RESEARCH

MTHFR functional polymorphisms and haplotypes are a risk factor for urinary bladder cancer – a case-control study and meta-analysis

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ABSTRACT

Background: A subtle modification in the genes coding for enzymes responsible for DNA synthesis, DNA repair and methylation can modify their activity, which eventually affects the susceptibility and sensitivity of an individual towards cancer development. Methylene-tetrahydrofolate reductase (MTHFR) plays a crucial role in DNA synthesis and methylation reactions and genetic variations in this gene may associate with the cancer susceptibility. The present study analyzed three polymorphisms (c.677C>T, c.1298A>C and c.203G>A) in the *MTHFR* gene for correlation with the risk of urinary bladder cancer (UBC).

Methods: Genotyping of the three polymorphisms was performed in 232 cases and 250 controls by direct DNA sequencing method. For meta-analysis, the relevant data were collected through literature search in "Pubmed", "ScienceDirect" and "Google Scholar" databases using the keywords,'methylene-tetrahydrofolatereductase', 'MTHFR', 'c.677C>T polymorphism', 'c.1298A>C polymorphism' and 'urinary bladder cancer' in various combinations.

Results: We observed a significant protective association of c.1298A>C with bladder cancer risk, but c.677C>T and c.203G>A were unrelated to the UBC risk. We also undertook a meta-analysis on 4171 cases and 4749 controls for c.677C>T polymorphism and on 3785 cases and 4205 controls for c.1298A>C polymorphism. The meta-analysis revealed a lack of association of these polymorphisms with the UBC risk.

Conclusion: *MTHFR* c.1298A>C substitution is protective against urinary bladder cancer in the study population; however, meta-analysis denied an association at the global level.

KEYWORDS: Methylenetetrahydrofolate reductase; MTHFR; c.677C>T polymorphism; c.1298 A>C polymorphism; urinary bladder cancer

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INTRODUCTION

Several epidemiological studies have found that urinary bladder cancer is the second most common genitourinary malignant disease that is affected by several genetic and environmental factors (Xu et al., 2013; Pakzad et al., 2015). The most common environmental factors are cigarette smoking, occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons (Wang et al., 2009; Otunctemur et al., 2013). A subtle modification in the genes coding enzymes responsible for DNA synthesis, DNA repair, and methylation may modify their activity, resulting in hypomethylation, activation of oncogenes and deactivation of tumor suppressor genes that may affect the susceptibility and sensitivity towards cancer (Weisberg et al., 1998). A number of genes are known to affect the risk of cancer, but MTHFR, a key enzyme involved in folate metabolism, has received great attention in the last decade (Izmirli et al., 2011; Kouidhi et al., 2011). MTHFR catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the latter serves as a substrate for re-methylation of homocysteine to methionine with the subsequent synthesis of S-adenosylmethionine (SAM). Thus, MTHFR plays a crucial role in DNA synthesis and methylation reactions (Xu et al., 2013). Variations in the *MTHFR* gene have been shown to correlate with the susceptibility to several cancers, such as cervical, lung, colorectal, breast, gastric, brain and urinary bladder cancers (Boccia et al., 2007; Galbiatti et al., 2012; Yin et al., 2004).

The three functional polymorphisms in the *MTHFR* gene, c.203G>A (rs2066472), c.677C>T (rs1801133) and c.1298A>C (rs1801131), result in Arg68Gln, Ala222Val and Glu429Ala amino acid substitutions, respectively. At least two of these substitutions (677C>T and 1298A>C) are associated with the production of labile forms of enzyme with reduced activity (Weisberg et al., 1998; Frosst et al., 1995). Eventually, the altered MTHFR activity could influence the level and availability of methyl donors, and potentially the methylation status of key tumor suppressor or promoter genes involved in the pathogenesis of urinary bladder cancer (Xu et al., 2013; Frosst et al., 1995). These two polymorphisms in the *MTHFR* gene have been extensively studied in different ethnic populations, but the results are inconsistent and inconclusive. Considering this, we undertook the present study to (i) investigate the association of *MTHFR* gene variants, c.677C>T, c.1298A>C and c.203G>A with the risk of urinary bladder cancer, (ii) execute haplotype analysis of the above polymorphisms to find the linkage disequilibrium between them, and (iii) perform a meta-analysis on c.677C>T and c.1298A>C to understand their effects on UBC risk.

MATERIALS AND METHODS

Ethics approval and consent

The study was approved by the Ethics Committee of King George's Medical University (REF. No.XLII ECM/B-P31). Informed written consents were obtained from all the subjects.

Study subjects

The study was approved by the Ethics Committee of King George's Medical University (REF. No.XLII ECM/B-P31). Informed written consents were obtained from all the subjects. The study recruited histopathologically confirmed urinary bladder

cancer patients who visited the Out Patient Department (OPD) of University Hospital at the King George's Medical University (KGMU), Lucknow, India. We recruited 237 cancer cases and 270 control subjects with no history of cancer or any serious illness. All the patients underwent a standardized clinical and laboratory evaluation. All enrolled controls were matched to cases by age, sex, and ethnicity. The data for demographical parameters (age, sex, and ethnicity), smoking or tobacco chewing habits and complete clinical investigations of the disease (stage and grade) were added to an organized data sheet. After collecting the subjects' details, peripheral blood from each subject was aspirated into the EDTA coated vial and stored at 4°C for DNA isolation and molecular analysis.

Genotyping

Genomic DNA was isolated from peripheral blood using standard salting out method. The DNA quality was assessed on the basis of spectrophotometric readings and agarose gel analysis. Five cases and 20 controls were excluded because of poor DNA quality that affected PCR amplification. Genotyping of the target SNPs was performed using direct DNA sequencing of the amplified PCR product using primers published in an earlier study (Gupta et al., 2013).

Statistical Analysis

Demographical parameters were statistically analyzed using the Student's t-test or Chi-square test. Fitness of the control data in the Hardy Weinberg Equilibrium (HWE) for each polymorphism was checked using the chi-square test by comparing the observed and expected frequencies of the genotypes. Vassarstats online calculator (http://www.vassarstats.net) was used to compare genotype frequencies and logistic regression was applied to compute ORs and 95% CIs adjusted for confounders. Linkage Disequilibrium (LD) was calculated using Haploview software (version 4.2). P value < 0.05 was considered significant for statistical inference.

Meta-analysis

A number of studies have analyzed c.677C>T and c.1298A>C polymorphisms for correlation with urinary bladder cancer; however, the results are heterogeneous. Hence, we performed a meta-analysis to estimate the effect of the *MTHFR* polymorphisms on urinary bladder cancer risk at a global level.

Identification of related studies

We performed a literature search in "Pubmed", "ScienceDirect" and "Google Scholar" databases for relevant articles using the keywords, 'methylene-tetrahydrofolatereductase', 'MTHFR', 'c.677C>T polymorphism', 'c.1298A>C' and 'urinary bladder cancer' in the various combinations. Broad search terms were used to assist the identification of all pertinent articles, with the last search performed on the 31st of December 2017. The search was restricted to the studies on human patients and the articles published in English. The full-text articles were obtained for all the studies. Authors were contacted via e-mail in case the full text of a relevant study was not available. All the studies thus retrieved were evaluated and filtered considering the following inclusion criteria.

Inclusion criteria: (i) It should be a case-control study, (ii) Study had analyzed *MTHFR*c.677C>T or c.1298A>C polymorphisms in correlation with urinary bladder cancer risk, (iii) Patients were recruited on the basis of histopathology reports, and controls had no malignancies, (iv) Genotype frequencies were available, (v) statistical analysis in terms of odds ratio (OR) and 95% confidence interval (CI) was available, (vi) control data were in Hardy-Weinberg equilibrium.

Exclusion criteria: Studies with irrelevant data and study design were excluded from the meta-analysis.

Data extraction and statistical analysis

The extraction of information from the selected studies was carefully done according to the inclusion criteria. From each related study, the following information was collected and tabulated: the first author's last name, year of publication, the ethnicity of the study population and allele and genotype frequencies. Meta-analysis was performed using the comprehensive meta-analysis software (version 2). Odd ratios (ODs) with 95% Confidence Intervals (CIs) were applied to measure the strength of association between *MTHFR* polymorphisms and the risk of UBC. Heterogeneity between the studies was observed utilizing the chi-square-based Q-test. High-resolution plot (forest plot) was generated to estimate the pooled odds ratio and p-value (p = <0.05, statistically significant). I^2 value was used to quantify the degree of heterogeneity between studies. Values for I^2 statistics as suggested by Higgins and Thompson were used to estimate the magnitude of heterogeneity; viz. 25%, 50% and 75%, which correspond to low, medium and high heterogeneity, respectively (Higgins and Thompson, 2002). Publication bias was evaluated using the funnel plot of precision (1/std error vs log odds ratio) and the Egger's regression test of significance. In the absence of significant heterogeneity, Mantel-Haenszel fixed-effect model (Peto method) and in the presence of significant heterogeneity, the DerSimonian-Laird random-effects model (DL method) was used (Petitti, 2001; DerSimonian and Laird, 2015). We used both fixed and random effects models to estimate the pooled effect size.

RESULTS

Demographical analysis

The study included a total of 482 subjects with a far higher number of males (86.72%) than females (13.28%). The mean age was comparable between cases (60.71, SD = 11.5) and controls (58.23, SD = 10.15). The BMI was not significantly different between cases and controls (p= 0.14). Other lifestyle habits like smoking and alcohol consumption also did not differ significantly between the cases and controls. Nevertheless, the percentage of non-vegetarian people was significantly higher (p = 0.016) in the cases (52.2%) in comparison to the controls (41.20%).

Case-control study

The genotype frequencies in the control group followed the Hardy-Weinberg equilibrium (p>.05) for all variants. The frequency of the homozygous rare genotype at c.1298A>C locus was significantly lesser in the patients as compared to the controls, suggesting its association with a reduced risk of urinary bladder cancer (p = 0.004, OR 2.19, 95% CI = 1.29-3.71) while the heterozygous

genotype did not show a significant association with the disease (p = 0.34, OR 1.24, 95% CI 0.79-1.95) (Table 1). These results remained identical after adjusting for age, smoking, tobacco and dietary habits (p =0.02, OR 1.99, 95% CI = 1.13-3.51 and p = 0.58, OR 1.14, 95% CI = 0.71-85) (Table 2). The other two polymorphisms, c.677C>T and c.203G>A did not show any significant relationship with urinary bladder cancer in either unadjusted or adjusted analysis.

Haplotype analysis

c.1298 A>C and c.203G>A polymorphisms were in statistically significant linkage disequilibrium (D' = 1.0, LOD = 0.9, r^2 = 0.004). LD was not observed between c.1298A>C and c.677C>T (D' = 0.037, LOD = 0.01, r^2 = 0.0) or between c.677C>T and c.203G>A polymorphisms (D' = 0.17, LOD = 0.17, r^2 = 0.001) (Figure 1). Four haplotypes were observed namely ACG, CCG, CTG and ATG with frequencies of 45.3%, 44.8%, 5% and 4.5%, respectively (Table 3).

The frequency of the major ACG haplotype was statistically higher in the cases than controls (P = 0.010). However, haplotype CCG was significantly less frequent in the cases than controls (P = 0.003).

Table 1. Distribution of *MTHFR* genotypes in urinary bladder cancer patients and controls.

SNP	Genotype	Controls N (%)	Cases N (%)	Dominant (11vs12+22)	Recessive (22vs12+11)	Co-dominant (12vs11+22)
c.677C>T	CC(11)	205(82.0)	187(80.6)	P= 0.73	P=0.61	P=0.83
	CT(12)	44(17.6)	43(18.5)			
	TT(22)	1(0.4)	2(0.9)			
c.1298A>C	AA(11)	46(18.4)	69(29.7)	P=0.004 OR=0.53 CI=0.34-0.81	P=0.08 OR=1.48 CI=0.96-2.26	P=0.36 OR=1.19 CI=0.83-1.70
	AC(12)	137(54.8)	117(50.4)			
	CC(22)	67(26.8)	46(19.8)			
c.203G>A	GG(11)	249(99.6)	230(99.2)	P=0.61	P=0.48	P=1
	GA(12)	1(0.4)	1(0.4)			
	AA(22)	0(0.0)	1(0.4)			

Table 2. Estimation of urinary bladder cancer risk in the context of *MTHFR* genotypes.

	Cases N (%)	Controls N (%)	Univariate OR (95%CI)	P-value	Multivariate OR (95%CI)	P-value
c.677C>T						
CC	187(80.6)	205(82.0)	0.46 (0.04-5.07)	0.52	0.71 (0.06-8.13)	0.79
CT	43(18.5)	44(17.6)	0.49 (0.04-5.59)	0.56	0.92 (0.08-10.9)	0.95
TT	2(0.9)	1(0.4)	Ref.		Ref.	
c.1298A>C						
AA	69(29.7)	46(18.4)	2.19 (1.29-3.71)	0.004	1.99 (1.13-3.51)	0.02

AC	117(50.4)	137(54.8)	1.24 (0.79-1.95)	0.341	1.14 (0.71-1.85)	0.58
CC	46(19.8)	67(26.8)	Ref.		Ref.	
c.203G>A						
GG	230(99.2)	249(99.6)	-	-	-	-
GA	1(0.4)	1(0.4)				
AA	1(0.4)	0(0.0)				

Table 3. Frequency distribution of common MTHFR haplotypes in cases and controls.

Haplotype	Frequency	Case/control frequency	Chi-square value	P value
ACG	0.453	0.495, 0.413	6.495	0.0108
CCG	0.448	0.399, 0.493	8.487	0.0036
CTG	0.050	0.051, 0.049	0.017	0.8978
ATG	0.045	0.048, 0.043	0.159	0.6905

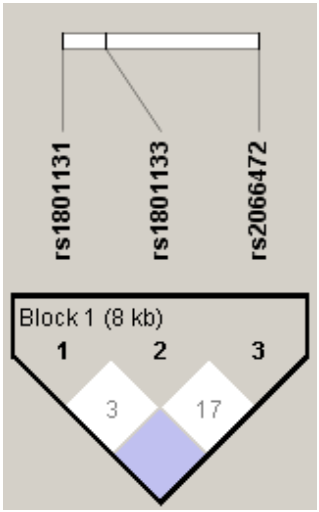

Figure1. Linkage Disequilibrium plot. The number of each cell represents D' and white color cells shows no LD between polymorphisms while blue color showed LD between polymorphisms. (c.1298 A>C and c.203G>A (D' = 1.0 , r2 = 0.004); c.1298A>C and c.677C>T (D' = 0.037, r2 = 0.0); c.677C>T and c.203G>A polymorphisms (D' = 0.17, r2 = 0.001))

Meta-analysis

Literature search and study characteristics:

A total of 1769 studies, found as a result of PubMed, Elsevier Science Direct, Medline, ScienceDirect, Embase and Google Scholar search with relevant keywords, were screened exhaustively. For c.677C>T polymorphism, fifteen studies qualified the inclusion criteria for meta-analysis. We used three different populations (European, Mexican-American, and African-American) as separate data sets from one study[13]. Thus, after the inclusion of the present study, a meta-analysis on c.677C>T included 16 studies and 18 data sets, making a total of 4171 cases and 4749 controls (Table 4). For c.1298A>C polymorphism, twelve studies qualified the inclusion criteria for meta-analysis. Thus, after the inclusion of the present study, a meta-analysis on c.1298A>G included 13 studies or 15 data sets with a total of 3785 cases and 4205 controls

(Table 5). In all these studies, control data were in HW equilibrium.

Table 4. Details and genotype data of the studies included in the meta-analysis on c.677C>T polymorphism

First Author	Year	Ethnicity	Sample Size		Cases			Allele		Controls			Allele	
			case	control	CC	CT	TT	C	T	CC	CT	TT	C	T
Kimura	2001	European	165	150	70	80	15	220	110	65	73	12	203	97
Sanyal	2004	European	309	246	173	113	23	459	159	121	102	23	344	148
Lin	2004	European	410	410	176	183	51	535	285	196	164	50	556	264
Moore	2004	Argentine-American	106	109	45	42	19	132	80	32	59	18	123	95
Lin	2004	Mexican-American	17	17	16	1	0	33	1	13	4	0	30	4
Lin	2004	African-American	21	21	7	13	1	27	15	9	9	3	27	15
Karagas	2005	USA-American	352	551	140	171	39	451	249	227	245	71	699	387
Moore	2007	European	1041	1049	418	478	145	1314	768	402	486	161	1290	808
Ouerhani	2007	African	111	131	43	58	10	144	78	58	56	17	172	90
Wang	2009	Asian	239	250	66	129	45	261	219	88	132	30	308	192
Ouerhani	2009	African	90	110	33	50	7	116	64	51	45	14	147	73
Rouissi	2009	African	185	191	87	86	12	260	110	81	90	20	252	130
Cai	2009	Asian	312	325	82	169	61	333	291	113	170	42	396	254
Chung	2010	Asian	150	300	80	57	13	217	83	141	123	36	405	195
Safarinejad	2011	Asian	158	316	67	74	17	208	108	144	142	30	430	202
Izmirli	2011	Asian	54	50	28	22	4	78	30	36	14	0	86	14
Dimmer	2012	USA	219	273	92	106	21	290	148	121	121	31	363	183
Present Study	2014	Asian	232	250	187	43	2	417	47	205	44	1	454	46

c.677C>T polymorphism: Meta-analysis was performed by adopting the dominant model ('CC' vs 'CT+ TT') for comparing the genotypes between case and control groups. Pooled data showed a moderate level of heterogeneity between the studies ($P_{heterogeneity}$ = 0.027, Q = 29.861, df(Q) = 17, I^2 = 43.069, variance = 0.001, τ^2 = 0.028, SE = 0.024, τ = 0.168). The difference in the genotype distribution between case and control groups was non-significant (Random effects model: OR = 1.06; 95% CI = 0.937-1.207; P = 0.34; Fixed effect model: OR = 1.043; 95% CI = 0.956-1.138; P = 0.34) (Figure 2).

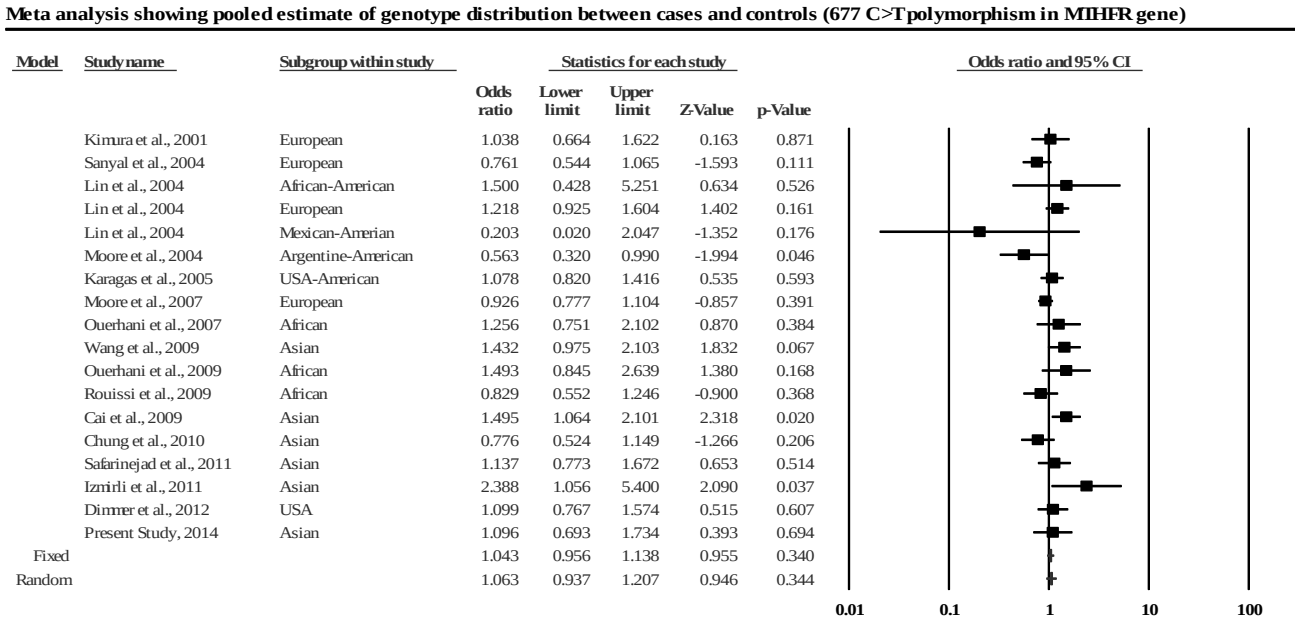

Figure 2. Forest plot for meta-analysis on c.677C>T polymorphism. The Z value shows the degree and direction of relationship, whereas the P value shows the significance of the relationship. The horizontal bar shows the range of OR with a square in the centre, the size of which is directly proportional to the weight given to each study. The direction of the projection of the horizontal bar shows the direction of the association.

Overall, neither random effects model (OR = 1.06; 95% CI = 0.937-1.207; P = 0.34) nor the fixed effect model (OR = 1.043; 95% CI = 0.956-1.138; P = 0.34) showed association of c.677C>T polymorphism with urinary bladder cancer.

c.1298A>C polymorphism: We observed a true high heterogeneity in the pooled data ($P_{heterogeneity}$ = 0.00, Q = 46.03, df(Q) = 14, I^2 = 69.58, variance = 0.003, τ^2 = 0.079, SE = 0.051, τ = 0.282).The meta-analysis did not reveal any significant relationship between c.1298A>C polymorphism and the risk of urinary bladder cancer. In the pooled data analysis, genotype as well as allele frequencies were found to be similar between cases and controls in random effects (genotype, OR = 1.07; 95% CI = 0.89-1.29; P = 0.43: allele, (OR= 0.99; 95% CI = 0.85-1.16; P = 0.93) as well as the fixed effect model (genotype, OR = 1.07; 95% CI = 0.97-1.17; P = 0.14: allele, (OR = 0.99; 95% CI = 0.92-1.06; P = 0.72) (Figure 3).

Publication bias: Begg's funnel plot and Egger's test were performed to analyze the publication bias in the pooled analysis. The distribution of studies on the funnel plot did not provide evidence of asymmetry, suggesting the absence of publication bias. Furthermore, Egger's test analysis confirmed the absence of publication bias as the observed p values were higher than 0.05.

Meta analysis showing pooled estimate of genotype distribution between cases and controls (1298 A>C polymorphism in MTHFR gene)

Model	Study name	Subgroup within study	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value
	Sanyal et al., 2004	European	0.933	0.667	1.306	-0.405	0.685
	Lin et al., 2004	African-American	0.813	0.229	2.877	-0.322	0.748
	Lin et al., 2004	European	0.975	0.741	1.284	-0.178	0.859
	Lin et al., 2004	Mexican-American	0.564	0.126	2.524	-0.749	0.454
	Moore et al., 2004	Argentine-American	1.078	0.630	1.842	0.273	0.785
	Karagas et al., 2005	USA-American	0.993	0.759	1.300	-0.049	0.961
	Moore et al., 2007	European	1.057	0.893	1.252	0.643	0.520
	Ouerhani et al., 2007	African	1.689	1.007	2.832	1.985	0.047
	Wang et al., 2009	Asian	0.897	0.610	1.318	-0.555	0.579
	Rouissi et al., 2009	African	1.568	1.038	2.368	2.139	0.032
	Cai et al., 2009	Asian	1.030	0.736	1.442	0.172	0.864
	Safarinejad et al., 2011	Asian	2.956	1.971	4.434	5.239	0.000
	Izmirli et al., 2011	Asian	0.573	0.245	1.339	-1.285	0.199
	Dimmer et al., 2012	USA	1.134	0.792	1.623	0.688	0.492
	Present Study, 2014	Asian	0.533	0.348	0.816	-2.897	0.004
Fixed			1.070	0.977	1.172	1.467	0.142
Random			1.077	0.895	1.296	0.785	0.432

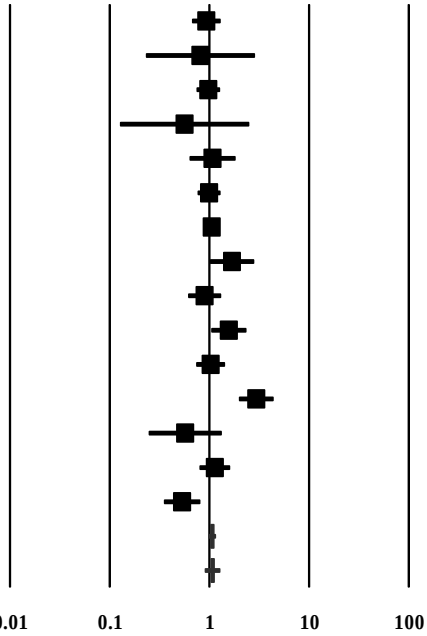

Figure 3. Forest plot for meta-analysis on c.1298 A>C polymorphism. The Z value shows the degree and direction of relationship, whereas the P value shows the significance of the relationship. The horizontal bar shows the range of OR with a square in the center, the size of which is directly proportional to the weight given to each study. The direction of the projection of the horizontal bar shows the direction of the association.

DISCUSSION

Several epidemiological and experimental studies have been undertaken to investigate the significance of *MTHFR* variants in urinary bladder cancer; however, contrasting results with respect to their relation with UBC have been reported (Wang wt al., 2009; Izmirli et al., 2011; Lin et al., 2004, Sanyal et al., 2004; Moore et al., 2004; Karagas et al., 2005; Moore et al., 2007; Cia et al., 2009; Beebe-Dimmer et al., 2012). We observed a significant protective effect of c.1298A>C substitution against the UBC risk. This is in contrast to other studies, which observed a significant direct correlation between this substitution and UBC risk in Asian (Safarinejad et al., 2011) and African populations (Ouerhani et al., 2009). A comprehensive review of the literature revealed a dramatic inconsistency in the frequency of c.1298A>C genotypes across different ethnic populations. The European and American populations showed an overall genotype frequency of 7-11% and 6-9%, respectively, which was as low as 1-2% in Asian populations.

Table 5.Details and genotype data of the studies included in the meta-analysis on 1298 A>C polymorphism.

Author	Year	Ethnicity	Sample Size		Cases			Allele		Controls			Allele	
			case	control	AA	AC	CC	A	C	AA	AC	CC	A	C
Sanyal	2004	European	311	245	145	133	33	423	133	110	111	24	331	159
Lin	2004	European	410	409	192	188	30	572	248	189	184	36	562	256
Moore	2004	Argentine-American	106	108	52	45	9	149	63	55	45	8	155	61
Lin	2004	African-American	21	21	14	7	0	35	7	13	8	0	34	8
Lin	2004	Mexican-American	17	17	13	4	0	30	4	11	5	1	27	7
Karagas	2005	USA-American	350	542	173	146	31	492	208	267	220	55	754	330
Moore	2007	European	1068	1078	537	457	74	1531	605	557	429	92	1543	613
Ouerhani	2007	African	111	131	58	47	6	163	59	85	37	9	207	55
Wang	2009	Asian	239	250	169	67	3	405	73	171	75	4	417	83
Rouissi	2009	African	185	191	97	78	10	272	98	121	60	10	302	80
Cai	2009	Asian	312	325	215	91	6	521	103	226	92	7	544	106
Safarinejad	2011	Asian	158	316	48	85	25	181	135	178	115	23	471	161
Izmirli	2011	Asian	47	50	19	25	3	63	31	14	29	7	57	43
Dimmer	2012	USA	218	272	95	109	14	299	137	127	111	34	365	179
Present Study	2014	Asian	232	250	69	117	46	255	209	46	137	67	229	271

The other most frequently studied *MTHFR* polymorphism, c.677C>T, results in a thermolabile form of the enzyme showing 35-50% reduced activity [11]. A large number of studies across the globe have variably reported a significant association of increased frequency of the TT genotype with urinary bladder cancer risk (Cai et al., 2009, Izmirli et al., 2011; Wang et al., 2009). Nevertheless, other studies have shown a protective association of the same against urinary bladder cancer. In the present study, we did not observe association of the c.677C>T substitution with urinary bladder cancer risk, which is consistent with other studies done on relatively larger sample sizes (Beebe-Dimmer et al., 2012; Lin et al., 2004; Sanyal., 2004; Moore et al., 2007). The genotype frequencies for the third variant, c.203G>A, were similar between cases and controls, suggesting no association with UBC risk; however, the percentage frequency of heterozygous and homozygous recessive genotypes was extremely low in the cases and controls. The haplotype analysis for all the three variants suggested a significant linkage disequilibrium (LD) between c.1298A>C and c.203G>A SNPs, with haplotype CCG showing a significant protective association with urinary bladder cancer. Furthermore, previous reports have suggested a strong linkage disequilibrium between c.677C>T and c.1298A>C (Wang et al., 2009; Cia et al., 2009), which was not seen in the present study. Among all the haplotypes, only CCG was found to be significantly associated with the UBC risk with a higher frequency in the controls as compared to cases, suggesting its protective effect.

Till date, a large number of the studies have investigated the role of the *MTHFR* polymorphisms in urinary bladder cancer; however, the results varied considerably from positive to negative or no effect. Therefore, in order to reach a consensus, we undertook a meta-analysis on data pooled from all eligible studies published till date. However, the meta-analysis revealed no significant association of any of the two variants with the UBC risk. This was consistent with some previous meta-analysis suggesting no association of the *MTHFR* polymorphisms with the UBC risk (Shi et al., 2014; Xu et al., 2013; Wang et al., 2009). The lack of publication bias further suggested the authenticity of the study. The lack of association in the meta-analysis suggests that *MTHFR* polymorphisms may show an association with the UBC risks in a few ethnic populations, but they are not bladder cancer risk factors globally.

CONCLUSIONS

We observed no association of *MTHFR* c.677C>T and c.203G>A polymorphisms with the risk of bladder cancer; however, a protective association of c.1298C>A polymorphism was evident. While a majority of the case-control studies performed so far have reported a significant association of c.1298A>C and c.677C>T polymorphism with urinary bladder cancer risk, this meta-analysis has denied any such association. Our strict statistical approach and a careful sensitivity analysis suggest that *MTHFR* variants, c.1298A>C and c.677C>T do not affect UBC risk. Nevertheless, other studies need to assess the significance of the protective association of c.1298C>A substitution against the UBC risk in Indian populations.

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Conflict of interest statement

All authors have stated that no conflict or competing interests exist.

Authors' contributions

KAG, PLS and SNS conceived and designed the study; PT, SNS contributed samples; KAG conducted the experiments. All authors have read and approved the manuscript for submission.

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