RESEARCH ARTICLE

Allele and Genotype Frequencies of the Polymorphic *Methylenetetrahydrofolate Reductase* and Colorectal Cancer among Jordanian Population

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Abstract

Background: Methylenetetrahydrofolate reductase (MTHFR) is involved in DNA synthesis and repair. We here aimed to investigate two common polymorphisms, C677T and A1298C, with genotype and haplotype frequencies in colorectal cancer (CRC) cases among Jordanian. Materials and Methods: 131 CRC cases were studied for MTHFR C677T and A1298C polymorphisms, compared to 117 controls taken from the general population, employing the PCR-RFLP technique. Results: We found the frequency of the three different genotypes of MTHFR C677T among Jordanians to be CC: 61.7%, CT: 35.2%, and TT 3.1% among CRC cases and 50.9%, 38.8% and 10.3% among controls. Carriers of the TT genotype were less likely to have CRC (OR=0.25; 95% CI: 0.076-0.811; p=0.021) as compared to those with the CC genotype. Genotype analysis of MTHFR A12987C revealed AA: 38.9%, AC: 45%, and CC 16% among CRC cases and 37.4%, 50.4% and 12.2% among controls. There was no significant association between genetic polymorphism at this site and CRC. Haplotype analysis of MTHFR polymorphism at the two loci showed differential distribution of the TA haplotype (677T-1298A) between cases and controls. The TA haplotype was associated with a decreased risk for colorectal cancer (OR=0.6; 95% CI: 0.4-0.9, p=0.03). Conclusions: The genetic polymorphism of MTHFR at 677 and the TA haplotype may modulate the risk for CRC development among the Jordanian population. Our findings may reflect an importance of genes involved in folate metabolism in cancer risk.

Keywords: Genetic polymorphism - colorectal cancer - methylene tetrahydrofolate - genomic DNA

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Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide. More than half of the cases (60%) occur in developed regions. The incidence varies considerably among different ethnicities (Parkin et al., 1999; Ferlay et al., 2008; Center et al., 2009). The age-standardized incidence (ASR) per 100,000 reaches its highest in Australia (38.7), Western Europe (33.1) and Northern America (30.1), and its lowest in Africa (5.9) (except Southern African Republic (14.5)) and South-Central Asia (4.5), and is intermediate in Latin America and Caribbean (11.4); China (16.3) and in Jordan (16.8). Incidence rates are substantially higher in men (20.3) than in women (14.6) worldwide (Ferlay et al., 2008). It is the fourth most common cause of death from cancer with the highest mortality rates (per 100,000) in both sexes estimated in Central and Eastern Europe (All: 15.1; Men: 20.1; Women: 12.2), Australia (All: 12.6; Men: 15.9; Women: 9.5) and North America (All: 9.1; Men: 10.4; Women: 7.9), and the lowest in Middle Africa (All: 3.1; Men: 3.5; Women: 2.7) (Ferlay et al., 2008). In Jordan, the ASR mortality is high (All: 12.5; Men: 14.2; Women: 10.8). Interestingly the ratio of ASR-mortality to ASR-incidence (0.74) is higher than that of Australia (0.33), Europe (0.36) and Northern America (0.3) (Ferlay et al., 2008).

Methylenetetrahydrofolate reductase (*MTHFR*) is an important enzyme involved in folate metabolism, which affects DNA methylation and synthesis (Lucock, 2000; 2004; Cicek et al., 2004). It converts 5,10-methylenetetrahydrofolate irreversibly to 5-methyltetrahydrofolate which in turn donates its methyl group to homocysteine in the generation of S-adenosylmethionine (SAM). SAM is a major source of methyl groups used for DNA methylation. The *MTHFR* maintains circulating levels of folate and methionine, and prevents the accumulation of homocysteine (Lucock,

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2000; 2004).

The *MTHFR* gene is located at the end of the short arm of chromosome 1 (1p36.3) (Goyette et al., 1998). The two common functional polymorphisms have been defined in the *MTHFR* gene - one is *C677T* (rs1801133; NT_021937.19: 7,861,110) and other is *A1298C* (rs1801131; NT_021937.19: 7,859,208). The two polymorphisms are located 1,903 bp apart.

MTHFR C677T polymorphism results from C-to-T transition at nucleotide 677 in exon 4 resulting in an amino acid substitution of alanine to valine at amino acid 222 (Frosst et al., 1995; Sharp and Little, 2004). The CC genotype of *MTHFR* gene at '677' is usually referred to as the wild type, CT as the heterozygous form, and TT as the homozygous variant. Subjects with the TT or CT genotype have lower levels of enzyme activity, 30% and 65%, respectively, relative to enzyme activity in subjects carrying the CC genotype (Frosst et al., 1995; Kono and Chen, 2005; Brockton, 2006). Additionally, this single nucleotide polymorphism (SNP) decreases the thermal stability of this enzyme (Cicek et al., 2004). It has been reported that this substitution may lower levels of 5-methyltetrahydrofolate, and increase plasma homocysteine levels (Frosst et al., 1995; Ma et al., 1997; Bagley and Selhub, 1998).

MTHFR A1298C polymorphism results from A-to-C transversion at nucleotide 1298 in exon 7 resulting in an amino acid substitution of alanine to glutamate at codon 429 of the protein (van der Put et al., 1998; Weisberg et al., 1998). The enzyme activity in vitro is decreased in homozygous variants (CC) and, to a lesser extent, in heterozygotes compared with those without the variant (van der Put et al., 1998). Studies of *A1298C* and plasma folate and homocysteine are inconsistent (Weisberg et al., 1998; Friedman et al., 1999; Chango et al., 2000; Lievers et al., 2001; Chen et al., 2002). Enzyme activity in vitro for compound heterozygotes (i.e., heterozygotes for *C677T* and for *A1298C*) is unclear (Lievers et al., 2001).

A meta-analysis of 29 studies evaluated the SNP at 677 position and risk of CRC. The overall odds ratio (OR) for CRC for persons with the TT genotype was 0.83 (95% CI: 0.77-0.90). The protective effect of the TT genotype was observed in whites (OR=0.83, 95%CI: 0.74-0.94) and Asians (OR=0.80, 95%CI: 0.67-0.96) but not in Latinos or blacks (Taioli et al., 2009).

A systematic review examined the association of genetic polymorphism at *A1298C* with CRC in 24 previously published studies (Zhou et al., 2012). In contrast to the significant association between *MTHFR C677T* and the risk of CRC, the *MTHFR A1298C* polymorphism was found to be not significantly related with the risk of CRC. On the other hand, Keku et al. reported that the *A1298C* cancer association was stronger among self-reported European-American, non-Hispanic white (CC genotype: OR: 0.5, 95%CI=0.3-0.9) than African-American subjects (Keku et al., 2002).

The genetic polymorphism of *MTHFR* seems to be related to the risk of CRC, but this may not hold true for all populations (Taioli et al., 2009; Zhou et al., 2012). The aim of this study was to determine if *MTHFR C677T* and *A1298C* polymorphism is/are associated with an altered risk of developing CRC among Jordanians.

Materials and Methods

Study population

Paraffin-embedded tissues from (131) unrelated Jordanian patients previously diagnosed with CRC with no evidence of familial colon cancer were collected from Jordan University Hospital (JUH). Cases consisted of patients with positive colonoscopic results for malignancy and histologically confirmed as adenocarcinomas of colon or rectum.

None of the cases were familial adenomatous polyposis (FAP) or non-polyposis colorectal cancer (HNPCC). All cases were adenocarcinoma of colon or rectum. All samples were retrieved from the archives of histopathology department at Jordan university hospital between 2009 and 2011.

The control group consisted of (117) healthy individuals from the same geographical area as the CRC patients who consented to analyze their biological samples. The study protocol was approved by the Institutional Review Board (IRB) at JUH. The study was exempted by the IRB from obtaining a formal written consent from patients.

Not all samples were successfully analyzed for the two SNPs because either we ran out of sample, failure of PCR or failure of RFLP. Three cases had data at 1298 but not at 677. Two controls had data at 677 position but not at 1298, and one control had data on 1298 but not 677.

MTHFR genotyping

DNA from paraffin-embedded blocks of patient and control samples was extracted using an FFPE kit (Qiagen, Germany) according to the manufacturer's instructions. Genotyping was performed for both polymorphisms, *C677T* and *A1298C*, by two separate uniplex polymerase chain reaction (PCR) reactions using a thermal cycler (model PTC-100; Bio-Rade MJ Research, Watertown, MA). Amplification of the *C677T* region was performed using the forward primer TGAAGGAGAAGGTGTCTGCGGGA (NT_021937.19: 7,861,134 to 7,861,112) and the reverse primer AGGACGGTGCGGTGAGAGTG (NT_021937.19: 7,860,937 to 7,860,956) yielding a 198-bp band, whereas for the *A1298C* region, the forward primer CAAGGAGGAGCTGCTGAAGA (NT_021937.19: 7,859,255 to 7,859,236), and the reverse primer CCACTCCAGCATCACTCACT (NT_021937.19: 7,859,128 to 7,859,147), were used yielding a 128-bp band. For both amplifications, the PCR conditions were described by Yi et al: 8 minutes of initial denaturation at 95°C, followed by 40 cycles of 95°C for 60 seconds, 63°C for 60 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 7 minutes (Yi et al., 2002).

The PCR products of *C677T* and *A1298C* were separately digested with the HinfI and MboII restriction enzymes (Promega), respectively. Resulting fragments were visualized using ethidium bromide staining and 3% agarose (Promega) gel electrophoresis. The digestion fragment sizes for the *C677T* genotypes were: a single 198-bp band for CC, 198 bp, 175 bp and 23 bp for CT, and 175 bp and 23 bp for TT. For *A1298C* genotypes, the

fragments were 72 bp and 28 bp (2 fragments) for AA, 28 bp, 72 bp, and 100 bp for AC, and 100 bp and 28 bp for CC.

Findings of the PCR-RFLP were validated by: *i*) Every time PCR reactions were done a negative control was run simultaneously. A negative control contains all PCR components except the DNA template; *ii*) Around 15% of all samples were repeated to confirm findings of the PCR-RFLP. The concordance between repeated samples was 100%; *iii*) Randomly selected 30 PCR-RFLP results were confirmed by direct DNA sequencing using BigDye Terminator Cycle Sequencing on 3730xl DNA sequencer (Macrogene® Co., Korea).

Haplotype analysis

The interaction between genetic polymorphism at the two loci was assessed by evaluating the combined-genotypes effects and haplotype analysis. We analyzed the haplotype frequencies of the two SNPs for colorectal cancer cases and compared them with those of controls. Haplotype frequencies were calculated using Golden Helix Tree® software and linkage disequilibrium was represented by D prime (D'). The Golden Helix software is enabled to estimate the haplotype frequencies even with presence of some missing data of one or two of the SNPs. Similar findings were obtained utilizing Multiallelic Interallelic Disequilibrium Analysis Software (University of Southampton, Highfield, Southampton, UK).

Statistical analysis

Statistical analysis was performed using SPSS© software (version 11.0; SPSS, Inc. Data were expressed as mean +/- standard deviation (SD), or as counts (%). Normal probability distribution of age was determined by examining the Kolmogorov-Smirnov-Lilliefors test (K-S test). The age difference between cases and controls was assessed by independent student t-test. Allele and genotype frequencies for different alleles among Jordanian population were estimated from the results of the above PCR-RFLP test. This estimation was according to formulas reported previously (Brooker, 2005). Genotype and allele frequency were analyzed for concordance to the Hardy-Weinberg equilibrium. Differences in allele/genotype frequencies between CRC cases and controls were assessed using chi square test or Fisher exact test as appropriate (Graph Pad Software Inc). Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated as a measure of association between *MTHFR* genotypes/alleles/diplotypes/haplotypes and CRC. A p-value below 0.05 was considered statistically significant throughout the population comparisons.

Results

A total of 131 CRC patients and 117 control subjects were included in this study. The patients comprised 64 males and 67 females (M/F ratio=0.96) and the control subjects consisted of 57 males and 60 females (M/F ratio=0.95). Mean age in patients and control groups was 57.8±13.8 and 47.4±18.7 years, respectively. No significant gender differences were observed between the groups (p=0.93). Patients were 10 years older than controls (p<0.0001) (Table 1).

The distribution of *MTHFR C677T* and *A1298C* genotypes and their alleles are presented in Table 2 and Table 3, respectively. There were statistically significant differences in the genotype frequency of *MTHFR C677T* between CRC cases and the controls. The frequency of the *MTHFR* T allele was lower in patients with CRC compared with healthy controls (Table 2). The TT genotype was associated with a 4-fold lower risk of CRC (p=0.021); the CT genotype was associated with a 1.3-fold lower risk, albeit not statistically significant (p=0.28). Subjects with the T allele were 1.6 time less likely to suffer from colorectal cancer (p=0.028). However, no significant difference was found in the 1298 SNP between colorectal cancer cases and controls (Table 2). The allelic distribution of the two SNPs was in Hardy Weinberg equilibrium (p>0.05).

Table 1. Age and Gender Characteristics of Cases and Controls

Parameter		No. (%)		p
		Colorectal cancer	Controls	
Gender	Male	64 (48.9%)	57 (48.7%)	0.93
	Female	67 (51.1%)	60 (51.3%)	
Age (years) (mean±SD)		57.8±13.8	46.4±18.3	<0.0001
	<40	12 (9.2%)	39 (33.3%)	
	40-49	26 (20%)	17 (14.5%)	
	50-59	26 (20%)	25 (21.4%)	
	60-69	47 (36.2%)	26 (22.2%)	
	≥70	19 (14.6%)	10 (8.5%)	

Table 2. *MTHFR C677T* and *MTHFR A1298C* Genotypes and Allele Types and Risk of Colorectal Cancer

Parameter	Colorectal cancer (N=131)	Controls (N=117)	Odds Ratio (95%CI)	p
MTHFR C677T:				
CC	79 (61.7%)	59 (50.9%)	1 (reference)	
CT	45 (35.2%)	45 (38.8%)	0.75 (0.44-1.27)	0.28
TT	4 (3.1%)	12 (10.3%)	0.25 (0.076-0.81)	0.021
CC+CT	124 (96.9%)	104 (89.7%)	1 (reference)	
TT	4 (3.1%)	12 (10.3%)	0.28 (0.088-0.89)	0.044
C	203 (79.3%)	163 (70.3%)	1 (reference)	
T	53 (20.7%)	69 (29.7%)	0.62 (0.41-0.93)	0.028
MTHFR A1298C:				
AA	51 (38.9%)	43 (37.4%)	1 (reference)	
AC	59 (45%)	58 (50.4%)	0.86 (0.5-1.5)	0.58
CC	21 (16%)	14 (12.2%)	1.27 (0.58-2.78)	0.56
AA+AC	110 (83.9%)	101 (87.8%)	1 (reference)	
CC	21 (16%)	14 (12.2%)	1.38 (0.66-2.85)	0.5
A	161 (61.5%)	144 (62.6%)	1 (reference)	
C	101 (38.5%)	86 (37.4%)	1.05 (0.73-1.51)	0.86

*Data are reported as number (N=actual numbers) with percent in parentheses

Table 3. Haplotype Frequencies of MTHFR among Colorectal Cancer Patients and Controls

Haplotype	Colrectal cancer N (%)	Control	Odds Ratio	95% CI	p
677C-1298A	115 (44.8)	86 (37.5)	1.3	0.9-1.9	0.1
677C-1298C	88 (34.5)	77 (33.9)	1.03	0.7-1.5	0.9
677T-1298A	42 (16.5)	56 (24.7)	0.6	0.4-0.9	0.03
677T-1298C	11 (4.2)	9 (3.8)	1.1	0.4-2.7	0.9

*Counts reflect the number of chromosomes

There was no statistically significant difference between *C677T* genotypes and either gender distribution (Men: 63CC, 48CT, 7TT; Women: 75CC, 42CT, 9TT, p=0.489) or age (CC: 53.2±17.7 years; CT: 53.2±15.4 years; TT: 45±19.8 years, p=0.178).

MTHFR haplotypes

Four different haplotypes appeared in our analysis. The most frequent haplotypes were CA (677C-1298A) (CRC: 44.8%; controls: 37.5%), and CC (CRC: 34.5%; controls: 33.9%) followed by TA (CRC: 16.5%; controls: 24.7%), while the rare haplotype was TC (CRC: 4.2%; controls: 3.8%) (Table 3). Our results indicated that the two loci 677 and 1298 show relatively strong linkage disequilibrium (Lewontin's coefficient [D']) (Controls: D'=0.65, r^2: 0.1; CRC: D'=0.48, r^2: 0.04). Carriers of the TA haplotype were 1.8-fold less likely to be associated with colorectal cancer (OR: 0.6; 95%CI: 0.4-0.9, p=0.03). Similar findings were obtained when haplotypes were compared with the most frequent haplaotype (TA vs. CA: OR=0.56; 95%CI: 0.34-0.91, p=0.027, "data not shown in Table 4"). None of the remaining haplotypes was associated with CRC.

Discussion

Prior to our investigation, based on Zhou et al. (2012) the studies that investigated *MTHFR C677T* were distributed by ethnicity as follows: 20 studies recruited Caucasians; 11 studies examined individuals of Asian descent; the remaining 10 studies were on Indians, Africans, Hawaiian, or mixed populations. There was a single study conducted in an Arab country which is Egypt (El Awady et al., 2009). This is the first study conducted in Jordan and the second in the Arab world. Jordanians are mostly descended from people of villagers and Bedouin descent originating in the Arabian Peninsula (Lowi, 1995); thus, ethnically, the Jordanians represent a mixed stock. Most of the population is Arab (approximately 98%) with 1% of the population, Armenian, and another 1%, Circassian. There are also Kurd, Druze, and Chechen minorities (The Royal Hashemite Court; Central Intelligence Agency, 2012).

We used paraffin-embedded tissues samples, while the majority of other studies used blood samples for the extraction of DNA. The only exception was for the study by Shannon et al. in which frozen tissues samples were utilized (Shannon et al., 2002).

In our sample of colorectal cancer cases and their healthy controls, we observed a good association for the *MTHFR C677T* variant and no association for the *A1298C* SNP. The observed association was not gender or age dependent. The results for the *C677T* variant are consistent with some but not all published studies. Numerous studies worldwide have reported on the association of *MTHFR C677T* polymorphism with the risk of colorectal carcinoma. Many studies were consistent with reduced risk in homozygous variant (TT) subjects compared with homozygotes for the common allele (Chen et al., 1996; Ma et al., 1997; Chen et al., 1998; Park et al., 1999; Slattery et al., 1999; Houlston and Tomlinson, 2001; Keku et al., 2002; Le Marchand et al., 2002; Marugame et al., 2003). Nevertheless, 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). Overall, non-concordant findings were obtained ranging from strong links to no association. The divergent outcomes regarding the effects of these genetic polymorphisms upon CRC risk may be attributed to the differences in racial origin of the population, the lifestyle, and the pattern of diet in distinct countries (Taioli et al., 2009; Guimaraes et al., 2011). A recent meta-analysis concluded that whites and Asians who carry the TT genotype were less susceptible to colorectal cancer, but the association did not hold for Latinos or blacks (Taioli et al., 2009). Alternatively, the apparent inconsistency may be due to small sample size, bias, a failure to control for confounders, or simply it could be due to chance (Sharp and Little, 2004).

The relative risk/odds ratio of 677TT ranged in published articles from 0.45 to 0.9, although most did not reach statistical significance. The strongest effects were found in the two earliest studies (Chen et al., 1996; Ma et al., 1997). Both were nested within cohort studies of predominantly white male populations in the United States. These populations are likely to have relatively high average intakes of total folate as a consequence of the mandatory folate fortification program in the U.S. since 1998 and comparatively frequent use of vitamin supplements (Klerk et al., 2002). Among Jordanians significant associations were found in the allele comparisons (T vs C. OR=0.62, 95%CI: 0.41-0.93) and in the homozygous genotype comparisons (TT vs. CC: OR=0.25, 95%CI: 0.076-0.81). We observed a trend of decreasing risk with increasing number of the T allele (OR_{CC}: 1.56 "data not shown in Table 2 because CC was considered as reference"; OR_{CT}: 0.75; OR_{TT}: 0.25). Similar findings were observed in previous studies (Le Marchand et al., 2002).

We also investigated the frequencies of the *MTHFR A1298C* genotypes in Jordanian patients with CRC and the healthy controls. Contrary to the apparent association between *MTHFR C677T* and the risk of CRC, the *MTHFR A1298C* polymorphism was not found to be significantly related with the risk of CRC. A marginal association was found in a meta-analysis of the allele and genotypes of *MTHFR A1298C* (C vs. A, CC vs. AA, and CC vs. CA+AA). The trend disappeared after exclusion of one study, in which the 95% CI did not overlap the lines of the pooled results (Zhou et al., 2012). A lack of association may be due to methodological reasons (e.g., non-population-based study, small sample size), or it may be that there is a relation that depends on the nutrient status of folate and/or related nutrients which we did not investigate. CRC risk seems to be inversely associated with higher folate intake, and the protective effect of the *MTHFR* polymorphisms may be dependent on adequate nutrients status (Bailey, 2003).

Haplotype analysis, in addition to the customary analysis of SNPs, may play an important role in the identification of genetic variations between cases and controls. Haplotype analysis of *MTHFR* is increasingly

employed in various diseases and conditions (Shen et al., 2001; Chen et al., 2002; Le Marchand et al., 2002; Caccamo et al., 2004; Terrazzino et al., 2006; Pardini et al., 2011). There is evidence of linkage disequilibrium between the *C677T* and the *A1298C* variants of the *MTHFR* gene (Chen et al., 2002; Shi et al., 2003) with the possibility that both SNPs may be dependent on each other from the genetic and functional point of view. The interdependence of the loci may imply requirement of a haplotype approach when assessing the value of *MTHFR C677T* and *A1298C* polymorphisms.

The physical distance between the two SNPs is short (1.9 kb), and thus one may expect that LD is evident as has been shown in German general population (Stegmann et al., 1999). Unfortunately, only very few studies described the strength of LD between the two SNPs in *MTHFR*. Although not stated, we calculated D' and r^2 among Indians (Controls: D'=0.76, r^2=0.07; CRC: D'=0.76, r^2=0.07) (Chandy et al., 2010); Chinese (Controls: D'=0.13, r^2=0.004; CRC: D'=0.17, r^2=0.015) (Li et al., 2011); self-reported whites (Controls: D'=0.93, r^2=0.19; CRC: D'=0.93, r^2=0.15) (Keku et al., 2002); self-reported African Americans (Controls: D'=1, r^2=0.03; CRC: D'=1, r^2=0.03) (Keku et al., 2002); and mixed-population Americans (Controls: D'=1, r^2=0.23; CRC: D'=1, r^2=0.21) (Curtin et al., 2004). Our results indicated that the two loci showed relatively strong LD ([D']=[0.65 (controls), 0.48 (CRC)]), albeit is somewhat lower than reported by a Czech study ([D']=0.94 and r^2=0.22) (Pardini et al., 2011).

According to a meta-analysis of 16 published articles that was based on large number of Caucasian populations, the most frequent haplotypes of *MTHFR* were CA (37%); CC (31%); and TA (32%) (Ogino and Wilson, 2003). A more recent study on Czek subjects reported CA (33.6%); CC (33.7%); and TA (31.9%) as the most common haplotypes (Pardini et al., 2011). Current study reports similar trend to that of Caucasians (CA (37.5%) ; CC (34.5%)), albeit a little bit lower TA (24.5%) and higher TC (3.8%) ($TC_{Caucasians}$=0%; TC_{Czek}=0.8%). Because of rarity of TT/CC (677TT and 1298CC) and TT/AC among Jordanians and in the world, the TC haplotype should be rare as well (Ogino and Wilson, 2003). The estimated TC haplotype among Caucasians-general populations was reported in less than 1% of the general public (0.3-0.8%) (Ogino and Wilson, 2003; Pardini et al., 2011). Some studies lacked the TC haplotype (Meisel et al., 2001; Terrazzino et al., 2006). Still, there may be an increased frequency of the very rare TC haplotype in some parts of the United Kingdom and Canada (Ogino and Wilson, 2003). Very few studies reported haplotype analysis of their data, but we managed to assess their haplotypes adopting the same methodology we used with our own data. Utilizing their published data, we calculated the TC haplotype among other ethnicities and found it rare as well (Indians: (CA (43.8%); CC (44%); TA (10.9%); TC (1.3%) (Chandy et al., 2010); Chinese: (CA (42.5%); CC (17.5%); TA (30.6%); TC (9.4%) (Li et al., 2011); African Americans : (CA (70.2%); CC (19%); TA (10.8%); TC (0%) (Keku et al., 2002)).

Few studies investigated the effect of *MTHFR* haplotypes on susceptibility to colorectal cancer (Pardini et al., 2011). Interestingly, the haplotype analysis based on the two investigated *MTHFR* polymorphisms (*C677T* and *A1298C*) showed that haplotype TA was less common in cases than controls (OR=0.6, p=0.03). This haplotype, when compared to the most common haplotype CA, was associated with a decreased risk for colorectal cancer. This outcome matches the results of the allele analysis for the individual *C677T* and *A1298C* polymorphism in the *MTHFR* gene. Similar findings were observed previously, where the TA haplotype, compared with the most common haplotype (CA), was less frequent among the cases than among the controls (OR, 0.84; 95%CI, 0.71-0.99) (Pardini et al., 2011). Furthermore, Terrazino et al found that *MTHFR* TA haplotype in genomic DNA has the potential to be a predictive marker of tumor response in rectal cancer patients submitted to preoperative chemoradiotherapy (Terrazzino et al., 2006). It was reported that the TA haplotype was the only variable associated with tumor regression (p=0.004). Interestingly, the TC haplotype was not associated with reduced risk of colorectal cancer, possibly because the frequency was too low to obtain statistical rigor.

In conclusion, the findings of the current study suggest that genetic polymorphism of *MTHFR* at *C677T* and its haplotype analysis at 677and 1298 modulates the risk of CRC in the Jordanian population. To our knowledge, this is being reported for the first time among this population.

In Limitations, this is a case-control study with relatively small number of patients and controls. One should keep in mind that the number of individuals with the CT/CC, TT/AC, or TT/CC genotype in published studies was always small, and therefore, a small error in genotyping, either false positive or false negative, can affect an *MTHFR* T/C haplotype frequency estimate significantly (Ogino and Wilson, 2003).

In a case-control study of 220 recently diagnosed Jordanian CRC cases and 220 age and gender matched healthy subjects as a control group, researchers concluded that sedentary lifestyle and a diet low in fibers, folate, vitamin B12, β-carotene, vitamin C, selenium, fruits and vegetables, and high in animal red meat and saturated fat, appeared associated with CRC among studied Jordanian subjects (Arafa et al., 2011). A larger scale prospective clinical study is needed to better define the predictive value of *MTHFR* haplotypes in CRC susceptibility and the effects of lifestyle, diet, smoking status and alcohol consumption on the association (Taioli et al., 2009).

An additional concern is the age distribution, where the control subjects were 10 years younger than patients. The fact that the incidence (per 100,000) of CRC among Jordanian is associated with age (40-44 years: 9.5; 45-49 years: 20.9; 50-54 years: 38.5; 55-59 years: 49.2; 60-64 years: 80.1; 65-69 years: 94.1; 70-74 years: 115.3; 75+ years: 101.0) (Ferlay et al., 2010) raises worrisome. A question arises on how many apparently healthy subjects could have theoretically developed cancer over these ten years? In worst case scenario, the 10 years difference in age may equate to future-to-develop incidence of 45 (per 100,000) which was calculated from the maximum difference in incidence between the "65-69 years" and "55-59 years" age categories. This calculated estimate

translates to an error rate of 0.05 in our 117 controls. Though the error is small, the probability that our findings were biased by age should not be underestimated.

Finally, in the present study CRC is considered as a uniform disease, whereas there are distinct phenotypes linked with the tumor location. Unavoidably, the small sample size will preclude subcategory analysis based on the tumor locations.

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