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Risk of colorectal cancer associated with the methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism in the Kashmiri population

A.S. Sameer[1,2], Z.A. Shah[1], S. Nissar[1], S. Mudassar[2] and M.A. Siddiqi[1]

[1]Department of Immunology and Molecular Medicine,
Sher-I-Kashmir Institute of Medical Sciences, Kashmir, India
[2]Department of Clinical Biochemistry,
Sher-I-Kashmir Institute of Medical Sciences, Kashmir, India

Corresponding author: M.A. Siddiqi
E-mail: vc.tmuk@gmail.com

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ABSTRACT. Methylenetetrahydrofolate reductase (*MTHFR*) is a critical enzyme in folate metabolism and is involved in DNA synthesis, DNA repair and DNA methylation. The two common functional polymorphisms of *MTHFR*, 677 C→T and 1298 A→C, have been shown to impact various diseases, including cancer. The 677 C→T polymorphism has been widely investigated in different cancers and has been implicated as a risk factor for the development of various cancers. We investigated *MTHFR C677T* genotype frequency in colorectal cancer cases in the Kashmiri population and correlated this information with the known clinicopathological characters of colorectal cancer, in a case-control study. Eighty-six colorectal cancer cases were studied for *MTHFR C677T* polymorphism, compared to 160 controls taken from the general population, employing the PCR-RFLP technique. We found the frequency of the three different genotypes of *MTHFR* in our ethnic Kashmir population, i.e., CC, CT and TT, to be

68.6, 20.9 and 10.4% among colorectal cancer cases and 75.6, 16.9 and 7.5% among the general control population, respectively. There was a significant association between the *MTHFR* TT genotype and colorectal cancer in the higher age group. We conclude that the *MTHFR* C677T polymorphism slightly increases the risk for colorectal cancer development in our ethnic Kashmir population.

Key words: Colorectal cancer; *MTHFR*; Polymorphism; RFLP; Restriction digestion; Kashmir

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the third leading cause of cancer death in the world (Center et al., 2009a,b). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. Colorectal cancer is the third most common cancer in men and the second most common cancer in women worldwide (Jemal et al., 2011). To date, Kashmir has been reported as a high-incidence area of gastrointestinal (GIT) cancers (Mir et al., 2005; Murtaza et al., 2006). In Kashmir Valley, CRC represents the third most common GIT cancer after esophageal and gastric (Sameer et al., 2010a,b).

Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme regulating the metabolism of folates, which are important nutrients required for both DNA synthesis and DNA methylation (Lucock, 2000; Cicek et al., 2004). *MTHFR* irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating folate and the one-carbon donor for remethylation processes (Bailey and Gregory III, 1999).

Two common functional polymorphisms have been defined in the *MTHFR* gene - one is *C677T* and other *A1298C*. *MTHFR C677T* polymorphism is the most important one regulating the function of this enzyme. This polymorphism results in an alanine-to-valine substitution at codon 222 of the protein (Frosst et al., 1995). This polymorphism has a profound effect on the MTHFR protein, not only does it decrease the thermal stability of this enzyme but also reduce its activity (Cicek et al., 2004). Individuals with the variant Val/Val genotype (TT) have no more than 30% of normal enzyme activity, and heterozygotes (CT) have 65% of normal enzyme activity (Frosst et al., 1995; Kono and Chen, 2005). This substitution also results in lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate and increased plasma homocysteine levels (Frosst et al., 1995; Ma et al., 1997; Bagley and Selhub, 1998).

Several studies from around the globe have reported on the association of *MTHFR C677T* polymorphism with the risk of colorectal carcinoma. While many studies have found the homozygous TT variant form to be inversely associated with the risk of colorectal carcinoma (Ma et al., 1997; Chen et al., 1996, 1998; Slattery et al., 1999; Houlston and Tomlinson, 2001; Marugame et al., 2003), others have reported lack of any association (Sachse et al., 2002; Shannon et al., 2002) or have associated the TT variant form with an increased risk of developing CRC (Park et al., 1999; Levine et al., 2000; Yin et al., 2004).

Therefore, we carried out a case-control study in our population to determine if this *MTHFR C677T* polymorphism is associated with an altered risk of developing CRC, as many

dietary habits (Siddiqi et al., 1992; Mir et al., 2005; Murtaza et al., 2006; Sameer et al., 2010b) of our population have already been established as risk factors for the development of GIT cancers. We also investigated whether there is a link between these risk factors and the *MTHFR C677T* genotype and CRC predisposition.

MATERIAL AND METHODS

Study population

This study included 86 consecutive primary CRC patients. All CRC patients were recruited from the Department of Surgery, Sher-I-Kashmir Institute of Medical Science from March 2008 to August 2009. Tumor types and stages were determined by two experienced pathologists. Blood samples of 160 age- and gender-matched cases with no signs of any malignancy were collected for controls. The mean age of both patient and control groups was 52 years old, and 56 patients and 104 controls were >50 years or older. See Table 1 for details.

Data on all CRC patients were obtained from personal interviews with patients and or guardians, medical records and pathology reports. The data collected included gender, age, dwelling, tumor location, Dukes stage, lymph node status, pesticide exposure, and rectal bleeding. All patients and or guardians were informed about the study and their consent to participate in this study was taken on predesigned questionnaire (available on request). The collection and use of tumor and blood samples for this study were previously approved by the appropriate Institutional Ethics Committee.

DNA extraction and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

DNA extraction was performed using any one of the previously described techniques. Previously reported primers, forward primer 5'-GGTCAGAAGCATATCAGTCAT GAG-3' and the reverse primer 5'-CTGGGAAGAACTCAGCGAACTCAG-3' (Cicek et al., 2004), were used for the amplification of the 494-bp target region within the *MTHFR* gene.

PCR was carried out in a final volume of 25 µL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools) with 2 mM $MgCl_2$, 0.4 µM of each primer (Genescript), 50 µM dNTPs (Biotools), and 0.5 U DNA polymerase (Biotools). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 7 min, followed by 35 cycles of denaturation for 30 s at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min. For RFLP, the PCR product of *MTHFR* was digested with *Hin*fI (2 U at 37°C for 16 h) (Fermentas). In the case of *MTHFR C677T* polymorphism, the wild-type Ala/Ala (CC) was identified by 394- and 100-bp bands, while the Val/Val (TT) variant was identified by 229-, 165- and 100-bp bands and the heterozygous Ala/Val (CT) variant displayed all four bands (394, 229, 165, and 100 bp) (Figure 1).

DNA fragments were electrophoresed through a 2-3% agarose gel for resolution. The genotypes of >20% of the samples were double blindly reassessed to confirm the results by two independent researchers. A positive control for each polymorphism was used for 50% of samples.

Statistical analysis

Observed frequencies of genotypes in CRC were compared to controls using chi-square or Fisher exact tests when expected frequencies were small. The chi-square test was used to determine whether genotype distributions were in Hardy-Weinberg equilibrium. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using the PASW version 18 software.

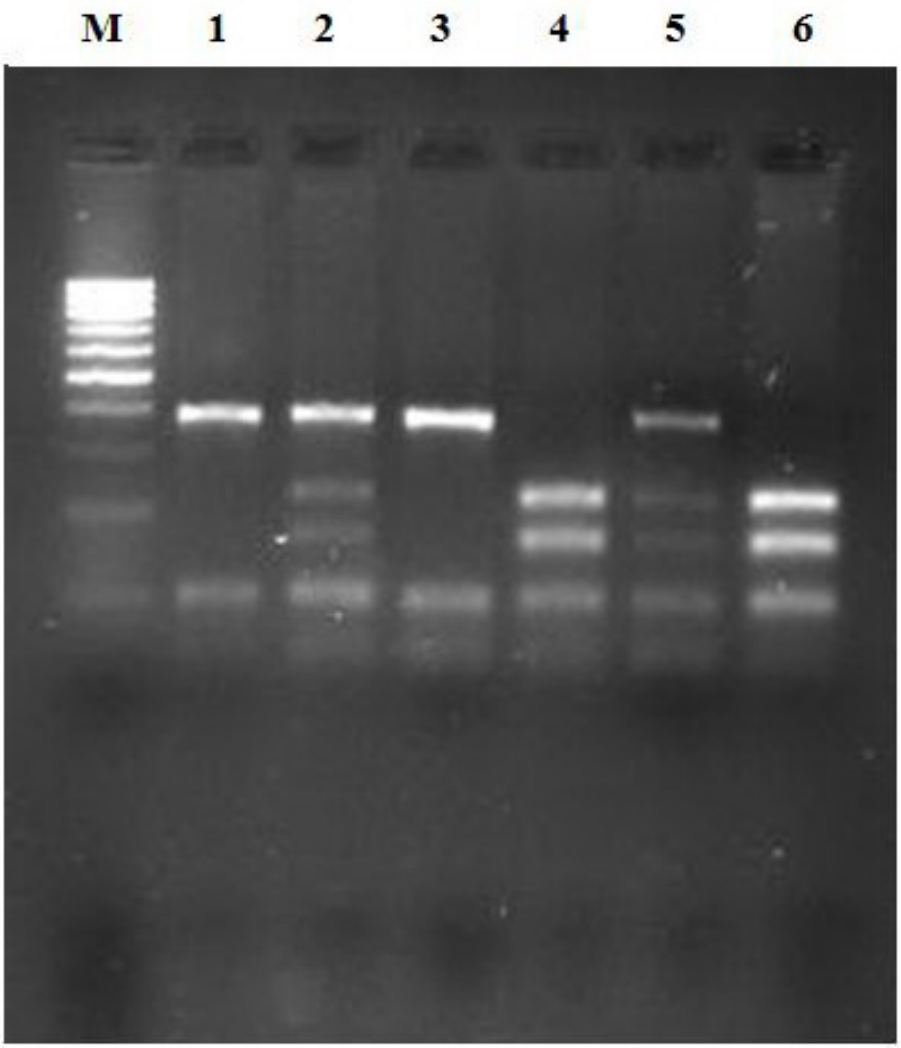

Figure 1. Representative gel of *MTHFR C677T* polymorphism, representing amplicon digest with *Hin*fI (G|ANTC/CTNA|G); where the variant TT is cleaved but not the wild-type CC. *Lane M* = 100-bp ladder. *Lanes 1* and *3* = wild-type (CC) form (394 and 100 bp). *Lanes 2* and *5* = heterozygous (CT) variant form (394, 229, 165, and 100 bp). *Lanes 4* and *6* = homozygous (TT) variant form (229, 165 and 100 bp).

RESULTS

A total of 86 CRC patients and 160 control subjects were included in this study. The patients comprised 49 males and 37 females (M/F ratio = 1.32) and the control subjects consisted of 88 males and 72 females (M/F ratio = 1.2). Mean age in patient and control groups was 52 years. No significant gender- or age-related differences were observed between the groups (P > 0.05). Furthermore, of 86 confirmed cases of CRC, 81 cases were sporadic, 4 were familial adenomatous polyposis (FAP) and one case was hereditary non-polyposis colorectal cancer (HNPCC). All cases were adenocarcinoma except one of squamous cell carcinoma (SCC) of the basal cell type; 59 were rural and 27 urban; 36 cases had carcinoma in the colon and 50 in the rectum, and 55 were smokers and 31 nonsmokers (Table 1).

Table 1. Frequency distribution analysis of selected demographics and risk factors in colorectal cancer cases and controls.

Variable	Cases (N = 86)	Controls (N = 160)	P
Age group (years)			
≤50	30 (34.9%)	56 (35.0%)	1.00
>50	56 (65.1%)	104 (65.0%)	
Gender			
Female	37 (43.0%)	72 (45.0%)	0.764177
Male	49 (67.0%)	88 (55.0%)	
Dwelling			
Rural	59 (68.6%)	104 (65.0%)	0.565659
Urban	27 (31.4%)	56 (35.0%)	
Smoking status			
Never	31 (36.0%)	75 (46.8%)	0.102256
Ever	55 (64.0%)	85 (53.2%)	
Pesticide exposure			
Never	33 (38.4%)	75 (46.8%)	0.200325
Ever	53 (61.6%)	85 (53.2%)	

Data are reported as numbers with percent in parentheses.

In our study, we also found a varied difference in the genotype frequency of *MTHFR C677T* between CRC cases and the matched controls. The incidence of the *MTHFR* Val allele was increased in patients with CRC compared with healthy controls (Table 2). The frequency of Ala/Val (CT) genotype in CRC cases was 20.9% and that of Val/Val (TT) was 10.4%, as compared to healthy controls, where it was 16.9 and 7.5%, respectively. The overall hazard ratio of the *MTHFR* Val allele in patients with CRC was 1.41 (95%CI = 0.79-2.54). Overall, both the heterozygous CT genotype (Ala/Val) and the homozygous variant TT genotype (Val/Val) were associated with a modestly elevated risk for CRC (OR = 1.36; 95%CI = 0.70-2.67 and OR = 1.53; 95%CI = 0.61-3.85, respectively). The overall hazard ratio for the T allele was 1.41 (95%CI = 0.79-2.54) (Table 2).

The correlation of *MTHFR C677T* polymorphic status with the clinicopathological characteristics was carefully analyzed. It was found that the Val/Val variant status increased the risk of CRC in the higher age group, smokers and the site of the tumor but not with the other variables (Table 3).

Table 2. Genotype frequencies of *MTHFR C677T* gene polymorphism in cases and controls and their associations with the risk of colorectal cancer.

MTHFR genotype	Cases (N = 86)	Controls (N = 160)	OR (95%CI)
Ala/Ala (CC)	59 (68.6%)	121 (75.6%)	1.00 (Ref.)
Ala/Val (CT)	18 (20.9%)	27 (16.9%)	1.36 (0.70-2.67)
Val/Val (TT)	9 (10.4%)	12 (7.5%)	1.53 (0.61-3.85)
Ala/Val (CT) + Val/Val (TT)	27 (31.3%)	39 (24.4%)	1.41 (0.79-2.54)

Data are reported as number with percent in parentheses.

DISCUSSION

This is the first study to report on the association of *MTHFR* genotype with the risk of development of CRC in our Kashmiri population. The Kashmir Valley is located in the northern part of India, walled by the mighty Himalayas, its unique ethnic population live in discrete temperate environmental conditions and have distinctive eating habits, which play an overwhelming role in the development of GIT cancers over the genetic factors (Murtaza et al., 2006; Salam et al., 2009; Sameer et al., 2010a,b). As previously reported (Siddiqi et al., 1992), the etiology and incidence of various GIT cancers in this population have been attributed to the probable exposure to nitroso compounds, amines and nitrates reported to be present in the local foodstuffs. The various unique eating habits of our population include the consumption of sun-dried and smoked fish and meat, dried and pickled vegetables, red chili, *Hakh* (a leafy vegetable of the *Brassica* family), and hot *noon chai* (salted tea) and are accompanied by *Hukka* (water pipe) smoking (Mir et al., 2005).

The *MTHFR* gene, located on 1p36.22, encompasses 19.3 kb of DNA and is composed of 11 exons. The gene codes for a 74.6-kDa protein of 656 amino acids (Saffroy et al., 2005). It is a cytosolic enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, a co-substrate for homocysteine remethylation to methionine with subsequent production of S-adenosyl methionine, the universal methyl donor in humans, required for DNA methylation. The methylation of homocysteine is catalyzed by the enzyme methionine synthase, which requires the co-factor vitamin B12. *MTHFR* is also linked to the production of dTMP via thymidylate synthase and to purine synthesis and, therefore, plays a role in the provision of nucleotides essential for DNA synthesis (Wagner, 1995). Thus, any defect in the *MTHFR* gene will be reflected in an imperfection in the methylation pattern of DNA as well as in its synthesis.

It has been suggested by Chen et al. (1996) that the low activity of *MTHFR 677TT* genotype is probably advantageous because it ensures an adequate thymidylate pool for DNA synthesis when the folate supply is sufficient. However, in the folate-depleted situation high activity of the *677CC* genotype may be disadvantageous because 5,10-methylenetetrahydrofolate is converted and the thymidylate pool is depleted as suggested by Keku et al. (2002). Also, as per Haghighi et al. (2009) increased risk for *677TT* versus *677CC* would be seen in the folate-depleted situation if aberrant DNA methylation is a primary mechanism.

In this study, we analyzed 86 CRC patients in relation to 160 healthy controls in order to examine the role of the *C677T* SNP in the *MTHFR* gene in CRC risk in the Kashmiri population. Cancer patients and healthy control subjects were well-matched for age, gender, ethnic distribution, and tobacco use. We found the frequency of the three different genotypes of *MTHFR* in our ethnic Kashmir population, i.e., CC, CT and TT, to be 68.6, 20.9 and 10.4%

Table 3. Association between *MTHFR C677T* gene polymorphism and clinicopathological characteristics.

Variables	Cases (N = 86)		OR (95%CI)
	Group I	Group II	
Age group (years)	≤50, N = 30 (34.9%)	>50, N = 56 (65.1%)	
Ala/Ala (CC); N = 59 (68.6%)	15	44	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	7	11	1.8 (0.6-5.7)
Val/Val (TT); N = 9 (10.4%)	6	3	5.8 (1.3-26.4)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	15	12	3.7 (1.4-9.5)
Gender	Male = 49 (67.0%)	Female = 37 (43.0%)	
Ala/Ala (CC); N = 59 (68.6%)	34	25	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	10	8	0.9 (0.3-2.7)
Val/Val (TT); N = 9 (10.4%)	5	4	0.9 (0.2-3.7)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	15	12	0.9 (0.3-2.3)
Dwelling	Rural = 59 (68.6%)	Urban = 27 (31.4%)	
Ala/Ala (CC); N = 59 (68.6%)	42	17	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	11	7	0.6 (0.2-1.9)
Val/Val (TT); N = 9 (10.4%)	6	3	0.8 (0.2-3.6)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	17	10	0.6 (0.3-1.8)
Smoking status	Never = 31 (36.0%)	Ever = 55 (64.0%)	
Ala/Ala (CC); N = 59 (68.6%)	20	39	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	6	12	0.9 (0.3-2.9)
Val/Val (TT); N = 9 (10.4%)	5	4	2.4 (0.6-10.1)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	11	16	1.3 (0.5-3.4)
Tumor location	Colon = 36 (41.9%)	Rectum = 50 (58.1%)	
Ala/Ala (CC); N = 59 (68.6%)	23	36	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	7	11	0.9 (0.3-2.9)
Val/Val (TT); N = 9 (10.4%)	6	3	3.1 (0.7-13.7)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	13	14	1.4 (0.5-3.6)
Nodal status	Involved = 48 (55.8%)	Not involved = 38 (44.2%)	
Ala/Ala (CC); N = 59 (68.6%)	33	26	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	10	8	0.9 (0.3-2.8)
Val/Val (TT); N = 9 (10.4%)	5	4	0.9 (0.2-4.0)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	15	12	0.9 (0.3-2.4)

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Table 3. Continued.

Variables	Cases (N = 86)		OR (95%CI)
	Group I	Group II	
Tumor grade	A+B = 38 (44.2%)	C+D = 48 (55.8%)	
Ala/Ala (CC); N = 59 (68.6%)	26	33	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	8	10	1.0 (0.4-2.9)
Val/Val (TT); N = 9 (10.4%)	4	5	1.0 (0.2-4.1)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	12	15	1.0 (0.4-2.5)
Pesticide exposure	Never = 33 (38.4%)	Ever = 53 (61.6%)	
Ala/Ala (CC); N = 59 (68.6%)	23	36	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	6	12	0.8 (0.2-2.3)
Val/Val (TT); N = 9 (10.4%)	4	5	1.2 (0.3-5.2)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	10	17	0.9 (0.3-2.35)
Rectal bleeding/constipation	Yes = 60 (69.8%)	No = 26 (30.2%)	
Ala/Ala (CC); N = 59 (68.6%)	41	18	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	12	6	0.8 (0.3-2.7)
Val/Val (TT); N = 9 (10.4%)	7	2	1.5 (0.3-8.13)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	19	8	1.0 (0.4-2.8)
Tumor type*	Mucinous = 33 (38.5%)	Non-mucinous = 52 (60.5%)	
Ala/Ala (CC); N = 58 (67.4%)	21	38	1.0 (Ref.)
Ala/Val (CT); N = 18 (20.9%)	8	10	1.4 (0.5-4.2)
Val/Val (TT); N = 9 (10.4%)	4	5	1.4 (0.4-5.9)
Ala/Val (CT) + Val/Val (TT); N = 27 (31.3%)	12	15	1.4 (0.5-3.6)

*One was squamous cell carcinoma. OR = odds ratio; CI = confidence interval.

among CRC cases and 75.6, 16.9 and 7.5% among the general control population, respectively. The results were similar to the previous study of Haghighi et al. (2009). This may be due to the fact that our population belongs to the same Persian genotypic pool as our ancestors have descended from the Persian migrants who have settled here during the 15th century.

Our results were somewhat different from the main Indian population (South), where the frequency of genotypes CC, CT and TT of *MTHFR* were reported to be 74, 25 and 1.0% among CRC cases and 76.7, 22.1 and 1.16% in controls (Chandy et al., 2010). The difference in frequency may be due to ethnicity and the special set of environmental factors to which our population is exposed to (Sameer et al., 2010a,b).

Furthermore, we also found a 1.36-fold greater incidence of the *MTHFR* CT genotype and 1.53-fold greater incidence of the *MTHFR* TT genotype in patients with CRC compared with the healthy control population (Table 1).

In this case-control study, conducted for the first time in this population, we found a modest increase in the risk of CRC in the individuals with the TT (Val/Val) genotype when compared with the general population (OR = 1.53; 95%CI = 0.61-3.85). These results were similar to previously reported studies (Ulrich et al., 1999; Shannon et al., 2002). These results also indicate that the *MTHFR* TT genotype, which is associated with lower functionality, does not play an avid protective role in the cell and also affects the methylation status of the cell by limiting the availability of 5,10-methylenetetrahydrofolate, which in turn, also affects thymidine synthesis.

We also found a significantly increased association of *MTHFR* TT (Val/Val) with age (>50) (OR= 5.8; 95%CI = 1.3-26.4), indicating the deleterious role of decreased function of *MTHFR* in cell metabolism, where these results were in agreement with similar results reported by Shannon et al. (2002). We also found a modest increased risk of CRC with smoking status (ever), tumor location (C+D) and pesticide exposure (ever) (OR = 2.4; 3.1; 1.2) (Table 3).

In a nutshell, this study revealed a significant correlation between the Val/Val variant genotype of *MTHFR* and various clinicopathological variables in this ethnic Kashmiri population, especially in the older age group. However, these correlations need to be authenticated in a large-sample study in the future, so as to help in better discernment of racial differences and in determining the aggressiveness of colorectal cancer.

CONCLUSION

Hence, in this study, which has been carried out for the first time in the Kashmir Valley, we observed a significant correlation between the Val/Val variant *MTHFR* status and various clinicopathological variables in this ethnic Kashmiri population.

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