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C3435T polymorphism of the *ABCB1* gene: impact on genetic susceptibility to peptic ulcers

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Abstract:
The functional single nucleotide polymorphism (SNP) C3435T in exon 26 of the *ABCB1* gene encoding the xenobiotic transporter P-glycoprotein (P-gp) may influence susceptibility to several diseases, as well as the clinical outcome of treatment with P-gp substrates. Exposure to environmental chemicals is thought to be involved in peptic ulcer pathogenesis and then later in stomach cancer development. About 80% of ulcers are associated with *Helicobacter pylori* infection, one of the risk factors of stomach cancer. P-gp-transported drugs are used in treatment of *H. pylori*. Therefore, a lack of effectiveness in eradication therapy can lead to chronic stomach inflammation and promote cancerogenesis.
In this study, 196 patients with peptic ulcers divided into two groups with and without *H. pylori* infection and combined with 96 healthy controls were genotyped for the *ABCB1* C3435T SNP. A trend towards higher incidence of the 3435TT genotype among peptic ulcer patients than in controls ($p = 0.0983$) was observed. Likewise, the 3435T allele was more frequent in groups suffering from peptic ulcers. The association was near to statistical significance ($p = 0.0538$). Between analyzed genotypes and *H. pylori* infection, statistically significant dependence was found ($p = 0.0372$). In addition, the CT genotype was associated with 1.56 times and the TT with 2.45 times higher prevalence of infection compared to the CC genotype. A similar association was present in a subgroup of peptic ulcer men ($p = 0.0090$).
The isolated C*3435T ABCB1* SNP is not a major factor for genetic susceptibility to peptic ulcer, but in a group of men who suffered from peptic ulcer, this polymorphism seemed to be a risk factor for *H. pylori* infection development.

Key words:
P-glycoprotein, *ABCB1,* single nucleotide polymorphism, peptic ulcer, *Helicobacter pylori*, susceptibility

Abbreviations: ABC – adenosine triphosphate-binding cassette, *ABCB1* – adenosine triphosphate-binding cassette subfamily B transporter 1 gene, *H. pylori* – *Helicobacter pylori*, HWE – Hardy-Weinberg equilibrium, P-gp – P-glycoprotein, RFLP – restriction fragment length polymorphism, SNP – single nucleotide polymorphism

Introduction

Multiple factors contribute to pathogenesis of gastric cancer. Environmental factors, including dietary habits, are important in its development. In 1994, the In-

ternational Agency for Research on Cancer classified *H. pylori* as carcinogenic to humans. It causes a chronic inflammation of the stomach mucosa and is strongly linked to the development of peptic ulcers and gastric cancer. However, it is important to emphasize that over 80% of individuals infected with the bacterium are asymptomatic and the development of gastric cancer in *H. pylori* positive patients is diagnosed at a low rate for this population [10, 34]. These data suggest that genetic factors may also influence the development of peptic ulcers and gastric cancer. A lot of publications have indicated that gastric cancer appearance depends, to some measure, on polymorphisms in genes which protein products are involved in immunological defense (e.g., cytokine IL-1, IL-10), drug metabolism (e.g., CYP2A6, CYP2E1, CYP2C19, glutathione S-transferase, N-acetyltransferase) [3] and transport (e.g., P-glycoprotein) [32].

P-glycoprotein (P-gp) is a multidrug resistant transporter (MDR1, ABCB1) encoded by the *ABCB1* gene. P-gp was originally isolated from cancer cells that became resistant to anticancer drugs. This protein is also expressed in normal tissues, including the liver, kidney, intestines, brain, placenta and adrenals [33]. P-gp is highly expressed on the apical (luminal) surface of organs that have excretory functions, such as the bile canalicular membrane of hepatocytes and the renal proximal tubule. Moreover, P-gp is significantly expressed on the luminal surface of tissues that serve as barriers, such as the brush border of the small intestine and the capillary endothelial cells of the blood-brain barrier. P-gp is involved in active transport of a large number of amphipathic molecules through lipid membranes. Another function is the export of unnecessary and toxic exogenous substances out of cells.

Tissue distribution suggests that P-gp protects the body from toxic xenobiotics by secreting them into the bile, urine, and intestinal lumen and by reducing their accumulation in the brain and testes. A wide range of structurally unrelated anti-cancer drugs can be actively extruded from tumor cells by this protein, thus P-gp is responsible for the multidrug resistance phenotype [11, 15]. Additionally, drugs recommended for the treatment of acid-related disorders with *H. pylori* infection, such as proton pump inhibitors (PPI) and antibiotics (i.e., amoxicillin and either clarithromycin or metronidazole), are substrates for P-gp and can lead to ineffective therapy. Recent investigations have also implicated P-gp in the system regulating cell differentiation, proliferation and survival [15].

In 2000, Hoffmeyer et al. [9] performed systemic screening for *ABCB1 (MDR1*) genetic polymorphisms and identified 15 single nucleotide polymorphisms (SNPs) in a Caucasian population. Recently, more than 50 single nucleotide polymorphisms of the *ABCB1* gene [18] and 3 insertion/deletion polymorphisms [11] have been listed. Of all identified SNPs, a silent single nucleotide polymorphism C3435T in exon 26 of *ABCB1* is the best characterized [19]. It is known that it is correlated with altered expression and function of P-gp [16], as well as altered pharmacokinetics and pharmacodynamics, but the mechanisms are unclear [27]. The mutant T-allele was found to be linked with lower P-gp expression and/or activity in the duodenum [9], natural killer cells [8], and renal parenchyma [28]. Recently, it has been reported that the C3435T polymorphism can change mRNA stability and influence P-gp expression [16]. In many studies, the effects of C3435T on susceptibility to cancer diseases were studied, but there are still discrepancies in the results.

In 2007, Tahara et al. [32] published research showing the patients with a TT genotype in the 3435 position of *ABCB1* gene were at low risk of gastric cancer development. The TT genotype was found less frequently in *H. pylori* positive and negative gastric cancer patients than in a control group. Different results were also obtained [29]. Sugimoto et al., [29] attempted to determine the association between the *ABCB1* C3435T polymorphism and susceptibility to gastric cancer and peptic ulcers in *H. pylori* positive patients. They concluded that there was a lack of dependence between this polymorphism and the risk for gastric cancer and peptic ulcer development.

The phenomenon of polymorphisms in *ABCB1* with functional consequences is expected to be of high clinical importance due to the wide spectrum of drugs and toxins that are P-gp substrates. Observations that this gene and its protein are involved in the pathogenesis of chronic stomach inflammation mean that it may be a risk factor for gastric cancer development. Because P-gp-substrate drugs are used in the treatment of peptic ulcers, evaluation of the impact of the C3435T *ABCB1* polymorphism in patients with this disease is critically important.

Materials and Methods

Materials

One hundred ninety-six patients suffering from gastric ulcers (71 males, median age of 52, range 17–84;

125 females, median age of 56, range 14–85) diagnosed in the Department of Surgery, District Hospital, Łęczyca, Poland were enrolled in the study. The control group consisted of 96 blood donors at a local blood bank, geographically and ethnically matched to the patients. The results of *ABCB1* genotyping of the control group were reported previously [14]. The data concerning exposure to carcinogens in patients and controls were not available. The investigation was in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of the Medical University of Łódź. All subjects included in the study gave informed consent.

Rapid urease test

Biopsy specimens were collected and then used to conduct rapid urease test (Instytut Żywności i Żywienia Warszawa, Poland) to detect *H. pylori* infection. The basis of this test is the ability of *H. pylori* to secrete the urease enzyme, which catalyzes the conversion of urea to ammonia and bicarbonate. This test was performed at the time of gastroscopy. Mucosa are taken during biopsy from the antrum of the stomach and placed into a medium containing urea and an indicator, such as phenol red. The urease produced by *H. pylori* then hydrolyzes urea to ammonia, which raises the pH of the medium and changes the color of the sample from yellow (negative) to red (positive).

Genotyping of C3435T *ABCB1* SNP

The C3435T *ABCB1* SNP was identified using PCR-RFLP (restriction fragment length polymorphism) as described previously [12]. Briefly, genomic DNA was isolated from peripheral blood cells (control subjects) or biopsy specimens of gastric mucosa (gastric ulcers patients) by standard methods. The reaction mixture for PCR amplification consisted of DNA template, 0.5 μM of each primer (TTGATGGCAAAGAAA TAA AGC; CTTACATTAGGCAGTGACTCG, 10X PCR buffer, 1.5 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, and 0.2 mM of each dNTP. PCR grade water was added to a final volume of 20 μl. PCR amplification consisted of three steps: denaturation at 94°C for 90 s, annealing at 54°C for 60 s and extension at 72°C for 90 s over 30 cycles. A negative control was included in each experiment. Amplified DNA fragments (206 bp) were cut by restriction enzyme *Mbo*I (Fermentas, Vilnius, Lithuania) for 16 h at 37°C. The genotypes were identified by electrophoresis of DNA fragments generated after digestion (two bands of 130 and 76 bp for 3435CC, one band of 206 bp for 3435TT and three bands of 206bp, 130 bp and 76 bp for heterozygous 3435CT genotype).

Statistical analysis

All statistical analyses were performed using the STATISTICA version 8.0 (StatSoft, Tulsa, OK, USA) software package. The χ^2 test was applied to evaluate conformity between the observed and expected genotype frequencies according to the Hardy-Weinberg rule and to determine the significance of differences in allele and genotype frequencies between cases and controls. An odds ratio (OR) with a 95% confidence interval (95% CI) was estimated by logistic regression. A p-value < 0.05 was assumed as significant in all tests conducted.

Results

In all 196 patients included in the study, genotyping of C3435T *ABCB1* SNP was successful. The genotypes in the group of patients with peptic ulcers and the group of healthy individuals (control) were dis-

Tab. 1. Comparison of C3435T *ABCB1* allele and genotype frequencies between peptic ulcer patients and healthy individuals

ABCB1 variants	Peptic ulcer n = 196	Control n = 96	p (χ^2 test)	Odds ratio	95% CI
CC	46 (23.5%)	27 (28.1%)		1	–
CT	83 (42.3%)	48 (50.0%)	0.0983	1.36	0.98–1.91
TT	67 (34.2%)	21 (21.9%)		1.86	0.95–3.63
C	175 (44.6%)	102 (53.1%)			
T	217 (55.4%)	90 (46.9%)	0.0538		
HWE: p	0.1339	0.9991			

tributed in accordance with the Hardy-Weinberg equilibrium (HWE) (Tab. 1). Allele and genotype frequencies were calculated and compared with those obtained earlier for the control group. There was a trend towards higher incidence of the 3435TT genotype among peptic ulcer patients than controls (p = 0.0983). Likewise, in the patient group, the 3435T allele was more frequently observed. The association was near to statistical significance (p = 0.0538).

Connection between the C3435T *ABCB1* SNP and *H. pylori* infection was assessed in the group of peptic ulcer patients (Tab. 2). A statistically significant dependence between the analyzed genotypes and *H. pylori* infection was found (p = 0.0372). The CT genotype was associated with 1.56 times and the TT genotype with 2.45 times higher risk for infection compared to the CC genotype. A similar association between the C3435T genotype and *H. pylori* infection was present in the subgroup of peptic ulcer men (p = 0.0090), but not in the subgroup of peptic ulcer women (p = 0.4011). In addition, TT homozygous men were proved to have 8.34 times greater and heterozygous men 2.90 times lower risk of *H. pylori* infection than CC male carriers.

A statistically significant link between the presence of the 3435T allele and *H. pylori* infection both in all peptic ulcer patients (p = 0.0133) and peptic ulcer men (p = 0.0014) was discovered. The 3435T allele was more frequent in *H. pylori*-positive patients when cases of both genders were analyzed and in *H. pylori*-positive men only. Surprisingly, no significant connection between the C3435T *ABCB1* allele frequency and *H. pylori* infection was found in the group of women with peptic ulcers (p = 0.4994).

Tab. 2. Comparison of C3435T *ABCB1* allele and genotype frequencies between *H. pylori* infected and uninfected peptic ulcer patients

ABCB1 variants	All cases		p (χ^2 test)	Odds ratio	95% CI
	Infected, n = 101	Uninfected, n = 95			
CC	20 (19.8%)	26 (27.4%)		1	–
CT	38 (37.6%)	45 (47.4%)	0.0372*	1.56	1.07–2.30
TT	43 (42.6%)	24 (25.2%)		2.45	1.13–5.37
C	78 (38.6%)	97 (51.1%)			
T	124 (61.4%)	93 (48.9%)	0.0133*		
HWE: p	0.1164	0.8785			
	Women				
	Infected, n = 65	Uninfected, n = 60			
CC	15 (23.1%)	13 (21.7%)		1	–
CT	23 (35.4%)	28 (46.8%)	0.4011	1.16	0.72–1.86
TT	27 (41.5%)	19 (31.7%)		1.35	0.53–3.44
C	53 (40.8%)	54 (45.0%)			
T	77 (59.2%)	66 (55.0%)	0.4994		
HWE: p	0.0980	0.9064			
	Men				
	Infected, n = 36	Uninfected, n = 35			
CC	5 (13.9%)	13 (37.1%)		1	–
CT	15 (41.7%)	17 (48.6%)	0.0090*	2.90	1.40–6.04
TT	16 (44.4%)	5 (14.3%)		8.34	1.95–36.52
C	25 (34.7%)	43 (61.4%)			
T	47 (65.3%)	27 (38,6%)	0.0014*		
HWE: p	0.8890	0.9891			

* Statistically significant

Discussion

Peptic ulcer disease has been a significant cause of morbidity and mortality over the past two centuries. Familiar occurrence of the disease has been observed and considered to be an effect of shared environment, but recent evidence also suggests genetic factors [25]. In light of the presented results, the polymorphism of the *ABCB1* gene coding membrane transporter glycoprotein P could be of relevance in explaining the inherited component of peptic ulcer pathogenesis.

There is strong evidence that P-gp plays an important role in the maintenance of homeostasis in the digestive tract. The interaction between bacterial toxins and food carcinogens and the gastric mucosa can lead to susceptibility to chronic peptic ulcer and gastric cancerogenesis. As such, P-gp as a plasma membrane pump may be involved in the clearance of carcinogens within the gastric mucosa. Several reports have suggested that SNPs of *ABCB1* are a risk factor for cancer development, including ALL [13], MM [14], renal cancer [28] and colon cancer [17, 22, 23]. However, such a correlation was not found in inflammatory bowel disease [4].

Genotype distribution showed no distortion from the Hardy-Weinberg rule, which suggested representative sampling for the investigated populations. In this study, the allele/genotype frequency of the polymorphism of *ABCB1* gene in healthy population was similar to other Caucasian populations from Europe [2, 5, 9, 35], as well as different from Asian populations [31].

In this study, no significant difference was found in the genotype distribution between group with peptic ulcers and control population, but there was a trend towards higher incidence of the 3435TT genotype among peptic ulcer patients than controls. Significant differences in frequency of the T allele in investigated populations were also observed.

In the majority of studies, people having the homozygous TT genotype had lower P-gp expression in various normal tissues [1, 30]. For example, Hitzl et al. [8] observed that P-gp activity and MDR1 mRNA expression in peripheral blood CD56+ NK cells was the highest in 3435CC carriers, intermediate in 3435CT carriers, and the lowest in 3435TT carriers. Our results (allele C – 44.6%) were different than in African populations, where this allele is most common (e.g., Kenyan 83%, Sudanese 73%, Ghanaian 83%), but similar to other Caucasian and Asian populations (e.g., Portuguese 43%, British Caucasian 48%, German 46–52%, Chinese 53%, Southwest Asian 34%, Japanese 51%) [1, 2, 8, 9, 30]. On the basis of these data, we could see that our population was similar to other Caucasian populations, but different than African populations for genotype and allele frequency of C3435T polymorphism. Because the TT genotype is associated with lower P-gp expression, patients that carry the T allele might be more sensitive to certain cytotoxic agents.

The great achievement in the understanding of peptic ulcer pathogenesis was the Noble prize-winning discovery of *H. pylori.* This Gram-negative bacteria persistently colonizes the human stomach, evoking innate and adaptive host immune response and consequently, chronic inflammation, which can predispose the carrier to peptic ulceration, gastric lymphoma, and gastric carcinoma. Mohammadi et al. [21] showed that infection induced with a single isolate of *Helicobacter felis* resulted in various patterns and intensities of gastric inflammation in different inbred mouse strains. This proved the significance of host response in disease outcome during *Helicobacter* infection. Additionally, a twin cohort study revealed that convergence of anti-*H. pylori* status was significantly higher in monozygotic than dizygotic twins, which firmly indicated a genetic basis for *H. pylori* infection [25].

The interaction between *H. pylori* and antigen presenting cells, such as monocytes or dendritic cells, leads to the secretion of cytokines (e.g., IL-1b, TNF-α, IL-8). These cytokines attract neutrophils, lymphocytes and plasma cells. Attracted cells are activated and increase the production of pro- and anti-inflammatory cytokines [24]. Many of the genes encoding cytokines important for inflammatory responses, including gastritis, are known to be polymorphic. Different gene alleles lead to different levels of that cytokine generated in response to the same antigenic stimulus. It is well established that IL-1B, IL-8, IL-10 and TNF-α gene polymorphisms are associated with *H. pylori* infection, gastric atrophy, and gastric cancer [7].

A statistically significant dependence between the C3435T *ABCB1* genotypes and *H. pylori* infection ($p = 0.0372$) was revealed. The CT genotype was found to be connected with 1.56 and the TT genotype with 2.45 times higher prevalence of infection compared to the CC genotype. Beside the tissue barriers and luminal surfaces of excretory organs, P-gp is con-

stitutively expressed in the natural killer (NK) cells, antigen-presenting dendritic cells, and T and B lymphocytes. It was proved that inhibition of P-gp efflux function using either specific monoclonal antibodies or pharmacological inhibitors, or a decrease of P-gp expression using antisense oligonucleotides, resulted in a reduction in NK and CD8 T-cell cytolytic activity [15].

Additionally, P-gp could be involved in the transendothelial migration of antigen-presenting dendritic cells and T lymphocytes during an immune response [26]. P-gp as a transporter of cytokines (e.g., IL-1b, IL-2, IL-4, IFN-γ, TNF-α) [20] could then regulate both their activation and migration processes. This hypothesis could also provide an explanation for the results reported here. Differences in P-gp expression levels connected with different C3435T *ABCB1* genotypes could thus influence immunological response and susceptibility to chronic infection caused by *H. pylori.*

High frequency of the TT genotype in a group with peptic ulcers could be important for other reasons. For example, Gawrońska-Szklarz et al. [6] reported that genotype 3435 TT was more often found in groups of patients who were cured after the first cycle of triple therapy. Moreover, 3435 polymorphism of the *ABCB1* gene together with polymorphism of CYP2C19 were recommended as independent predictive determinants of the efficacy of triple therapy, including proton pump inhibitors.

In conclusion, it was found that the C*3435T ABCB1* SNP alone was not a major factor for genetic susceptibility to peptic ulcers. In the group of men suffering from peptic ulcers, rather, this polymorphism of the *ABCB1* gene seemed to be a risk factor for *H. pylori* infection development.

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