# Association of Human Methionine Synthase-A2756G Transition With Prostate Cancer: A Case-Control Study and in Silico Analysis

Arezou Ebrahimi<sup>1,2</sup>, Abasalt Hosseinzadeh Colagar<sup>2</sup>, and Mohammad Karimian<sup>1</sup>

<sup>1</sup> Department of Biology, Faculty of Sciences, Islamic Azad University, Damghan Branch, Damghan, Iran
<sup>2</sup> Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran

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**Abstract**- Methionine synthase (MTR) is one of the key enzymes of folate pathway, which play a key role in the construction, repair, and methylation of DNA. In this study, an association of MTR A2756G gene transition with prostate cancer in men populations of Kashan-Iran was investigated by a case-control study and an in silico analysis. The 200 samples including 100 patients with prostate cancer, as case group and 100 healthy men, as control group included in this study. MTR-A2756G genotyping was performed by PCR-RFLP technique. Some in silico tools used to evaluate the effects of A2756G transition on the structure and function of MTR. Results showed that the AG genotype (OR: 2.4014, 95% CI: 1.3216-4.3636, P=0.0040), and GG genotype (OR: 3.6324, 95% CI: 1.2629-10.4475, P=0.0167) and G allele (OR: 2.0120, 95% CI: 1.3098-3.0905, P=0.0014) were associated with prostate cancer. In silico analysis showed that polymorphisms of the enzyme protein might change properties of MTR such as relative mutability and flexibility, which leads to alteration of stability and function of the enzyme. Based on the results, an MTR-A2756G polymorphism which changes activity and stability of the methionine synthase associated with prostate cancer in men. It is a preliminary study and is presenting data for future comprehensive study for making a clinical conclusion that this gene transition is a biomarker for susceptibility to prostate cancer. © 2017 Tehran University of Medical Sciences. All rights reserved. Acta Med Iran 2017;55(5):297-303.

**Keywords:** Prostate cancer; *MTR* gene; A2756G transition; PCR-RFLP

## Introduction

The prostate cancer is one of the most commonly diagnosed solid malignancies and the sixth leading cause of cancer-related deaths in males in the worldwide (1). Prostate cancer as a multifactorial disorder interacts with environmental, ethnic, hormonal, and lifestyle factors (2). The carcinogenesis mechanisms in the prostate, like other cancers, remain unclear yet. Folate metabolizing genes are thought to play an important role in tumorigenesis through its involvement in both DNA methylation and repair (3). One of the key enzymes in folate pathway is methionine synthase (MTR) which located on chromosome 1 (1q43) of human (4,5). This gene is composed of 34 exons, and encodes a protein with 1265 amino acids with molecular weight 140.5 kDa (Figure 1A), is composed of 34 exons, and encodes a protein with 1265 amino acids in molecular weight 140.5 kDa (6). There is a common polymorphism in MTR gene at position 2756 (MTR A2756G; rs1805087) which result in an aspartic acid to glycine substitution at location 919 (D919G) (7). The remethylation of homocysteine to methionine is catalyzed by MTR in a reaction depending on vitamin B12 as an intermediate carrier of the methyl group (8). MTR polymorphisms can reduce the activity of an enzyme that it is associated with a defect of numerous functions in human cells. For instance, this transition may cause homocysteine elevation and DNA hypomethylation (9). However, some studies suggested a modest inverse association between 2756GG polymorphism and homocysteine levels, indicating an increased enzymatic activity of the variant genotype (10). Moreover, the polymorphism may result in CpG hypermethylation in tumor suppressor genes (11). Association of MTR-A2756G transition has been reported in various diseases, including coronary heart disease (12) and several cancers such as cancers of the esophagus, stomach, and liver (13) and some studies demonstrated the association of MTR A2756G

polymorphism with prostate cancer risk (8). However, the association between polymorphism and cancer risk is still controversial. The aim of this study was to investigate the association of *MTR* A2756G with prostate cancer in Iranian men which followed by an *in silico* approach.

## **Materials and Methods**

## Collection and analysis of blood samples

In this experimental work, 200 samples consisted of 100 patients with prostate cancer as case group and 100 healthy men as control group included in this study. All of the participants were referred to Shahid Beheshti Hospital (Kashan; Iran), during the May 2013 to January 2015. The patients histologically verified for prostate cancer. The patients' clinical records had important pathological characteristics for this research such as age, BMI, smoking status, and level of the PSA, before treatment. Control subjects were selected to the study without any history of prostate cancer. The whole of the case and control samples had the same geographical origin. Finally, blood samples collected into tubes containing EDTA $_{\rm Na2}$  as an anticoagulant.

Written informed consent was obtained from all cases and controls. This study was approved by the Medical Research Ethics Committee of the Kashan University of Medical Sciences.

## DNA extraction and PCR-RFLP

Genomic DNA was extracted from blood leukocytes by the salting-out procedure (14). Extracted total DNA, were checked in agarose gel at voltage 90 V for 40 min, with 1X TBE buffer (contain: 10.8 g of Tris base. 5.5 g of boric acid. 4 ml of 0.5 M EDTA pH 8.0), and DNA bands were visualized by UV light transilluminator after staining with 1  $\mu$ g/mL ethidium bromide at 25° C for 5 min.

For primer designing, the entire sequence of MTR gene was obtained from NCBI (Accession No. NC\_000001). The primer sequences were designed by the oligo7 software. The forward and reverse primer sequences were *MTR*-f: 5'-AAGCCCACTGAGTTTACCTTTTC -3' and *MTR*-r: 5'-ATCCAAAGCCTTTTACACTCCTC -3', respectively. To amplify the *MTR* fragments, a polymerase chain reaction (PCR) was performed in 25 μL PCR reaction containing 0.2 μL *Taq* DNA polymerase, 2.5 μL AMS, 2.5 μL DMSO (10%V/V), 5 μL betaine (5M), 0.5 μL dNTPs mix, 0.35 μmol/l each of forward and reverse primers, 2 μL MgCl2 and 2.5μL template DNA. The

PCR was performed in a thermal cycler (Eppendorf, Hamburg, Germany) with the following program: the initial denaturation step at 94° C for 10 min was followed by 35 repetitive cycles including, denaturation temperature: 94°C for 45 sec, annealing temperature: 61° C for 45 sec and extension temperature: 72° C for 45 sec, and final extension at 72° C for 5 min. PCR Products were separated on a 1% agarose gel at 70 V for 1 h, with 1X TBE buffer and DNA bands were visualized by transillumination under UV light after staining with 1 µg/mL ethidium bromide (Merck) at 25° C for 5 min. To detect the MTR A2756G genotype, the amplified fragments were digested with HaeIII restriction enzyme. Each restriction digestion reaction contained 6 µl of the PCR product, 0.5 µl of HaeIII, and 0.7 µl R buffer in a total volume of 10 µl. The enzymatic mixture was then incubated at 37° C for 16 hours. Finally, PCR-RFLP products were electrophoresed in 1% agarose gel.

## **DNA** sequencing

The PCR product (381-bp) which amplified by using the *MTR*-f and *MTR*-r primer pairs were purified with the PCR product recovery kit from Roche Applied Science (Mannheim, Germany). Direct sequencing of the purified PCR product was done with the Bioneer Co. (Korea). The electropherograms were analyzed by using Chromas software ver. 1.45 (http://www.technelysium.com.au/chromas.html).

## **Bioinformatics tools**

The entire genomic sequences of human *MTR* gene was deduced from NCBI (accession No: NC\_000001) and analyzed with GeneRunner software 5.1.02 Beta. The coding sequence domain of MTR detected and translated to amino acid sequence by Expasy web server (web.expasy.org/translate). The Average flexibility and Relative mutability scores were calculated for normal and mutant MTR by ExPASy server (http://web.expasy.org/protscale/). Finally, the network of gene-gene interaction for *MTR* gene was obtained from String online server (http://string-db.org/).

## Statistical analysis

The Hardy-Weinberg equilibrium in case and control groups was calculated by Oege online server (http://www.oege.org/software/Hardy-Weinberg.html). The odd ratios (OR) and 95% confidence intervals (95% CI) were calculated for each allele and genotypes. Differences between cases and healthy controls were evaluated by *Chi*-square test. The *P* less than 0.05

(P<0.05) was considered as statistically significant. All of the statistical analyses were performed by using the SPSS software version 19.0 (SPSS, Chicago, IL, USA).

#### Results

## MTR-A2756G genotyping

Total human genome showed a major band with low mobility in agarose gel. By use of the isolated genome as a template, MTR fragment containing A2756G transition with the size of 381-bp was amplified by using the forward and reverse primers. After electrophoresis of the enzymatic mixture, the AA genotype showed one band (381-bp) on an agarose gel; the AG genotype showed three bands (381-bp, 251-bp, and 130-bp) on agarose gel whereas GG genotype showed two bands (251-bp and 130-bp) on an agarose gel (Figure 1B). PCR-RFLP results were confirmed by DNA sequencing. Partial DNA sequence data at the A2756G location is shown in figure 1C. To final confirmation of PCR-RFLP results, PCR products were checked by DNA sequencing. Data showed that the sequence of DNA was bonafide at 2756A>G location. Partial sequence data at the location of 2756 is depicted in figure 1D.

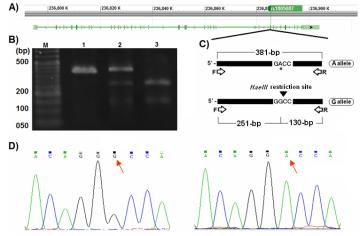


Figure 1. Polymorphism analysis of 2756A>G: (A) MTR Gene map composed of 34 exons; (B) The PCR products which digested with HaeIII restriction enzyme (lane M, DNA marker; Lane 1, AA genotype; Lane 2, AG genotype; Lane 3, GG genotype); (C) Schematic of the PCR product digestion in the RFLP technique; (D) MTR sequence in sample with the genotype GG (left) and sample with genotype AA (right).

### Distribution of alleles and genotypes frequencies

The MTR genotypes distribution for the A2756G transition was in Hardy-Weinberg equilibrium in the case and control groups. The genotypes and alleles frequencies for the A2756G in case and control groups are shown in Table 1. The AA, AG and GG genotypes frequencies in cases were 34%, 53%, and 13%, whereas these ratios in controls were 57%, 37%, and 6%, respectively. The A and G allele frequencies for total cases were 60.5% and 39.5%, whereas these ratios in controls were 75.5% and 24.5%, respectively. Genotype analyses showed a significant association of AG (OR 2.4014; 95% CI 1.3216 to 4.3636; P=0.0040) and GG (OR 3.6324; 95% CI 1.2629 to 10.4475; *P*=0.0167) genotypes with prostate cancer. Furthermore, carriers of the G allele (AG+GG) were at highest risk of prostate cancer (OR 2.5732; 95% CI 1.4513 to 4.5622; P=0.0012). Also, allele analysis showed a significant association of G allele with prostate cancer (OR 2.0120; 95% CI 1.3098 to 3.0905; *P*=0.0014).

Table 1. Genotype and allele frequencies of the MTR-2756A>G in case and control groups

Genotype -		No. and Percentage		OR (95% CI)	P
		Control(n=100) 57 (57%)	Case (n=100) 34 (34%)	- OK (95% CI)	- r
GG		6 (6%)	13 (13%)	3.6324 (1.2629 to 10.4475)	0.0167
AG+GG		43 (43%)	66 (66%)	2.5732 (1.4513 to 4.5622)	0.0012
Allele	$\mathbf{A}$	151 (75.5%)	121 (60.5%)	-	-
	G	49 (24.5%)	79 (39.5%)	2.0120 (1.3098 to 3.0905)	0.0014

OR, odds ratio; CI, confidence interval.

## In silico analysis

The A2756G transition causes an Asp to Gly substitution at codon 919 (D919G) in the sequence of MTR protein. The data from Protparam server showed that the D919G substitution changes physicochemical properties of MTR. The phenotype of MTR-919D with  $C_{6268}H_{9906}N_{1676}O_{1872}S_{59}$  molecular formula and molecular weight of 140587.2 Da has an isoelectric point (pI) of 5.39. While the molecular weight of 919G phenotype was reduced to 140529.2 Da. The average flexibility of the MTR protein was 0.472

(Expasy/ProtParam server; http://web.expasy.org/cgibin/protparam/protparam). The average flexibility of the 2756G mutant protein increased to 0.476 (Figure 2). The relative mutability of the normal MTR protein was 49.02, compared with 49.05 in the mutant protein (Figure 2). Gene-gene and gene-environment interactions are effective in most cellular activity. MTR gene interacts with a number of genes, which each of these genes may affect the activity of MTR. The network of gene-gene interaction obtained from String server for MTR gene is shown in figure 3.

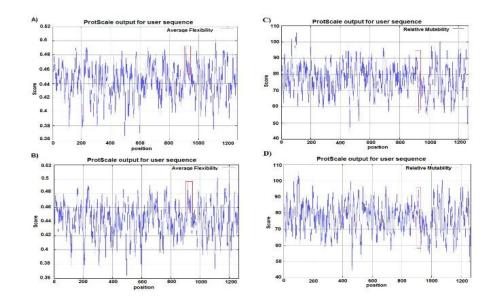


Figure 2. The results of an average protein flexibility and mutability. A and B) Protein flexibility of 919D and 919G phenotype, respectively6; C and D) Protein relative mutability of 919D and 919G, respectively. The location of 919 is shown with a red box.

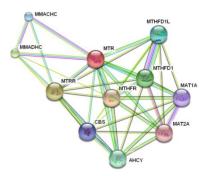


Figure 3. Human MTR-interactions network obtained from String server. MTR gene linked with 10 other genes: MTR; 5-methyltetrahydrofolatehomocysteine methyltransferase. MTHFR; methylenetetrahydrofolate reductase. MTRR; 5-methyltetrahydrofolate-homocysteine methyltransferase reductase. MMADHC; methylmalonic aciduria cblD type, with homocystinuria. MTHFD1; methylenetetrahydrofolate dehydrogenase 1. AHCY; adenosylhomocysteinase. MTHFD1L; methylenetetrahydrofolate dehydrogenase 1-like. MMACHC; methylmalonic aciduria cblC type, with homocystinuria. CBS; cystathionine-beta-synthase. MATIA; methionine adenosyltransferase I, alpha. MAT2A; methionine adenosyltransferase II, alpha.

## **Discussion**

Prostate cancer is the second most prevalent cancer among men globally, and it has markedly high rates in developed countries (15). Some risk factors instance of age, ethnicity and family history could influence the process of the prostate carcinogenesis (16). Many putative gene markers have been reported to be involved in prostate cancer susceptibility, including, ANPEP, ABL1, PSCA, EFNA1, HSPB1, INMT, and TRIP13 (17). Furthermore, MTR is a folate-metabolizing gene which involved in prostate cancer (18). In this study, we investigated the relationship between MTR-A2756G polymorphism and prostate cancer risk. Our results showed that G allele and GG genotype might be genetic risk factors for prostate cancer in Iranian men. Marchal et al., reported that G allele in MTR A2756G polymorphism acts as a tumor aggressiveness factor in prostate (18). However, there are some studies showed no association between MTR A2756G polymorphism and risk of prostate cancer (19). The possible causes for the inconsistent result in different studies may be due to differences in ethnicity and folate dietary intake. Folate, a form of water-soluble vitamin B9, is one of the important substances in human's body which is found mainly in vegetables. The human's body needs folate as a cofactor for many functions, such as DNA methylation, synthesis, and repair (20). Deficiency of the diet in consumption of fruit and other plant foods have an important role in a low density of folate and other nutrients which cause amounts of DNA damage and high rates of cancer (21). Shannon et al., explain the association between dietary folate intake and prostate cancer risk (22). Hultdin et al., demonstrated that higher folate concentrations increased the risk of prostate cancer (23). Also, latest research reported that serum folate concentration has a positive association with prostate cancer (24). Also, Ma et al., have suggested that the MTR A2756G allele is a protective factor or a risk factor depends on dietary folate intake. The contribution of MTR gene is alterations in the levels of serum folate and plasma homocysteine; specifically, subjects with the 2756AG and GG genotypes have lower levels of plasma homocysteine or higher levels of serum folate than those with the 2756AA genotype (25).

Hypomethylation may reduce tumorigenesis by decreasing regulatory controls on proto-oncogenes and some other genes. In contrast, down-regulation of tumor suppressor genes as a protective gene resulted by hypermethylation (26). The high frequency of alterations in DNA methylation patterns found in prostatic

carcinoma (27). In addition, Hypermethylation of the CpG promoter sequences of the tumor suppressor genes is probably the most frequent alteration in prostatic carcinoma cells and may be associated with prostatic tumorigenesis (28). Folate is a basic pathway in the cell that may affect processes such as DNA methylation. MTR is a key regulatory enzyme in this pathway. Therefore, any changes in the structure of MTR may affect DNA methylation process, and also may induce carcinogenesis. Exonic A2756G polymorphism may affect the function of this enzyme by replacing the amino acid glycine instead of aspartic acid. Our previous studies showed that bioinformatics is an efficient tool to investigate the influence of gene polymorphisms on the gene expression (29) structure of RNA (30) and function of proteins (31-33). Also, our bioinformatics analysis in this study showed that the A2756G MTR polymorphism might change in some properties of the enzyme. For example, this polymorphism may change protein flexibility and relative mutability. The existence of multiple domains in protein structure causes a great deal of flexibility and mobility (34). Domain motions and flexibility are important for catalysis, regulatory activity, and cellular transportation (35). The mutability of individual sites, which estimates the tolerance of a given position to amino acid replacement, is derived from evolutionary protection (36). Our findings showed that these two parameters change for MTR protein after the A2756G transition. Also, our previous study revealed that this transition changes secondary structure of MTR at residue 919 (4).

We were confronted with some limitations in our study. For instance, the interactions of *MTR* with other genes and environmental factor may influence the studied genetic variants. As depicted in figure 3, the *MTR* gene interacts with 10 other genes. So as to achieve more accurate results, we must also consider the interaction of these genes with these 10 genes. Another restriction in the present study was the lack of data for Hcy and folate status in the cases and controls.

In conclusion, this is a preliminary study. So to achieve more accurate results, further studies with larger sample sizes in different ethnic groups and with regard to the interaction of gene-gene and gene-environment are required.

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