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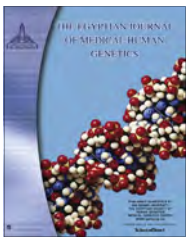

ORIGINAL ARTICLE

Do the MTHFR gene polymorphism and Down syndrome pregnancy association stands true? A case–control study of Indian population and meta-analysis

Srinivasan Muthuswamy, Sarita Agarwal *

Department of Medical Genetics, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, India

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KEYWORDS

MTHFR
gene polymorphism;
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Abstract *Background:* Down syndrome, the most common trisomy 21 arises from abnormal chromosomal segregation. The etiology includes genetic and acquired factors. The main genetic factor that is well appreciated for onset of Down syndrome pregnancy is *MTHFR* gene polymorphism. But till date, no final conclusion has arrived despite multiple studies on this gene polymorphism.

Aim: To investigate the risk of *MTHFR* gene polymorphisms, C677T and A1298C, with Down syndrome pregnancies and a meta-analysis of published literature.

Subjects and methodology: PCR-RFLP method was used to genotype C677T and A1298C polymorphism. For meta-analysis the literature was retrieved from PubMed database with the key words, *MTHFR* polymorphism; C677T; A1298C and Down syndrome.

Results: Mothers carrying C677T polymorphism had a risk of 2.48 times compared with control subjects while A1298C polymorphism carriers had 1.60 times and 2.12 times increased risk under assumption of dominant and recessive model. However, meta-analysis of published studies resulted in 1.26 times and 1.32 times increased risk of Down syndrome pregnancies among the C677T carries under the assumption of recessive and dominant models of inheritance. Considering A1298C polymorphism, dominant model predicated no risk; recessive model resulted in 1.34 times increased risk in CC genotype individuals. In subgroup analysis, Indian studies had a risk of 1.61 times and 1.44 times under recessive and dominant model of C677T polymorphism inheritance while A1298C polymorphism carriers had a risk of 1.75 and 1.46 under the assumption of recessive and dominant inheritance.

Conclusion: Our study suggests that both C677T and A1298C polymorphisms are significantly associated with the risk of DS pregnancy.

Abbreviations: DS, Down syndrome; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase remethylation of homocysteine to methionine; MTRR, methionine synthase reductase; CBS, cystathionine beta-synthase; QF-PCR, Quantitative fluorescent PCR; OR, odds ratio; HWE, Hardy Weinberg equilibrium

* Corresponding author.
E-mail address: saritasgpgi@gmail.com (S. Agarwal).

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1. Introduction

Down syndrome (DS) is the most common trisomy in live born with a prevalence of 1 in 1000 to 1 in 1100 (WHO report, http://www.who.int/genomics/public/geneticdiseases/en/index1.html). It is characterized by the presence and expression of three copies of chromosome 21. The extra copy arises from abnormal segregation during meiosis [1–3,22]. Meiotic nondisjunction is attributed to multifactorial etiology-genetic and acquired factors; well-studied etiologies were maternal age and genetic polymorphism of folate metabolic pathway. However, recent studies reported that DS children are born to mothers of age lesser than 25 years [4].

Thirty four years of extensive research into folate metabolism pathway has instanced its indispensable function in almost all cellular process, nucleotide synthesis, amino acid synthesis and synthesis of S-adenosyl methionine, DNA and lipid methylation reactions [5].

Folate metabolism is governed by multiple enzymes: methylenetetrahydrofolate reductase (MTHFR), converts 5,10-methylenetetrahydrofolate to 5 methylenetetrahydrofolate; methionine synthase (MTR), remethylation of homocysteine to methionine; methionine synthase reductase (MTRR), regenerates methionine synthase; cystathionine beta-synthase (CBS), transsulfuration of homocysteine to cystathionine; and finally, folate transporting protein (RFC-1), transporting 5-methyltetrahydroflate into cells [6–10].

Various clinical as well as experimental evidences have linked DNA hypomethylation to chromosomal instability and aneuploidy, such examples are ICF syndrome and tumors having DNA hypomethylation [2]. Genetic variants of these enzymes are reported to modulate folate pathway; *MTHFR*-C677T and A1298C polymorphisms remains as most commonly studied enzyme for their association with DS pregnancy [2–4,11–13,18,22–24,28,29].

In-vitro studies have demonstrated that C677T nucleotide transition modulates enzyme activity by approximately 50% and the enzyme becomes thermolabile. Still, the enzyme activity can be retained by folate supplement [14,15]. The second most common variant, A1298C (exon 7), point mutation causes substitution of alanine in place of glutamate. Upon mutation the activity of enzyme is reduced to 68% of the wild-type enzyme. Though, the enzyme is not thermolabile and does not appear to have higher serum homocysteine levels [16,17].

Yet, a clear reason for the association of *MTHFR* polymorphism with DS pregnancies has to be ascertained. Various individual studies have found an increased frequency of 677-TT genotype in mothers of DS children compared to controls along with elevated homocysteine and reduced methionine levels [2,18]. Provided this background, it was hypothesized that reduced MTHFR enzyme activity (C677T polymorphism) will result in DNA hypomethylation at pericentromeric region of chromosome. The hypomethylation might cause abnormal chromosomal segregation, predisposing to trisomy 21 [2]. Alternative hypothesis supports the survival advantage of CBS overexpression which counteracts the effect of *MTHFR* variant in fetus thereby maintaining minimal supply of folate for vital functions [18–21].

In light of the provided evidences linking *MTHFR* polymorphisms to the DS pregnancies, many researchers are testing the hypothesis that variability of the *MTHFR* gene sequence might be a general mechanism predisposing women to DS pregnancy [22–24,11–13,25,2,18]. However, the results in the publications are in support [45,36,24,25,2] and against this hypothesis [39,32,38,47,37]. Controversies may be due to relatively small number of participants in individual studies that is statically underpowered to detect the effect. Meta-analysis is a powerful tool for summarizing the results from multiple different studies by producing a single estimate of the major effect with enhanced precision. In the present study, therefore, we perform the current meta-analysis to examine whether the *MTHFR* polymorphisms are associated with DS pregnancies or not.

2. Subjects and methods

2.1. Study subjects

It was a hospital based case–control study conducted between 2012 and 2014. All the participants were of Indian origin. Case cohort includes 87 mothers of individuals with DS-ascertained by Quantitative fluorescent PCR (QF-PCR), while control cohort constitutes 110 mothers with no history of DS pregnancies. 2 ml of venous blood samples were collected on EDTA vial from all mothers and preserved at 4 °C till analysis.

Genomic DNA was isolated from the samples by standard phenol chloroform method; quality and quantity were assured by agarose gel electrophoresis and Nanodrop reading. The study was cleared by the institutional ethics committee. The work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Genotyping

MTHFR C677T genotyping [26] was performed by PCR amplification of target sequence of 203 bp in a reaction containing 50 ng genomic DNA, 10 pmol each of forward primer "5′-GCACTTGAAGGAGAAGGTGTC-3′" and reverse primer "5′-AGGACGGTGCGGTGAGAGTG-3′", 1.5 mM $MgCl_2$, 200 μM each dNTPs and 1 unit Taq Polymerase

Table 1 Frequency of MTHFR C677T and A1298C polymorphism in study subjects.

Genotype/Allele	Cases (n = 87)	Controls (n = 110)	Odds ratio (CI)
C677T Polymorphism (in %)			
CC	51.8	72.7	1
CT	48.2	27.3	2.48 (1.37–4.5)
TT	0	0	P = 0.0025
C	75.9	86.4	
T	24.1	13.6	
A1298C Polymorphism (in %)			
AA	36.8	48.2	1
AC	50.5	45.5	1.60 (0.89–2.83)*@
CC	12.7	6.3	2.12 (0.79–5.75)*#
A	62	71	
C	38	29	

* Statistically not significant.
@ AA Vs AC + CC.
# AA + AC Vs CC.

Table 2 Summary of included studies.

Study id	Population	Case/control no.	C677T polymorphism							A1298C polymorphism						
			Case			Control			HWE	Case			Control			HWE
			CC	CT	TT	CC	CT	TT		AA	AC	CC	AA	AC	CC	
Acácio [22]	Brazil	70/88	35	30	5	54	25	9	0.03	30	37	3	50	32	6	0.77
Biselli [23]	Brazil	72/194	29	35	8	100	77	17	0.69	40	27	5	108	74	12	0.88
Boduroğlu [24]	Turkey	152/91	86	55	11	58	30	3	0.71	44	97	11	21	60	10	0.00
Brandalize [31]	Brazil	239/197	94	113	32	86	93	18	0.31	143	84	12	113	76	8	0.27
Chango [32]	French	119/119	43	64	12	49	58	12	0.38	59	49	11	52	56	12	0.58
Coppede [33]	Italian	80/111	20	43	16	39	54	18	0.92	37	29	3	46	48	6	0.15
Coppede [34]	Italy	94/113	25	52	17	40	55	18	0.90	44	38	6	43	53	7	0.08
Coppede [35]	Italian	29/32	5	19	5	11	17	4	0.51	14	15	0	13	19	0	0.01
da [36]	Brazil	154/158	67	72	15	84	67	7	0.15	99	49	6	101	50	7	0.79
Hobbs [18]	Whites	157/144	51	84	22	67	59	14	0.84	0	0	0	0	0	0	0
James [2]		57/50	15	34	8	24	22	4	0.73	0	0	0	0	0	0	0
Kokotas [37]	Danish	177/1084	92	72	13	545	449	90	0.85	0	0	0	0	0	0	0
Martinez-Frias [38]	Spain	146/188	61	61	24	76	85	27	0.68	76	57	13	91	78	19	0.70
Meguid [25]	Egyption	42/48	20	17	5	33	12	3	0.21	8	20	14	18	29	1	0.00
O'leary [39]	Irish	48/192	18	21	2	90	84	18	0.80	0	0	0	0	0	0	0
Sadiq [40]	Jordan	53/29	23	27	3	23	5	1	0.31	10	18	1	24	29	0	0.00
Santos-Reboucas [41]	Brazil	103/108	51	43	9	49	47	12	0.88	58	40	5	57	49	2	0.01
Scala [42]	Italy	94/264	31	39	24	74	125	57	0.75	38	39	17	128	108	25	0.74
Stuppia [43]	Italy	64/112	20	32	12	27	62	23	0.25	0	0	0	0	0	0	0
Vranekovĭc [44]	Caucasian	111/141	49	49	13	66	64	11	0.40	48	56	7	63	68	10	0.14
Wang [45]	China	64/70	14	32	18	36	29	5	0.79	0	0	0	0	0	0	0
Zampieri [47]	Brazil	105/185	40	55	10	94	73	18	0.49	51	48	6	101	73	9	0.36
Indian studies																
Cyril [11]	India	36/60	33	3	0	60	0	0	0	14	19	3	26	21	13	0.03
Kaur [13]	India	110/111	86	22	2	89	22	0	0.24	0	0	0	0	0	0	0
Kohli [12]	India	104/109	74	29	0	71	32	6	0.35	0	0	0	0	0	0	0
Mohanty [45]	India	52/52	44	8	0	49	3	0	0.83	0	0	0	0	0	0	0
Rai [46]	India	89/70	60	23	6	54	16	0	0.28	28	39	22	28	37	5	0.12
Present Study	India	87/110	45	42	0	80	30	0	0.09	32	44	11	53	50	7	0.28

HWE-Hardy–Weinberg equilibrium of controls.

Table 3 Metaanalysis of C677T polymorphism and A1298C polymorphism.

Model	Population	Sample		P heterogeneity	Effect model	Test of association	
		Case	Control			OR (95% CI)	*P* value
C677T Polymorphism							
Recessive	Others	2222	3706	0.67	Random	1.25 (1.05–1.50)	0.01*
	Indian	477	512	0.04		1.61 (0.07–35.27)	0.76
	Overall	2699	4218	0.45		1.26 (1.05–1.50)	0.01*
Dominant	Others	2222	3706	0.006	Fixed	1.31 (1.17–1.46)	$< 1 \times 10^{-5}$*
	Indian	477	512	0.03		1.44 (1.07–1.95)	0.02
	Overall	2699	4218	0.002		1.32 (1.19–1.47)	$< 1 \times 10^{-5}$*
A1298C Polymorphism							
Recessive	Others	1406	1741	0.14	Fixed	1.25 (0.95–1.65)	0.12
	Indian	212	240	0.01		1.75 (1.0–3.07)	0.54
	Overall	1618	1981	0.02		1.34 (1.04–1.72)	0.02*
Dominant	Others	1622	2065	0.36	Random	1.00 (0.87–1.14)	0.99
	Indian	212	240	0.86		1.46 (0.99–2.14)	0.05*
	Overall	1834	2305	0.34		1.04 (0.92–1.18)	0.53

* Statistical significance.

(Bangalore Genei, India) in a total volume of 50 µl. PCR conditions were as follows: denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; the terminal elongation was performed at 72 °C for 5 mins. The PCR products were checked on 2% agarose gel followed by restriction digestion of 10 µl of PCR

product with 10 U of *HinfI* restriction enzyme (Newengland biolabs, UK) and checked on 4% agarose gel (Wildtype: 203 bp, Variant allele: 173 bp + 30 bp).

A1298C genotyping [27] was carried out with the set of forward "5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′" and reverse "5′-CAC TTT GTG ACC ATT CCG GTT TG-3′" primers. The reaction consisted of 50 ng genomic DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM each dNTP's and 1 U Taq polymerase (Bangalore Genei, India) in a total volume of 50 µl. PCR profile was similar to that of C677T polymorphism. Amplified products (163 bp) were checked on 2% agarose gel. The PCR products (10 µl) were restriction digested with 10 µ of MboII enzyme and checked on 4% agarose gel (Wildtype: 56, 31, 30, 28 and 18 bp, Variant allele: 84, 31, 30 and 18 bp).

2.3. Literature search

Studies published before April 1st 2014 were identified through a search on Pubmed and Web of Science databases using the keywords "MTHFR polymorphism" or "C677T" or "A1298C" and DS. All references cited in retrieved studies were also reviewed to identify additional literature that was not indexed in Pubmed.

2.4. Inclusion and exclusion criteria of literature

Studies included in the study met the following criteria: case–control study design, focused on association between *MTHFR* polymorphism (C677T or A1298C or both) and

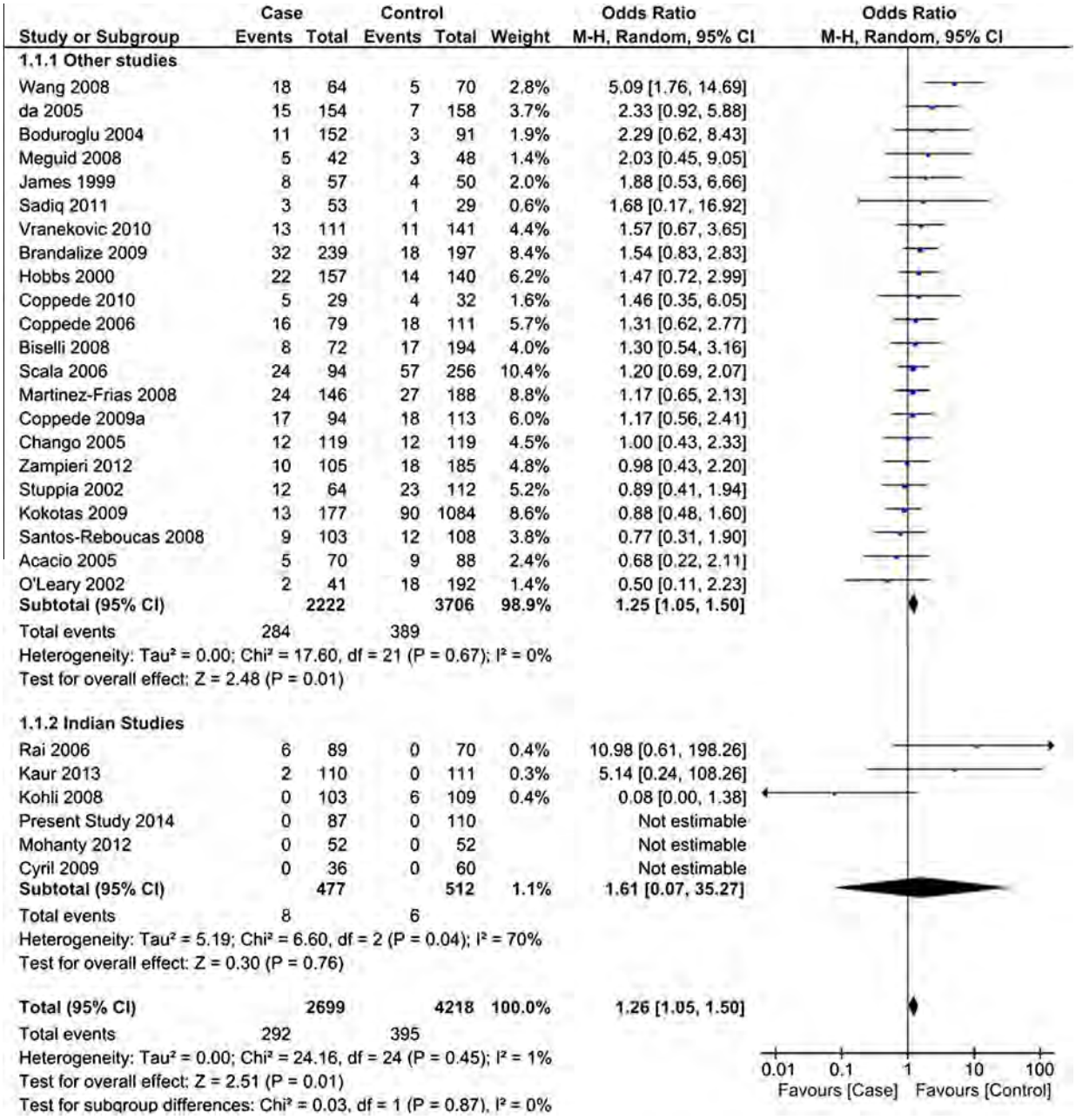

Figure 1 Results from meta-analysis of MTHFR C677T polymorphism under assumption of recessive model.

DS pregnancies, published data should contain genotype frequencies of polymorphism. The excluded studies were of non case–control study design, articles in language other than English, publications with insufficient data and the following literature type meta-analysis, review, editorial and commentary.

2.5. Data extraction

Two authors independently extracted data from the published literature using a standard format. Each study was looked for the first author, year of publication, country, study design, number of cases and controls, and genotype frequencies of studied polymorphism. Any disagreement in evaluation was resolved by discussion.

2.6. Statistical analysis

Initially Data were manually fed in Microsoft Excel followed by statistical analysis. The pooled ORs were performed for dominant model and recessive model. The potential for publication bias was tested by a Begg's test (funnel plot method) and heterogeneity assumption was evaluated by a chi-square based Q-test. All the above analysis was performed with

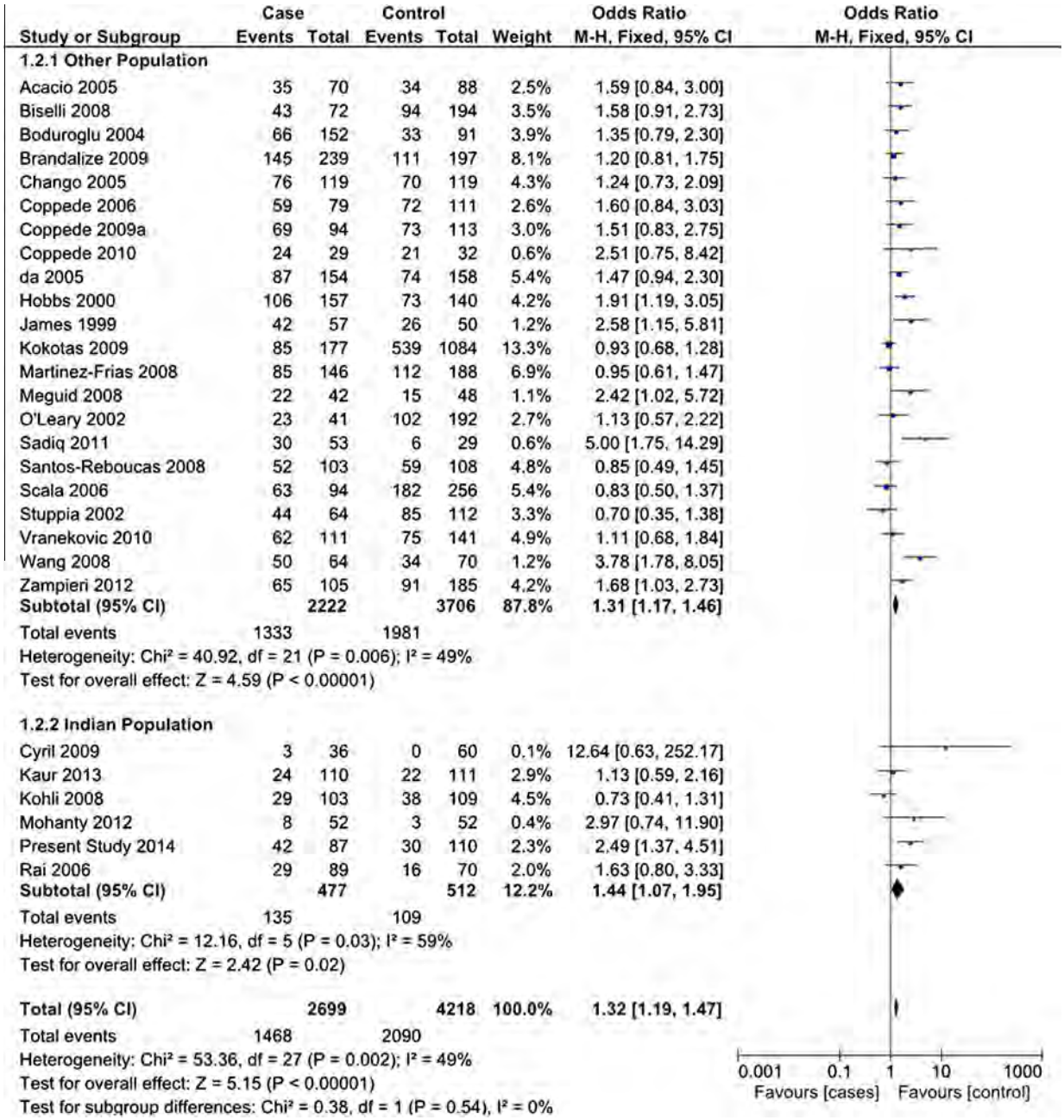

Figure 2 Results from meta-analysis of MTHFR C677T polymorphism under assumption of dominant model.

Review Manager 5.2 (Review Manager (RevMan) Version 5.2. Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2008). Departure from the Hardy–Weinberg equilibrium (HWE) for the C677T genotype distribution of the *MTHFR* gene both in the case and control groups was tested by chi square analysis with exact probability, and $p < 0.05$ was considered as departure from HWE. A subgroup analysis was carried out based on country of publication, India and other countries.

3. Results

3.1. Prevalence of MTHFR polymorphisms

The frequencies of the CC, CT, and TT genotypes among the cases were 51.8%, 48.2%, and 0%, respectively. The corresponding frequencies among the controls were 72.7% (CC), 27.2% (CT), and 0% (TT) (Table 1). These data indicated that the risk of having a child with DS was 2.48 fold higher in mothers with the 677C → T substitution than in mothers without the T substitution (OR 2.48 [1.37–4.5], $P = 0.0025$).

Concerning the frequencies of A1298C polymorphism variants, AA, AC and CC genotypes among the cases were 36.8%, 50.5% and 12.7%, respectively. In the control cohort it was found as 48.2% (AA), 45.5% (AC) and 6.3% (CC) (Table 1). Mothers who had a *MTHFR* AC or CC genotype had a 1.60-fold increased risk of having a child with DS compared with those who had AA genotype (OR 1.60 [0.89 to 2.83], $P = 0.11$). When the data were analyzed in the dominant model, mothers carrying CC genotype have a 2.12-fold, 0.5 increased risk compared to recessive model, increased risk compared with AA or AC genotype (OR 2.12 [0.79 to 5.78] $P = 0.13$).

The Hardy–Weinberg equilibrium analysis of the control cohort implied that the frequencies of C677T and A1298C genotype were randomly distributed ($P = 0.097$ and $P = 0.28$). In sum, these data indicate that the presence of either the 677C → T *MTHFR* polymorphism or A1298C polymorphism on one or both alleles increased the risk of having a child with DS.

4. Meta-analysis

4.1. Characteristics of studies

The literature search identified 88 potential relevant articles. Twenty-nine articles were excluded after initial screening. The remaining 59 studies were further reviewed by reading the full text, and 29 additional articles were excluded due to study design and study subjects. Finally out of 30 studies, 3 studies in Chinese language were excluded due to non-availability of literature in English version. Distribution of data in the included studies is shown in Table 2.

4.2. Quantitative data synthesis

4.2.1. C677T polymorphism

The estimated polled OR was 1.26 (95% CI: 1.05–1.50, $P = 0.01$) and 1.32 (95% CI: 1.19–1.47, $P = <1 \times 10^{-5}$) with respect to recessive and dominant model (Table 3). In subgroup analysis by source of study subjects, studies from other than Indian population showed a significant association with OR's of 1.25 (95% CI: 1.05–1.50, $P = 0.01$) in recessive model (Table 3). The dominant model based analysis showed association in both the subgroups (Other studies: 1.31 (95% CI: 1.17–1.46, $p = 0.01$) and Indian studies: 1.44 (95% CI: 1.07–1.95, $p = 0.02$) (Table 3, Figs. 1 and 2).

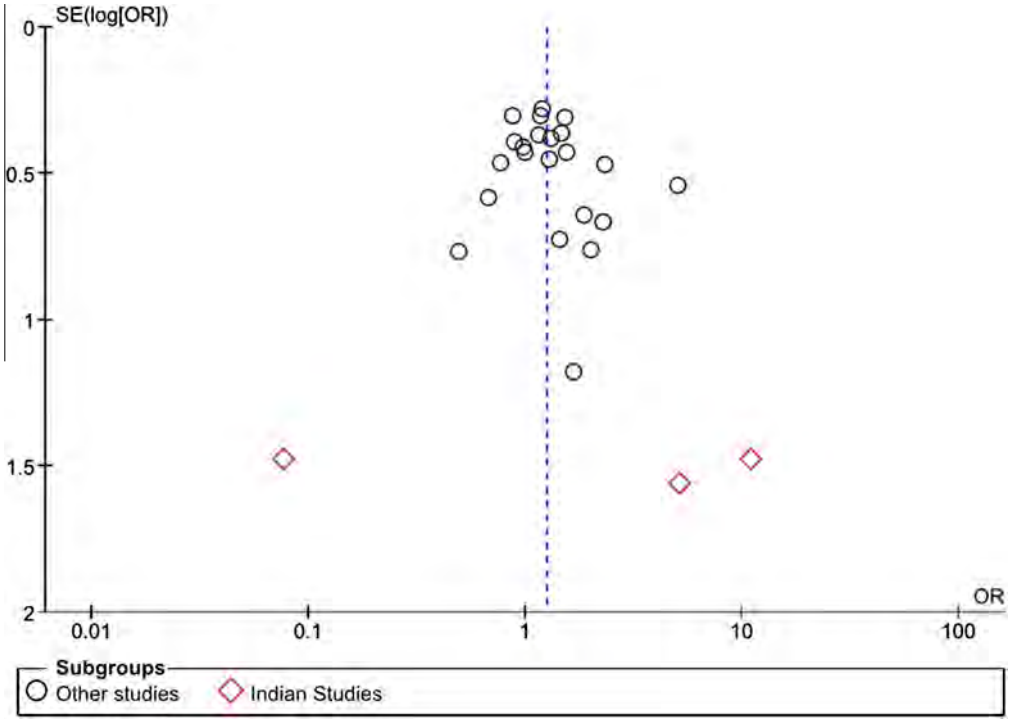

Figure 3 Funnel plot analysis for publication bias with studies related to C677T polymorphism.

We performed sensitivity analysis to evaluate the stability of the meta-analysis. The statistical significance of the result was not altered when a single study was omitted, which is not in HWE. The funnel plot revealed only a very mild asymmetry (Fig. 3).

4.2.2. A1298C polymorphism

Nineteen studies identified the association between A1298C polymorphism and DS pregnancy risk. A total of 1834 cases and 2305 controls were included in this meta-analysis. Under the assumption of recessive model, the pooled OR was 1.34 (95% CI: 1.04–1.72, $P = 0.02$) while assumption of dominant model resulted in no association (OR: 1.04 [0.92–1.18], $P = 0.53$). However, subgroup analysis of dominant model showed 1.46 fold increased risk of DS pregnancies among the Indian subjects with AC and CC genotype compared with AA genotype subjects (OR: 1.46 [0.99–2.14], $P = 0.05$). Similarly, in recessive model Indian population showed 1.75 times increased risk of DS pregnancy (OR: 1.75 [1.0–3.07], $P = 0.54$) (Table 3, Figs. 4 and 5).

In the sensitivity analysis, we found that there was no substantial modification of our estimate after exclusion of 6 studies whose data are not in HWE. The shape of the funnel plot did not reveal evidence of asymmetry (Fig. 6).

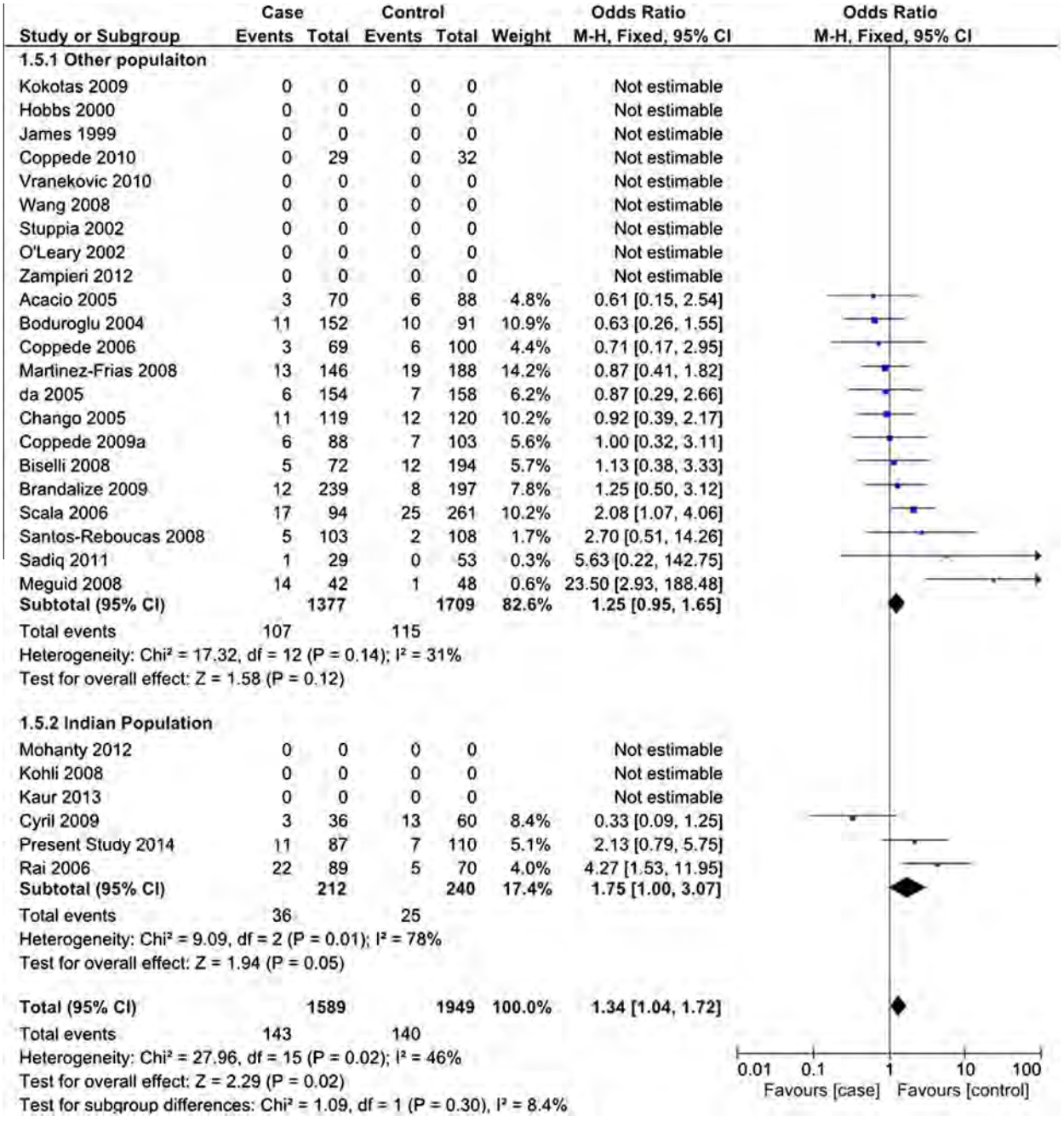

Figure 4 Results from meta-analysis of MTHFR A1298C polymorphism under assumption of recessive model.

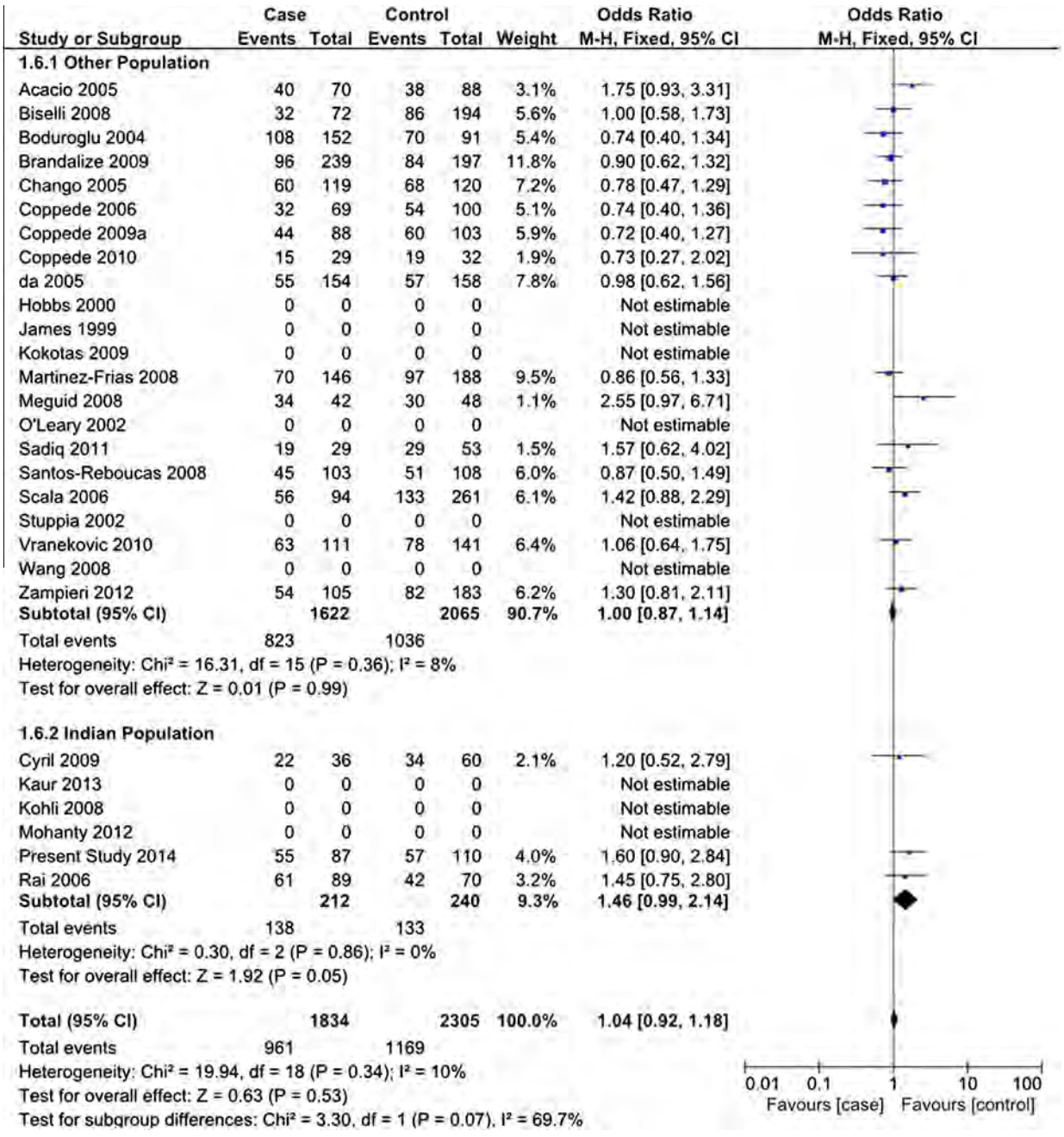

Figure 5 Results from meta-analysis of MTHFR A1298C polymorphism under assumption of dominant model.

5. Discussion

The present study examined the association of C677T and A1298C polymorphisms with DS pregnancies and a meta-analysis of all published literature. Since the sample size of most of the published studies was smaller, results from such individual study would result in false-negative or false-positive association. Therefore, meta-analysis will provide results based on a huge accumulated data to detect significant differences.

In cohorts of case (87) can controls (110) C677T and A1298C polymorphism were studied by PCR-RFLP method. It was observed that CT heterozygous genotype carrier mothers were at 2.48 fold risk of having DS child. Female's positive for CC genotype of A1298C polymorphism had a risk of 1.6 fold under the recessive model assumption but in the dominant model it was elevated to 2.12 fold risk of having DS child.

In the meta-analysis, the effect of C677T and A1298C polymorphisms was analyzed in assumption of both recessive and dominant models. In our results, the combined evidence suggests that C677T polymorphism is significantly associated with the risk of DS pregnancy. A1298C polymorphism showed a significant risk in recessive model while in the dominant model only India population had a risk with marginal significance similar to previous reports [28–30]. No association or marginal association of A1298C polymorphism in other populations as

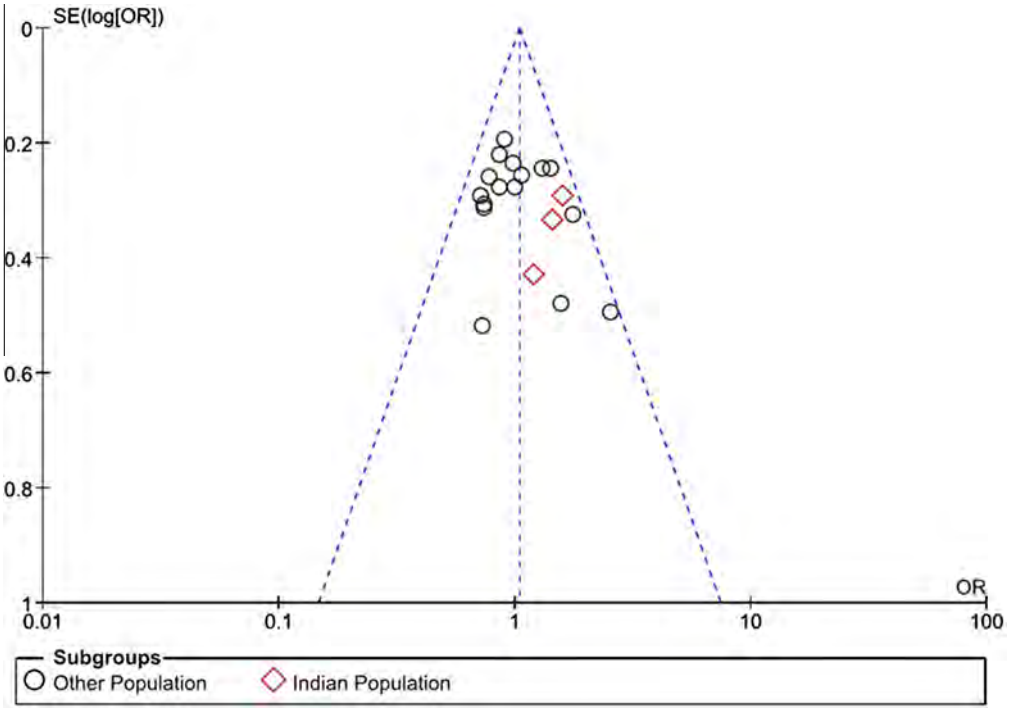

Figure 6 Funnel plot analysis for publication bias with studies related to A1298C polymorphism.

well as in Indian population can be speculated because of 68% retention of enzyme activity compared to 50% enzyme activity in C677T polymorphism [16,17]. Since we didn't measure folate levels in the present study, a conclusion can be derived after performing a large scale study with serum folate levels. Also, other confounding factors should be controlled.

Our meta-analysis has also showed significant interstudy heterogeneity in the dominant model of C677T polymorphism and the recessive model of A1298C polymorphism. Various confounding factors, such as population stratification, covariates and deviation from Hardy–Weinberg equilibrium, could be the source of heterogeneity. Therefore, we did sensitivity analysis by excluding the studies that are not in HWE and did not find any contribution of such study in heterogeneity. The funnel plot analysis revealed no publication bias.

Some limitation in our meta-analysis should be mentioned: the effect of gene-gene or gene-environment interaction, other gene in folate metabolism like MTR, MTRR and RFC-1 were not analyzed [6–10]. Other factors like maternal age and folate supplement could also affect the pregnancy but these factors were not considered in our analysis [4]. Three studies of Chinese population were also eliminated due to the non-availability of the literature in English. Taken together, our study suggested that both C677T and A1298C polymorphisms were significantly associated with the risk of DS pregnancy.

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Declaration of interest

The authors report no declaration of interest.

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Further reading

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