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Haplotype Analysis of *ABCB1* in Patients with Peptic Ulcer–Predisposition to Diseases Development

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ABSTRACT The aim of this study was to determine the significance of *ABCB1* haplotypes composed of the three most common single-nucleotide polymorphisms (c.1236C>T: rs1128503, c.2677G>T/A: rs2032582, c.3435C>T: rs1045642) in the context of peptic ulcer risk and *Helicobacter pylori* infection development in this condition. The *ABCB1* gene product P-glycoprotein is a membrane protein that functions as an ATP-dependent exporter of xenobiotics from cells. The function of the transporter is determined by the assembles of *ABCB1* polymorphism called haplotypes. Particular haplotypes have been reported as determining individual susceptibility to conditions such as inflammatory bowel diseases, colorectal cancer.202 peptic ulcer patients and 96 healthy subjects were genotyped. Genotyping was performed for c.1236C>T and c.2677G>T/A by automated sequencing and for c.3435C>T by polymerase chain reaction-restriction fragment length polymorphism method. Haplotypes and degree of linkage disequilibrium (LD) were inferred using PHASE 2.1 and EMLD software. The groups of peptic ulcer and healthy subjects differed significantly in haplotype frequencies (p=0.04). Moreover, there was a statistically significant difference in haplotype frequencies between the *H. pylori*-infected and -uninfected peptic ulcer cases (p=0.01), and between *H. pylori*-infected and -uninfected peptic ulcer men (p=0.03). In the peptic ulcer group 1236-2677 locus pair was observed to be in modest LD (D'=0.187), 1236-3435 and 2677-3435 pairs were almost in linkage equilibrium (D'=0.036 and 0.051, respectively). Haplotype structure could be responsible for individual differences in peptic ulcer predisposition and in development of *H. pylori* infection in peptic ulcer patients.

INTRODUCTION

The *ABCB1* gene product P-glycoprotein (P-gp) is a membrane protein that functions as an ATP-dependent exporter of xenobiotics from cells. It was recognized as a gene with a product that is involved in the development of MDR (multidrug resistance) of cultured tumor cells against various anticancer agents. P-gp as a transporter is not only expressed in tumor cells, but also in normal tissues with excretory function (intestine, liver, kidney). P-glycoprotein has a very broad substrate specificity, it determines disposition of a broad variety of drugs.

Systematic screening of the *ABCB1* gene has identified multiple single nucleotide polymorphisms that are associated with altered transporter function and expression. *ABCB1* polymorphisms can influence P-glycoprotein tissue expression, drug disposition, treatment outcome and disease risk (Kim et al. 2006).

According to the SNP database, there are more than 50 SNPs in the human *ABCB1* gene. These SNPs result in both synonymous and non-synonymous mutations. No nonsense mutations have been found. Most of the mutations are translated into amino acids located in the intracellular region.

Polymorphisms of *ABCB1* are also located in the intronic region. The SNPs have been confirmed as being associated with a younger onset age of mood disorder, with sporadic colorectal cancer (Qian et al. 2006) Furthermore, the 52 UTR and the 32 UTR are also polymorphic (Gow et al. 2008).

All of these mutations are factors that affect P-gp expression. Investigations of the mutations have confirmed that *ABCB1* function is affected by changes in crucial amino acid in the transmembrane domains, ATP-binding domains, Walker-A and Walker-B motifs, or the signature motif (Kim et al. 2006; Sauna et al. 2002). Folding pathway or protein conformation may be disrupted by mutations (Loo et al. 2004).

Studying *ABCB1* haplotypes could further the understanding of the structure and function of the gene. The first evidence that SNPs are re-

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lated to each other was the discovery of the synonymous 3435C>T (I1145I) SNP (silent mutation) which changes *ABCB1* expression (Hoffmeyer et al. 2000). This led to the assumption that other non-synonymous polymorphisms may be linked to the C3435T SNP. Linkage analysis confirmed that this SNP is associated with several other SNPs in *ABCB1*. Such panels of mutations, known as haplotypes (ranging in number from 3 to 55), have been presented in several studies (Kroetz et al. 2003; Leschziner et al. 2006).

The most frequently observed haplotype consists of the C3435T polymorphism combined with G2677T/A, and/or C1236T (Kroetz et al 2003). Strong linkage disequilibrium among these SNPs has been found in multiple studies of different populations (Jeannesson et al. 2007; Sai et al. 2006) and in different diseases e.g. AML, MM and colon cancer (Illmer et al. 2002; Jamroziak et al. 2009; Panczyk et al. 2009).

Haplotype analysis suggests that each of the alleles that built the complete haplotype may have a small, but together, significant impact on protein folding and function. As a result of this, many researchers suggest that the functional effects of P-gp may be haplotype-dependent and do not necessarily need to be defined nor explained by a single polymorphism. In this study, after separate investigation of three previously described SNPs at positions 1236, 2677 and 3435 (Sa³agacka et al. 2011; Zebrowska et al. 2012). *ABCB1* haplotype analysis was carried out in a group of patients with peptic ulcers. The study could elaborate on information about the role of the gene in the development of this disease.

METHODOLOGY

The investigated group comprised 129 women and 83 men suffering from peptic ulcer. The control group was geographically and ethnically matched to the patients and consisted of 96 blood donors at a local blood bank. The results of *ABCB1* genotyping of the control group have been reported by us previously (Jamroziak et al. 2009).

For diagnosis of *Helicobacter pylori* infection a rapid urease test at the time of gastroscopy was used (Instytut Zywnoœci i Zywienia, Warsaw, Poland).

DNA was isolated according to "Genomic DNA Prep Plus" protocol (A&A Biotechnology, Gdynia, Poland) from biopsy specimens of gastric membrane mucosa.

PCR was conducted according to the "AccuTaq™ LA DNA Polymerase Kit" protocol (Sigma Aldrich, Germany). The primer design was based on previously published sequences for genotyping procedure of c.1236C>T, c.2677G>T/A, and c.3435C>T *ABCB1* polymorphisms using genomic DNA (Panczyk et al. 2009).

c.1236C>T and c.2677G>T/A polymorphisms were analyzed by automated sequencing. (Zebrowska et al. 2012). c.3435C>T was analyzed by RFLP (Salagacka et al. 2011).

Haplotypes were statistically inferred using the PHASE v. 2.1 software. The PHASE program implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. Linkage disequilibrium (LD) was estimated using EMLD software with algorithm EM (The Expectation-Maximization Algorithm).

RESULTS

Haplotype analysis was carried out using genotyping data concerning three common SNPs of *ABCB1* (c.1236C>T: rs1128503, c.2677G>T/A: rs2032582, c.3435C>T: rs1045642), that were obtained for each SNP individually (Salagacka et al. 2011; Zebrowska et al. 2012).

First, haplotype frequencies were estimated and compared in groups of peptic ulcer patients and healthy subjects to determine the significance of the *ABCB1* haplotypes to disease risk (Table 1) The most common haplotype in the group of healthy subjects was C-G-C (19.1%), and in the group of peptic ulcer cases it was C-G-T (14.9%). Among healthy individuals, the rarest haplotype recorded was T-T-T (7.6%). The compared groups differed significantly in haplotype frequencies (p=0.04).

Subsequently, to ascertain the importance of *ABCB1* haplotype structure to *Helicobacter pylori* infection development in peptic ulcer cases, haplotype frequencies were estimated and compared in two subgroups of peptic ulcer patients: those infected with the pathogen and those uninfected. T-T-T (13.7%) was present in this subgroup as comparably often as the T-G-C (13.8%), C-T-T (13.6%), and CGT (13.3%) variants. Contrarily, the most commonly recorded haplotypes in the subgroup of *H. pylori*- infected patients were C-G-T (18.8%) (Table 2). There

Table 1: Comparison of estimated *ABCB1* haplotype frequencies (1236-2677-3435) in health subjects and peptic ulcer patients

	Whole group (n=298)		*Healthy subjects (n=96)*		*Peptic ulcer patients (n=202)*		*p-value (permutation test)*
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
C-G-C	0.1479	0.0091	0.1909	0.0158	0.1275	0.0109	0.04
C-G-T	0.1449	0.0085	0.1364	0.0154	0.1489	0.0103	
C-T-C	0.1008	0.0078	0.1062	0.0140	0.0982	0.0103	
C-T-T	0.1278	0.0082	0.1395	0.0140	0.1222	0.0098	
T-G-C	0.1329	0.0085	0.1599	0.0138	0.1201	0.0103	
T-G-T	0.1301	0.0082	0.1118	0.0127	0.1388	0.0106	
T-T-C	0.0947	0.0074	0.0795	0.0132	0.1019	0.0095	
T-T-T	0.1211	0.0086	0.0759	0.0126	0.1425	0.0108	

was a statistically significant difference in haplotype frequencies between infected and uninfected patients (p=0.01).

Parallel to the aforementioned, analysis was also carried out after splitting the peptic ulcer patient cohort according to gender. The observed difference in *ABCB1* haplotype frequencies between women with and without *H. pylori* infection was not statistically significant (p=0.34), (Table 2).

Table 2: Comparison of estimated *ABCB1* haplotype frequencies (1236-2677-3435) in *H. pylori*-infected and -uninfected peptic ulcer patients

	Peptic ulcer patients						*p-value (permutation test)*
	Whole group (n=202)		*H. pylori-uninfected (n=101)*		*H. pylori-infected (n=101)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
C-G-C	0.1252	0.0105	0.1333	0.0150	0.1172	0.0128	0.01
C-G-T	0.1526	0.0106	0.1168	0.0150	0.1884	0.0124	
C-T-C	0.1004	0.0105	0.1259	0.0155	0.0749	0.0120	
C-T-T	0.1168	0.0097	0.1335	0.0150	0.1002	0.0114	
T-G-C	0.1187	0.0093	0.1360	0.0136	0.1013	0.0133	
T-G-T	0.1382	0.0111	0.1041	0.0157	0.1722	0.0140	
T-T-C	0.1030	0.0095	0.1135	0.0147	0.0924	0.0119	
T-T-T	0.1450	0.0107	0.1368	0.0167	0.1533	0.0128	
	Peptic ulcer women						
	Whole group (n=129)		*H. pylori-uninfected (n=65)*		*H. pylori-infected (n=64)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
C-G-C	0.1116	0.0108	0.1135	0.0180	0.1096	0.0141	0.34
C-G-T	0.1541	0.0130	0.1301	0.0201	0.1786	0.0153	
C-T-C	0.1073	0.0118	0.1237	0.0194	0.0906	0.0128	
C-T-T	0.1350	0.0126	0.1559	0.0209	0.1137	0.0159	
T-G-C	0.1004	0.0106	0.1088	0.0179	0.0917	0.0143	
T-G-T	0.1304	0.0117	0.1020	0.0200	0.1591	0.0144	
T-T-C	0.1097	0.0140	0.1130	0.0217	0.1065	0.0135	
T-T-T	0.1515	0.0138	0.1529	0.0222	0.1501	0.0160	
	Peptic ulcer men						
	Whole group (n=73)		*H. pylori-uninfected (n=36)*		*H. pylori-infected (n=37)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
C-G-C	0.1460	0.0168	0.1604	0.0211	0.1322	0.0251	0.03
C-G-T	0.1478	0.0181	0.0958	0.0223	0.1983	0.0287	
C-T-C	0.0866	0.0130	0.1291	0.0191	0.0453	0.0175	
C-T-T	0.0904	0.0146	0.1004	0.0215	0.0807	0.0197	
T-G-C	0.1574	0.0172	0.1945	0.0240	0.1213	0.0246	
T-G-T	0.1515	0.0184	0.1049	0.0226	0.1969	0.0285	
T-T-C	0.0882	0.0134	0.1133	0.0186	0.0639	0.0195	
T-T-T	0.1320	0.0142	0.1016	0.0181	0.1615	0.0219	

When subgroups of men infected with the bacteria and those uninfected were compared, they varied considerably in particular haplotype frequencies (p=0.03). Among men without the infection, the most common haplotype was T-G-C (19.5%). The second and third most common were C-G-C and C-T-C (16.0% and 12.9%, respectively). These findings differed from the three most prevalent variants found in the compared subgroup of men with *H. pylori* infection: C-G-T and T-G-T were of similar prevalence (19.8% and 19.7%, respectively), and haplotype T-T-T, containing only mutant-type allele (16.2%), (Table 2).

Finally, the pair-wise linkage disequilibrium pattern of *ABCB1* gene was inferred in the group of peptic ulcer patients. Only the 1236-2677 locus pair was stated to be in modest LD (D'=0.187), whilst 1236-3435 and 2677-3435 locus pairs were almost in linkage equilibrium (D'=0.036 and 0.051, respectively), (Table 3).

Table 3: Measures of linkage disequilibrium between pairs of *ABCB1* loci in the group of peptic ulcer patients

ABCB1 locus pair	*D'*	r^2
1236 - 2677	0.187	0.0294
1236 - 3435	0.036	0.0010
2677 - 3435	0.051	0.0019

DISCUSSION

Nowadays, haplotype analysis is attracting a great deal of attention as a powerful tool for pinpointing the disease-causing loci in both family-based and population-based studies. It provides a more complete picture than a single-locus approach to the genome points, which could be relevant, either alone or collectively, in the practice of personalized medicine. In the *ABCB1* gene 64 potential haplotypes were identified (Kroetz et al. 2003). In various populations, the most common haplotypes are 1236C-2677G-3435C and 1236T-2677T-3435T. For example haplotype TTT occures 2-5-fold often in Caucasian population than in Afro-American population (Hodges et al. 2011). It is thought that haplotypes, rather than single sites alone, are required to produce a particular phenotype (Crawford et al. 2005). The occurrence of *ABCB1* polymorphisms may provide a selective advantage in particular populations in response to environmental toxins or when subject to certain diseases (Fung et al. 2009). There is growing evidence that phenotypic effects in P-gp activity may be related to haplotypes of *ABCB1* gene.

Previously, *ABCB1* haplotypes have been associated with various conditions. Panczyk (Panczyk et al. 2009) revealed a significant difference in *ABCB1* haplotype distribution between Polish colorectal patients and the healthy population. Potocnik (Potocnik et al. 2008) stated that haplotypes of the gene might modify susceptibility to colorectal cancers with high micro-satellite instability.

The high constitutive level of P-gp expression in the gut reflects its role in protection not only against xenobiotics but also against bacterial products. Predisposition to ulcerative colitis and Crohn's disease, common chronic inflammatory disorders characterized by a dysregulated mucosal immune response, have, several times, been stated to be connected with specific *ABCB1* haplotype variants (Ho et al. 2005) but other authors were not able to confirm these observations (Oostenbrug et al. 2006).

To the best of the researchers' knowledge, theirs is the first attempt to determine the importance of *ABCB1* haplotype structure in the context of peptic ulcer disease and *H. pylori* infection development in patients with the disease. They found that the frequencies of inferred haplotypes vary significantly between healthy subjects and peptic ulcer patients (p=0.04). This, to some extent, reflects the researchers' recent findings where they carried out *ABCB1* single-locus analysis of peptic ulcer patients and healthy subjects (Salagacka et al. 2011; Zebrowska et al. 2012). In this study, C-G-C haplotype was observed more frequently in the control group. This might suggest that the C-G-C haplotype and P-gp have a protective role in the healthy population. Furthermore, the haplotype composed of mutant alleles (T-T-T) was estimated to occur more frequently among peptic ulcer patients. Potocnik et al. showed that TTT haplotype was connected with higher risk of ulcerative colitis and Crohn's disease (Potocnik et al. 2004). Also Wu et al. showed association between TTT haplotype and increased risk of breast cancer development in Chinese population (Wu et al. 2012). This gene variant is connected with lower P-gp expression and loss of its protective function can increase risk of peptic ulceration. Modest LD was observed in SNPs 1236 and 2677, and, similarly, near equilibrium in 1236 and 3435 as well as 2677 and 3435. Such results conflict with Tanabe

(Tanabe et al. 2001). who observed strong association between 1236, 2677 and 3435 alleles and Qui (Qui et al. 2012) who observed in Turkish population strong LD between 2677-3435 but are, however, in agreement with data published by Zhang (Zhang et al. 2008), who proved minor LD existence between SNPs 2677-3435 and SNPs 1236 - 2677.

A distinct prevalence of particular haplotypes was also noted in the sub-division of peptic ulcer cases that were infected with *H. pylori* when compared with the group of uninfected cases (p=0.01). Similarly, prevalence of particular haplotypes was also recorded when only men with peptic ulcers were analyzed (p=0.03). This finding is in agreement with previously reported results that revealed significant dependence between c.3435C>T genotypes and *H. pylori* infection among peptic ulcer patients and also in the subgroup of peptic ulcer men (Salagacka et al. 2011). Mutant homozygous 1236TT of this SNP occurred more frequently in the group of all infected subjects. A higher prevalence of 2677GG genotype and 2677G allele tended to be associated with *Helicobacter pylori* infection in the whole group (Zebrowska et al. 2012). Juyal et al. showed that TTT haplotype association with ulcerative colitis development (Juyal et al. 2009). After that, Panczyk et al. observed that ocurrence of TTT haplotype was connected with 9,61 times higher risk of colon cancer development (Panczyk et al. 2009).

CONCLUSION

Haplotype structure could be responsible for individual differences in peptic ulcer predisposition and in development of *H. pylori* infection in peptic ulcer patients.

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