Linkage Between a New Splicing Site Mutation in the *MDR3* alias *ABCB4* Gene and Intrahepatic Cholestasis of Pregnancy

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Intrahepatic cholestasis of pregnancy (ICP) is defined as pruritus and elevated bile acid serum concentrations in late pregnancy. Splicing mutations have been described in the multidrug resistance p-glycoprotein 3 (*MDR3, ABCB4*) gene in up to 20% of ICP women. Pedigrees studied were not large enough for linkage analysis. Ninety-seven family members of a woman with proven ICP were asked about pruritus in earlier pregnancies, birth complications and symptomatic gallstone disease. The familial cholestasis type 1 (*FIC1, ATP8B1*) gene, bile salt export pump (*BSEP, ABCB11*) and *MDR3* gene were analyzed in 55 relatives. We identified a dominant mode of inheritance with female restricted expression and a new intronic *MDR3* mutation c.3486+5G>A resulting in a 54 bp (3465–3518) inframe deletion via cryptic splicing site activation. Linkage analysis of the ICP trait versus this intragenic *MDR3* variant yielded a LOD score of 2.48. A Bayesian analysis involving *MDR3*, *BSEP*, *FIC1* and an unknown locus gave a posterior probability of >0.9966 in favor of *MDR3* as causative ICP locus. During the episode of ICP the median γ-glutamyl transpeptidase (γ-GT) activity was 10 U/l (95% CI, 6.9 to 14.7 U/l) in the index woman. Four stillbirths were reported in seven heterozygous women (22 pregnancies) and none in five women (14 pregnancies) without *MDR3* mutation. Symptomatic gallstone disease was more prevalent in heterozygous relatives (7/21) than in relatives without the mutation (1/34), ($P = 0.00341$). *Conclusion:* This study demonstrates that splicing mutations in the *MDR3* gene can cause ICP with normal γ-GT and may be associated with stillbirths and gallstone disease. (HEPATOLOGY 2007;45:150-158.)

Intrahepatic cholestasis of pregnancy (ICP) is a cholestatic disorder of pregnant women which was first described by Ahlfeld in 1883.[1] The incidence of ICP in Europe is approximately 0.1 to 1.5% of pregnancies.[2] In contrast, incidence rates as high as 16% have been described in Chile and Bolivia.[3-5] ICP is generally characterized by a benign course of disease with a good prognosis. However, vehement itching, jaundice and fat malabsorption may impair the mother's quality of life. On the other hand, an increased perinatal fetal risk with possible lethal outcome has been described.[2,6-11]

Early studies on ICP considered its etiology as idiopathic.[12] However, even in these early studies several cases of familial recurrent ICP were identified in various regions of the world. Reyes et al. described a family from Chile in which 10 of 32 multiparous women developed ICP over two generations.[13] In 1983 Holzbach et al. presented their analysis on dominant familial ICP in three generations of a pedigree comprising more than 50 individuals.[14] These studies were mainly descriptive and did not allow conclusions on the pathophysiology of ICP. This changed when Jacquemin and co-worker discovered that heterozygosity for a non-sense mutation in the *MDR3* alias name *ABCB4* gene was associated with ICP in family members of a child with a severe form of progressive familial intrahepatic cholestasis type three (PFIC 3).[10] This observation fostered the assumption that *MDR3* gene variants may be associated with a familial

Abbreviations: 5′ ss, 5′ splicing sites; γ-GT, γ-glutamyl transpeptidase; BRIC, benign recurrent intrahepatic cholestasis; bp, base pares; BSEP, bile salt export pump; FIC1, familial cholestasis type 1; ID, identification number; ICP, intrahepatic cholestasis of pregnancy; LC, liability class; LOD score, decadic logarithm of a likelihood ratio; MDR3, multidrug resistance p-glycoprotein 3; nt, nucleotides; PFIC, progressive familial intrahepatic cholestasis; S&S, Shapiro and Senapathy; SNP, single nucleotide polymorphisms.

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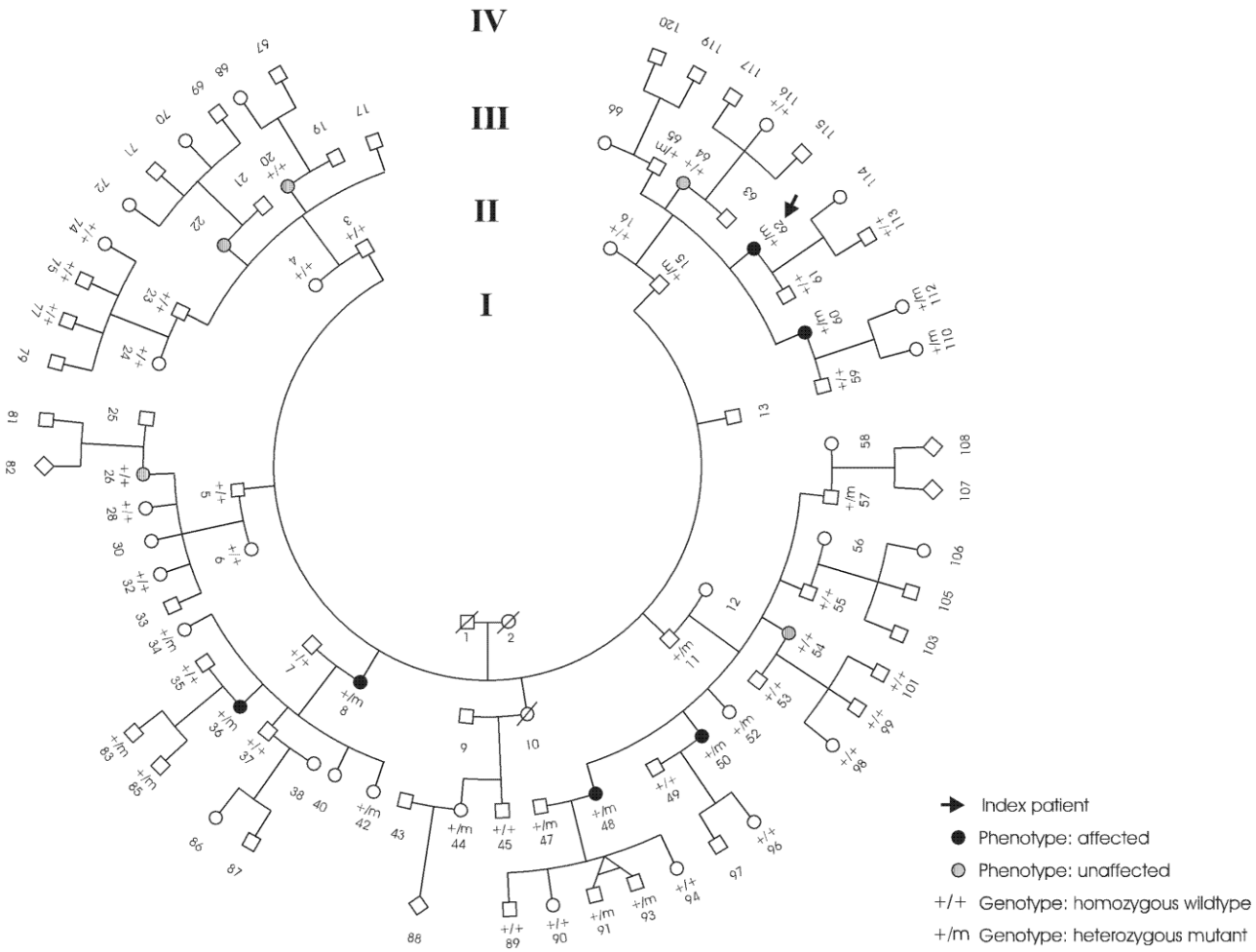

Fig. 1. Pedigree of a familiy with intrahepatic cholestasis of pregnancy. Squares indicate males, circles females and rhombus unknown gender. Black filled circles represent affected females, and grey shaded circles represent proven unaffected females, respectively. A diagonal line through symbol indicates deceased individuals. Below each symbol is the identification number, ID, in arabic numerals, and the genotype for the *MDR3* c.3486+5G>A variant, if analysed (n = 55). The index case (ID62) is marked with an arrow.

form of ICP. Further support came from several studies identifying *MDR3* gene variants as being presumably associated with ICP in small families.[15-21] However, the majority of studies were retrospective in design and the pedigrees studied were usually not large enough to allow a linkage analysis or genetic differential diagnosis.[22] Thus, the question whether linkage can be demonstrated between *MDR3* gene variants and familial forms of ICP remained to be answered conclusively.[10,15,17,23,24] In addition, we demonstrated in an earlier study that 4 of 21 ICP women harbored intronic *MDR3* variants presumably associated with aberrant splicing.[16] However, aberrant splicing was not verified in those cases.[16] In this study, we present evidence that dominant familial ICP is linked to a newly identified intronic *MDR3* gene splicing mutation resulting in a 54 bp (3465–3518) inframe deletion via cryptic splicing site activation.

Materials and Methods

Characterization of the Index Patient. A 27-year-old pregnant women (23rd week of gestation) suffering from intense pruritus was included in a prospective study on the genetic background of ICP.[16] After a 12 hour fasting period, serum and whole blood was collected for liver function tests, various routine laboratory tests and DNA extraction. The γ-glutamyl transpeptidase (γ-GT) activity was expressed as IU at 25°C. The reference values (2.5 to 97.5 percentile) for γ-GT activity were 6.7 to 15 U/l in our laboratory. These were assessed in 17 women without liver disease and ICP in the last trimenon of their pregnancy. Overall two consecutive pregnancies were studied in the index patient.

Family Study. Our patient was found to be a member of a Mennonite kinship, comprising more than 97 family members (Fig. 1), living in Germany, Paraguay, Canada

and Kazakhstan. Blood specimens were drawn from 55 family members: 44 individuals are related by blood, and 11 individuals were related by married. DNA was extracted using the QIAamp DNA Blood Mini Kit (Quiagen, Hilden, Germany). Whenever DNA extraction could not be performed directly, DNA was stabilized by using the QIAamp ASI and ASII buffer system (Quiagen) and samples were stored until DNA extraction. Like the index patient identification number (ID) 62 all family members included were interviewed according to a standardized questionnaire asking about outcome and complications during previous pregnancies, symptomatic liver and gallstone disease, operations on the biliary tract, episodes of pruritus during pregnancy and clinical signs of liver disease during use of medications or oral contraception. The interviewer was blinded to the individuals genotype. The study was approved by the Ethics committee of the University of Bonn and written informed consent was obtained from all participating individuals.

Sequencing. Genomic and cDNA sequences were derived from known sequences (BSEP: GenBank accession number AC008177 for promoter and exon 1 - 21, AC069165 for exon 22 – 28 and NM_003742 for cDNA; MDR3: AC005068.2 for noncoding exons -3 to 1 and coding exon 2 and 3, AC006154 for exons 4 - 12, AC0005045 for exons 13 - 28 and NM_000443 for cDNA; FIC1: AC027097.10 for exons 2, 6, 7, 8, 9, 15, 17, 20, 21 and 25. Primers for genomic DNA were designed to span all exons and at least 100 base pares (bp) of flanking intronic sequences at the 5′ and 3′ end of each exon in case of BSEP and MDR3. In case of FIC1 all primers were designed to span exons and their branch and splicing sites known to be mutated in cholestatic liver diseases i.e. exons 2, 6, 7, 8, 9, 15, 17, 20, 21, 25.[25-29] The DNA sequences were analyzed on an ABI3700 capillary sequencer (ABI, Weiterstadt, Germany) using the phredPhrap, consed and polyphred software (University of Washington, Seattle, WA). Details are available by e-mail from: info@epidauros.com.

Analysis of Variants of the MDR3 Gene. The genetic variability of the *MDR3* gene in the index patient (ID62) (Table 1) was compared with results of earlier studies.[16,30] For previously unreported intronic variants in the vicinity of authentic splicing sites (5′ss), the Shapiro and Senapathy (S&S) consensus values were calculated.[31] Identification and verification of cryptic splicing sites was performed using splice-junction primer combinations designed according to NM_000443. The aberrant bands were cut out from agarose gels, purified (High Pure PCR product Purification Kit, Roche, Germany) and sequenced in both directions by manual sequencing using semi automated sequencers (PE Biosystems, Foster City, CA) after probe preparation according to the manufacturer's protocol.

Statistical Analysis. For linkage analysis the following assumptions were made. All women with a positive history of pruritus during pregnancy were classified as affected and labeled with the highest liability class (LC1). Women with three pregnancies and a negative history of pruritus were classified as unaffected with LC2. The liability decreased to the lowest LC4 in women with a negative history of pruritus and only one pregnancy carried to term. All male family members were typed as of unknown affection status. The genotype-phenotype relationship was described by a liability class dependent penetrance vector. The penetrance for the homozygous wild-type is the phenocopy rate and was assumed to vary from 0.01 (LC1) to 0.04 (LC4), whereas the penetrance for the heterozygous genotype was assumed to be 0.95 (LC1) to 0.50 (LC4), and for the homozygous mutant a penetrance of 1.0 was assumed for all liability classes (LC1-4). These assumptions are in accordance with a standard dominant genetic model with a reduced penetrance graded according to diagnostic information.

Table 1. *MDR3* Genetic Variability in the Index Patient

Variant	DNA Position	Nucleotide Reference	Nucleotide Variant	Effect
c.−3335_−3336insAG*	Prom A5		insAG	n.a.
c.−316A>T*	Prom B6	A	T	n.a.
c.345−50_−46 delGAAAA	Intron 5	GAAAA	del GAAAA	n.a.
c.504C>T	Exon6	C	T	syn
c.1231−81delT	Intron11		del T	n.a.
c.1954A>G	Exon 16	A	G	R652G
c.2064+55A>G	Intron 16	A	G	n.a.
c.2478+40A>G*	Intron 20	A	G	n.a.
c.3486+5G>A*	Intron 26	G	A	splicing
c.3634−72T>C	Intron 27	T	C	n.a.

NOTE. Coding DNA numbers are relative to the ATG site and based on the cDNA sequence from GenBank accession number NM_000443. The promoter sites (Prom) are relative to noncoding exon 1. Nucleotide changes in the intronic region are from the accession numbers AC005068.2 (Promoter and non-coding exon 1-3), AC006154.1 (exon 4-12) and AC0005045.2 (exon 13-28). The amino acid position is indicated for those variants in the coding exonic sequence. Syn: synonym; n.a.: not applicable; splicing: if located in a splicing region. Variants not published before are marked by "*".

Table 2. Linkage Analysis and Genetic Differential Diagnosis

Locus Chromosomal location	*MDR3 #7q21.1*	*BSEP #2q24*	*FIC1#18q21*	*Unknown Elsewhere*
Individuals studied	**n = 55**	**n = 5**	**n=2**	**n=0**
SNPs studied	**n = 1**	**n = 11**	**n=17**	**n=0**
	c.3486+5G>A*	**c.−2078T>C**	**c.555−167A>G**	
		c.−1365G>A	**c.698+20C>T****	
		c.−963A>G	**c.696T>C**	
		c.1036−15A>G	**c.699−52C>T**	
		c.1331T>C	**c.811A>C**	
		c.1561+70C>T	**c.3262−40C>T**	
		c.1765+35T>C		
		c.2306−17C>A		
		c.2471−17T>C		
		c.3084G>A		
		c.3893−34G>A		
prior probabilities	**0.25**	**0.25**	**0.25**	**0.25**
max. LOD-scores	**2.48**	**− 2.77**	**− 1.64**	**0.00**
antilog$_{10}$ (conditional weights)	**302.00**	**0.00169**	**0.00229**	**1.00**
posterior probabilities	**> 0.9966**	**$< 10^{-5}$**	**$< 10^{-4}$**	**< 0.0033**

NOTE. The SNPs shown and variants for the three known candidate loci *FIC1, BSEP and MDR3* were entered into the linkage analysis and genetic differential diagnosis. The ten *BSEP* SNPs not marked by asterisk were already described in earlier studies.(16) Newly identified variants are marked by asterisk. In case of the *FIC1*-gene, a total of 17 SNPs and variants were entered into the multipoint linkage analysis. The new identified variants are marked by asterisk. One asterisk (*) if identified in the DNA of ID62 and two asterisks (**) if identified in ID36. The following *FIC1*-variants known to be associated with FIC1-disease were not found in the individuals (ID62, ID36) studied: c.863T>C, c.886C>T, c.923G>T, c.1660G>A, c.1982T>C, c.2081T>A, c.2097+2T>C, c.2286−4_2286−3 delCT>insAA, c.2384_2392 delGAAACCGTG, c.2674B>A, c.3391C>T. The identified wild-type genotypes at these positions, although not shown in the table, were also entered into the multipoint linkage analysis, and the subsequent genetic differential diagnosis.

Implying these assumptions, we performed a parametric, standard decadic logarithm of a likelihood ratio (LOD score) analysis involving the three candidate gene loci *MDR3* (OMIM *171060, located on chromosome #7q21.1, alias name *ABCB4*), *BSEP* (OMIM *603201, #2q24, alias *ABCB11*) and *FIC1* (OMIM *602397, #18q21 alias *ATP8B1*). A two-point linkage analysis was performed using the new intronic variant c.3486+5G>A at the *MDR3* gene as marker. For *BSEP* and *FIC1* we performed a multipoint linkage analysis. Involving 11 intragenic *BSEP* variants from the family members ID8, ID48, ID50, ID60, ID62 and 17 intragenic *FIC1* variants from the family members ID36 and 62 (Table 2). Linkage analyses were performed using the program MLINK of the LINKAGE package version 5.2, and the GENEHUNTER program (version 2.0 beta r2).[32-35] In order to assess the linkage informativity of the pedigree and its phenotypic segregation we calculated the maximum reachable LOD score (LODmax). For this, the disease model and the individual phenotypic assignments are not changed. A fully informative marker locus with zero distance to the putative disease locus was assumed.

To allow for a joint statement involving the three candidate loci simultaneously, we additionally performed a "genetic differential diagnosis" implying a Bayesian analysis under the neutrality principle. For reasons of completeness, an additional fourth unknown locus mapping elsewhere in the genome, and thus taken as unlinked to any of the markers, was included into the genetic differential diagnosis. The antilog of the maximum LOD score in a candidate gene region was taken as a conditional probability for each candidate locus to harbour the causative gene variant. The neutrality principle provided for equal prior probabilities.

The protocols of the interviews of all genotyped individuals (n = 55) were evaluated by investigators blinded to the genotype of the individuals studied. Differences in prevalence of gallstone disease and stillbirths in individuals of different genotypes were compared by the Fisher's Exact Test.

Results

Follow up of the Index Patient. The fasting total bile acid serum concentrations were 304 μM (normal <8.9 μM) on admission in the 23rd week of her first pregnancy and 90 μM in the 21st week of her second pregnancy. Pruritus started in the 22nd week of gestation during her first and in the 8th week during her second pregnancy. In both pregnancies pruritus was so intense that the patient had multiple excoriations on her lower limbs due to itching. Several measurements of liver function tests were elevated: alkaline phosphatase (AP) maximum 439 U/l (normal <50 U/l), alanine amino transferase (ALT) maximum 569 U/l (normal <19 U/l), aspartate aminotransferase (AST) maximum 224 U/l (normal <15 U/l) and

Table 3. Phenotype and Genotype of All Consanguineous Women with Positive History of Pregnancy

ID	Pregnancies Carried to Term	Stillbirths	Episodes of ICP	Phenotype	Genotype MDR3c.3486+5G>A
8	5	0	2	a	+/m
36	2	0	1	a	+/m
48	4	1	4	a	+/m
50	2	2	4	a	+/m
60*	2	1	2	a	+/m
62*	2	0	2	a	+/m
44	1	0	0	ua	+/m
20	2	0	0	ua	+/+
22	4	0	0	ua	+/+
26	2	0	0	ua	+/+
54	3	0	0	ua	+/+
64	3	0	0	ua	+/+

NOTE. The table gives an overview over all pregnancies of ICP affected and unaffected consanguineous women including the indexpatient (ID62). Overall four of these women did take birth control pills (ID8, 54, 60 and 62) of which two experienced pruritus during intake (ID60 and 62 marked by an asterisks "*"). The other women of the pedigree denied intake of oral contraceptives. a: affected of ICP; ua: unaffected of ICP; +/+: Genotype homozygous wild-type; +/m: genotype heterozygous mutant.

serum bilirubin concentrations maximum 1.7 mg/dl (normal <1.2 mg/dl). Almost similar values were found in the second pregnancy. Repeated measurements between the 23rd and 39th week of gestation revealed a median γ-GT activity of 10 U/l (95% confidence interval, 6.9 to 14.7 U/l) during this first episode of ICP. During the second episode of ICP the maximum γ-GT activity was 15 U/l. Only on one occasion, seven weeks after the first pregnancy the γ-GT was found to be 21 U/l but returned to normal before the second pregnancy. Ursodeoxycholic acid (UDCA, 250 mg 3 times a day) was tried. However, due to diarrhea occurring on the third day of treatment and only limited success in suppressing pruritus the treatment was changed to cholestyramine (4 g two times a day). The diarrhea ceased within 3 days after discontinuation of UDCA and the intensity of pruritus fell from 10 to 3 on a scale with a maximum of 10. After both pregnancies the index woman delivered a healthy child without further complications. Six years after start of the study the index patient is well without any symptoms of liver disease.

Formal Genetics of the Pedigree. The index patient (ID62) and additional five women (ID8, ID36, ID48, ID50, ID60) of the pedigree met the phenotypic criteria for the affection status "affected". Furthermore, six women (ID20, ID22, ID26, ID44, ID54 and ID64) met the phenotypic criteria of the affection status "unaffected" (Table 3).

Applying the phenotype definitions, the trait segregation was found to be consistent with a regular Mendelian segregation, characterized by a dominant mode of inheritance with female restricted expression, depending on the number of pregnancies carried to term (Fig. 1).

Characterization of a New Splicing Mutation in the MDR3 Gene. Overall ten variants were identified in the *MDR3* gene of our index patient (Table 1). Six of these variants were identified in earlier studies and found not to be associated with the occurrence of ICP.[16] Two variants c.3335_3336insAG and c.-316A>T were promoter variants. Another new intronic variant c.2478+40A>G was located 40 nucleotides (nt) downstream the exon/intron boundary and thus highly unlikely to be of any significance. Examining the remaining new variant c.3486+5G>A it became evident that this variant was located in the vicinity of the authentic 5′ss between exon 26 and intron 26 (Fig. 2). The S&S score was 87.6 for the authentic 5′ss and 72.1 for the mutated 5′ss (Fig. 2). The S&S consensus value for a cryptic 5′ss 54 nt upstream of the authentic 5′ss was 76.1 (Fig. 2). To verify this assumed cryptic 5′ss RNA was obtained from four women heterozygous for the mutated 5′ss (ID48, ID50, ID60, ID62) and from two women homozygous for the authentic 5′ss (ID20, ID64). Amplification of cDNA using splice junction primers revealed aberrant bands for the cDNA of women heterozygous for the mutant 5′ss (Fig. 3). After sequencing the obtained aberrant bands a 54 bp exon 26 (3465 – 3518) inframe deletion could be verified.

Overall, four women tried oral contraceptives. Three (ID60, 62 and 8) were heterozygous for c.3486+5G>A and one did not harbour the mutation (Table 3). Two of three heterozygous women ID60 and the index patient ID62 reported pruritus during oral contraceptives whereas ID8 did not (Table 3). The other women of the pedigree denied intake of oral contraceptives. Other forms of symptomatic drug induced cholestasis, symptomatic liver injury or chronic liver disease where not reported. Only the father of the index patient (ID15) reported of episodes of recurrent choledocholithiasis despite cholecystectomy. In four of 22 pregnancies (20%) of

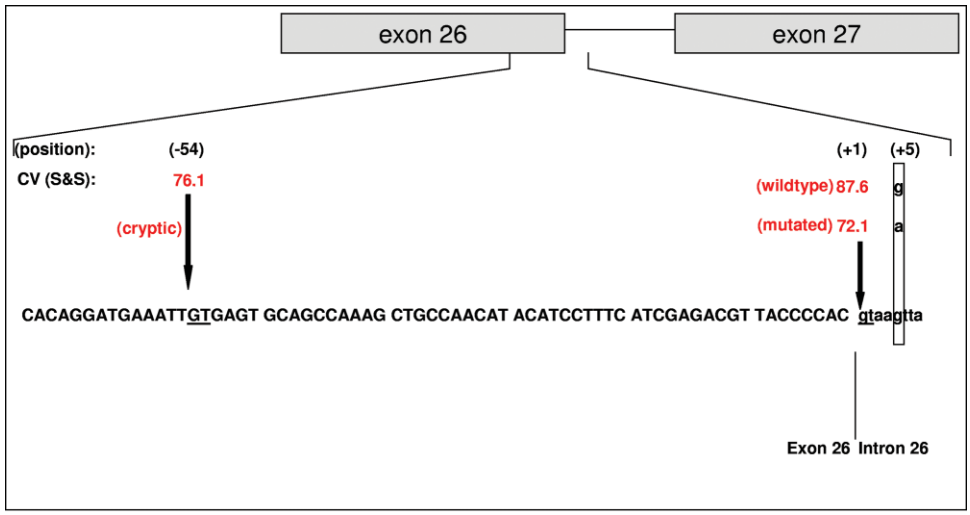

Fig. 2. Schema of cryptic 5′ss activation in human *MDR3* gene due to the c.3486+5G>A variant. The diagram shows exon 26 and 27 (grey box) and intron 26 (thin line). The sequence around the exon26/intron26 splicing site is shown. Vertical arrows indicate the 5′ss, whose GT dinucleotides are underlined. The number in parenthesis is the relative position of the nucleotides relative to the authentic 5′ss at +1. At position +5 the intronic c.3486+5G>A mutation is located. Red numbers above the splicing sites are their S&S consensus values.

women heterozygous for the c.3486+5G>A mutation stillbirths occurred, whereas no stillbirth was recorded in the 14 pregnancies of four women not harboring this mutation. However, this difference was not significant ($P = 0.203$, Fisher′s exact test). Furthermore, seven of 21 family members heterozygous for c.3486+5G>A had a positive history of gall stone disease whereas this prevalence was significantly lower in family members negative for the c.3486+5G>A mutation (one of 34) ($P = 0.00341$).

Genetic Analysis. First, we analyzed whether the mutated 5′ss (c.3486+5G>A) segregated with the history of ICP in the family of our index patient. Not mutated women did not experience ICP. Furthermore, all women who fulfilled the criteria for the phenotypic affection status "affected" (ID8, ID36, ID48, ID50, ID60, ID62) (Fig. 1, Table 3) were heterozygous for the mutated 5′ss

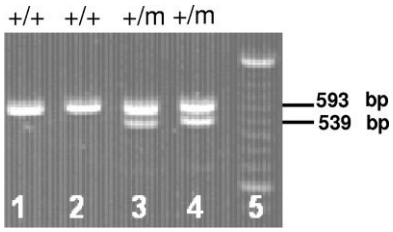

Fig. 3. Verification of the 54bp deletion caused by the new *MDR3* mutation c.3486+5G>A. After reverse transcription cDNA was studied on agarose gel by bromide staining. The genotype for c.3486+5G>A is shown above each lane. The homozygous wild-type genotype (+/+) corresponds to the presence of one fragment (sized at 593bp). Heterozygosity (+/m) results in two fragments, one sized at 593bp corresponding to the wild-type allele and one sized at 539bp corresponding to the mutated allele. *Lane 1*: Healthy control not related to the kindred, wild-type homozygous genotype (+/+). *Lane 2*: Family member (ID20), negative phenotype definition, wild-type homozygous (+/+). *Lane 3:* Index patient (ID62), ICP, heterozygous genotype (+/m). *Lane 4:* Family member (ID60), positive phenotype definition, heterozygous genotype (+/m). *Lane 5: 50 bp* standard.

(c.3486+5G>A). On the other hand, of the six women (ID20, ID22, ID26, ID44, ID54 and ID64) who met the criteria for "unaffected" only one (ID44) was mutated (Table 3). Interestingly, she was cholecystectomized due to symptomatic gallstone disease at the age of 32. Furthermore, she experienced only one pregnancy, thus the apparent unaffected phenotype was believed to be due to limited phenotypic information.

Thus, for *MDR3* the variant c.3486+5G>A was entered as marker into a two-point linkage analysis. Multipoint linkage analysis for *BSEP* involved the 11 single nucleotide polymorphisms (SNPs) identified (Table 2). Ten of these have been described by Pauli-Magnus.[16] Genotyping four affected women for these 11 SNPs revealed inconsistent results for the ten variants already known to be of non disease causing type.[16] In all four affected women the newly detected promoter variant could not be detected. Thus, it is very unlikely that this new variant may be disease causing.

The *FIC1*-gene was analysed in the index patient (ID62) and one other affected woman (ID36). Both women were homozygous wild-type at each of the eleven known polymorphisms (Table 2). Additionally in the sequence of ID62 homozygous mutations for two intronic and two silent exonic variants were detected (*). Sequence analysis in ID 36 for the above stated genomic region revealed heterozygositiy for two new intronic variants (**). Overall, 17 SNPs and variants were entered into the multipoint linkage analysis for *FIC1*.

The linkage informativity of the pedigree and its phenotypic segregation expressed as LOD_{max} was found to be 2.70.

The linkage analysis revealed the following LOD scores: for *MDR3* (marker locus c.3486+5G>A) LOD score 2.48, for *FIC1* (marker loci see Table 2) LOD score

−1.64 and *BSEP* (marker loci see Table 2) LOD score −2.77. The antilogs of these LOD scores went into the Bayesian analysis as conditional probabilities. With equal prior probabilities (motivated by the so-called neutrality principle). The genetic differential diagnosis involving all three candidate loci and an additional unknown locus gave a posterior probability of >0.9966 for *MDR3* indicating overwhelming evidence in favour of *MDR3* as the causative candidate locus.

Discussion

Our family study demonstrates evidence for linkage of the dominant form of familial ICP to heterozygosity for a new intronic *MDR3* mutation. Earlier studies had already identified *MDR3* mutations in four ICP families.[10,17,21,23] The size of our kindred enabled us to identify a regular Mendelian segregation characterized by a dominant mode of inheritance (MOI) with female restricted expression (Fig. 1). With one exception (ID47), there were no consanguineous marriages in the pedigree. Thus, a monogenic nature of the ICP phenotype in this pedigree was found to be very likely. The same MOI has been observed in earlier studies.[10,14]

Furthermore, we demonstrate that the new identified *MDR3* mutation led to a 54 bp exon 26 (3465 – 3518) inframe deletion in women with the ICP phenotype (Fig. 2; Fig. 3). As mechanism responsible for this deletion an activation of a cryptic 5′ splicing site 54 bp upstream the mutated authentic 5′ splicing site (Fig. 2) was found. This is the first time that cryptic splicing site activation due to a disease related mutation has been verified in the *MDR3* gene. However, in an earlier study Pauli-Magnus in collaboration with our group identified ICP specific splicing site mutations in four out of 21 ICP women.[16] Neither these earlier described splicing site mutations nor our newly identified c.3486+5G>A *MDR3* splicing site mutation were found in 80 controls.[16] Thus, it is highly unlikely that particularly our new identified mutation is a common polymorphism in healthy adults. Interestingly, similar to the findings in our index patient, a significant decrease in the S&S consensus value can be found for all splicing site mutations described by Pauli-Magnus and coworkers (data not shown). Thus, it is tempting to speculate that cryptic splicing site activation may have caused functional relevant *MDR3* gene deletions in almost 20% of ICP patients in the Swiss/German collective studied by Pauli-Magnus.[16] It is interesting to note that the majority of individuals in our pedigree are members of the Mennonite church which in part originates from Switzerland. Thus, the high prevalence of splicing site mutations found by Pauli Magnus and our findings in Mennonites may reflect this common genetic background. Furthermore, it is known that cholestatic diseases like the benign recurrent intrahepatic cholestasis (BRIC) — a FIC1 disease — are more prevalent in members of the Amish Mennonite church which separated from the Mennonite church in Switzerland in 1693.[36] In this context, it is very important to note, that we did not find any of the known genetic variants linked to FIC1 disease in individuals from our pedigree (Table 2). The same holds true for the *BSEP* gene which was sequenced completely (Table 2).

In addition, the multipoint linkage analysis for *BSEP* and *FIC1* did not show any linkage (negative LOD scores) to ICP. In contrast to these negative findings the two point linkage analysis using the *MDR3* gene mutation c.3486+5G>A as intragenic marker gave a positive 2.48 LOD score which was very near to the LOD_{max} score of 2.70 achievable in our kindred (Table 2). The low LOD_{max} in such a large kindred reflects the fact that LOD score analysis is highly dependent on the number of informative meioses going into the analysis. Furthermore, only two generations and four segregating sibships could be studied in our pedigree (Fig. 1); and the female restricted expression of the trait further limited the available phenotypic information. Despite this limitations, we present overwhelming evidence that the new identified *MDR3* gene mutation c.3486+5G>A is linked to ICP in the pedigree presented.

As no generally accepted *in vitro MDR3* expression system exists we were not able to test for the functional relevance of the 54 bp exon 26 (3465 – 3518) inframe deletion caused by the *MDR3* gene mutation. Furthermore, *in vivo* expression studies were not possible, as for ethical reasons no liver biopsy was performed. However, if the position of the putative amino acid deletion del 1145-1162 corresponding to the 54 bp (3465 – 3518) inframe deletion in exon 26 is analyzed in the transmembrane topology scheme it seems to be possible that the function of the intracellular ATP binding sites of the *MDR3* gene may be modified by this deletion (Fig. 4).

The anamnestic data revealed, that individuals heterozygous for the *MDR3* gene mutation had a significantly higher prevalence of symptomatic gall stone disease irrespective of gender. This finding is in accordance with earlier findings that identified *MDR3* as a so called lithgene[37,38]and demonstrated a higher prevalence of symptomatic gall stone disease in women with ICP.[19,39,40] In this respect it appears noteworthy that the father (ID15) of our index patient is heterozygous for the c.3486+5G>A mutation and suffers from recurrent choledocholithiasis despite cholecystectomy (Fig. 1). Furthermore, we speculate that c.3486+5G>A may be responsible for the rather high rate of stillbirths (20%) reported from heterozygous women. Such an elevated rate

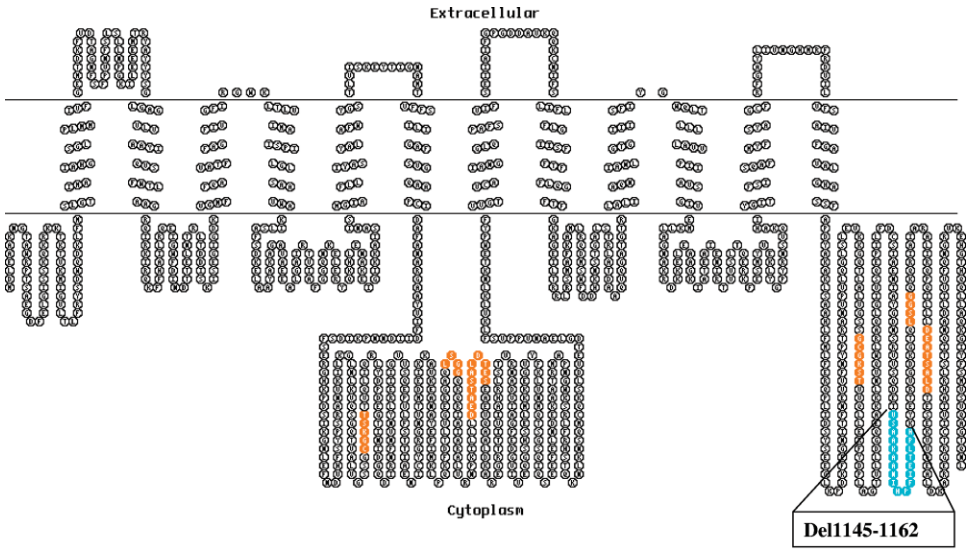

Fig. 4. Secondary structure of the MDR3 transmembrane protein. The transmembrane topology schematic was rendered using TOPO (S.J. Johns and R.C. Speth, transmembrane protein display software, **http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl). Shown in orange are putative Walker A, c-signature element and Walker-B. Shown in blue is the putative aminoacid deletion from position 1145 to 1162 resulting from the proven 54 bp exon 26 (3465 – 3518) inframe deletion caused by the c.3486+5G>A mutation.

of stillbirths has also been observed in earlier ICP studies especially if the bile acid serum concentrations were as high as in our index patient.[2,10,11] Furthermore, two of three heterozygous women reported of pruritus during intake of oral contraceptives. Although not conclusive this may support the hypothesis that cholestasis linked to MDR3 defects may be induced by birth control pills.[24] Despite all these risks associated with the mutation our index patient and the other heterozygous family members did not show any signs of symptomatic liver disease six years after start of the study. Furthermore, γ-GT activity during ICP was normal in two pregnancies of the index patient. Thus, the type of ICP observed in our index patient would best be described as normal γ-GT ICP of the benign type or classical ICP.[41]

In case of PFIC1, 2 and 3 it has been stated in different studies and reviews that γ-GT is a reliable marker to differentiate between MDR3, FIC1 and BSEP defects.[42] The normal γ-GT values found in our index patient support the view that normal γ-GT values (≤15 U/l in pregnant women) do not exclude ICP linked to *MDR3* mutations. Of note, no general agreement exists concerning gender specifc reference values for γ-GT. Data of Bacq et al. obtained in women and our data in women in late pregnancy support lower reference values for γ-GT in women.[43]

In conclusion our study demonstrates that the dominant familial form of ICP observed in this large Mennonite pedigree is linked to the *MDR3* mutation c.3486+5G>A. We further demonstrate that this intronic mutation results in an 54 bp (3465 – 3518) inframe deletion in exon 26 due to cryptic splicing site activation, a mechanism not described so far in MDR3 disease. Furthermore, we speculate from our present and from earlier studies,[16] that the prevalence of ICP caused by *MDR3* splicing site mutations may be rather high up to 20% at least in women suffering from ICP who share a Swiss Mennonite genetic background.

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