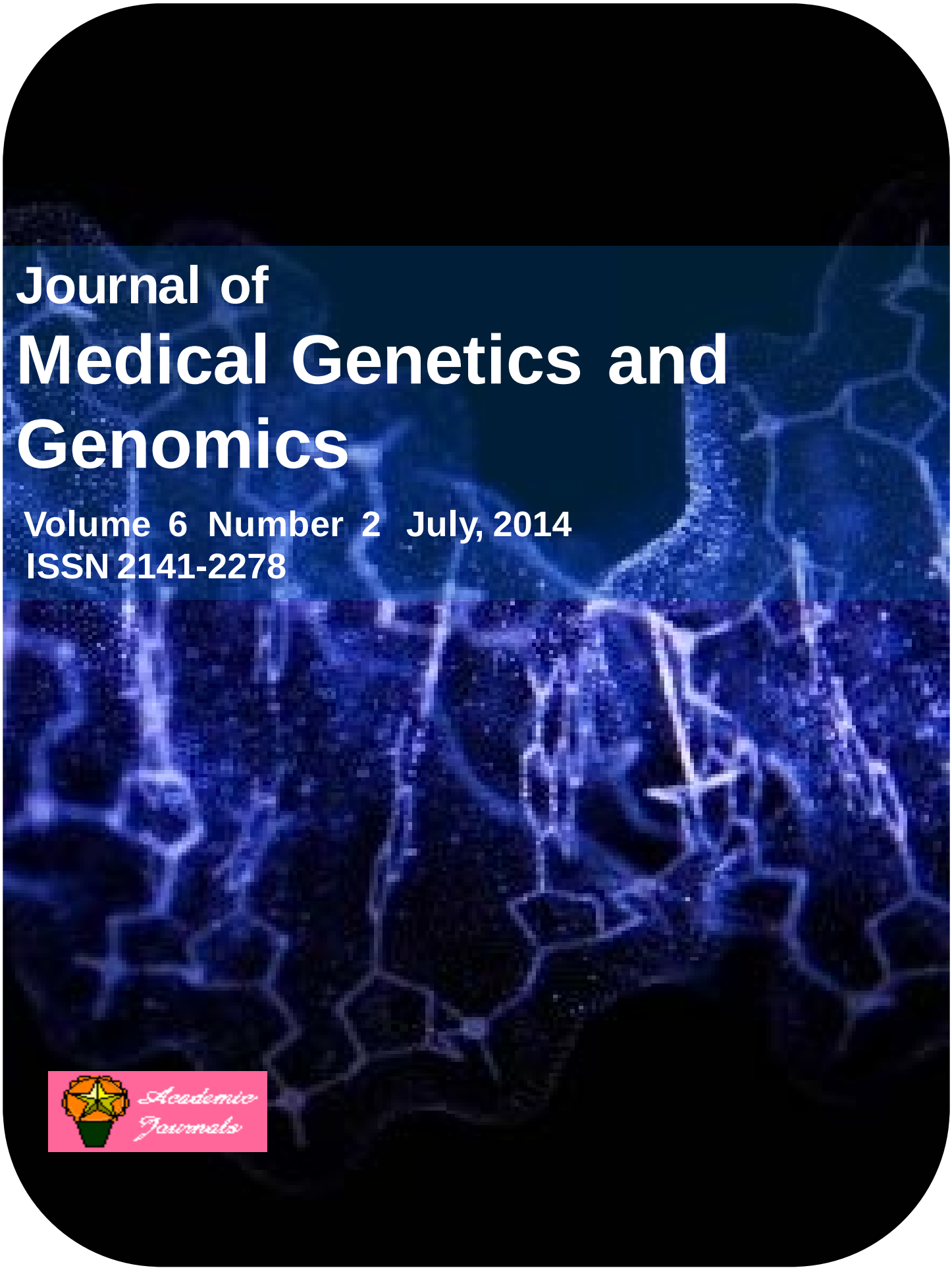

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Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Journal of Medical Genetics and Genomics

Table of Contents: Volume 6 Number 2 July 2014

ARTICLES

Research Article

0
.
9
2
2

0
.
8
5
2

0
.
6
2
8

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Full Length Research Paper

Meta-analysis of methylenetetrahydrofolate reductase (MTHFR) A1298C polymorphism and risk of orofacial cleft

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Polymorphisms in key genes involving the folate pathway have been reported to be associated with the risk of orofacial cleft (OFC) and several studies were published with conflicting results. A meta-analysis of the previous studies of allelic association between OFC with A1298C polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) gene was carried out. Odds ratios (ORs) with 95% confidence intervals (CIs) were estimated to assess the association between MTHFR A1298C polymorphism and OFC risk. A total of 11 studies including 1628 cases and 2676 controls were involved in this meta-analysis. No statistical relationship was found with any genetic model (C vs. A (Additive): OR = 1.14, 95%CI = 0.76-1.65, P = 0.47; CC vs. AA (homozygote): OR = 0.90, 95%CI = 0.72-1.15, P = 0.0.41; AC vs. AA (co-dominant): OR = 0.97, 95%CI = 0.85-1.11, P = 0.0.63; CC+AC vs. AA (Dominant): OR = 0.96, 95%CI = 0.84-1.1 , P = 0.51; CC vs. AC+AA (Recessive): OR = 0.93, 95%CI = 0.74-1.16, P = 0.52). The present meta-analysis supports that the common A1298C polymorphism of MTHFR gene is not risk factor for OFC.

Key words: Orofacial cleft, cleft lip, cleft palate, methylenetetrahydrofolate reductase (MTHFR), A1298C, folic acid.

INTRODUCTION

Approximately 90% of craniofacial congenital abnormalities comprised orofacial cleft (OFC) or cleft lip and/or palate (CL/P). According to the world health organization (WHO) data, the frequency of this pathology in the world is 0.6 to 1.6 cases per 1000 newborns (Shaw et al., 2001; Chorna et al., 2011). Prevalence rate varies according to geographical origin, sex, racial background, ethnicity, and socio-economic status (Vanderas et al., 1987; Croen et al., 1998; Clark et al., 2003; Brito et al., 2011; Aslar et al., 2013). Prenatal folic acid supplementation to pregnant women has been shown to reduce the incidence of CL in many (van Rooij et al., 2004; Badovinac et al., 2007; Rouget et al., 2005; Yazdy et al., 2007; Wilcox et al., 2007), but not all (Ray et al., 2003) populations studied (Sozen et al., 2009). Several studies established that polymorphisms in genes implicated

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in folate metabolism may play a significant role in the OFC etiology. Among several genes that take part in folate metabolism, the methylenetetrahydrofolate reductase gene (MTHFR) has been the most frequently reported to be associated with OFC.

MTHFR (EC.1.5.1.20) is a key enzyme in folate and homocysteine metabolism and catalyzes the reduction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which provides the methyl group for the remethylation of homocysteine to methionine. Methionine is in turn converted to S-adenosylmethionine (SAM), the common methyl donor for the methylation processes of DNA, proteins, phospholipids and neurotransmitters (Finkelstein, 1990; Bailey and Gregory, 1999; Ozarda et al., 2009). The MTHFR gene is localized on chromosome 1p36.3 and two common and clinically important polymorphisms (C677T and A1298C) identified in the MTHFR gene (Frosst et al., 1995; Weisberg et al., 1998) are implicated in the development of OFC. MTHFR C677T polymorphism is very well studied but A1298C polymorphism is less explored. A1298C influences specific activity of the enzyme, homocysteine levels, and plasma folate concentration, but to a lesser extent than the C677T polymorphism does (Blount et al., 1997; Shen et al., 2005).

Substitution at nucleotide 1298 (exon 7) results in an amino acid substitution of glutamate for alanine at codon 429. A1298C (glutamate to alanine) polymorphism, has been associated with decreased enzyme activity (40%), although to a lesser extent than C677T (Weisberg et al., 1998). Those who have the AC or CC genotype present with a decreased ability to produce the methyl form of folate, which together with cobalamin, is essential for the remethylation of homocysteine to methionine. The resulting abnormality in folate metabolism and the resultant increase in homocysteine levels may be a direct cause of the observed teratogenicity, homocysteine itself may be toxic to the embryo or it may be an indicator of reduced availability of SAM for the methylation of DNA. Animal studies suggest that a decreased conversion of homocysteine to methionine could be a crucial step in causing neural tube defects. It has been shown that rat embryos in culture require methionine for neural tube closure (Mills et al., 1999).

A1298C allele frequency differs greatly in various ethnic groups of the world. The prevalence of the A1298C homozygote variant genotype ranges from 7 to 12% in White populations from North America and Europe. Lower frequencies have been reported in Hispanics (4 to 5%), Chinese (1 to 4%) and Asian populations (1 to 4%) (Botto and Yang, 2000; Rabein and Ulrich, 2003). A number of molecular epidemiological studies have been conducted to investigate the associations of the MTHFR A1298C polymorphism with OFC. However, the results remain conflicting rather than conclusive. Hence, a meta-analysis to derive a more precise estimation of this association is needed. In light of the above facts, a meta-analysis of all available studies relating the A1298C polymorphism of MTHFR gene to the risk of having cleft lip was conducted.

METHODOLOGY

Selection of studies

All studies that investigate the association of the A1298C polymorphism in the MTHFR gene with CLP published before October 2013 were considered in the meta-analysis. The studies were identified by extended computer-based searches of the PubMed, Google Scholar, Elsevier and Springer Link databases. As a search criterion, the following terms were used: 'MTHFR', 'orofacial cleft', 'OFC', 'cleft lip', 'cleft lip and palate', 'A1298C'.

The following inclusion criteria were used: (i) studies must have a case–control study, (ii) study must be published as full papers, (iii) authors must investigate patients with cleft lip and palate cases and healthy control subjects, (iv) authors must provide information on genotype frequencies of the MTHFR A1298C polymorphism or sufficient data for the calculation, (iv) studies with overlapping cases and/or controls, the largest study with extractable data was included. The major reasons for exclusion of studies were (1) only case studied, (2) review papers, editorial, letter to editor and (3) containing overlapping data.

Data extraction

Following information was extracted from each study: first author, journal, year of publication, racial descent of study population, demographics, matching, validity of the genotyping method, and the number of cases and controls for MTHFR A1298C. The frequencies of A and C alleles were calculated for cases and controls from the corresponding genotype distributions.

Meta-analysis

The meta-analysis examined the overall association of the C allele with the risk of OFC relative to the A allele, the additive model for C allele (C vs. A), the co-dominance model (AC vs. AA), the homozygote model for allele C (CC vs. AA), the dominant model for C allele (CC + AC vs. AA), and the recessive model for C allele (CC vs. AC + AA). All associations were indicated as odds ratios (OR) with the corresponding 95% confidence interval (CI). A pooled OR was estimated based on the individual ORs. Heterogeneity between studies was tested using the Q statistic (Cochran, 1954). Heterogeneity was considered statistically significant if $P<0.05$. Heterogeneity was quantified with the I^2 metric, which is independent of the number of studies in the meta-analysis (I^2<25%: no heterogeneity; I^2=25–50%: moderate heterogeneity; I^2=50–75%: large heterogeneity; I^2>75%: extreme heterogeneity (Higgins and Thompson, 2002). The pooled OR was estimated using fixed effects (Mantel and Haenszel, 1959) and random effects (Dersimonian and Laird, 1986) models (Whitehead, 2002). Random effects modeling assume a genuine diversity in the results of the studies, and it incorporates the calculations of between-study variability; it therefore tends to provide wider CIs (Zintzaras and Hadjigeorgiou, 2005).

Publication bias

An estimate of the potential publication bias was carried out by

Table 1. Characteristics of eleven studies included in the present meta-analysis.

Study	Country	Year	Case	Control	Reference
Tolarova et al.	Argentina	1998	108	103	Am J Hum Genet, 63:A27.
Grunert et al.,	Germany	2002	65	184	Mund Kiefer Gesichtschir 6:131– 133.
Shoteresuk et al.	Thailand	2003	109	202	J Med Genet, 40:e64.
van Roij et al.	The Netherlands	2003	94	115	Am J Epidemiol, 157:583–591.
Pezzetti et al.	Italy	2004	110	289	Hum Mutat, 24:104–105.
Mills et al.	Ireland	2008	407	1050	Birth Defects Research, 82:636–643.
Ali et al.	India	2009	323	214	Genetic Testing and Molecular Biomarkers, 13(3).
Sozen et al.	Venezuela	2009	179	138	J. Genet. Genomics, 36: 283-288.
Chorna et al.	Ukraine	2011	33	50	Cytology and Genetics, 45: 177–181
Semic-Jusufagic et al.	Turkey	2012	56	76	The Turkish Journal of Pediatrics, 54: 617-625
Kumari et al.	India	2013	467	469	J. Biosci., 38: 21–26.

funnel plot, in which the standard error (SE) of log (OR) of each study was plotted against its log (OR). An asymmetric plot suggested a possible publication bias. The funnel plot asymmetry was assessed by Egger's test, and P=0.05 was considered representative of statistically significant publication bias (Egger et al., 1997). All analyses were performed using the computer program MIX version 1.7 (Bax et al., 2006). A p value less than 0.05 was considered statistically significant, and all the p values were two sided.

RESULTS

Eligible studies

Eleven articles were found to be eligible for the inclusion in the present meta-analysis (Tolarova et al., 1998; Grunert et al., 2002; Shoteresuk et al., 2003; van Roij et al., 2003; Pezzetti et al., 2004; Mills et al., 2008; Ali et al., 2009; Sozen et al., 2009; Chorna et al., 2011; Semic-Jusufagic et al., 2012; Kumari et al., 2013). All these eleven studies were performed in different countries: Argentina (Tolarova et al., 1998), Germany (Grunert et al., 2002), India (Ali et al., 2009; Kumari et al., 2013), Ireland (Mills et al., 2008), Italy (Pezzetti et al., 2004), Netherlands (van Roij et al., 2003), Thailand (Shoteresuk et al., 2003), Ukraine (Chorna et al., 2011), and Venezuela (Sozen et al., 2009). In one study, Ali et al. (2009) reported only allele numbers (Table 1).

Statistical analysis

Overall, eleven studies provided 1628/2676 cases/controls for MTHFR A1298C. The frequencies of the genotypes MTHFR 1298AA and AC were the highest in both OFC cases and controls, and allele A was the most common. In all eleven studies, total cases were 1628 with AA (825), AC (668) and CC (135), and controls were 2676 with AA (1285), AC (1140), and CC (251) genotypes. In controls genotypes, percentage of AA, AC and CC were 48, 42.6, and 9.38%, respectively. In total cases, genotype percentage of AA, AC, and CC was 50.6, 41 and 8.3%, respectively. The genotype and allele distributions are as shown in Table 2. Only in four studies (Shoteresuk et al., 2003; van Roij et al., 2003; Semic-Jusufagic et al., 2012; Kumari et al., 2013), OR was above one and in other seven studies did not show any association between MTHFR A1298C polymorphism and OFC. The distribution of genotypes in the control groups were in Hardy-Weinberg equilibrium (HWE) in all studies. Lack of HWE indicates possible genotyping errors and/or population stratification (Zintzaras, 2007).

Allele contrast meta-analysis

The main results of this meta-analysis and the heterogeneity test were shown in Tables 3. Mutant allele (C vs. A) did not show significant association with OFC in both fixed effect (OR=1.06, 95% CI=0.96-1.16, P=0.25, P_{hetero}<0.0001, I^2=92.62%, P_{Pb}=0.60) and random effect (OR=1.14, 95% CI=0.79-1.65, P=0.47) models (Table 3 and Figure 1).

Genotype contrast meta-analysis

Overall, no significantly elevated cleft lip risk was detected in any genetic models when all studies were pooled into the meta-analysis. Homozygote model (CC vs. AA) did not show significant association with OFC in both fixed effect (OR=0.97, 95% CI=0.72-1.15, P=0.41, P_{hetero}=0.04, I^2=47.04%, P_{Pb}=0.57) and random effect (OR=0.88, 95% CI=0.59-1.28, P=0.49) models (Table 3 and Figure 2). Similarly dominant model (CC+AC vs. AA) also did not show any association between A1298C polymorphism and risk of OFC either with fixed effect (OR=0.96, 95% CI=0.84-1.08, P=0.51, P_{hetero}=0.13, I^2=34.65%, P_{Pb}=0.61) or random effect (OR=0.94, 95% CI=0.78-1.12, P=0.47) model. Meta-analysis result using

Table 2. The distributions of MTHFR A1298C genotypes and allele frequencies for CLP cases and controls.

Study ID	Genotype						Alleles			
	AA		AC		CC		A		C	
	Case	Control	Case	Control	Case	Control	Case	Control	Case	Control
Tolarova et al. (1998)	67	63	39	33	2	7	173	43	159	47
Grunert et al. (2002)	28	77	30	80	7	27	86	44	234	134
Shoteresuk et al. (2003)	55	108	48	80	6	14	158	60	296	108
van Roij et al. (2003)	48	61	34	43	12	11	130	58	165	65
Pezzetti et al. (2004)	56	95	46	151	8	43	158	62	341	237
Mills et al. (2008)	202	519	172	439	33	92	576	238	1477	623
Ali et al. (2009)	-	-	-	-	-	-	483	295	163	133
Sozen et al. (2009)	138	101	37	33	4	4	313	235	45	41
Chorna et al. (2011)	19	24	12	22	2	4	50	70	16	30
Semic-Jusufagic et al. (2012)	21	36	25	36	10	4	67	108	45	44
Kumari et al. (2013)	191	201	225	223	51	45	607	625	327	313

Table 3. Summary estimates for the odds ratio (OR) of MTHFR A1298C in various allele/genotype contrasts, the significance level (p value) of heterogeneity test (Q test), and the I^2 metric: overall analysis, subgroup analyses, and publication bias p-value (Egger test).

Genetic models	Fixed effect OR (95% CI), p	Random effect OR (95% CI), p	Heterogeneity p-value (Q test)	I^2 (%)	Publication Bias (p of Egger's test)
Allele contrast (C vs. A)	1.06 (0.96-1.16), 0.25	1.14 (79-1.65), 0.47	<0.0001	92.62	0.60
Co-dominant (AC vs. AA)	0.97 (0.85-1.107), 0.63	0.96 (0.83-1.11), 0.61	0.38	6.12	0.56
Homozygote (CC vs. AA)	0.90 (0.72-1.15), 0.41	0.88 (0.59-1.28), 0.49	0.04	47.04	0.57
Dominant (CC+AC vs. AA)	0.96 (0.84-1.08), 0.51	0.94 (0.78-1.12), 0.47	0.13	34.65	0.61
Recessive (AA+AC vs. CC)	0.93 (0.74-1.16), 0.52	0.91 (0.66-1.26), 0.58	0.14	33.64	0.59

co-dominant and recessive genetic models were also not significant: (AC vs. AA: OR=0.96, 95% CI=0.85-1.11, P=0.63, P_{hetero}=0.38, I^2=6.16%, P_{Pb}=0.56 and CC vs. AC+AA: OR=0.93, 95% CI=0.74-1.16, P=0.52, P_{hetero}=0.14, I^2=33.64%, P_{Pb}=0.59), the pooled ORs were performed using fixed-effect model.

Table 3 lists the main results of the meta-analysis.

Sensitivity analysis

Sensitivity analysis was performed by sequential omission of individual studies from various contrasts.

The associations of the A1298C polymorphism with cleft lip did not change during the sensitivity analysis.

Publication bias

Funnel plots using standard error and precision values for allele and genotypes using fixed effect model were generated (Figure 3). Symmetrical distribution of studies in the funnel plots suggests absence of publication bias. This is also supported by Beggs and Eggers test (Begg's

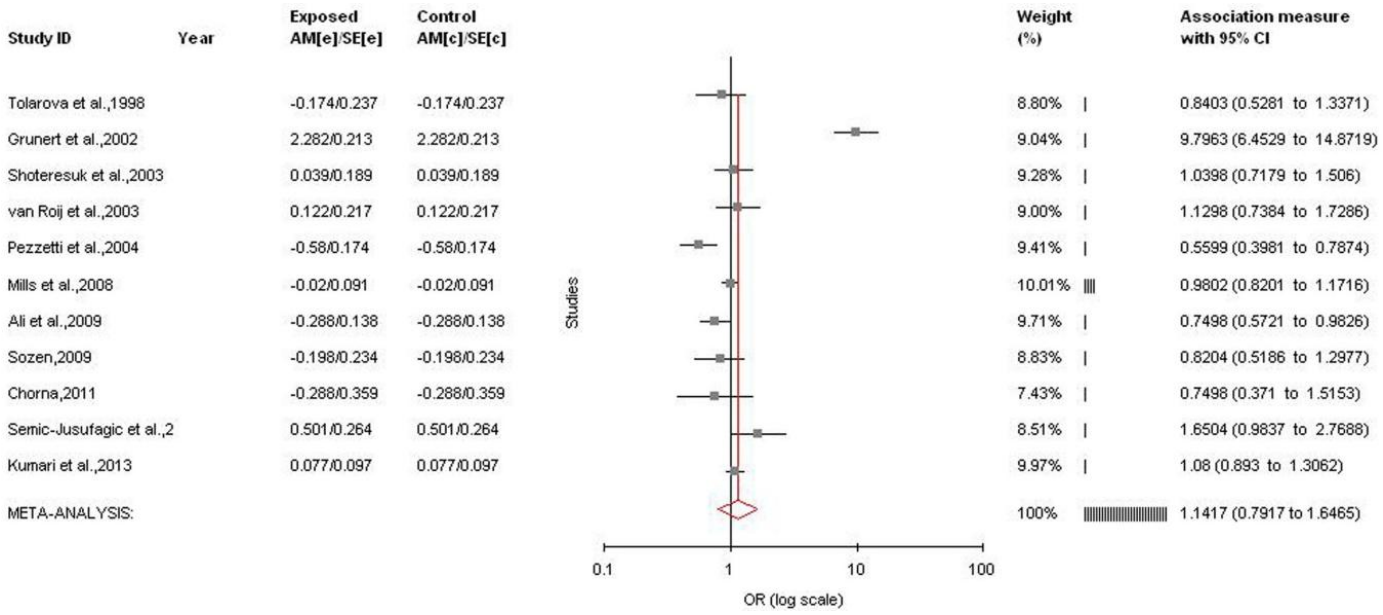

Figure 1. Forest plots for the association between MTHFR C677T polymorphism and orofacial cleft for additive model (C vs. A) with random effect model

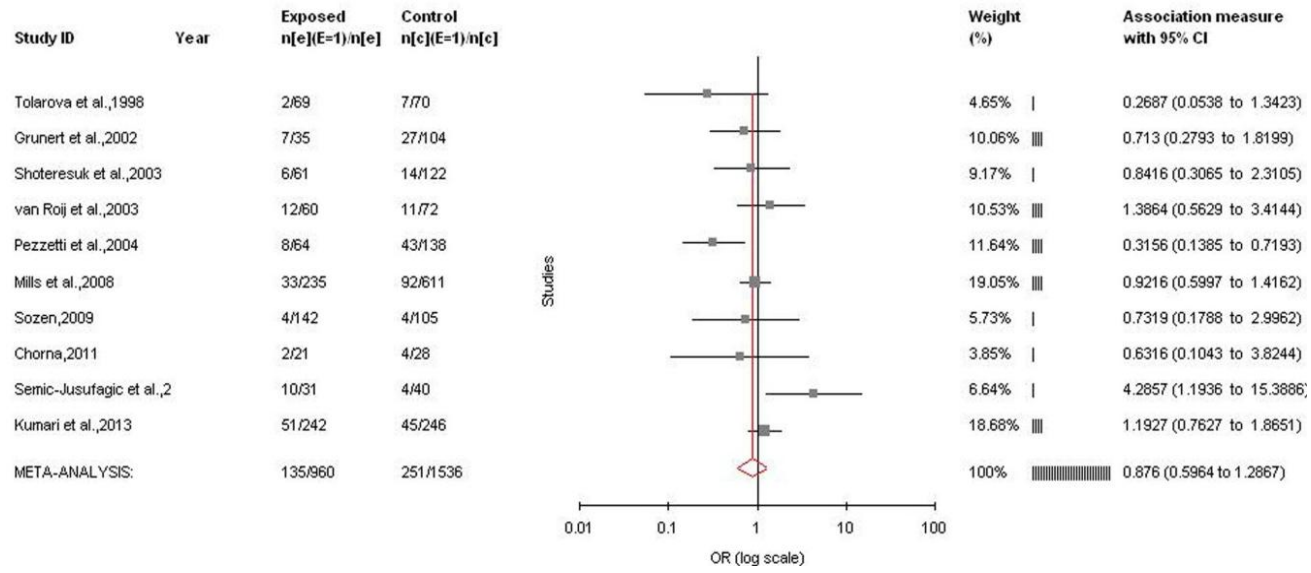

Figure 2. Forest plots for the association between MTHFR C677T polymorphism and orofacial cleft for homozygote model (CC vs. AA) with fixed effect model.

p=0.43, Egger's p=0.60 for C vs. A; Begg's p=0.37, Egger's p=0.57 for CC vs. AA; and Begg's p=0.72, Egger's p=0.56 for AC vs. AA; Begg's p=0.47, Egger's p=0.61 for CC+AC vs. AA; Begg's p=0.85, Egger's p=0.59 for CC vs. AC+AA) (Table 3).

DISCUSSION

Normal MTHFR activity is crucial to maintain the pool of circulating folate and methionine and to prevent the accumulation of homocysteine (Frosst et al., 1995). Homocysteine considered as a useful and important metabolic marker of the overall folate status. Folic acid derivatives provide essential single carbon units fro nucleic acid synthesis and methylation reactions both of which are essential for cell division, gene expression and maintenance of chromosome structure during fetal development. It is interesting to note that the case control

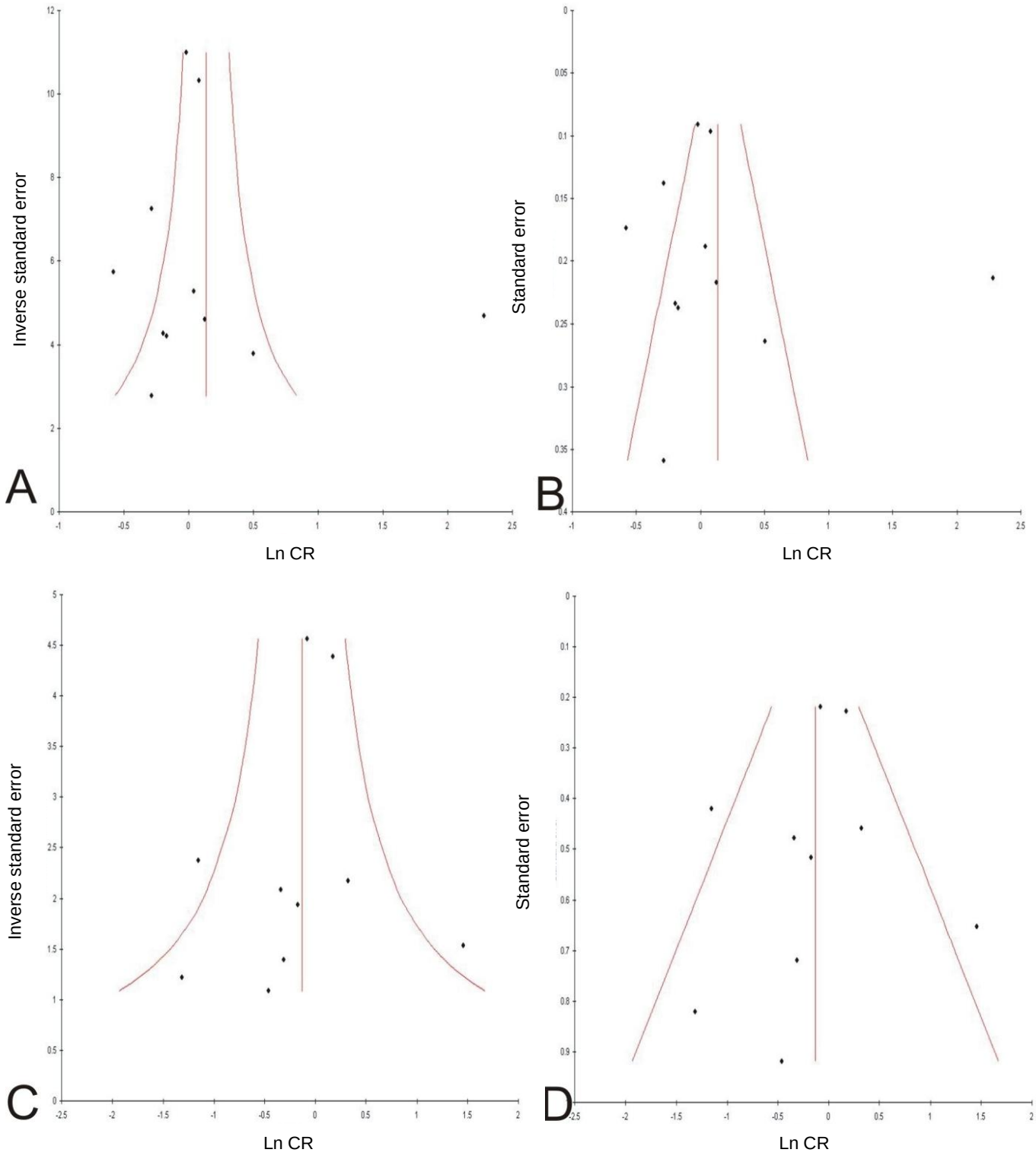

Figure 3. (A) Funnel plots A. precision versus OR (C vs. A), (B) standard error versus OR (C vs. A), (C) precision versus OR (CC vs. AA), (D) standard error versus OR (CC vs. AA).

studies have indicated an effect of the maternal MTHFR genotype rather than that of the affected child (Martinelli et al., 2001; Prescott et al., 2002; Pezzetti et al., 2004). The association of MTHFR polymorphisms with the

increased risk of OFC supports the protective effect of maternal use of multivitamins containing folic acid with respect to the occurrence of orofacial clefts (Bailey et al., 2005).

Several meta-analysis studies illustrate the utility of the technique in identifying genes of small effects like MTHFR with phenotypes like-NTD (Zhang et al., 2013); down syndrome (Zintzaras, 2007; Wu et al., 2013); cardiovascular disease (Xuan et al., 2011), stroke (Yadav et al., 2013); migraine (Shurks et al., 2010), Alzheimer's (Zhang et al., 2010), bipolar disorder (Rai, 2011), and depression (Zintzaras, 2006; Wu et al., 2013). Author identified one meta-analysis (Verkleij-Hagoort et al., 2007) published in 2007 concerning similar topic during the literature search. Verkleij-Hagoort performed a meta-analysis based on eight studies and find meager between MTHFR C677T polymorphisms and orofacial cleft (OR=1.01; 95% CI=0.87–1.16; I^2=0%). They investigated MTHFR C677T polymorphism and did not investigate A1298C polymorphism.

This study has some limitations and strength also. The main strength was the absence of publication bias and except additive model, low heterogeneity was observed. The insignificant and inconclusive result of the present meta-analysis may be due to (i) small number of studies (only eleven studies), (ii) small sample size, (iii) different ethnic backgrounds of the individuals included in the study, (iv) widely spread exclusion and inclusion criteria which might complicate the comparison between the studies.

In conclusion, result of the present meta-analysis demonstrated that MTHFR A1298C polymorphism did not show any association with CLP and is not a risk factor for oral facial cleft. Oral facial cleft has not been studied as extensively. Further research on facial cleft associations with this MTHFR polymorphism is needed.

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Conflict Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

Abbreviations

OFC, Orofacial cleft; **MTHFR,** methylenetetrahydrofolate reductase C677T ; **SAM S**, adenosylmethionine.

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