

## Contribution of *ABCG2* gene polymorphisms (G34A and C376T) in the prognosis of colorectal cancer

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This study aims to assess the association of two single nucleotide polymorphisms (SNPs), G34A and C376T, in the *ABCG2* gene with the risk of developing CRC. To the best of our knowledge, this is the first study that determined the role of genetic variations in the *ABCG2* gene with the risk of CRC in Saudi Arabia. The gDNA was extracted from the blood of 58 CRC patients and 48 healthy subjects. The DNA sequencing was used to determine the distribution of genotypes. The results showed that CRC patients carried a heterozygous (GA) genotype for SNP G34A had a low risk of developing CRC (odds ratio=0.015, 95% CI [0.00–0.12]; risk ratio=0.35, 95% CI [0.25–0.12],  $P < 0.0001$ ). On the other hand, patients that carried a heterozygous (CT) genotype for SNP C376T had a high risk of developing CRC (odds ratio=13.83, 95% CI [4.31–44.38]; risk ratio=4.88, 95% CI [1.95–12.24],  $P < 0.0001$ ). In conclusion, the results indicated that a heterozygous (GA) genotype in SNP G34A may decrease the risk of CRC development, whereas, the heterozygous (CT) genotype in SNP C376T may increase the risk of CRC. The results may suggest a protective role of *ABCG2* SNP G34A against CRC and a deleterious effect of *ABCG2* SNP C376T for increasing the risk of CRC.

**Keywords:** ABC transporter, C376T variant, G34A variant, Polymorphism

Nearly one to two million new cases of colorectal cancer (CRC) are diagnosed every year. This high incidence rate makes CRC the third most common type of cancer and the fourth most common cause of cancer-related deaths in the world. By gender, CRC represents the second most common tumor in women (9.2%) and the third most common tumor in men (10%)<sup>1,2</sup>. In the Kingdom of Saudi Arabia (KSA), CRC represents the first and third most common malignancy in men and women, respectively<sup>3</sup>. CRC survival is highly dependent on the stage of the disease at diagnosis. In general, diagnosis at an early stage gives a high greater chance of survival, and the five-year survival rate is 95%, 82%, 61%, and 8% for CRC patients in stages I, II, III, and IV, respectively<sup>4,5</sup>.

There are several etiologic factors that are linked with the incidence of CRC, such as age, lifestyle, and

genetic mutations<sup>4</sup>. Colorectal carcinomas are classified as sporadic, inherited, or familial carcinoma, according to the source of the mutation. More than 70% of all diagnosed CRC cases are sporadic<sup>2,6</sup>. One of the major gene families that contribute to cancer development or treatment response is the ATP-binding cassette (ABC) transporter family, and the breast cancer resistance protein (*BCRP/ABCG2*) is a member of the ABC transporter super family.

The *ABCG2* gene is located on chromosome 4q22, spans more than 66 kb, and consists of 16 exons ranging from 60 to 532 base pairs<sup>7</sup>. It is prominently expressed in the placenta, colonic epithelium, small intestine, liver, lungs, and kidneys<sup>8-10</sup>. The expression and activity of the human *ABCG2* protein differ between individuals due to genetic polymorphisms, and the analysis and validation of single nucleotide polymorphisms (SNPs) occurring in the *ABCG2* gene are of clinical importance<sup>10</sup>. The two most frequently occurring SNPs, G34A in exon 2 and C376T in

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exon 4, were found to alter the transporter function, alter sensitivity to several anticancer drugs, and disrupt protein expression. Therefore, they may affect the response of cancer cells to therapy and result in increased susceptibility to different types of cancers<sup>11</sup>. Studies have revealed that carriers with the minor allele (A) of both SNP G34A and SNP C421A in the *ABCG2* gene had a significantly increased risk of breast carcinoma<sup>12</sup> and diffuse large B-cell lymphoma (DLBCL)<sup>13</sup>. Furthermore, one study indicated that heterozygous carriers of the G alleles of rs2622621 and rs1481012 in the *ABCG2* gene had a decreased risk of CRC<sup>10</sup>. A combination of SNPs C376T and C421A would have a negative effect on *ABCG2/BCRP* activity, and the combined variant is expected to show reduced *ABCG2/BCRP* activity<sup>14</sup>.

Based on the previous background, SNPs in the gene encoding *ABCG2* were found to alter the functional significance of the transporter. Therefore, the present study aimed to illustrate the effect of two SNPs (G34A and C376T) in the *ABCG2* transporter gene on the prognosis for CRC in Saudi patients.

## Materials and Methods

### Subjects and samples

This case-controlled study was conducted on 106 volunteers. The study comprised of 48 healthy control subjects and 58 CRC patients. The whole blood of the control samples was collected from King Fahad General Hospital (KFGH) – blood bank unit in Jeddah, KSA, while the blood samples of the CRC patients were collected from oncology centers at King Abdulaziz University (KAU) Hospital and King Abdullah Medical City (KAMC) in Jeddah, KSA. All study subjects agreed to participate in this study, signed informed consent, and answered a questionnaire that included information about their physical characteristics and medications they were taking. The study was approved by the Unit of Biomedical Ethics at the Faculty of Medicine (reference no. 261-15), KAU, Jeddah, KSA. DNA was extracted from the whole blood samples of patients and healthy controls

using the QIAamp DNA Blood Mini Kit (catalog no. 51306; QIAGEN, Germantown, MD, USA).

### Amplification of SNPs (G34A and C376T) in the *ABCG2* gene

To prepare a 25  $\mu$ L PCR reaction, 1  $\mu$ L genomic DNA (100 pmol/ $\mu$ L), 12.5  $\mu$ L HotStarTaqMaster Mix (catalog no. 71156; Affymetrix, Santa Clara, CA, USA), 9.5  $\mu$ L RNase free water (catalog no. 71156; Affymetrix, Santa Clara, CA, USA), and 1  $\mu$ L of each primer were used. The PCR thermocycler conditions for both SNPs are shown in (Table 1).

### Genotyping of SNPs (G34A and C376T) in the *ABCG2* gene

The different genotypes of SNPs G34A and C376T were determined with a DNA sequencing technique. The sequencing was done at the Centre of Excellence in Genomic Medicine Research (CEGMR) at King Fahd Medical Research Center (KFMRC), KAU, Jeddah, Saudi Arabia.

### Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The unpaired t-test was used to compare one variable between the two groups. Using a  $2 \times 2$  contingency table, the chi-square ( $\chi^2$ ) test was used to determine the allele frequency, genotype distribution, odds ratio (OR), risk ratio (RR), and Fisher's exact *P* values according to the Hardy–Weinberg equilibrium assumption. All *P* values <0.05 were considered statistically significant.

## Results

### Comparison of the physical characteristics between patients and controls

As shown in (Table 2), the results indicated that there were significant differences between the patients and the controls about weight ( $P=0.003$ ) and body mass index (BMI) ( $P=0.003$ ). The differences in weight and BMI are due to the loss of appetite in patients because of chemotherapy.

### Genotypes and allele frequencies of *ABCG2* SNP G34A in patients and controls

The genotype frequencies of the patients were 40% ( $n=23$ ) normal (GG), 59% ( $n=34$ ) heterozygous (GA),

Table 1 — PCR primers and cycling conditions for *ABCG2* SNPs amplification

Locus	Primers (5'-3')	Cycling conditions
Exon 2 SNP G34A	For: CAGTAATGTCTGAAGTTTTTATCGCA Rev: AAATGTTTCATAGCCAGTCTTGGA	95°C/1 min, followed by 25 cycles of 95°C/30 sec, 60°C/30 sec, and 68°C/1 min.
Exon 4 SNP C376T	For: TAGGGAAAGGAAGCAGAAGATGTAAGTAAA Rev: TAAGCTAGGGGAAAAGAAAGGTGAAGGA	Final extension at 68°C/5 min

and 2% (n=1) homozygous (AA), and the frequency of the G and A alleles were 70% and 30%, respectively. The genotype distribution of CRC patients was out of Hardy–Weinberg equilibrium ( $\chi^2=4.84$ , degree of freedom [DF]=1,  $0.05 < P < 0.025$ ). The genotype frequencies of controls were 92% (n=44) normal (GG), 2% (n=1) heterozygous (GA), and 6% (n=3) homozygous (AA), and the allele frequencies for G and A were 93% and 7%, respectively. The genotype distribution of controls was out of Hardy–Weinberg equilibrium ( $\chi^2=36.31$ , DF=1,  $P < 0.05$ ). In comparing the results for the patients and the controls, shown in (Table 3), the occurrence of the normal (GG) to heterozygous (GA) genotype was significantly different ( $P < 0.0001$ ); however, the calculated OR and RR were not high. The results indicate that SNP G34A protects against CRC development.

#### Genotypes and allele frequencies of *ABCG2* SNP G34A in male and female patients

The genotype frequencies of G34A in male patients were 41.30% (n=19) normal (GG), 58.70%

(n=27) heterozygous (GA), and 0% (n=0) homozygous (AA), and the G and A allele frequencies were 70.65% and 29.35%, respectively. The genotype distribution of CRC male patients was out of Hardy–Weinberg equilibrium ( $\chi^2=7.93$ , DF=1,  $0.005 < P < 0.002$ ). In female CRC patients, the results showed 33.33% (n=4) normal (GG), 58.33% (n=7) heterozygous (GA), and 8.33% (n=1) homozygous (AA) genotypes, and the G and A allele frequencies were 29.17% and 70.83%, respectively. The genotype distribution was out of Hardy–Weinberg equilibrium ( $\chi^2=13.72$ , DF=1,  $P < 0.05$ ). Although the frequency of genotypes was out of Hardy–Weinberg equilibrium in both male and female CRC patients, none of them showed a significant difference in an increased risk of CRC development.

#### Genotypes and allele frequencies of *ABCG2* SNP G34A in male and female controls

The genotype frequencies of male controls were 95.75% (n=30) normal (GG), 3.13% (n=1) heterozygous (GA), and 3.13% (n=1) homozygous (AA), and the G and A allele frequencies were 95.32% and 4.68%, respectively. The genotype distribution was out of Hardy–Weinberg equilibrium ( $\chi^2=13.58$ , DF=1,  $P < 0.05$ ). In female controls, the results showed 87.50% (n=14) normal (GG), 0% (n=0) heterozygous (GA), and 12.5% (n=2) homozygous (AA) genotypes, and the G and A allele frequencies were 87.50% and 12.50%, respectively. The genotype distribution of female controls regarding SNP G34A was out of Hardy–Weinberg equilibrium ( $\chi^2=16$ , DF=1,  $P < 0.05$ ). The results indicate a protective role of SNP G34A in the *ABCG2* gene against the development of CRC.

Table 2 — Mean values and standard errors of physical characteristics comparison between patients and controls

Physical characteristics	Patients (n=58)	Controls (n=48)	Unpaired <i>t</i> -test <i>P</i> value
Age (years)	56.14 ± 1.70	52.54 ± 1.90	0.15
Height (cm)	165.50 ± 1.24	166.30 ± 1.44	0.67
Weight (kg)	73.33 ± 2.06	84.13 ± 3.00	0.003
Waist (cm)	100.60 ± 2.70	99.85 ± 2.29	0.84
Hip (cm)	110.0 ± 2.55	109.90 ± 2.41	0.98
Waist to hip ratio (WHR)	0.921 ± 0.02	0.915 ± 0.02	0.82
Body mass index (BMI) (kg/m <sup>2</sup> )	26.82 ± 0.75	30.54 ± 0.97	0.003

Table 3 — Genotypes and allele frequencies of the *ABCG2* gene SNPs G34A and C376T for patients and controls

SNP G34A					
	Patients, n (%)	Controls, n (%)	<i>P</i> value	Odds ratio (95% CI)	Risk ratio (95% CI)
GG	23 (40)	44 (92)		1.0 (Reference)	1.0 (Reference)
GA	34 (59)	1 (2)	<0.0001	0.02 (0.002-0.12)	0.35 (0.25-0.50)
AA	1 (2)	3 (6)	1	1.57 (0.15-15.94)	1.37 (0.24-7.74)
G	40 (70)	44 (93)		1.0 (Reference)	1.0 (Reference)
A	18 (30)	4 (7)	<0.0001	0.18 (0.07-0.42)	0.53 (0.42-0.67)
SNP C376T					
	Patients, n (%)	Controls, n (%)	<i>P</i> value	Odds ratio (95%CI)	Risk ratio (95% CI)
CC	53 (91.38)	23 (47.92)		1.00 (Reference)	1.0 (Reference)
CT	4 (6.90)	24 (50)	<0.0001	13.83 (4.31-44.38)	4.88 (1.95-12.24)
TT	1 (1.72)	1 (2.1)	1	2.30 (0.14-38.46)	1.39 (0.35-5.62)
C	55 (94.83)	35 (72.92)		1.0 (Reference)	1.0 (Reference)
T	3 (5.17)	13 (27.1)	<0.0001	7.03 (2.58-19.14)	3.62 (1.60-8.18)

#### Genotypes and allele frequencies of *ABCG2* SNP C376T for patients and controls

The genotype frequencies of patients were 91.38% (n=53) normal (CC), 6.9% (n=4) heterozygous (CT), and 1.72% (n=1) homozygous (TT), and the frequency of the C and T alleles were 94.83% and 5.17%, respectively. The genotype distribution of CRC patients was out of Hardy–Weinberg equilibrium ( $\chi^2=5.13$ , DF=1,  $0.025 < P < 0.02$ ). In controls, the results showed 47.92% (n=23) normal (CC), 50% (n=24) heterozygous (CT), and 2.1% (n=1) homozygous (TT), and the allele frequencies for C and T were 72.92% and 27.10%, respectively. The genotype distribution of controls was out of Hardy–Weinberg equilibrium ( $\chi^2=23.14$ , DF=1,  $P < 0.05$ ).

In comparing the results for the patients and controls, shown in (Table 3), surprisingly, the occurrence of the normal (CC) to heterozygous (CT) genotype was significant and greatly different ( $P < 0.0001$ ), which is compatible with the calculated OR (13.83) and RR (4.88). Moreover, when the occurrence of the normal (CC) to homozygous (TT) genotype was calculated, the RR was equal in the two groups, which indicates a similarity in the ratio of the probability of exposure and outcome in the patient and control groups. However, the OR was slightly increased (OR=2.3), but since the *P*-value (Fisher's exact probability test) was not significant ( $P=1$ ) due to small sample size, we cannot confirm the role of the homozygous (TT) genotype in increasing the risk of CRC development. To confirm our findings, the allele frequencies were also compared. The results from a chi-square analysis showed that most of the patients and controls carried the major allele (C) as compared to the number of patients and controls that carried the minor allele (T). However, the OR and RR were extremely high (OR=7.03, 95% CI [2.58-19.14], and RR=3.62, 95% CI [1.60-8.18]), with a significant high *P*-value ( $< 0.0001$ ). These results confirm that subjects with the minor allele (T), whether heterozygous (CT) or homozygous (TT), are at a greater risk of developing CRC compared to the subjects carrying the major allele (C) in the form of a normal genotype (CC).

#### Genotypes and allele frequencies of *ABCG2* SNP C376T for male and female patients

The genotype frequencies of the C376T variant in male patients showed 93.48% (n=43) normal (CC), 4.35% (n=2) heterozygous (CT), and 2.17% (n=1) homozygous (TT), and the C and T allele frequencies

were 95.66% and 4.34%, respectively. The genotype distribution of male patients was out of Hardy–Weinberg equilibrium ( $\chi^2=10.47$ , DF=1,  $0.002 < P < 0.001$ ). In females, the results showed 83.33% (n=10) normal (CC), 16.67% (n=2) heterozygous (CT), and 0% (n=0) homozygous (TT) genotypes, and the C and T allele frequencies were 91.67% and 8.33%, respectively. The genotype distribution of female patients was within Hardy–Weinberg equilibrium ( $\chi^2=0.10$ , DF=1,  $0.98 < P < 0.20$ ). Results from a chi-square analysis showed that there was no significant difference in genotype distribution and the risk of developing CRC regarding to gender.

#### Genotypes and allele frequencies of *ABCG2* SNP C376T for male and female controls

The genotype frequencies of the C376T variant in male controls showed 50% (n=16) normal (CC), 46.88% (n=15) heterozygous (CT), and 3.13% (n=1) homozygous (TT), and the C and T allele frequencies were 73.44% and 26.56%, respectively. The genotype distribution of male controls was within Hardy–Weinberg equilibrium ( $\chi^2=1.30$ , DF=1,  $0.98 < P < 0.20$ ). In females, the results showed 43.75% (n=7) normal (CC), 56.25% (n=9) heterozygous (CT), and 0% (n=0) homozygous (TT) genotypes, and the C and T allele frequencies were 71.88% and 28.12%, respectively. The genotype distribution of female controls was within Hardy–Weinberg equilibrium ( $\chi^2=2.45$ , DF=1,  $0.20 < P < 0.10$ ). Results from a chi-square analysis showed that there was no significant difference in genotype distribution and the risk of developing CRC in relation to gender.

#### Discussion

CRC is one of the most commonly diagnosed cancers worldwide. In 2015, CRC ranked as the third most common cancer in males and the second most common type in females, giving rise to an estimated 753000 deaths worldwide<sup>15,16</sup>. In Saudi Arabia, CRC represents the first most common cancer in males and the third in females<sup>3</sup>. One of the studied mechanisms that explain drug resistance and increases the risk of developing cancer is the expression of *ABCG2*, a drug efflux pump and a member of the ABC drug transporter family<sup>17</sup>. *ABCG2* is widely expressed on the surfaces of many tissues, including epithelial cells in the colon<sup>8,10,18</sup>. SNPs in the *ABCG2* gene have been found to change the expression and activity of the corresponding gene and its product. Therefore, it may increase the susceptibility to different types of

cancers<sup>7,19</sup>. The *ABCG2* SNPs, G34A, which resulted in a V12M change in exon 2, and C376T (substituting stop codon for Gln-126) in exon 4, are two of the most studied SNPs<sup>13,20</sup>. The *ABCG2* SNP G34A is related to disturbed transporter membrane localization of *ABCG2* and drug efflux<sup>21,22</sup>. Moreover, SNP C376T has been found to affect protein expression due to the premature stop codon<sup>20</sup>. For this reason, experiments are currently performed to overcome this obstacle by studying the response of CRC patients towards many chemotherapeutic agents such as 5-fluorouracil (5-FU) and thymidine phosphorylase inhibitors as well as to identify new biomarkers to assess drug response in cancer<sup>23-25</sup>.

Several studies have related *ABCG2* SNPs with the risk of different types of diseases, most notably cancers. Wu and his team found that carriers with the *ABCG2* G34A A allele and the C421A A allele and haplotype G34A A-C421A C or G34A G-C421A A had a significantly increased risk of breast carcinoma<sup>12</sup>. The variants 421C>A and 34G>A were associated with an increased risk of DLBCL<sup>12,13</sup>. Furthermore, a study published by Campa *et al.*<sup>10</sup> indicated that heterozygous carriers of the G alleles of rs2622621 and rs1481012 in the *ABCG2* gene had a decreased risk of CRC. However, more studies regarding *ABCG2* SNPs have focused on drug resistance because this transporter contributes greatly to treatment response.

In the current study, we were concerned about the contribution of allele frequencies and genotype distributions of two SNPs (G34A and C376T) in the *ABCG2* transporter gene to an increased risk of CRC (as it is among the most common types of cancer in males and females in the KSA). Interestingly, for the first time in the KSA, to the best of our knowledge, we found that CRC subjects carrying the heterozygous (GA) genotype of the G34A variant showed a low risk of CRC, as determined by their calculated OR, whereas, patients with the homozygous (AA) genotype had a slightly higher degree of risk compared to heterozygous (GA) subjects, although the *P*-value was not significant due to the small sample size used in the comparison. This result might suggest a protective role of the heterozygous (GA) genotype of *ABCG2* SNP G34A against CRC development. Our results disagree with another study, which was done on breast cancer patients that found the G34A GA/AA genotypes were significantly associated with an increased risk of

breast cancer<sup>12</sup>. A recent study performed on Polish myeloma patients found that SNP C421A in *ABCG2* gene may be used as a predictor for multiple myeloma, whereas, SNP G34A was expressed normally in the study population and therefore, cannot be used as a predictor marker for myeloma<sup>26</sup>. Concerning the C376T variant in this study, the results for the patients and controls with the heterozygous (CT) genotype indicated a high risk of CRC. Our results indicate that the heterozygous genotype (CT) of the C376T variant increased the risk of CRC development more than the normal genotype (CC). Interestingly, there have been less studies done to indicate the correlation of SNP C376T with a risk of cancer, however, a recent study was performed on 580 advanced CRC patients to assess the role of polymorphisms in DNA repair genes and *ABCG2* gene. They have found that the XPA DNA repair gene and *ABCG2* gene had significant interaction with environmental factors and prognosis of CRC, especially in CRC patients receiving oxaliplatin-based chemotherapy<sup>27</sup>.

## Conclusion

The results of this study suggest a protective role of the heterozygous (GA) genotype in SNP G34A against CRC development. Our data also suggest an increased risk of developing CRC in subjects with the heterozygous (CT) genotype in SNP C376T. However, this study had some limitations. These results need to be investigated with a larger number of patients, and an *in vitro* study on patients' tissues should be performed to determine the expression of the transporter using an immunohistochemistry technique.

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## Conflict of Interest

All authors declare no conflict of interest.

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