Association between *MDR1* gene polymorphisms and the risk of Crohn's disease in a cohort of Algerian pediatric patients

Amira Bouzidi[1], Hamida Mesbah-Amroun[1], Aziza Boukercha[1], Fadila Benhassine[2], Réda Belboueb[3], Karima Berkouk[4], Wassila Messadi[5] and Chafia Touil-Boukoffa[1]

BACKGROUND: The multi-drug resistance gene (*MDR1)* has raised increasing interest as a susceptibility gene for Crohn's disease (CD). The role of *MDR1* single-nucleotide polymorphisms (SNPs) in the predisposition and behavior of CD in the pediatric population is still elusive. Here, we investigated whether SNPs in *MDR1* are associated with CD in Algerian pediatric patients.

METHODS: A case-control study was conducted enrolling 47 pediatric CD patients and 100 controls. All subjects were genotyped for the most common *MDR1* SNPs (C3434T, C1236T, and G2677A/T) using PCR-RFLP method. We also explored the association between polymorphisms and clinical sub-phenotypes.

RESULTS: We have detected no significant association of C3435T SNP and pediatric CD. However, we observed a significantly higher frequency of the risk alleles, 1236T and 2677T/A among the CD patients compared to controls. Moreover, the risk allele 1236T was associated to a higher risk for resective surgery.

CONCLUSION: Our data suggest that the C1236T and G2677A/T SNPs in the *MDR1* gene are associated with CD and the C1236T risk allele with a more severe course of disease in Algerian pediatric patients. Further analysis using larger patients group and functional studies would be interesting to elucidate the role of *MDR1* gene in pediatric CD.

Inflammatory bowel disease (IBD) is a disorder characterized by chronic inflammation of the gastrointestinal tract. There are two common clinical subtypes, Crohn's disease (CD) and ulcerative colitis (UC). CD can affect any part of the intestine and is associated with discontinuous transmural lesions of the gut wall (1). CD affects both adult and children with the peak age range of diagnosis in the second and third decades of life. In childhood, rates of CD increase from the first year of life, with highest rates in teenage years (2).

The etiology of CD involves environmental and genetic factors that lead to dysfunction of the epithelial barrier with consequent deregulation of the mucosal immune system and responses to gut microbiota (1).

Over the last 15 y, linkage analysis, positional candidate gene studies, and genome-wide association studies (GWAS) provided the most convincing evidence of a genetic link, identifying a number of susceptibility genes for CD. In particular, the *NOD2/CARD15* (3,4), *IL23R* (5), *ATG16L1* (6) genes have been widely implicated in the pathogenesis of IBD in different geographical populations. Numerous data have shown that the underlying cause of susceptibility to CD is a complex interplay between autophagy, innate immunity, and adaptive immunity. Most genetic analyses in CD have been performed in patients with adult-onset disease (7). Pediatric CD, however, has unique characteristics of phenotype and severity (8), features that provide support for the search for genetic loci that may be specific to pediatric disease. In addition, pediatric CD has a stronger familial component than the adult disease. Thus, targeting pediatric patients potentially provide additional power to identify molecular factors that contribute to pathogenesis of IBD, as illustrated by a transatlantic collaboration, which analyzed a cohort of 3426 childhood-onset IBD patients and identified five new loci associated with pediatric IBD (9).

The multi-drug resistance gene (*MDR1*) spans 209 kb and is located in the chromosome 7q21.1. It is composed of 28 exons and the cDNA is 4.5 kb; thus, its coding region accounts for less than 5% of the total gene (10). The *MDR1* gene codes for the P-glycoprotein 170 (P-gp170), a member of the adenosine triphosphate (ATP) binding cassette family. P-gp170 functions as a transmembrane efflux pump, thereby moving drugs from the intracellular to the extracellular domain (11). In the human gastrointestinal tract (GI), P-gp170 is found at high concentrations on apical surfaces of superficial columnar epithelial cells of the colon, distal small bowel, small biliary ductules, and small pancreatic ductules (12). The putative role of the P-gp170 in the GI tract is to decrease the absorption of endogenous and exogenous hydrophobic amphipathic toxins (13).

Evidences at functional and genetic levels point to *MDR1* gene as an attractive candidate for the pathogenesis and therapeutic responses in patients with IBD (14). First, the *MDR1* gene is present in a region of the human genome (7q21.1) that

may harbor a disease gene involved in susceptibility to IBD (15). Second, *MDR1a* knockout mice develop a spontaneous colitis when maintained under specific pathogen-free conditions (16). The colitis is prevented and reversed by the administration of antibiotics, suggesting that the intestinal flora is necessary to initiate and perpetuate the inflammation. This model implies that loss of the xenobiotic efflux mechanism may promote the development of the colitis.

Three single-nucleotide polymorphisms (SNPs) in *MDR1* have been most commonly studied: C3435T (rs1045642, synonymous), C1236T (rs1128503, synonymous), and G2677T/A (rs2032582, nonsynonymous).

Despite *in vivo* and *in vitro* studies, epidemiological evidence on *MDR1* gene-associations with CD is largely inconsistent. Some studies have found associations between these variants and CD (17,18); however, others have not (19,20). Furthermore, two meta-analyses evaluating the potential association of C3435T and G2677A/T polymorphisms with IBD failed to show an association between these variants and CD (21,22).

Pediatric CD has a distinct phenotype and the difference from adult CD makes it a unique subgroup (8). It can thus be expected that *MDR1*-related genetic associations among pediatric CD patients may also differ from those observed among adults. Only two studies had investigated *MDR1*-gene associations with CD exclusively among pediatric CD patients (23,24). However, there is no report of the frequency distribution of the major allelic variants or haplotypes of *MDR1* gene in the Algerian pediatric patients with CD. Therefore, we aimed to investigate the frequencies of *MDR1* gene polymorphisms and haplotypes in an Algerian cohort of pediatric CD patients, and evaluate the association between those polymorphisms and the susceptibility to CD in the Algerian population. A genotype–phenotype analysis was performed by comparing the genotype distribution among specific CD phenotype vs. that among controls.

RESULTS
Clinical Findings
A total of 47 pediatric CD cases and 100 controls were studied. The main clinical characteristics of patients included in the study are summarized in **Table 1**. The mean age at diagnosis of the cases was 10.78 ± 3.79 y. Most of the cases (70.21%) belongs to A1b age category. 14.89% of the patients have a positive family history of CD, 27.65% had resective surgery, and 23.40% presents extra-intestinal manifestations (in particular articular). Analysis of the disease location revealed the following distribution: L3 ± L4 (ileo-colonic with or without upper tract): 53.19%, L1 ± L4 (ileal with or without upper tract): 34.04% and L2 ± L4 (colonic with or without upper tract): 12.76%. No patient had disease affecting only the upper tract. Disease behavior was: B1 ± p = 61.70%, B2 ± p = 8.51%, B3 ± p = 6.38%, B2B3 ± p = 23.4%.

Genotype Analysis
Distributions of *MDR1* gene polymorphisms in CD patients and controls are shown in **Table 2**. Genotype frequencies for

each SNP were in Hardy-Weinberg equilibrium. We detected no significant association with CD in our population for the *MDR1* C3435T SNP in comparison to the control group, since the frequency of wild-type, heterozygous, and mutated homozygous genotypes did not differ among the two study groups.

In contrast, the risk allele 1236T of the *MDR1* gene was significantly increased in patients with CD (52.1 vs. 34.5% in controls, $P = 0.005$; OR = 2.067; $(CI)_{95\%} = 1.255–3.405$). Homozygous and heterozygous carriers of the risk allele 1235T are significantly more frequent ($P = 0.016$) in the cases group (25.53 and 53.19%, respectively) compared to the controls (14 and 41%, respectively).

In CD patients, the frequency of G2677A/T mutation was significantly higher than in controls (allele T: 42.5 vs. 25%; allele A: 2.12 vs. 0.5%; $P = 0.0012$; OR = 2.360; $(CI)_{95\%} = 1.408–3.955$). Homozygous and heterozygous carriers of the mutated allele 2677T are significantly more frequent ($P = 0.0022$) in the cases group (17.02 and 51.06% respectively) compared to the controls (3 and 43% respectively).

We detected no homozygous carrier for the mutated allele 2677A in the two studied groups.

Haplotypes Calculation
There are 12 possible haplotypes of the 3 *MDR1* loci listed in **Table 3** with their estimated frequencies. We did not observe a statistical significant difference when comparing the overall haplotypes distribution between CD patients and controls ($P = 0.47$). However, there was a weak evidence ($P = 0.03$) that the haplotype composed of only wild-type alleles at position 1236, 2677, 3435 (CGC) is more frequent in the control group. In the CD patients group, this haplotype is in second position ($P = 0.14$) and the most frequent haplotype bears a mutated allele at position 1236 and wild type at position 2677 and 3435 (TGC).

Frequencies of haplotypes for MDR1 SNPs pairs are listed in **Table 4**. When comparing haplotype pairs between patients and controls, frequency of 3435C-2677G and 2677G-1236C pairs were significantly higher in the control group compared to patients ($P = 0.04$ and $P = 0.01$ respectively). In the patients group, the frequency of 2677T-1236T haplotype was significantly higher compared to controls ($P = 0.02$).

Phenotype/Genotype Analysis
When the contribution of each SNP of the *MDR1* gene was investigated (**Table 1**), the strongest correlation could be ascribed to the C1236T SNP. In CD patients, the occurrence of one risk allele of C1236T SNP was significantly associated with resective surgery ($P = 0.028$, r = 0.321). No correlation was found for age at diagnosis, family history, disease behavior, or extra-intestinal manifestations.

DISCUSSION
We carried out a case–control study to examine associations between the *MDR1* gene and CD among Algerian pediatric patients. Furthermore, we compared the genotyping results with phenotypic characteristics of CD patients to identify a possible genotype-phenotype correlation. Of the three SNPs

Table 1. Genotypic distribution of *MDR1* polymorphisms according to CD patient's demographic and clinical characteristics

Parameters	Total cases	C1236T n (%)				C3435T n (%)				G2677A/T n (%)				
		CC	CT	TT	P value[a]	CC	CT	TT	P value[a]	GG	GT	TT	GA	P value[a]
Gender n (%)														
Female	29 (61.70)	5 (17.24)	16 (55.17)	8 (27.58)	0.402	5 (17.24)	19 (65.51)	5 (17.24)	0.402	6 (20.68)	18 (62.06)	5 (13.79)	–	0.183
Male	18 (38.29)	5 (27.77)	9 (50)	4 (22.22)		5 (27.77)	11 (61.11)	2 (11.11)		7 (38.88)	6 (33.33)	3 (16.66)	2 (11.11)	
Age category[b]														
A1a	14 (29.78)	4 (28.57)	8 (57.14)	2 (14.28)	0.437	4 (28.57)	9 (64.28)	1 (7.14)	0.437	5 (35.71)	8 (57.14)	1 (7.14)	–	0.432
A1b	33 (70.21)	6 (18.18)	17 (51.51)	10 (30.30)		6 (18.18)	21 (63.63)	6 (18.18)		8 (24.24)	16 (48.48)	7 (21.21)	2 (6.06)	
Location[b]														
L1±L4	16 (34.04)	6 (37.5)	9 (56.25)	1 (6.25)	0.133	5 (31.25)	8 (50)	3 (18.75)	0.057	5 (31.25)	8 (50)	1 (6.25)	2 (12.5)	0.386
L2±L4	6 (12.76)	–	3 (50)	3 (50)		3 (50)	2 (33.33)	1 (16.66)		3 (50)	2 (33.33)	1 (16.66)	–	
L3±L4	25 (53.19)	4 (16)	13 (52)	8 (32)		2 (8)	20 (80)	3 (12)		5 (20)	14 (56)	6 (24)	–	
Behavior[b]														
B1±p	29 (61.70)	8 (27.58)	13 (44.82)	8 (27.58)	0.155	5 (17.24)	18 (62.06)	6 (20.68)	0.612	7 (24.13)	15 (51.72)	6 (20.68)	1 (3.44)	0.598
B2±p	4 (8.51)	1 (25)	3 (75)	–		2 (50)	2 (50)	–		2 (50)	2 (50)	–	–	
B3±p	3 (6.38)	–	3 (100)	–		–	3 (100)	–		–	3 (100)	–	–	
B2+B3±p	11 (23.40)	1 (9.09)	6 (54.54)	4 (36.36)		3 (27.27)	7 (63.63)	1 (9.09)		4 (36.36)	4 (36.36)	2 (18.18)	1 (9.09)	
EIM[c]														
Yes	11 (23.40)	3 (27.27)	6 (54.54)	2 (18.18)	0.588	–	11 (100)	–	0.050	3 (27.27)	5 (45.45)	2 (18.18)	1 (9.09)	0.975
No	36 (76.59)	7 (19.44)	19 (52.77)	10 (27.77)		10 (27.7)	19 (52.77)	7 (19.44)		10 (27.77)	19 (52.77)	6 (16.66)	1 (2.77)	
Family history														
Yes	7 (14.89)	2 (28.57)	4 (57.14)	1 (14.28)	0.618	2 (28.57)	3 (42.85)	2 (28.57)	0.618	3 (42.85)	3 (42.85)	1 (14.28)	–	0.341
No	40 (85.10)	8 (20)	21 (52.5)	11 (27.5)		8 (20)	27 (67.5)	5 (12.5)		10 (25)	21 (52.5)	7 (17.5)	2 (5)	
Surgery														
Yes	13 (27.65)	–	10 (76.92)	3 (23.07)	0.028*	2 (15.38)	10 (76.92)	1 (7.69)	0.552	3 (23.07)	7 (53.84)	2 (15.38)	1 (7.69)	0.672
No	34 (72.34)	10 (29.4)	15 (44.11)	9 (26.47)		8 (23.52)	20 (58.82)	6 (17.64)		10 (29.41)	17 (50)	6 (17.64)	1 (2.94)	

A1a: age between birth and 10 y (0 < age < 10 y).
A1b: age between 10 and 17 y (10 < age < 17 y).
L1±L4: distal 1/3 ileum with or without upper digestive tract involvement; L2±L4: colonic with or without upper digestive tract involvement; L3±L4: ileocolonic with or without upper digestive tract involvement.
B1±p: non-stricturing, non-penetrating with or without perianal involvement; B2±p: stricturing with or without perianal involvement; B3±p: penetrating with or without perianal involvement B2B3±p: both penetrating and stricturing with or without perianal involvement.
[a]P value for Pearson correlation test. Correlation between phenotypes and carrying at least one risk allele (*$P < 0.05$). [b]Age at diagnosis, disease location and behavior were classified according to Paris classification (34). [c]Extra intestinal manifestations.

Table 2. *MDR1* SNPs allele and genotype frequencies of early-onset CD patient's vs. control group

SNP	Amino acid change	Allele	Controls $n = 100$ (%)	CD $n = 47$ (%)	P value[a]	CI$_{95\%}$	OR	Genotype	Controls	P for HWE[b]	CD	P for HWE[b]	P value[b]
rs1128503		C	131 (65.5)	45 (47.87)	0.005	(1.255–3.405)	2.067	CC	45 (45)	0.3533	10 (21.27)	0.6521	0.016
C1236T	G412G	T	69 (34.5)	49 (52.12)	**			CT	41 (41)		25 (53.19)		*
								TT	14 (14)		12 (25.53)		
rs2032582		G	149 (74.5)	52 (55.31)				GG	53 (53)		13 (27.65)		
G2677A/T	S893A/T	T	50 (25)	40 (42.55)		(1.408–3.955)		GT	43 (43)		24 (51.06)		
		A	1 (0.5)	2 (2.12)	0.0012**		2.360	TT	3 (3)	0.1278	8 (17.02)	0.59	0.0022**
								AT	1 (1)		–		
								GA	–		2 (4.25)		
								AA	–		–		
rs1045642	I1145I	C	126 (63)	51 (54.25)	0.161		1.436	CC	37 (37)		11 (23.4)		0.2526
C3435T		T	74 (37)	43 (45.74)	NS	(0.8732–2.360)		CT	52 (52)	0.248	29 (61.70)	0.0957	NS
								TT	11 (11)		7 (14.89)		

CI, confidence interval; HWE: Hardy-Weinberg equilibrium; OR, odd ratio.
[a]P values for Fisher's exact tests with a confidence interval of 95% (*P < 0.05, **P < 0.01). [b]P values for Chi square test with a confidence interval of 95% (*P < 0.05, **P < 0.01).

Table 3. Comparison of haplotype frequencies for *MDR1* (1236/2677/3435) between CD patients and controls under the null hypothesis (H0) assuming independence among all the loci

	Haplotype frequencies		
Haplotype	Controls (%) under H0	CD (%) under H0	P value[a]
CGC	30.7424	14.0865	0.0311*
CGT	18.0551	12.3961	0.3873
CAC	0.2063	0.5418	0.779
CAT	0.1212	0.4768	0.6789
CTC	10.3163	10,8357	0.9239
CTT	6.0588	9.5355	0.4470
TGC	16,1926	15.3386	0.8946
TGT	9.5099	13.4980	0.4684
TAC	0.1087	0.5899	0.5958
TAT	0.0638	0.5192	0.5735
TTC	5.4338	11.7989	0.1724
TTT	3.1913	10.3831	0.0887
Overall			0.4700

[a]P values for Chi square test with a confidence interval of 95% (*P < 0.05).

studied, associations with C1236T and G2677A/T SNPs were evident, whereas associations with C3435T were not significant. Moreover, our data suggest that the C1236T SNP correlates with the increased risk for surgery.

There is no published data on the epidemiology of CD in the Algerian pediatric population. However, our study provides first insights on the prevalence of the disease in Algiers as we enrolled 47 pediatric CD patients from 4 different pediatric departments during 2 y. Pediatric UC patients were also enrolled but the very small number of six patients did not allow us to conduct a case–control study.

Previous studies investigating *MDR1* gene polymorphisms and its predisposition to CD, have shown conflicting results. In a multicenter North American cohort with 444 IBD trios, Brant *et al.* investigated the G2677T/A and the C3435T SNPs (17). They reported a significant association of the G2677 variant with IBD confirmed by PDT (pedigree disequilibrium test) analysis, whereas no association was observed for the C3435T SNP. In contrast, Potocnik *et al.* reported no significant associations between both, C3435T and G2677T/A SNPs in a Slovenian cohort (25). However, they have documented positive associations of two other intronic SNPs (rs2235035 and rs1922242) with refractory CD. Furthermore, Urcelay *et al.* reported a significant association of the C3435Twild-type genotype in Spanish CD patients, but data were not in Hardy-Weinberg equilibrium (18).

In contrast, other studies failed to demonstrate an association between *MDR1* variants and CD. In a German case–control study and in a large Scottish cohort, the authors reported a significant increase of the 3435 TT genotype and T-allelic frequencies in patients with UC but no association was seen with CD (26,27).

Reports in pediatric CD are limited. Only two studies had investigated associations of the *MDR1* gene with CD exclusively among the pediatric population (23,24). Cucchiara *et al.* have not found an association between the C3435T SNP and either predisposition to early onset CD, clinical features, or response to medical therapy (23).The study by Krupoves *et al.* revealed that the SNP rs17327442 was significantly associated with an overall susceptibility to CD. Analysis of the genotype-phenotype correlation indicated that two SNPs (rs10248420 and rs2032583) were significantly associated with colonic disease and that five SNPs (rs1128503, rs1202184, rs1202186, rs2091766, and rs2235046) were associated with structuring and/or penetrating disease (24). The reason for such variability of results in the literature is not clear. However, population ethnic heterogeneity and/or variability of exposure to environmental factors between populations might be plausible explanations.

Table 4. Comparison of haplotype frequencies for pairs of *MDR1 SNPs* between CD patients and controls

Haplotype	C3435T-G2677A/T				G2677A/T-C1236T				C3435T-C1236T		
	Controls	Patients	*P* value[a]		Controls	Patients	*P* value[a]		Controls	Patients	*P* value[a]
C-G	46.93	29.42	0.0451*	G-C	48.79	26.48	0.0108*	C-C	41.26	25.46	0.0664
T-G	27.56	25.89	0.8322	G-T	25.7	28.83	0.6901	C-T	21.73	27.72	0.4270
C-T	15.75	22.63	0.3124	T-C	16.37	20.37	0.5543	T-C	24.23	22.4	0.8082
T-T	9.25	19.91	0.0708	T-T	8.62	22.18	0.0229*	T-T	12.76	24.4	0.0771
C-A	0.315	1.13	0.5438	AC	0.32	1.01	0.5959				
T-A	0.1085	0.99	0.4943	AT	0.17	1.1	0.4422				

[a]*P* values for Chi square test with a confidence interval of 95% (**P* < 0.05).

In our study, we described for the first time the distribution of *MDR1* gene polymorphisms in a cohort composed of pediatric CD patients and healthy controls from Algeria.

Similar to the study by Cucchiara *et al.*, our results indicated that the C3435T polymorphism is not a risk factor for CD in our cohort.

To the best of our knowledge, this is the first study reporting a significant association of the G2677A/T (*P* = 0.0012) SNPs with CD in the early onset population. Previous studies investigating the influence of the coding polymorphism G2677A/T on both mRNA and P-gp170 expression have reached conflicting results. Kim *et al.* reported an association of 2677T allele with higher levels of P-gp170 expression (28). However, Kimchi-Sarfaty *et al.* documented no influence of this polymorphism on expression level and the intracellular localization of P-gp170 (29).

Moreover, our results indicated that the C1236T is a risk factor for CD in our cohort of Algerian pediatric CD patients (*P* = 0.005). This result is consistent with a study carried out in a Caucasian population from Canterbury, New Zealand. Based on stratification of all IBD patients with respect to age, the authors noted that individuals under 17 y of age carrying the variant 1236T allele were at an increased risk for CD development (30). The influence of C1236TSNP on the expression and P-gp170 activity still remains under investigation. In a study by Hemauer *et al.*, it was indicated that in human placenta the 1236CT and 1236TT genotypes were related to a decreased P-gp170 expression in comparison to the wild-type 1236CC genotype (31). However, Goto *et al.* did not observe any correlation between the change in the intestinal mRNA expression of *MDR1* or P-gp170 protein and the C1236T SNP in recipients of liver transplantation (32). It is suggested that *MDR1* SNPs might exert their *effect* not by influencing expression level of P-gp170 but by altering the timing of co-translational folding and insertion of P-gp170 into the membrane, thereby altering the structure of substrate and inhibitor interaction sites (29). Consequently, the SNP might affect the affinity of theP-gp170 to toxins, which might lead to increased absorption of toxins in the gut and inflammation. Interestingly, the frequency of haplotype pairs 2677T-1236T was significantly higher in the patients group, compared to controls (*P* = 0.02), confirming thus their status as risk allele in the CD patients.

When comparing the genotype results with clinical characteristics of CD patients, the occurrence of one risk allele of C1236T SNP was moderately correlated with resective surgery (*P* = 0.028, *r* = 0.321) which suggest a more aggressive course of the disease in carriers of at least one risk allele. Krupoves *et al.* analyzed children with CD (12.1 ± 3.5 y) from a Canadian population and observed a relationship between the C1236T polymorphism and stricture formation as well as penetrating disease (*P* = 0.02) (24). As far as we know, our study is the first report correlating the C1236T SNP with an increased risk for surgery, which could suggest a more severe disease course. In **Table 1**, we observe in one hand, that 1236TT genotype frequency is higher in the group of patients that did not have surgery. Following the outcome of this group of patients to see if they will need surgery would be interesting to confirm our results. In the other hand, none of patients with surgery had a 1236CC genotype which comfort our suggestion that 1236T allele is a risk allele for surgery.

It is important to acknowledge the strengths and limitations of the study prior to interpretation. First, because of the difficulty to get enrolled healthy children in our study, cases and controls are not matched regarding their age. However, enrolling adult controls does not impact our result because the matching factor (age in this case) is associated with disease (CD) but not exposure (*MDR1* SNPs). Second, findings of association between *MDR1* and pediatric CD disease susceptibility and risk for surgery should be treated with caution, as they were based on a small sample of subjects. Further studies with larger sample sizes are needed to fully confirm these findings. However, our result should get *MDR1* SNPs, mainly C1236T and G2677A/T more interest, as there are very few studies investigating MDR1 SNPs in the pediatric CD population. To date, no meta-analysis investigating the association of *MDR1* with pediatric CD was performed. As a perspective, a meta-analysis, including our results would be interesting in order to cumulate and summarize the contribution of *MDR1* gene contribution in pediatric CD patients. Moreover, further functional studies are required to address whether this SNP has an impact on the susceptibility to pediatric CD.

Conclusion

In this study, we have, for the first time assessed the association between the *MDR1* polymorphisms and susceptibility to CD

in an Algerian pediatric cohort. Our results indicate that the C1236T and G2677A/T SNPs might represent relevant genetic factors/modifiers that may predispose towards pediatric CD. In order to better understand the relevance of these *MDR1* gene polymorphisms in CD, further functional studies are required to evaluate the P-gp170 gene expression and activity in the gastrointestinal tract.

METHODS

Subjects

To study associations between the *MDR1* gene and CD, we carried out a case–control study.

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was reviewed and approved by the Ethic Committee of the Agence Thématique de la Recherche Scientifique en Santé (ATRSS, ex ANDRS). Forty-seven unrelated pediatric CD patients with a mean age of 10.78 ± 3.79 y were enrolled prospectively from four independent pediatric departments in Algiers, from January 2012 to June 2014. This cohort was not analyzed before in any IBD genetic studies. The diagnosis of CD was based on standard clinical, endoscopic, radiologic, and histopathological criteria (33). The Paris classification was used for CD disease type (34). In all of the patients, the following clinical features were recorded: family history, age at diagnosis, localization of CD (ileum, ileocolonic, colonic, upper GI tract), disease behavior (penetrating, stricturing, inflammatory (nonpenetrating and nonstricturing) presence of perianal fistulas, extraintestinal manifestations, and previous abdominal surgery (bowel restriction). The control group consisted of 100 unrelated healthy blood donors (59 female, 41 male) with a mean age of 23.6 ± 5.2 y without history of immune-mediated diseases. Prior to study enrollment, a voluntary written informed consent was obtained from all participants and legal guardians of patients under 16 y of age. All groups studied originated from different regions of North Algeria and confirmed the Algerian origin of their parents and grandparents.

Molecular Methods

DNA was isolated from peripheral blood samples by phenol/chloroform extraction method according to standard protocols (35).

Three polymorphisms of the *MDR1* (MIM*171050) gene including C3435T (exon 26, rs1045642), C1236T (exon 12, rs1128503), and G2677T/A (exon 21 rs2032582) were analyzed by PCR-RFLP method.

The following primers were used for PCR amplification. For C3435T, forward primer: 5′-GATGTCTTGTGGGAGA GGGA-3′ and reverse primer: 5′-GCATGTATGTTGGCCTCCTT-3′ (36).

For C1236T, forward primer: 5′-TATCCTGTGTCTGTGAATT GCC-3′ and reverse primer: 5′-CCTGACTCACCACACCAATG-3′ (37).

For G2677A/T, forward primer: 5′-TGACAAACGTTAGGCTT AAAATTACA-3′ and reverse primer: 5′-AAGATTGCTTTGAGG AATGGTT-3′ (38).

The PCR reactions were carried out using a T100 thermal cycler (Bio-Rad, Richmond, CA). PCR assays were performed using 200 ng of genomic DNA, 10× buffer, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTP, 10 pmol each primer and 1U Taq DNA polymerase (Promega, Madison, WI) in a final volume of 50 μl.

PCR conditions were as follows: predenaturation for 2 min at 96 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s by primer-specific temperature, primer extension for 30 s at 72 °C, the final extension at 72 °C for 5 min. Annealing temperature for the C3435T, C1236T, and G2677T/A primers were 56, 60, and 63 °C, respectively.

The restriction enzyme reactions were conducted following the manufacturer protocol. Twenty microliters of the PCR product were digested at 37 °C by MboI (EC: 3.1.21.4), (New England Biolabs, Ipswich, MA) and HaeIII (EC: 3.1.21.4), (Promega) for the polymorphisms C3435T and C1236T respectively. For the tri-allelic polymorphism G2677T/A, a double digestion was performed using RsaI and BseYI (New England Biolabs).

Data Analysis

Allele and genotype frequencies for *MDR1* SNPs were assessed for deviation from the Hardy-Weinberg equilibrium using a chi-square test. The allele with the lower frequency among the controls was denoted as the "risk-conferring allele", whereas the common allele was denoted as the "wild-type" allele. Genotype and allele frequencies were compared between cases and controls using chi-square tests and Fisher's exact tests where appropriate. Odds ratios (OR) and 95% confidence intervals ($CI_{95\%}$) were estimated. All statistical tests and *P* values were two-tailed. Haplotypes frequency was calculated using the Estimated Haplotype (EH) program which is based on maximum likelihood calculations (39). The overall haplotype frequencies distribution and each single haplotype were compared between cases and controls using chi squared test.

Genotype–phenotype associations were measured using the Pearson correlation test. Statistical analyses were performed applying GraphPad prism (version 6.01 for Windows, GraphPad Software, La Jolla, CA).

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