MTHFR 677C>T Polymorphism and the Risk of Breast Cancer: Evidence from an Original Study and Pooled Data for 28031 Cases and 31880 Controls

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Abstract

Background

Methylenetetrahydrofolate reductase (MTHFR) acts at an important metabolic point in the regulation of cellular methylation reaction. It assists in the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The latter aids in remethylation of homocysteine to *de novo* methionine that is required for DNA synthesis. The objective of this study was to examine the effect of MTHFR 677 C>T polymorphism on the risk of breast cancer in the Indian sub-continent.

Methods and Results

We genotyped 677 C>T locus in 1096 individuals that were classified into cases (N=588) and controls (N=508). Genotype data were analyzed using chi-square test. No significant difference was observed in the distribution of genotypes between cases and controls in north Indian (P = 0.932), south Indian (P = 0.865), and pooled data (P = 0.680). To develop a consensus regarding the impact of 677C>T polymorphism on breast cancer risk, we also conducted a meta-analysis on 28031 cases and 31880 controls that were pooled from sixty one studies. The overall summary estimate upon meta-analysis suggested no significant correlation between the 677C>T substitution and breast cancer in the dominant model (Fixed effect model: OR = 0.97, P=0.072, Random effects model: OR = 0.96, P = 0.084) or the recessive model (Fixed effect model: OR = 1.05, P = 0.089; Random effects model: OR= 1.08, P= 0.067).

Conclusion

677 C>T substitution does not affect breast cancer risk in the Indo-European and Dravidian populations of India. Analysis on pooled data further ruled out association between the 677 C>T polymorphism and breast cancer. Therefore, 677 C>T substitution does not appear to influence the risk of breast cancer.

Introduction

Breast cancer has become the most common cancer among women with a consistent increase in frequency. The genetic damage caused by endogenous metabolites and exogenous risks might explain nature of the disease [1]. The exact causes of breast cancer are unknown, but a number of factors may contribute to the development of the disease, such as age of menarche and menopause, diet and exposure to high estrogen levels [2]. The etiology of the disease links to various genetic and epigenetic processes, including DNA synthesis, methylation, and repair [3]. Two important mechanisms that might lead to the risk of malignancy are: 1) DNA hypomethylation and activation of proto-oncogenes. 2) Misincorporation of uracil during DNA synthesis, leading to catastrophic DNA repair and chromosome damage [4]. Folate, an important dietary component, is found in legumes, green leafy vegetables, and liver, and the role of this B vitamin involves the transmission of one carbon group to carry out necessary biological reactions [5]. Deficiency of folate caused by low dietary intake, diminished metabolism or no auxiliary intake may result in breakage of DNA strands, increased rate of mutagenesis and changes in the DNA methylation patterns, ultimately affecting the expression of a number of genes [6, 7]. Lack of folate is believed to affect the risk of cancer through the processes described above.

The methylenetetrahydrofolate reductase (MTHFR) gene is mapped to chromosome 1p36.3 and consists of a 2 kbp coding region divided into eleven exons [8]. It plays an important role in the regulation of cellular methylation by assisting the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [9]. The latter aids in the remethylation of homocysteine to de novo methionine [10], which serves as a precursor for the S-adenosylmethionine, a universal methyl donor for methylation reactions [11]. It also functions as a coenzyme in purine and thymidylate synthesis. Two functional polymorphisms in the *MTHFR* gene, 677C>T (ala→val) and 1298A>C (glu→ala), have a profound effect on the activity of enzyme, producing more labile forms with reduced activity [12]. The 677C>T is a common SNP, which converts an alanine to valine at codon 225 of the folate binding site of methylenetetrahydrofolate reductase [13]. The enzyme with homozygous and heterozygous substitutions exhibit 30% and 65% activity, respectively, in comparison to the wild type [9]. Since low dietary folate intake is correlated with an increase in the rate of breast cancer, MTHFR 677C>T may affect breast cancer risk by negatively modifying folate levels [14]. In this case-control study, we investigated if MTHFR 677 C>T polymorphism affects breast cancer risk. A few meta-analyses have reported an association between 677 C>T polymorphism and breast cancer risk [10, 15, 16, 17, 18]; however, none of these has addressed the issue using a meticulous plan taking into consideration sensitivity analysis that may significantly affect the outcome. Repeated meta-analysis using similar strategy does not add new information to the literature. The principal of statistics that odd observations should not be favoured unless the evidence is very compelling, propelled us to undertake a stringent statistical approach and sensitivity analysis to critically look into the relationship between the 677 C>T polymorphism and breast cancer risk.

Materials and Methods

Case-control study

Subjects. The study and the protocol for sample collection were approved by the Institutional Human Ethics Committee of the King George's Medical University (KGMU), Lucknow, India. Informed written consents of the participants were obtained, and no minor subjects were enrolled in the study. The consent procedure was approved along with the study protocol by the Institutional Ethics Committee. The study included two ethnically different case-control groups from the Indian sub-continent. A pre-defined set of recruitment/exclusion protocol was followed for both groups.

The north Indian group consisted of breast cancer cases (N = 331) and controls (N = 181) of the Indo-European linguistic group from Uttar Pradesh. The subjects were recruited from the Department of Surgery and Oncology, KGMU, Lucknow, India. The age of the patients varied from 22 to 90 years with a mean age of 42.11 years (SD 14.21). In this group, 191 cases were pre-menopausal and 140 were post-menopausal. One hundred fifty-five patients had cancer in the right breast, 160 patients in the left breast, and only 16 patients in both the breasts. The size of tumours varied from a minimum of 3 cm^3 to a maximum of 1150 cm^3 with a mean value of 125.93 cm^3 (SD, 324.43). The staging of tumours was done according to the TNM classification. Three patients (0.91%) were in stage I, 123 patients (37.16%) were in stage II, 159 patients (48.03%) were in stage III, and 46 patients (13.89%) were in stage IV. Grading of tumours was done according to the Bloom-Richardson grading system, where the tumor grade was decided according to the overall score that a tumor got upon analyzing for the degree of tumor tubule formation, tumor mitotic activity, and tumor nuclear grade. Healthy controls were recruited from the out-patient department and staff members of the Department of Surgery and Oncology. The controls had no family history of breast cancer and all had undergone a recent mammogram confirming that there was no detectable breast cancer at the time of sampling. Age of the controls ranged from 28 to 70 years with a mean age of 40 years (SD, 12.40). It was ensured that patients and controls were enrolled from the populations of same ethnicity.

The south Indian group consisted of patients (N = 257) and controls (N = 327) of the Dravidian linguistic group. The age of the patients ranged from 24 to 82 years with a mean age of 48.32 years (SD 12.25). One hundred and two cases were pre-menopausal and 155 were post-menopausal. One hundred and nineteen patients had cancer in the right breast, 128 patients in the left breast, and 10 in both breasts. Size of the tumours varied from a minimum of 6 cm^3 to a maximum of 1310 cm^3 with a mean value of 131.23 cm^3 (SD, 347.12). The staging of the tumours was done according to the TNM classification. Two patients (0.78%) were found to be in stage I, 103 patients (40.07%) were in stage II, 142 patients (55.25%) were in stage III, and 10 patients (3.89%) were in stage IV. Ethnically matched controls were recruited from the out-patient department and staff members who had no family history of breast cancer. The controls had undergone a recent mammogram confirming absence of breast cancer at the time of sampling. Age of the controls ranged from 32 to 70 years with a mean age of 48 years (SD 12.37). Further details of the patients and controls are presented in Table 1.

Isolation of genomic DNA. DNA from the peripheral blood samples was extracted using the phenol-chloroform-isoamyl method. The 677C>T polymorphism was genotyped using the PCR-RFLP method. Primers in the vicinity of polymorphic site were designed with the GENE-TOOL software. PCR reactions of 10μl volume were performed in thin walled PCR tubes consisting of 1.0μl of PCR buffer (10X), 1.0μl of dNTPs (10mM), 2.0μl of each of the forward (5′ CATCCCTATTGGCAGGTTACCC3′) and reverse (5′ GGGAAGAACTCAGCGAACTCAG3′) primers, 0.2 μl of Taq DNA polymerase enzyme (Applied Biosystems), and 40ng of genomic DNA. PCR was carried out in ABI Veriti thermal cycler (Applied Biosystems, USA). PCR

Table 1. Descriptive data of cases and controls.

Variables	North Indian		South Indian	
	Cases	Controls	Cases	Controls
Age (mean±SD)	42.11±14.21	40±12.40	48.32±12.25	48 ± 12.37
BMI (Kg/m^2)	22.41+5.87	23.21 ± 5.81	22.19±5.21	22.36± 5.21
Age at menarche (years, mean ± SD)	13.76 ± 1.72	13.54 ± 1.78	13.91 ± 1.18	13.52 ± 1.29
Age at diagnosis for cases or at interview for controls				
≤ 30 years	54	16	4	34
31–45 years	134	87	81	114
46–60 years	97	53	117	119
61–75 years	36	17	50	50
76–90 years	10	8	5	10
Family history				
Positive	23	0	16	0
Negative	308	181	241	327
Tobacco chewing/smoking habit				
Yes	20	10	31	12
No	311	171	226	315

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conditions consisted of: denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 66°C for 30 seconds, polymerization at 72°C for 20 seconds, and a final stage polymerization at 72°C for 7 minutes. The products were digested with *HinfI* in a total volume of 10 μl, and the fragments were separated on a 3.0% agarose gel. The C>T substitution created a restriction site for *HinfI* that produced fragments of 225bp and 93bp upon restriction digestion. Representative samples of each genotype were sequenced by direct DNA sequencing to confirm genotyping results produced by RFLP.

Statistical analysis. Genotype data for control population was studied for fitness in the Hardy Weinberg Equilibrium (HWE). For this purpose, data was analyzed using calculator available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Chi-square analysis was done to compare the genotype data between cases and controls. Data were analyzed using the online statistical tool available at Vassar Stats online calculator http://faculty.vassar.edu/lowry/VassarStats.html. Significance was present if p values were less than 0.05.

Meta-analysis

677C>T in breast cancer has been studied in several ethnic groups, making it valuable to conduct a meta-analysis. We have used the Comprehensive Meta Analysis software (version 2) for this purpose.

Identification of studies. A thorough electronic search of the published literature was done in 'Google Scholar (scholar.google.co.in)', 'Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/)' and 'Sciencedirect (www.sciencedirect.com)' databases up to August 2014 as the publication date, using the following keywords: breast cancer, *MTHFR* 677C>T polymorphism, folate metabolism, and breast cancer in different combinations. Detailed information regarding data presentation, design and purpose of the study, method of genotyping, and inclusion and exclusion criteria of the subjects were collected. The authors were contacted by e-mail when published information was inadequate for inclusion in meta-analysis. Meta analyses published to date suggest a significant correlation of 677 C>T polymorphism with breast cancer. Most of these pooled data analysis lack quality control and sensitivity analysis. We have

undertaken a meta-analysis on published data in order to look into the association between 677 C>T polymorphism and breast cancer risk.

Inclusion and exclusion criteria. The inclusion criteria comprised of the following: i) Each study was an independent case-control study ii) The statistical methods and purpose of all the studies were similar iii) The given information was enough to calculate the odds ratio iv) SNP genotyping was done using standard genotyping techniques v) Patients were recruited in accordance with the standard diagnostic parameters. The exclusion criteria included: the raw data were unavailable in the article or the authors did not respond after three requests by e-mail.

Data extraction and statistical approach. The genotype data for *MTHFR* 677C>T polymorphism in relation to breast cancer risk were collected. The required information such as the first author, ethnicity of the study population, publication year, number of cases and controls, and the frequency of genotypes were gathered.

Statistical analysis. Meta-analysis was conducted using the Comprehensive Meta-Analysis (CMA) software (version 2), which allows data entry in various formats. The 'effect size' was considered an important criterion to design and interpret the results of meta-analysis that compared CC versus CT+TT genotypes in the dominant model and CC+CT versus TT in the recessive model. We chose the effect size calculated in the form of 'odds ratio' for data interpretation. To calculate heterogeneity quantitatively, Thompson and Higgins classification index, I^2, was taken into account, where a proposed range of 25%, 50%, and 75% is set that corresponds to low, medium, and high magnitudes of heterogeneity [19]. In the absence of heterogeneity, fixed effect model using the Mantel-Haenzel method was used for pooled data analysis, else the random effects model using the Der Simonian and Laird method was applied [19,20]. High resolution plot (Forest plot) was produced to estimate the pooled odds ratio and p value. The p values less than 0.05 were considered statistically significant. The results and the robustness of the methodology were checked by sensitivity analysis, whereby, studies using small sample size (<100) in either of the study groups were excluded followed by re-analysis of the data. Sensitivity analysis aims at identifying the studies that are sensitive enough to significantly bias the results of pooled analysis. A study may be sensitive due to a variety of reasons, such as the use of small sample size, large variation in the number of cases and controls analyzed and poor methods, of which the use of a small sample size is one of the main reasons. The presence of publication bias was assessed from the funnel plot of precision by log odds ratio method and statistically tested using Egger's regression test.

Results

Case—Control study

We have analyzed MTHFR 677 C>T polymorphism in 588 patients and 508 controls (Table 2). There was no significant difference in the distribution of genotypes between cases and controls in north Indian (P = 0.932) or south Indian (P = 0.865) groups (Table 2).

Table 2. Genotypes distribution between cases and controls.

Population	Cases			Controls		
	CC	CT	TT	CC	CT	TT
North Indian	229	89	13	127	48	6
South Indian	208	45	4	259	63	5
Total	437	134	17	386	111	11

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Statistical analysis using dominant, co-dominant, and recessive models also detected no significant association of c.677C>T polymorphism with breast cancer in north Indian or south Indian groups (Table 3).

Meta—Analysis

Literature search. A total of 141 studies were retrieved upon literature search. After removal of one duplicate [21], 140 records were screened for inclusion in the study. Seventy four of these were found to be relevant to our study on 677C>T substitution and breast cancer. Eight studies were excluded due to lack of direct relation to the 677C>T SNP and breast cancer [22, 23, 24, 25, 26, 27, 28, 29], while six others were excluded as they lacked information required for meta- analysis [30, 31, 32, 33, 34, 35]. Hence, a total of 60 studies [1, 3–7, 9–13, 36–84] following strict selection criteria were included in the meta-analysis. Along with our data from India, data for a total of 28031 cases and 31880 controls were included in the meta-analysis (Fig. 1). Genotype data for all the studies are tabulated in S1 Table.

Pooled analysis. The pooled data showed a low level of heterogeneity based on the Thompson and Higgins classification index ($P_{Heterogeneity}$ = 0.02, I^2 = 29.55). Meta-analysis suggested no significant association between c.677 C>T and breast cancer risk in the dominant model (Fixed effect model: OR = 0.97, P = 0.072; Random effects model: OR = 0.96, P = 0.084, Fig. 2) or the recessive model (Fixed effect model: OR = 1.05, P = 0.089; Random effects model: OR = 1.08, P = 0.067, figure not shown). In the sub-group analysis, only dominant model was adopted. In the Caucasian group, the data were homogeneous ($P_{heterogeneity}$ = 0.19, I^2 = 17.96), and both Fixed effect (odds ratio = 1.007, P = 0.808) and Random effects models (odds ratio = 1.009, P = 0.791) suggested a lack of association between the study polymorphism and the disease risk (Fig. 3). Similarly, the data for East Asians ($P_{heterogeneity}$ = 0.01, I^2 = 47.21) showed low level of heterogeneity, and no correlation between c.677 C>T substitution and breast cancer risk was evident in this group (Fixed effects model: OR = 0.974, P = 0.457 and Random effects model: OR = 0.933, P = 0.196) (Fig. 4).

Sensitivity analysis based on sample size. To identify sensitive studies affecting the results of meta-analysis, thirteen studies based on small sample size (<100) in either of the study groups were excluded [4, 10, 42, 44, 47, 49, 54, 59, 67–69, 74, 81]. Re-analysis of the data showed more homogeneity ($P_{Heterogeneity}$ = 0.06, I^2 = 25.58), but the substitution did not correlate with breast cancer (fixed effect model: odds ratio = 0.975, P = 0.142; random effects model: odds ratio = 0.970, P = 0.174).

Publication bias. The distribution of studies on the funnel plot was almost symmetrical, suggesting the absence of publication bias in the overall analysis (S1 Fig.). This was further confirmed by Egger's regression intercept test (P = 0.259). Similarly, a symmetrical distribution of studies on the funnel plot for the Caucasian population showed absence of bias that was

Table 3. Statistical comparison of genotypes distribution between cases and controls.

Comparisons	North Indian			South Indian			Pooled		
	OR	95%CI	P	OR	95%CI	P	OR	95%CI	P
CC vs. (CT+TT)	1.04	0.70–1.55	0.82	0.89	0.59–1.35	0.6	1.09	0.83–1.43	0.52
CC vs. CT	1.02	0.68–1.55	0.88	0.88	0.58–1.35	0.59	1.06	0.80–1.41	0.66
CC vs. TT	1.2	0.44–3.23	0.71	0.99	0.26–3.75	1	1.36	0.63–2.95	0.42
TT vs. CT	0.85	0.30–2.39	0.76	0.89	0.22–3.51	1	0.78	0.35–1.73	0.54
(CC+CT)vs. TT	1.19	0.44–3.19	0.72	1.01	0.27–3.83	1	1.34	0.62–2.89	0.44
CC vs. CT vs. TT	-	-	0.93	-	-	0.86	-	-	0.68

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PRISMA 2009 Flow Diagram
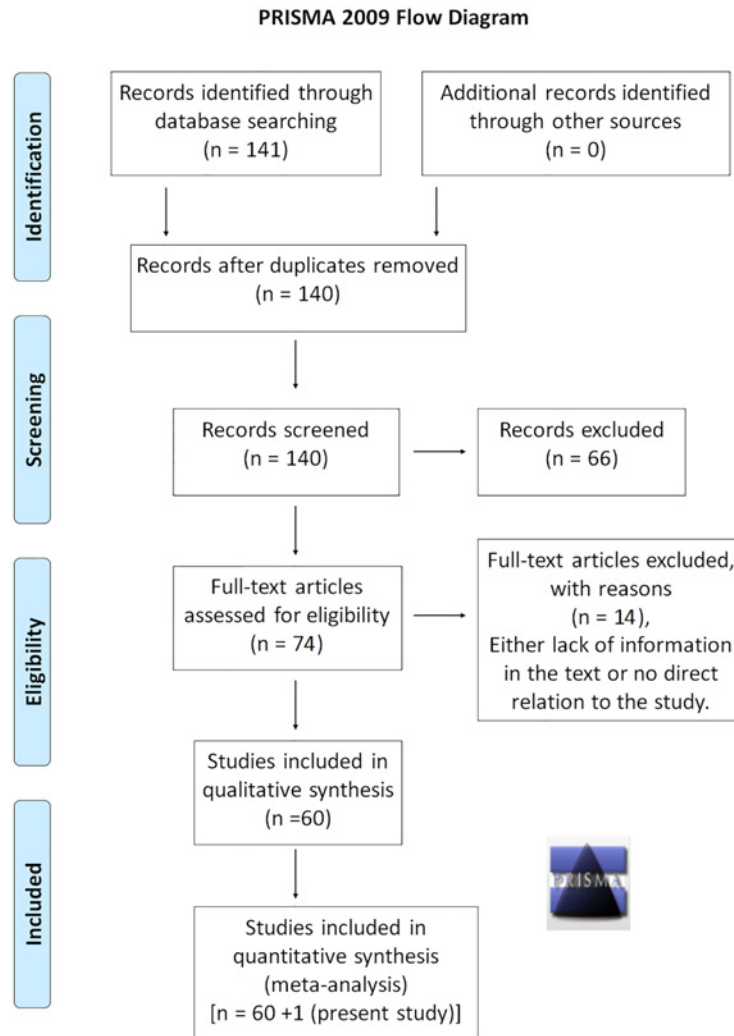
Fig 1. PRISMA flow diagram. The chart shows systematic review of the literature for inclusion/exclusion of the studies in meta-analysis.

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confirmed by Egger's regression intercept test (P = 0.555). But, the East Asian data also showed the presence of publication bias, confirmed by the Egger's regression intercept test (P = 0.017).

Discussion

In the present case-control study on 588 patients and 508 healthy controls, we found no association between *MTHFR* 677 C>T gene polymorphism and breast cancer amongst Indian women. Among other studies on Indian populations, Mir et al. showed that individuals carrying 677 C>T substitution had a 3.5 fold less risk of breast cancer (OR = 3.41, 95%CI = 3.1–3.7, P<0.02) in a north Indian Caucasian population [59]. On the other hand, Kalyankumar et al. (2006) and Prasad et al. (2011) reported a lack of association between MTHFR variants and the risk of breast cancer in south Indian populations [47, 75]. However, Naushad et al. (2010) suggested that the c.677C>T substitution is an independent risk factor for breast cancer in Indian women of Dravidian ethnicity (OR = 1.74, 95% CI = 1.11–2.73) [72]. The authors suggested that the risk is related to thermolabile MTHFR enzyme that has the tendency to lose its active

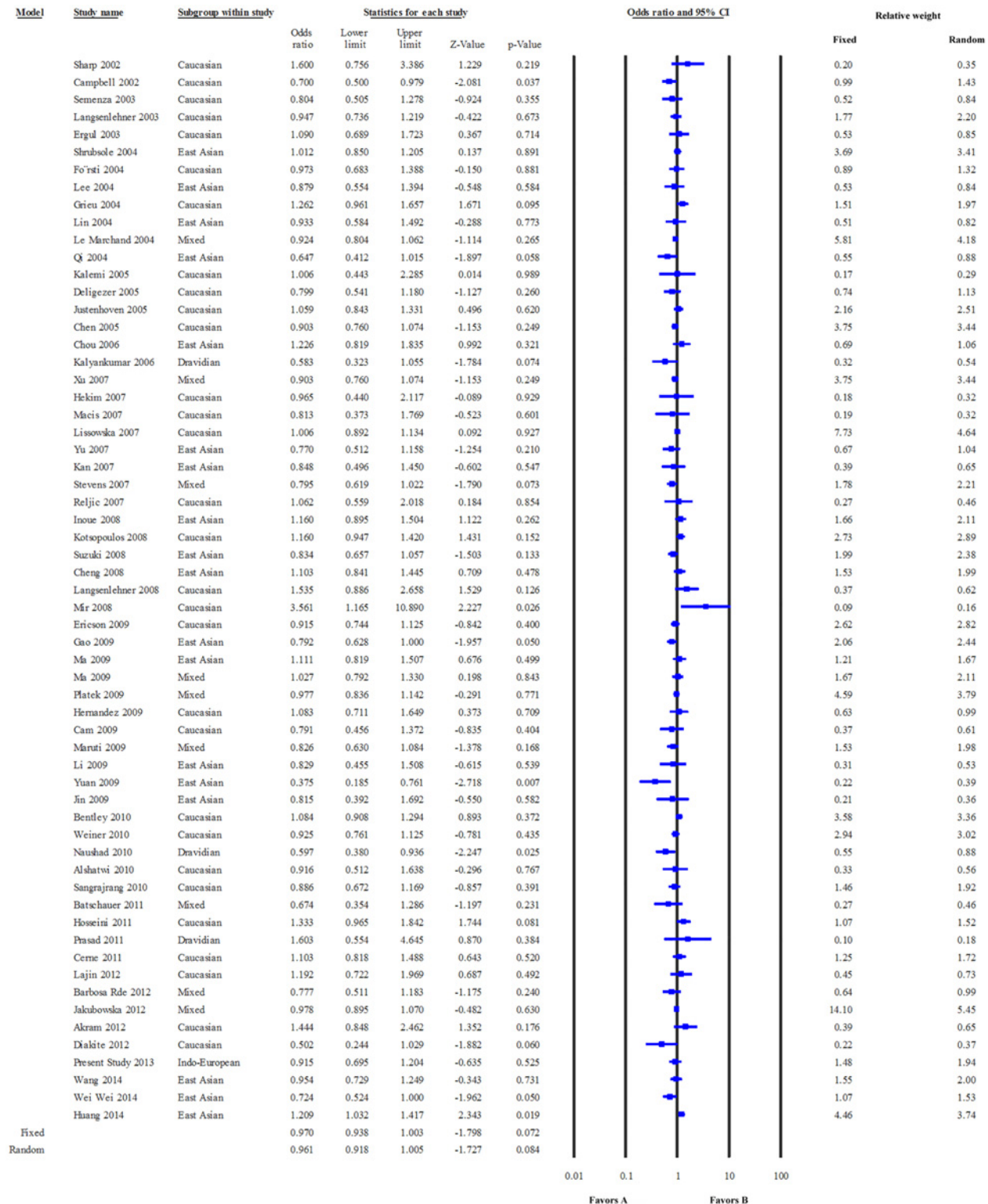| Model	Study name	Subgroup within study	Odds ratio	Lower limit	Upper limit	Z-Value	p-Value	Odds ratio and 95% CI	Fixed	Random
	Sharp 2002	Caucasian	1.600	0.756	3.386	1.229	0.219		0.20	0.35
	Campbell 2002	Caucasian	0.700	0.500	0.979	-2.081	0.037		0.99	1.43
	Semenza 2003	Caucasian	0.804	0.505	1.278	-0.924	0.355		0.52	0.84
	Langsenlehner 2003	Caucasian	0.947	0.736	1.219	-0.422	0.673		1.77	2.20
	Ergul 2003	Caucasian	1.090	0.689	1.723	0.367	0.714		0.53	0.85
	Shrubsole 2004	East Asian	1.012	0.850	1.205	0.137	0.891		3.69	3.41
	Fo`rsti 2004	Caucasian	0.973	0.683	1.388	-0.150	0.881		0.89	1.32
	Lee 2004	East Asian	0.879	0.554	1.394	-0.548	0.584		0.53	0.84
	Grieu 2004	Caucasian	1.262	0.961	1.657	1.671	0.095		1.51	1.97
	Lin 2004	East Asian	0.933	0.584	1.492	-0.288	0.773		0.51	0.82
	Le Marchand 2004	Mixed	0.924	0.804	1.062	-1.114	0.265		5.81	4.18
	Qi 2004	East Asian	0.647	0.412	1.015	-1.897	0.058		0.55	0.88
	Kalemi 2005	Caucasian	1.006	0.443	2.285	0.014	0.989		0.17	0.29
	Deligezer 2005	Caucasian	0.799	0.541	1.180	-1.127	0.260		0.74	1.13
	Justenhoven 2005	Caucasian	1.059	0.843	1.331	0.496	0.620		2.16	2.51
	Chen 2005	Caucasian	0.903	0.760	1.074	-1.153	0.249		3.75	3.44
	Chou 2006	East Asian	1.226	0.819	1.835	0.992	0.321		0.69	1.06
	Kalyankumar 2006	Dravidian	0.583	0.323	1.055	-1.784	0.074		0.32	0.54
	Xu 2007	Mixed	0.903	0.760	1.074	-1.153	0.249		3.75	3.44
	Hekim 2007	Caucasian	0.965	0.440	2.117	-0.089	0.929		0.18	0.32
	Macis 2007	Caucasian	0.813	0.373	1.769	-0.523	0.601		0.19	0.32
	Lissowska 2007	Caucasian	1.006	0.892	1.134	0.092	0.927		7.73	4.64
	Yu 2007	East Asian	0.770	0.512	1.158	-1.254	0.210		0.67	1.04
	Kan 2007	East Asian	0.848	0.496	1.450	-0.602	0.547		0.39	0.65
	Stevens 2007	Mixed	0.795	0.619	1.022	-1.790	0.073		1.78	2.21
	Reljic 2007	Caucasian	1.062	0.559	2.018	0.184	0.854		0.27	0.46
	Inoue 2008	East Asian	1.160	0.895	1.504	1.122	0.262		1.66	2.11
	Kotsopoulos 2008	Caucasian	1.160	0.947	1.420	1.431	0.152		2.73	2.89
	Suzuki 2008	East Asian	0.834	0.657	1.057	-1.503	0.133		1.99	2.38
	Cheng 2008	East Asian	1.103	0.841	1.445	0.709	0.478		1.53	1.99
	Langsenlehner 2008	Caucasian	1.535	0.886	2.658	1.529	0.126		0.37	0.62
	Mir 2008	Caucasian	3.561	1.165	10.890	2.227	0.026		0.09	0.16
	Ericson 2009	Caucasian	0.915	0.744	1.125	-0.842	0.400		2.62	2.82
	Gao 2009	East Asian	0.792	0.628	1.000	-1.957	0.050		2.06	2.44
	Ma 2009	East Asian	1.111	0.819	1.507	0.676	0.499		1.21	1.67
	Ma 2009	Mixed	1.027	0.792	1.330	0.198	0.843		1.67	2.11
	Platek 2009	Mixed	0.977	0.836	1.142	-0.291	0.771		4.59	3.79
	Hernandez 2009	Caucasian	1.083	0.711	1.649	0.373	0.709		0.63	0.99
	Cam 2009	Caucasian	0.791	0.456	1.372	-0.835	0.404		0.37	0.61
	Maruti 2009	Mixed	0.826	0.630	1.084	-1.378	0.168		1.53	1.98
	Li 2009	East Asian	0.829	0.455	1.508	-0.615	0.539		0.31	0.53
	Yuan 2009	East Asian	0.375	0.185	0.761	-2.718	0.007		0.22	0.39
	Jin 2009	East Asian	0.815	0.392	1.692	-0.550	0.582		0.21	0.36
	Bentley 2010	Caucasian	1.084	0.908	1.294	0.893	0.372		3.58	3.36
	Weiner 2010	Caucasian	0.925	0.761	1.125	-0.781	0.435		2.94	3.02
	Naushad 2010	Dravidian	0.597	0.380	0.936	-2.247	0.025		0.55	0.88
	Alshatwi 2010	Caucasian	0.916	0.512	1.638	-0.296	0.767		0.33	0.56
	Sangrajrang 2010	Caucasian	0.886	0.672	1.169	-0.857	0.391		1.46	1.92
	Batschauer 2011	Mixed	0.674	0.354	1.286	-1.197	0.231		0.27	0.46
	Hosseini 2011	Caucasian	1.333	0.965	1.842	1.744	0.081		1.07	1.52
	Prasad 2011	Dravidian	1.603	0.554	4.645	0.870	0.384		0.10	0.18
	Cerne 2011	Caucasian	1.103	0.818	1.488	0.643	0.520		1.25	1.72
	Lajin 2012	Caucasian	1.192	0.722	1.969	0.687	0.492		0.45	0.73
	Barbosa Rde 2012	Mixed	0.777	0.511	1.183	-1.175	0.240		0.64	0.99
	Jakubowska 2012	Mixed	0.978	0.895	1.070	-0.482	0.630		14.10	5.45
	Akram 2012	Caucasian	1.444	0.848	2.462	1.352	0.176		0.39	0.65
	Diakite 2012	Caucasian	0.502	0.244	1.029	-1.882	0.060		0.22	0.37
	Present Study 2013	Indo-European	0.915	0.695	1.204	-0.635	0.525		1.48	1.94
	Wang 2014	East Asian	0.954	0.729	1.249	-0.343	0.731		1.55	2.00
	Wei Wei 2014	East Asian	0.724	0.524	1.000	-1.962	0.050		1.07	1.53
	Huang 2014	East Asian	1.209	1.032	1.417	2.343	0.019		4.46	3.74
Fixed			0.970	0.938	1.003	-1.798	0.072			
Random			0.961	0.918	1.005	-1.727	0.084			

0.01   0.1   1   10   100

Favors A   Favors B

Fig 2. Meta-analysis. Forest plot on data pooled from all eligible studies. The Z value shows the degree and direction of relationship, wheras 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 projection of the horizontal bar shows the direction of association.

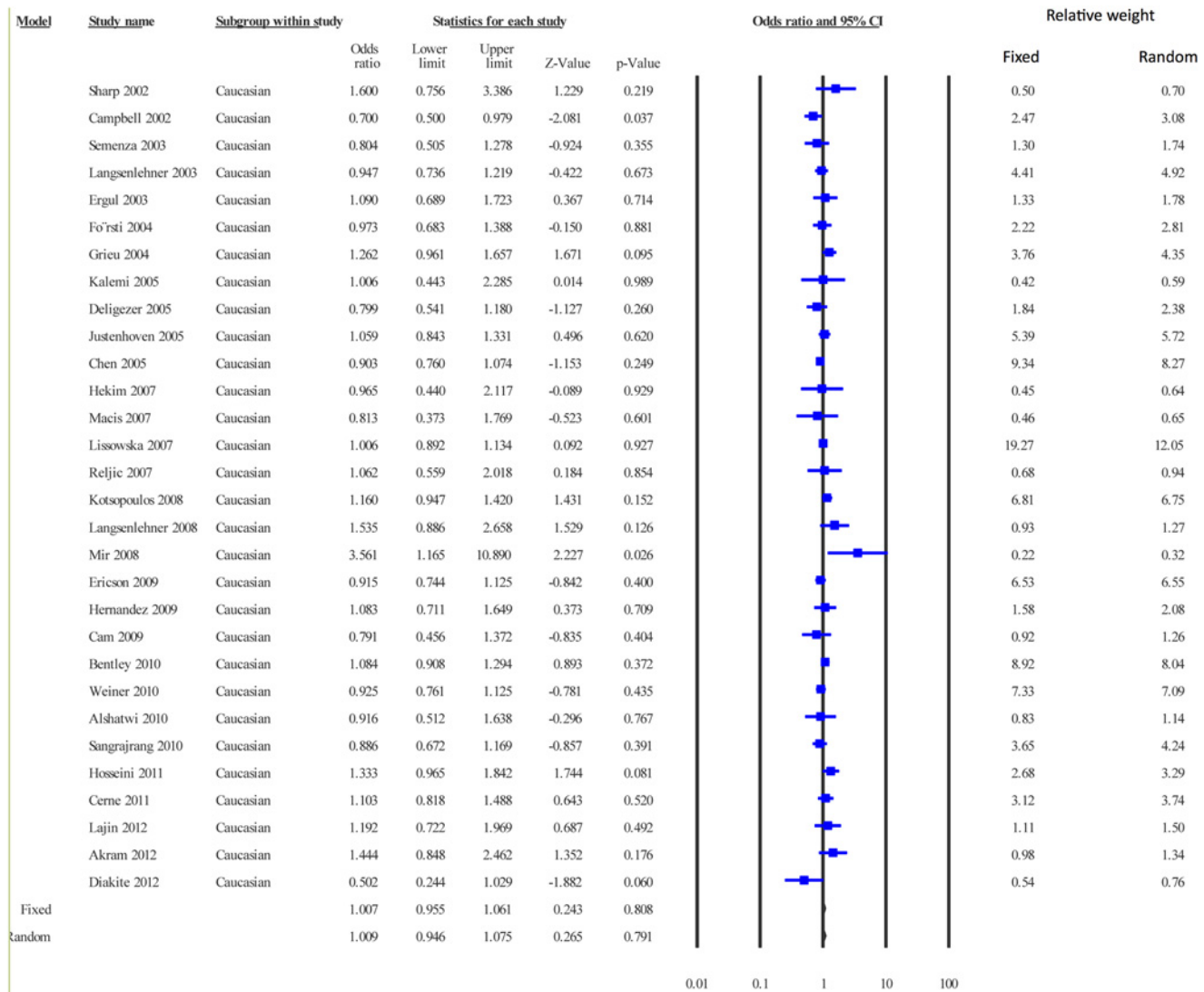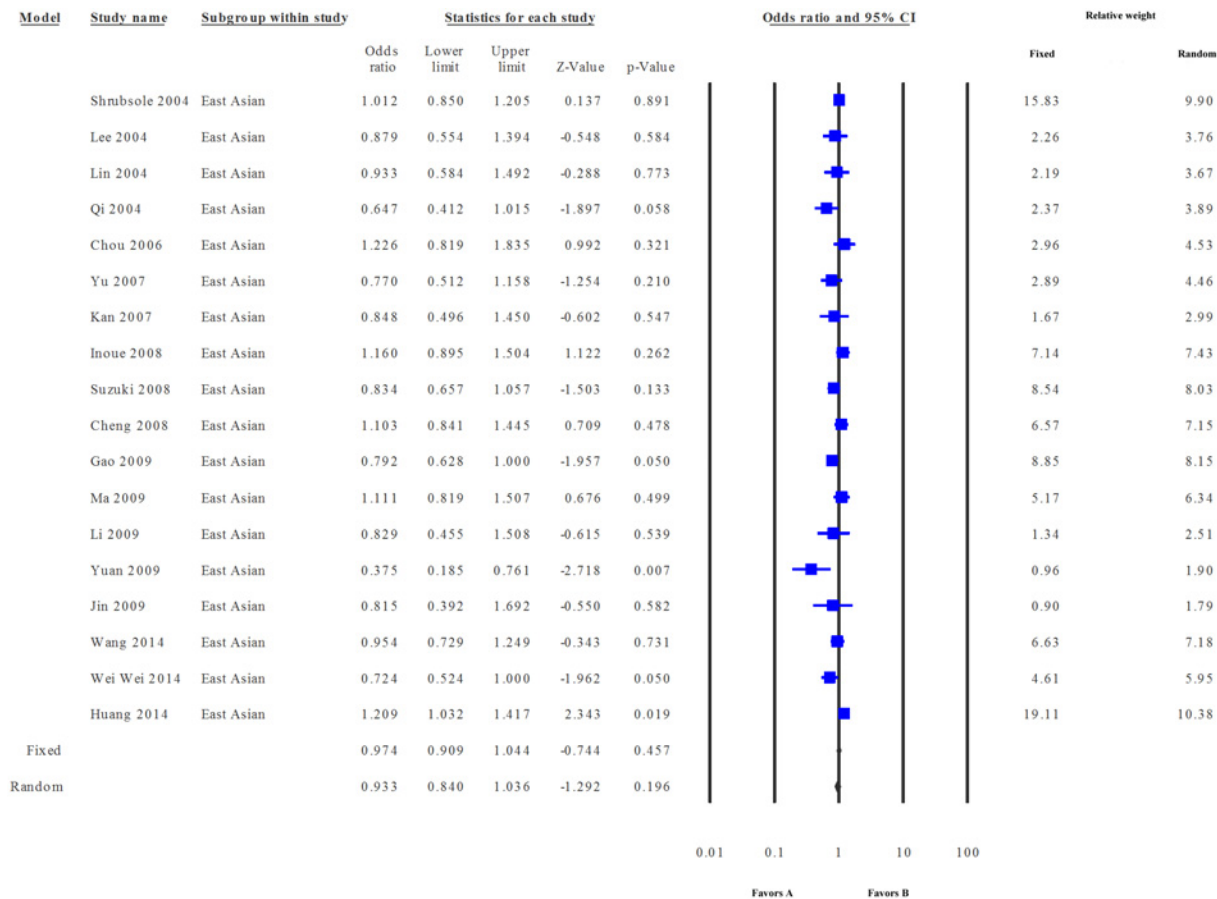**Fig 3. Meta-analysis.** Forest plot on data pooled from studies on Caucasian populations. All other parameters are as detailed in Fig. 2.

dimer form with a reduction in the FAD- binding capacity and loss in specific activity. The same contrast in the results of case-control studies is seen in other studies on diverse ethnicities; however, a relatively large number of studies support lack of association between 677 C>T substitution and breast cancer risk. Among studies on Chinese populations, only two out of 13 showed association of 677 C>T substitution with breast cancer risk, whereas all others stated no such correlation. Ten out of 30 studies on Caucasians reported an association between 677 C>T substitution and breast cancer risk, while others stated lack of such a correlation.

Meta-analysis is a powerful tool to reach consensus on heterogeneous data reported across studies. At-least seven meta-analyses have been conducted to pool genotype data in order to reach a consensus. However, interestingly, even meta-analysis on the relation of 677 C>T substitution with breast cancer has been equally heterogeneous with respect to the analysis models, stringency, and the outcomes. Zintzaras (2006) compared CC versus TT genotypes in a meta-analysis on `18 studies (5476 cases and 7336 controls) and found that 677C>T polymorphism

Model	Study name	Subgroup within study	Statistics for each study					Odds ratio and 95% CI	Relative weight	
			Odds ratio	Lower limit	Upper limit	Z-Value	p-Value		Fixed	Random
	Shrubsole 2004	East Asian	1.012	0.850	1.205	0.137	0.891		15.83	9.90
	Lee 2004	East Asian	0.879	0.554	1.394	-0.548	0.584		2.26	3.76
	Lin 2004	East Asian	0.933	0.584	1.492	-0.288	0.773		2.19	3.67
	Qi 2004	East Asian	0.647	0.412	1.015	-1.897	0.058		2.37	3.89
	Chou 2006	East Asian	1.226	0.819	1.835	0.992	0.321		2.96	4.53
	Yu 2007	East Asian	0.770	0.512	1.158	-1.254	0.210		2.89	4.46
	Kan 2007	East Asian	0.848	0.496	1.450	-0.602	0.547		1.67	2.99
	Inoue 2008	East Asian	1.160	0.895	1.504	1.122	0.262		7.14	7.43
	Suzuki 2008	East Asian	0.834	0.657	1.057	-1.503	0.133		8.54	8.03
	Cheng 2008	East Asian	1.103	0.841	1.445	0.709	0.478		6.57	7.15
	Gao 2009	East Asian	0.792	0.628	1.000	-1.957	0.050		8.85	8.15
	Ma 2009	East Asian	1.111	0.819	1.507	0.676	0.499		5.17	6.34
	Li 2009	East Asian	0.829	0.455	1.508	-0.615	0.539		1.34	2.51
	Yuan 2009	East Asian	0.375	0.185	0.761	-2.718	0.007		0.96	1.90
	Jin 2009	East Asian	0.815	0.392	1.692	-0.550	0.582		0.90	1.79
	Wang 2014	East Asian	0.954	0.729	1.249	-0.343	0.731		6.63	7.18
	Wei Wei 2014	East Asian	0.724	0.524	1.000	-1.962	0.050		4.61	5.95
	Huang 2014	East Asian	1.209	1.032	1.417	2.343	0.019		19.11	10.38
Fixed			0.974	0.909	1.044	-0.744	0.457			
Random			0.933	0.840	1.036	-1.292	0.196			

0.01   0.1   1   10   100

Favors A   Favors B

Fig 4. Meta-analysis. Forest plot on data pooled from studies on East Asian populations. All other parameters are as detailed in Fig. 2.

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and breast cancer are very closely associated with each other in pre-menopausal women [15]. Macis et al. (2007) investigated the relationship by pooling data from 18 case-control studies and found that 677C>T is strongly associated with breast cancer in both dominant and recessive genetic models [10]. Supporting the conclusions further, Zhang et al. (2010), Qi et al. (2010), and Liang et al (2013) conducted meta-analyses on 37 studies (15260 cases and 20411 controls), 41 studies (16480 cases and 22388 controls), and 22 studies (6103 cases and 7913 controls), respectively, and reported significant association in comparison of CC versus TT and in recessive model [16–18]. Rai (2014) and Li et al (2014) conducted meta-analyses on 36 studies (8040 cases and 10008 controls) and 57 studies (25877 cases and 29781 controls), respectively, and found a significant association across all genetic models in Asian population [85, 86]. Interestingly, all the above described meta-analyses suggested that c.677C>T polymorphism is a risk factor for breast cancer.

We undertook a meta-analysis on data pooled from all eligible studies that fitted a strictly defined inclusion and exclusion criteria. The present meta-analysis pooled data for 28031 cases and 31880 controls from sixty one studies. Our results suggest that *MTHFR* 677C>T polymorphism is not associated with the risk of breast cancer in either dominant (P = 0.084) or recessive genetic model (P = 0.067). It must be appreciated that pooling small studies into the meta-analysis resolves the issues related to sample size, but the biasness introduced by inappropriate representation of population-wise genotypes ratio would fail to correct. Therefore, we

conducted a sensitivity analysis after excluding studies using a sample size smaller than 100 in either of the case/control groups. Interestingly, we failed to detect an association between 677 C>T substitution and breast cancer, suggesting robustness of the method used in pooled analysis. Lack of publication bias further suggests that the results have not been influenced by any missing study. Analysis ethnicity-wise was considered so as to uncover the association in a particular ethnic population. A majority of the published studies were conducted on Caucasian and East-Asian populations with a small number on other populations. Therefore, we undertook two sub-analysis on Caucasian and East-Asian data; however, lack of a correlation between 677 C>T substitution and breast cancer risk was consistent. It is interesting to note the reported association of this substitution with breast cancer in a number of case-control studies; however, it has not been suggested what could be the mechanism leading to cancer in relatively poor one carbon metabolism as the nutritional deficiencies have not been reported to directly raise cancer risk.

In conclusion, while a majority of the case-control studies deny an association between the 677 C>T polymorphism and breast cancer, meta-analyses till date have consistently supported existence of an association. Our stringent statistical approach and thorough sensitivity analyses have suggested that 677C>T does not affect breast cancer risk. About 50% of the studies pooled in this analysis had been undertaken on populations of Caucasian ethnicity and 30% on East Asian populations. Therefore, our results would be more relevant to the populations of these ethnicities and caution must be ensured while extrapolating them to other populations.

Supporting Information

S1 Table. Studies included in the meta-analysis. All the studies that were included in the meta-analysis have been listed with details of the observed genotypes.
(DOCX)

S1 Fig. Publication bias. Funnel plot of precision by log odds ratio. Each empty dot represents one study included in the analysis and each solid dot represents one imputed study.
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: SP JC DS NG Sandeep Kumar KT SR. Performed the experiments: SP JC DS AF NG SR. Analyzed the data: SP JC DS Sandeep Kumar SR. Contributed reagents/materials/analysis tools: SP JC DS AF Sandeep Kumar Surender Kumar KT SR. Wrote the paper: SP JC DS AF NG RK Sandeep Kumar Surender Kumar SR.

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