

ABCB1 variants C3435T and T129C are not associated with colorectal cancer risk

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Abstract

Background: Colorectal cancer (CRC) is one of the most prevalent cancers in Saudi Arabia that is highly characterized with poor survival rate and advanced metastasis. Many studies contribute this poor outcome to the expression of ABC transporters on the surface of cancer cells.

Objectives: In this study, two *ABCB1* variants, C3435T and T129C, were examined to evaluate their contribution to CRC risk.

Methods: 125 subjects (62 CRC patients and 63 healthy controls) were involved. The DNA was isolated and analyzed with PCR-RFLP to determine the different genotypes. The Hardy-Weinberg equilibrium was performed to determine genotype distribution and allele frequencies. Fisher's exact test (two-tailed) was used to compare allele frequencies between patients and control subjects.

Results: The study showed that for SNP C3435T, the population of both CRC patients and controls were out of Hardy-Weinberg equilibrium. Genotype distribution for CRC patients was (Goodness of fit $\chi^2 = 20$, $df = 1$, $P \leq 0.05$), whereas, for the controls the genotype distribution was (Goodness of fit $\chi^2 = 21$, $df = 1$, $P \leq 0.05$). For SNP T129C, all subjects showed normal (TT) genotype.

Conclusion: There was no significant association between *ABCB1* 3435C>T and 129T>C polymorphisms with CRC risk.

Keywords: Colorectal cancer, *ABCB1* gene, SNP C3435T, SNP T129C, PCR-RFLP, Saudi Arabia.

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Introduction

In the Kingdom of Saudi Arabia (KSA), colorectal cancer (CRC) represents the second most common cancer type among Saudi and Non-Saudi patients. It ranks the first and the third most common cancer types among males and females, respectively^{1,2}. Surgery remains the main treatment option for CRC along with chemotherapeutic drugs³. In chemotherapy, the tumor is treated with

multiple types of anticancer agents based on the stage of the tumor⁴. However, in some patients, the tumor, with time, may tend to resist certain types of drugs and this may lead to the failure of the treatment (multidrug resistance)⁴. Multidrug resistance (MDR) is one of the major causes of cancer chemotherapy failure⁵. In some cases of CRC patients, cancer cells survive, even under the presence of several chemotherapeutic agents, due to the alterations of specific proteins within the cancer cells resulted in MDR formation⁴. The phenotype of MDR in cancer cells plays a major role in decreasing the sensitivity to anticancer drugs through several mechanisms such as drug efflux activation, drug uptake reduction, cell growth and survival signaling activation, DNA repair promotion, apoptosis inhibition, and finally, the overexpression of several proteins such as ATP-binding cassette (ABC) transporters family⁶.

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ABC family is composed of many subclasses of membrane transport proteins that hydrolyze ATP to transport solutes across biological membranes⁷. Members of this family are present in both prokaryotes and eukaryotes and have more roles beyond the transportation and these include signal transduction, xenobiotic secretion, immunological functions, and drugs resistance⁸. Among them is the multidrug resistance member 1 (*MDR1*) gene (*ABCB1* gene), which is located on chromosome 7 and encodes a P-glycoprotein (Pgp) that is responsible for the active efflux of drugs from cells⁵. The Pgp is widely expressed on the plasma membrane, Golgi membrane, and in several normal human tissues including the liver, kidney, colon, adrenal glands, intestines, placenta, hematopoietic precursor cells, and endothelial cells of the blood-brain barrier⁶. As most ABC transporters, its structure contains six trans-membrane domains and an ATP binding/ utilization domain, separated by a flexible linker polypeptide⁴. The Pgp is frequently over-expressed in cancer cells. Overexpression of *ABCB1* is believed to be one of the major causes for chemotherapy resistance and is associated with reduced patient survival^{5,8}. In tumor cells, the Pgp overexpression was found to increase MDR against antineoplastic agents, and was connected with inhibited apoptosis and increased neoplasm transformation⁹. *ABCB1* gene is highly varied in different ethnicities and this has remarkable differences among them⁶. Most of the studies suggested that *ABCB1* is an important factor that contributes to several diseases development, progression and toxicity and also affects the response of the patients to the currently used drugs. Indeed, many studies that have been performed on patients with CRC, found that several *ABCB1* variants could increase the risk of colorectal cancer¹⁰⁻¹⁵. Although many advances have been made to diagnose and treat CRC in KSA, the latest cancer incidence report from the National Cancer Registry (NCR) in Saudi Arabia indicates the remarkable increase in the percentages of patients with CRC. Therefore, more studies are still needed to determine different genotype biomarkers that can be used to predict the degree of risk for CRC in Saudi patients. To the best of our knowledge, this is the first study performed on CRC patients in Saudi Arabia in order to determine the allele frequencies and genotype distributions of two SNPs (C3435T and T129C) in *ABCB1* gene, and correlate them with the risk of developing CRC.

Methods

Subjects

In this study, 125 male and female volunteers (45-90 years) were included. The study was approved by the Research Committee of the Unit of Biomedical Ethics at Faculty of Medicine, King Abdulaziz University (reference no. 261-15) and the Directorate of Health Affairs (reference no. A00221) in Jeddah, KSA. The CRC subjects (n=62) were comprised of 48 males and 14 females selected from patients who were routinely visiting the Oncology Centers at King Abdullah Medical City and King Abdulaziz University Hospital (KAUH) in Jeddah, KSA. The healthy subjects (n=63) included 43 males and 20 females who were interested in the study. Those healthy subjects were visiting the blood banks at King Fahad General Hospital in Jeddah, KSA. The practical work of this study was conducted at King Fahd Medical Research Centre (KFM-RC) King Abdulaziz University, Jeddah, KSA.

Determination of genomic DNA concentration and purity

From each subject, 5 ml blood sample was collected in EDTA tube, to be used for DNA extraction. DNA was extracted from whole blood by using QIAamp DNA Mini kit (Qiagen, catalog no: 51306) following the manufacturer's instructions. The DNA concentration was determined by reading the absorbance at a wavelength of 260 nm using the Thermo Scientific NanoDrop 2000 spectrophotometer. Purity was determined by calculating the ratio of absorbance at 260/280 nm and 260/230 nm. The ideal concentration was typically 4-12 µg of DNA in 200 µl elution buffer (30 ng/µl) with an A260/A280 ratio of 1.7-1.9.

Amplification of SNPs C3435T and T129C in *ABCB1* gene

Polymerase chain reaction (PCR) was used to amplify the regions in *ABCB1* gene that contains SNPs C3435T and T129C for both CRC patients and control subjects using the extracted gDNA. The PCR primers that were used in PCR amplification were mentioned in Table 1 and were previously described^{13,16}. For a 25 µl PCR reaction, 1 µl genomic DNA (100 ng/µl), 12.5 µl USB® HotStart-IT® Fidelity™ PCR Master Mix (2X) (Affymetrix, catalog no: 71156), 9.5 µl RNase free water, and 1 µl of (100 nmol) of each primer (Table 1) were used. The PCR thermocycler conditions that were used for amplification

were as follows: an initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C for SNP C3435T and for 40 seconds at 58°C for SNP T129C, and extension for 1 minute at 72°C. The final extension step was

done for 5 minutes at 72°C. The final PCR product with sizes, 207 bp and 258 bp for SNPs C3435T and T129C, respectively were run on 2% agarose gel with a GelPilot 100 bp ladder (Qiagen, catalog no: 239035) to confirm the amplification of the desired region of *ABCB1* gene that contains the SNP.

Table 1: Primers and restriction endonucleases used for SNPs genotyping

<i>ABCB1</i> SNPC3435T				
Forward primer (5'-3')	Reverse primer (5'-3')	PCR product length (bp)	Restriction enzyme	Genotypes (bp)
TTGATGGCAAAGAAATAAAGC	CTTACATTAGGCAGTGACTCG	207 bp	<i>MboI</i> (NEB: R0147L)	(CC) 62,145 (CT) 62,145,207 (TT) 207
<i>ABCB1</i> SNPT129C				
TTTCACTACTTGCCCTTTCTAGAG	CGGCCTCTGCTTCTTTGAG	258 bp	<i>MspAII</i> (NEB: R0577L)	(TT) 258 (TC) 36,226,258 (CC) 32,226

Genotyping of SNPs C3435T and T129C in *ABCB1* gene Restriction fragments length polymorphisms (RFLP) technique was used to digest the amplified PCR products to reveal the different genotypes for SNPs C3435T and T129C among CRC patients and healthy controls. For both SNPs, In Eppendorf tube, 5 µl PCR product, 16.5 µl nuclease-free water, 2.5 µl cutsmart buffer and 1 µl of the corresponding restriction enzyme (Table 1) were added. Then the mixture was mixed by pipetting gently, and was spun down for few seconds. The incubation and inactivation temperatures for each restriction enzyme were exactly as mentioned in the manufacturer's instructions (For both SNPs the incubation temperature was 37°C for 1 hour whereas, the inactivation temperature was 65°C for 20 minutes). The digested samples were separated on 2% agarose gel at 70 volts for 1 hour. After the separation, the gel was placed in the ultraviolet imaging device and a picture was taken to reveal the different genotypes.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism version 7. Mann-Whitney U/Wilcoxon Rank Sum Test (Z test) was used to compare between

two groups regarding one non-parametric variable. Moreover, Hardy-Weinberg Equilibrium was performed to determine allele frequencies and genotype distributions in a population. Fisher's exact test (two-tailed) was used to compare allele frequencies between patients and control subjects. P-value was recognized statistically significant when $P \leq 0.05$.

Results

Demographic characteristics of patients and controls

In this study, 125 subjects were included. The demographic characteristics of the patients and the control groups are presented in Table 2. In comparing the CRC patients to the controls, there were no significant differences in height ($P=0.77$), waist circumference ($P=0.23$), hip circumference ($P=0.60$), and waist to hip ratio (WHR) ($P=0.38$). However, the results also showed a highly significant difference between patients and controls regarding age ($P \leq 0.0001$, patients were higher than controls), whereas, in weight and body mass index (BMI) ($P=0.0006$ and 0.0002 , respectively higher in controls compared to patients).

Table 2: Demographic characteristics of patient and control groups

Demographic characteristics	CRC patients (n=62)	Controls (n=63)	P value
	Mean ± SD	Mean ± SD	
Age (years)	55.89 ± 12.6	44.11 ± 9.65	<0.0001
Height (cm)	165.5 ± 9.38	165.0 ± 9.82	0.77
Weight (kg)	73.37 ± 15.83	84.48 ± 19.13	0.0006
Hip (cm)	109.8 ± 19.15	110.7 ± 12.51	0.60
Waist (cm)	100.1 ± 19.99	106.7 ± 22.95	0.23
WHR	0.918 ± 0.14	0.964 ± 0.17	0.38
BMI (kg/m ²)	26.80 ± 5.71	31.15 ± 6.35	0.0002

WHR: waist to hip ratio; BMI: body mass index

Genotype and allele frequencies of C3435T variant in the *ABCB1* gene in patients and controls

PCR-RFLP analysis was used to amplify the region (exon 26) that contains SNP C3435T for all the collected samples (n=125). The genotype and allele frequencies of C3435T variant were examined (Table 3).

The genotypic frequencies of the patients were 29% (n=18) normal (CC), 21% (n=13) heterozygous (CT), and 50% (n=31) homozygous (TT). In controls, the results showed that the genotypes were as follow: 25.40% (n=16) normal (CC), 17.50% (n=11) heterozygous (CT), and 57.14% (n=36) homozygous (TT). In patient subjects,

the frequencies of the C and T alleles were 40% and 60%, respectively. In controls, the frequencies of the C and T alleles were 34% and 66%, respectively. The calculated χ^2 for both patients and controls were higher than the χ^2 value in the χ^2 distribution table. This indicates that there was a significant difference between what we observed and what we expected under Hardy-Weinberg equilibrium. Therefore, the population of both CRC patients and controls were out of Hardy-Weinberg equilibrium. Genotype distribution for CRC patients was (Goodness of fit $\chi^2 = 20$, degree of freedom (df) = 1, $P \leq 0.05$), whereas, for the controls the genotype distribution was (Goodness of fit $\chi^2 = 21$, df = 1, $P \leq 0.05$).

Table 3: Genotypes and allele frequencies of *ABCB1* gene C3435T for patients and controls

C3435T polymorphism	Frequencies %		P value	Odds ratio (95% CI)	Risk ratio (95% CI)
	Patients (n=62)	Controls (n=63)			
Genotypes					
CC	29.0% (n=18)	25.4% (n=16)		1.00 (Reference)	1.00 (Reference)
CT	21.0% (n=13)	17.5% (n=11)	0.92	1.05 (0.37-2.99)	1.03 (0.56-1.90)
TT	50.0% (n=31)	57.1% (n=36)	0.54	0.77 (0.33-1.75)	0.91 (0.69-1.21)
CT + TT	71.0% (n=44)	75.0% (n=47)	0.79	0.83 (0.38-1.83)	0.95 (0.77-1.18)
Alleles					
C	40.0%	34.0%		1.00 (Reference)	1.00 (Reference)
T	60.0%	66.0%	0.46	0.77 (0.43-1.37)	0.91 (0.73-1.13)

Genotype and allele frequencies of C3435T variant in *ABCB1* gene in male and female controls

Genotype and allele frequencies of the male and female volunteers are presented in Table 4. The genotype frequencies of C3435T variant in males showed 21% (n=9) normal (CC), 21% (n=9) heterozygous (CT), and 58.12% (n=25) homozygous (TT). Allele frequencies for C and T were 32% and 68%, respectively. The genotype distri-

bution for male volunteers was out of Hardy-Weinberg equilibrium (Goodness of fit $\chi^2 = 10$, df = 1, $P \leq 0.05$). In females, the results showed 35% (n=7) normal (CC), 10% (n=2) heterozygous (CT), and 55% (n=11) homozygous (TT) genotype. Allele frequencies for C and T were 40% and 60%, respectively. The genotype distribution for female volunteers was within Hardy-Weinberg equilibrium (Goodness of fit $\chi^2 = 6.71$, df = 1, $P > 0.05$).

Table 4: Genotypes and allele frequencies of *ABCB1* gene C3435T for male and female controls

C3435T polymorphism	Frequencies %		P value	Odds ratio (95% CI)	Risk ratio (95% CI)
	Males (n=43)	Females (n=20)			
Genotypes					
CC	21.0% (n=9)	35.0% (n=7)		1.00 (Reference)	1.00 (Reference)
CT	21.0% (n=9)	10.0% (n=2)	0.23	3.50 (0.57-21.66)	2.25 (0.61-8.31)
TT	58.12% (n=25)	55.0% (n=11)	0.52	1.77 (0.52-5.96)	1.20 (0.79-1.83)
CT + TT	79.10% (n=34)	65.0% (n=13)	0.35	2.03 (0.63-6.59)	1.22 (0.85-1.74)
Alleles					
C	32.0%	40.0%		1.00 (Reference)	1.00 (Reference)
T	68.0%	60.0%	0.30	1.42 (0.79-2.53)	1.13 (0.92-1.39)

Genotype and allele frequencies of C3435T variant in *ABCB1* gene male and female patients

Genotype and allele frequencies of the male and female patients are presented in Table 5. The genotype frequencies of C3435T variant in male patients showed that among the male patients (n=48), 33.33% (n=16) were normal (CC), 18.75% (n=9) were heterozygous (CT), and 47.92% (n=23) were homozygous (TT). The C and T allele frequencies were 42.71% and 57.29%, respectively.

The genotype distribution was out of Hardy-Weinberg equilibrium (Goodness of fit $\chi^2 = 18.27$, $df = 1$, $P \leq 0.05$). In female patients, the results showed that female patients (n=14) were as follow: 14% (n=2) were normal (CC), 29% (n=4) were heterozygous (CT), and 57% (n=8) were homozygous (TT). The C and T allele frequencies were 29% and 71%, respectively. The genotype distribution was within Hardy-Weinberg equilibrium (Goodness of fit $\chi^2 = 1.238$, $df = 1$, $P > 0.05$).

Table 5: Genotypes and allele frequencies of *ABCB1* gene C3435T for male and female patients

C3435T polymorphism	Frequencies %		P value	Odds ratio (95% CI)	Risk ratio (95% CI)
	Males (n=48)	Females (n=14)			
Genotypes					
CC	33.33% (n=16)	14.0% (n=2)		1.00 (Reference)	1.00 (Reference)
CT	18.75% (n=9)	29.0% (n=4)	0.4	0.28 (0.043-1.849)	0.54 (0.25-1.167)
TT	47.92% (n=23)	57.0% (n=8)	0.3	0.36 (0.067-1.920)	0.74 (0.49-1.106)
CT + TT	66.66% (n=32)	85.71% (n=12)	0.2	0.33 (0.07-1.672)	0.78 (0.58-1.042)
Alleles					
C	42.71%	29.0%		1.00 (Reference)	1.00 (Reference)
T	57.29%	71.0%	0.05	0.55 (0.31-0.98)	0.81 (0.66-0.997)

Genotype and allele frequencies of T129C variant in the *ABCB1* gene in patients and controls

The PCR-RFLP analysis was used to amplify the region (exon 1b) that contains SNP T129C for all the collected

samples (n=125). The genotype and allele frequencies of the T129C variant were examined. The results showed 100% normal (TT) genotype in both patients and controls. Heterozygous (TC) and homozygous (CC) genotypes were not detected in all patients and controls.

Discussion

CRC is one of the most aggressive and prevalent cancers worldwide¹⁷.

In KSA, according to the latest statistical analysis from National Cancer Registry in King Faisal Specialist Hospital and Research Centre, CRC is the most common cancer (11.9%) among Saudis males and females for all ages². Major reasons behind the higher incidence of CRC are the development of drug resistance, life styles, and instability in many important proteins and genes¹⁸. One of the major factors that contribute to CRC tumorigenesis and chemoresistance is the expression of ABC transporters on the surface of cancer cells¹⁹. Among this family, the *ABCB1* is the most studied member that correlates to cancer drug resistance and development risk. *ABCB1* gene is highly varied in different ethnicities and this has remarkable differences among them⁶. In this study, the genotype distributions and allele frequencies of two major variants in *ABCB1* gene, C3435T and T129C, were studied and correlated with the risk of CRC in Saudi Arabia. The data revealed that variant C3435T was not considered a risk factor for CRC in our study population as shown by the calculated odds ratio. However, patients with 3435 homozygous (TT) genotype had less risk of developing CRC (as shown by the low calculated OR<1). Interestingly, the comparison performed between male and female controls regarding the effect of carrying heterozygous (CT) or homozygous (TT) genotype showed a remarkable increased risk for male. Moreover, for the T129C variant, no risk could be assigned due to its rarity in the study population (all subjects had normal (TT) genotype).

Data on *ABCB1* variants showed contradictory results. In agreement with our results, a study performed on CRC German patients in 2012, showed that individuals carrying (rs1202168_T and rs868755_T) alleles have higher chance to develop CRC compared to individuals with the rs1045642_C allele (C3435T)²⁰. Another study performed on Polish breast cancer patients, showed that 3435C/T variant was not a good predictive factor as shown by the non-significant frequencies²¹. Moreover, Kim et al.²² found that breast cancer patients with 3435 TT genotype had longer overall survival rate compared to patients with 3435 CC or CT genotypes. In addition, in a meta-analysis study on eight SNPs in *ABCB1* gene, Zhang and his colleagues found that SNP 3435 C/T had no association with the development of CRC²³.

In contrast to our findings, other studies that were performed on several variants in *ABCB1* gene showed that *ABCB1* is an important factor that contributes to CRC development and progression as well as, it affects the response of the patients to the currently used chemotherapeutic agents. Many studies found that several *ABCB1* variants can increase the risk of CRC¹⁰⁻¹⁵ and may affect the functions of the P-glycoprotein and therefore, may have an effect on the development of CRC⁹.

In another study performed on the Turkish population in 2013, the *ABCB1* variant (T1236C) was found to be associated with CRC risk and it showed that the *ABCB1* haplotype (C1236-G2677-T3435) was more common in patients than in controls²⁴. Moreover, the *ABCB1* polymorphisms might be considered as a pharmacogenomic factor that plays an important role in the evaluation and diagnosis of CRC and determines the appropriate chemotherapy for CRC patients²⁵.

Additionally, *ABCB1* C3435T polymorphism was found to influence the susceptibility of CRC development and the clinical outcomes of colorectal cancer patients¹⁵.

Regarding T129C SNP, few studies were performed on cancer patients. Most studies were performed to correlate this SNP with drug resistance and patients' response. Two studies found that T129C variant in *ABCB1* gene was associated with epilepsy risk but not with drug-resistance^{13,26}. Moreover, SNP T/C was found to be positively related with PGP expression in placenta tissue in Japanese patients¹⁶.

Conclusion

This study to the best of our knowledge is the first study that correlates two variants in *ABCB1* gene (C3435T and T129C) with the risk of CRC in Saudi Arabia. Neither of the two SNPs showed a high contribution risk to CRC. However, this study has number of limitations. Most importantly, the small number of subjects which might affect the strength of statistical associations. In addition, the results could be confirmed by DNA sequencing analysis and needs to be done on a larger population size and to be performed on tissue samples rather than blood samples to confirm the findings and to elucidate the possible mechanisms behind the protective role.

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Competing interests

The author declares no competing interests

Author's contributions

Designed the experiments: Ayat B. Al-Ghafari. Performed the experiments: Areej M. Al Qahtani. Collected patient data: Anas H. Alzahrani. Analyzed the data: Ayat B. Al-Ghafari. Contributed reagents/materials/analysis tools: Ulfat M. Omar and Sawsan A. Rahimulddin. Wrote the paper: Ayat B. Al-Ghafari and Huda A. Al Doghaither.

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