RESEARCH ARTICLE

Allele and Genotype Frequencies of the Polymorphic Methylenetetrahydrofolate Reductase and Lung Cancer in ther Jordanian Population: a Case Control Study

Yousef Al-Motassem[1]*, Maha Shomaf[2], Ismail Said[3], Sondra Berger[4], Nidaa Ababneh[3], Ola Diab[1], Nathir Obeidat[5], Abdallah Awidi[6]

Abstract

Background: Methylenetetrahydrofolate reductase (*MTHFR*) is involved in amino acid synthesis and DNA function. Two common polymorphisms are reported, *C677T* and *A1298C*, that are implicated in a number of human diseases, including cancer. Objective: The association between *MTHFR C677T* and *A1298C* genotype and haplotype frequencies in risk for lung cancer (LC) was investigated in the Jordanian population. Materials and Methods: A total of 98 LC cases were studied for *MTHFR C677T* and *A1298C* polymorphisms, compared to 89 controls taken from the general population, employing the PCR-RFLP technique. Results: The frequency of the genotypes of *MTHFR C677T* among Jordanians was: CC, 59.6%, CT, 33%; and TT, 7.4% among LC cases and 49.4%, 40.2% and 10.3% among controls. No significant association was detected between genetic polymorphism at this site and LC. At *MTHFR* A12987C, the genotype distribution was AA, 29.5%; AC, 45.3%, and CC 25.3% among LC cases and 36.8%, 50.6% and 12.6% among controls. Carriers of the CC genotype were more likely to have LC (OR=2.5; 95%CI: 1.04-6; p=0.039) as compared to AA carriers. Smokers and males with the CC genotype were 9.9 and 6.7 times more likely to have LC, respectively ($OR_{smokers}$=9.9; 95%CI: 1.2-84.5, p=0.018; OR_{men}=6.6; 95%CI: 1.7-26.2, p=0.005). Haplotype analysis of *MTHFR* polymorphism at the two loci showed differential distribution of the CC haplotype (677C-1298C) between cases and controls. The CC haplotype was associated with an increased risk for lung cancer (OR=1.6; 95% CI: 1.03-2.4, p=0.037). Conclusions: The genetic polymorphism of *MTHFR* at *1298* and the CC haplotype (risk is apparently lower with the C allele at position *677*) may modulate the risk for LC development among the Jordanian population. Risk associated with the 1298C allele is increased in smokers and in males. The results indicate that a critical gene involved in folate metabolism plays a modifying role in lung cancer risk, at least in the Jordanian population.

Keywords: Lung cancer - methylene tetrahydrofolate reductase - polymorphism - Jordanian population

Asian Pac J Cancer Prev, **16 (8)**, 3101-3109

Introduction

Lung cancer (LC) is the most common cancer in the world with an estimated 1.8 million new cases in 2012. It is also the most common cause of death from cancer, with 1.59 million deaths. Incidence depends on gender and ethnicity. LC is the most frequent cancer in men and the fourth in women. The majority of the cases are in the developing countries (55%) and the lowest rates are estimated in Middle and Western Africa. Patients with LC suffer from high fatality with the ratio of mortality to incidence is 0.87 irrespective of geographical location ranging from 0.75 (USA) to 0.94 (Africa)(Ferlay et al., 2012).

In Jordan, cancer is the second most frequent cause of death after heart disease. A total of 6820 new cancer cases were registered by Jordan Cancer Registry in 2010, of these, 4921 cases (72.2%) were among Jordanians, and 1899 cases were non-Jordanians. Out of a total of 4921 new cases of cancer recorded amongst Jordanians in the 2010, of these, 2330 cases (48.1%) were males and 2519 cases (51.9%) were females (Jordan Cancer Registry, 2010). The rank order of the five most common cancers affecting Jordanians are: Breast, Colorectal, Lymphoma, Lung, and Prostate. The crude incidence rate of all cancer among Jordanians was 79.4 per 100,000 population (74.0 for males and 85.1S for females). The lung cancer age-standardized rate (ASR) for incidence/mortality in men (27.0/24.1) is substantially higher than women (4.1/3.7) (Ferlay et al., 2012).

[1]*Department of Biopharmaceutics and Clinical Pharmacy, Faculty of Pharmacy,* [2]*Department of Pathology,* [3] *Department of Biochemistry, Molecular Biology Research Lab,* [5]*Department of Internal Medicine,* [6]*Thrombosis and Molecular Hematology Laboratory, Department of Medicine and Hematology, Faculty of Medicine, The University of Jordan, Amman, Jordan,* [4]*Department of Pharmaceutical and Biomedical Sciences, South Carolina College of Pharmacy, University of South Carolina, Columbia, USA* **For correspondence: ayousef@ju.edu.jo*

Folate metabolism is thought to play a critical role in carcinogenesis through its involvement in DNA methylation and repair. Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme involved in folate metabolism (Cicek et al., 2004; Lucock, 2004). It converts 5,10-methylenetetrahydrofolate irreversibly to 5-methyltetrahydrofolate (5-MeTHF) 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. *MTHFR* maintains circulating levels of 5-MeTHF and methionine, and prevents the accumulation of homocysteine (Lucock, 2004).

A number of molecular epidemiological studies have been conducted to investigate the associations of the *MTHFR C677T* and *A1298C* polymorphisms with lung cancer predisposition. However, the results remain conflicting rather than conclusive (Mao et al., 2008; Boccia et al., 2009; Zhang et al., 2012).

Genetic polymorphism in *MTHFR* may be related to the risk for lung cancer, but this may not hold true for all populations (Mao et al., 2008; Boccia et al., 2009; Zhang 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 lung cancer among Jordanians.

Materials and Methods

Study design and population

In this case-control study, paraffin-embedded tissues from histologically confirmed lung cancer cases from (98) unrelated Jordanian patients previously diagnosed with lung cancer were collected from Jordan University Hospital (JUH). Histologically, 88 cases were non-small-cell carcinoma mainly squamous cell carcinoma (N=35) and adenocarcinoma (N=30). The remaining 10 cases were small-cell carcinoma.

The control group consisted of (89) healthy individuals from the same geographical area as the lung cancer patients who consented to analysis of 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 for paraffin-embedded samples.

Not all samples were successfully analyzed for the two SNPs due to sample depletion, failure of PCR or failure of endonuclease digestion. Four cases had data at 1298 but not at 677, and three cases had data at 677 but not at 1298. One control had data at 1298 but not at 677, and two controls had data at 677 but not at 1298.

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 polymerase chain reaction (PCR) reactions using a thermal cycler (model PTC-100; Bio-Rad-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. (2002) 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.

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: 1) inclusion of a negative control in all PCR analyses; negative controls contain all PCR components except DNA template; 2) repeat analysis of approximately 15% of all samples; concordance between repeated samples was 100%; and 3) direct DNA sequencing of 30 randomly selected PCR-RFLP digests 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-genotype effects by haplotype analysis. We analyzed the haplotype frequencies of the two SNPs for lung 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, Chicago, IL). Data was expressed as mean ± SD or as counts and percentages. Data was subdivided between two groups (LC and controls). 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). Age was compared between the two groups by independent student t-test or Mann Whitney as appropriate. Age was compared between the

different genotypes by ANOVA or Kruskal Wallis. Data of categorical nature (gender, smoking behavior, genotypes and haplotypes) were compared with tissue type by chi square 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/haplotypes and LC. Genotype and allele frequency were analyzed for concordance to the Hardy-Weinberg equilibrium. Multiple logistic regressions was utilized to discern the relationship between significant variables and the susceptibility to lung cancer. We conducted multivariate logistic regression analysis for variables which demonstrated significance (p<0.05) in bivariate analysis. Adjusted odds ratio (aOR) and 95% confidence intervals (CI) for the probability of LC control were calculated for each predictor to explain the strength of association. A p-value below 0.05 was considered statistically significant throughout the population comparisons.

Results

Demographics

A total of 98 lung cancer patients and 89 control subjects were included in this study. The patients comprised 78 males and 20 females (M/F ratio = 3.9) and the control subjects consisted of 46 males and 43 females (M/F ratio=1.1). Significant gender differences were observed between the groups (p< 0.001). Lung cancer cases were 2.5 years older than controls (p=0.16). More than half of the recruited sample was smokers (99; 52.9%). Smoking was more likely in lung cancer patients than controls (LC: 77; (78.6%) *vs* Controls: 22 (24.7%); OR: 11.2 (95%CI: 5.6-22.1); p<0.001). Men were more likely to smoke than women (men: 89 (71.8%); women: 10 (15.9%), OR: 13.5(95%CI: 6.2-29.4), p<0.001). The same relationship was seen in lung cancer patients and in controls [lung cancer patients: (men: 70 (89.7%) *vs* women: 7 (35%), OR: 16.3 (95%CI: 5.0-52.6), p<0.001)] and controls [men: 19 (41.3%) *vs* women: 3 (7%), OR: 9.4, (95%CI: 2.5-34.8), p<0.001].

Genetics

The distribution of *MTHFR C677T* and *A1298C* genotypes and their alleles are presented in Table 1. Neither *C677T* genotypes nor alleles was statistically different in lung cancer cases and controls (table 1). However, there were statistically significant differences in the genotype frequency of *MTHFR A1298C* between LC cases and the controls. The frequency of the *MTHFR* C allele was higher in patients with LC compared with healthy controls (Table 1). The CC genotype was associated with a 2.3 to 2.5-fold higher risk of LC (p=0.031); the AC genotype was associated with a 1.1-fold higher risk, albeit not statistically significant (p=0.74). Subjects with the C allele were 1.5 time more likely to suffer from lung cancer (p=0.057).

The allelic distribution of the two SNPs was in Hardy Weinberg equilibrium [(C677T: LC (X^2: 0.84, p=0.4); controls: (X^2:0.22, p=0.6)), (A1298C: LC (X^2: 0.82, p=0.4); controls: (X^2:0.48, p=0.5))].

Genetics and age

There was no statistically significant difference between *C677T* genotypes and age (CC: 57.4±12.5 years; CT: 58.1±11.0 years; TT: 53.9±15.4 years, p=0.47) and further sub-classification between cases and controls resulted in similar outcomes [(Controls: CC: 57.2±14.8 years; CT: 55.6±12.6 years; TT: 49.8±18.1 years, p=0.38); (Lung cancer: CC: 57.6±10.6 years; CT: 60.9±8.0 years; TT: 59.0±10.0 years, p=0.32)]

There was statistically significant difference between *A1298C* genotypes and age (AA: 55.6±14.3 years; AC: 59.7±10.1 years; CC: 53.9±12.9 years, p=0.027) and further sub-classification revealed age interaction with *A1298C* genotypes among controls but not cases [(Controls: AA: 52.7±16.2 years; AC: 60.0±10.6 years; CC: 49.7±18.3 years, p=0.026); (Lung cancer: AA: 59.0±11.2 years; AC: 59.5±9.7 years; CC: 55.8±9.4 years, p=0.37)]. The CC carriers were younger in the whole sample, controls and cases.

When comparing age between cases and control within each genotype, no significant differences were observed [($AA_{control}$ *vs* AA_{LC}, p=0.084); ($AC_{control}$ *vs* AC_{LC}, p=0.8); ($CC_{control}$ *vs* CC_{LC}, p=0.32)].

Genetics and smoking

The interaction between genotype and smoking was assessed for the two SNPs. There was no statistically significant difference between *C677T* genotypes and smoking (smokers: 54CC, 35CT, 7TT; non-smokers: 45CC, 31CT, 9TT, p=0.73) and between *A1298C* genotypes and smoking (smokers: 31AA, 47AC, 19CC; non-smokers: 29AA, 40AC, 16CC, p=0.95).

Stratification of data based on sample type (controls or LC) yielded no significant differences when comparing *C677T* genotypes to smoking [Lung cancer: (smokers: 42CC, 27CT, 5TT; non-smokers: 14CC, 4CT, 2TT, p=0.37); (Control: (smokers: 12CC, 8CT, 2TT; non-smokers: 31CC, 27CT, 7TT, p=0.86)]. Similar findings

Table 1. Genotypes and Allele Frequency of MTHFR C677T and A1298C and Risk of Lung Cancer

Parameter	Lung cancer Count	%	Controls Count	%	OR (95% CI)	p
677						
CC	56	59.6	43	49.4	Reference	
CT	31	33	35	40.2	0.68 (0.36-1.3)	0.23
TT	7	7.4	9	10.3	0.6 (0.21-1.7)	0.34
CC+CT	87	92.6	78	89.7	Reference	
TT	7	7.4	9	10.3	0.7 (0.25-2)	0.45
C	143	76	121	69.5	Reference	
T	45	24	53	30.5	0.72 (0.45-1.1)	0.19
1298						
AA	28	29.5	32	36.8	1 (reference)	
AC	43	45.3	44	50.6	1.1 (0.58-2.2)	0.74
CC	24	25.3	11	12.6	2.5 (1.04-6)	0.039
AA+AC	71	74.7	76	87.4	1 (reference)	
CC	24	25.3	11	12.6	2.3 (1.06-5.1)	0.031
A	99	52.1	108	62.1	1 (reference)	
C	91	47.9	66	37.9	1.5 (0.99 to 2.3)	0.057

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

Table 2. Association of Lung Cancer and Genetic Polymorphism of *MTHFR* with Smoking Status

	Smoking Behavior								Gender							
	Smokers				Non-smokers				Men				Women			
C677T	LC	Controls	OR (95%CI)	P	LC	Controls	OR (95%CI)	P	LC	Controls	OR (95%CI)	P	LC	Controls	OR (95%CI)	P
CC	42	12	Reference		14	31	Reference		40	20	Reference		16	23	Reference	
CT	27	8	0.96 (0.35-2.7)	1	4	27	0.33 (0.1-1.1)	0.1	28	21	0.67 (0.31-1.5)	0.33	3	14	0.31 (0.076-1.2)	0.13
TT	5	2	0.71 (0.12-4.2)	0.66	2	7	0.63 (0.11-3.4)	0.71	6	4	0.75 (0.19-3.0)	0.73	1	5	0.29 (0.031-2.7)	0.38
CC+CT	69	20	Reference		18	58	Reference		68	41	Reference		19	37	Reference	
TT	5	2	0.72 (0.13-4.0)	0.66	2	7	0.92 (0.18-4.8)	0.99	6	4	0.90 (0.24-3.4)	1	1	5	0.19 (0.020-1.8)	0.66
CC	42	12	Reference		14	31	Reference		40	20	Reference		16	23	Reference	
CT+TT	32	10	0.91 (0.35-2.4)	0.85	6	34	0.39 (0.13-1.1)	0.08	34	25	0.68 (0.32-1.4)	0.31	4	19	0.30 (0.087-1.1)	0.054
A1298C																
AA	20	11	Reference		8	21	Reference		19	20	Reference		9	12	Reference	
AC	37	10	2.0 (0.74- 5.6)	0.2	6	34	0.46 (0.14-1.5)	0.24	38	23	1.7 (0.77-3.9)	0.22	5	21	0.32 (0.087-1.2)	0.11
CC	18	1	9.9 (1.2-84.5)	0.018	6	10	1.6 (0.43-5.8)	0.52	19	3	6.7 (1.7-26.2)	0.005	9	8	1.5 (0.41-5.4)	0.74
AA+AC	57	21	Reference		14	55	Reference		57	43	Reference		14	33	Reference	
CC	18	1	6.6 (0.83-52.8)	0.064	6	10	2.4 (0.73-7.6)	0.19	19	3	4.8 (1.3-17.2)	0.01	9	8	2.7 (0.85-8.3)	0.74
AA	20	11	Reference		8	21	Reference		19	20	Reference		9	12	Reference	
AC+CC	55	11	2.8 (1.03-7.3)	0.039	12	44	0.72 (0.25-2.0)	0.53	57	26	2.3 (1.1-5.0)	0.034	14	29	0.64 (0.22-1.9)	0.17

were obtained for *A1298C* [Lung cancer: (smokers: 20AA, 37AC, 18CC; non-smokers: 8AA, 6AC, 6CC, p=0.29); (Control: (smokers: 11AA, 10AC, 1CC; non-smokers: 21AA, 34AC, 10CC, p=0.22)]

We analyzed genotype data for the two tissue types once among smokers and then among non-smokers. The 677 genotypes of *MTHFR* were distributed similarly between smokers of either tissue type (LC-smokers: 42 CC, 27 CT, 5 TT *vs* controls-smokers: 12 CC, 8 CT, 2 TT, p= 0.9). Similar findings were observed among the non-smokers.

There were statistically significant differences in 1298 genotypes of *MTHFR* among smokers (LC-smokers: 20 AA, 37 AC, 18 CC *vs* controls-smokers: 11 AA, 10 AC, 1 CC, p= 0.044). There was no statistically significant difference in any of 1298 genotypes of *MTHFR* among non-smokers. 1298CC genotype of *MTHFR* presented 9.9 times higher risk of LC in smokers as compared with the AA carriers (Table 2).

Genetics and gender

There was no statistically significant difference between *C677T* genotypes and gender distribution (Men: 60CC, 49CT, 10TT; Women: 39CC, 17CT, 6TT, p=0.19). Similarly, stratification of data based on sample type (controls or LC) yielded no significant differences when comparing *C677T* genotypes to gender [(Controls: Men: 20CC, 21CT, 4TT; Women: 23CC, 14CT, 5TT, p=0.43); (Lung cancer: Men: 40CC, 28CT, 6TT; Women: 16CC, 3CT, 1TT, p=0.11)]. We analyzed genotype data for the two tissue types once among men and then among women. There was no statistically significant difference in any of 677 genotypes of *MTHFR* among men (controls-men: 20 CC, 21 CT, 4 TT *vs* LC-men: 40 CC, 28 CT, 6 TT, p=0.6). Similar findings were observed among women (controls-women: 23 CC, 14 CT, 5 TT *vs* LC-women: 16 CC, 3 CT, 1 TT, p=0.2).

There was no statistically significant difference between *A1298C* genotypes and gender distribution (Men: 39AA, 61AC, 22CC; Women: 21AA, 26AC, 13CC, p=0.68). Similarly, stratification of data based on sample type (controls or LC) yielded no significant differences when comparing *A1298C* genotypes to gender [(Controls: Men: 20AA, 23AC, 3CC; Women: 12AA, 21AC, 8CC, p=0.13); (Lung cancer: Men: 19AA, 38AC, 19CC; Women: 9AA, 51AC, 5CC, p=0.11)]. We analyzed genotype data for the two tissue types once among men and then among women. There were statistically significant differences in 1298 genotypes of *MTHFR* among men (controls-men: 20 AA, 23 AC, 3 CC *vs* LC-men: 19 AA, 38 AC, 19 CC, p=0.014). There was no statistically significant difference in any of 1298 genotypes of *MTHFR* among women. Carriers of the 1298CC genotype were 6.7 times higher risk of LC in men as compared with the AA carriers (Table 2).

Genetics, smoking and gender

We analyzed 677 genotypes among "men who were smokers" in the two tissue categories. Different genotypes were distributed similarly between LC and controls (LC: CC= 37, CT= 26, TT= 4 *vs* Controls: CC= 9, CT= 8, TT= 2; p=0.7).

We analyzed 1298 genotypes among "men who were smokers" in the two tissue categories. The genotypes were distributed differently (LC: AA= 18, AC= 35, CC = 16 *vs* Controls: AA= 10, AC= 9, CC = 0, p= 0.02). Carriers of

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

Haplotype	Lung Cancer Count	%	Control Count	%	OR	95% CI	p
677C-1298A	60	33.1	64	36.9	0.83	(0.54-1.3)	0.4
677C-1298C	80	43.9	57	33.4	1.6	(1.03-2.4)	0.037
677T-1298A	39	21.3	42	24.7	0.84	(0.52-1.4)	0.53
677T-1298C	3	1.7	9	5	0.3	(0.081-1.1)	0.06
D'	0.84		0.56				
r^2	0.18		0.08				

*Counts reflect the number of chromosomes

the CC genotype among "men who were smokers" were at higher risk of LC (CC *vs* AA: OR=7.3, 95% CI=1.7-31.1; p=0.007).

Logistic regression

Backward stepwise logistic regression with conditional likelihood was used to find out the best subset of variables to predict lung cancer. The logistic regression was specified for significance level of 0.05 for entry and at level of 0.1 for removal. The different models were compared by conditional logistic regression. The Wald statistic was used in comparing the fitness of combined models. As a further check, -2 log-likelihood ratio statistic was utilized. Only when the two statistics agree, then the predictor is decided to be useful for the model. The following variables were evaluated as predictors of lung cancer: gender; smoking; age; and *A1298C* genotypes (CC *vs* others). The Hosmer-Lemeshow statistic indicated that the model adequately fits the data (p=0.685). The Nagelkerke's R^2 of the prediction model was 0.383. As a further check, the model was built using forward stepwise methods. The two methods chose the same variables. Smoking behavior and the 12988CC genotype remained statically significant with adjusted odds ratio of 12.1 (95% CI: 6.0-25; p<0.001) and 3.2 (95% CI: 1.3-8.2; p=0.015), respectively.

The logistic regression was repeated again utilizing *A1298C* genotypes CC *vs* AA rather than CC *vs* others. The other predictors were retained (gender; smoking, age). The Hosmer-Lemeshow statistic was (p=0.223) and the Nagelkerke's R^2 was (0.36). Smoking behavior and the 12988CC genotype remained statically significant with adjusted odds ratio of 8.1 (95% CI: 3.1-22.7; p<0.001) and 3.5 (95% CI: 1.2-10.1; p=0.02), respectively.

MTHFR haplotypes

All four different haplotypes appeared in our analysis. The most frequent haplotypes were CA (677C-1298A) (LC: 33.1%; controls: 36.9%), and CC (LC: 43.9%; controls: 33.4%) followed by TA (LC: 21.3%; controls: 24.7%), while the rare haplotype was TC (LC: 1.7%; controls: 5%) (Table 3). Our results indicated that the two loci 677 and 1298 show relatively strong linkage disequilibrium (Lewontin's coefficient [D']) (LC: D'=0.84, R^2: 0.18; Controls: D'=0.56, r^2: 0.08). Carriers of the CC haplotype were 1.6-fold more likely to be associated with lung cancer (OR: 1.6; 95% CI: 1.03-2.4, p=0.037). None of the remaining haplotypes was associated with LC.

Discussion

The mechanism of lung carcinogenesis is, like other cancers, still not fully understood. Among the lifestyle related causes of lung cancer, smoking is the primary risk factor for lung cancer. However, lung cancer develops in less than 20% of people who smoke throughout their life(Shields, 2002) This suggests that other factors including genetic susceptibility and nutritional habits contribute to carcinogenesis of lung cancer (Perera, 1998). Examination of genetic polymorphisms may explain individual differences in cancer risk.

Folate metabolism is thought to play a critical role in carcinogenesis through its involvement in both DNA methylation and repair. Previous studies revealed higher risk of breast and esophagus cancer but not colorectal cancer with less functional forms of *MTHFR* (Ergul et al., 2003; Stolzenberg-Solomon et al., 2003; Pardini et al., 2011). 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 another is *A1298C* (rs1801131; NT_021937.19: 7,859,208).

MTHFR C677T polymorphism results in substitution of alanine by valine at amino acid 222 in exon 4 (Frosst et al., 1995; Sharp and Little, 2004). Studies indicated that 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).

Generally speaking, the number of studies that evaluated the relationship between *MTHFR* genetic polymorphism and LC risk is few and moreover their findings have been conflicting where some yielded an increased risk (Siemianowicz et al., 2003; Shen et al., 2005; Hung et al., 2007; Vineis et al., 2007; Arslan et al., 2011), a decreased risk (Jeng et al., 2003; Shi et al., 2003; Suzuki et al., 2007; Liu et al., 2009; Cui et al., 2011) or no association (Shen et al., 2001; Heijmans et al., 2003; Liu et al., 2008; Truong et al., 2010).

Prior to our investigation, based on Zhang et al, the studies that investigated *MTHFR C677T* and *A1298C* were distributed by ethnicity as follows: 5 studies recruited Caucasians (USA, The Netherlands, Poland and France); 9 studies examined individuals of Asian descent (China, Taiwan, Korea, and Japan); the last study was from Turkey (Zhang et al., 2012). This is the first study conducted in Jordan and the 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 (Central Intelligence Agency, 2012; The Royal Hashemite Court, 2012).

In 2008, Mao et al. performed a meta-analysis based on eight studies and did not find a relationship between the *MTHFR* polymorphisms and the risk of lung cancer (Mao et al., 2008). In 2009, another meta-analysis aggregated with ten studies and also did not find a significant relationship between the polymorphisms and lung cancer risk, but an increased risk for individuals with low folate intake (Boccia et al., 2009). In 2012, a meta-analysis of 14 publications found no significant association between the *MTHFR C677T* polymorphism and LC risk irrespective of race, Stratified analysis by histological type indicated significantly increased non-small-cell lung cancer (NSCLC) risk among the T allele carriers (T-allele *vs* C-allele: OR=1.11, 95%CI=1.03-1.19; TT *vs* CC: OR=1.24, 95%CI=1.09-1.41; TC *vs*CC: OR=1.11, 95%CI=1.03-1.20 and TT+TC *vs* CC: OR=1.09, 95%CI=1.03-1.15) (Hou et al., 2012). A more recent meta-analysis of 20 studies was published in 2013 that evaluated the association between *MTHFR* C677CT polymorphism and lung cancer risk (8 Caucasians and 12 Asians) (Liu et al., 2013). Seven studies suggested that *MTHFR C677T* polymorphism was associated with an increased lung cancer risk. After stratification by ethnicity (Asian and Caucasian), a significant association between *MTHFR C677T* polymorphism and lung cancer risk was observed in Asian subjects (OR=1.31, 95% CI =1.01–1.71, p=0.045, TT *vs* CC), while no significant association was found among Caucasians (OR=1.16; 95% CI =0.91–1.49,p=0.224, TT *vs* CC) (Liu et al., 2013). The association between lung cancer and *MTHFR C677T* polymorphism has been recently confirmed (Rai, 2014).

In our study, none of the 677 genotypes or allele types was distributed differentially between cases and controls. The lack of association persisted even after stratification by gender or smoking status (Table 2 and 3). Logistic regression (data not presented) confirmed the lack of association.

The discrepancies in the results may be explained by differences in ethnicity (Cheng et al., 2012), dietary intake, exposure to environmental carcinogens and sample size. Folate intake has been suggested to modulate cancer risk associated with *MTHFR* genetic polymorphism (Chen et al., 1996; Ma et al., 1997). Kiyohora et al have proposed that excessive folate supplementation increases folic acid levels, reduce homocysteine levels and then restore normal methionine levels, particularly in these individuals with the TT genotype of the *MTHFR* polymorphism (Paul et al., 2004; Kiyohara et al., 2011).

MTHFR A1298C polymorphism results in an amino acid substitution of alanine to glutamate at codon 429 of the protein in exon 7 (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 the relationship between *A1298C* genotype 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 less active form of *MTHFR* leads to lower S-adenosyl-L-methionine levels and consequently to hypomethylation; this phenomenon would be expected to increase the risk of some cancers (Stern et al., 2000). It is expected that 1298CC carriers are at higher risk for cancer development.

In our study, we also investigated the frequencies of the *MTHFR A1298C* genotypes in Jordanian patients with LC and the healthy controls. Contrary to the apparent lack of association between *MTHFR C677T* and the risk of LC, the *MTHFR A1298C* polymorphism was found to be significantly related with the risk of LC. There was significant interaction between genetic polymorphism at 1298 and both smoking status and subjects' gender. Carriers of the 1298CC genotype as compared with the AA carriers presented 9.9, 6.7 and 7.3 times higher risk of LC in smokers, men, "men who were smokers", respectively. Because of the unequal distribution of gender and smoking among cases and controls, logistic regression was conducted, and confirmed the association between 1298CC and susceptibility to lung cancer. The interaction between *A1298C* and smoking maybe explained by the reported decreased circulating folate levels due to low folate intake in smokers (Dastur et al., 1972; Witter et al., 1982).

The results for the *A1298C* variant are consistent with some but not all published studies. A case-control study evaluated the role of the *MTHFR A1298C* polymorphisms in 462 lung cancer cases and 379 controls in a Japanese population. The 1298CC genotype was only associated with increased risk among ever-smokers (aOR= 3.2, 95% CI= 1.2-8; p=0.02) non-smokers (aOR=2.83, 95% CI= 1.02-7.8; p=0.05) and excessive drinkers (aOR=7.24, 95% CI=1.89-27.7, p<0.001) (Kiyohara et al., 2011). The 1298CC genotype was associated with a significantly increased risk of lung cancer in women in one study (Shi et al., 2005).

A Turkish study involved 64 lung cancer patients and 61 controls. Lung cancer risk was 1.5 times higher in 1298CC genotype though it was not statistically significant. Numerous other studies worldwide have reported lack of association of *MTHFR A1298C* polymorphism with the risk of lung carcinoma (Zhang et al.; Mao et al., 2008; Boccia et al., 2009; Liu et al., 2013)

The physical distance between the two SNPs is short (1.9 kb), and thus one may expect that LD is evident. There is evidence of linkage disequilibrium between the *C677T* and the *A1298C* variants of the *MTHFR* gene with the possibility that both SNPs may be dependent on each other from the genetic and functional point of view (Chen et al., 2002; Shi et al., 2003). The interdependence of the two loci may imply that haplotype analysis plays an important role in the identification of genetic variations between cases and controls in addition to the customary analysis of SNPs. Although not stated, we calculated D' and r^2 among different ethnicities: Chinese (Controls: D'=0.13, r^2=0.004) (Li et al., 2011); Indians (Controls: D'=0.56, r^2=0.08) (Chandy et al., 2010); and three sets of Americans: non-Hispanic whites (Controls: D'=0.24,

100.0
6.3
75.0
56.3
50.0
25.0
31.3
0
Newly diagnosed without treatment

r^2=0.043; LC: D'=0.25, r^2=0.045) (Shen et al., 2001); African Americans (Controls: D'=1, r^2=0.03) (Keku et al., 2002); and mixed-population Americans (Controls: D'=1, r^2=0.23) (Curtin et al., 2004). A study from central Europe involved 2250 lung cancer cases, 811 upper aero-digestive tract cases and 2899 controls reported relatively strong linkage disequilibrium (D'= -0.99, r^2=0.21) (Hung et al., 2007). Our results indicated that the two loci showed relatively strong LD among cases and controls ([D'] = [0.56 (controls), 0.84 (LC)]).

The most frequent haplotypes of *MTHFR* among Caucasians were reported as 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 a similar trend, albeit a slightly lower TA distribution (24.7%) and higher TC (5%). Because of rarity of TT/CC (677TT and 1298CC) and TT/AC among Jordanians and worldwide, 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, Canada (Ogino and Wilson, 2003) and USA (Shen et al., 2001). Utilizing published data, we calculated the TC haplotype among other ethnicities and found it rare as well (non-Hispanic white Americans 13.2% (Shen et al., 2001);Chinese 9.4 % (Li et al., 2011) and African Americans 0% (Keku et al., 2002)). Interestingly, the haplotype analysis based on the two investigated *MTHFR* polymorphisms (*C677T* and A1298C) showed that haplotype CC was more common in Jordanians with lung cancer than controls (OR=1.6, 95% CI= 1.03-2.4, p=0.037). Examining the literature revealed few studies that investigated the effect of *MTHFR* haplotypes on susceptibility to lung cancer (Shen et al., 2005; Hung et al., 2007). The TA haplotype, rather than the CC haplotype, was associated with in increased risk of lung cancer in a Central European study (OR=1.16, 95% CI=1.04–1.29) (Hung et al., 2007) and a Chinese study (OR= 1.67, 95% CI: 1.10-2.52) (Shen et al., 2005). In the Chinese study the CC haplotype was associated with increased risk though not statistically significant.

In conclusion, the findings of the current study suggest that genetic polymorphism of *MTHFR* at *A1298C* and its haplotype analysis at 677and 1298 modulates the risk of LC in the Jordanian population. To our knowledge, this is being reported for the first time among this population.

Acknowledgements

This study was supported by unconditional grant from the Deanship of Scientific Research (University of Jordan, Jordan). The study sponsors had no part in the study design, data collection, data analysis, data interpretations or in the writing of the manuscript. The sponsors had no role in the decision to submit the paper for publication.

References

Arslan S, Karadayi S, Yildirim ME, et al (2011). The association between methylene-tetrahydrofolate reductase gene polymorphism and lung cancer risk. *Mol Biol Rep*, **38**, 991-6.

Bagley PJ, Selhub J (1998). A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. *Proc Natl Acad Sci USA*, **95**, 13217-20.

Boccia S, Boffetta P, Brennan P, et al (2009). Meta-analyses of the methylenetetrahydrofolate reductase *C677T* and *A1298C* polymorphisms and risk of head and neck and lung cancer. *Cancer Lett*, **273**, 55-61.

Brockton NT (2006). Localized depletion: the key to colorectal cancer risk mediated by *MTHFR* genotype and folate? *Cancer Causes Control*, **17**, 1005-16.

Brooker R (ed.) (2005). Genetics, Analysis and Principles, McGraw-Hill, USA.

Central Intelligence Agency (2012). The world factbook [Online]. Available: https://www.cia.gov/library/publications/the-world-factbook/fields/2075.html [Accessed Feb 18th 2012].

Chandy S, Sadananda Adiga MN, Ramachandra N, et al (2010). Association of methylenetetrahydrofolate reductase gene polymorphisms & colorectal cancer in India. *Indian J Med Res*, **131**, 659-64.

Chango A, Boisson F, Barbe F, et al (2000). The effect of 677C-->T and 1298A-->C mutations on plasma homocysteine and 5,10-methylenetetrahydrofolate reductase activity in healthy subjects. *Br J Nutr*, **83**, 593-6.

Chen J, Giovannucci E, Kelsey K, et al (1996). A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res*, **56**, 4862-4.

Chen J, Ma J, Stampfer MJ, et al (2002). Linkage disequilibrium between the 677C>T and 1298A>C polymorphisms in human methylenetetrahydrofolate reductase gene and their contributions to risk of colorectal cancer. *Pharmacogenetics*, **12**, 339-42.

Cheng Z, Wang W, Dai LL, et al (2012). *MTHFR* C667T polymorphism association with lung cancer risk in Henan province: a case-control study. *Asian Pac J Cancer Prev*, **13**, 2491-4.

Cicek MS, Nock NL, Li L, et al (2004). Relationship between methylenetetrahydrofolate reductase *C677T* and *A1298C* genotypes and haplotypes and prostate cancer risk and aggressiveness. *Cancer Epidemiol Biomarkers Prev*, **13**, 1331-6.

Cui LH, Shin MH, Kim HN, et al (2011). Methylenetetrahydrofolate reductase *C677T* polymorphism in patients with lung cancer in a Korean population. *BMC Med Genet*, **12**, 28.

Curtin K, Bigler J, Slattery ML, et al (2004). *MTHFR C677T* and *A1298C* polymorphisms: diet, estrogen, and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev*, **13**, 285-92.

Dastur DK, Quadros EV, Wadia NH, et al (1972). Effect of vegetarianism and smoking on vitamin B12, thiocyanate, and folate levels in the blood of normal subjects. *Br Med J*, **3**, 260-3.

Ergul E, Sazci A, Utkan Z, et al (2003). Polymorphisms in the *MTHFR* gene are associated with breast cancer. *Tumour Biol*, **24**, 286-90.

Ferlay J, Shin HR, Bray F, et al (2012). GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide 2012 [Online]. Lyon, France: International Agency for Research on Cancer; 2012. Available: http://globocan.iarc.fr [Accessed Jan 2014 2014].

Friedman G, Goldschmidt N, Friedlander Y, et al (1999). A common mutation *A1298C* in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine

and folate concentrations. *J Nutr*, **129**, 1656-61.

Frosst P, Blom HJ, Milos R, et al (1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*, **10**, 111-3.

Goyette P, Pai A, Milos R, et al (1998). Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). *Mamm Genome*, **9**, 652-6.

Graph Pad Software Inc. Available: http://www.graphpad.com/quickcalcs/chisquared1.cfm [Accessed 20th February 2011].

Heijmans BT, Boer JM, Suchiman HE, et al (2003). A common variant of the methylenetetrahydrofolate reductase gene (1p36) is associated with an increased risk of cancer. *Cancer Res*, **63**, 1249-53.

Hong W, Wang K, Zhang YP, et al (2013). Methylenetetrahydrofolate reductase *C677T* polymorphism predicts response and time to progression to gemcitabine-based chemotherapy for advanced non-small cell lung cancer in a Chinese Han population. *J Zhejiang Univ Sci B*, **14**, 207-15.

Hou XH, Huang YM, Mi YY (2012). Methylenetetrahydrofolate reductase gene *C677T* polymorphism and lung cancer: an updated meta-analysis. *Asian Pac J Cancer Prev*, **13**, 2025-9.

Hung RJ, Hashibe M, McKay J, et al (2007). Folate-related genes and the risk of tobacco-related cancers in Central Europe. *Carcinogenesis*, **28**, 1334-40.

Jeng YL, Wu MH, Huang HB, et al (2003). The methylenetetrahydrofolate reductase 677C-->T polymorphism and lung cancer risk in a Chinese population. *Anticancer Res*, **23**, 5149-52.

Jordan Cancer Registry (2010). Cancer incidence in Jordan [Internet]. [Online]. Amman, Jordan: Jordan National Cancer Registry; 2008. Available: http://www.moh.gov.jo/AR/Documents/Annual%20Incidence%20of%20cancer%20in%20Jordan%202010.pdf [Accessed January 2014].

Keku T, Millikan R, Worley K, et al (2002). 5, 10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. *Cancer Epidemiol Biomarkers Prev*, **11**, 1611-21.

Kiyohara C, Horiuchi T, Takayama K, et al (2011). Methylenetetrahydrofolate reductase polymorphisms and interaction with smoking and alcohol consumption in lung cancer risk: a case-control study in a Japanese population. *BMC Cancer*, **11**, 459.

Kiyohara C, Horiuchi T, Takayama K, et al (2013). Methylenetetrahydrofolate reductase polymorphisms and interaction with smoking and alcohol consumption in lung cancer risk: a case-control study in a Japanese population. *BMC Cancer*, **11**, 459.

Kono S, Chen K (2005). Genetic polymorphisms of methylenetetrahydrofolate reductase and colorectal cancer and adenoma. *Cancer Sci*, **96**, 535-42.

Li H, Xu WL, Shen HL, et al (2011). Methylenetetrahydrofolate reductase genotypes and haplotypes associated with susceptibility to colorectal cancer in an eastern Chinese Han population. *Genet Mol Res*, **10**, 3738-46.

Lievers KJ, Boers GH, Verhoef P, et al (2001). A second common variant in the methylenetetrahydrofolate reductase (MTHFR) gene and its relationship to *MTHFR* enzyme activity, homocysteine, and cardiovascular disease risk. *J Mol Med (Berl)*, **79**, 522-8.

Liu CS, Tsai CW, Hsia TC, et al (2009). Interaction of methylenetetrahydrofolate reductase genotype and smoking habit in Taiwanese lung cancer patients. *Cancer Genomics Proteomics*, **6**, 325-9.

Liu H, Jin G, Wang H, et al (2008). Association of polymorphisms in one-carbon metabolizing genes and lung cancer risk: a case-control study in Chinese population. *Lung Cancer*, **61**, 21-9.

Liu ZB, Wang LP, Shu J, et al (2013). Methylenetetrahydrofolate reductase 677TT genotype might be associated with an increased lung cancer risk in Asians. *Gene*, **515**, 214-9.

Lowi MR 1995. Water and power: the politics of a scarce resource in the Jordan River basin, Cambridge, Cambridge University Press.

Lucock M (2004). Is folic acid the ultimate functional food component for disease prevention? *BMJ*, **328**, 211-4.

Ma J, Stampfer MJ, Giovannucci E, et al (1997). Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res*, **57**, 1098-102.

Mao R, Fan Y, Jin Y, et al (2008). Methylenetetrahydrofolate reductase gene polymorphisms and lung cancer: a meta-analysis. *J Hum Genet*, **53**, 340-8.

Meisel C, Cascorbi I, Gerloff T, et al (2001). Identification of six methylenetetrahydrofolate reductase (MTHFR) genotypes resulting from common polymorphisms: impact on plasma homocysteine levels and development of coronary artery disease. *Atherosclerosis*, **154**, 651-8.

Ogino S, Wilson RB (2003). Genotype and haplotype distributions of MTHFR677C>T and 1298A>C single nucleotide polymorphisms: a meta-analysis. *J Hum Genet*, **48**, 1-7.

Pardini B, Kumar R, Naccarati A, et al (2011). *MTHFR* and MTRR genotype and haplotype analysis and colorectal cancer susceptibility in a case-control study from the Czech Republic. *Mutat Res*, **721**, 74-80.

Paul RT, McDonnell AP, Kelly CB (2004). Folic acid: neurochemistry, metabolism and relationship to depression. *Hum Psychopharmacol*, **19**, 477-88.

Perera FP (1998). Molecular epidemiology of environmental carcinogenesis. *Recent Results Cancer Res*, **154**, 39-46.

Rai V (2014). Folate Pathway Gene *MTHFR C677T* polymorphism and risk of lung cancer in Asian populations. *Asian Pac J Cancer Prev*, **15**, 9259-64.

Sharp L, Little J (2004). Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. *Am J Epidemiol*, **159**, 423-43.

Shen H, Spitz MR, Wang LE, et al (2001). Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer: a case-control study. *Cancer Epidemiol Biomarkers Prev*, **10**, 397-401.

Shen M, Rothman N, Berndt SI, et al (2005). Polymorphisms in folate metabolic genes and lung cancer risk in Xuan Wei, China. *Lung Cancer*, **49**, 299-309.

Shi M, Caprau D, Romitti P, et al (2003). Genotype frequencies and linkage disequilibrium in the CEPH human diversity panel for variants in folate pathway genes MTHFR, MTHFD, MTRR, RFC1, and GCP2. *Birth Defects Res A Clin Mol Teratol*, **67**, 545-9.

Shi Q, Zhang Z, Li G, et al (2005). Sex differences in risk of lung cancer associated with methylene-tetrahydrofolate reductase polymorphisms. *Cancer Epidemiol Biomarkers Prev*, **14**, 1477-84.

Shields PG (2002). Molecular epidemiology of smoking and lung cancer. *Oncogene*, **21**, 6870-6.

Siemianowicz K, Gminski J, Garczorz W, et al (2003). Methylenetetrahydrofolate reductase gene *C677T* and *A1298C* polymorphisms in patients with small cell and non-small cell lung cancer. *Oncol Rep*, **10**, 1341-4.

Stern LL, Mason JB, Selhub J, et al (2000). Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the *C677T* polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev*, **9**, 849-53.

Stolzenberg-Solomon RZ, Qiao YL, Abnet CC, et al (2003). Esophageal and gastric cardia cancer risk and folate- and vitamin B(12)-related polymorphisms in Linxian, China. *Cancer Epidemiol Biomarkers Prev*, **12**, 1222-6.

Suzuki T, Matsuo K, Hiraki A, et al (2007). Impact of one-carbon metabolism-related gene polymorphisms on risk of lung cancer in Japan: a case control study. *Carcinogenesis*, **28**, 1718-25.

Swartz MD, Peterson CB, Lupo PJ, et al (2013). Investigating multiple candidate genes and nutrients in the folate metabolism pathway to detect genetic and nutritional risk factors for lung cancer. *PLoS One*, **8**, 53475.

Terrazzino S, Agostini M, Pucciarelli S, et al (2006). A haplotype of the methylenetetrahydrofolate reductase gene predicts poor tumor response in rectal cancer patients receiving preoperative chemoradiation. *Pharmacogenet Genomics*, **16**, 817-24.

The Royal Hashemite Court. 2012. Keys to the Kingdom-The People of Jordan. [Online]. Available: http://www.kinghussein.gov.jo/people.html [Accessed Feb 18th 2012].

Truong T, Sauter W, McKay JD, et al (2010). International Lung Cancer Consortium: coordinated association study of 10 potential lung cancer susceptibility variants. *Carcinogenesis*, **31**, 625-33.

van der Put NM, Gabreels F, Stevens EM, et al (1998). A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet*, **62**, 1044-51.

Vineis P, Veglia F, Garte S, et al (2007). Genetic susceptibility according to three metabolic pathways in cancers of the lung and bladder and in myeloid leukemias in nonsmokers. *Ann Oncol*, **18**, 1230-42.

Weisberg I, Tran P, Christensen B, et al (1998). A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab*, **64**, 169-72.

Witter FR, Blake DA, Baumgardner R, et al (1982). Folate, carotene, and smoking. *Am J Obstet Gynecol*, **144**, 857.

Yi P, Pogribny I, Jill James S (2002). Multiplex PCR for simultaneous detection of 677 C-->T and 1298 A-->C polymorphisms in methylenetetrahydrofolate reductase gene for population studies of cancer risk. *Cancer Lett*, **181**, 209.

Zhang Y, Chen GQ, Ji Y, et al (2012). Quantitative assessment of the effect of *MTHFR* polymorphisms on the risk of lung carcinoma. *Mol Biol Rep*, **39**, 6203-11.