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MTR-A2756G and breast cancer risk: a study of Iranian women with a meta-analysis

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ABSTRACT

Polymorphisms in folate metabolizing genes have been revealed to be associated with the risk of breast cancer. One of the key regulatory enzymes in the folate metabolism pathway is methionine synthase (MTR) enzyme. The aim of this study was to investigate the association of A2756G polymorphism in MTR gene with breast cancer risk followed by a meta-analysis. In a case-control study, 157 women with sporadic breast cancer and 188 healthy women were included. *MTR*-A2756G genotyping was performed by using PCR-RFLP method. In our meta-analysis, a total of 22 studies reflecting 14037cases and 16621 healthy controls were included. Our case-control study revealed that GG genotype is associated with breast cancer risk (OR: 2.89, 95%CI: 1.06-7.90, p=0.039). In meta-analysis, a significant association was observed between *MTR*-A2756G and breast cancer risk within Asian population. While we observed a protective association of *MTR*-A2756G polymorphism with breast cancer risk. Based on result, MTR-A2756G may be a genetic risk factor and a protective factor for breast cancer in Asian and Caucasian populations, respectively. This different effects of *MTR*-A2756G polymorphism in breast cancer risk may arise from ethnicity.

KEY WORDS: BREAST CANCER; *MTR* GENE; A2756G POLYMORPHISM; META-ANALYSIS

INTRODUCTION

Breast cancer is one of the main causes of cancer-related mortality in women worldwide. Every year about 1.2 million women suffer from breast cancer in the world and this number is increasing. The etiology of the breast cancer is poorly understood. Some factors such as reproductive history, age of menopause, changes in estrogen level, diet, smoking, familiar history, and genetic factors can be involved in this cancer. The greatest obstacle for the treatment of this disease is a highly variable outcome of breast cancer among patients, even women with the same biological characteristics and stage. It seems some genes may provide a possible explanation for

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breast cancer predisposition and could be essential for treatment choices and improve patients' survival. Genes contributed to DNA repair, synthesize and methylation such as genes involved in folate metabolizing pathway, are good candidates for this purpose. Failures in folate metabolizing genes, may be associated with the risk of breast cancer (Parkin, 2001; Stover, 2004, Ferlay et al., 2004, Hankinson et al., 2004; Dumitrescu et al., 2005, Babyshkina et al., 2013 and Wu et al., 2014).

Methionine synthase (*MTR*) which catalyzes the remethylation of homocysteine to methionine is a regulatory enzyme in folate metabolism (Fodinger et al., 2000). Mutations or Single nucleotide polymorphisms (SNPs) in coding sequence of *MTR* gene may lead to decrease modification of DNA methylation profiles due to reduce in the efficiency of purine nucleotides and thymidylate synthesis (Chen et al., 2001, Ma et al, 2009b; Suzuki et al, 2008; Kakkoura et al, 2015; Lopez_Cortes et al, 2015).

There is a common SNP (A2756G; rs1805087) in *MTR* sequence which result in substitution of aspartic acid to glycine residue at position 919 (D919G) in the protein sequence (Yu et al., 2007; Ma et al., 2009[a]). To date, the association between *MTR*-A2756G and breast cancer risk started to receive attention. A numerous studies, investigate the association of this polymorphism with breast cancer in different populations however the results are inconsistent. Thus, in the present study we investigate the association of *MTR*-A2756G with the risk of breast cancer followed by a meta-analysis.

MATERIAL AND METHODS

SUBJECTS

A total of 345 women comprised of 157 women with sporadic breast cancer as case group and 188 healthy women as control group were included in this study. All the participants were Iranian, and lived in the Kashan city (Kashan, Iran). Patients (with mean age 54.40±10.12 years) were women referred to the Shahid Beheshti hospital (Kashan, Iran) from 2011 to 2013. They were with a histologically confirmed diagnosis of breast cancer. Controls (with mean age 58.30±5.87) were women with no history of oncological disease, who contributed in the local mammography screening plan, and they had no positive result. Finally from each subjects, 2mL blood collected. All the participants' informed written consent and this study confirmed by the principles outlined in the Declaration of Helsinki and approved by the Hospital's Ethics Committee.

SNP GENOTYPING

Genomic DNA was isolated from blood samples by DNG-plus buffer (Cinnagen Co., Iran). *MTR*-A2756G genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For PCR purpose the primer oligonucleotide were designed by Oligo7 software. The forward and reverse primer sequences were 5'-AAGCCCACTGAGTTTAC-CTTTTC-3' and 5'-ATCCAAAGCCTTTTACACTCCTC-3', respectively. The PCR reaction was done in a 20µl total volume containing, 10µl PCR master mix buffer (Cinnagen Co., Iran), 0.35µM each of forward and reverse primers, and 50ng genomic DNA. The thermal cycling program for PCR porous were: initial denaturation for 5 min at 94°C, followed by 35 repetitive cycles of 45s at 94°C, 45s at 63°C, and 45s at 72°C, with a final extension of 5 min at 72°C. The amplified PCR fragments were digested by *Hae*III restriction enzyme (Fermentas, Lithuania) and visualized by ethidium bromide after electrophoresis in 1% agarose gel. The accuracy of PCR-RFLP data was ensured by DNA sequencing. For this purpose, three PCR products with different genotypes were selected and sequenced by Bioneer Company (Korea).

META-ANALYSIS

The PubMed, SienceDirect, and Google Scholar databases were searched for published reports examining associations between *MTR*-A2756G and breast cancer risk by using the following keywords: breast cancer, MTR, A2756G, and polymorphism. The literature search was performed up to October 2015. Original articles with sufficient information to compute odd ratio (OR) with 95% Confidence Interval (CI) were selected. The inclusion criteria for studies were as follows: (i) investigation of the *MTR*-A2756G polymorphism and breast cancer risk (ii) studied on human beings; (iii) in a case-control study design. The characteristics of included studies in meta-analysis are introduced in Table 1. Also the association between *MTR*-A2756G and breast cancer was further stratified by ethnicity.

STATISTICAL ANALYSIS

Odds ratio (OR) with 95% confidence interval (95%CI) was calculated for various alleles and genotypes in case and control groups. The Chi-square test was used to compare the allele and genotype frequencies between the case and control groups. Also, Hardy-Weinberg equilibrium (HWE) in the case and control groups was calculated. The *p*-value less than 0.05 ($p<0.05$) considered as statistically significant. These statistical calculations were done by SPSS Statistical Package version 19.

In meta-analysis a Chi square based 'Q' test was used to evaluate the heterogeneity. When there was no heterogeneity among the studies ($P_{heterogeneity}>0.1$), the pooled ORs were calculated by a fixed-effects model (the Man-

Table 1: Characteristics of included studies in meta-analysis

Country (ethnicity)	Allele frequencies				Genotype frequencies						Author. Year
	Control		Case		Control			Case			
	A	G	A	G	AA	AG	GG	AA	AG	GG	
Germany	1095	247	929	241	451	193	27	366	197	22	Justenhoven et al. 2005
Chinese	2059	217	1935	197	932	195	11	877	181	8	Shrubsole et al. 2006
Polish	3534	1034	3150	820	1350	834	100	1244	662	79	Lissowka et al. 2007
Taiwan	740	96	192	24	324	92	2	85	22	1	Yu et al. 2007
USA	1776	428	1723	385	714	348	40	705	313	36	Xu et al. 2007
Canada	1230	320	1543	335	489	252	34	635	273	31	Kutsopoulous et al. 2008
Taiwan	895	171	586	118	384	127	22	246	94	12	Cheng et al. 2008
USA	2970	780	1673	425	1184	602	89	678	317	54	Platek et al. 2009
Japan	1501	319	737	173	616	269	25	301	135	19	Suzuki et al. 2008
Brazil	737	179	723	191	294	149	15	294	135	28	Ma et al. 2009 a
Japan	635	139	611	165	261	113	13	237	137	14	Ma et al. 2009 b
Brazil	282	66	255	93	109	64	1	82	91	1	Carvalho et al. 2012
Russian	1116	300	1293	357	443	230	35	505	283	37	Weiner et al. 2012
Chinese	421	341	325	295	127	167	87	97	131	82	He et al. 2014
Chinese	1089	257	808	262	479	131	63	344	120	71	Jiang-hua et al. 2014
Chinese	1494	206	1425	191	662	170	18	631	163	14	Xi et al. 2014
Chinese	455	177	408	184	176	103	37	149	110	37	Weiwei et al.2014
Chinese	151	19	153	39	69	13	3	59	35	2	Wu et al. 2014
Greek-Cypriot	1772	540	1708	440	684	404	68	679	350	45	Kakkoura et al.2015
Ecuadorian Mestizo	308	82	206	22	123	62	10	94	18	2	Lopez-cortes et al. 2015
European American	1007	263	1021	239	408	191	36	410	201	19	Gong et al. 2015
African American	1047	371	865	295	387	273	49	325	215	40	Gong et al. 2015
Iran	298	78	231	83	116	66	6	87	57	13	This study

tel-Haenszel method). To sensitivity analysis, each study was excluded at a time to determine the magnitude of effect on the total summary assessment. Begg's funnel plot and Egger's test were used to evaluate the publication bias (Begg and Mazumdar 1994; Egger et al., 1997). These calculations were performed by using Comprehensive Meta-Analysis software (version 2.0).

RESULTS

A2756G GENOTYPING

The *MTR* fragment containing A2756G with size of 381 bp was amplified by using the forward and reverse primers. After PCR-RFLP procedure, electrophoresis of the products on agarose gel showed the AA, AG, and GG genotypes with one band (381 bp), three bands (381 bp, 251 bp, and 130 bp), and two bands (251 bp, and 130 bp), respectively. PCR-RFLP results were confirmed by DNA sequencing.

DISTRIBUTION OF A2756G

The *MTR* genotypes distribution for 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 presented in Table 2. The AA, AG and GG genotypes frequencies of cases were 55.41%, 36.31%, and 08.28%, while these ratios in controls were 61.70%, 35.11%, and 03.19%, respectively. The A and G allele frequencies for case group were 73.57% and 26.43%, while these ratios in controls were 79.26% and 20.74%, respectively. Genotype analysis showed a significant association of AG genotype with breast cancer (OR= 2.89, 95%CI= 1.06-7.90, p= 0.039). But, we observed no significant association between A allele and breast cancer risk (OR= 1.37, 0.96-1.96, p=0.079).

META-ANALYSIS

After screening of the articles, a total of 21 eligible studies were included in our meta-analysis (Justenhoven et

Table 2: Genotype and allele frequencies of A2756G in cases and controls

Genotype	No. and Percentage		OR (95% CI)	p-value
	Control (n=188)	Case (n=157)		
AA	116 (61.70%)	87 (55.41%)	-	-
AG	66 (35.11%)	57 (36.31%)	1.15 (0.73-1.81)	0.539
GG	6 (03.19%)	13 (08.28%)	2.89 (1.06-7.90)	0.039
AG+GG	72 (38.30%)	70 (44.59%)	1.30 (0.84-1.99)	0.238
Allele				
A	298 (79.26%)	231 (73.57%)	-	-
G	78 (20.74%)	83 (26.43%)	1.37 (0.96-1.96)	0.079

OR: odds ratio, CI: confidence interval
Significant differences between the case and control groups are bolded

al., 2005; Shrubsole et al., 2006; Lissowka et al., 2007; Yu et al., 2007; Xu et al., 2007; Kotsopolous et al., 2008; Cheng et al., 2008; Suzuki et al., 2008; Platek et al., 2009; Ma et al[a,b]., 2009; Carvalho et al., 2012; Weiner et al., 2012; He et al., 2014; Jiang-hua et al., 2014. Xi et al., 2014; Weiwie et al., 2014; Wu et al., 2014; Kakkoura et al., 2015; Lopez-Cortes et al., 2015; Gong et al., 2015). Also the data from our case-control study was added to the meta-analysis. The studies selection procedure is introduced in Figure 1. As a result, 22 studies were included in the meta-analysis, reflecting 14037cases and 16621 healthy controls. There were 5 studies of Caucasians, 11 studies of Asians and 6 studies of other ethnicities. The total results of the meta-analysis are represented in Table 3. When meta-analysis performed for the 22 pooled studies, no significant association was observed between *MTR*-A2756G and breast cancer risk in any of genetic models. Results of meta-analysis for

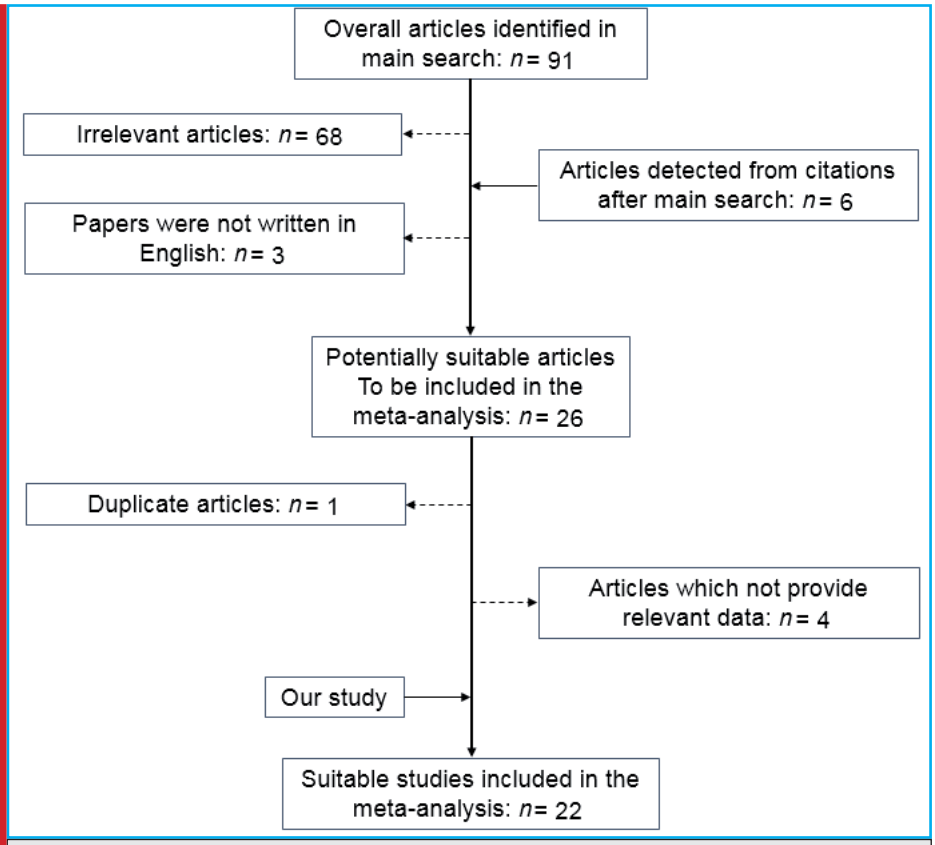

FIGURE 1. Flow diagram of the study selection process.

Table 3: Results of meta-analysis for MTR-A2756G polymorphism and breast cancer risk

ethnicity	Genetic model	Analysis model	OR (95%CI)	P-value	tau2	Ph	I2	PE
Total populations	G vs. A (Allelic)	Random effect	1.024 (0.951-1.103)	0.522	0.019	< 0.001	65%	0.052
	GG vs. AA (Codominant)	Random effect	0.999 (0.862-1.159)	0.992	0.040	0.053	35%	0.915
	AG vs. AA (Codominant)	Random effect	1.025 (0.941-1.117)	0.571	0.023	< 0.001	60%	0.022
	AG+GG vs. AA (Dominant)	Random effect	1.057 (0.962-1.161)	0.245	0.033	< 0.001	70%	0.045
	GG vs. AA+AG (Recessive)	Fixed effect	1.000 (0.898-1.114)	1.000	-	0.114	27%	0.813
Caucasian population	G vs. A (Allelic)	Random effect	0.929 (0.837-1.032)	0.170	0.008	0.051	58%	0.336
	GG vs. AA (Codominant)	Fixed effect	0.809 (0.672-0.976)	0.026	-	0.693	0%	0.814
	AG vs. AA (Codominant)	Random effect	0.952 (0.829-1.092)	0.481	0.015	0.030	63%	0.193
	AG+GG vs.AA (Dominant)	Random effect	0.994 (0.814-1.215)	0.955	0.043	< 0.001	84%	0.295
	GG vs. AA+AG (Recessive)	Fixed effect	0.832 (0.692-1.001)	0.051	-	0.821	0%	0.872
Asian populations	G vs. A (Allelic)	Fixed effect	1.133 (1.053-1.220)	< 0.001	-	0.159	30%	0.099
	GG vs. AA (Codominant)	Fixed effect	1.270 (1.054-1.530)	0.012	-	0.571	0%	0.392
	AG vs. AA (Codominant)	Fixed effect	1.126 (1.024-1.238)	0.014	-	0.158	31%	0.006
	AG+GG vs.AA (Dominant)	Fixed effect	1.139 (1.043-1.244)	0.004	-	0.160	30%	0.014
	GG vs. AA+AG (Recessive)	Fixed effect	1.216 (1.018-1.452)	0.031	-	0.554	0%	0.351

OR, odds ratio; CI, confidence interval; Ph, P-values for heterogeneity from Q test; PE, PEgger

Asian and Caucasian subgroups are presented in Table 3. The results showed that the *MTR*-A2756G was associated with the breast cancer risk within Asian population in G *vs*. A (OR: 1.133, 95%CI: 1.053-1.220, $p< 0.001$), GG *vs*. AA (OR: 1.270, 95%CI: 1.054-1.530, $p=0.012$), AG *vs*. AA (OR: 1.126, 95%CI: 1.024-1.238, $p=0.014$), AG+GG *vs*. AA (OR: 1.139, 95%CI: 1.043-1.244, $p=0.004$), and GG *vs*. AA+AG (OR: 1.216, 95%CI: 1.018-1.452, $p=0.031$) genetic models (Figure 2). This despite the fact that we observed a protective association between *MTR*-A2756G and breast cancer risk in GG *vs*. AA (OR: 0.809, 95%CI: 0.672-0.976, $p=0.026$) genetic model (Figure 2). Also we observed a protective association in GG *vs*. AA+AG model, but this association was not statistically significant (OR: 0.832, 95%CI: 0.692-1.001, $p=0.051$).

As depicted in Table 3 in overall meta-analysis, there was a high heterogeneity for G vs. A, AG *vs*. AA, and AG+GG vs. AA genetic model ($P_{heterogeneity}< 0.001$). In Caucasian population, a high heterogeneity was found in AG+GG vs. AA genetic model with $P_{heterogeneity}< 0.001$ (Table 3). While, we don't observed a true heterogeneity within Asian population in any of five genetic models (Table 3). Egger's test and Funnel plot were used to evaluate the publication bias in meta-analysis. The Egger's test suggested a publication bias for the G *vs*. A (PEgger= 0.052), AG *vs*. AA (PEgger= 0.022), and AG+GG *vs*. AA (PEgger= 0.045) genetic models. In subgroup analysis, also we observed a publication bias in AG vs. AA (PEgger= 0.006) and AG+GG *vs*. AA (PEgger= 0.014) models within Asian population (Table3). Whereas, the shapes of funnel plot for other genetic models in Asian subgroup and for all of the five genetic models in Caucasian subgroup seemed approximately symmetrical, suggesting the lack of publication bias (Figure 3). The lack of publication bias was confirmed by Egger's test (Table 3). Sensitivity analysis was done by excluding

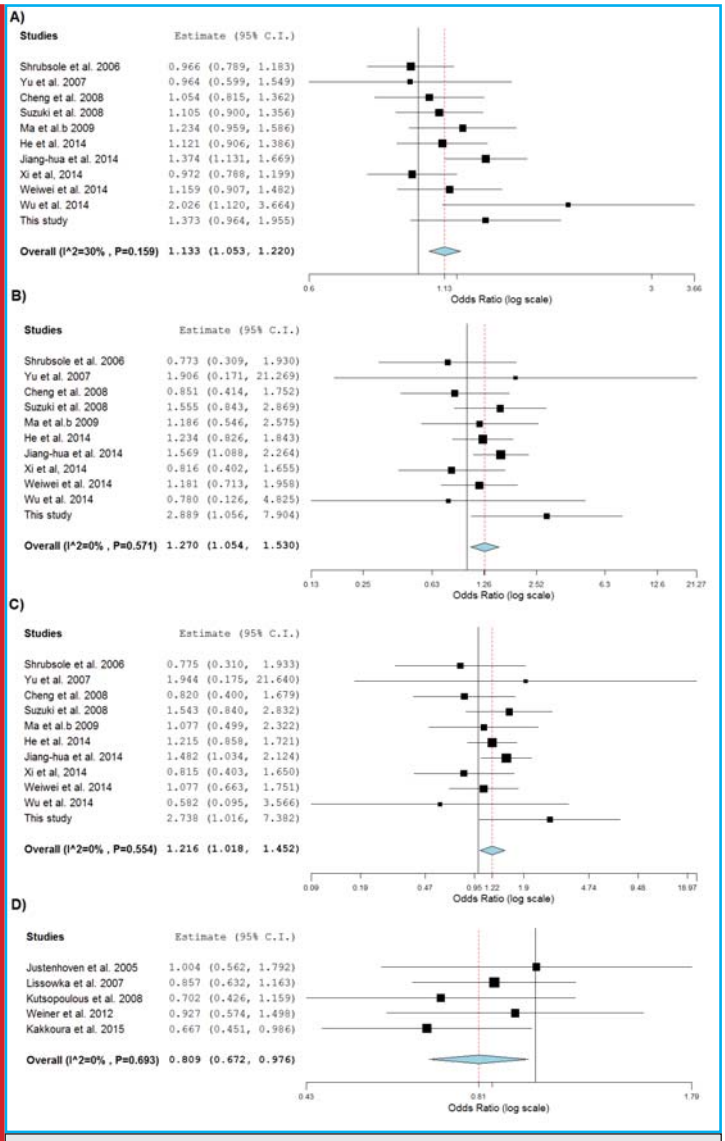

FIGURE 2. Forest plot for the association of *MTR*-A2756G with breast cancer. Findings of quantitative data synthesis under G *vs.* A (A); GG *vs.* AA (B); GG *vs.* AA+AG (C) models in Asian population; and GG *vs.* AA model in Caucasian population (D).

a study at one time. The results of sensitivity analysis showed that the estimates before and after the omission of every study were similar, suggesting this meta-analysis were stable (data not shown).

DISCUSSION

Genetic polymorphisms in folate metabolizing enzymes may be involved in the susceptibility breast cancer predisposition. MTR is one of key regulatory enzymes in folate metabolism. The A2756G transition in the *MTR* gene lead to aspartic acid to glycine residue substitution at position 919 near the cobalamin-binding domain of the *MTR* enzyme (Chen et al, 1997; van der Put et al, 1998). In the present study we investigated the association of *MTR*-A2756G polymorphism with breast cancer risk in Iranian population followed by a meta-analysis. Our case-control study revealed that GG genotype is associated with breast cancer risk (OR: 2.89, 95%CI: 1.06-7.90, p= 0.039). Some studies reported a similar association between this variety and breast cancer risk (Yu et al., 2007; Ma et al., 2009[a]). While some other studies reported no significant association between this variety and breast cancer risk (Justenhoven et al., 2005; Platek et al., 2009). To help clarify the inconsistent findings,

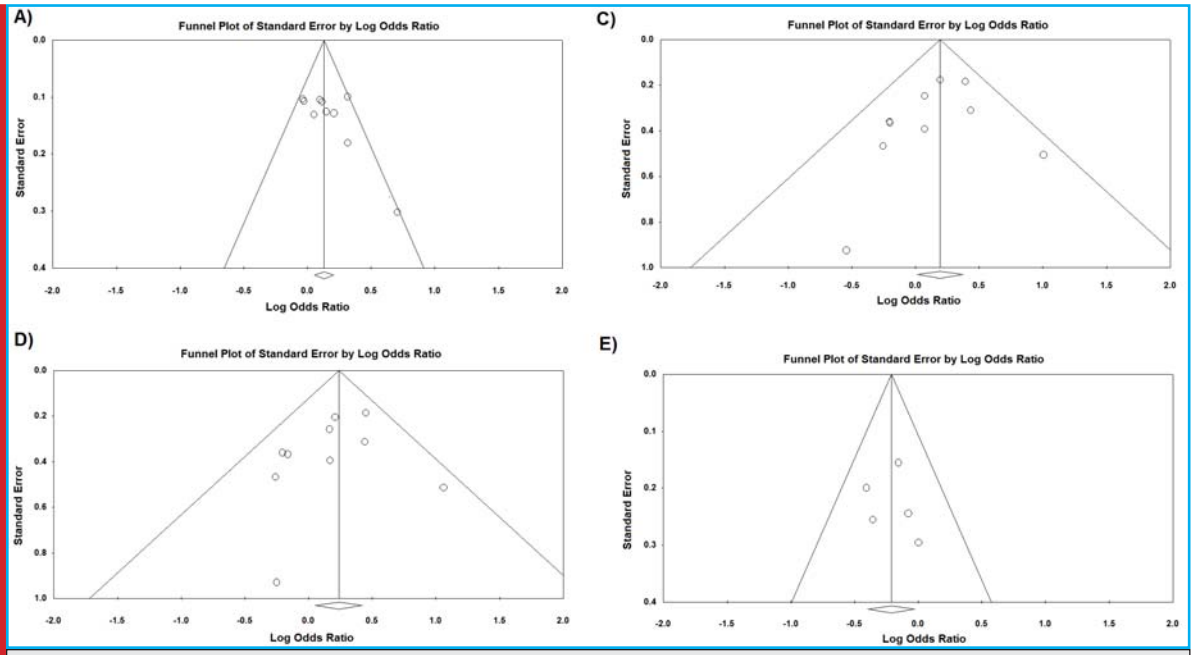

FIGURE 3. Funnel plot of breast cancer risk associated with A2756G. The plots under G *vs.* A (A); GG *vs.* AA (B); GG *vs.* AA+AG (C) models in Asian population; and GG *vs.* AA model in Caucasian population (D).

we performed a meta-analysis to obtain a competitive result via combining more eligible studies, enlarging the sample size, and performing a subgroup analysis. Results of overall meta-analysis, revealed no significant association between *MTR*-A2756G and breast cancer risk in any of five genetic models. But the meta-analysis in ethnical subgroups revealed different results. We observed a significant association between *MTR*-A2756G and breast cancer risk within Asian population in G *vs.* A, GG *vs.* AA, AG *vs.* AA, AG+GG *vs.* AA, and GG *vs.* AA+AG genetic models. While we observed a protective association of *MTR*-A2756G with breast cancer risk in GG *vs.* AA (OR: 0.809, 95%CI: 0.672-0.976, p=0.026) genetic model. The geographic and ethnic variations could explain the conflicting data between different studies.

DNA methylation is a key procedure for regulation of genome integrity and gene expression. Role of abnormal DNA methylation in carcinogenesis is complex. Hypermethylation of special genes and hypomethylation of global DNA are two most common mechanism detected in several tumors (Pufulete et al., 2003). In the human genome, cytosines are mostly methylated in CpG islands (Jones and Takai, 2001; Takai and Jones, 2002). The CpG dinucleotide are frequently located around and in the start sites of transcription of nearly half of the human genes. The CpG islands in a several genes, which are generally unmethylated in normal tissues, are methylated in human cancers including breast cancer, with variable degrees (Yanget al., 2001a). Methionine synthase with vitamin B12 as a cofactor catalyzes the remethylation of homocysteine to methionine. Methionine synthase play a crucial role in maintaining suitable intracellular methionine, normal homocysteine and folate concentrations. Methionine as a crucial amino acid and a precursor of S-adenosylmethionine is a common methyl group donor involved in reactions of methylation including DNA methylation (Ma et al., 1999).

Missense mutations are responsible for several credited to single gene disorders. Some studies stated that non-synonymous SNPs (nsSNP) are dangerous for structure of the proteins (Karimian and Hosseinzadeh Colagar, 2014; Nikzad et al., 2015). Wang and Moult reported that some nsSNPs disrupt the function of protein by alteration of the protein hydrophobicity (Wang and Moult, 2001) or by affecting the three-dimensional structure of the protein depending on the location of nsSNP (Sunyaev et al., 2001). A2756G as an nsSNPs may alter the MTR function, therefor we suggests that future studies focus on it.

There are three similar meta-analyses about the association of the *MTR*-A2756G polymorphism and the breast cancer risk (Lu et al., 2010; Weiner et al., 2012, Zhong et al., 2013). Lu et al. (2012) reported that there is no significant association between *MTR*-A2756G gene polymorphism and risk of breast cancer, overall. But, in the stratified analysis, they found significantly decreased risk of breast cancer in Europeans (Lu et al., 2010). Similarly, Zhong et al. reported a protective association in Caucasian population (Zhong et al., 2013). Weiner et al. (2012) represented a deferent results. They observed no significant association between *MTR*-A2756G poly-

morphism and breast cancer risk in any ethnical groups (Weiner et al., 2012). The data which reported Naushad et al. were wrong (Naushad et al. 2011), but Zhong et al. included this study in their meta-analysis (Zhong et al., 2013).

There are some limitations in our meta-analysis that must be considered. First, the lack of original data from the studies restricted our more assessment of the possible interactions such as gene-gene and gene-environment which may modulate cancer risk. Second, restriction of the study to English language articles may potentially lead to language bias. Third, this meta-analysis is absence of adequate data from African populations. Last, as the *MTR* gene has some polymorphisms except the A2756G, our analysis cannot state the role of other polymorphisms in the breast cancer risk.

CONCLUSION

Our genetic association study suggests that the *MTR*-A2756G polymorphism is associated with the risk of breast cancer. Supplementary studies with large sample size are necessary to confirm our findings. Future studies must include homogeneous breast cancer patients with well-matched controls. Furthermore, other *MTR* gene polymorphisms and specific haplotypes may contribute to the risk of breast cancer. More studies investigating gene-environment and gene-gene interactions should be performed to better understand the role of *MTR*-A2756G transition in breast cancer predisposition.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest in this article.

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