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ORIGINAL ARTICLE

ABCB1/MDR1 gene polymorphisms as a prognostic factor in colorectal cancer

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Abstract
Objective To analyse the single-nucleotide polymorphisms (SNPs): *ABCB1*$_{1236C>T}$, *ABCB1*$_{2677G>T/A}$, *ABCB1*$_{3435C>T}$ and haplotypes in the *ABCB1/MDR1* gene, which could contribute to genetic risk of colorectal cancer (CRC). Disease association between the *ABCB1/MDR1* genotype, allele, haplotype frequencies and histological features, such as TNM classification, localization of primary carcinoma, grade of malignancy, histological type of tumour, lymphoid infiltration and vessel invasion were estimated. In this study, the potential role of SNPs of the *ABCB1/MDR1* gene as a prognostic marker for CRC was analysed.

Materials and methods Tumour specimens of 95 patients with CRC were studied. Using automated sequencing or PCR-RFLP method, DNA for three common SNPs of *ABCB1/MDR1* was extracted and analysed. The results of genotyping and haplotype analysis with histopathological features, grading and clinical staging of neoplasms were correlated.

Results A statistically significant higher frequency of T_{1236} allele in T1/T2 (89.7%), M0 groups (81.6%) and I/II clinical staging (82.7%) in comparison with T3/T4 (68.2%), M1 groups (47.4%) and III/IV clinical staging (65.1%) was detected. Furthermore, multivariate analysis according to Cox's proportional hazard model indicated that the T_{1236} allele is a good, independent prognostic factor and the presence of this allele decreases the risk of death in comparison with a group without this allele (HR=0.26; p=0.0424). In addition, a statistically significant higher frequency of C_{3435} allele and significant differences in the C_{3435} allele distribution in N1/N2 group (91.7% and 62.5%, respectively) than N0 group (71.2% and 44.9%, respectively) was found. Each of the eight possible haplotypes was noted in M0 or I/II group and only seven in M1 or III/IV group. Haplotype T_{1236}-G_{2677}-C_{3435} only in less advanced CRC subjects (9.6% in I/II and 9.2% in M0 group) was detected. In addition, significant differences in haplotype distributions between M0 or I/II and M1 or III/IV group were found (p=0.01 and p=0.05, respectively).

Conclusions These results suggest association between T_{1236} allele and T_{1236}-G_{2677}-C_{3435} haplotype and less advanced CRC, so these genetic markers may play a role as potentially good prognostic factors. Differences in haplotype distributions and degree of clinical staging may suggest that some other potential SNPs, especially in regulatory region of *ABCB1/MDR1* gene, may influence P-glycoprotein function and CRC progression.

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Introduction

Colorectal cancer (CRC) is one of the most frequent neoplasms and is the main reason for the high mortality ratio among different type cancer sufferers in industrial countries [1]. Every year, in the European Union, there are approximately 220,000 new cases of CRC diagnosed. The number of deaths each year approaches 112,000 [2].

It is well documented that single nucleotide polymorphism (SNP) of some genes may be related to an increased or decreased cancer risk. Among them, the *ABCB1/MDR1* gene seems to play an important role in tumour progression [3]. This gene belongs to ATP-binding cassette family and encodes P-glycoprotein (P-gp), which is an efflux pump protein of 170-kDa [3]. Overexpression of P-gp in tumour cells leads to multidrug resistance against antineoplastic agents [4–7]. 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 [8, 9]. Authors have implicated P-gp in the system regulating cell differentiation, proliferation [6], apoptosis [10] and immune response [11].

The role of P-gp in carcinogenesis was described in animal models of colon [12], breast [13] and liver [14] cancers. Overexpression of P-gp was connected with apoptosis inhibition and increasing possibility of neoplasm transformation in an mdr1a mouse model [12]. Moreover, high expression of P-gp at the atypical surface of differentiated tubular structures was identified in previously non-treated CRC [15], and its high expression at the leading edge of CRC has been associated with tumour progression [16]. A transcription factor complex TCF4/ß-catenin responsive element was identified recently in the *ABCB1/MDR1* promoter region, pointing to a direct link between the *ABCB1/MDR1* gene and the Wnt signalling pathway, the most important pathway that is altered in CRC [17]. In vitro study indicated that the *ABCB1/MDR1* gene expression is activated in cells with the *P53* gene mutation [18]. The promoter of the human *ABCB1/MDR1* gene was shown to be a target for the *P53* tumour suppressor gene products. Mutant *P53* specifically stimulated the *ABCB1/MDR1* promoter and wild-type *P53* exerted specific repression [18]. Prevalence of *P53* mutations in CRC is around 50% [19].

Intensive studies into the implications of genetically determined differences in P-gp function for drug disposition, therapeutic outcome, risk for development of certain diseases and tumour progression are ongoing.

There exist multiple mutations in the *ABCB1/MDR1* gene. Analysis of all 28 exons of the *ABCB1/MDR1* gene demonstrated 48 single-nucleotide polymorphisms (SNPs), including promoter and the intron–exon region [20]. The most frequent SNP $ABCB1_{2677G>T/A}$ in exon 21 (RefSNP ID: rs2032582), leads 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 [21]. The third common polymorphism of *ABCB1/MDR1* gene is a silent mutation in exon 12 $ABCB1_{1236C>T}$ (RefSNP ID: rs1128503). The relationships between the SNPs of *ABCB1/MDR1* gene are not clear. Perhaps these three polymorphisms are closely related to linkage disequilibrium (LD), but an unknown genetic variant is located on the same LD block or haplotype [20, 22]. Several studies show that polymorphisms of *ABCB1/MDR1* gene can influence susceptibility to cancer development. It was suggested that $ABCB1_{3435C>T}$ SNP is connected with susceptibility to renal epithelial tumours [23] and acute lymphoblastic leukaemia [24]. These SNPs were also reported in patients diagnosed with CRC [25–29] and may contribute to the susceptibility and progression of CRC [30].

The aim of this study is to determine the significance of three SNPs of *ABCB1/MDR1*, namely $ABCB1_{1236C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$, in the progression of CRC. We analysed the potential role of SNPs or haplotypes of the *ABCB1/MDR1* gene as a prognostic marker for CRC.

Materials and methods

Tissue samples from 95 colorectal carcinoma patients from a region in Central Poland (48 women and 47 men, ratio 1:0.98, median age is 6) operated on in the Oncological Center of Lodz, Poland were obtained. CRC was diagnosed by histopathological examination using the established clinical criteria (TNM classification by Jass with latest revision Cancer Staging Manual by AJCC, 1997 [31]) at the Department of Pathology, Medical University of Lodz, Poland. Primary colorectal carcinoma and normal colorectal mucosa (tissue taken from a site several centimetres away from the tumour) in the study (estimated resection status of all patients: R0) were used. Furthermore, 40 patients (42.1%) qualified for combination adjuvant chemotherapy 5-fluorouracil and leucovorin (5-FU/LV), and 15 patients (15.8%) were subjects to preoperative radiotherapy. Samples were frozen in liquid nitrogen immediately after surgical resection and stored in the freezer at−80°C until processed. All subjects were of Slavic origin. Detailed information for the colorectal cancer group is summarised in Table 1. All experiments were carried out with local ethical committee approval (No KE/813/07) and patient's informed consent.

Table 1 Detailed information on the colorectal cancer group

		N	Median age	±SD[a]
Depth of tumour invasion	T1	3	54	15.1
	T2	26	67	9.1
	T3	56	59.5	11.5
	T4	10	61	10.5
Lymph node involvement	N0	59	63	11.7
	N1	16	61	10.4
	N2	20	63	10.4
Distant metastases	M0	76	63	10.8
	M1	19	63	12.5
pTNM	I	26	67	9.9
	II	26	56.5	12.0
	III	24	62	8.8
	IV	19	63	12.5
Grade of malignancy	G1	10	56	14.2
	G2	57	65	9.6
	G3	28	57.5	11.9
Tumour localization	Rectum	36	64	10.2
	Different location	59	66	13.0
Lymphoid infiltration	(+)	43	63.5	10.6
	(−)	52	59	11.5
Vessel invasion	Not involved	36	67	9.1
	Involved	59	58	11.7
Histological type	adenocarcinoma	64	59.5	11.4
	mucinous adenocarcinoma and medullary adenocarcinoma	31	65	10.4
Radiotherapy	(+)	15	59	9.9
	(−)	68	63.5	11.2
	Data missing	12	61	11.9
Adjuvant chemotherapy (5-FU/LV)	(+)	40	57.5	9.0
	(−)	43	69	11.2
	Data missing	12	61	11.9
Family predispositions	(+)	11	64.5	8.5
	(−)	21	54	11.7
	Data missing	64	63	11.2

Resection status of all patients estimated as R0

(5-FU/LV) 5-fluorouracil and leucovorin

[a] Standard Deviation

DNA isolation

DNA was isolated according to "Genomic DNA Prep Plus" protocol (A&A Biotechnology, Gdynia, Poland) from the frozen tissue slides of colon cancer. 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) was conducted according to the "AccuTaq™ LA DNA Polymerase Kit" protocol (Sigma–Aldrich, Germany). The reaction mixture for PCR amplification consisted of 50 ng of DNA template, 0.5 μM of each primer, 10X 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). Primer design based on published sequences for genotyping procedure of $ABCB1_{1236C>T}$, $ABCB1_{2677G>T/A}$ and $ABCB1_{3435C>T}$ polymorphisms using genomic DNA [21, 32, 33].

Restriction fragment length polymorphism

After checking PCR product in 2% agarose gel, amplified DNA was cut for $ABCB1_{3435C>T}$ mutation by restriction enzyme MboI (Fermentas, Vilnius, Lithuania) over 16 h at 37°C according to Panczyk [32]. DNA fragments generated after digestion were separated on 2% agarose gel and visualised with ethidium bromide. The 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.

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, WI, USA).

The reaction mixture for sequencing-PCR amplification consisted of amplified DNA, 0.2 μM of primer, 3.5X Sequencing Buffer, 5 U of SequiTherm EXCEL™ II DNA Polymerase, 0.2 mM each dNTP/dideoxyribonucleotide triphosphate 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 primer sequences for automated sequencing genotyping procedure of $ABCB1_{1236C>T}$ and $ABCB1_{2677G>T/A}$ were planned by using software Primer3: WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). SeqPCR products after denaturation were separated in polyacrylamide gels. Sequencing was performed with the use of automated sequencer LI-COR® 4000.

Statistical analysis

Statistical significance of the observed genotype frequencies compared to genotype frequencies expected according to the Hardy–Weinberg rule was evaluated. Data were analysed using STATISTICA version 8.0 (data analysis software system, StatSoft Inc.). The differences in allele or genotype frequencies according to TNM classification, grade of malignancy, localization of primary carcinoma, lymphoid infiltration, vessel invasion, gender and family predispositions to tumours were calculated using Pearson's chi-square test or Yates' chi-square test. Survival probability of CRC patients according to genotypes and histological types were estimated on the basis of the Kaplan–Meier method and compared between groups using the F Cox test. The multivariate analysis was performed according to the Cox's proportional hazards model. The odds ratio (OR), hazard ratio (HR) and 95% confidence intervals (CI) were calculated on the basis of logistic regression and the Wald test. Haplotypes were statistically inferred using PHASE v. 2.1 software. PHASE implements a Bayesian statistical method for reconstructing haplotypes from population genotype data [34]. For all analyses, *p*-values at the level of 0.05 were considered statistically significant.

Results

The observed genotype frequency distribution did not show any significant deviation from Hardy–Weinberg equilibrium (data not shown).

To assess the clinical utility of *ABCB1/MDR1* genotyping, the results were compared with several clinicopathological parameters such as depth of tumour invasion (T), lymph node metastases (N) distant metastases (M) and clinical staging (pTNM).

The vast majority of cases investigated in this study (69.5%) belonged to the group with deep-wall penetration (T3/T4). Only 30.5% belonged to the T1 and T2 groups. Higher frequency of T_{1236} allele (genotype CT_{1236} or TT_{1236} vs. CC_{1236}) was observed in the less advanced T1/T2 groups than in the T3/T4 groups (89.7% and 68.2%, respectively; p=0.0498, OR=4.04 (95% CI=1.14–14.32), measure of correlation: Fi–Yule coefficient=0.228). There were no statistically significant differences in haplotype distribution between the T1/T2 and T3/T4 groups. Statistical dependencies are presented in Table 2.

Genotyping of *ABCB1/MDR1* gene in cases with and without lymph node metastases was also evaluated. In patients without lymph node metastases (N0 group) the $ABCB1_{3435C>T}$ wild-type genotype (CC_{3435}) was observed in 18.6% of patients, whereas 52.6% were heterozygous (CT_{3435}) and 28.8% were homozygous for the mutation (TT_{3435}). In patients with lymph node metastases (N1/N2 groups) the frequencies of $ABCB1_{3435C>T}$ genotypes were different: 33.3% (CC_{3435}), 58.4% (CT_{3435}) and 8.3% (TT_{3435}) (p=0.037). Moreover, a higher frequency of C_{3435} allele (genotype CC_{3435} or CT_{3435} vs. TT_{3435}) was found in the N1/N2 groups than the N0 group (91.7% and 71.2%, respectively; p=0.0344, OR=4.45 (95% CI=0.82–24.29), measure of correlation: Fi–Yule coefficient=0.244). In addition, significant differences in C_{3435} allele distribution were found (62.5% and 44.9%, respectively; p=0.0186, OR=2.04 (95% CI=1.07–3.90), measure of correlation: Fi–Yule coefficient=0.171). There were no statistically significant differences in haplotype distribution between N0 and N1/N2 groups. Statistical dependencies are summarised in Table 2.

Genotypes of *ABCB1/MDR1* gene were also analysed in cases with and without distant metastases. Significant differences in $ABCB1_{1236}$ genotypes distribution were found (p=0.0057). CC_{1236} was detected in 18.4% of subjects without distant metastases (M0 group) and in 52.6% of subjects with distant metastases (M1 group) (OR=0.20 (95% CI=0.07–0.58)). The frequencies of CT_{1236} and TT_{1236} were higher in the M0 group (71.1% and 10.5%, respectively) in comparison with the M1 group (47.4% and 0.00%, respectively) (OR=2.73 (95% CI=0.96–7.78)). Moreover, a higher frequency of T_{1236} allele (genotype TT_{1236} or CT_{1236} vs. CC_{1236}) was found in the M0 than the M1 group (81.6% and 18.4%, respectively; p=0.0021, OR=0.20 (95% CI=0.07–0.58), measure of correlation: Fi–Yule coefficient=0.315). In addition, significant differences in T_{1236} allele distribution were found (46.1% and 23.7%, respectively; p=0.0123, OR=0.36 (95% CI=0.20–0.67), measure of correlation: Fi–Yule coefficient=0.182).

Haplotype analysis showed that each of the eight possible haplotypes was noted in M0 group and only seven in M1 group. Haplotype T_{1236}-G_{2677}-C_{3435} presented only in the M0 group (frequency 9.2%). However, the frequencies of C_{1236}-G_{2677}-C_{3435} and T_{1236}-T_{2677}-T_{3435} haplotypes were higher in the M0 group in comparison with the M1 group (36.2% vs. 15.8%, OR=3.02 (95% CI=0.93–9.83) and 22.4% vs. 5.3%, OR=5.17 (95% CI=0.70–38.20, respectively)). In addition, the frequencies of C_{1236}-G_{2677}-T_{3435} and C_{1236}-T_{2677}-C_{3435} haplotypes were higher in M1 group in comparison with M0 group (36.8% vs. 6.6%, OR=0.12 (95% CI=0.05–0.32) and 18.4% vs 0.7%, OR=0.03 (95% CI=0.00–0.27), respectively). Furthermore, three of four possible haplotypes (T_{1236}-T_{2677}-T_{3435}, T_{1236}-G_{2677}-T_{3435} and T_{1236}-G_{2677}-C_{3435}) containing the T_{1236} allele were more often registered in the M0 group than in the M1 group (total frequency of three haplotypes is 0.382 vs. 0.079). In the case of haplotypes with the C_{1236} allele, two of them

Table 2 *ABCB1/MDR1* genotype/allele/haplotype frequencies analysis in investigated populations divided according to the pTNM classification

Genotypes Alleles Haplotypes	T1/T2 freq	T3/T4 freq	*p*	N0 freq	N1/N2 freq	*p*	M0 freq	M1 freq	*p*	I/II freq	III/IV freq	*p*
CC_{1236}	0.103	0.318	0.085[a]	0.237	0.278	0.701[a]	0.184	0.526	0.005[a]	0.173	0.349	0.099[a]
CT_{1236}	0.794	0.606		0.661	0.667		0.711	0.474		0.712	0.604	
TT_{1236}	0.103	0.076		0.102	0.056		0.105	0.000		0.115	0.047	
CC_{1236}/CT_{1236}	0.897	0.924	0.963[b]	0.898	0.944	0.686[b]	0.895	1.000	0.310[b]	0.885	0.953	0.405[b]
TT_{1236}	0.103	0.076		0.102	0.056		0.105	0.000		0.115	0.047	
CT_{1236}/TT_{1236}	0.897	0.682	0.049[b]	0.723	0.722	0.660[a]	0.816	0.474	0.002[a]	0.827	0.651	0.049[a]
CC_{1236}	0.103	0.318		0.237	0.278		0.184	0.526		0.173	0.349	
C_{1236}	0.500	0.621	0.119[a]	0.568	0.611	0.557[a]	0.539	0.763	0.012[a]	0.529	0.651	0.089[a]
T_{1236}	0.500	0.379		0.432	0.389		0.461	0.237		0.471	0.349	
CC_{3435}	0.172	0.273	0.431[a]	0.186	0.333	0.037[a]	0.237	0.263	0.969[a]	0.192	0.302	0.215[a]
CT_{3435}	0.552	0.545		0.526	0.584		0.553	0.526		0.538	0.558	
TT_{3435}	0.276	0.182		0.288	0.083		0.210	0.211		0.270	0.140	
CC_{3435}/CT_{3435}	0.724	0.818	0.301[a]	0.712	0.917	0.034[b]	0.790	0.789	0.753[b]	0.730	0.860	0.123[a]
TT_{3435}	0.276	0.182		0.288	0.083		0.210	0.211		0.270	0.140	
CT_{3435}/TT_{3435}	0.828	0.727	0.429[b]	0.814	0.667	0.105[a]	0.763	0.737	0.952[b]	0.808	0.698	0.213[a]
CC_{3435}	0.172	0.273		0.186	0.333		0.237	0.263		0.192	0.302	
C_{3435}	0.448	0.545	0.217[a]	0.449	0.625	0.018[a]	0.513	0.526	0.885[a]	0.462	0.581	0.099[a*]
T_{3435}	0.552	0.455		0.551	0.375		0.487	0.474		0.538	0.419	
C_{1236}-T_{2677}-T_{3435}	0.086	0.076	0.22[c]	0.085	0.083	0.13[c]	0.105	0.053	0.01[c]	0.125	0.058	0.05[c]
C_{1236}-T_{2677}-C_{3435}	0.000	0.083		0.051	0.097		0.007	0.184		0.010	0.081	
C_{1236}-G_{2677}-T_{3435}	0.069	0.152		0.178	0.097		0.066	0.368		0.077	0.163	
C_{1236}-G_{2677}-C_{3435}	0.345	0.311		0.254	0.333		0.362	0.158		0.317	0.349	
T_{1236}-T_{2677}-T_{3435}	0.293	0.212		0.254	0.194		0.224	0.053		0.240	0.198	
T_{1236}-T_{2677}-C_{3435}	0.052	0.045		0.051	0.014		0.079	0.158		0.087	0.035	
T_{1236}-G_{2677}-T_{3435}	0.103	0.015		0.034	0.000		0.092	0.000		0.096	0.000	
T_{1236}-G_{2677}-C_{3435}	0.052	0.106		0.093	0.181		0.066	0.026		0.048	0.116	

[a] Pearson's chi-square test

[b] Yates' chi-square test

[c] Permutation test

(C_{1236}-T_{2677}-C_{3435} and C_{1236}-G_{2677}-T_{3435}) were observed much more frequently in the M1 than the M0 group (total frequency of three haplotypes is 0.552 vs. 0.073). There were significant differences in haplotype distributions between M0 and M1 group (p=0.01). Statistical dependencies are presented in Table 2.

The genotyping results of *ABCB1/MDR1* gene we obtained were compared with clinical staging of neoplasms. Of the cases investigated in this study, 54.7% belonged to the group with less clinically advanced neoplasms (I/II degree), whereas, 45.3% belonged in the III/IV degree group according to TNM clinical staging classification. A higher frequency of T_{1236} allele (genotype TT_{1236} or CT_{1236} vs. CC_{1236}) was detected in I/II than III/IV group (82.7% and 65.1%, respectively; p=0.0497, OR=0.39 (95% CI=0.14–1.08), measure of correlation: Fi–Yule coefficient=0.201). Each of the eight possible haplotypes was noted in I/II group and only seven in III/IV group. Haplotype T_{1236}-G_{2677}-C_{3435} was presented only in I/II group (frequency 9.6%). In I and II stage three haplotypes with the T_{1236} alelle (T_{1236}-T_{2677}-T_{3435}, T_{1236}-G_{2677}-T_{3435} and T_{1236}-T_{2677}-C_{3435}) were observed more frequently than in III and IV stage (0.423 vs. 0.233). Haplotypes with the C_{1236} allele (C_{1236}-T_{2677}-C_{3435}, C_{1236}-G_{2677}-T_{3435} and C_{1236}-G_{2677}-C_{3435}) were observed more often in more advanced stages (0.593 vs. 0.404). There were significant differences in haplotype distributions between investigated groups (p=0.05). Statistical dependencies are presented in Table 2.

There was no statistically significant correlation between *ABCB1/MDR1* genotype/allele/haplotype frequencies and gender, family predispositions to tumours, grading, localization of primary carcinoma, lymphoid infiltration, or vessel invasion (Table 3).

The multivariate analysis with the Cox's proportional hazards model indicated that some of the clinicopathological features (approved prognostic factors and the presence of the studied alleles of the *ABCB1/MDR1* gene) have independent influence on overall survival time of the

Table 3 Multivariate analysis of clinicopathological features influence on overall survival of 95 patients with CRC disease (Cox's proportional hazard model)

	Number of deaths in %	HR[a]	CI (95%)[b]	Wald statistic	*p*-value
Gender					
Women	39.6	1.00	(−1.87)–(+4.96)	0.7912	0.3737
Men	36.2	1.55			
Family predispositions					
Negative	38.1	1.00	(−1.99)–(+3.99)	0.4341	0.5060
Positive	18.2	1.00			
Tumour localization					
Different location	33.9	1.00	(−1.08)–(+4.77)	1.5381	0.2149
Rectum	44.4	1.84			
Depth of tumour invasion					
T1 or T2	27.6	1.00	(+1.89)–(+7.63)	10.5496	0.0012
T3 or T4	42.4	4.76			
Lymph node involvement					
N0	28.8	1.00	(−4.17)–(+7.50)	0.3122	0.5763
N1 or N2	52.8	1.66			
Distant metastases					
M0	26.3	1.00	(+6.87)–(+23.93)	12.5597	0.0004
M1	84.2	15.40			
pTNM classification					
I or II	25.0	1.00	(−0.04)–(+0.31)	2.3573	0.1247
III or IV	52.5	0.14			
Grade of malignancy					
G1 or G2	38.8	1.00	(−0.78)–(+2.22)	0.8893	0.3457
G3	35.7	0.72			
Histological type					
adenocarcinoma	34.4	1.00	(+0.52)–(+3.15)	7.5484	0.0060
mucinous adenocarcinoma and medullary adenocarcinoma	45.2	1.83			
Vessel invasion					
Not involved	30.6	1.00	(−2.65)–(+4.22)	0.2009	0.6540
Involved	42.4	0.79			
Lymphoid infiltration					
(−)	44.2	1.00	(−2.83)–(+4.39)	0.1786	0.6726
(+)	30.2	0.78			
Allele C_{1236}					
TT_{1236}	10.5	1.00	(−4.10)–(+5.62)	0.0936	0.7597
CC_{1236} or CT_{1236}	44.7	0.76			
Allele T_{1236}					
CC_{1236}	22.0	1.00	(0.01)–(+0.52)	4.1204	0.0424
CT_{1236} or TT_{1236}	50.0	0.26			
Allele G_{2677}					
TT_{2677}	38.1	1.00	(−1.13)–(+5.44)	1.6514	0.1988
GG_{2677} or GT_{2677}	35.1	2.15			
Allele T_{2677}					
GG_{2677}	37.1	1.00	(−1.15)–(+5.25)	1.5757	0.2093
GT_{2677} or TT_{2677}	36.7	2.05			
Allele C_{3435}					
TT_{3435}	45.0	1.00	(−0.83)–(+2.07)	0.7072	0.4004
CC_{3435} or CT_{3435}	36.0	0.62			
Allele T_{3435}					
CC_{3435}	32.0	1.00	(−18.40)–(+20.30)	0.0093	0.9232

Table 3 (continued)

	Number of deaths in %	HR[a]	CI (95%)[b]	Wald statistic	*p*-value
CT_{3435} or TT_{3435}	40.0	0.95			
Adjuvant combined chemotherapy (5-FU/LV)					
No	39.5	1.00	(−3.02)–(+5.67)	0.3570	0.5502
Yes	45.0	1.32			
Radiotherapy					
No	42.6	1.00	(−1.57)–(+3.06)	0.3977	0.5283
Yes	40.0	0.74			

Cox's proportional hazard model: chi-square test (χ^2)=40.4963; degrees of freedom (df)=19; *p*-value=0.0028

(5-FU/LV) 5-fluorouracil and leucovorin

[a] hazard ratio

[b] 95% confidence interval for hazard ratio

patients with colorectal cancer (χ^2=40.4963; df=19; p=0.0028).

As prognostic factors, the following maintained their independence: depth of tumour invasion (HR=4.76; p=0.0012), distant metastases (HR=15.40; p=0.0004), histological type of tumour (HR=1.83; p=0.0060) and the presence of the T_{1236} allele (HR=0.26; p=0.0424). Furthermore, it was demonstrated that, with regard to the objective, the T_{1236} allele is the important one and seems to be a good independent prognostic factor because patients possessing it (TT_{1236} or CT_{1236} genotype) can be characterised by lower risk of death in comparison to patients without this allele (CC_{1236} gentotype) (HR < 1). The results of our multivariate analysis of cancer factors influence on overall survival of the 95 patients with CRC disease are presented in Table 3. Kaplan–Meyer curves presenting survival probability with time depending on T_{1236} allele (CC_{1236} vs. TT_{1236} or CT_{1236} genotype) and histological type (adenocarcinoma vs. mucinous adenocarcinoma and medullary adenocarcinoma) based on statistical analysis with the use of F Cox test are presented in Figs. 1 and 2.

Discussion

Relations between the presence of different SNPs and the risk of CRC development is being investigated in over 35 different genes [35]. Among them, *ABCB1*/*MDR1* is studied extensively.

Gaikovitch indicated a higher risk (1.65-fold) of colorectal cancer development among CC_{3435} genotype carriers than among T_{3435} allele carriers. Similarly, the presence of T_{2677} allele decreases the risk of colorectal cancer development in relation to G_{2677} allele [36]. A 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 cell death pathways suppression. Robinson indicated that apoptosis of cells transfected with the *ABCB1/MDR1* gene is reversible through P-gp verapamil inhibition [37]. Other studies also indicate the antiapoptotic function of P-gp visible in its cell protection against cytotoxic compounds activating a caspase pathway and also against pro-apoptotic influence of TNF [38]. It was indicated that P-gp can protect cells against apoptosis by its influence on the sphingomyelin–ceramide pathway [39, 40].

In this study, three polymorphisms were analysed, one leading to amino acid exchange ($ABCB1_{2677G>T/A}$) and two silent ones, which have no influence on the amino acid sequence of P-gp but, surprisingly, may influence P-gp

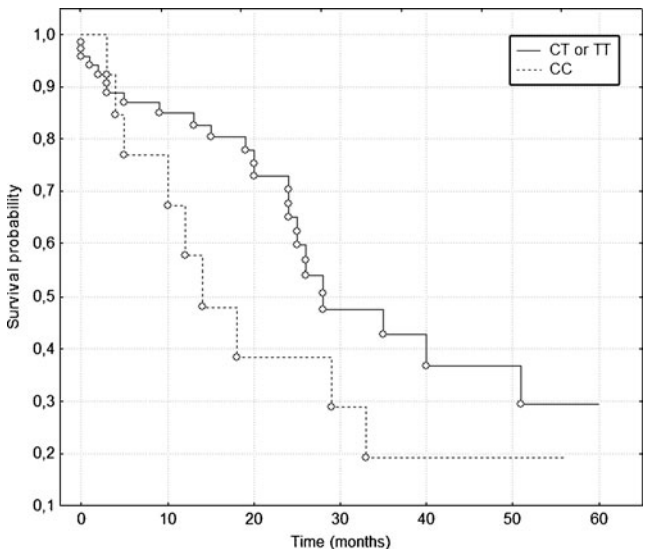

Fig. 1 Adjusted survival probability of colorectal cancer patients according to T_{1236} allele (CC_{1236} vs. TT_{1236} or CT_{1236} genotype) F Cox test: T1=33.16845, T2=9.831555, F(60.24)=1.349469, p=0.05

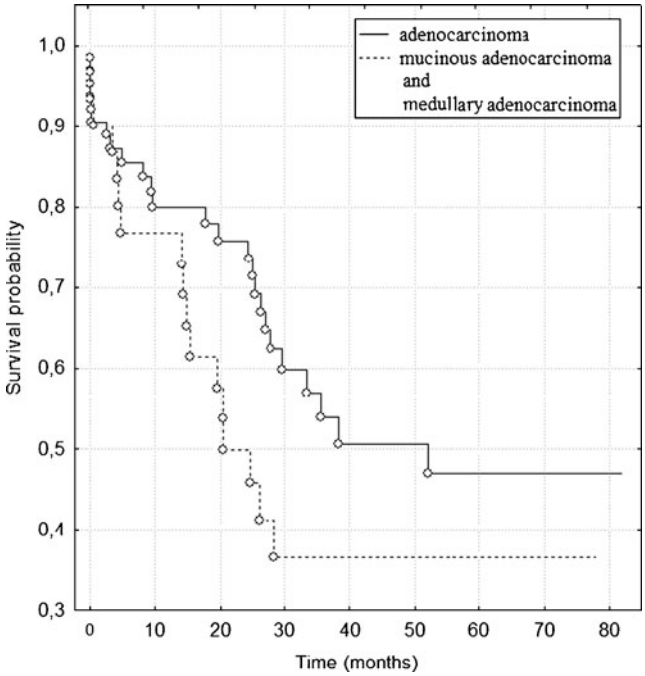

Fig. 2 Adjusted survival probability of colorectal cancer patients according to histological type (adenocarcinoma vs. mucinous adenocarcinoma and medullary adenocarcinoma) F Cox test: T1=30.52871, T2=12.47129, F(50. 34)=1.664585, p=0.05

function(*ABCB1*$_{1236C>T}$ and *ABCB1*$_{3435C>T}$). There are several hypotheses regarding the influence of a silent polymorphism on phenotypically revealed features and predispositions. One hypothesis assumes the possibility of influence of a synonymous triplet on the efficiency of translation [41]. Other authors suggest that the allele-specific differences in the RNA secondary structure may influence the splicing process or the translation process control [42]. It is also possible that some of SNPs increase the mRNA stability and in consequence leads to increased protein expression [43] and/or a change of the substrate's affinity to the P-gp transporter [44]. The changed function of P-gp could be a risk and progression factor of CRC due to it facilitating intracellular penetration of DNA damaging factor of both exo- and endogenous origin.

The impact of *ABCB1*$_{1236C>T}$ and *ABCB1*$_{3435C>T}$ polymorphisms on the function ofP-gp can also be explained by the importance of LD. In our previous study it was proven that three investigated SNPs of the *ABCB1/MDR1* gene (*ABCB1*$_{1236C>T}$, *ABCB1*$_{2677G>T/A}$ and *ABCB1*$_{3435C>T}$) are located in one haploblock [32]. The presence of the mentioned haplotype structure within the *ABCB1/MDR1* gene was also described by other authors [22]. The haplotype may often provide more useful information than the genotype about interindividual and interethnic differences [45]. Kroetz defined 32 haplotypes and their subtypes in *ABCB1/MDR1* gene (64 distinct haplotypes obtained for 28 variant sites) [20]. In our study, haplotype T_{1236}-G_{2677}-C_{3435} was presented only in M0 or I/II groups. Furthermore, three of four possible haplotypes (T_{1236}-T_{2677}-T_{3435}, T_{1236}-G_{2677}-T_{3435} and T_{1236}-G_{2677}-C_{3435}) containing the T_{1236} allele were more often registered in the M0 than in the M1 group. In instances of haplotypes with the C_{1236} allele, two of them (C_{1236}-T_{2677}-C_{3435} and C_{1236}-G_{2677}-T_{3435}) were observed much more frequently in the M1 than M0 group. A similar trend was observed when comparing groups regarding haplotype frequency in relation to clinical stage of the disease. In stages I and II, three haplotypes with the T_{1236} allele (T_{1236}-T_{2677}-T_{3435}, T_{1236}-G_{2677}-T_{3435} and T_{1236}-T_{2677}-C_{3435}) were observed more frequently than in stages III and IV. Haplotypes with the C_{1236} allele (C_{1236}-T_{2677}-C_{3435}, C_{1236}-G_{2677}-T_{3435} and C_{1236}-G_{2677}-C_{3435}) were observed more often in more advanced stages. There were significant differences in haplotype distributions between groups I/II and III/IV. Since the presence of the T_{1236} allele is related to lower disease development risk and lower disease advancement, a protective effect of this allele can be deducted, especially in the context of the possible influence of this SNP on P-gp function related to the cell cycle control. To the best of our knowledge, this is the first report of frequent polymorphism *ABCB1*$_{1236C>T}$ affecting the genetic factor for progression of CRC.

ABCB1/MDR1 is highly expressed in lymphocytes, including CD8+, CD4+ T cells, and DC, and it is able to transport several cytokines and chemokines. Lymphocytes play an important role in the response of the immunological system to the presence of tumour cells through releasing cytokines with possible P-gp mediation (secretion of IL-2, IL-4 and interferon-γ) [46]. In this paper, a higher frequency of C_{3435} allele in N1/N2 groups in comparison to N0 group is reported. Moreover, differences in C_{3435} allele distribution and genotypes distribution between cases with and without lymph node metastases were noticed.

This study shows that patients possessing the T_{1236} allele (TT_{1236} or CT_{1236} genotype) have a higher survival chance in comparison to patients without this allele. The results confirm the previously described possible protective function of this allele. However, there is no data regarding the possible direct influence of the *ABCB1*$_{1236C>T}$ polymorphism on P-gp function, especially on this protein expression level. Kimchi-Sarfaty indicated that there is dependency between the *ABCB1*$_{3435C>T}$ silent polymorphism and posttranslational protein folding and its acquiring proper transport activities [44]. It is also necessary to determine the influence of the P-gp protein activity on CRC progression. The level of P-gp expression is known to correlate with worse prognosis in the course of leukaemia (AML) [47, 48]. Some researches indicate that P-gp expression level influences disease-free survival [30], however, no relationship was seen between P-gp expression, genotypes of *ABCB1*$_{2677G>T/A}$ and *ABCB1*$_{3435C>T}$ and long-term prognosis of CRC [25].

On the basis of this and previously published data, it could be suggested that other potential SNPs, especially in the regulatory region of the *ABCB1/MDR1* gene, may also influence P-gp expression and function, e.g. *ABCB1*$_{-2410T>C}$, *ABCB1*$_{-1910T>C}$, *ABCB1*$_{-692T>C}$ and *ABCB1*$_{-129T>C}$ [27, 47, 49]. Mutations in the promoter region of *ABCB1/MDR1* gene were shown to be associated with haematological malignancies [50]. Potocnik identified the promoter polymorphism (+8 T>C) and located it in intron 1 (IVS1-81 delG) of the *ABCB1/MDR1* gene, which was related to low expression of P-gp and the presence of the lymphoid infiltration [29].

In conclusion, correlations between *ABCB1*$_{1236C>T}$ polymorphism and haplotypes with allele T_{1236} and CRC progression were identified. Differences between haplotype distributions at different clinical stages of the disease suggest that other potential SNPs, especially in the regulatory region of the *ABCB1/MDR1* gene, may influence progression of CRC via functional changes in P-gp. This research also supports the role of P-gp in the initiation and progression of CRC development, thus reinforcing the idea of multiple physiological functions for P-gp.

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