with the provisions of the Declaration of Helsinki.[17] Diagnostic criteria were persistent pruritus and elevated liver function tests in the absence of any other known liver disease.[10] Family and clinical history, pedigree drawing and blood samples of each patient were obtained after informed consent. Table 1 is summarizing the details of patients regarding parental consanguinity, clinical and family history. To serve as controls, we used 20 samples taken from healthy pregnant women with no history of ICP or any other pregnancy related complication.

Blood samples from each affected and control female were drawn and stored in EDTA containing vials. Genomic DNA was extracted by a non-organic method.[18]

Sequencing of exon 6 and 14 of *ABCB4* gene: Specific primer pairs were designed using primer3 software for two coding exons i.e., exon 6 and 14 of *ABCB4* gene to screen the patients for already known as well as novel sequence variant/s. Primer sequences were as follows: Primer 6F, 5'-GTATGGTGGTGCATGCCT-3'; Primer 6R, 5'-GCTGCCAGATGATCGATTTC-3' and Primer 14F, 5'-ACTTCAAGAGCTGATCCATGT-3'; Primer 14R, 5'-ATGAGGTAGCTCCATTTGCT-3'.Amplification of exon 6 and 14 was done in a reaction mixture containing 40 ng of genomic DNA, 2.5 µl of 10X reaction buffer, 2.5 µl of 10 mM deoxynucleoside triphosphate (dNTP) mix (Thermo Scientific, USA), 2.5 mM MgCl2 (Thermo Scientific, USA), 0.3µl of 10 pmol/µl of each primer (forward and reverse), 0.3µl of 5U/µl Taq DNA Polymerase (Thermo Scientific, USA) and a total volume of 25 µl was made by addition of distilled water. The PCR was done using Bio-Rad T100 thermal cycler with a cycling programme of 95^oC for 5 min, followed by 35 cycles of 95^oC for 45 sec, 54^oC or 55^1C (according to melting temperature of each primer pair) for 45 sec, 72^oC for 90 sec and a final extension at 72^oC for 10 min followed by a final hold at 25^oC. Amplified PCR products were loaded on the 1.5% agarose gel along with 1kb ladder to evaluate the fragment size. Amplified products were purified with ethanol. The sequencing reaction was performed using Big Dye Terminator Ready reaction mix (Applied Biosystems) following manufacturer instructions. Sequencing was done on an automated ABI 3100 genetic analyzer and Sequencher software (version 5.4.6) was used for data analysis. Both identified sequence variants were screened using DNA samples of 20 ethnically matched controls.

MutationTaster (www.mutationtaster.org) programme was used to predict disease-causing potential of identified sequence variations. HSF (Human Splice Site Finder) software version 3.0 (www.umd.be/HSF3/) was used to determine effects of sequence variations on exonic splicing signals.[19]To compare the allele frequencies of identified sequence variants i.e., c.504C>T and c.1686A>G of the *ABCB4* in ICP patients and controls, Fisher's exact test with a Bonferroni correction for adjusted p-value was used.

Results

During the four months study period 20 out of 1150

Table-1: Data of ICP patients regarding parental consanguinity, clinical profile and family history.

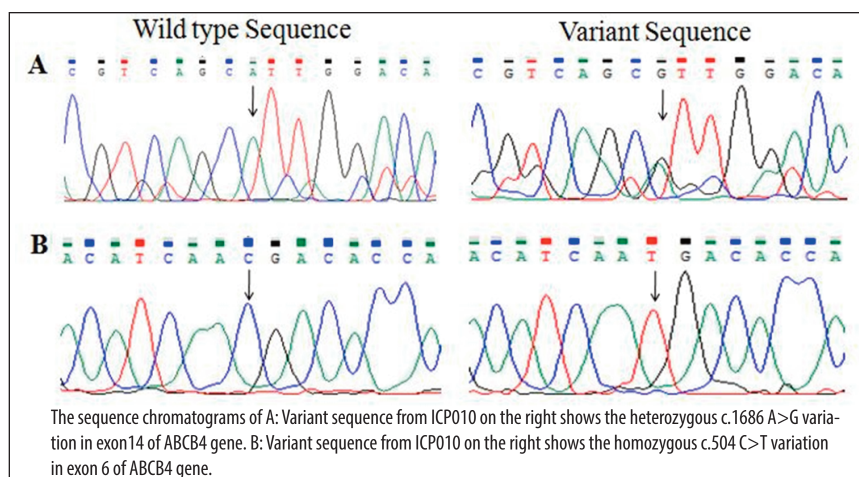
Sr#	PatientID	PC	Family History	Ethnicity	Pruritus	ALT (u/L)	BG/Rh Factor	Other findings	Age (Y)
1	ICP01	yes	no	Pathan	mild	29.00	AB+	SB	35.00
2	ICP02	yes	no	Punjabi	mild	227.00	0+	MC/ SB	25.00
3	ICP03	no	no	Punjabi	severe	65.00	AB+	Nil	35.00
4	ICP04	yes	no	Punjabi	mild	134.00	B+	HRD	31.00
5	ICP05	yes	no	Punjabi	mild	79.00	0+	Nil	30.00
6	ICP06	no	no	Punjabi	severe	150.00	B+	Nil	25.00
7	ICP07	yes	no	Punjabi	severe	18.00	0+	MC	30.00
8	ICP08	yes	no	Punjabi	severe	85.00	0+	Nil	23.00
9	ICP09	no	no	Punjabi	mild	98.00	B+	Nil	26.00
10	ICP10	no	no	Punjabi	severe	73.00	B+	MC/ SB	31.00
11	ICP11	yes	no	Punjabi	severe	560.00	AB+	Nil	32.00
12	ICP12	yes	no	Punjabi	mild	100.00	0+	Nil	26.00
13	ICP13	no	no	Punjabi	severe	263.00	0+	Nil	27.00
14	ICP14	yes	no	Pathan	mild	114.00	B+	MC	28.00
15	ICP15	yes	yes	Punjabi	severe	248.00	A+	MC	38.00
16	ICP16	yes	no	Punjabi	severe	115.00	0+	MC/ SB	35.00
17	ICP17	no	no	Punjabi	severe	410.00	0+	Nil	35.00
18	ICP18	no	no	Pathan	no	159.00	B+	Nil	34.00
19	ICP19	yes	no	Punjabi	mild	81.00	B+	MC	30.00
20	ICP20	no	yes	Punjabi	severe	117.00	B+	Nil	32.00

PC: Parental Consanguinity; ALT: Alanine transaminase; Reference Normal value of ALT: up to 43u/L; BG: Blood Group; MC: Miscarriage; SB: Still Birth, HRD: History of Respiratory Distress, Y: years.

obstetric patients (1.74%) presented with ICP. Mean age of the ICP patients was 30.4±4 years. Mean values of liver enzymes i.e., alanine transaminase and alkaline phosphatase was 156.2500±132.11194 and 372.3500±96.59643 respectively. Varying degree of pruritus was present in (19/20) 95% of cases (Table 1). All patients had onset of ICP symptoms in third trimester of pregnancy. Among enrolled patients, (2/20) 10% cases had familial history of ICP whereas (18/20) 90% were sporadic. Parental consanguinity was positive for (12/20) 60% ICP patients.

Table-2: Genetic variations identified in exon 6 and 14 of ABCB4 gene in ICP patients.

Patient ID	Exon	Nucleotide change	Status	Variant type
ICP02	6	c.504C>T	Heterozygous	synonymous
	14	c.1686A>G	Heterozygous	synonymous
ICP04	6	c.504C>T	Homozygous	synonymous
	14	c.1686A>G	Heterozygous	synonymous
ICP06	6	c.504C>T	Heterozygous	synonymous
ICP07	14	c.1686A>G	Heterozygous	synonymous
ICP08	6	c.504C>T	Homozygous	synonymous
	14	c.1686A>G	Heterozygous	synonymous
ICP09	6	c.504C>T	Heterozygous	synonymous
ICP10	6	c.504C>T	Homozygous	synonymous
	14	c.1686A>G	Heterozygous	synonymous
ICP11	6	c.504C>T	Heterozygous	synonymous
ICP12	6	c.504C>T	Heterozygous	synonymous
ICP14	6	c.504C>T	Heterozygous	synonymous
ICP15	6	c.504C>T	Heterozygous	synonymous
ICP16	6	c.504C>T	Homozygous	synonymous
ICP17	6	c.504C>T	Homozygous	synonymous
ICP18	6	c.504C>T	Heterozygous	synonymous



The sequence chromatograms of A: Variant sequence from ICP010 on the right shows the heterozygous c.1686 A>G variation in exon14 of ABCB4 gene. B: Variant sequence from ICP010 on the right shows the homozygous c.504 C>T variation in exon 6 of ABCB4 gene.

Figure: DNA Sequence chromatograms for Single Nucleotide Polymorphisms (SNPs) of ABCB4 gene identified in our study. Wild type sequences are on left side, whereas variant sequences are on right side.

Table-3: *ABCB4* sequence variantsdetected in cholestasispatients.

Nucleotide Change	Exon	Amino Acid Change	Variant Type	Minor Allele Frequency %		*p*-value
				PCG patients	Controls	
c.504C>T	6	p.(=)	silent	16/40 (40)	0/40 (0)	0.0001*
c.1686A>G	14	P.(=)	silent	5/40 (12.5)	0/40 (0)	0.05*

One patient i.e., ICP04 reported previous history of neonatal respiratory distress and her one baby died at age 7 days. A history of miscarriage and stillbirth was recorded for eight 8/20 (40%) and four cases 4/20 (20%) respectively (Table. 1).

Sequencing of *ABCB4* coding exon 6 and 14 in twenty ICP patients revealed two sequence variations of which a known variant i.e., c.504C>T (rs ID 1202283) was found in exon 6 in thirteen patients and a novel variant c.1686A>G was detected in exon 14 of five patients (table 2 and figure). c.504C>T variant was present in heterozygous state in nine cases and in homozygous state in five cases i.e., ICP04, ICP08, ICP10, ICP16 and ICP17 whereas novel variant c.1686A>G was present in heterozygous state in each respective case as listed in table. 2. Both of these variations i.e., c.504C>T and c.1686A>G were predicted by in-silico analysis tools as polymorphisms (Table 2 and Figure). Results of Fisher's exact test showed a significant difference in frequencies of both variants between ICP patients and controls (Table 3).

Using bio-informatic tool (www.umd.be/HSF3/) c.504C>T variant of exon 6 was analysed for potential splicing effects. The results predicted that this variation occurs in late exonic position and may break wild type Exonic Splicing Enhancer site (ESEs), hence may affect pre-mRNA splicing. The other novel variant c.1686A>G of exon 14 was predicted as disease causing variant by Mutation taster (www.mutationtaster.org/). Analysis by HSF software (www.umd.be/HSF3/) provided two predictions that it could create a potential new donor site or new Exonic Splicing Silencer site (ESSs). HSF matrices prediction algorithm showed that c.1686A>G variant might activate an exonic cryptic donor site, whereas according to second algorithm based on hnRNP (heterozygous ribonucleoprotein) it might play an inhibitory role causing exon skipping as it may create ESSs.

Discussion

Bile acids synthesize and secrete from the hepatocytes and flow through biliary tree to gallbladder.[9] Increased amount of pregnancy hormones decrease bile flow in ICP causing hepatic inflammation and increased serum bile acids secretion. Elevated serum bile acids might result in foetal complications ranging from increased risk of premature

delivery, meconium excretion causing staining of amniotic fluid, respiratory distress, and foetal intrauterine death.[9-11] Etiology of ICP is multifactorial. Genetic alterations of various transport protein encoding genes i.e., *ABCB4*,[1] ABCB11,[12] ATP8B1[13]and ABCC2 have been identified to be associated with ICP.[2] Incidence rate of this pregnancy related liver disorder was significantly higher in the Pakistani and Indian subgroups compared to the whites i.e., 1.46%, 1.24% and 0.62% respectively.[20] In a recent study conducted by Hafeez et al., at CMH Kharyan, Punjab, Pakistan, an incident rate of 3.1 % was recorded.[5] However, in a study conducted by Rasheed et al., at Mother and Child Health Center, Pakistan Institute of Medical Sciences, Islamabad, Pakistan, a frequency of 0.4% for ICP was reported.[21] Whereas an incidence of 2% was reported by Masood et al., from Karachi, Pakistan.[22] Here in present study the frequency of ICP in obstetric patients visiting Department of Gynaecology and Obstetrics, Holy Family Hospital Rawalpindi during February 2017- May 2017 was 1.74% (twenty out of 1150 obstetric patients). This rate is less than those reported from Kharian and Karachi i.e., 3.1% and 2% respectively, while higher than Rasheed et al., and Abedin et al., reports.[5,20-22] Previously increased prevalence of ICP during winter season was reported[7] and that could be possible reason behind finding of low ICP incidence in our study as compared to Hafeez et al., study, which was conducted during February 2017-May 2017.

Contribution of genetic risk factors to etiology of ICP is evident from reported familial cases.[1] ICP exhibits an autosomal dominant and sex-limited inheritance pattern. In the present study, two (10%) patients had a family history of ICP while 90% were sporadic cases. Out of twenty ICP cases, parental consanguinity was positive for 12 (60%) patients. Parental consanguinity may contribute to risk of ICP as in a study by Gotthardt et al., missense mutation in *ABCB4* gene in an inbred family was associated with liver disease ranging in severity from ICP to cirrhosis in carriers of mutated allele.[23] Hence, *ABCB4* mutations and variations are linked to a spectrum of cholestatic diseases of varying severity. Exon 6 and 14 of *ABCB4* gene was analyzed in 20 enrolled ICP cases. A single nucleotide substitution (c.504 C>T) was identified in 13 out of 20 ICP affected females (Table. 2, Figure). This silent variant was present in homozygous state in five cases i.e., ICP04, ICP08, ICP10, ICP16 and ICP17 whereas in heterozygous state in eight cases i.e., ICP02, ICP06, ICP09, ICP11-12, ICP14-15 and ICP18. Previously c.504 C>T was detected in ICP patients of different ethnicities including Caucasians and Japanese patients by Gendrot et al., Bacq et al.,and Kamimura et al.[24-26] Fathy et al., identified c.504 C>T variant in PFIC3 patients.[27] Hence, identification of this c. 504 C>T variant in 13 ICP patients in this study is the first report from Pakistan and reaffirms its involvement in ICP phenotype.

Various in silico analysis tools are available to analyze effect of sequence variant/s on the splicing mechanism. In the present study, HSF3.0 in silico analysis of the exonic variant c.504 C>T predicted to cause breakage of Exonic Splicing Enhancers (ESEs) and creation of new ESE site 3 nucleotide downstream. Therefore it might affect wild type pre mRNA splicing due to alterations in putative exonic splicing enhancer binding site. For precise recognition of exons, nuclear spliceosome recognises the exon-intron junctions on pre-mRNAs along with exonic and intronic splicing enhancers/silencers.[28,29] These are stretches of largely degenerated sequences present in exon/intron and are sites for trans-acting RNA-binding proteins which positively or negatively impact pre-mRNA splicing.[28]

In five ICP patients (i.e., ICP02, ICP04, ICP07, ICP08 and ICP10) a heterozygous novel synonymous variation i.e., c.1686A>G in exon 14 of *ABCB4* was detected. Out of these five patients, three patients i.e., ICP04, ICP08 and ICP10 were also carrying homozygous c.504 C>T variation of exon 6. Two of these patients (ICP08 and ICP10) had severe pruritus. Mutation taster predicted c.1686A>G to be disease causing and it was not detected in ethically matched controls. Further in silico analysis by HSF3.0 showed that c.1686A>G substitution may affect *ABCB4* pre-mRNA splicing either by creating a potential new donor site or by promoting new Exonic Splicing Silencer site (ESSs). ESSs bind negative regulators of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and could regulate splicing mechanism by inhibition of exon inclusion and inhibition of intron-retention.[30]

Limitation of the study

The reason to use 1:1 cases and controls was unavailability of funds for commercial sequencing of a large number of control samples, so after consulting previously published literature[3,31] we used equal number of controls in our study.

Conclusion

Our study highlighted ICP burden i.e., 1.74% in obstetric patients visiting Department of Gynaecology and Obstetrics, Holy Family Hospital Rawalpindi during February 2017- May 2017. Awareness of disease incidence in different areas of country is necessary to educate obstetric care providers and pregnant females for in time presentation, diagnosis and better management of disease to reduce adverse outcomes i.e., spontaneous preterm labour, foetal morbidity and mortality. Furthermore the present study identified one previously known ICP linked *ABCB4* variant in 13 patients and a novel synonymous variant in five ICP analyzed cases. These findings suggested hereditary predisposition of disease in local population and

necessitates inquiry about positive personal or family history of ICP/PFIC3 from every pregnant women at first antenatal visit and screening test for ICP at start of third trimester to reduce ICP associated complications. We could not record details regarding treatment of enrolled patients and foetal parameters including gestational age, mode of delivery, birth weight, bradycardia, meconium staining, respiratory distress etc which is a major limitation of our study. To, the best of our knowledge, this is first report of molecular analysis of *ABCB4* in ICP from Pakistan. These results reaffirm genetic etiology of intrahepatic cholestasis of pregnancy.

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