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Original article

The protective effects of the methylenetetrahydrofolate reductase rs1801131 variant among Saudi smokers

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ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) polymorphism plays a fundamental role in susceptibility to various diseases, including cancers and autoimmune diseases. In the current study, we aimed to compare genotype and allele frequency variations of rs1801131, one of the most common variants found in the MTHFR gene, among Saudi smokers and non-smokers. We hypothesized that genetic variations of this gene are responsible for many diseases, particularly those caused by cigarette smoking (CS) such as pulmonary diseases, oral cancer and lung cancer. We performed a case-control study on a sample of 235 healthy smokers and 239 healthy non-smokers in Saudi Arabia. The rs1801131 SNP genotypes were determined using a genotyping assay and multiple *in silico* algorithmic software programs were used to identify the effects and structural functions of the rs1801131 (Glu429Ala) mutation. Using chi-squared tests, we found that, among smokers, TG and GG genotype carriers had 0.209-fold (OR = 0.209, $P < 0.005$) and 0.427-fold (OR = 0.427, $P = 0.003$) lower risks of CS-related disease compared to TT reference genotypes. In addition, this protective effect was observed in Saudi smokers independent of age, gender, types of smoking, duration, and average daily smoking consumption. Filling a research gap by exploring this topic in the Saudi population, the current findings indicate that genotype and allele distributions of MTHFR rs1801131 polymorphism present fundamental protective effects against the risk of CS-related disease. These findings should be verified in future studies with larger sample sizes, different ethnicities, and patients suffering from CS-related diseases, such as oral cancer and lung cancer.
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1. Introduction

Environmental factors, such as cigarette smoking (CS), contribute to the prevalence of serious global health problems. According to a World Health Organization (WHO) report, smoking kills approximately six million people around the world every year; more than five million of those deaths are due to CS use (2011). The prevalence of smoking decreased significantly from 1990 to 2015 (Collaborators, 2017); however, the number of smoking-related deaths is expected to rise annually, reaching approximately eight million deaths worldwide by 2030 (2011).

Cigarette smoke comprises carcinogenic and toxic substances that can lead to the development of cancer and other diseases (Luetragoon et al., 2018). CS can exacerbate asthma (Uh et al., 2018), and it contributes to immune response changes and induces chronic inflammation in the human body. Moreover, CS can lead to genetic alterations, which are important factors in the pathogenesis of smoking-related illnesses (Andersson et al., 2018). CS results in human DNA damage and causes DNA double-strand breaks; this damage can increase an individual's risk of cancer if not quickly repaired. Furthermore, a previous study has reported that CS can lead to the reduction of multiple DNA repair gene expressions (Romanowicz-Makowska et al., 2012). CS may also be associated

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with changes in global DNA methylation due to changes in the metabolism of homocysteine (Semmler et al., 2015).

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme that plays fundamental roles in the folic acid metabolic process (folate metabolic genes) (Goyette et al., 1998), DNA methylation, and DNA synthesis/repair (Lee et al., 2012, Kiyohara et al., 2011). THFR catalyzes the conversion of methylenetetrahydrofolate to methyltetrahydrofolate (the main circulating form of folate), which adds methyl groups that contribute to converting homocysteine to methionine (Zhao et al., 2018). The MTHFR gene is located on human chromosome 1 at 1p36.3 and contains 11 exons and 10 introns (Goyette et al., 1998).

Single nucleotide polymorphisms (SNPs) contribute to the genetic differences among humans, altering the DNA genome by substituting one of the four nitrogen bases (adenine, thymine, guanine, or cytosine) for another, which influences the gene expression profile (Lindgren, 2014). Two significant functional polymorphisms, C677T and A1298C, are the most common genetic variants identified in the MTHFR gene (Noori et al., 2017, Nassereddine et al., 2015). A1298C (rs1801131) involves a change from adenine to cytosine at position 1298 of exon 7, and C677T (rs1801133) involves a change from cytosine to thymine at position 677 of exon 4. In MTHFR, this substitution contributes to an alanine-to-valine conversion at amino acid 222, which results in the loss or reduction of MTHFR enzyme activity (Irfan et al., 2016, Al-Shahrani et al., 2016).

Studies have shown that MTHFR activity decreased by 70% in individuals with the homozygous TT genotype compared to those with the CC genotype (wild-type) (Vranekovic et al., 2010, Aly et al., 2014). Meanwhile, epidemiological investigations have examined correlations between MTHFR polymorphism and various diseases, including ischemic stroke, depressive disorders, and hypertension as well as many types of cancers, such as colorectal cancer, acute lymphoblastic leukemia, breast cancer, non-small cell lung cancer, and stroke (Zhang et al., 2017, Waseem et al., 2016, Li et al., 2015, Zhi et al., 2016, Bondarenko et al., 2016, Frikha et al., 2018, Ding et al., 2017, Fan et al., 2016, Kumar et al., 2020). Kiyoharal et al. observed that smokers with a MTHFR genotype linked with reduced folate levels could be susceptible to the development of lung cancer (Kiyohara et al., 2011). Other studies identified MTHFR rs1801131 prevalence among many ethnic groups (Kaya et al., 2016, Ramos-Silva et al., 2015, Romero-Sanchez et al., 2015). Meanwhile, rs1801131 polymorphism has been correlated with an increased risk for various diseases, such as type 2 diabetes (Poodineh et al., 2019), breast cancer (Ericson et al., 2009), bladder cancer (Xu and Zuo, 2020), myeloid leukemia (Dong et al., 2014), rheumatoid arthritis (Boughrara et al., 2015), coronary atherosclerosis (Gu et al., 2013b), and stroke (Kumar et al., 2020). However, the effects of this polymorphism among the Saudi population remain unclear, especially among patients suffering from smoking-related diseases. Consequently, the aim of this article was to compare the effects of genotype and allele distributions of MTHFR rs1801131 polymorphism among Saudi smokers and non-smokers.

2. Materials and methods

2.1. Ethics statement and study participants

The present study was approved by the Research Ethics Committee of the Applied Medical Sciences College at King Saud University in Riyadh, Saudi Arabia (reference number: 13/3536). After receiving this approval, we obtained blood samples from 235 cigarette and shisha smokers (211 males and 24 females) from the Blood Donation Center at Aleman Public Hospital in Riyadh, Saudi Arabia. In addition, 239 volunteers (142 males and 97 females) who had never smoked were recruited from the same hospital from August 2016 to August 2018.

This can be considered a single-center study because samples were only collected from Riyadh city of Saudi Arabia. All samples were collected from Self-reported healthy smokers and non-smokers (controls) who had signed informed consent forms confirming their participation in the present. We excluded any potential participants who self-reported having symptoms, such as metabolic disorders, inflammatory diseases, autoimmune diseases, cancer, or blood diseases. Detailed information, including age, medical history, and smoking consumption (average daily consumption and packs/year) was collected from each participant using a self-report questionnaire. A summary of the demographic characteristics of the smoker and non-smoker participants is given in Table 1.

2.2. DNA sample processing

As described by our previous works (Almutairi et al., 2019, Semlali et al., 2019a, Semlali et al., 2019b, Semlali et al, 2019c, Semlali et al., 2017, Semlali et al., 2018, Semlali et al., 2016). A peripheral blood (4 ml) was collected from each participant and placed in vacutainer vials with EDTA (anticoagulant vials). Then, genomic DNA was extracted from peripheral blood using the QIAmp DNA Blood Mini Kit, according to the manufacturer's protocol (Qiagen, catalogue number 69504). Before the genotyping reaction, the optical density and DNA concentration were calculated using NanoDrop 8000 devices, and an A_{260}/A_{280} ratio between 1.8 and 2.0 was required before the genotyping assay could be used.

2.3. SNP selection and genotyping assessment

We used two selection criteria for the MTHFR gene polymorphism: (1) position on the MTHFR gene (MTHFR rs1801131 polymorphism involves a change from glutamic acid to alanine (Glu429Ala) on an exonic region (functional consequence: missense) of chromosome 1) (S1 Table) and (2) correlation with many diseases, such as cardiovascular disease and cancers, among diverse ethnic groups (Boughrara et al., 2015, Dong et al., 2014, Ericson et al., 2009, Gu et al., 2013b, Poodineh et al., 2019, Xu and Zuo, 2020).

The concentrations of the DNA samples for smokers and non-smokers were diluted using a stock concentration of 10 ng/μl.

Table 1
Clinical characteristics of the individuals participating in the this study.

Variable	Non-Smokers n (%)	Smokers n (%)
Number of individuals	239 (100%)	235 (100%)
Mean of age ± SD	27.9 ± 8.63	28.5 ± 5.36
Age of individuals		
Below 29	169 (71%)	143 (61%)
Above 29	70 (29%)	92 (39%)
Gender		
Male	142 (59%)	211 (90%)
Female	97 (41%)	24 (10%)
Types of smoking		
Cigarette	–	185 (79%)
Shisha (Hookah)	–	50 (21%)
Years of smoking		
Smoking since < 10yrs	–	167 (71%)
Smoking since > 10yrs	–	68 (29%)
Average of Smoking/ Day		
<7 cig per day	–	99 (42%)
>7 cig per day	–	136 (58%)

N: Number, SD: Standard Deviation.

The genotyping assay was performed using a 10 μl-reaction mixture of each sample in 96-well plates. Each reaction mixture (10 μl) comprised 0.2 μl of 40 × TaqMan® Genotyping SNP Assay (Thermo Fisher Scientific; catalogue number 4351379), 6 μl of TaqMan® Genotyping Master Mix (Thermo Fisher Scientific; catalogue number 4371355), 2 μl of 10 ng genomic DNA, and a 10 μl-volume of ddH2O. All of the materials used in the genotyping were purchased from Applied Biosystems (Grand Island, NY, USA).

The PCR amplification protocol began with a denaturation initiation of 1 cycle at 96 °C for 7 min, followed by three 40-cycle stages: 96 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. Finally, the PCR reaction mixture underwent a 1-cycle extension at 72 °C for 7 min. The DNA genotyping was accomplished using the QuantStudio™ 7 Flex Real-time PCR System platform (Applied Biosystems Company, Grand Island, NY, USA), which is based on TaqMan genotyping analyses.

2.4. In silico analysis

Multiple *in silico* algorithmic software programs—Mutation Taster, PolyPhen-2.0 (Adzhubei et al., 2010), SIFT (Kumar et al., 2009), Mutation Assessor (Reva et al., 2011), FATHMM (Shihab et al., 2013), and CONDEL (Gonzalez-Perez and Lopez-Bigas, 2011) were used to identify the sequence and structural effects of the rs1801131 (Glu429Ala) mutation. 3D homology modelling of MTHFR was performed using the SWISS-MODEL template library (SMTL version 2017–10-23, PDB release 2017–10-13) (Biasini et al., 2014). Molecular graphics and 3D structure visualization were performed using PyMOL software (PyMOL Molecular Graphics System, v1.2r3pre, Schrödinger, Inc., http://www.pymol.org/).

2.5. Statistical analysis

The collected data was analyzed using SPSS statistical software, version 20.0 (Chicago, IL, USA). Continuous variables and non-continuous variables were estimated via means ± standard derivation (SD) and frequency counts (N, %), respectively. The Hardy–Weinberg Equilibrium (HWE) was estimated by the χ^2 test (two-tailed). Associations between the genetic variations and their interactions with CS were evaluated according to the odds ratio (OR) with a 95% confidence interval (CI) using logistic regression analyses with adjustments for age, gender, and smoking status. A P-value of<0.05 was used to represent statistical significance.

3. Results

3.1. Demographic distributions among the study participants

In total, blood samples were obtained 474 Saudi individuals living in the city of Riyadh. Table 1 shows the demographic distributions for the study sample. Among the smokers, 211 (90%) were male and 24 (10%) were female; among the non-smokers, 142 (59%) were males and 97 (41%) were female. The mean age ± SD of the non-smokers and smokers were similar (27.9 ± 8.63 and 28.5 ± 5.36, respectively). The smokers were divided into two age groups (61% was under 29 years old and 39% was over 29 years old). The non-smokers were also divided into two age groups (71% was under 29 years old and 29% was over 29 years old). Regarding smoking materials, 79% of smokers used cigarettes and 21% of smokers consumed shisha. The average smoking duration among the smokers was 10 years (71% was < 10 years, 29% was > 10 years). Lastly, 42% of the smokers had smoked less than seven times per day, while 58% had smoked more than seven times per day.

3.2. Global genotype and allelic frequencies among smokers and non-smokers

To analyze the global genetic frequency associations between MTHFR polymorphism and smoking, only rs1801131 (T/G) SNP (missense variant) was used. The genotype and allele frequencies among the smokers and non-smokers are shown in Table 2A. Briefly, the homozygous ancestral allele—TT—was used as a reference in the Saudi population in order to identify potential risks related to CS. The results showed that rs1801131 was statistically significantly correlated with the smoker population. Among the smokers, rs1801131 TG genotype carriers had a 0.209-fold lower risk of CS compared to TT genotype carriers (TG vs. TT: OR = 0.209, 95% CI = 0.117–0.373, P < 0.005). In addition, among the smokers, GG genotype carriers had a 0.427-fold lower risk of CS compared to TT genotype carriers (GG vs. TT: OR = 0.427, 95% CI = 0.240–0.759, P = 0.003). The G allele frequency in the smoking group was 56%, which was significantly higher than that of the control group. Among the smokers, G allele carriers had a 0.752-fold lower risk of CS compared to T allele carriers (G vs. T: OR = 0.752, 95% CI = 0.567–0.996, P = 0.047).

3.3. Relationship between MTHFR SNP rs1801131 and age

We also investigated how age affected the correlations between the MTHFR SNP and genotype and phenotype differences among smokers. Given the average age of the study population (~29 years old), we stratified the smokers and non-smokers into two groups: individuals older than 29 years (39% smokers and 29% non-smokers) and individuals younger than 29 years (61% smokers and 71% non-smokers) (Table 2B). Our analysis identified associations between MTHFR polymorphism and genotypic allocations among smokers in both age groups. Among smokers younger than 29 years old, TG genotype carriers had a 0.275-fold lower risk of CS effects compared to TT genotype carriers (TG vs. TT: OR = 0.275, 95% CI = 0.128–0.594, P = 0.00074). However, no correlations were found for the GG genotype or the G allele among the smokers compared to the non-smokers. In contrast, among smokers older than 29 years old, the risk of CS was 0.153 times and 0.369 times lower among TG and GG genotype carriers, respectively, compared with TT genotype carriers (TG vs. TT, P = 0.00002; GG vs. TT, P = 0.02427). Finally, no correlations were found for the G allele compared with the T reference allele in either age group (shown in Table 2B).

3.4. Relationship between MTHFR SNP rs1801131 and gender

We also examined how gender affected the MTHFR variant relationships; the genotype prevalence and allele distributions of the examined SNP in smokers and non-smokers based on gender are shown in Table 2C. There were correlations between the MTHFR variant and smokers of both genders. Genotype frequency associations were observed in the TG genotype among males and in the TG and GG genotypes and the G allele among females. These frequencies were 0.262, 0.007, 0.008, and 0.007 times lower, respectively, than those of the TT genotype and the T allele: TG among males (OR = 0.262; CI = 0.125–0.546; *P* = 0.00022); TG among females (OR = 0.007; CI = 0.001–0.127; P < *0.005*; GG among females (OR = 0.008; CI = 0.001–0.147; P < 0.005, and G allele among females (OR = 0.007; CI = 0.001–0.114; *P* < 0.005 (Table 2C).

3.5. Relationship between MTHFR SNP rs1801131 and types of smoking

To study the link between types of smoking and the MTHFR variant, we classified the smokers into cigarette and shisha users.

Table 2
Genotype frequencies of rs1801131polymorphism in smokers and controls (non-smokers) according to different clinical parameters.

SNP	Variant	Smokers	Controls	OR	95% CI	χ^2 Value	P- Value
2a. Global genotype frequencies in smokers and non-smokers							
rs1801131	TT	59 (0.30)	23 (0.11)	Ref			
	TG	58 (0.29)	108 (0.53))	0.209	0.117–0.373	30.17	**<0.005***
	GG	81 (0.41)	74 (0.36)	0.427	0.240–0.759	8.60	**0.00336***
	T	139 (0.44)	182 (0.54)	Ref			
	G	176 (0.56)	154 (0.46)	0.752	0.567–0.996	3.95	**0.04694***
2b. Smokers and non-smokers age							
Below 29	TT	27 (0.26)	14 (0.11)	Ref			
	TG	34 (0.33)	64 (0.52)	0.275	0.128–0.594	11.40	**0.00074***
	GG	43 (0.41)	45 (0.37)	0.495	0.230–1.069	3.25	0.07129
	T	77 (0.47)	109 (0.54)	Ref			
	G	88 (0.53)	92 (0.46)	0.815	0.559–1.188	1.14	0.28667
Above 29	TT	32 (0.34)	9 (0.11)	Ref			
	TG	24 (0.26)	44 (0.54)	0.153	0.063–0.374	18.72	**0.00002***
	GG	38 (0.40)	29 (0.35)	0.369	0.152–0.892	5.08	**0.02427***
	T	62 (0.41)	73 (0.54)	Ref			
	G	88 (0.59)	62 (0.46)	0.691	0.451–1.058	2.90	0.08838
2c. Smokers and non-smokers gender							
Male	TT	38 (0.21)	12 (0.10)	Ref			
	TG	58 (0.33)	70 (0.56)	0.262	0.125–0.546	13.63	**0.00022***
	GG	81 (0.46)	42 (0.34)	0.609	0.288–1.287	1.70	0.19170
	T	139 (0.51)	112 (0.54)	Ref			
	G	134 (0.49)	94 (0.46)	1.002	0.717–1.400	0.001	0.99004
Female	TT	21 (1.00)	11 (0.14)	Ref			
	TG	0 (0.00)	37 (0.46)	0.007	0.001–0.127	34.90	**<0.005***
	GG	0 (0.00)	32 (0.40)	0.008	0.001–0.147	31.26	**<0.005***
	T	0 (0.00)	69 (0.54)	Ref			
	G	42 (1.00)	59 (0.46)	0.007	0.001–0.114	53.02	**<0.005***
2d. Types of smoking							
Cigarette	TT	36 (0.25)	23 (0.11)	Ref			
	TG	42 (0.29)	108 (0.53)	0.248	0.132–0.468	19.73	**<0.005***
	GG	66 (0.46)	74 (0.36)	0.570	0.307–1.059	3.20	0.07373
	T	108 (0.49)	182 (0.54)	Ref			
	G	114 (0.51)	154 (0.46)	0.918	0.674–1.251	0.29	0.58861
Shisha	TT	20 (0.69)	23 (0.11)	Ref			
	TG	7 (0.24)	108 (0.53)	0.075	0.028–0.197	36.10	**<0.005***
	GG	2 (0.07)	74 (0.36)	0.031	0.007–0.143	35.09	**<0.005***
	T	9 (0.16)	182 (0.54)	Ref			
	G	47 (0.84)	154 (0.46)	0.141	0.071–0.280	39.19	**<0.005***
2e. Years of smoking							
Smoking since < 10yrs	TT	44 (0.42)	23 (0.11)	Ref			
	TG	28 (0.27)	108 (0.53)	0.136	0.070–0.261	39.86	**<0.005***
	GG	33 (0.31)	74 (0.36)	0.233	0.122–0.447	20.26	**<0.005***
	T	61 (0.34)	182 (0.54)	Ref			
	G	116 (0.66)	154 (0.46)	0.487	0.348–0.683	17.65	**0.00003***
Smoking since > 10yrs	TT	12 (0.18)	23 (0.11)	Ref			
	TG	22 (0.32)	108 (0.53)	0.390	0.169–0.900	5.08	**0.02418***
	GG	34 (0.50)	74 (0.36)	0.881	0.393–1.975	0.10	0.75759
	T	56 (0.55)	182 (0.54)	Ref			
	G	46 (0.45)	154 (0.46)	1.177	0.783–1.769	0.61	0.43308
2f. Average of smoking/day							
<7 cig per day	TT	32 (0.32)	23 (0.11)	Ref			
	TG	35 (0.36)	108 (0.53)	0.233	0.121–0.450	20.16	**<0.005***
	GG	32 (0.32)	74 (0.36)	0.311	0.158–0.612	11.85	**<0.005***
	T	67 (0.40)	182 (0.54)	Ref			
	G	99 (0.60)	154 (0.46)	0.602	0.427–0.848	8.50	**0.00355***
>7 cig per day	TT	25 (0.32)	23 (0.11)	Ref			
	TG	18 (0.23)	108 (0.53)	0.153	0.072–0.326	26.69	**<0.005***
	GG	34 (0.45)	74 (0.36)	0.423	0.211–0.849	6.00	**0.01433***
	T	52 (0.43)	182 (0.54)	Ref			
	G	68 (0.57)	154 (0.46)	0.761	0.523–1.108	2.04	0.15322

*P < 0.05, SNP: Single Nucleotide Polymorphism, Ref = Reference allele, OR: Odd Ratio.

The MTHFR polymorphism distributions at the genotype and allele levels among smokers and non-smokers are described in Table 2D. Among cigarette smokers, only TG genotype frequencies were highly associated with a decreased risk of CS effects (OR = 0.248; CI = 0.132–0.468; *P* < 0.005); however, TG and GG genotypes and the G allele were correlated with a decreased risk of CS effects among shisha users compared to the controls: TG (OR = 0.075; CI = 0.028–0.197; P < 0.005); GG (OR = 0.031; CI = 0.007–0.143; P < 0.005), and G (OR = 0.141; CI = 0.071–0.280; *P* < 0.005) (Table 2D).

3.6. Relationship between MTHFR SNP rs1801131 and years of smoking

We also studied whether MTHFR SNP was connected with smoking duration in order to identify variations in the genotype

and allele frequencies. In Saudi Arabia, people start smoking at a very early age (before 16 years old among males) (Almutairi, 2014), which increases the rate of oral diseases, especially those related to smoking. The smokers were distributed into short-term smokers (<10 years) and long-term smokers (10 years or more). Table 2E shows the distribution of the MTHFR variant in the smokers and non-smokers. Among short-term smokers, the risk of CS was approximately 0.136, 0.233, and 0.487 times lower compared to non-smokers among the TG and GG genotype and G allele carriers, respectively (P < 0.005, P < 0.005, and $P = 0.00003$, respectively); however, among long-term smokers, genetic variation was associated with a decreased risk of CS of about 0.390 fold among TG genotype carriers compared with non-smokers ($P = 0.02418$), as presented in Table 2E.

3.7. Relationship between MTHFR SNP rs1801131 and average daily smoking consumption

Since smoking shisha is very popular in the Middle East, we investigated the connection between MTHFR genetic and allelic variations and average daily smoking consumption of cigarettes and shisha. We classified the smokers into two categories: moderate smokers consumed less than seven cigarettes and/or shisha per day (Category A) and heavy smokers smoked more than seven cigarettes and/or shisha daily (Category B). Table 2F compares the genotype frequencies for the participants' categories A and B with those of the controls.

The TG and GG genotypes and G alleles for Category A and the TG and GG genotypes for Category B were statistically correlated with decreased risks of CS compared to the non-smokers. For Category A, TG and GG genotype and the G allele carriers had 0.233-fold, 0.311-fold, and 0.602-fold lower risks, respectively, than the TT genotype and T allele carriers ($P < 0.005$ for TG and GG; $P = 0.00355$ for G). For Category B, TG and GG genotype carriers had 0.153-fold ($P < 0.005$) and 0.423-fold ($P = 0.01433$) lower risks than the TT reference genotype (shown in Table 2F).

3.8. In silico sequence and structure-based analysis

The 3D-structure visualization of the MTHFR protein (UniPro: P42898) was performed by PyMOL using the Protein Data Bank (PDB) 6FCX x-ray structure with 2.5 Å resolution. The PyMOL mutagenesis tool was used to mutate the native residue glutamic acid to alanine at position 429. The wild-type and smaller-sized mutant amino acids differed in charge. In addition, the mutation introduced a more hydrophobic residue (Fig. 1). Multiple *in silico* algorithms were used to predict protein stability and functionality; most of them strongly predicted that the mutation of a glutamic acid to alanine at position 429 was "tolerated" and had no obviously damaging consequences except CONDEL (Table 3). The p. Glu429Ala substitution occurred at a moderately conserved residue with a medium functional impact score. The allele frequency of the MTHFR variant observed in the genome aggregation database (gnomAD) among different ancestry was 0.2902 (male = 0.3019 and female = 0.2759), as shown in Table 4.

4. Discussion

CS is a major environmental risk factor for a wide range of serious diseases, and associations have been found between the genetic influences caused by CS and chronic obstructive pulmonary disease, cardiovascular disease, oral and other cancers, and periodontal disease (Lutz et al., 2018, Ramoa et al., 2017). However, the specific effects of these genetic risk factors are still largely unknown (Lutz et al., 2018). Other studies have shown that CS has become a major public health issue among Saudi adolescents (Algorinees et al., 2016) and that smoking tobacco is to a potential risk factor for oral cancer (Alharbi and Quadri, 2018).

Genetic polymorphisms are responsible for inter-individual variability in susceptibility to cancer and other disorders (Saifullah and Tsukahara, 2018). As noted above, MTHFR is a critical enzyme involved in the conversion of methylenetetrahydrofolate to methyltetrahydrofolate, and it affects DNA synthesis and methylation (Yousef et al., 2013). Multiple causes of malignancy have been reported, including isolate deficiency in cardiovascular, coronary artery, neurological, and infertility diseases (Liew and Gupta, 2015). In a previous study, A1298C was correlated with vascular diseases (Hankey and Eikelboom, 2005), the risk of ischemic and hemorrhagic stroke (Kumar et al., 2020). To the best of our knowledge, no studies have evaluated whether the presence/absence of the A1298C variant in the *MTHFR* gene interferes with smoking risk. Hence, the main aim of this study was to compare *MTHFR* polymorphism among Saudi smokers and non-smokers. This work may contribute to the detection of a genetic marker for preventing CS-related diseases.

We found a decreased significant association between global genotype frequencies of *MTHFR* rs1801131 polymorphism and risk of CS effects in the study population compared to non-smoking individuals. Interestingly, there were lower genotypic and allelic differences between the MTHFR variant and risk of CS based on age, gender, types of smoking, years of smoking, and average daily smoking consumption among smokers compared to non-smokers. These findings suggest that the genotype and allele distributions were less represented in the smokers, which may indicate a fundamental protective Of MTHFR rs1801131 polymorphism against the risk of CS-related disease.

The results of this study are consistent with those of previous studies of various diseases and ethnicities, such as liver cancer among Caucasian individuals (Liang et al., 2014), Parkinson's disease in the Chinese Han population (Yuan et al., 2016), breast cancer in the Chinese population (Lu et al., 2015), acute lymphoblastic leukemia among Chinese children (Xia et al., 2017), breast cancer risk in the northern Sardinian population (Floris et al., 2020), and acute lymphoblastic leukemia in the Iranian population (Bahari et al., 2016).

However, a few studies showed that MTHFR polymorphism was not associated with the development of lung cancer (Bailey et al., 1999, Liu et al., 2009). Meanwhile, another study found that the prevalence of lung cancer in the Saudi population increased significantly due to the increased incidence of CS in both genders (Alamoudi, 2010). According to the Saudi Cancer Registry, in 2015, lung cancer was ranked fifth among males and fifteenth among females (Registry, 2015). The age-standardized rate (ASR) was 4.8/100,000 among Saudi men and 1.3/100,000 among Saudi women. However, the ASR rate per 100,000 females was 20.8 in China, 33.7 in the United States (US), 25.8 in the United Kingdom (UK), 5.1 in Bahrain, 5.3 in Qatar, 2.7 in Kuwait, 1.4 in the United Arab Emirates (UAE), and 3.2 in Oman. The ASR rate per 100,000 males was 52.8 in China, 44.2 in the US, 34.9 in the UK, 14.1 in Bahrain, 14 in Qatar, 9.5 in Kuwait, 8.9 in the UAE, and 4.9 in Oman (Registry, 2015). Therefore, the Saudi Cancer Registry data showed that each gender in Saudi Arabia had the lowest ASR rate for lung cancer compared to other countries, including Arab Gulf countries. This could be explained by one of the findings of the present study, namely our conclusion that MTHFR rs1801131 polymorphism was a protective factor against the risk of CS-related disease in the study population.

Interestingly, this study showed that gender affected the association between *MTHFR* rs1801131 polymorphism and the risk of CS-related disease. For example, MTHFR polymorphism offered more protection against the risk of CS among females than males,

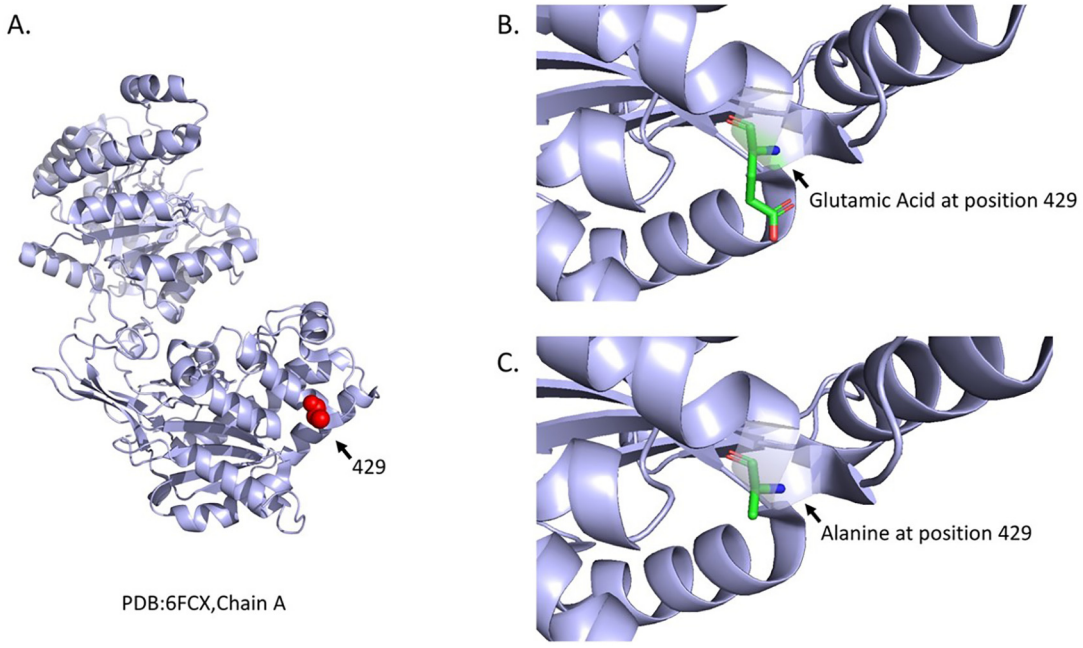

Fig. 1. (A) The structure of MTFHR, PDB:6FCX, chain A, and the red sphere amino acid indicate position 429. (B) and (C) show the structures of the wild-type residues (glutamic acid and mutant, respectively), which differ in size, charge, and hydrophobicity.

Table 3
Predict the damaging and pathogenicity effects of the MTHFR rs1801131 (Glu429Ala) mutation.

In silico prediction	Prediction	Score
MutationTaster	Polymorphism automatic	Accuracy 0.00000165
FATHMM	Tolerated	−0.78
MetaSVM	Tolerated	−1.0759
MetaLR	Tolerated	0
MutationAssessor	Low Functional effect	1.45
SIFT	Tolerated	0.18
PolyPhen	Benign	0.021
CONDEL	**Damaging**	**0.530**
CADD	**Damaging**	**22.1**
FATHMM	Tolerated	−0.78

although the number of female smokers was not sufficient given their low prevalence compared to male smokers and the social traditions in Saudi Arabia. This gender difference was also found in colon and lung cancer risk studies. Specifically, Curtin et al. found that the TG, GG, and T genotypes of the MTHFR rs1801131 variant were associated with a significantly decreased risk of colon cancer in women (Curtin et al., 2004). Furthermore, the MTHFR rs1801133 variant was associated with a significantly reduced risk of lung cancer among women (Shi et al., 2005). These findings suggest that gender may play a vital role in the correlation between MTHFR polymorphism and the risk of cancer or other diseases.

Two possible hypotheses have been suggested to explain this correlation. The first hypothesis is related to homocysteine levels. Studies have shown that there is an interaction between homocysteine levels and estrogen status (Bruschi et al., 2005, Smolders et al., 2003). Homocysteine was detected at lower levels among pregnant and premenopausal women on hormonal replacement therapy than among age-matched men (Smolders et al., 2003). In addition, smokers exhibited higher progesterone and testosterone levels (Duskova et al., 2012) and lower estrogen balances than non-smokers (Duskova et al., 2012, Gu et al., 2013a). The second hypothesis is related to methylation capacity. A previous work showed that there was a significant gender difference in the methylation pattern of the MTHFR gene at higher levels of methylation (Sarter et al., 2005).

Meanwhile, the present findings show that the mutant genotype among smokers in both age groups had a protective role against the risk of CS-related disease. Similarly, Almutairi et al. (2019) found a correlation between the TDG rs4135050 variant and a decreased smoking risk among smokers younger than 29 years old (Almutairi et al., 2019). Another study result showed that the TLR2 rs3804100 variant had decrease significance

Table 4
Predict the allele frequency of the MTHFR rs1801131 variant in population databases gnomAD Exomes Version: 2.0.2 = 0.29.

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
African	2417	15,304	204	0.1579
Ashkenazi Jewish	2906	9850	439	0.295
East Asian	3780	17,246	441	0.2192
European (Finnish)	7139	22,298	1143	0.3202
European (Non-Finnish)	35,620	111,706	5780	0.3189
Latino	5373	33,580	497	0.16
South Asian	12,658	30,782	2665	0.4112
Other	1565	5484	231	0.2854
Total	**71,458**	**246,250**	**11,400**	**0.2902**
Male	40,732	134,896	6679	0.3019
Female	30,726	111,354	4721	0.2759

(protective effect) among short-term smokers (<16 years of smoking) and long-term smokers (>16 years of smoking), while the TLR2 rs3804099 variant only played a protective role among long-term smokers (Kohailan et al., 2016).

The 3D-structure visualization of the MTHFR protein conducted in the present study showed that mutating the wild-type residue can cause the loss of interactions with other residues or molecules, such as hydrogen, due to charge, size, and hydrophobicity differences between the wild-type and mutant amino acids. Multiple *in silico* algorithms confirmed that such a change was tolerated and had a benign effect on the structure. Furthermore, the identified p.Glu429Ala substitution occurred at a moderately conserved residue with a medium functional impact score. High allele frequency has been observed in population databases, also indicating the familiarity of this variant. The variant has been annotated as benign in ClinVar in more than eight entries, has been labelled as a common polymorphism, and has been associated with a decreased risk of adult acute leukemia.

5. Conclusion

In summary, the results of this study demonstrate that MTHFR rs1801131 polymorphism might be a potential genetic marker that can help prevent the risk of CS-related diseases. Further genetic and functional studies with sufficiently larger samples using different ethnicities are recommended to confirm the correlation between smoking effect and the *MTHFR* genetic variant.

6. Ethics approval and participant consent:

This study was approved by the Research Ethics Committee of the Applied Medical Sciences College at King Saud University in Riyadh, Saudi Arabia (reference number: 13/3536). All study participants signed informed consent forms confirming their participation.

Author contributions

MHA wrote the first draft of the manuscript and conducted the genotyping experiments, NSA performed the *in silico* analysis, NRP analyzed the genotyping data, BOA and AFA collected the samples and the clinical data, MR interpreted the results and contributed to the writing of the manuscript, and AS designed all experiments and wrote the final manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.04.011.

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