INFLAMMATORY BOWEL DISEASE

Genetic polymorphisms in biotransformation enzymes in Crohn's disease: association with microsomal epoxide hydrolase

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Background: Mucosal biotransformation enzymes can modify toxic compounds in the gut. As chemical or oxidative stress may be involved in the aetiology of Crohn's disease, genes encoding for enzymes involved in the prevention of such stress may be candidates for genetic susceptibility to Crohn's disease.

Aim: To assess the association of Crohn's disease with genetic polymorphisms in cytochrome P450 1A1, glutathione S-transferases mu-1, pi-1, and theta-1, and epoxide hydrolase.

Methods: χ^2 square analysis was used to compare frequencies of polymorphisms between 151 patients with Crohn's disease and 149 healthy controls.

Results: In patients, a genetic polymorphism in exon 3 of the microsomal epoxide hydrolase gene was distributed significantly different compared with controls ($\chi^2=23.7$; $p<0.0001$). All other polymorphisms tested were equally distributed between patients and controls.

Conclusions: Microsomal epoxide hydrolase may play a role in the pathophysiology of Crohn's disease. Furthermore, the epoxide hydrolase gene is located on chromosome 1q, close to a region previously linked to Crohn's disease.

Crohn's disease is a chronic disorder characterised by inflammation of the intestinal mucosa. Both genetic and environmental factors may play a role in its aetiology. Epidemiological data provide evidence that genetic predisposition to Crohn's disease depends on the contribution of multiple genes instead of a single genetic factor. Recently, the CARD15 gene (also named NOD2) on chromosome 16 was found to display a strong association with Crohn's disease.¹⁻³ However, mutations within the CARD15 gene were absent in the majority of patients, resulting in a population attributable risk for CARD15 in Crohn's disease of 25-30%.^{4,5} Therefore, additional susceptibility genes, coding for immune regulatory proteins, may be present. The mechanism of inflammation which leads to tissue injury in the mucosa is still unknown. Intraluminal antigens may trigger the immune system, ultimately resulting in tissue damage. Reactive oxygen species (ROS) may play a pivotal role in mediating tissue injury. ROS may be products of endogenous metabolism or bacterial fermentation within the intestinal lumen but are also produced by activated immune cells. In several studies, increased production of ROS in the inflamed intestine was demonstrated.^{6,7} ROS are highly toxic to cells and their formation in excess of physiological amounts may overload the intestinal antioxidant defence system, generating oxidative injury. An imbalance between pro-oxidant and antioxidant mechanisms may play a role in the aetiology of Crohn's disease.⁶ Furthermore, therapeutic interventions with known antioxidant properties, such as 5-aminosalicylates, have been shown to be beneficial in patients with Crohn's disease.⁸

Biotransformation enzymes play a key role in the metabolism of ROS and many other toxic molecules.^{9,10} Cytochrome P450 enzymes often modify xenobiotics to highly reactive intermediates such as epoxides. These intermediates may bind to cellular components and may lead to tissue damage. Detoxification of these epoxides may occur by conjugation with glutathione, catalysed by glutathione S-transferases¹¹ or by hydration, catalysed by epoxide hydrolases.¹² In the genes

coding for these enzymes, several polymorphisms have been described, resulting in enzymes with reduced or enhanced enzyme activity. Such genetic polymorphisms may modulate the risk of developing certain diseases. As chemical or oxidative stress may be involved in the aetiology of Crohn's disease, polymorphic genes encoding for these biotransformation enzymes may be putative candidates for genetic susceptibility to Crohn's disease.

The aim of the study was to determine whether genetic polymorphism rates in biotransformation enzymes in patients with Crohn's disease differ from those in healthy controls. Polymorphisms in genes encoding for cytochrome P450 1A1 (CYP1A1), glutathione S-transferase mu-1 (GSTM1), theta-1 (GSTT1), and pi-1 (GSTP1), and microsomal epoxide hydrolase (EPXH) were investigated. Furthermore, correlations between polymorphisms associated with Crohn's disease and several phenotypes of Crohn's disease were determined.

METHODS

Patients and controls

A total of 151 consecutive patients with Crohn's disease (97 females, 54 males; all Caucasian) consulting the outpatient clinic of the Department of Gastroenterology, University Medical Centre Nijmegen, the Netherlands, were included in the study. Patient data were obtained from the medical records. Patient subgroups with respect to phenotypes were formed based on location of disease, age at diagnosis, and behaviour of Crohn's disease. For age at diagnosis, patients

Abbreviations: Arg, arginine; CYP1A1, cytochrome P450 1A1 gene; EPXH, microsomal epoxide hydrolase gene; GSTM1, glutathione S-transferase mu-1; GSTP1, glutathione S-transferase pi-1; GSTT1, glutathione S-transferase theta-1; His, histidine; Ile, isoleucine; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ROS, reactive oxygen species; Tyr, tyrosine; Val, valine.

were divided into two groups on the basis of median age of onset. Behaviour of disease was divided into fistulising or non-fistulising disease. Furthermore, a distinction was made for bowel resections in the past as a consequence of stricturing or refractory disease.

Healthy Dutch Caucasians matched for age and sex were used as controls. In two of 151 healthy controls, DNA isolation was unsuccessful. The investigations were approved by the local ethics committee on human experimentation.

Genomic DNA isolation

Blood samples were collected by venepuncture in sterile EDTA tubes (Greiner, Kremsmünster, Austria or Becton Dickinson, San Jose, California, USA). Whole blood was stored at -80°C until use. The method of Miller and colleagues¹³ was used for genomic DNA isolation from blood lymphocytes of patients. In controls, genomic DNA was isolated from whole blood using the Wizard genomic DNA purification kit, according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

CYP1A1

For demonstrating genetic polymorphism in the 3'-flanking region of *CYP1A1*, a primer set 5'-TAG GAG TCT TGT CTC ATG CCT-3' and 5'-CAG TGA AGA GGT GTA GCC GCT-3' was used so that the presence of the rare C nucleotide resulted in the appearance of an MspI restriction enzyme site.¹⁴ According to this method, a single 340 base pair fragment indicates the presence of homozygous wild-type, 340, 200, and 140 base pair fragments demonstrate heterozygote alleles, whereas 200 and 140 base pair fragments indicate the presence of homozygous rare alleles. Genetic polymorphism in exon 7 of *CYP1A1* was detected using the primer set 5'-GAA CTG CCA CTT CAG CTG TCT-3' and 5'-CCA GGA AGA GAA AGA CCT CCC AGC GGG CCA-3'. The presence of the rare A to G mutation resulted in loss of a NcoI restriction enzyme site.¹⁵ Analysis by gel electrophoresis (3% Pronagarose D-ILE) revealed 163 and 32 base pair fragments in individuals with two wild-type alleles (isoleucine (Ile)) and a single 195 base pair fragment when the polymorphism (A to G, Ile to valine (Val)) was present in both alleles. Heterozygotes were indicated by the presence of 195, 163, and 32 base pair fragments.

Glutathione S-transferases M1, T1, and P1

Genetic polymorphism in *GSTM1* was determined by PCR according to the method of Brockmöller and colleagues.¹⁶ PCR primers (G2/G3: 5'-CTG GAT TGT AGC AGA TCA TGC-3'/5'-CTC CTG ATT ATG ACA GAA GCC-3') were designed according to the known *GSTM1* cDNA sequence. The PCR amplificates were subjected to electrophoresis on 1% agarose gels (Agarose MS) and analysed for the presence of a 650 base pair product, which was indicative of a functional *GSTM1* gene. Lack of an amplification product is consistent with the null genotype. The procedure to detect the polymorphism in *GSTT1* is very similar to that of *GSTM1* and has been described by Pemble and colleagues.¹⁷ The primers used were: 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3'. Visualisation of a 480 base pair fragment indicates the presence of a functional *GSTT1* gene. Samples showing negative results for *GSTT1* or *GSTM1* were always repeated in another series to exclude misinterpretation of results, and results in the second analysis were found to be 100% identical. To detect genetic polymorphisms at the *GSTP1* locus, a PCR assay was used based on the method by Harries and colleagues.¹⁸ The following primers were used: P105F (5'-ACC CCA GGG CTC TAT GGG AA-3') and P105R (5'-TGA GGG CAC AAG AAG CCC CT-3'). This method revealed a single 176 base pair fragment (homozygous wild-type), 176, 91, and 85 base

Table 1 Characteristics of patients with Crohn's disease and controls

	Patients (n=151)	Controls (n=149)
Sex (M/F)	54/97	54/95
Age (y)	38 (1)	38 (1)
Age of onset of Crohn's disease (y)	25 (1)	
Location of disease*		
Ileum	38 (26%)	
Colon	31 (21%)	
Ileocolonic	79 (53%)	
Fistulising disease*	79 (53%)	
History of bowel resections*	89 (60%)	

Data are expressed as mean (SEM) or number.

*In three patients these detailed data were missing in the medical records.

pair fragments (heterozygote), and 91 and 85 base pair fragments (homozygous polymorphic).

Microsomal epoxide hydrolase

Separate PCR assays were used to detect two distinct polymorphisms in the *EPXH* gene. In exon 3, a T to C polymorphism, changing tyrosine (Tyr) 113 to histidine (His), was tested. The assay used the primer pair 5'-CTT GAG CTC TGT CCT TCC CAT CCC-3' and 5'-AAT CTT AGT CTT GAA GTG ACG GT-3'. The reverse primer contains a mismatched C residue that creates a Tht 111 I restriction site.¹⁹ The Tyr 113 allele was identified by an undigested single DNA band of 231 base pairs whereas the His 113 allele gave two bands of 209 and 22 base pairs. In exon 4, an A to G polymorphism was tested, changing His 139 to arginine (Arg). The primer pair 5'-GGG GTA CCA GAG CCT GAC CGT and 5'-AAC ACC GGG CCC ACC CTT GGC was used to assay this polymorphism.²⁰ His 139 alleles were identified by two DNA bands (295 and 62 bp) whereas Arg 139 alleles produced three bands (174, 121, and 62 bp) after digestion. Heterozygous individuals displayed all four DNA fragments (295, 174, 121, and 62 bp).

Statistical analysis

Data are expressed as mean (SEM). The statistical significance of differences in the distribution of polymorphisms between groups was tested using χ^2 analysis with Yates' correction. Where appropriate, 2x2, 2x3, or 3x3 contingency tables were used. Based on the observed allele frequencies, odds ratios with 95% confidence intervals (95% CI) were calculated. In order to detect differences of at least 20% between groups with 80% power, and an anticipated genotype frequency of 50% for the genotype of interest in the control group, 150 subjects per group were needed. In total, seven different genetic polymorphisms were analysed. To take into account the number of comparisons, a two tailed p value of less than 0.007 instead of 0.05 was considered to represent statistical significance (0.05 divided by 7).

RESULTS

Patients and controls

Clinical characteristics of patients and controls are given in table 1. Mean age of patients was 38 (1) years at the time of venepuncture, and age at onset of Crohn's disease was 25 (1) years. Crohn's disease was confined to the ileum in 38 patients (26%) and to the colon in 31 patients (21%). Both ileal and colonic disease was present in 79 patients (53%). In three patients no detailed data on disease location and/or disease behaviour were available in the medical records. Bowel resections had been performed in 89 patients (60%) and fistulae were present in 79 patients (53%). Mean age of the healthy controls was 38 (1) years.

Table 2 Frequencies of genetic polymorphisms in genes encoding for biotransformation enzymes in patients with Crohn's disease and controls

Genetic polymorphism	No of patients (%)	No of controls (%)	Odds ratio	95% CI
<i>CYP1A1</i> (3'-flanking region)				
T/T	130 (86)	125 (84)		
T/C	20 (13)	24 (16)	0.87	0.30–2.49
C/C	1 (1)	0 (0)		
<i>CYP1A1</i> (exon7)				
Ile/Ile	129 (86)	122 (82)		
Ile/Val	20 (13)	22 (15)	0.70	0.27–1.83
Val/Val	2 (1)	5 (3)		
<i>GSTM1</i>				
Wild-type	69 (46)	75 (50)	1.17	0.67–2.05
Deletion	82 (54)	74 (50)		
<i>GSTT1</i>				
Wild-type	128 (85)	119 (80)	0.71	0.34–1.47
Deletion	23 (15)	30 (20)		
<i>GSTP1</i>				
Ile/Ile	75 (49)	87 (58)		
Ile/Val	63 (42)	55 (37)	1.37	0.72–2.58
Val/Val	13 (9)	7 (5)		
<i>EPXH</i> (exon 3)				
His/His	20 (13)	59 (40)		
Tyr/His	60 (40)	58 (39)	2.92*	1.64–5.20
Tyr/Tyr	71 (47)	32 (21)		
<i>EPXH</i> (exon 4)				
His/His	90 (60)	98 (66)		
His/Arg	53 (35)	48 (32)	1.36	0.68–2.72
Arg/Arg	8 (5)	3 (2)		

CYP1A1, cytochrome P450 1A1; *GSTM1*, glutathione S-transferase mu-1; *GSTT1*, glutathione S-transferase theta-1; *GSTP1*, glutathione S-transferase pi-1; *EPXH*, microsomal epoxide hydrolase; Ile, isoleucine; Val, valine; Tyr, tyrosine; His, histidine; Arg, arginine; 95% CI, 95% confidence interval.

*2x3 table, $\chi^2=23.7$; $p<0.0001$.

Table 3 Frequencies of genetic polymorphisms in the *EPXH* gene in subgroups of patients with Crohn's disease, divided by disease location and disease behaviour

	<i>EPXH</i> exon 3			<i>EPXH</i> exon 4		
	Tyr/Tyr	Tyr/His	His/His	His/His	His/Arg	Arg/Arg
Disease location						
Ileum	18 (50)	13 (36)	5 (14)	22 (61)	12 (33)	2 (6)
Colon	15 (49)	10 (32)	6 (19)	17 (55)	12 (39)	2 (6)
Ileocolonic	33 (43)	35 (45)	9 (12)	45 (58)	28 (36)	4 (5)
Age of onset (y)						
≤23	36 (47)	32 (41)	9 (12)	41 (53)	32 (42)	9 (5)
>23	30 (45)	26 (39)	11 (16)	42 (63)	21 (31)	4 (6)
Fistulae present						
Yes	36 (46)	33 (42)	9 (12)	40 (51)	34 (44)	4 (5)
No	30 (45)	25 (38)	11 (17)	44 (67)	18 (27)	4 (6)
Bowel resection						
Yes	42 (49)	31 (37)	12 (14)	46 (54)	36 (42)	3 (4)
No	24 (41)	27 (46)	8 (13)	38 (64)	16 (27)	5 (9)

Results are given as number of patients (%).

EPXH, epoxide hydrolase gene; Tyr, tyrosine; His, histidine; Arg, arginine.

The statistical significance of differences for polymorphisms between different groups were analysed by χ^2 analysis in 2x3 or 3x3 contingency tables. No significant differences were observed.

Genetic polymorphisms

The distribution of polymorphic variants in *CYP1A1*, *GSTM1*, *GSTT1*, *GSTP1*, and *EPXH* genes in patients with Crohn's disease and healthy controls is summarised in table 2. In exon 3 of the epoxide hydrolase gene, we found a statistically significant higher rate of the Tyr 113/Tyr 113 genotype among patients with Crohn's disease (47%) compared with controls (21%; $\chi^2=23.7$, $p<0.0001$). In the patient group, the Tyr 113 allele in exon 3 was more common than in controls, with allele frequencies of 0.67 versus 0.41, respectively. The corresponding odds ratio for the Tyr 113 allele was 2.9 (95% CI 1.64–5.20). The genetic polymorphism in exon 4 of the *EPXH* gene was equally distributed among patients with Crohn's disease and healthy controls, with the rare Arg 139/Arg 139 genotype

present in 2% of controls and 5% of patients with Crohn's disease. The distribution of genetic polymorphisms in exons 3 and 4 of the *EPXH* gene in Crohn's disease was not different for subgroups of patients with regard to location of disease, age of onset, presence of fistulae, or a history of bowel resections (table 3).

The rare alleles in the 3'-flanking region and exon 7 of *CYP1A1* were equally distributed among patients and controls. Furthermore, *GSTM1* and *GSTT1* null genotypes were found in 50% and 20% of controls, respectively, and these frequencies were not statistically different from those in the patient population (54% and 15%, respectively). Also, the combination of both *GSTM1* null and *GSTT1* null genotypes was found equally as often in patients and controls (7% and 13%, respectively;

$p=0.6$). The Ile to Val substitution in the *GSTP1* gene was found equally as often in patients and controls.

DISCUSSION

A imbalance between toxic compounds and detoxifying substances on the luminal side of the gut may play a role in the aetiology of Crohn's disease. Biotransformation enzymes play a pivotal role in a variety of detoxification processes. In this case control study, we found a highly significant difference in allele frequency of the Tyr113 variant in exon 3 of the *EPXH* gene between patients with Crohn's disease (0.67) and controls (0.41), resulting in an odds ratio of 2.9 for the Tyr 113 allele. An increased frequency of Tyr 113 in exon 3 in patients may indicate a role for epoxide hydrolase in the genetic susceptibility to Crohn's disease. In vitro, substitution of Tyr for His has been shown to increase EPXH activity by approximately 40%.²⁰ Previously, the presence of epoxide hydrolase activity was demonstrated in the normal human gastrointestinal mucosa.^{21, 22} An increase in enzyme activity may result in either enhanced activation of endogenous or exogenous substrates to more reactive diol- epoxide derivatives or may lead to a more efficient deactivation. In patients with Crohn's disease, altered formation of highly reactive metabolites in the gastrointestinal mucosa due to variations in the *EPXH* gene may contribute to chronic inflammation and injury to the intestinal wall. An association of variations in the *EPXH* gene with a variety of malignancies (ovarian, larynx, lung, hepatocellular cancer) and with preeclampsia has been published.^{19, 23–26} An association of this variation in the *EPXH* gene and Crohn's disease has not been reported previously.

In exon 3 of the *EPXH* gene, remarkable variation in polymorphic rates has been reported for different ethnic and geographic populations, with frequencies for the Tyr113 allele in 58–94% of controls.^{19, 20, 24, 25, 27–29} All patients and controls in the present study were Caucasian, originating from the Netherlands, a small geographic region. Furthermore, no differences were found for the other genetic polymorphisms determined in the control group in this study compared with other Caucasian control groups. Therefore, bias due to differences in genetic background between patients and controls is unlikely.

Interestingly, the *EPXH* gene is located on the distal portion of chromosome 1q,^{30, 31} close to a region linked to inflammatory bowel disease in a report by Hampe and colleagues.³²

Genetic polymorphisms in the 3'-flanking region and in exon 7 of *CYP1A1*, both leading to a more active enzyme,³³ were found equally often in patients with Crohn's disease and controls. Therefore, we could not demonstrate an association between *CYP1A1* genotype and the development of Crohn's disease. According to literature databases, no other studies have been performed concerning genetic polymorphisms in *CYP1A1* in Crohn's disease.

GSTs are involved in the detoxification of a wide variety of toxic compounds, and four main subclasses have been identified in humans.¹¹ Previous studies in Crohn's disease demonstrated no differences in the *GSTM1* gene between patients and controls.^{34, 35} Duncan *et al* determined the *GSTM1* genotype, while Hertervig *et al* investigated the *GSTM1* phenotype. Both studies showed that approximately 60% of patients lack a functional *GSTM1* gene or do not express the *GSTM1* enzyme. In accordance with these studies, no difference was demonstrated between patients and controls. In our population, a deletion in the *GSTM1* gene was present in 54% of patients and 50% of controls. These results indicate that absence of the *GSTM1* gene does not play an important role in the pathophysiology of Crohn's disease.

In agreement with Duncan and colleagues,³⁴ our *GSTT1* null frequencies did not differ significantly between patients with Crohn's disease (15%) and controls (20%), which indicates that lack of this enzyme is not crucial for the development of

Crohn's disease. The frequencies of the null genotype in controls was comparable with that reported in other studies.^{10, 17, 36–39}

In the present study, an Ile to Val substitution at codon 105 of the *GSTP1* gene (*GSTP1b* variant), resulting in reduced enzyme activity, was demonstrated equally often in patients with Crohn's disease and controls. According to literature databases, no other studies have been performed which have investigated this genetic polymorphism in relation to Crohn's disease. Control populations in previous reports demonstrated very similar frequencies of wild-type, heterozygous, and homozygous genotypes compared with controls in our study.^{18, 36, 40, 41} The results in the present study indicate that genetic polymorphisms in GST enzymes do not play an important role in the development of Crohn's disease.

In conclusion, we found no evidence for a possible genetic predisposition to Crohn's disease due to genetic polymorphisms in the *CYP1A1*, *GSTM1*, *GSTT1*, and *GSTP1* genes. We demonstrated a clear difference in the distribution of variants in the *EPXH* gene that may lead to an increase in EPXH enzyme activity in patients with Crohn's disease compared with healthy controls. Furthermore, the *EPXH* gene is located in a region previously linked to Crohn's disease in a genome wide mapping strategy. Although our results strongly indicate a role for the *EPXH* gene in the genetic susceptibility to Crohn's disease, confirmation of data in an independent cohort of patients and controls would greatly strengthen our conclusion.

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