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ORIGINAL ARTICLE

ABCB1 gene polymorphisms and haplotype analysis in colorectal cancer

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Abstract
Purpose The aim of this study was to determine the significance of three most common single-nucleotide polymorphisms (SNPs) of *ABCB1* gene in the development of colorectal cancer and to estimate the influence of these SNPs to surviving patients' treatment combination adjuvant therapy 5-fluorouracil/leucovorin. Haplotype structure of *ABCB1* was analysed, and degree of linkage disequilibrium (LD) between SNPs of *ABCB1* was estimated.
Materials and methods Tumour specimens of 95 patients with colorectal cancer and blood samples of 95 healthy cases were studied. Genotyping of *ABCB1* gene was performed by automated sequencing or polymerase chain reaction-restriction fragment length polymorphism method. Comparison of frequencies of alleles/genotypes/haplotypes between the studied group (colorectal cancer samples) and the control group (blood samples) were analysed. These results were correlated with the surviving patients after treatment of adjuvant chemotherapy.
Results Significant differences in $ABCB1_{1236C>T}$ (p=0.00043) and $ABCB1_{2677G>T/A}$ (p=0.04) genotype distribution and T_{1236} allele distribution (CT_{1236} or TT_{1236} vs CC_{1236}; p=0.0499, OR=0.55, Fi–Yule coefficient=0.14) were found. A strong LD between $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ SNPs (D'=0.621, r^2=0.318) was detected. All SNPs were located in one haplotype block. There were significant differences in haplotype distributions between colorectal cancer patients and healthy population (p=0.03). Significant differences in survival probability of colorectal cancer patients' treatment chemotherapy according to allele of $ABCB1_{3435C>T}$ was observed. Survival probability of patients with wild-type C_{3435} allele were higher than among patients without this allele (p=0.04572).
Conclusions These results suggested that three studied SNPs of *ABCB1* were located in one haplotype block. Differences in $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ genotypes and T_{1236} allele distribution between investigated populations indicate significant impact of these SNPs on risk of development of colorectal cancer. Polymorphism $ABCB1_{3435C>T}$ may be a prediction marker of cancer chemotherapy effectiveness. Differences in haplotype distributions between colorectal cancer patients and healthy population suggested that other potential SNPs, especially in regulatory region of *ABCB1* gene, may influence P-glycoprotein expression and function.

Keywords *ABCB1* gene · *MDR1* gene · Polymorphism · Haplotype analysis · Colorectal cancer

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Introduction

The gene product of *ABCB1* (*ATP-binding cassette*, *sub-family B*, *member 1*, also named *MDR1*, *multidrug resistance gene 1*), P-glycoprotein, (P-gp) is an efflux pump protein of 170 kDa. It belongs to ABC membrane transporters superfamily of [1]. Overexpression of P-gp in tumour cells leads to multidrug resistance against antineoplastic agents [1–5]. P-gp is expressed in the apical membranes of excretory tissues, such as liver, kidney and intestine. This contributes to the elimination of toxic exogenous substances or metabolites and drugs into bile and urine or limits drug absorption from the gastrointestinal tract [6, 7]. P-gp was implicated in the system regulating cell differentiation, proliferation and survival [4].

Relation between the presence of different single-nucleotide polymorphisms (SNPs) and the risk of colon cancer development is being investigated in over 35 different genes [8]. One of them is *ABCB1* which is already known to accompany colon cancer development in cases with high microsatellite instability characteristics (MSI-H) [9].

Multiple mutations in the *ABCB1* gene have been identified. Analysis of all 28 exons of the *ABCB1* gene demonstrated 40 SNPs, including promotor and the intron–exon region [10]. The most frequent SNP $ABCB1_{2677G>T/A}$ in exon 21 (refSNP ID: rs2032582), leading to amino acid exchange from Ala to Ser or Thr. The silent mutation in exon 26 $ABCB1_{3435C>T}$ (refSNP ID: rs1045642) is associated with altered protein function [11]. Subjects with the TT_{3435} genotype had higher plasma concentration of digoxin, which is P-gp substrate, compared to subjects with the wild-type genotype [12]. Of all SNPs, the $ABCB1_{3435C>T}$ is best characterised for its association with expression and function in the tissues [13, 14]. The third common polymorphism of *ABCB1* gene is mutation in exon 12 $ABCB1_{1236C>T}$ (refSNP ID: rs1128503). This SNP similarly to $ABCB1_{3435C>T}$ mutation is silent. Relation between SNPs of *ABCB1* gene is not clear. Maybe these three polymorphisms are closely related to disequilibrium (LD), but unknown genetic variant is located on the same LD block or haplotype [10, 15]. The study performed by Kim has pointed that there is a correlation between bioavailability of drugs and mutations in positions of $ABCB1_{3435C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{1236C>T}$ [16].

P-gp plays an important role in the detoxification systems of normal tissues. Several studies have shown that polymorphisms of *ABCB1* gene can influence susceptibility to cancer. Siegsmund and Jamroziak suggested that $ABCB1_{3435C>T}$ SNP combined with susceptibility to renal epithelial tumours and acute lymphoblastic leukaemia [17, 18]. Such SNP was also reported in colorectal patients [9, 19–22].

Colon and rectum cancer are two of the most frequent neoplasms and are the main reasons of high mortality ratio among all suffering from different types of cancer [23]. Genetic factors together with influence of xenobiotics which are present in a diet, cigarette smoke, drugs, bacterial toxins and other biological and chemical factors may increase the risk of colon and rectum cancer development. A significant relation between the risk of colon cancer development determined by genetic variants of *ABCB1* gene and environmental factors such as cigarette smoking was indicated by Osswald [22].

The role of P-gp in carcinogenesis was described in animal models of colon [24], breast [25] and liver [26] cancers. P-gp overexpression was connected with apoptosis inhibition and increasing possibility of neoplasm transformation in an mdr1 mouse model [24]. The implications of genetically determined differences in P-gp function for drug disposition, therapeutic outcome and risk for development of certain diseases are being intensively studied.

The potential mechanism of the *ABCB1* polymorphisms affecting susceptibility to colorectal cancer has not been satisfactorily described yet. The aim of this study was to determine the significance of three SNPs of *ABCB1*, namely $ABCB1_{1236C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ in the development of colorectal cancer. Also, haplotype structure analysis of this gene in colorectal cancer group comparison and frequencies of haplotypes between the studied group was carried out. In this study, we investigated the impact of *ABCB1* gene polymorphisms on surviving patients after adjuvant combined chemotherapy with 5-fluorouracil/leucovorin (5-FU/LV).

Materials and methods

Tissues

Tissue samples were obtained from 95 colorectal carcinomas patients in 43 cases with III or IV pTNM (Table 1) operated on in the Oncological Center of Lodz, Poland. Colorectal cancer was diagnosed by histopathological examination using established clinical criteria at the Department of Pathology, Medical University of Lodz, Poland. Primary colorectal carcinoma and normal colorectal mucosa taken from a site distant several centimetres from the tumour were used in the study. Samples were frozen in liquid nitrogen immediately after surgical resection and stored at −80°C until processed. The control group constituted of 95 unrelated peripheral blood samples (46 men and 49 women, ratio 1:0.94, the controls were matched to patients by gender) from Lodz region in central Poland. All subjects were of Slavic origin. All experiments were carried out with the local ethical committee approval.

Table 1 Detailed information for the colorectal cancer group

Feature	Number of patients (n=95)	
Median age	61 years of age	
Gender	Women	48
	Men	47
Tumour localization	Rectum	36
	Different location	59
Depth of tumour invasion	T_1	3
	T_2	26
	T_3	56
	T_4	10
Lymph node involvement	N_0	59
	N_1	16
	N_2	20
Distant metastases	M_0	76
	M_1	19
pTNM	I	26
	II	26
	III	24
	IV	19
Grade of malignancy	G_1	10
	G_2	57
	G_3	28
Histological type	Tubular	64
	Mucinous adenocarcinoma	31
Vessel invasion	Involved	59
	Not involved	36
Adjuvant combined chemotherapy	Yes	40
	No	43
	Data missing	12

Resection status of all patients were estimated as R0

DNA isolation

The studying group: DNA was isolated according to protocol "Genomic DNA Prep Plus" (*A&A Biotechnology*, Gdynia, Poland) from the frozen tissue slides of colon cancers.

"Blood Mini Kit" (*A&A Biotechnology*) was used for DNA isolation from blood. The purity and concentration of DNA samples were estimated spectrophotometrically. The samples were stored at −20°C until analysis.

Polymerase chain reaction

Polymerase chain reaction (PCR) reaction was conducted according to protocol "AccuTaq™ LA DNA Polymerase Kit" (*Sigma Aldrich,* Germany). The reaction mixture for PCR amplification consisted of 50 ng of DNA template, 0.5 μM of each primer, 10× AccuTaq Buffer, 0.5 U of AccuTaq LA DNA Polymerase Mix, 0.2 mM each deoxyribonucleotide triphosphate (dNTP) and water to a final volume of 20 μl. Negative control was included in each experiment (sample without DNA template). The primers design were based on published sequences for genotyping procedure of $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ polymorphisms using genomic DNA [11, 27]. The primer sequences for genotyping procedure of $ABCB1_{1236C>T}$ was planned by using software Primer3: WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and GeneBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Selected primer sequences and conditions of PCR reaction are summarised in Table 2.

Restriction fragment length polymorphism

After checking PCR product in 2% agarose gel (Fig. 1a), DNA fragments were cut by restriction enzyme MboI (*Fermentas*, Vilnius, Lithuania) for $ABCB1_{3435C>T}$ mutation during 16 h at 37°C. DNA fragments generated after digestion were separated on 2% agarose gel and visualised with ethidium bromide. Electrophoretic pattern showed two bands (130 and 76 bp) for homozygous wild-type C allele, one band (206 bp) for homozygous mutant T allele and three bands (206, 130 and 76 bp) for heterozygous CT genotype (Fig. 1b).

Sequencing analysis

Genotyping of $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ was performed by automated sequencing. Sequencing PCR reaction was performed according to "SequiTherm EXCEL™ II DNA Sequencing Kit-LC" protocol (*Epicentre Technologies*, Madison, USA).

The reaction mixture for sequencing-PCR amplification consisted of DNA fragments generated after sequencing, 0.2 μM of primer, 3.5× Sequencing Buffer, 5 U of SequiTherm EXCEL™ II DNA Polymerase, 0.2 mM each dNTP/ddNTP and distilled water to a final volume of 11 μl. Sequencing primers were labelled by IRD 700 or IRD 800 on 5′ end. Stop/Loading Buffer was used after sequencing-PCR amplification. The primers sequences for automated sequencing genotyping procedure of $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ was planned by using software Primer3: WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and GeneBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html; Table 2). SeqPCR products after denaturation were separated in polyacrylamide gels. Sequencing was performed with the use of automated sequencer LI-COR®4000. Example of $ABCB1_{1236C>T}$ sequencing analysis are summarised in Fig. 2.

Table 2 Primer sequences, number of cycles and PCR conditions

Primer for PCR or PCR_{seq}	Sequences	Number of cycles	PCR conditions
$F_{1236C>T}$	5′ TAT CCT GTG TCT GTG AAT TGC C 3′	35	Denaturation, 94°C (45 s)
$R_{1236C>T}$	5′ CCT GAC TCA CCA CAC CAA TG 3′		Annealing, 56°C (45 s)
			Extension, 72°C (45 s)
$F_{2677G>T/A}$	5′ CAT GAA AAA GAT TGC TTT GA 3′	30	Denaturation, 94°C (90 s)
$R_{2677G>T/A}$	5′ TAT GGT TGG CAA CTA ACA CT 3′		Annealing, 54°C (60 s)
			Extension, 72°C (90 s)
$F_{3435C>T}$	5′ TTG ATG GCA AAG AAA TAA AGC 3′	30	Denaturation, 94°C (90 s)
			Annealing, 54°C (60 s)
$R_{3435C>T}$	5′ CTT ACA TTA GGC AGT GAC TCG 3′		Extension, 72°C (90 s)
$Seq_{1236C>T}$	IRD-800-5′GCC TTG AAG TTT TTT TCT CAC 3′	30	Denaturation, 94°C (30 s)
			Annealing, 56°C (60 s)
			Extension, 70°C (60 s)
$Seq_{2677G>T/A}$	IRD-700-5′ TAT GGT TGG CAA CTA ACA CT 3′	30	Denaturation, 94°C (30 s)
			Annealing, 54°C (60 s)
			Extension, 70°C (60 s)

Statistical analysis

Statistical significance of the observed genotype frequencies compared to genotype frequencies expected according to Hardy–Weinberg rule was evaluated. Data were analysed using STATISTICA version 8.0. (data analysis software system, StatSoft). The differences in allele or genotype frequencies between studying group (colorectal cancer samples) and control group (blood samples) were calculated using chi-square test. Survival probability of colorectal cancer patients according to genotypes was estimated on the basis of the Kaplan–Meier method and compared between groups by the log-rank test. Haplotypes were statistically inferred using the PHASE v. 2.1 software. Program PHASE implements a Bayesian statistical method for reconstructing haplotypes from population genotype data [28]. Linkage disequilibrium (LD) was estimated according to EMLD software [29] with the Expectation-Maximization Algorithm.

For all analyses, p values at the level of 0.05 were considered as statistically significant.

Result

Analysing for three SNPs of *ABCB1* by PCR-restriction fragment length polymorphism (RFLP) method or automated sequencing was successful in all specimens (Figs. 1 and 2).

In our recent study for *ABCB1* gene genotyping, we have used DNA isolated from frozen tissue (the colorectal cancer group, Table 1), and for the control group, DNA was isolated from leukocytes [18]. Recent studies have proven

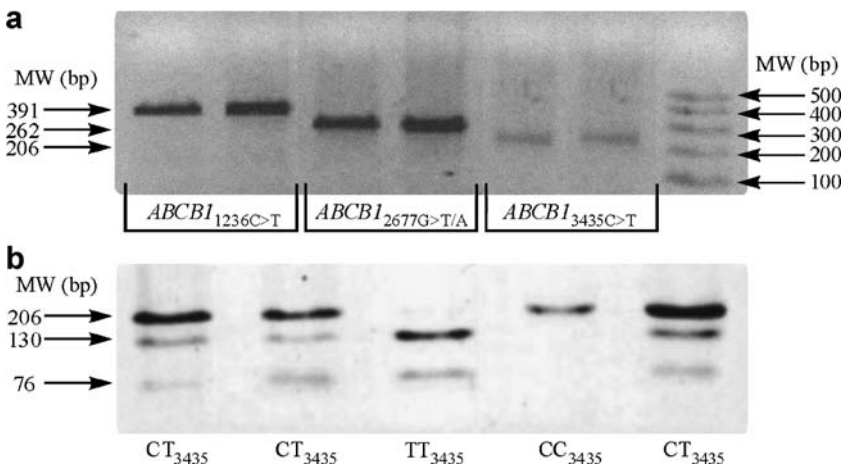

Fig. 1 Electrophoretic pattern of PCR products containing $ABCB1_{1236C>T}$. $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ polymorphic sits (**a**) and $ABCB1_{3435C>T}$ polymorphism evaluated by PCR-RFLP (**b**)

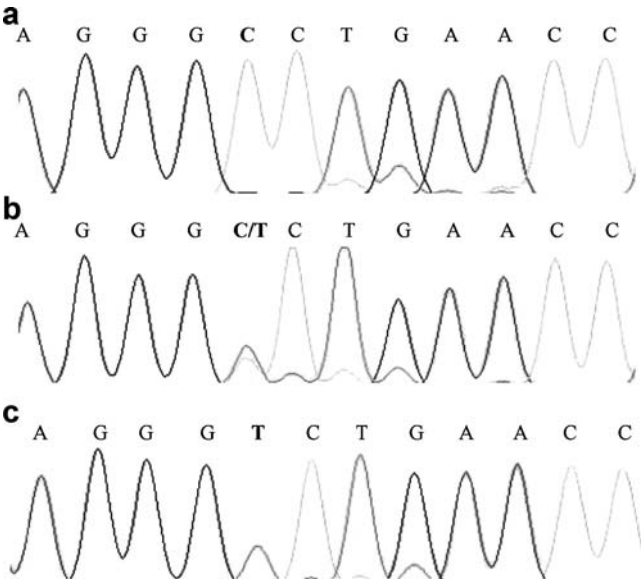

Fig. 2 Sequencing analysis of *ABCB1*$_{1236C>T}$ polymorphism. Wild-type genotype CC_{1236} (**a**). Heterozygous CT_{1236} (**b**) and homozygous for the mutation TT_{1236} (**c**)

that genotyping based on DNA isolated from frozen tissues is equal to DNA isolated from blood [30].

The observed genotype frequency distribution did not show significant deviation from Hardy–Weinberg equilibrium (Table 3). The comparison of results from genotyping studies in colorectal cancer and control group is presented in Table 4. Higher frequency of T_{1236} allele (genotype CT_{1236} or TT_{1236} vs CC_{1236}) in the control than colorectal cancer group was found (70.5% and 56.8%, respectively; p=0.0499, OR=0.55, measure of correlation: Fi–Yule coefficient = 0.14). Moreover, the *ABCB1*$_{1236C>T}$ wild-type genotype (CC_{1236}) was observed in 43.2% of patients, whereas 36.8% was heterozygous (CT_{1236}) and 20.0% patients were homozygous for the mutation (TT_{1236}). In healthy population, the frequencies of *ABCB1*$_{1236C>T}$ genotypes were different compared with colorectal cancer group: 29.5% (CC_{1236}), 56.8% (CT_{1236}) and 13.7% (TT_{1236}) for controls (p=0.00043). In addition, significant differences in *ABCB1*$_{2677G>T/A}$ genotype distribution were found (p=0.04). The GT_{2677} was detected in 41.1% subjects of colorectal cancer and 52.6% subjects of healthy population. The frequencies of TT_{2677} and GG_{2677} were higher in colorectal cancer (22.1% and 36.8%, respectively) in comparison with control group (14.8% and 32.6%). Statistical analysis did not reveal any differences in *ABCB1*$_{3435C>T}$ genotype/allele frequencies between investigated populations.

Figure 3 shows pair-wise linkage disequilibrium patterns for the *ABCB1* gene. We detected a strong LD between *ABCB1*$_{1236C>T}$ and *ABCB1*$_{2677G>T/A}$ SNPs (D'=0.621, r^2=0.318). We also observed remains pair-wise LD between *ABCB1*$_{1236C>T}$ and *ABCB1*$_{3435C>T}$ (D'=0.394, r^2=0.104) as well as *ABCB1*$_{2677G>T/A}$ and *ABCB1*$_{3435C>T}$ SNPs (D'=0.384, r^2=0.120). All SNPs were located in one haplotype block.

Each of the eight possible haplotypes was noted with frequencies above 3%. However, the frequencies of T_{1236}–T_{2677}–T_{3435} haplotype were higher in colorectal cancer in comparison with control group (24.0% vs 3.0%, OR=9.61). There were significant differences in haplotype distributions between colorectal cancer patients and healthy population (p=0.03; Table 5).

We estimated influence of *ABCB1* genotypes on outcome of colorectal cancer therapy.

Analysis included 32 patients subjected to adjuvant combine chemotherapy with 5-FU/LV. Cases with the presence of metastases were excluded from survival analysis despite the fact that they were also treated with adjuvant chemotherapy.

Table 3 Statistical significance of observed genotype frequencies in investigated populations

Genotypes	Control (%)	H-W[a] (%)	p value[b] CI(95%)[c]	Colorectal cancer (%)	H-W[a] (%)	p value[b] CI(95%)[c]
CC_{1236}	29.00	33.06	0.252 (0.169–0.347)	43.00	37.82	0.091 (0.042–0.164)
CT_{1236}	57.00	48.88		37.00	47.36	
TT_{1236}	14.00	18.06		20.00	14.82	
GG_{2677}	33.00	34.40	0.273 (0.186–0.368)	37.00	33.06	0.273 (0.186–0.368)
GT_{2677}	53.00	48.20		41.00	48.88	
TT_{2677}	14.00	16.40		22.00	18.06	
CC_{3435}	35.00	33.64	0.856 (0.776–0.921)	27.00	28.62	0.809 (0.729–0.882)
CT_{3435}	46.00	48.72		53.00	49.76	
TT_{3435}	19.00	17.64		20.00	21.62	

[a] Genotype frequencies expected according to Hardy–Weinberg rule

[b] Chi-square test

[c] 95% confidence interval

Table 4 *ABCB1* genotype and allele frequencies in investigated groups

Genotypes/alleles	Colorectal cancer (n=95)		Control group (n=95)		p value[a] CI(95%)[b]	OR CI(95%)[b]
	N	Frequency	N	Frequency		
CC_{1236}	41	0.432	28	0.295	**0.00043*** (0.00001–0.0381)	1.82 (0.94–3.52)
CT_{1236}	35	0.368	54	0.568		0.44 (0.25–0.78)
TT_{1236}	19	0.200	13	0.137		1.57 (0.63–3.95)
CC_{1236}/CT_{1236}	76	0.800	82	0.863	0.244 (0.183–0.309)	0.63 (0.35–1.14)
TT_{1236}	19	0.200	13	0.137		1.57 (0.63–3.95)
CT_{1236}/TT_{1236}	54	0.568	67	0.705	**0.0499*** (0.0255–0.0947)	0.55 (0.32–0.93)
CC_{1236}	41	0.432	28	0.295		1.82 (0.94–3.52)
C_{1236}	117	0.616	110	0.579	0.464 (0.391–0.537)	1.17 (0.79–1.73)
T_{1236}	73	0.384	80	0.421		0.86 (0.56–1.31)
GG_{2677}	35	0.368	31	0.326	**0.040*** (0.0116–0.1043)	1.20 (0.63–2.31)
GT_{2677}	39	0.411	50	0.526		0.63 (0.36–1.10)
TT_{2677}	21	0.221	14	0.148		1.63 (0.68–3.98)
GG_{2677}/GT_{2677}	74	0.779	81	0.853	0.190 (0.116–0.283)	0.61 (0.34–1.08)
TT_{2677}	21	0.221	14	0.147		1.65 (0.68–3.98)
GT_{2677}/TT_{2677}	60	0.632	64	0.674	0.542 (0.442–0.650)	0.79 (0.48–1.43)
GG_{2677}	35	0.388	31	0.326		1.27 (0.63–2.31)
G_{2677}	109	0.574	112	0.589	0.755 (0.685–0.812)	0.94 (0.64–1.38)
T_{2677}	81	0.426	78	0.411		1.06 (0.70–1.63)
CC_{3435}	25	0.263	33	0.347	0.225 (0.142–0.318)	0.67 (0.35–1.30)
CT_{3435}	50	0.526	44	0.463		1.29 (0.72–2.30)
TT_{3435}	20	0.211	18	0.190		1.14 (0.50–2.59)
CC_{3435}/CT_{3435}	75	0.789	77	0.811	0.7168 (0.614–0.804)	0.87 (0.49–1.57)
TT_{3435}	20	0.211	18	0.189		1.15 (0.50–2.59)
CT_{3435}/TT_{3435}	70	0.737	62	0.653	0.208 (0.134–0.306)	1.49 (0.84–2.65)
CC_{3435}	25	0.263	33	0.347		0.67 (0.35–1.30)
C_{3435}	100	0.526	110	0.579	0.302 (0.236–0.371)	0.81 (0.55–1.19)
T_{3435}	90	0.474	80	0.421		1.24 (0.81–1.89)

OR odds ratio

[a] Chi-square test

[b] 95% confidence interval

*p<0.05

No significant prognostic influence of $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ SNPs in terms of surviving patients' treatment with adjuvant chemotherapy was detected. However, significant differences in survival probability of colorectal cancer patients according to allele of $ABCB1_{3435C>T}$ were observed (Fig. 4). Survival probability of patients with wild-type C_{3435} allele was higher than among patients without this allele (p=0.04572; Fig. 4b). Higher survival probability of patients without mutant T_{3435} allele than patients with this allele was detected (p= 0.08605; Fig. 4a).

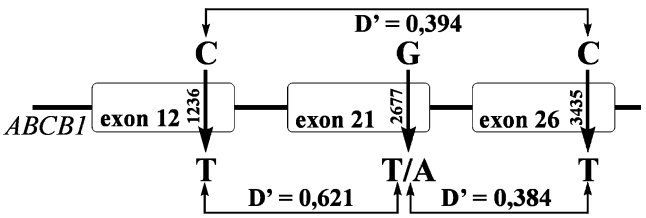

Fig. 3 Pair-wise linkage disequilibrium of *ABCB1* gene (*D'* Lewontin coefficient)

Discussion

It is well documented that P-gp plays an important role in the maintenance of intestinal homeostasis. The interaction between toxic agents (for example bacterial toxin or carcinogens) contained in food and intestinal epithelium

Table 5 Frequencies of haplotypes of *ABCB1* gene in investigated populations (combinations of the three SNPs of *ABCB1* gene)

Haplotypes	Colorectal cancer		Control group		*p* value[a]	OR CI(95%)[b]
	N	Frequency	*N*	Frequency		
C_{1236}–T_{2677}–T_{3435}	23	0.08	74	0.26	0.03*	0.25 (0.15–0.43)
C_{1236}–T_{2677}–C_{3435}	11	0.04	20	0.07		0.53 (0.22–1.26)
C_{1236}–G_{2677}–T_{3435}	31	0.11	17	0.06		1.92 (0.89–4.15)
C_{1236}–G_{2677}–C_{3435}	112	0.39	54	0.19		2.77 (1.77–4.33)
T_{1236}–T_{2677}–T_{3435}	68	0.24	9	0.03		9.61 (3.67–25.18)
T_{1236}–T_{2677}–C_{3435}	17	0.06	11	0.04		1.58 (0.60–4.16)
T_{1236}–G_{2677}–T_{3435}	14	0.05	34	0.12		0.38 (0.19–0.78)
T_{1236}–G_{2677}–C_{3435}	9	0.03	66	0.23		0.11 (0.05–0.23)

OR odds ratio

[a] Permutation test for significant differences in colorectal cancer and control group

[b] 95% confidence interval

*$p<0.05$

can play a significant role in susceptibility to the development of ulcerative colitis and colon cancerogenesis. It is well established that ulcerative colitis predisposes to tumorgenesis. P-gp as a plasma membrane pump may be involved in the clearance of carcinogens within intestinal epithelium. Xenobiotic-dependent modification of cellular defence against dietary carcinogens via *ABCB1* expression might differ in *ABCB1* genotypes [12, 22]. SNPs in transporters for xenobiotic agents may contribute to the susceptibility and progression of colorectal cancer [31].

Genotype distribution showed genetic stability and no distortion from Hardy–Weinberg rule, which suggests representative sampling for investigated populations. Furthermore, the allele/genotype frequency of the polymorphisms of *ABCB1* gene in healthy population was in agreement to other Caucasian populations from Europe [11, 12, http://www.ncbi.nlm.nih.gov/SNP/]. Widely reported ethnical differences in *ABCB1* allele/genotype frequencies were seen especially in African, Asian and African-American populations [32, http://www.ncbi.nlm.nih.gov/SNP/]. Much higher frequencies of the CC_{3435} genotype in African population (Ghanaian and Kenyan) compared with Caucasian population are due to an advantage offered by this SNP ($ABCB1_{3435C>T}$) against gastrointestinal tract infections [33]. Variable susceptibility to cancer incidences can be connected with inter-ethnic differences in frequencies of the *ABCB1* genotypes [17]. One of the reasons for development of neoplastic diseases is described mutations. Probably, changes of function and structure of genes simultaneously with influence of environmental factors lead to colon cancerogenesis [34]. Significant differences in $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ genotype distribution between colorectal cancer group and healthy population were found. In addition, significant differences in frequency of T_{1236} allele in investigated populations were found. There was a tendency for a higher

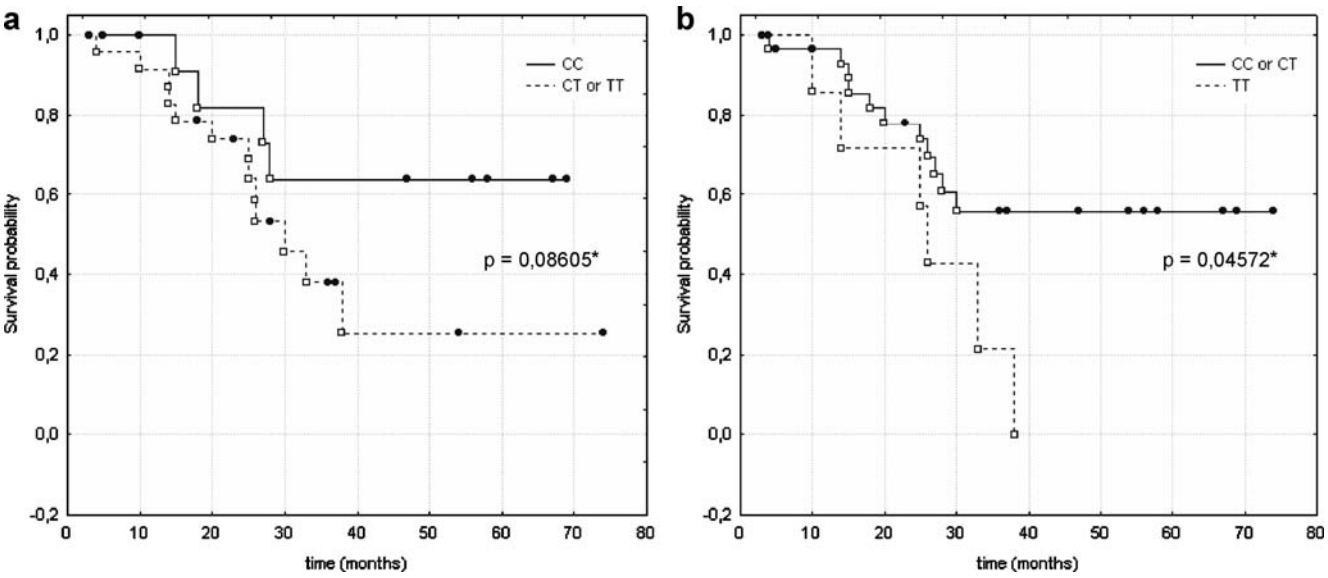

Fig. 4 Adjusted survival probability of colorectal cancer patients treatment chemotherapy according to *ABCB1* alleles: T_{3435} (genotype CT_{3435} or TT_{3435} vs CC_{3435}) (**a**) and C_{3435} (genotype CC_{3435} or CT_{3435} vs TT_{3435}) (**b**). *log-rang test

frequency of the T_{1236} allele, CT_{1236} and GT_{2677} genotypes in healthy population compared to colorectal patients (results statistically significant). Several reports have suggested that SNPs of *ABCB1* are a risk factor for cancer development, including colorectal cancer [9, 17–22]. Extensive studies have been carried out for $ABCB1_{3435C>T}$ since the Hoffmeyer report [12]. Changed function of P-gp could be a risk factor of colon cancer due to facilitated intracellular penetration of DNA damaging factor of both exo- and endogenous origin which, in consequence, may lead to the development of colon cancer [21]. In this study, it was found that $ABCB1_{3435C>T}$ was not associated with colorectal cancer in Polish population. Similar results have been presented by Petrova who has not found relation between SNPs $ABCB1_{3435C>T}$, $ABCB1_{2677G>T/A}$ and the risk of sporadic colorectal cancer development in Bulgarian population [30].

Earlier researches indicate that TT_{3435} genotype develops more frequently among ulcerative colitis patients but not among Crohn disease patients. Both diseases are considered to be factors predisposing to colorectal cancer development [35, 36]. Furthermore, it was noticed that earlier-diagnosed colorectal cancer patients (below the age of 50) have TT_{3435} genotype and T_{3435} allele more frequently (2.7-fold and 1.7-fold higher risk) [21]. However, other researches indicate rather a protective role associated with the presence of T_{3435} allele. Gaikovitch indicated a higher risk (1.65-fold) of colorectal cancer development among CC_{3435} genotype carriers than among T_{3435} allele (p=0.01) carriers. Similarly, the presence of T_{2677} allele decreases the risk of colorectal cancer development in relation to G_{2677} allele (OR=0.65, p=0.02) [37]. Protective role of T_{3435} and T_{2677} alleles (possibly also T_{1236} allele) may be associated with the function of P-gp protein, which influences functions of *c-Myc* and *cyclin D1* and contributes to unblocking of cell death pathways suppression.

Impact of $ABCB1_{1236C>T}$ and $ABCB1_{3435C>T}$ polymorphisms on the function of P-gp can be explained by importance of LD of $ABCB1_{1236C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$. The $ABCB1_{1236C>T}$ and $ABCB1_{3435C>T}$ are "silent" SNPs, and therefore it may be linked with the causal polymorphisms. We detected a strong LD between $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ SNPs (the LD value D'=0.621, r^2=0.318) but also observed the remains of pairwise LD between $ABCB1_{1236C>T}$ and $ABCB1_{3435C>T}$ (D'=0.394, r^2=0.104) as well as $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ SNPs (D'=0.384, r^2=0.120). Also, a strong association between $ABCB1_{1236C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ alleles was found by Tanabe [27]. Strong linkage disequilibrium of SNPs: $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ (D'=0.739, r^2=0.428) was also described by Petrova [30].

These results suggested that three studied SNPs of *ABCB1* were located in one haplotype block. $ABCB1_{1236C>T}$ and $ABCB1_{3435C>T}$ are in linkage disequilibrium with other common functional non-synonymous polymorphisms such as $ABCB1_{2677G>T/A}$. In fact, these SNPs are a part of a common haplotype [15]. It is very probable that other potential SNPs, especially in regulatory region of *ABCB1* gene, influence P-gp expression and function, e.g. $ABCB1_{-2410\ T>C}$, $ABCB1_{-1910\ T>C}$, $ABCB1_{-692\ T>C}$ and $ABCB1_{-129\ T>C}$ [38, 39]. Rund proved that mutations in the promotor region of *ABCB1* gene were associated with haematological malignancies, so screening for SNPs and other types of polymorphism in the promotor region of this gene is important for studying human cancers [40].

Haplotype may often provide more useful information than genotype about interindividual and interethnic differences [41]. Kroetz defined 32 haplotypes and their subtypes (64 distinct haplotypes obtained for 28 variant sites) [10]. Estimates in our laboratory of *ABCB1* haplotype frequencies showed that T_{1236}–T_{2677}–T_{3435} (24.0% vs 3.0%, OR=9.61) and C_{1236}–C_{2677}–C_{3435} (39.0% vs 19.0%, OR=2.77) of eight haplotypes found in colorectal cancer patients were significantly higher than in a control group. Moreover, haplotype analysis showed that haplotypes C_{1236}–T_{2677}–T_{3435} and T_{1236}–G_{2677}–C_{3435} were found higher in healthy population compared to colorectal patients (26.0% vs 8.0%, OR=0.25 and 23.0% vs 3%, OR=0.11). In addition, statistically significant differences were found in haplotype distributions between investigated populations. Potocnik identified relationship between haplotypes of *ABCB1* gene ($1236_{C>T}$–rs2235035–$2677_{G>T/A}$–$3435_{C>T}$) and the presence of high microsatellite instability. It also suggests that changes of *ABCB1* gene function may contribute to the initiation and development of MSI-H tumours. Most frequently noticed haplotype T_{1236}–$C_{rs2235035}$–T_{2677}–T_{3435} is associated with significant risk of MSI-H occurrence (p=0.004, OR=0,48) [42]. A transcription factor complex TCF4/β catenin responsive element was identified in the *ABCB1* promoter region pointing to a direct link between the *ABCB1* gene and the Wnt signalling pathway, the most important pathway altered in colorectal cancers [43]. Therefore, somatic mutations and functional polymorphisms within the *ABCB1* gene in colorectal tumours and corresponding normal mucosa were previously characterised for microsatellite instability and lymphoid infiltration [44].

Several haplotypes of *ABCB1* gene have clinical relevance, e.g. the C_{1236}–C_{2677}–C_{3435} haplotype has been associated with pharmacoresistance [45]. This haplotype correlated with treatment failure indicated that the degree of pharmacoresistance may be modulated by *ABCB1* gene, for example drug resistance in temporal lobe epilepsy [46]. On the other hand, the T_{1236}–T_{2677}–T_{3435} haplotype was shown

to be associated with high risk of developing refractory Crohn's disease [44].

These results suggested that *ABCB1* haplotypes based on three or more sites may be useful for colorectal cancer patient characterization. In the future, large-scale studies are warranted to appropriately investigate this possibility.

Tumour cells could become resistant to anticancer drugs by a variety of mechanisms. Several factors affect *ABCB1* gene expression or the MDR (multidrug resistance) phenotype such as: inhibitors of P-gp, calmodulin inhibitors, X-ray, cytotoxic agents, proapoptotic agents and others. Allelic variants in *ABCB1* gene have attracted attention as a possible explanation of inter-individual differences in drug response. Also, in this study, the influence of *ABCB1* genotypes on outcome of colorectal cancer therapy was estimated. Survival probability of patients with wild-type C_{3435} allele was higher than among patients without this allele present. Because 5-FU is probably not the substrate for P-gp, some indirect mechanisms than transports via membrane could have impact on efficiency of chemotherapy in colorectal cancer. Moreover, silent mutation in a haplotype, mammalian membrane transport protein alters the substrate specificity [47]. P-gp was implicated in the system regulating cell differentiation, proliferation and survival [4]. On the other hand, P-gp may play a role in regulating some caspase-dependent apoptotic pathways, a function completely independent of its drug transport properties [48]. The combined actions of 5-FU metabolites are associated with inhibition of DNA biosynthesis, altered DNA stability, production of DNA damages and interference with DNA repair. The genotoxic stress resulting from 5-FU administration may activate apoptosis in susceptible cells via activated P53 or inactivated cyclin-dependent kinases [49]. On the other hand, protection function of P-gp against carcinogens may reduce the degree of accumulation of mutations in genes (e.g. *P53* gene) of cell, thus increasing sensitivity of cancer cell to action of anticancer agents.

The incidence of colorectal cancer is increasing in Poland, and the results of treatment are some of the worst among other western European countries [50]. The reason of that is late diagnosis, which in consequence hinders treatment. A few coexisting SNPs may influence the therapeutic outcome and the risk of cancer disease development. Larger number of samples must be examined to reach more reliable conclusions. Therefore, further studies are needed to determine whether environmental factors, unexamined *ABCB1* genotypes and genes other than *ABCB1* were more predominant for the development of colorectal cancer.

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