

CYP19A1 gene polymorphism and colorectal cancer etiology in Saudi population: case–control study

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Background: Considerable interest is directed toward the enzyme aromatase (CYP19A1) and the development of cancer, due to CYP19A1's role in estrogen biosynthesis. Several cancers display excessive intra-tumor accumulation of estrogens, and aromatase inhibitors are used for treatment. The CYP19A1 gene exhibits polymorphism and mutations that can alter its expression or aromatase activity and influence estrogen production. We designed this study to investigate the link between CYP19A1 polymorphism and susceptibility to colorectal cancer (CRC) development in Saudis.

Patients and methods: Blood samples from 100 CRC patients and 100 healthy controls were drawn for DNA extractions. Three polymorphic sites, rs4774585, rs936308, and rs4775936, were genotyped using Taqman genotyping by real-time polymerase chain reaction. Allelic and genotype frequencies were calculated and compared in the two groups.

Results: All single nucleotide polymorphisms (SNPs) were polymorphic in Saudis, and comparison of allele frequencies showed several differences when compared to other populations. None of the SNPs were associated with the risk of CRC development in Saudis ($P > 0.05$). Some gender and location (colon or rectal) differences were observed.

Discussion: The results of this study highlighted the genetic heterogeneity existing between populations in the prevalence of different SNPs and their relation to disease state. It showed that, although rs4774585, rs936308, and rs4775936 are involved in CRC development in several populations, their role is not significant in the etiology of CRC in Saudis; however, some SNPs do increase susceptibility or resistance to CRC development as judged from the odds ratio. Further large-scale studies are warranted to clarify the role of the CYP19A1 development in CRC.

Keywords: aromatase, CYP19A gene, polymorphism, SNPs, colorectal cancer, rs4774585, rs936308, rs4775936

Introduction

In Saudi Arabia, the second most common cancer, in both men and women, is colorectal cancer (CRC) and it also comprises the second leading cause of cancer-related deaths in this population.¹ The clinical characteristics of CRC are variable and depend largely on the location, that is, colon or the rectum, where cancer develops.² The malignant transformation initiates on the inner lining of the colon or the rectum, as a noncancerous growth called a polyp, which develops slowly, over a period of 10–20 years to the malignant state.^{2,3}

During the last few years, interest has been directed toward the role of estrogens in the development of CRC. Some of the earlier reports pointed to a protective role as it was shown that men have lower estrogen levels and a higher susceptibility to colon cancer compared to women,⁴ and women on hormone replacement therapy (HRT) had

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even lower susceptibility compared to their counterparts not receiving HRT.⁵ Studies showed the presence of different isoforms of the estrogen receptor (ER) and the wild type, ER β , was shown to be overexpressed in the colonic tissue.⁶ The ER β was protective against cancer development, and an inverse relationship was reported to exist between tumor progression and ER β expression in the CRC cells compared to the normal cells in the same patient.⁷ More recent studies demonstrated that total estrogens (estrone and estradiol) were 2- to 2.4-fold higher in the CRC tissue compared to the adjacent normal tissue.^{8,9} The intra-tumoral estrogens were locally synthesized and were shown to be significantly associated with poorer survival.⁶ Two major pathways were reported to be involved in the local synthesis of estrogens, that is, the aromatase pathway and the sulfatase pathway.^{6,10} Though the major intra-tumoral synthetic partway was the sulfatase pathway, the levels of aromatase (EC 1.14.14.1; CYP19A1) were shown to be significantly elevated in the CRC tissue.⁸ English et al¹¹ demonstrated that intra-tumoral aromatase was very active in the colon epithelial and carcinoma tissue and was responsible for the conversion of androstenedione to estrone. A significant association was demonstrated between the levels of total estrogens and the clinical outcome in CRC.⁶ Similar finding in other malignancies, including breast cancer,^{12–14} gastric cancer,¹⁵ ovarian cancer,¹⁶ and others,¹⁷ points to the significance of estrogens in cancer development. Since the intra-tumoral aromatase is elevated, a treatment strategy using aromatase inhibitors to decrease the level of estrogens was adopted for several of the cancers.^{18,19}

Aromatase is considered in several studies as a possible candidate involved in the development of CRC.^{20–24} The enzyme is located in the estrogen-producing cells on the endoplasmic reticulum and brings about the conversion of the C19 androgens: androstenedione to estrone and testosterone to estradiol. Several studies discuss its clinical significance.^{25,26} It is encoded by the CYP19A1 gene (cytochrome P450 family 19 subfamily A member 1) located at chromosome 15q21.2.^{27–29} Several polymorphisms have been reported in the promoter and intronic regions of CYP19A1 gene that may affect the gene product resulting in either increased or decreased aromatase activity. The role of CYP19A1 polymorphisms has been evaluated in breast cancer, endometrial cancer, prostate cancer, Alzheimer's disease, cardiovascular disease, and obesity.^{30–35} Certain single nucleotide polymorphisms (SNPs) show a positive correlation, whereas others show a protective effect against the development of these pathological states.

We hypothesized that polymorphisms in CYP19A1 might be associated with the development and outcome in CRC. We designed the present case–control study to investigate three SNPs in the CYP19A1, that is, rs4774585, rs936308, and rs4775936, in Saudi CRC patients. The SNPs were selected based on database NCBI/dbSNP (<https://www.ncbi.nlm.nih.gov/snp>)³⁸ and previous population studies that have focused on other types of cancers and diseases in which these SNPs were reported to show a functional effect on the development of these diseases.³⁶ Selection of each SNP was also based on their location in the gene, where it may play a role in the regulation of CYP19A gene expression. In this paper, we report frequencies of three polymorphisms in the CYP19A1 in normal healthy Saudis and patients suffering from CRC. We also show the differences that exist in the frequency of different alleles of these SNPs in Saudis compared to other populations. We believe this is the first report exploring genetic variations in CYP19A1 in CRC in Saudis.

Patients and methods

Study population

The study was reviewed and approved by the institutional review board of the ethics committee at King Khalid University Hospital (KKUH), Riyadh, Saudi Arabia. The study population comprised 100 cases diagnosed with CRC at the endoscopy service at KKUH, Riyadh. A group of 100 age-matched normal healthy individuals, also attending the endoscopy unit for other reasons and with normal colonoscopy, were recruited as controls. The objectives of the study were explained to both groups, and written informed consent was obtained. Demographic data and clinical data of the patients were collected from the participants and entered on specially designed spreadsheets.

SNP selection and genotyping

Blood was collected from patients and controls, by venepuncture, in EDTA tubes and stored at 4°C until required for analysis. To extract genomic DNA from whole blood, QIAmp DNA blood mini kits (Qiagen, Valencia, CA, USA) were used following the protocol provided by the manufacturer. TaqMan genotyping assay kits (Thermo Fisher Scientific, Waltham, MA, USA) were used for genotyping of the three SNPs (rs4774585, rs936308, and rs4775936). All probes used were those designed by Thermo Fisher Scientific (C_27892984_10, C_1664163_10 and C_11301451_10) following the protocol provided by the manufacturer. The reaction mixture (11 μ L) consisted of 5.5 μ L of TaqMan PCR master mix, 0.26 μ L of SNP assay, and 2 μ L of DNA (20 ng)

and the volume was made up by adding 3.25 μ L of water. For the PCR amplification, LightCycler 480 Instrument II Real-Time PCR System was used, following the protocol: initial denaturation at 94°C for 15 minutes, followed by 45 cycles at 94°C for 15 seconds and at 60°C for 1 minute.

Statistical methods used for data analysis

The genotypes and allele frequencies of the three SNPs were calculated manually. Fisher's exact test (two-tailed) was applied to obtain the χ^2 , odds ratios (OR), and 95% CI, to determine the significance of the difference between the results of the CRC patients and controls. The results were subjected to statistical analysis using the Statistical Package for the Social Sciences (version 21) (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to determine the significance of the difference between demographic data in the patients and controls.

Results

The demographic and clinical characteristics of CRC patients and the control group were obtained from interviewing the patients and from their hospital files and are presented in Table 1.

The Hardy–Weinberg model was applied to the data, and the results showed that the genotype distributions were consistent with the expected values ($P>0.05$). The OR of CRC association were calculated using the homozygous wild-type allele as a reference for the other two genotypes. The genotype and allele frequencies of the analyzed SNPs, the OR and the significance levels (P) for each SNP investigated are presented in Table 2. The genotype frequencies of GG, AG, and AA for rs4774585 were 46%, 46%, and 8%, respectively, in the CRC patients, and 48%, 41%, and 11%, respectively, in the control group. The frequencies of GG, GC, and CC for rs936308 were 5%, 35%, and 60%, respectively, in CRC patients and 9%, 34%, and 57%, respectively, in the control group. Although the frequencies of the CC,

CT, and TT genotypes of rs4775936 were 37%, 44%, and 19%, respectively, in CRC cases, and 35%, 47%, and 18% respectively, in the control group. These genotype and allele frequencies for rs4774585, rs936308, and rs4775936 did not show a statistically significant difference between the CRC patients and the healthy control group.

The patients were stratified according to whether the cancer was in the colon or the rectum, and for each location, the genotype and allele frequencies were calculated, separately for each SNP. Table 3 presents the results for the three SNPs in colon cancer compared to the results in rectal cancer patients. It also shows the results in the colon cancer patients and rectal cancer patients, compared to the results in the control group. The AG genotype of rs4774585 occurred at a significantly higher frequency in the rectal cancer patients compared to the colon cancer patients. None of the other genotypes or alleles of the studied SNPs showed a significant difference between the two groups. When compared to the results in the control group, the CG genotype for rs936308 occurred at significantly higher frequency in the rectal cancer patients. Frequencies of all other genotypes and alleles, for the three SNPs, did not differ in the colon cancer and rectal cancer patients when compared to the results in the control group.

In an attempt to determine gender influence on the frequency of CYP19A genotypes in CRC, the male and female patients were grouped separately, and the frequency of occurrence of the genotype and allele was calculated and compared to the results in the control groups of the same gender. Table 4 presents the findings and shows the significantly lower frequency of the genotype “AA” of rs4774585 in the male CRC patients compared to the control ($P=0.04$), and the genotype “CT” of rs4775936 in female CRC patients compared to the controls. Frequencies of the other genotypes and alleles of the three studied SNPs were similar in the two genders, compared to their sex-matched controls.

The allele frequencies of the three SNPs in healthy Saudis were compared to those reported for other populations in the NCBI SNP database. The results are presented in Table S1.

Discussion

Extensive investigations have reported genetic variations in different genes that may be related to cancer susceptibility and many positive associations have been identified. The aromatase (CYP19A1) gene has been investigated in several studies, where some of the common polymorphisms have been investigated but the results obtained are inconsistent

Table 1 Demographic characteristics of colorectal cancer patients and normal control group

Parameters	Normal control (N=100)	Cancer patients (N=100)	P-value
Age (years) (mean \pm SD)	53.06 \pm 1.5	55.50 \pm 1.3	>0.05
Sex			>0.05
Male	58	60	
Female	42	40	
Smoking	5	5	>0.05
Non-smoking	95	95	

Table 2 Genotype and allele frequencies of three SNPs in CYP19A1 gene in the CRC patients and controls

SNP	Variation	Case	Control	OR	CI	χ^2	P-value
rs4774585	GG	46 (46)	48 (48)	Ref			
	AG	46 (46)	41 (41)	1.17	0.65–2.09	0.28	0.59
	AA	8 (8)	11 (11)	0.76	0.28–2.05	0.30	0.58
	AG + AA	54 (54)	52 (52)	1.08	0.62–1.88	0.08	0.77
	G	138 (69)	137 (68.5)	Ref			
rs936308	A	62 (31)	63 (31.5)	0.977	0.64–1.49	0.01	0.91
	GG	5 (5)	9 (9)	Ref			
	CG	35 (35)	34 (34)	0.97	0.53–1.77	0.01	0.94
	CC	60 (60)	57 (57)	1.89	0.59–5.99	1.21	0.27
	CG + GG	40 (40)	43 (43)	0.88	0.50–1.55	0.19	0.66
rs4775936	G	45 (22.5)	52 (26)	Ref			
	C	155 (77.5)	148 (74)	1.21	0.76–1.91	0.67	0.41
	CC	37 (37)	35 (35)	Ref			
	CT	44 (44)	47 (47)	0.88	0.47–1.60	0.15	0.70
	TT	19 (19)	18 (18)	0.99	0.45–2.20	0.00	0.99
	CT + TT	63 (63)	65 (65)	0.91	0.51–1.63	0.09	0.76
	C	118 (59)	117 (58.5)	Ref			
T	82 (41)	83 (41.5)	0.98	0.65–1.45	0.01	0.92	

Abbreviations: CRC, colorectal cancer; OR, odds ratio; Ref, reference; SNP, single nucleotide polymorphism; CI, confidence interval.

in regard to their possible association with CRC risk, the location of the tumor, levels of sex hormones, and survival rate of the patients. Population-related variations are also frequent. Hence, aromatase has become the target for considerable research in the exploration of cancer association and a potential target for therapeutic interventions due to the significant role it plays in the synthesis of estrogens.^{25,26} The intra-tumoral concentration of estrogens is elevated in several cancers, including colon cancer. Estrogen levels are higher in aromatase positive cancer cells, and a study by Niikawa et al³⁷ confirmed that aromatase levels were higher in the men compared to women, even those in their postmenopausal state. This elevated level of aromatase may be a consequence of elevated gene expression in the cancer tissue and the increase in amount, or enzyme activity may result in increased production of estrogens. Elevated aromatase immunoreactivity in the colon cancer tissue was demonstrated, confirming an increased amount of aromatase. Furthermore, a close association was shown between aromatase mRNA and the levels of estradiol in the tissue. Niikawa et al³⁷ and Sato et al⁸ showed that there was a five fold increase in aromatase mRNA in cancerous compared to the adjacent noncancerous tissue. All of these point to an elevated aromatase gene expression, and this may be due to mutations or SNPs in the regions of the gene responsible for control of gene expression. With this background, we have explored three polymorphisms in aromatase gene and have selected two SNPs in the promoter region and one in the intronic region of the CYP19A1 gene. The selection was

based on the fact that these regions in the gene may influence the rate of gene expression.

Of the three SNPs investigated, two of the SNPs, rs4774585, an A > G transition and rs936308, C > G transversion, are located in the promoter region; while the third SNP rs4775936 is located in the intronic region and is a C > T transition.

The SNPs, rs4774585, and rs936308 were studied to detect if the promoter region mutation has an influence on the gene expression of CYP19A1 gene and whether it plays a role in the development of CRC. Our results showed that these SNPs exhibit polymorphism in Saudis; however, the frequency of the genotypes did not differ when the CRC patients were compared to controls. When stratified by sex, AA genotype of rs4774585 showed a significant difference between CRC patients and controls in the male group (P -value = 0.04). The presence of “A” allele in the homozygous state (AA) decreased the OR to 0.28, compared to 3.55 in the homozygous GG and 1.52 in the heterozygous AG state, suggesting a negative association with CRC development in males and exhibits a recessive inheritance. Comparison of the frequencies of G and A in Saudis with those reported by NCBI³⁶ in different population groups showed several differences compared to the Hispanic and Asian populations, but the frequencies in Saudis were similar to those reported for Europeans and Sub-Saharan Africans.³⁶ For the SNP rs936308, the ancestral allele G was found to occur at a low frequency in the Saudi population both the CRC and the control group, compared to the results in other populations.

Table 3 Comparison of the genotype and allele frequencies of three SNPs in CYP19A1 gene in the colon and rectum cancer patients and the control group

SNP	Variation	Case ^a		Control	Colon vs rectal				CRC vs control				
		Colon	Rectal		OR	CI	χ^2	P-value	OR	CI	χ^2	P-value	
rs4774585	GG	46 (70.7)	–	48 (48)	Ref				Ref				
		–	15 (45.4)										
	AG	11 (16.9)	–	41 (41)	4.46	1.70–11.6	9.86	0.001	1.05	0.54–2.04	0.03	0.86	
		–	16 (48.4)						1.24	0.55–2.83	0.28	0.59	
	AA	8 (14)	–	11 (11)	0.76	0.14–4.01	0.10	0.75	0.84	0.28–2.51	0.09	0.76	
		–	2 (6.06)						0.58	0.11–2.92	0.44	0.50	
AG + AA	19 (29.2)	–	52 (52)	2.90	1.21–6.92	5.97	0.014	1.21	0.42–3.46	0.13	0.71		
rs936308	GG	4 (0.06)	–	9 (9)	Ref				Ref				
		–	1 (3.03)										
	CG	22 (33.8)	–	34 (34)	3.81	0.39–37.0	1.50	0.22	0.94	0.48–1.85	0.03	0.87	
		–	21 (63.6)						3.201	1.37–7.44	7.65	0.005	
	CC	38 (58.4)	–	57 (57)	1.15	0.11–11.4	0.02	0.90	1.53	0.44–5.35	0.47	0.49	
		–	11 (33.3)						1.73	0.19–15.12	0.26	0.61	
CG + GG	27 (41.5)	–	43 (43)	1.15	0.11–11.4	0.02	0.90	1.50	0.44–5.11	0.44	0.50		
rs4775936	G	30 (23.0)	–	52 (26)	Ref				Ref				
		–	23 (34.8)										
	C	98 (75.3)	–	148 (74)	0.57	0.29–1.09	2.86	0.09	1.17	0.69–1.96	0.36	0.54	
		–	43 (65.1)						0.65	0.36–1.19	1.92	0.16	
	CC	31 (47.6)	–	35 (35)	Ref				Ref				
		–	16 (48.4)										
CT	22 (33.8)	–	47 (47)	0.96	0.37–2.48	0.00	0.94	0.88	0.47–1.60	0.15	0.70		
TT	12 (18.4)	–	18 (18)	0.96	0.30–3.06	0.00	0.95	0.51	0.21–1.23	2.24	0.13		
	–	6 (18.1)						0.99	0.45–2.20	0.00	0.99		
CT + TT	34 (52.3)	–	65 (65)	0.96	0.41–2.24	0.01	0.94	0.72	0.24–2.18	0.32	0.57		
C	84 (64.6)	–	117 (58.5)	Ref				0.91	0.51–1.63	0.09	0.76		
	–	43 (65.1)						0.57	0.25–1.26	1.91	0.16		
T	46 (35.3)	–	83 (41.5)	0.97	0.52–1.81	0.01	0.94	0.77	0.48–1.21	1.24	0.26		
	–	23 (34.8)						0.75	0.42–1.34	0.92	0.33		

Note: ^aThe total number of patients is not 100, since the location was not available for a few patients.

Abbreviations: CRC, colorectal cancer; OR, odds ratio; Ref, reference; SNP, single nuclear polymorphism; CI, confidence interval.

When the frequencies of G and C in Saudis were compared with different populations, several differences were identified with the Asians, and Sub-Saharan African populations, although a similarity was seen to the reports for Europeans and Hispanic populations.³⁶ When the patients were grouped based on the location of cancer, that is, colon or rectum, the GC genotype showed a significant difference between the patients with rectal cancer compared to the control group (P -value = 0.0056). The presence of homozygous GG state decreased the OR to 0.57 compared to 1.73 in the homozygous CC. Hence, the allele “G” seems to have a negative association with rectal cancer development.

For rs4775936, when the samples were grouped according to the gender, the frequency of CT heterozygous showed a statistically significant difference between female CRC patients and the controls ($P=0.029$). The presence of homozygous TT state decreased the OR to 0.68, whereas the OR was 1.4 in the homozygous CC state, thus showing a negative association of the T allele and CRC development in females.

Conclusion

This study has shown that the studied SNPs exhibit polymorphism in Saudis. It has confirmed our earlier observations during our studies that the frequencies of different SNPs

Table 4 Genotype and allele frequencies of three SNPs in *CYP19A1* gene in females and males colorectal cancer patients and the control group

SNP	Variation	Case ^a	Control	OR	CI	χ^2	P-value
rs4774585	Females						
	GG	18 (42.85)	29 (49.15)	Ref			
	AG	20 (47.61)	12 (28.33)	1.11	0.49–2.52	0.06	0.80
	AA	4 (9.52)	1 (1.69)	6.44	0.66–62.3	3.22	0.07
	AG + AA	24 (57.14)	13 (22.03)	1.28	0.58–2.85	0.39	0.53
	G	56 (66.66)	70 (59.32)	Ref.			
	A	28 (33.33)	14 (11.86)	1.40	0.76–2.58	1.18	0.27
	Males						
	GG	27 (46.55)	19 (46.34)	Ref			
	AG	26 (44.82)	12 (29.26)	1.52	0.61–3.75	0.85	0.35
	AA	4 (6.89)	10 (24.39)	0.28	0.07–1.03	3.90	0.04
	AG + AA	29 (50)	22 (53.65)	0.96	0.42–2.14	0.01	0.91
	G	80 (68.96)	50 (60.97)	Ref			
	A	56 (48.27)	32 (39.02)	0.66	0.36–1.20	1.81	0.17
rs936308	Females						
	GG	3 (7.14)	4 (6.77)	Ref			
	CG	11 (26.19)	23 (38.98)	0.54	0.22–1.31	1.83	0.17
	CC	28 (66.66)	32 (54.23)	1.16	0.24–5.66	0.04	0.84
	CG + GG	13 (30.95)	27 (45.76)	0.59	0.26–1.34	1.57	0.20
	G	17 (20.23)	31 (26.27)	Ref			
	C	67 (79.76)	69 (58.47)	1.40	0.71–2.74	0.99	0.32
	Males						
	GG	3 (5.17)	5 (12.19)	Ref			
	CG	23 (39.65)	11 (26.82)	1.63	0.67–3.97	1.18	0.27
	CC	32 (55.17)	25 (60.97)	2.13	0.46–9.79	0.98	0.32
	CG + GG	26 (44.82)	22 (53.65)	1.27	0.56–2.86	0.33	0.56
	G	29 (25.38)	21 (25.60)	Ref			
	C	87 (75.61)	61 (74.39)	1.03	0.53–1.97	0.01	0.92
rs4775936	Females						
	CC	20 (47.61)	17 (28.81)	Ref			
	CT	14 (33.33)	32 (54.23)	0.37	0.15–0.91	4.73	0.02
	TT	8 (19.04)	10 (16.94)	0.68	0.21–2.21	0.45	0.50
	CT + TT	22 (52.38)	42 (71.18)	0.44	0.19–1.01	3.74	0.05
	C	54 (64.28)	66 (55.93)	Ref.			
	T	30 (35.71)	52 (44.06)	0.70	0.39–1.25	1.42	0.23
	Males						
	CC	18 (31.03)	18 (43.9)	Ref			
	CT	29 (50)	16 (39.02)	1.81	0.74–4.43	1.71	0.19
	TT	11 (18.96)	7 (17.07)	1.57	0.49–4.96	0.60	0.44
	CT + TT	40 (68.96)	23 (56.09)	1.73	0.75–3.99	1.72	0.18
	C	65 (56.03)	52 (63.41)	Ref			
	T	51 (43.96)	30 (36.58)	1.36	0.76–2.42	1.08	0.29

Note: ^aThe total number of patients is not 100, since the gender was not recorded for a few patients.

Abbreviations: OR, odds ratio; Ref, reference; SNP, single nucleotide polymorphism; CI, confidence interval.

in Saudi population differ considerably when compared to other populations thus any potential therapeutic development targeting this pathway in other populations might not be applicable to this population and emphasizes the notion of personalized treatment targets. Studies on a larger population-based levels are required to confirm our findings and investigations on ethnic groups within Saudi Arabia may provide insight into the role of polymorphisms in *CYP19A1* gene in CRC development. Furthermore, Saudis may be

carrying SNPs that are specific for this population and to identify these SNPs in *CYP19A1* gene, future studies have to be directed to the sequencing of the gene.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 Allele frequencies in the three studied SNPs in Saudis compared to frequencies in populations reported in NCBI SNP database¹

Populations	rs4774585			rs936308			rs4775936		
	Chromosome number	A	G	Chromosome number	C	G	Chromosome number	C	T
Saudi Arabian	200	0.315	0.685	200	0.740	0.260	200	0.585	0.415
European	1,006	0.219	0.780	1,006	0.8399	0.1600	1,006	0.567	0.432
African	1,322	0.150	0.849	1,322	0.3026	0.697	1,322	0.892	0.1074
American	694	0.273	0.726	694	0.7839	0.216	694	0.716	0.2839
South American	978	0.181	0.819	978	0.672	0.3272	978	0.6933	0.3067
Asia	172	0.005	0.994	170	0.5882	0.4117	172	0.6220	0.3779

Note: *This study.

Abbreviation: SNP, single nucleotide polymorphism.

Reference

1. NCBI SNP database. Available from: <https://www.ncbi.nlm.nih.gov/snp>. Accessed September 5, 2016.

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