RESEARCH ARTICLE

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Genetic Polymorphisms of Gene Methionine Synthase Reductase (MTRR) and Risk of Urinary Bladder Cancer

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Abstract

Methionine synthase reductase (MTRR) gene involved in the signaling for production of enzyme called methionine synthase reductase that use for the synthesis of methionine, which further used in DNA replication and repair. Genetic variation in MTRR gene may alter the susceptibility of developing urinary bladder cancer. The present study undertaken to identify the contribution of genetic polymorphisms in the MTRR gene on the selected polymorphic sites including c.66A>G and c.524C>T towards urinary bladder cancer risk. Direct-DNA sequencing method was applied for the observation of genotyping distribution of MTRR c.66A>G and c.524C>T polymorphisms in 232 histopathological confirmed cases of transitional cell carcinoma (TCC) of urinary bladder cancer and 250 age-, sex- and ethnicity-matched cancer free controls. With significant difference (p = 0.05) of genotype analysis further corresponding Odds ratio (OR) and 95% confidence interval (CI) were calculated. Multivariable logistic regression analysis was applied for adjusting significant confounder variables. Haploview software (version 4.2) was used to perform pairwise Linkage Disequilibrium (LD) analysis. Age (p = 0.01), Habit of smoking (p = 0.05), tobacco consumption (p = 0.001) and diet (p = 0.02) were significantly differed between cases and controls. Both the MTRR substitution showed higher risk of developing urinary bladder cancer (p = <0.001), although this effect alters in multivariable logistic regression analysis in a protective association for both the substitution. No LD observed between the c.66A>G and c.524C>T substitutions. In conclusion, MTRR c.66A>G and c.524C>T substitutions showed a joint effect with the other associated risk factors. Further studies with a greater number of subjects of different ethnicity and polymorphisms are recommended for the better understanding urinary bladder cancer etiology and to screen the population who are at higher risk of developing urinary bladder cancer.

Keywords: Urinary bladder cancer- MTRR- Methionine synthase reductase- genetic polymorphisms

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Introduction

Urinary bladder cancer is the 10th most common cancer in the world (Bray et al., 2018). According to the GLOBOCAN data, in 2018, estimated incidence of urinary bladder cancer was 5,55,000 (Bray et al., 2018). The most common urothelial carcinoma or transitional cell carcinoma comprises about 90% of all primary tumors of the urinary bladder (Longe., 2005). Smoking tobacco is accounting for approximately 50-60% of urinary bladder cancer incidence each year as a result relative risk of urinary bladder cancer mortality is second only to lung cancer (Freedman et al., 2011).

Epidemiological studies observed a relationship between low folate consumption or folate deficiency with an increased risk of developing urinary bladder cancer. The major genes involved in folate (one-carbon) metabolism involved methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), cystathionine β-synthase (CBS), and thymidylate synthase (TS). The important role of one-carbon metabolism is in the synthesis of purines, pyrimidines and S-adenosylmethionine (SAM) (Sharp and Little, 2004), these key components genes and associated proteins are involved in DNA replication, DNA repair and methylation of DNA, RNA and protein (Hannibal and Blom, 2017). Methionine synthase reductase (MTRR) gene is located on short arm of chromosome 5 (5p15.2-15.3). This gene involved in the cell-signaling for the production of enzyme called methionine synthase reductase that use for the synthesis of methionine by regenerating methionine synthase (MS) enzyme that converts homocysteine (Hcy)

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to methionine in a cobalamin-dependent (folate) manner. MTRR plays an important for providing methyl group in folate metabolism.

Genetic variations in MTRR gene directly affects functions of the expressed proteins which ultimately affects the process of DNA synthesis, repair and methylation (Wang et al., 2008). Genetic polymorphism of MTRR c.66 A>G results in amino acid substitution of isoleucine by a methionine at codon 22 position (Wilson et al., 1999). This polymorphism marginally reduces the protein's biological activity (Dong et al., 2012). The variant genotype GG is associated with a decrease level of Hcy in plasma (Gaughan et al., 2001). Other investigated MTRR polymorphism showed a C>T substitution at position 524 results in a Ser→Leu change at codon 175 has been reported, and to date no relationship with the risk of cancer has been identified (Ravel et al., 2009).

Association data for the genetic polymorphisms of MTRR c.66A>G and c.524C>T with the risk of urinary bladder cancer have still inconsistent. Therefore, this prompts us to execute this case-control study with the aim to identify the contribution of genetic polymorphisms in the MTRR gene on the selected polymorphic sites including c.66A>G and c.524C>T towards urinary bladder cancer risk.

Materials and Methods

Study Design and Subjects characteristics

This case-control study was conducted in the Department of Urology, King George's Medical University, Lucknow and Division of Endocrinology, Central Drug Research Institute, Lucknow. A total of 498 subjects were recruited based on inclusion and exclusion criteria. Study comprises 232 histopathological confirmed cases of transitional cell carcinoma (TCC) of urinary bladder cancer and 250 age-, sex- and ethnicity-matched cancer free controls. Subjects with any chronic disease or any kind other cancer were excluded from the study. Demographical, personal habits and clinical data of the recruited subjects were collected using questionnaire and arranged in excel sheet for further data analysis. After collecting subject's details 3 ml peripheral blood sample was aspirated and transferred into the EDTA vial and stored at -20˚C for the DNA isolation and genotype analysis. This study was approved by the Ethics Committee of King George's Medical University, Lucknow (Ref. No. XLIIECM/B-P31). Detailed description about the study was given to subjects and written consent form was taken for their recruitment.

Genetic Polymorphisms analysis

Genomic DNA was isolated from the stored peripheral blood samples. For the DNA isolation phenol-chloroform isoamyl alcohol (PCI) extraction method was adopted. DNA quality was quantified by measuring absorbance at 260nm using Nano-Drop spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA). The quality of DNA was assessed by agarose gel-electrophoresis. Subjects with good quality of DNA extracted were included for the further study. Primer used for the amplification of c.66A>G and c.524C>T sites are design using Primer3 online tool and custom synthesized by Eurofins (Bengaluru, India). Primer used for c.66 A>G was forward: GCAAAGGCCATCGCAGAAGACAT and reverse: GTGAAGATCTGCAGAAAATCCATGTA with product size of 296 bp and for c.524 C>T was forward: TTGTGGTTGAGCCGTGGATTG reverse: GAGAGTGGGGGTACCGAAC with product size 375 bp.

The amplicons were directly sequenced using dideoxy chain terminator cycle sequencing protocol (Big Dye V3.1, Applied Biosystems, Foster City, Ca, USA) on ABI 3730 DNA analyzer. In Brief, the sequencing PCR reaction consisted of Big-Dye 3.8 µl, PCR product 0.5-1.0 µl, forward or reverse primer 0.1-0.5 µl and variable volume of Milli-Q to make the reaction volume to 5 µl. The sequencing PCR was conducted under PCR conditions consisting of initial denaturation at 96°C for 1 minute followed by 30 cycles of denaturation at 96°C for 10 seconds, annealing at 55˚C for c.524 C>T and 58˚C for c.66 A>G for 7 seconds and polymerization at 60°C for 4 minutes. After PCR amplification, the amplicons were purified by precipitation method. For precipitation, 25 µl of sodium acetate: ethanol (1:25) mixture was added to each well of the plate and incubated at room temperature for 10 minutes. Afterwards, the plate was centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was removed with slow motion of hand shaking. The precipitated PCR product was purified by washing with alcohol. The products were washed with 70% ethanol twice. The plate was left at room temperature to facilitate evaporation of the remaining alcohol. After 20 minutes, 10ul of 50% Hi-Di formamide was added to each well and the plate was loaded on ABI 3730 DNA analyzer for reading the sequences of the amplicons. The sequencing results were edited as required using the sequence analysis software (Applied Biosystems, USA). After editing, the sequences were aligned using Auto-assembler software (Applied Biosystems, USA) for identification of the mutations/polymorphisms.

Statistical Analysis

Chi-square (χ^2) test for categorial data and Student's 't' test for continuous data were applied. Continuous data were summarized as Mean ± SD (standard deviation) while discrete (categorical data) in number and percentage (%). The statistical significance for deviation from the Hardy-Weinberg Equilibrium (HWE) was tested using Pearson's Chi-square test in the control population. Genotype distribution analysis between cases and controls was done using 2 x 3 chi-square contingency table calculator available online at VassarStats: Statistical Computation Web Site (http://www.vassarstat.net). With the significant difference of genotype analysis further corresponding Odds ratio (OR) and 95% confidence interval (CI) were calculated. P-value (two-tailed tests) and 95% CI were used to assess the strength of association. Multivariable logistic regression analysis was applied for adjusting significant confounder variables (Gautam et al., 2016). Haploview software (version 4.2) was used to perform pairwise Linkage Disequilibrium (LD) analysis

of the eligible polymorphisms. D’ and r^2 were used to observe the magnitude of LD between sequenced variants. Haplotype frequencies were calculated to find if allelic combination of these substitution influenced the risk of urinary bladder cancer

Results

Subjects Characteristic

The mean age of urinary bladder cancer (58.28 ± 10.18) patients was significantly differed (p = 0.01) with healthy subjects (60.74 ± 11.50). The present study found a statistically significant difference of smoking tobacco and dietary habit of cases and controls that was p = 0.05, p = 0.001 and p = 0.02, respectively (Table 1). There was no difference of other parameters including sex, BMI, occupation and alcohol consumption habit between cases and controls. Table 1 shows the selected characteristics of cases and controls.

MTRR c.66A>G and c.524C>T substitution and urinary bladder cancer risk

Polymorphisms at the two positions were analyzed and c.66A>G was in the HWE (p = 0.99) , while c.524C>T genotype distribution did not consistent with the HWE law at the level of significance that is 0.05. The present study found that MTRR (c.66 A>G) substitution was statistically significantly differed between cases and controls (p = 0.0001; OR = 2.03; 95% CI 1.41-2.92), it showed 2.03-fold higher risk of UBC in cases as compared to controls (Table 2). The distribution of MTRR (c.524 C>T) genotypes was also significantly associated with increased risk of UBC (p = 0.001; OR = 1.32; 95% CI 1.06-1.89) (Table 2).

On applying multivariable logistic regression analysis for the significant confounder factors, we found that both genetic substitutions showed a protective association against the risk of urinary bladder cancer (Table 3). In c.66 A>G polymorphism for AA versus GG - unadjusted OR = 0.20, p = <0.001; adjusted OR = 0.21, p = <0.001 and for AG verses GG- unadjusted OR = 0.30, p = <0.001; adjusted OR = 0.30, p = <0.001 showed significant difference between cases and control (Table 3). Similarly, in c.524 C>T polymorphism for CC versus TT - unadjusted OR = 0.15, p = <0.001; adjusted OR = 0.15, p = <0.001 and for CT verses TT- unadjusted OR = 0.15, p = <0.001; adjusted OR = 0.16, p = <0.001 showed significant difference between the cases and controls although the association was protective against the risk of urinary

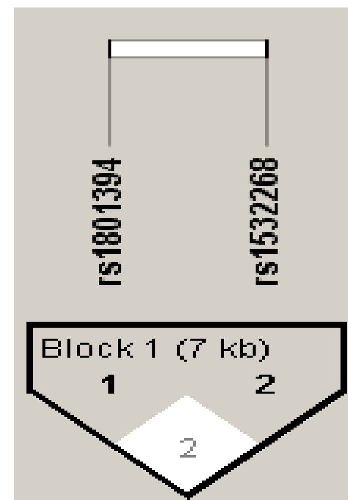

Figure 1. Linkage Disequilibrium Plot. The number of each cell represents D' and white colour cells shows no LD between polymorphisms The rs numbers are SNP IDs taken from National Center for Biotechnology Information (NCBI).

Table 1. Demographic and Subject’s Personal Characteristics of Urinary Bladder Cancer Cases and Healthy Controls

Characteristics	Controls n = 250 (%)	Cases N = 232 (%)	p value
Age (years)			
Mean ± SD	60.74 ± 11.50	58.28 ± 10.18	0.01*
Sex:			
Female	36 (14.4)	28 (12.1)	0.45
Male	214 (85.6)	204 (87.9)	
BMI (kg/m^2)			
Mean ± SD	23.98 ± 3.36	24.44 ± 3.71	0.14
Occupation			
Sedentary	114 (45.6)	103 (44.4)	0.79
Hard	130 (54.4)	120 (55.6)	
Smoking:			
No	126 (50.4)	96 (41.4)	0.05*
Yes	124 (49.6)	136 (58.6)	
Tobacco:			
No	165 (66.0)	98 (42.2)	<0.001*
Yes	85 (34.0)	134 (57.8)	
Alcohol:			
No	219 (87.6)	203 (87.5)	0.97
Yes	31 (12.4)	29 (12.5)	
Diet:			
Vegetarian	147 (58.8)	111 (47.8)	0.02*
Non-vegetarian	103 (41.2)	121 (52.2)	

*Significantly differed between cases and controls

Table 2. Comparison of Frequency of Genotype Distribution of MTRR Gene Polymorphisms between Cases the Cases of Urinary Bladder Cancer and Healthy Controls.

SNPs	Controls (n=250) (%)	Cases (n=232) (%)	p value
MTRR c.66A>G			
AA	135 (54.0)	85 (36.6)	<0.001*
AG	97 (38.8)	91 (39.2)	
GG	18 (7.2)	56 (24.1)	
MTRR c.524C>T			
CC	135 (54.0)	109 (47.0)	<0.001*
CT	109 (43.6)	90 (38.8)	
TT	6 (2.4)	33 (14.2)	

*Significantly differed between cases and controls

Table 3. Multivariable Logistic Regression Analysis of Urinary Bladder Cancer Risk and MTRR Genotypes

SNP	Genotype	Unadjusted OR (95%CI)	Adjusted* OR (95%CI)
MTRR c.66 A>G	AA	0.20 (0.11-0.37)	0.21 (0.11-0.40)
	AG	0.30 (0.17-0.55)	0.30 (0.16-0.56)
	GG	Ref	Ref
MTRR c.524 C>T	CC	0.15 (0.06-0.36)	0.15 (0.06-0.37)
	CT	0.15 (0.06-0.37)	0.16 (0.06-0.40)
	TT	Ref	Ref

bladder cancer (Table 3).

MTRR haplotypes and risk of urinary bladder cancer risk

Haplotyping of the two substitutions of MTRR generated four different haplotypes as detailed in Table 4. The distribution of the haplotype ‘GC’ was significantly different between cases and controls (p = 0.001), and associated with high risk of UBC as its frequency was higher in cases than controls. The frequencies of other haplotypes were almost similar between cases and controls. Further, the c.66A>G and c.524C>T polymorphisms of MTRR were not in significant LD (D’ = 0.02, LOD = 0.05, r^2 = 0.0 Figure 1.

Discussion

Folate and methionine metabolism are the key component for DNA methylation (Sharp and Little, 2004) . Several studies have found evidences between hypomethylation and hypermethylation and link with different form of cancers including colorectal, gastric cancer, esophagus cancer (Frigola et al., 2005; Miranti et al., 2017; Waki et al., 2002). DNA synthesis, repair and methylation is an important mechanism of gene regulation and expression and may play crucial roles in urinary bladder cancer initiation and its development if abnormality in these mechanisms occur due to altered activity of associated enzymes (Sharp and Little, 2004). The present case-control study has been carried out to investigate the influence of genetic polymorphisms in MTRR gene on the risk of urinary bladder cancer risk. MTRR c.66 A>G polymorphism results an enzyme with lower affinity to MTR therefore; as a result, homocysteine levels increases and methionine levels decreases, ultimately affects the process of DNA methylation (Miller et al., 2013; Wang et al., 2007). Till date only two genetic polymorphisms studies on MTRR c.66A>G (Moore et al., 2007; Rouissi et al., 2009) and only one study on MTRR c.524C>T (Rouissi et al., 2009) has investigated the association with urinary bladder cancer risk.

Moore et al., in 2007 conducted a study on Caucasian population with a sample size of 1150 cases and 1149 controls, to investigate the role of c.66 A>G polymorphism and risk of urinary bladder cancer and found that variant genotype GG or AG did not have any significant association with urinary bladder cancer risk (Moore et al., 2007). A similar association was observed by Rouissi et al., in 2009 on Tunisian population however; the sample size (185 cases and 191 controls) was relatively very small from the previous study (Rouissi et al., 2009). For the pooled data, the present study showed a contradictive result from previously published reports. The study observed a statistically significant association of c.66 A>G polymorphism with the risk of urinary bladder cancer in which variant genotype (GA+GG) showed a 2.03-fold higher risk of urinary bladder cancer in cases as compared to controls. Although, this finding was totally contrary when we applied adjusted analysis for the confounder factors, now this substitution showed a protective association with the risk of urinary bladder cancer. A study reporting c.524 C>T polymorphism and risk of urinary bladder cancer did not find any relationship between c.524 C>T polymorphism and risk of urinary bladder cancer (Rouissi et al., 2009). In contrast, the present study found a statistically significant association of c.524 C>T polymorphism with the risk of urinary bladder cancer. Although, the effect did not remain same after applying the multivariable logistic regression analysis. The finding of the present study showed a strong affect of confounders that might be associated with the risk of urinary bladder cancer and may support the cumulative effect of genetic polymorphisms with other associated risk factors.

Table 4. Common MTRR Haplotypes Distribution in Relation with Urinary Bladder Cancer

Haplotype	Frequency (overall)	Frequency (cases, controls)	P value
AC	0.47	0.39, 0.41	0.21
CG	0.24	0.29, 0.20	0.001*
AT	0.18	0.15, 0.17	0.36
GT	0.1	0.14, 0.06	0.91

*Significantly differed between cases and controls

The present study is first to identify the effect of haplotyping analysis and linkage disequilibrium between the selected substitution. These two polymorphisms of MTRR gene did not show any LD between them. Nevertheless, four haplotypes were generated in haplotype analysis and one (GC) of them was significantly differed between cases and controls. Present study considered some limitations like the subjects were recruited from the single hospital, therefore, the genotypes in the cases and controls may not be the true representatives of the population at large. In addition, pathological characteristics of the tumor were not studied with reference to the genotype distribution among the cases of urinary bladder cancer.

In conclusion, the pooled data results of this study showed statistically significant evidence that supports the interaction between genetic polymorphism in MTRR gene and risk of urinary bladder cancer, although this association deviate after applying multivariable logistic regression analysis for the significant confounder factors. This may support the joint effect of genetic polymorphisms and other risk factors. Further studies with a greater number of subjects of different ethnicity and polymorphisms are recommended for the better

understanding urinary bladder cancer etiology and to screen the population who are at higher risk of developing urinary bladder cancer.

Author Contribution Statement

Singh R and Sankhwar SN: supervision, conceptualization and methodology. Gautam KA and Raghav A: experiment, result interpretation, analysis. Gautam KA and Tripathi P: manuscript writing. All authors have read and approved the final draft of manuscript

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Conflict of interest

Authors have no conflict of interest to declare.

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