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Intrahepatic cholestasis of pregnancy: new insights into its pathogenesis

Annarosa Floreani[1], **Diego Caroli**[1], **Roberta Lazzari**[1], **Alessia Memmo**[2], **Elisa Vidali**[2], **Davide Colavito**[3], **Antonello D' Arrigo**[3], **Alberta Leon**[3], **Roberto Romero**[4,5], and **Maria Teresa Gervasi**[2]

[1]Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy

[2]Department of Obstetrics and Gynaecology, Azienda Ospedaliera, Padova, Italy

[3]Research and Innovation, Padova, Italy

[4]Perinatology Research Branch, NICHD/NIH/DHHS, Bethesda, Maryland and Detroit, MI, USA

[5]Department of Obstetrics and Gynaecology, Wayne State University School of Medicine, Detroit, MI, USA

Abstract

Aim: To search a specific gene expression profile in women with intrahepatic cholestasis of pregnancy (ICP) and to evaluate the maternal and foetal outcome.

Methods: We consecutively enrolled 12 women with ICP and 12 healthy pregnant controls. The gene expression profile was assayed with the microarray technique including a panel of 5541 human genes. Microarray data were validated by real-time PCR technique.

Results: Caesarean delivery was performed in eight patients with ICP versus three controls ($p = 0.05$). ICP women delivered at earlier gestational age than control ($p < 0.001$). Foetal distress was recorded in two babies, but we failed to find any correlation between bile salt concentration and foetal distress. Twenty genes potentially correlated with ICP were found differentially expressed ($p < 0.05$). Among these, three belong to genetic classes involved in pathogenic mechanisms of ICP: (1) pathophysiology of pruritus (*GABRA2*, cases versus controls = 2, upregulated gene); (2) lipid metabolism and bile composition (*HLPT*, cases versus controls = 0.6, down-regulated gene); and (3) protein trafficking and cytoskeleton arrangement (*KIFC3*, cases versus controls = 0.5, down-regulated gene).

Conclusions: Different gene expression may contribute to the complex pathogenesis of ICP. An upregulation of *GABRA2* receptor may indicate that GABA may play a role in the pathogenesis of pruritus in this condition.

Keywords

Genetics; intrahepatic cholestasis of pregnancy; microarray

Correspondence: Prof. Annarosa Floreani, Department of Surgical and Gastroenterological Sciences, Via Giustiniani, 2 – 35128 Padova, Italy. Tel: 0039-0498212894. Fax: 00390498760820. annarosa.floreani@unipd.it.

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INTRODUCTION

Intrahepatic cholestasis of pregnancy (ICP) is a peculiar hepatic disorder in pregnancy, which usually occurs at the last trimester [1]. It is characterized by severe pruritus enough to affect quality of life, associated with an increase in serum bile acids and transaminases [1]. The incidence of ICP ranges between 0.5% and 1.8% of pregnancies in Europe, but the highest peak of incidence has been reported in Chile (up to 28% in the Araucanic population) and in Scandinavia [2]. This geographical distribution clearly indicates a genetic predisposition. The *MDR3* gene was first reported to be involved in ICP by De Vree et al. [3]. In a large consanguineous family, subjects affected by PFIC3 were heterozygous for a nonsense mutation in exon 23 (R957X) and women affected by ICP were heterozygous. Subsequently, other heterozygous mutations in *MDR3* gene have been reported in affected women with a wide variability according to the geographical area [4–7]. Recently, we performed two multicentre prospective case-control studies in Italy, enrolling 96 women with ICP. At overall, an association with *MDR3* variant was present in 7% of cases only [8,9]. Therefore, the involvement of different pathogenic genes in this condition may be suggested.

The *ABCB11* gene coding for bile salt export pump (BSEP) is another gene implicated in the development of ICP [10]. In contrast to *MDR3*, evidence for a pathogenic involvement of hereditary BSEP mutations in ICP is controversial. While high GGT values were present in the majority of ICP patients with *MDR3* mutations, BSEP mutations were postulated in low GGT cases [11,12].

A study by Savander et al. [6] in individuals from Finnish ICP families could not find any association between ICP and BSEP noted mutations, which was in-line with another recent study that did not find any evidence of a strong role for BSEP in the pathogenesis of ICP [13]. It is possible, however, that combined variants of *MDR3* and BSEP may cause a severe phenotypic expression of ICP [14].

Till now, the genetic studies on ICP have been performed with the association study protocol of candidate genes. However, association-based studies carry several bias, particularly the sample size, the lack of independent replication in different populations, the complex variability of phenotypes, the penetrance, and environmental factors. Microarray technology provides a powerful tool to assay differential expression of gene products [15] for microarray analysis of gene expression. In fact, the simultaneous expression of thousands of transcripts allows the demonstration of gene expression patterns associated with this condition.

The aim of the present study was to evaluate the contribution of possible genes involved in the multifactorial pathogenesis of ICP using the microarray technology.

Material and Methods

Patients

Twelve consecutive pregnant women diagnosed as affected by ICP and 12 age-matched controls (pregnant women without ICP) were enrolled in the study.

The diagnostic criteria were as follows: (1) pruritus; (2) high total bile acid concentration and serum transaminases values; (3) absence of viral infections (hepatitis A virus [HAV], hepatitis B virus [HBV], hepatitis C virus [HCV], cytomegalovirus); (4) negativity for markers of autoimmunity (organ- and non-organ-specific autoantibodies, i.e. thyroid autoantibodies, nuclear, smooth muscle, mitochondrial, liver-kidney microsomal autoantibodies); (5) no extrahepatic causes of pruritus (allergies, thyroid dysfunction); and (6) normalization of pruritus and liver function tests after delivery.

The control group included 12 pregnant women without ICP, with a mean age of 32 ± 3.1 years, normal function tests and matched for parity, trimester of pregnancy, age and geographical region.

This investigation was conducted after the approval of the local Ethical Committee, and informed consent was obtained by each subject.

Microarray procedures

Six milliliters of peripheral blood were collected from each subject and frozen at −80° C until utilized. Duplicated arrays comprising 5541 humancDNAs were assembled onto mirrored, aminosilane-coated slides (Amplislide, GeneWave, Paris, France) by a Lucidea Spotter (GE Healthcare, Milan, Italy).

Total RNA was extracted using a TRIzol-chlorophorm method (Bibco, St Louis, MO). RNAwas resuspended in water (E.E.P.C.) and its integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Noventa Padovana, Italy).

A dendrimer-based labelling system (Array-50 version 2; Genisphere Inc., Hatfield, PA, USA) was used for the mRNA reverse transcription, preparation of cDNA probes and slide hybridization. Briefly, 10 μg of total RNA was reverse transcribed into cDNA using 5′ tagged oligo (dt) primers and murine leukemia virus (MLV) reverse transcriptase (Promega, Madison, WI, USA). The tagged cDNAwas hybridized for 16 h at 50° C in a humidified chamber, followed by sequential slide washing as follows: 2 × saline sodium citrate (SSC) + 0.2% sodium dodecyl sulphate (SDS) at 50° C, 2 × SSC at room temperature, and 0.2 × SSC at room temperature (10 min per washing step). At the end, the slides were incubated with DNA dendrimers containing cyanine dyes and including sequences complementary to the cDNAtags. The slides were scanned by a Generation III Laser Scanner (Amersham-Pharmacia, Molecular Dynamics). For image visualization and signal quantification, an Array Vision software was utilized (Imaging Research, St. Catharine, ON, Canada).

Data analysis

For statistical analysis, the Genespring software was employed (Agilent, Santa Clara, CA). Data were normalized by LOWESS [16], baseline transformed for between-slide normalization and then filtered on the basis of their signal intensity: only spot values above background in both duplicate arrays were considered for further analysis. Data were analyzed by Welch *t* test, setting the *p* value threshold at 0.05. The *p* values were corrected by Bonferroni post hoc test.

Real-time PCR

To validate microarray data, real-time (RT) PCR was used to detect the expression of randomly selected genes in the same RNA samples used for microarray. Quantitative RT-PCR was performed in duplicate using the SYBR Green I assay on ABI PRISM Step One Plus (Applied Biosystems, Foster City, CA, USA) in a total volumes of 10 μl containing 5 μl of 2 × SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 5 μM gene-specific forward and reverse primers (MWG Operon) and 10 ng cDNA.

The following thermal cycling protocol was used: 10 min at 95° C followed by 45 cycles of 15 s at 95° C and 30 s at 60° C. DNA amplification was verified by melt curve analysis. The threshold fluorescence was set within the exponential range of the amplification curve and used to calculate the threshold cycle (Ct) values. Ct values were normalized for the housekeeping gene, porphobilinogen deaminase (PBGD).

The following primers were used for selected genes: SYT7, forward 5′-TCAAAGCCATGGACATCGG-3′, reverse 5′-TCCTCTTCATCGTCACCGTCTT-3′; *GABRA2*, forward 5′-TGTCCTCTGAAATTTGGCAGCT-3′, reverse 5′-GATTGGCCCAGCAGGTCA-3′; PFKP, forward 5′-ATGGCTTTGACGGCTTCG-3′, reverse 5′-CGTTTTGTCCCAAGAATGG-3′; *PLTP*, forward 5′-TGCGCAGGTTCCGAATCTA-3′, reverse 5′-ATCACCCCAATCTGCAGC-3′; *KIFC3*, forward 5′-TGCGGCTGAAAGGGAACA-3′, reverse 5′-GGCATCGAAAGTCACAGCATT-3′; AKAP10, forward 5′-GGCTTTTTGTCCAGCAATCT-3′; reverse 5′-GAGCAGTCAGCGACACGTTC-3′; BFAR, forward 5′-TAGCTCCTAACACAGGCCGAG-3′, reverse 5′-TAGACGAGCAGGACCACTGC-3′; SNAPC4, forward 5′-GATCTACCGATGGACCAAGAGC-3′, reverse 5′-CCCCGTATTTGGCAACAGC-3′; PBGD, forward 5′-GAACAGAGCAAAGGAAGCGC-3′, reverse 5′-TGGAACATTGCGACAGTGGA-3′. The expression of detected genes was calculated by Welch's *t* test, and *p* values of < 0.05 were regarded as significant.

RESULTS

Clinical characteristics of subjects

Clinical and biochemical characteristics of ICP women were reported in Table 1. ICP women had high total bile acid concentration and elevated serum transaminases values in accordance with diagnostic criteria, while levels of total bilirubin and gamma glutamyl

transferase were normal and comparable with the control group. Only one woman had family history of ICP.

Pregnancy outcome of ICP was presented in Table 2. Caesarean delivery was performed in eight patients with ICP versus three controls (67% versus 25%, $p = 0.05$). ICP women delivered at earlier gestational age than control (37.5 ± 1.44 versus 40.60 ± 0.97; $p < 0.001$). Weight at birth was similar in ICP group with respect to controls (3040 ± 563 versus 3425 ± 521 g, p = n.s.). Neonatal intensive care was required in one baby in the study group, but no obstetric problems were recorded in the controls. Overall, foetal distress was recorded in two babies, but we failed to find any correlation between bile salt concentration and foetal distress.

Differentially expressed genes in ICP

Twenty genes potentially correlated with ICP were found differentially expressed between ICP patients and controls ($p < 0.05$) and were reported in Table 3.

Among these, three belong to genetic classes involved in pathogenic mechanisms of ICP: (1) GABA receptor alpha-2 subunit gene (*GABRA2*), which was twofold upregulated in ICP group compared with controls, may be included in pathophysiology of pruritus; (2) gene coding for human phospholipid transfer protein (*PLTP*), which was downregulated of 0.6-fold than controls, may belong to lipid metabolism and bile composition; and (3) kinesin family member C3 gene (*KIFC3*), which was down-regulated of 0.5 fold in ICP group compared with controls, may be included in protein trafficking and cytoskeleton arrangement.

Gene expression results from microarray were confirmed by RT-PCR for three selected genes. We have found that the expression of *SYT7, GABRA2* and *PFKP* was significantly elevated in ICP group, 1.5, 1.77 and 3.80 fold, respectively, compared with control group ($p < 0.05$), and these results were consistent with the microarray results.

DISCUSSION

Using the microarray technique, we obtained a profile of differentially expressed genes in women with ICP that have been never pointed before. It was found that 6 genes were upregulated and 14 down-regulated. A previous study using the same technology has been conducted byWei et al. in placental samples from women with ICP [17]. Some genes may be classified into a complex category, which can be named as “immunity and cell growth”. Among this category, the B-cell growth factor precursor A has been found as more upregulated. Indeed, T lymphocytes routinely possess an intracytoplasmic pool of the precursor proteins, which are stimulated under a variety of conditions [18]. Therefore, a relationship between precursor and products exists for the processing of B-cell growth, analogous to that described for several cytokines [18]. Abnormal immunity has been described in ICP, including abnormalities in innate and adaptive immunity [19,20].

Other genes, although with a less power of significance, might have a role in this complex alteration of immunity.

It is also interesting to note that at least three other genes (coding for GABA receptor alpha 2 subunit, human PLTP, and kinesin family member C3) can be restricted into pathogenic mechanisms involved in ICP: (i) pathophysiology of pruritus; (ii) lipid metabolism and bile formation and (iii) protein trafficking and cytoskeleton arrangement.

GABA receptor alpha-2 subunit

GABA is an important neurotransmitter, which regulates the neuronal excitability in the nervous system. The GABA receptors comprise three classes: $GABA_A$, $GABA_B$ and $GABA_C$. *GABRA2* coding for GABA A receptor alpha 2 subunit is located on chromosome arm 4p12 [21].

GABA receptors have been implicated in multiple neurological functions including pruritus. Interestingly, GABA A receptors have been found highly expressed in skin biopsies from patients with psoriasis and pruritus [22], suggesting an active role of GABA system in pruritus.

Gabapentin, a synthetic analogue of GABA, has been studied as a therapeutic agent for treating pruritus. Its mechanism of action relates to its binding on the $\alpha_2\delta$ subunit of voltage-gated calcium channels and thereby inhibiting high threshold neuronal calcium channels [23]. A pilot study investigating the use of gabapentin in children with healing burn wounds demonstrated a marked reduction in reported itching and the reduction of antihistamine intake [24]. Unfortunately, in another double-blind randomized, placebocontrolled trial, gabapentin did not provide significant advantage over the placebo in patients with pruritus of cholestasis [25].

Indeed, the up-regulation of *GABRA2* gene in ICP indicates that the GABA system is actively involved in the pathophysiology of this condition. The change in GABRA1 expression in ICP group was confirmed by RT PCR.

Phospholipid transfer protein

PLTP is a 476 aminoacid protein (M281 kDA) with six N-linked glycosilation sites, which has a key role in the intravascular remodelling of high density lipoprotein (HDL) [26]. *PLTP* gene expression is controlled by nuclear receptors such as farnesoid X receptor and liver X receptor [27]. *PLTP* promotes transferring of phospholipids from very low-density lipoprotein (VLDL) and chylomicrons into HDL; moreover, it may contribute to the remodelling of HDL particles [28]. To date, no genetic deficiency has been reported for *PLTP*. Interestingly, a genetic variant in *PLTP* has been shown to modulate lipoprotein profiles in hyper-alphalipoproteinemia [29]. Another variant in the *PLTP* gene has been demonstrated to be associated with higher HDL and lower triglycerides [30]. Our results add another prospective in the elucidation of the role of this protein in human lipoprotein metabolism in pregnancy.

Kinesin family member C3

KIFC3 is one of the kinesin-14 family members which are molecular motors that sort and transport proteins and lipids along microtubules [31]. In an elegant study, Japanese authors

found that *KIFC3* is concentrated at the zonula adherens of the epithelial cells, the apical most part of cadherin-based adherens junctions, via a protein that they termed Zezha [32]. Moreover, *KIFC3* plays an essential role in Golgi positioning under the cholesterol-depleted condition [33]. Therefore, *KIFC3* should be regarded as a novel regulatory mechanism of cholesterol in membrane trafficking. We found *KIFC3* down-regulated in ICP, probably for the increased synthesis in cholesterol. Total bile acids are increased in ICP, representing one of the hallmarks in this condition. In particular, the levels of cholic acid or the ratio of cholic acid to chenodeoxycholic acid have been shown to be the most sensitive indicators of ICP [34]. Bile salts are polar derivatives of cholesterol, thus a downregulation of the *KIFC3* might be a protective mechanism toward the increase in bile salts. However, this is only a preliminary hypothesis that needs to be verified in future. Cholesterol is also the source of hormones, including progesterone (which prepares uterus lining for implantation of ovum) and estrogens. Hormonal abnormalities have been found in ICP with a typical pattern of steroid hormone metabolites in urine constitutes by disulphates of pregnanediols [35].

CONCLUSIONS

Our study confirmed that ICP has a multifactorial pathogenesis involving a genetic susceptibility, an altered immunity, abnormalities in hormone metabolism and possibly environmental factors.

The microarray technique is allowed to point out several genes which might contribute the pathogenic mechanisms, in particular genes involved in the pathophysiology of pruritus, of lipid metabolism and bile formation, and in the synthesis of protein trafficking and cytoskeleton arrangement.

The *GABRA2* gene upregulation in ICP suggests an active role of GABA system in the pathophysiology of pruritus of ICP. The down-regulation of *PLTP* gene supposes a role of *PLTP* in HDL metabolism and in reverse cholesterol transport pathways at the fetoplacental barrier. The down-regulation of *KIFC3* in ICP proposes a likely protective mechanism to counteract the increase of bile salts.

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Table 1

Clinical details of the patients with ICP

Points	Age (years)	Parity	Family history of ICP	Peak of AST IU/L	Peak of ALT IU/L	Peak of GGT IU/L	Peak of total bilirubin (mmol/L)	Peak of bile salts (μmol/L)
1	45	0000	Yes	43	60	9	9.6	100
2	29	0001	No	481	470	35	15.4	100
3	33	0001	No	58	122	13	8.8	27.5
4	34	0000	No	74	70	15	9.9	10
5	38	0001	No	542	584	27	13.8	32
6	24	0000	No	50	74	13	12.8	32
7	35	0000	No	484	600	27	13.4	41
8	39	0001	No	80	103	31	7.4	10
9	25	0000	No	80	113	22	20.6	16.7
10	34	0000	No	143	369	17	6.4	34.4
11	28	0001	No	80	234	9	7.7	10
12	35	0000	No	201	291	29	16.4	100
Total n = 12								
Mean ± SD	33 ± 6			188.8 ± 195.3	250.5 ± 202	21.3 ± 7.6	11.7 ± 4.5	42.8 ± 36

Normal value: AST: <40 IU/L; ALT: <45 UI/L; GGT: <65 UI/L; total bilirubin: <17 mmol/L; bile salts: <6 μmol/L.

Table 2

Outcome of pregnancy of the patients with ICP

Points	Delivery	Week of delivery	Neonatal gender	Neonatal weight (g)	Fetal distress	Meconium-stained AF	AF Color	Apgar 1′	Apgar 5′	NICU
1	C	37.6	F	3.700	No	No	Clear	9	10	0
2	C	36.0	F	3.100	Yes	No	Clear	9	10	0
3	C	38.9	M	3.430	No	No	Clear	9	10	0
4	C	38.9	F	3.650	No	No	Clear	9	10	0
5	C	38.3	F	3.650	No	Yes	Yellow	7	8	0
6	I	37.9	F	2.740	No	No	Clear	9	10	0
7	I	38.6	F	2.665	No	No	Clear	9	10	0
8	C	38.1	M	3.020	No	No	Clear	9	10	0
9	I	39.0	F	3.590	No	No	Clear	9	10	1
10	I	36.0	F	2.315	No	No	Clear	9	10	0
11	C	35.6	M	2.450	No	No	Clear	9	9	0
12	C	35.0	M	2.180	Yes	Yes	Green/ Brown	8	8	0
Total n = 12										
Mean ± SD		37.5 ± 1.44		3040 ± 562						

C: cesarean; I: induced; NICU: neonatal intensive care unit; AF: amniotic fluid

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Table 3

Differentially expressed genes in ICP

Spot labels	Gene symbol	GENBANK ID (Homo sapiens)	Description	Molecular function / Biological process	Regulation	RATIO cases/control	P value
1		AI668565.1	Transcribed locus		Up	6.57	0.0178
2		AA436716	Transcribed locus		Up	2.91	0.0321
3	*SYT7*	NM_004200.2	Synaptotagmin VII mRNA	Synaptic exocytosis and neurotransmitter; plasma membrane repair; regulation of insulin secretion	Up	2.44	0.0127
4	*GABRA2*	NM_000807.2	Gamma-aminobutyric acid A receptor alpha 2 subunit	Gamma-aminobutyric acid signaling pathway with chloride channel activity; regulation of neurotransmitter levels	Up	1.99	0.0066
5	*PFKP*	NM_002627.3	Phosphofructokinase, platelet	Nucleotide and protein binding, kinase activity, glycolysis	Up	1.89	0.0460
6	*CA3*	NM_005181.3	Carbonic anhydrase III, muscle specific	Metal ion binding, lyase activity, metabolic response to oxidative stress	Up	1.62	0.0372
7	*NME3*	NM_002513.2	Non-metastatic cells 3, protein expressed in	Synthesis of nucleoside triphosphates other than ATP (CTP, GTP, UTP); induction of apoptosis	Down	0.72	0.0005
8	*BCL3*	NM_005178.4	B-cell CLL/lymphoma 3	Transcription regulator activity as transcriptional co-activator	Down	0.66	0.0295
9	*PLTP*	NM_006227.2	Human phospholipid transfer protein mRNA	Cholesterol metabolism; lipid transport	Down	0.61	0.0296
10	*PTMA*	NM_001099285.1	Prothymosin, alpha	Transcription	Down	0.57	0.0221
11	*ID1*	NM_002165.2	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Protein binding, transcription repressor activity, angiogenesis, apoptosis	Down	0.52	0.0389
12	*KIFC3*	NM_001130099	Kinesin family member C3	Microtubule motor activity; Golgi organization	Down	0.51	0.0466
13	*NDUFV1*	NM_007103	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51kDa	Mitochondrial electron transport, NADH to ubiquinone	Down	0.47	0.0398
14	*NRN1*	NM_016588.2	Neuritin 1	Axonogenesis; nervous system development	Down	0.49	0.0247
15	*CLK2*	NM_003993.2	CDC-like kinase 2	Phosphorylation of amino acid proteins in RNA splicing	Down	0.45	0.0100
16	*MYCL1*	NM_001033081.2	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	Regulation of transcription, DNA-dependent	Down	0.42	0.0144
17	*FKBP11*	NM_001143781.1	FK506 binding protein 11, 19 kDa	Protein folding	Down	0.42	0.0137
18	*AKAP10*	NM_007202.2	Homo sapiens protein kinase A anchoring protein mRNA	Signal transduction; protein localization	Down	0.41	0.0328
19	*BFAR*	NM_016561	Human bifunctional apoptosis regulator (BAR) mRNA	Anti-apoptosis; apoptosis	Down	0.39	0.0035

Spot labels	Gene symbol	GENBANK ID (Homo sapiens)	Description	Molecular function / Biological process	Regulation	RATIO cases/control	P value
20	*SNAPC4*	NM_003086.2	Small nuclear RNA activating complex, polypeptide 4, 190kDa	Regulation of transcription	Down	0.38	0.0007