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

Arezou Ebrahimi[1,2], Abasalt Hosseinzadeh Colagar[2], and Mohammad Karimian[1]

[1] *Department of Biology, Faculty of Sciences, Islamic Azad University, Damghan Branch, Damghan, Iran*

[2] *Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran*

Received: 04 Aug. 2016; Accepted: 29 Jan. 2017

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.

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* gene 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 island 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

Corresponding Author: A. Hosseinzadeh Colagar
Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran
Tel: +98 11 3532452, Fax: 98 11 3532452, E-mail address: acolagar@yahoo

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 $EDTA_{Na2}$ 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 µg/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 *Hae*III restriction enzyme. Each restriction digestion reaction contained 6 µl of the PCR product, 0.5 µl of *Hae*III, 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.

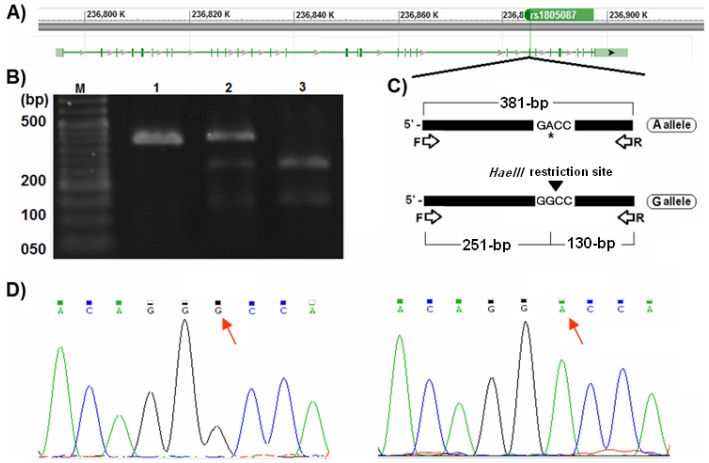

Figure 1. Polymorphism analysis of 2756A>G: (A) MTR Gene map composed of 34 exons; (B) The PCR products which digested with *HaeIII* restriction enzyme (lane M, DNA marker; Lane 1, AA genotype; Lane 2, AG genotype; Lane 3, GG genotype); (C) Schematic of the PCR product digestion in the RFLP technique; (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)	Case (n=100)		
AA		57 (57%)	34 (34%)	-	-
AG		37 (37%)	53 (53%)	2.4014 (1.3216 to 4.3636)	**0.0040**
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	**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 some 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/cgi-bin/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**.**

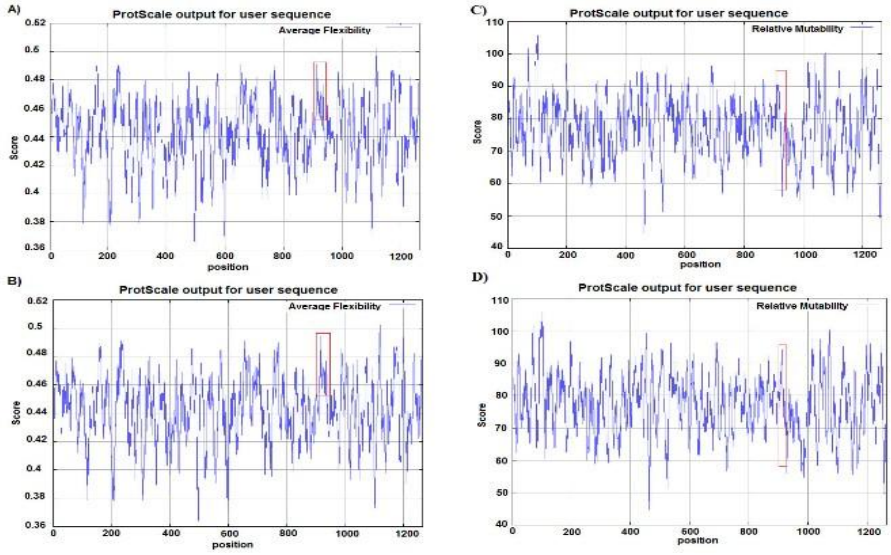

Figure 2. The results of an average protein flexibility and mutability. A and B) Protein flexibility of 919D and 919G phenotype, respectively6; C and D) Protein relative mutability of 919D and 919G, respectively. The location of 919 is shown with a red box.

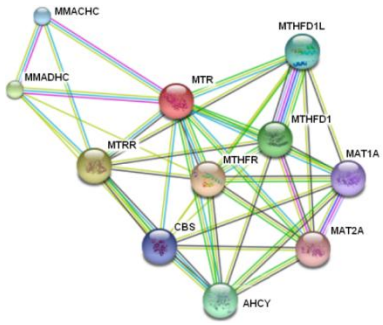

Figure 3. Human *MTR*-interactions network obtained from String server. *MTR* gene linked with 10 other genes: *MTR*; 5-methyltetrahydrofolate-homocysteine methyltransferase. *MTHFR;* methylenetetrahydrofolate reductase. *MTRR;* 5-methyltetrahydrofolate-homocysteine methyltransferase reductase. *MMADHC; 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. *MAT1A*; 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.

Acknowledgments

This work was supported by individual funding in the University of Mazandaran, Iran. We are tremendously grateful to all clinic staffs of Shahid

Beheshti Hospital, Kashan- Iran, for recruiting the case and control volunteers. The authors have declared that no competing interests exist.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Muslumanoglu MH, Tepeli E, Demir S, Uludag A, Uzun D, Atli E, et al. The analysis of the relationship between A1298C and C677T polymorphisms of the MTHFR gene with prostate cancer in Eskisehir population. Genet Test Mol Biomarkers 2009;13:641-5 .
3. Kim KC, Simonetta F, Choi SW. DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. J Nutr Biochem 2009;20:917–26.
4. Karimian M, Hosseinzadeh Colagar A. Methionine synthase A2756G transition might be a risk factor for male infertility: Evidences from seven case-control studies. Mol Cell Endocrinol 2016;425:1-10.
5. Chen LH, Liu ML, Hwang HY, Chen LS, Korenberg J, Shane B. Human methionine synthase. cDNA cloning, gene localization, and expression. J Biol Chem 1997;272:3628-34.
6. Goulding CW, Postigo D, Matthews RG. Cobalamin-dependent methionine synthase is a modular protein with distinct regions for binding homocysteine, methyltetrahydrofolate, cobalamin, and adenosylmethionine. Biochemistry 1997;36:8082-91.
7. Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, et al. Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci 1998;95:3059-64.
8. Collin SM, Metcalfe C, Zuccolo L, Lewis SJ, Chen L, Cox A, et al. Association of Folate-Pathway Gene Polymorphisms with the Risk of Prostate Cancer: a Population-Based Nested Case-Control Study, Systematic Review, and Meta-analysis. Cancer Epidemiol Biomarkers Prev 2009;18:2528-39.
9. Matsuo K, Suzuki R, Hamajima N, Ogura M, Kagami Y, Taji H, et al. Association between polymorphisms of folateand methionine-metabolizing enzymes and susceptibility to malignant lymphoma. Blood 2001;97:3205-09.
10. Goode EL, Potter JD, Bigler J, Ulrich CM. Methionine synthase D919G polymorphism, folate metabolism, and colorectal adenoma risk. Cancer Epidemiol Biomarkers Prev 2004;13:157-62.
11. Paz MF, Avila S, Fraga MF, Pollan M, Capella G, Peinado MA, et al. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res 2;62:4519-24.
12. Klerk M, Lievers KJ, Kluijtmans LA, Blom HJ, den Heijer M, Schouten EG, et al. The 2756A >G variant in the gene encoding methionine synthase: its relation with plasma homocysteine levels and risk of coronary heart disease in a Dutch case-control study. Thromb Res 2003;110:87–91.
13. Chang SC, Chang PY, Butler B, Goldstein BY, Mu L, Cai L, et al. Single nucleotide polymorphisms of one-carbon metabolism and cancers of the esophagus, stomach, and liver in a Chinese population. Plos one 2014;9:e109235.
14. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215 .
15. Wang R, Zheng Y, Huang JY, Zhang AQ, Zhou YH, Wang JN. Folate intake, serum folate levels, and prostate cancer risk: a meta-analysis of prospective studies. BMC Public Health 2014;14:1.
16. Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O, et al. International Variation in Prostate Cancer Incidence and Mortality Rates. Eur Urol 2012;61:1079-92 .
17. Larkin SE, Holmes S, Cree IA, Walker T, Basketter V, Bickers B, et al. Identification of markers of prostate cancer progression using candidate gene expression. Br J Cancer 2012;106:157-65.
18. Marchal C, Redondo M, Reyes-Engel A, Perea-Milla E, Gaitan MJ, Machuca J, et al. Association between polymorphisms of folate-metabolizing enzymes and risk of prostate cancer. Eur J Surg Oncol 2008;34:805-10.
19. Cai D, Ning L, Pan C, Liu X, Bu R, Chen X, et al. Association of polymorphisms in folate metabolic genes and prostate cancer risk: a case-control study in a Chinese population. J Genet 2010;89:263-67.
20. Donkena KV, Yuan H, Young CY. Vitamin Bs, one carbon metabolism and prostate cancer. Mini Rev Med Chem 2010;10:1385-92.
21. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. J Med Sci 1997;94:3290-95.
22. Shannon J, Phoutrides E, Palma A, Farris P, Peters L, Forester A, et al. Folate intake and prostate cancer risk: a case-control study. Nutr Cancer 2009;61:617-28.
23. Hultdin J, Guelpen BV, Bergh A, Hallmans G, Stattin P. Plasma folate, vitamin B12, and homocysteine and

prostate cancer risk: a prospective study. Int J Cancer 2005;113:819-24.

24. Jackson MD, Tulloch-Reid MK, McFarlane-Anderson N, Watson A, Seers V, Bennett FI, et al. Complex interaction between serum folate levels and genetic polymorphisms in folate pathway genes: biomarkers of prostate cancer aggressiveness. Genes Nutr 2013;8:199-207.
25. Ma E, Iwasaki M, Junko I, Hamada GS, Nishimoto IN, Carvalho SM, et al. Dietary intake of folate, vitamin B6, and vitamin B12, genetic polymorphism of related enzymes, and risk of breast cancer: a case-control study in Brazilian women. BMC Cancer 2009;9:1-10.
26. Schalinske KL, Nieman KM. Disruption of methyl group metabolism by ethanol. Nutr Rev 2005;63:387-91.
27. Majumdar S, Buckles E, Estrada J, Koochekpour S. Aberrant DNA methylation and prostate cancer. Curr Genomics 2011;12:486-505.
28. Lin X, Nelson WG. Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. Cancer Res 2003;63:498-504 .
29. Jamali S, Karimian M, Nikzad H, Aftabi Y. The c.− 190 C> A transversion in promoter region of protamine1. Mol Biol Rep 2016:43:795-802.
30. Karimian M, Nikzad H, Azami-Tameh A, Taherian A, Darvishi FZ, Haghighatnia MJ. SPO11-C631T gene polymorphism: association with male infertility and an in silico-analysis. J Family Reprod Health 2015;9:155-63.
31. Nikzad H, Karimian M, Sareban K, Khoshsokhan M, Colagar AH. MTHFR-Ala222Val and male infertility: a study in Iranian men, an updated meta-analysis and an in silico-analysis. Reprod Biomed Online 2015;31:668-80.
32. Karimian M, Hosseinzadeh Colagar A. Association of C677T transition of the human methylenetetrahydrofolate reductase (MTHFR) gene with male infertility. Reprod Fertil Dev 2016;28:785-94.
33. Raygan F, Karimian M, Rezaeian A, Bahmani B, Behjati M. Angiotensinogen-M235T as a risk factor for myocardial infarction in Asian populations: a genetic association study and a bioinformatics approach. Croat Med J 2016;57:351-62.
34. Bu ZI, Callaway DJ. Proteins move! Protein dynamics and long-range allostery in cell signaling. Adv Protein Chem Struct Biol 2011;83:163-221.
35. Huang F, Nau WM. A conformational flexibility scale for amino acids in peptides. Angew. Chem 2003;115:2371-74.
36. Pavelka A, Chovancova E, Damborsky J. HotSpot Wizard: a web server for identification of hot spots in protein engineering. Nucleic Acids Res 2009;37:376-83.