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Original Paper

Two Common MTHFR Gene Polymorphisms (C677T and A1298C) and Fetal Congenital Heart Disease Risk: An Updated Meta-Analysis with Trial Sequential Analysis

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Key Words
MTHFR • Polymorphism • Fetal congenital heart disease risk • Meta-analysis

Abstract
Background/Aims: Published studies indicated that the *MTHFR* gene polymorphisms C677T and A1298C are associated with congenital heart disease (CHD) risk in children, but obtained inconsistent results. Our study aims to reach a more accurate association between these two polymorphisms and CHD risk. ***Methods:*** Eligible studies were obtained by screening the PubMed, Embase, China National Knowledge Infrastructure, Wan Fang and VIP databases based on designed searching strategy. The odds ratio (OR) and 95% confidence interval (CI) were calculated. Moreover, a trial sequential analysis was introduced to confirm the positive results and an RNA secondary structure analysis was also applied to discover the potential molecular mechanism. ***Results:*** Based on thirty-two published articles, involving 6988 congenital heart disease subjects and 7579 healthy controls, the pooled results from the C677T polymorphism in the fetal population showed increased risks in allelic model (OR=1.32, 95%CI=1.14-1.53), recessive model (OR=1.69, 95%CI=1.25-2.30), dominant model (OR=1.35, 95%CI=1.11-1.64), heterozygote model (OR=1.20, 95%CI=1.01-1.41) and homozygote model (OR=1.75, 95%CI=1.31-2.33). An increased risk was only detected in the A1298C polymorphism in the overall fetal popalation in a recessive model (OR=1.42, 95%CI=1.10-1.84). In the subgroup stratified by region, sample size, genotyping method and source of controls, the increased risks were widely observed in both the C677T and A1298C polymorphisms with CHD risk. Furthermore, trial sequential analysis confirmed our positive results, and the RNA secondary structure analysis detected the changes in the RNA secondary structure caused by the mutant 677T allele and 1298C allele. ***Conclusion:*** In summary, we found that the *MTHFR* C677T polymorphism is associated with a significant increased risk in congenital heart disease

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in the fetal population. Moreover, an increased risk in the CC genotype of MTHFR A1298C polymorphism was observed, but the protective role of the 1298C allele needs further study.

Introduction

Congenital heart disease (CHD) is the most frequently occurring congenital disorder in newborns and the most common type of structural malformation of the heart and lager blood vessels [1, 2]. The aetiology of CHD is unclear and CHD is multifactorial in its derivation. Different related genes interacting with each other or with environmental factors may contribute to development of CHD [3]. Folate plays a crucial role in the ontogeny of the cardiovascular system [4]. Insufficient folic acid and a high level of homocysteine (Hcy) caused by a defective folic acid pathway are described as risk factors for CHD [5]. Therefore, common polymorphisms of folate-metabolizing enzymes have gained great attention.

The *MTHFR* gene, located on 1p36.3, encodes the vital enzyme involved in the folate/homocysteine metabolic pathway. Its transcription product is a 77 kDa protein, that catalyses the reduction of 5, 10-methylenetetrahydrofolate to 5-methytetrahydrofolate, which as a methyl donor induces Hcy remethylation to methionine [6]. Two common functional polymorphisms in the *MTHFR* gene are widely studied. The first one is the *MTHFR* C677T mutation at exon 4, which results in the conversion of the amino acid alanine to valine at position 226 in the protein [6]. The other mutation (*MTHFR* A1298C) is located at exon 7, within the presumptive regulatory domain, and results in a glutamate-to-alanine change with decreased enzyme activity *in vitro* [7].

Associations between these two *MTHFR* gene polymorphisms and CHD risk were firstly analyzed by Wenstrom et al. [8]., more and more studies were conducted to perfect this work in the recent years. However, previous case-control reports or meta-analyses have drawn inconsistent results and many biases exist in these studies. Therefore, we performed an updated meta-analysis to investigate the associations between *MTHFR* polymorphisms (C677T and A1298C) and the susceptibility to CHD. Moreover, a trial sequential analysis and a RNA secondary structure analysis were introduced in our meta-analysis to confirm our positive results and identify the potential possible mechanism respectively.

Materials and Methods

Based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) checklist [9], we organized our update meta-analysis. Ethical approval was not necessary for the type of the study (meta-analysis) [10].

Identification of related Studies

A literature search was conducted by the first two investigators in the PubMed, Embase, China National Knowledge Infrastructure, Wan Fang and VIP databases before August 2017 without a language limitation. The terms "*MTHFR*," "methylenetetrahydrofolate reductase," "congenital heart disease," "CHD," "ventricular septal defect," "atrial septal defect," "tetralogy of Fallot," "patent ductus arteriosus," "polymorphism," "variant," "mutant," and "polymorphisms" were used. The data that we failed to retrieve during the electronic search were obtained by reviewing the citations or contacting the corresponding author of the potential eligible articles.

Inclusion and Exclusion criteria

The included studies needed to meet the following inclusion criteria: (1) studies of the association between the *MTHFR* gene polymorphisms and congenital heart disease; (2) case-control study or cohort design in fetal population; and (3) detailed genotype data could be acquired to calculate the odds ratios (ORs), and 95% confidence intervals (CIs); The exclusion criteria were as follows: (1) duplication of previous publications; (2) comment, review and editorial; (3) study without detailed genotype data or

without a control group to conduct the Hardy-Weinberg equilibrium test; and (4) study departure from Hardy Weinberg equilibrium. The selection of the studies was achieved by two investigators independently. Any dispute was solved by a discussion with the corresponding author or group debates between all the authors.

Data Extraction

From each study, the following data were independently extracted by the first two investigators using a standardized form: first author's last name; year of publication; study country; region; genotyping methods; source of controls; number of cases and controls; genotype frequency in the cases and controls for the *MTHFR* gene; and results of the Hardy-Weinberg equilibrium test. Disagreements were resolved through a group discussion between authors.

Statistical analysis

The Hardy–Weinberg equilibrium (HWE) was evaluated for each study by a Chi-square test in the control group, and $P < 0.05$ was considered a significant departure from HWE. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated among five genetic models (allelic model (C677T: C versus T; A1298C: A versus C), recessive model (C677T: TT versus TC+CC; A1298C: CC versus CA+AA), dominant model (C677T: TT+TC versus CC; A1298C: CC+CA versus AA), heterozygote model (C677T: TC versus CC; A1298C: CA versus AA), and homozygote model (C677T: TT versus CC; A1298C: CC versus AA), respectively). Heterogeneity was evaluated by the Q statistic (significance level of $P < 0.1$) and I^2 statistic (greater than 50% as evidence of significant inconsistency). A sensitivity analysis was performed to detect the heterogeneity by omitting one study in each turn. Additionally, subgroup analyses were stratified by region, sample size, genotyping method and source of controls. The publication bias was assessed with a Begg's funnel plot and an Egg's test. Review Manager, Version 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration; Copenhagen, Denmark) and STATA 12.0 (STATA Corp, LP) was used for all the analyses. Multiple comparisons were adjusted by the Bonferroni method and the false discovery rate (FDR) was calculated [11]. The statistically significant level was determined by a Z-test with P value less than 0.05.

Trial sequential analysis (TSA)

TSA (The Copenhagen Trial Unit, Center for Clinical Intervention Research, Denmark) is a methodology that combines an information size calculation (cumulated sample sizes of all included trials) to reduce type I errors and type II errors for a meta-analysis with the threshold of statistical significance (http://www.ctu.dk/tsa). Therefore, we introduced TSA into our meta-analysis, and the required information size was calculated in adhere to an overall type I error of 5%, a power of 90% and a relative risk reduction (RRR) assumption of 10%.

RNA secondary structure analysis

The RNAfold WebServer is one of the core programs of the Vienna RNA package (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) [12], which can be used to predict secondary structures of single stranded RNA or RNA sequences by computing the minimum free energy (MFE) of single sequences based on the dynamic programming algorithm originally proposed by Zuker and Stiegler [13]. Therefore, we input the RNA sequence of the *MTHFR* C677T and A1298C polymorphisms into the RNAfold WebServer to analyse the potential secondary structure modification caused by the mutant allele.

Results

Characteristics of the Included Studies

One hundred and fifty-one articles were obtained by the online and manual search. After removing duplicates, screening the title and abstract and reading the full-text articles, forty-two articles were included in the qualitative synthesis, and then, nine articles were excluded for departure from the Hardy Weinberg Equilibrium. Finally, a total of thirty-three published articles [4, 14-45] involving 6988 cases and 7579 controls, were included in this meta-analysis (Fig. 1).

The characteristics of all the included articles are summarized in Table 1. For the C677T variant, thirty-two studies are included with 4848 cases and 5524 controls, and twelve studies with 2140 cases and 2055 controls, were included for the A1298C variant.

Results of the meta-analysis of the associations between MTHFR polymorphisms and congenital heart disease risk.

Table 2 shows the pooled results of this meta-analysis and the heterogeneity of the *MTHFR* gene polymorphisms and congenital heart disease risk.

For the C677T polymorphism, significant associations were observed in all the genetic models in the overall population but with high heterogeneity as follows: T versus C (OR=1.32, 95%CI=1.14-1.53, P=0.0003); TT+TC versus CC (OR=1.35, 95%CI=1.11-1.64, P=0.002); TT VS TC+CC (OR=1.69, 95%CI=1.25-2.30, P=0.000); TC VS CC (OR=1.20, 95%CI=1.01-1.41, P=0.03); and TT VS CC (OR=1.75, 95%CI=1.31-2.33, P=0.000) (Fig. 2). However, when an adjusted p value test was conducted, the heterozygote model (TC VS CC) was a false positive. In addition, for the A1298C polymorphism, a significant association was only found in the recessive genetic model in the overall population (CC versus CA+AA: OR=1.42, 95%CI=1.10-1.84, P=0.008) (Fig. 3).

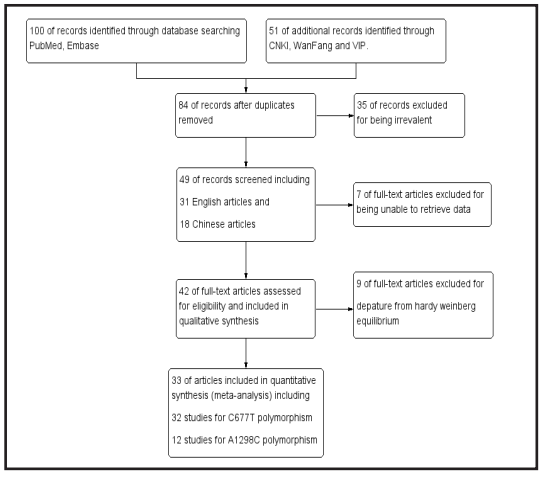

Fig. 1. Flowchart of literature search in our meta-analysis.

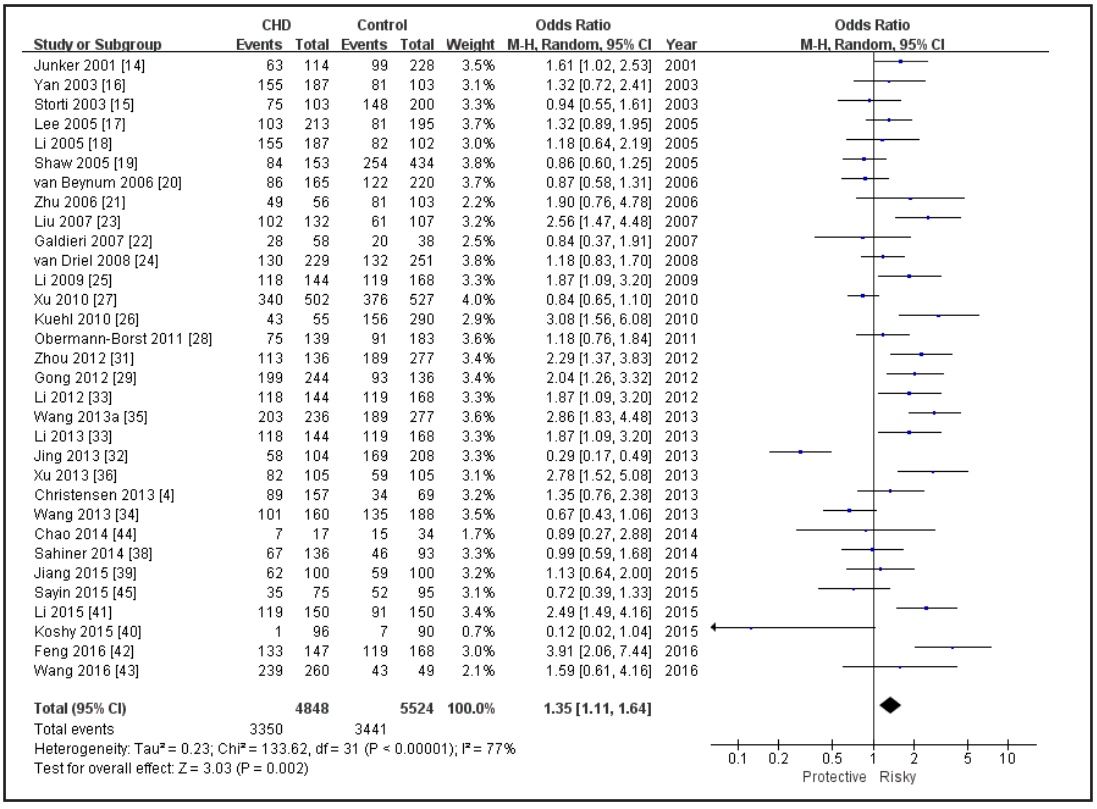

Fig. 2. Pooled analysis of C677T polymorphism and CHD risk in children population.

Table 1. Characteristic of included studies of MTHFR C677T and A1298C polymorphisms associated with congenital heart disease. HB=Hospital Based; PB=Population Based; PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism; PCR-TAQMAN = polymerase chain reaction with Taqman probe. ; PCR-ABD = polymerase chain reaction using Assay by Design (ABD) kits from Applied Biosystems (Carlsbad, CA, USA): PCR-MassaArray Assay= Polymerase Chain Reaction with MassArray Assay. PCR-SSCP = Polymerase Chain Reaction-Single Strand Conformation Polymorphism; PCR-SNaPShot = polymerse chain reaction with SNaPShot; PCR-GeXP = Polymerse Chain Reaction with GeXP. HWE = Hardy-Weinberg equilibrium; * P value for Hardy–Weinberg equilibrium test in controls

				Genotyping	Controls			Case			Control			
First author	Year	Country	Region	method	Source	case	control	CC	CT	TT	CC	CT	TT	HWE
MTHFR C677T Polymorphism														
Junker [14]	2001	Germany	Middle Europe	PCR-SSCP	HB	114	228	51	42	21	129	78	21	0.075
Yan [16]	2003	China	East Asian	PCR-RFLP	HB	187	103	32	97	58	22	57	24	0.277
Storti [15]	2003	Italy	South Europe	PCR-SSCP	HB	103	200	28	55	20	52	108	40	0.235
Shaw [19]	2005	American	North America	PCR-TAQMAN	PB	153	434	69	68	16	180	202	52	0.684
Li [18]	2005	China	East Asian	PCR-RFLP	HB	187	102	32	94	61	20	57	25	0.224
Lee [17]	2005	China	East Asian	PCR-SSCP	HB	213	195	110	89	14	114	68	13	0.513
Zhu [21]	2006	China	East Asian	PCR-RFLP	PB	56	103	7	22	27	22	57	24	0.277
van Beynum [20]	2006	Netherlands	North Europe	PCR-SSCP	PB	165	220	79	66	20	98	104	18	0.184
Liu [23]	2007	China	East Asian	PCR-RFLP	HB	132	107	30	68	34	46	48	13	0.930
Galdieri [22]	2007	Brazil	South America	PCR-SSCP	HB	58	38	30	21	7	18	14	6	0.263
van Driel [24]	2008	Netherlands	North Europe	PCR-TAQMAN	PB	229	251	99	103	27	119	107	25	0.895
Li [25]	2009	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Xu [27]	2010	China	East Asian	PCR-SSCP	HB	502	527	162	244	96	151	261	115	0.911
Kuehl [26]	2010	American	North America	PCR-TAQMAN	PB	55	290	12	33	10	134	124	32	0.682
Obermann-Borst [28]	2011	Netherlands	North Europe	PCR-TAQMAN	PB	139	183	64	66	9	92	76	15	0.900
Zhou [31]	2012	China	East Asian	PCR-SSCP	HB	136	277	23	60	53	88	126	63	0.168
Li [30]	2012	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Gong [29]	2012	China	East Asian	PCR-MassArray Assay	HB	244	136	45	123	76	43	72	21	0.309
Li [33]	2013	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Jing [32]	2013	China	East Asian	PCR-RFLP	HB	104	208	46	42	16	39	114	55	0.139
WangLN [35]	2013	China	East Asian	PCR-SSCP	HB	236	277	33	92	111	88	126	63	0.168
WangBJ [34]	2013	China	East Asian	PCR-SNaPShot	HB	160	188	59	76	25	53	100	35	0.312
Christensen [4]	2013	Canada	North America	PCR-RFLP	HB	157	69	68	61	28	35	26	8	0.360
Xu [36]	2013	China	East Asian	PCR-SSCP	HB	105	105	23	54	28	46	40	19	0.059
Sahiner [38]	2014	Turkey	West Asian	PCR-SSCP	HB	136	93	69	53	14	47	39	7	0.779
Chao [44]	2014	China	East Asian	PCR-RFLP	HB	17	34	10	5	2	19	12	3	0.586
Sayin [45]	2015	Turkey	West Asian	PCR-RFLP	HB	75	95	40	33	2	43	44	8	0.484
Li [41]	2015	China	East Asian	PCR-SSCP	HB	150	150	31	78	41	59	66	25	0.376
Koshy [40]	2015	Indian	South Asian	PCR-RFLP	HB	96	90	95	1	0	83	7	0	0.701
Jiang [39]	2015	China	East Asian	PCR-RFLP	HB	100	100	38	46	16	41	48	11	0.583
Feng [42]	2016	China	East Asian	PCR-GeXP	HB	260	49	21	114	125	6	22	21	0.949
Wang [43]	2016	China	East Asian	PCR-RFLP	HB	147	168	14	73	60	49	84	35	0.928
MTHFR A1298C Polymorphism														
Storti [15]	2003	Italy	South Europe	PCR-SSCP	HB	103	200	45	47	11	101	86	13	0.347
Galdieri [22]	2007	Brazil	South America	PCR-SSCP	HB	57	38	35	21	1	19	16	3	0.884
van Driel [24]	2008	Netherlands	North Europe	PCR-TAQMAN	PB	229	251	112	90	27	97	129	25	0.057
Xu [27]	2010	China	East Asian	PCR-SSCP	HB	502	527	316	168	18	326	185	16	0.091
Obermann-Borst [28]	2011	Netherlands	North Europe	PCR-TAQMAN	PB	139	183	69	57	13	75	90	18	0.227
WangBJ [34]	2013	China	East Asian	PCR-SNaPShot	HB	170	188	115	45	10	133	47	8	0.155
Christensen [4]	2013	Canada	North America	PCR-RFLP	HB	157	69	78	67	12	38	26	5	0.849
Sahiner [38]	2014	Turkey	West Asian	PCR-SSCP	HB	137	93	45	68	24	31	54	8	0.223
Huang [37]	2014	China	East Asian	PCR-MassArray Assay	HB	170	208	111	56	3	146	56	6	0.823
Sayin [45]	2015	Turkey	West Asian	PCR-RFLP	HB	69	99	20	36	13	51	37	11	0.288
Li [41]	2015	China	East Asian	PCR-SSCP	HB	150	150	114	36	0	131	19	0	0.408
Feng [42]	2016	China	East Asian	PCR-GeXP	HB	257	49	194	51	12	35	14	0	0.243

Table 2. Pooled ORs and 95%CIs of the relationship between MTHFR polymorphisms and congenital heart disease risk. CI= confidence interval.a P value for between-study heterogeneity based on Q test;Bon= p value in Bonferroni test; FDR= false discovery rate. Significant results are marked in bold

		Allelic genetic model			Dominant genetic model			Recessive genetic model			Heterozygote genetic model			Homozygote genetic model		
Subgroup	N	OR[95%CI	P*	I2	OR[95%CI]	P*	I2	OR[95%CI]	P*	I2	OR[95%CI]	P*	I2	OR[95%CI]	P*	I2
MTHFR C677T																
Region																
Middle Europe	1	1.63 [1.16,	0.005	N	1.61 [1.02,	0.040	N	1.23 [0.63, 2.38]	0.550	N	1.36 [0.83,	0.220	N	2.53 [1.27,	0.008	N
East Asian	20	1.48 [1.21,	0.000	87	1.55 [1.17,	0.002	82	2.29 [1.63, 3.23]	0.000	88	1.29 [1.02,	0.030	70	2.10 [1.43,	0.000	84
South Europe	1	0.97 [0.69,	0.840	N	0.94 [0.55,	0.820	N	0.62 [0.34, 1.14]	0.120	N	0.95 [0.54,	0.850	N	0.93 [0.46,	0.840	N
North America	3	1.30 [0.81,	0.270	80	1.46 [0.72,	0.290	81	0.81 [0.17, 3.83]	0.790	92	1.40 [0.71,	0.340	77	1.63 [0.68,	0.280	72
South America	1	0.83 [0.45,	0.560	N	0.84 [0.37,	0.680	N	1.33 [0.42, 4.21]	0.630	N	0.90 [0.37,	0.820	N	0.70 [0.20,	0.570	N
North Europe	3	1.08 [0.91,	0.390	0	1.07 [0.85,	0.540	0	1.08 [0.73, 1.60]	0.690	0	1.04 [0.79,	0.760	21	1.21 [0.81,	0.350	0
West Asian	2	0.89 [0.60,	0.550	37	0.87 [0.58,	0.480	0	0.83 [0.10, 6.95]	0.870	81	0.87 [0.58,	0.750	0	0.69 [0.14,	0.640	65
South Asian	1	0.13 [0.02,	0.060	N	0.12 [0.02,	0.050	N	Not estimable	NA	N	0.12 [0.02,	0.050	N	Not estimable	NA	N
Sample Size																
≤300	13	1.32 [1.08,	0.006	59	1.35 [1.01,	0.040	58	2.41 [1.81, 3.21]	0.000	40	1.24 [0.95,	0.110	47	1.91 [1.36,	0.000	38
>300	19	1.34 [1.10,	0.004	88	1.36 [1.06,	0.020	83	1.49 [0.98, 2.26]	0.070	92	1.18 [0.96,	0.130	70	1.73 [1.17,	0.006	86
Genotyping																
PCR-RFLP	14	1.36 [1.03,	0.030	85	1.20 [0.84,	0.300	75	2.26 [1.51, 3.39]	0.000	80	1.07 [0.77,	0.690	67	1.95 [1.16,	0.010	81
PCR-SSCP	11	1.34 [1.06,	0.020	85	1.25 [0.94,	0.120	75	1.48 [1.07, 2.06]	0.020	72	1.18 [0.91,	0.210	65	1.77 [1.13,	0.010	81
PCR-TAQMAN	4	1.17 [0.88,	0.270	69	1.29 [0.85,	0.230	71	0.58 [0.30, 1.10]	0.100	71	1.29 [0.86,	0.210	66	1.27 [0.71,	0.420	59
PCR-MassArray	1	1.79 [1.33,	0.000	N	2.04 [1.26,	0.004	N	5.26 [3.12, 8.86]	0.000	N	1.63 [0.98,	0.060	N	3.46 [1.83,	0.000	N
PCR-SNaPShot	1	0.79 [0.58,	0.120	N	2.86 [1.83,	0.000	N	0.85 [0.48, 1.49]	0.560	N	1.95 [1.20,	0.007	N	0.64 [0.34,	0.170	N
PCR-GeXP	1	1.24 [0.79,	0.360	N	3.91 [2.06,	0.000	N	11.46 [6.91,	0.000	N	1.48 [0.54,	0.450	N	1.70 [0.61,	0.310	N
Source of																
PB	6	1.22 [0.96,	0.100	68	1.22 [0.89,	0.210	62	0.82 [0.45, 1.49]	0.510	78	1.15 [0.85,	0.370	57	1.46 [0.92,	0.110	55
HB	26	1.34 [1.12,	0.001	85	1.37 [1.08,	0.009	79	2.03 [1.47, 2.80]	0.000	87	1.20 [0.99,	0.070	65	1.81 [1.29,	0.000	82
MTHFR A1298C Polymorphism																
Region																
South Europe	1	1.30 [0.90,	0.160	N	1.31 [0.82,	0.260	N	0.95 [0.40, 2.22]	0.900	N	1.23 [0.74,	0.420	N	1.90 [0.79,	0.150	N
South America	1	0.62 [0.32,	0.170	N	0.63 [0.27,	0.270	N	0.34 [0.03, 3.36]	0.360	N	0.71 [0.30,	0.440	N	0.18 [0.02,	0.150	N
North Europe	2	0.82 [0.67,	0.070	0	0.68 [0.51,	0.006	0	1.04 [0.66, 1.65]	0.850	0	0.64 [0.47,	0.003	0	0.88 [0.54,	0.590	0
East Asian	5	1.13 [0.93,	0.220	26	1.17 [0.88,	0.280	45	1.31 [0.54, 3.19]	0.560	55	1.13 [0.83,	0.430	55	1.20 [0.72,	0.490	0
West Asian	2	1.50 [0.96,	0.070	56	1.61 [0.64,	0.310	78	2.32 [0.95, 5.70]	0.070	54	1.44 [0.52,	0.490	81	2.48 [1.28,	0.007	0
Sample Size																
≤300	5	1.31 [0.93,	0.130	60	1.36 [0.87,	0.180	63	2.05 [1.02, 4.10]	0.040	39	1.36 [0.86,	0.190	59	1.58 [0.71,	0.260	40
>300	7	0.99 [0.87,	0.890	8	0.94 [0.76,	0.570	40	1.09 [0.75, 1.57]	0.660	20	0.90 [0.72,	0.360	47	1.11 [0.80,	0.550	0
Genotyping																
PCR-RFLP	2	1.49 [0.91,	0.120	59	1.67 [0.76,	0.200	75	1.84 [0.93, 3.62]	0.080	0	1.72 [0.89,	0.110	53	1.96 [0.78,	0.150	38
PCR-SSCP	5	1.15 [0.88,	0.310	56	1.13 [0.82,	0.440	52	1.37 [0.64, 2.95]	0.420	60	1.10 [0.80,	0.580	49	1.40 [0.78,	0.260	32
PCR-TAQMAN	2	0.82 [0.67,	0.070	0	0.68 [0.51,	0.006	0	1.04 [0.66, 1.65]	0.850	0	0.64 [0.47,	0.003	0	0.88 [0.54,	0.590	0
PCR-SNaPShot	1	1.17 [0.80,	0.410	N	1.24 [0.70,	0.460	N	1.33 [0.51, 3.45]	0.560	N	1.11 [0.69,	0.680	N	1.45 [0.55,	0.450	N
PCR-MassArray	1	1.14 [0.78,	0.490	N	1.25 [0.81,	0.310	N	0.51 [0.13, 2.07]	0.350	N	1.32 [0.84,	0.230	N	0.66 [0.16,	0.560	N
PCR-GeXP	1	1.03 [0.55,	0.940	N	0.81 [0.41,	0.550	N	26.22 [1.54,	0.020	N	0.66 [0.33,	0.230	N	4.56 [0.26,	0.300	N
Source of																
PB	2	0.82 [0.67,	0.070	0	0.68 [0.51,	0.006	0	1.04 [0.66, 1.65]	0.850	0	0.64 [0.47,	0.003	0	0.88 [0.54,	0.590	0
HB	10	1.19 [1.01,	0.040	38	1.20 [0.97,	0.090	44	1.49 [0.91, 2.43]	0.110	47	1.16 [0.93,	0.190	45	1.50 [1.04,	0.030	73

Subgroup analysis

To excavate the potential associations and underlying heterogeneity source from the pooled results, a subgroup analysis was performed and four subgroups (Region, Sample size, Genotyping method and source of controls) were stratified (Table 3).

In the subgroup analysis of Region, significant associations were found for these two polymorphisms. In the Middle Europe subgroup, the allelic genetic model (OR=1.63, 95%

Table 3. Subgroup analysis of the associations of MTHFR polymorphisms with congenital heart disease risk. OR=odd ratio; CI=Confidence Interval; HB=Hospital Based; PB=Population Based; # P value for Hardy–Weinberg equilibrium test in controls; * P value for meta-analysis. PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism. PCR-TAQMAN = polymerase chain reaction with Taqman probe; PCR-ABD = polymerase chain reaction using Assay by Design (ABD) kits from Applied Biosystems (Carlsbad, CA, USA): PCR-MassaArray Assay= Polymerase Chain Reaction with MassArray Assay. PCR-SSCP = Polymerase Chain Reaction-Single Strand Conformation Polymorphism; PCR-SNaPShot = polymerse chain reaction with SNaPShot; PCR-GeXP = Polymerse Chain Reaction with GeXP. Significant results are marked in bold

				Genotyping	Controls			Case			Control			
First author	Year	Country	Region	method	Source	case	control	CC	CT	TT	CC	CT	TT	HWE
MTHFR C677T Polymorphism														
Junker [14]	2001	Germany	Middle Europe	PCR-SSCP	HB	114	228	51	42	21	129	78	21	0.075
Yan [16]	2003	China	East Asian	PCR-RFLP	HB	187	103	32	97	58	22	57	24	0.277
Storti [15]	2003	Italy	South Europe	PCR-SSCP	HB	103	200	28	55	20	52	108	40	0.235
Shaw [19]	2005	American	North America	PCR-TAQMAN	PB	153	434	69	68	16	180	202	52	0.684
Li [18]	2005	China	East Asian	PCR-RFLP	HB	187	102	32	94	61	20	57	25	0.224
Lee [17]	2005	China	East Asian	PCR-SSCP	HB	213	195	110	89	14	114	68	13	0.513
Zhu [21]	2006	China	East Asian	PCR-RFLP	PB	56	103	7	22	27	22	57	24	0.277
van Beynum [20]	2006	Netherlands	North Europe	PCR-SSCP	PB	165	220	79	66	20	98	104	18	0.184
Liu [23]	2007	China	East Asian	PCR-RFLP	HB	132	107	30	68	34	46	48	13	0.930
Galdieri [22]	2007	Brazil	South America	PCR-SSCP	HB	58	38	30	21	7	18	14	6	0.263
van Driel [24]	2008	Netherlands	North Europe	PCR-TAQMAN	PB	229	251	99	103	27	119	107	25	0.895
Li [25]	2009	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Xu [27]	2010	China	East Asian	PCR-SSCP	HB	502	527	162	244	96	151	261	115	0.911
Kuehl [26]	2010	American	North America	PCR-TAQMAN	PB	55	290	12	33	10	134	124	32	0.682
Obermann-Borst [28]	2011	Netherlands	North Europe	PCR-TAQMAN	PB	139	183	64	66	9	92	76	15	0.900
Zhou [31]	2012	China	East Asian	PCR-SSCP	HB	136	277	23	60	53	88	126	63	0.168
Li [30]	2012	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Gong [29]	2012	China	East Asian	PCR-MassArray Assay	HB	244	136	45	123	76	43	72	21	0.309
Li [33]	2013	China	East Asian	PCR-RFLP	HB	144	168	26	52	66	49	84	35	0.928
Jing [32]	2013	China	East Asian	PCR-RFLP	HB	104	208	46	42	16	39	114	55	0.139
WangLN [35]	2013	China	East Asian	PCR-SSCP	HB	236	277	33	92	111	88	126	63	0.168
WangBJ [34]	2013	China	East Asian	PCR-SNaPShot	HB	160	188	59	76	25	53	100	35	0.312
Christensen [4]	2013	Canada	North America	PCR-RFLP	HB	157	69	68	61	28	35	26	8	0.360

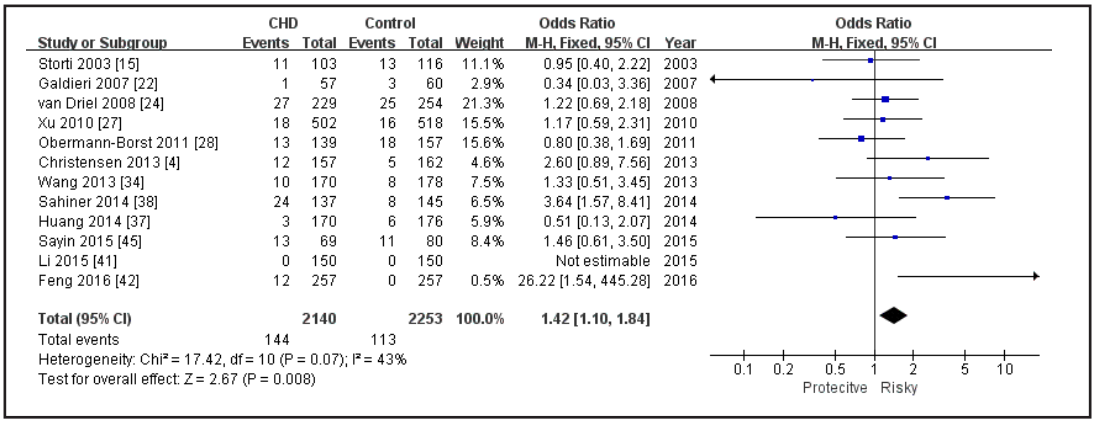

Fig. 3. Pooled analysis of A1298C polymorphism and CHD risk in children population.

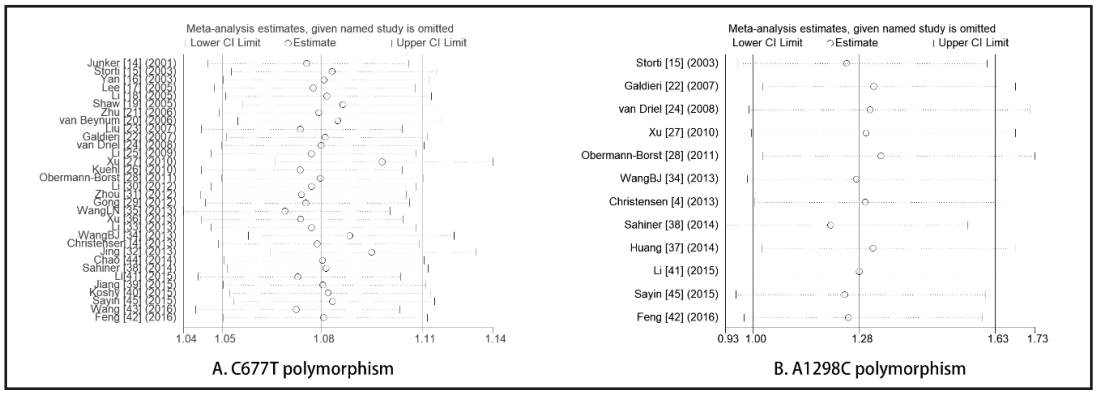

Fig. 4. Sensitivity analysis of C677T and A1298C polymorphism and CHD risk.

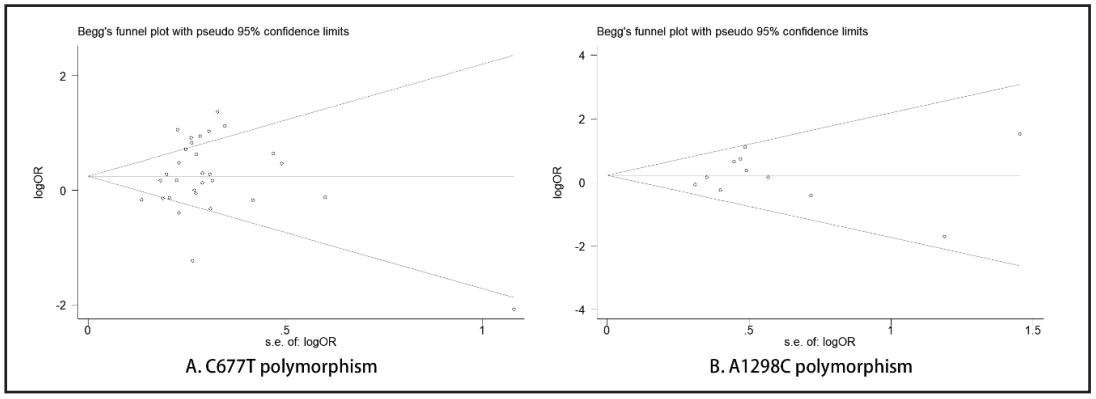

Fig. 5. Publication bias of C677T and A1298C polymorphism and CHD risk.

CI=1.16-2.30, P=0.005), dominant genetic model (TT+TC versus CC (OR=1.61, 95% CI=1.02-2.53, P=0.04) and homozygote genetic model (OR=2.53, 95% CI=1.27-5.02, P=0.008) of the C677T polymorphism were associated with CHD risk. All five-genetic model of the C677T polymorphism in the East Asian were observed to be associated with CHD risk but also with high heterogeneity. For A1298C polymorphism, the dominant genetic model (OR=0.68, 95% CI=0.51-0.90, P=0.006) and heterozygote genetic model (OR=0.64, 95%CI=0.47-0.86, P=0.003) in the North Europe subgroup and the homozygote genetic model (OR=2.48, 95% CI=1.28-4.81, P=0.007) in the West Asian subgroup were related to CHD risk.

In the subgroup analysis of Sample size, for the C677T polymorphism, wide significant associations with reduced heterogeneity were observed in the no more than 300 subgroup as follows: the allelic genetic model (OR=1.32, 95% CI=1.08-1.80, P=0.006); the dominant genetic model (OR=1.35, 95% CI=1.01-1.80, P=0.04); the recessive genetic model (OR=2.41, 95% CI=1.81-3.21, P=0.000); and the homozygote genetic model (OR=1.91, 95% CI=1.36-2.67, P=0.0002)). As for more than 300 subgroup, the allelic genetic model (OR=1.34, 95% CI=1.10-1.64, P=0.004), dominant genetic model (OR=1.36, 95% CI=1.06-1.76, P=0.02) and homozygote genetic model (OR=1.73, 95% CI=1.17-2.56, P=0.006) were associated with CHD risk. However, for the A1298C polymorphism, a significant association with reduced heterogeneity was only detected in the recessive genetic model (OR=2.05, 95% CI=1.02-4.10, P=0.04) in the no more than 300 subgroup.

As for the subgroup analysis stratified by the genotyping method, extensive significant associations were found in the C677T polymorphism by PCR-RFLP for the allelic genetic model (OR=1.36, 95% CI=1.03-1.80, P=0.03), recessive genetic model (OR=2.26, 95% CI=1.51-3.39, P=0.0001), homozygote genetic model (OR=1.95, 95% CI=1.16-3.29, P=0.0001), by PCR-SSCP for the allelic genetic model (OR=1.34, 95% CI=1.06-1.71, P=0.02), recessive genetic model (OR=1.48, 95% CI=1.07-2.06, P=0.02), homozygote genetic model

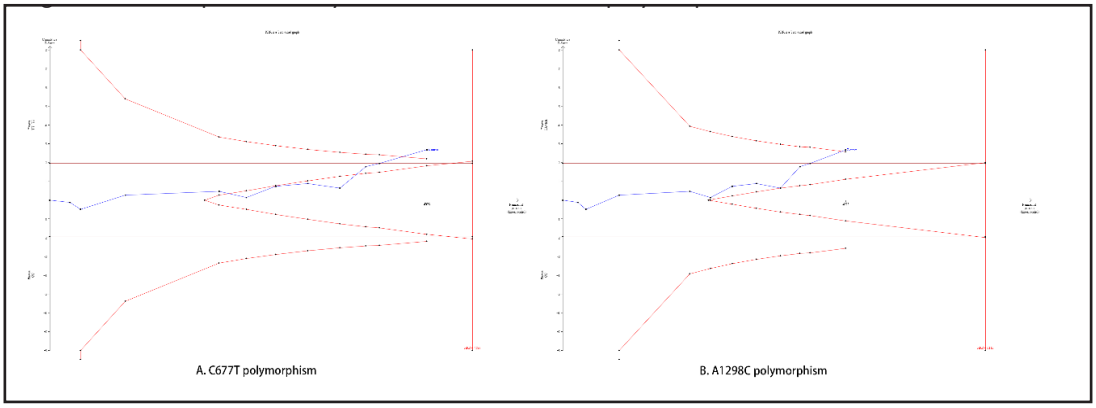

Fig. 6. Trial sequential analysis of C677T and A1298C polymorphism and CHD risk.

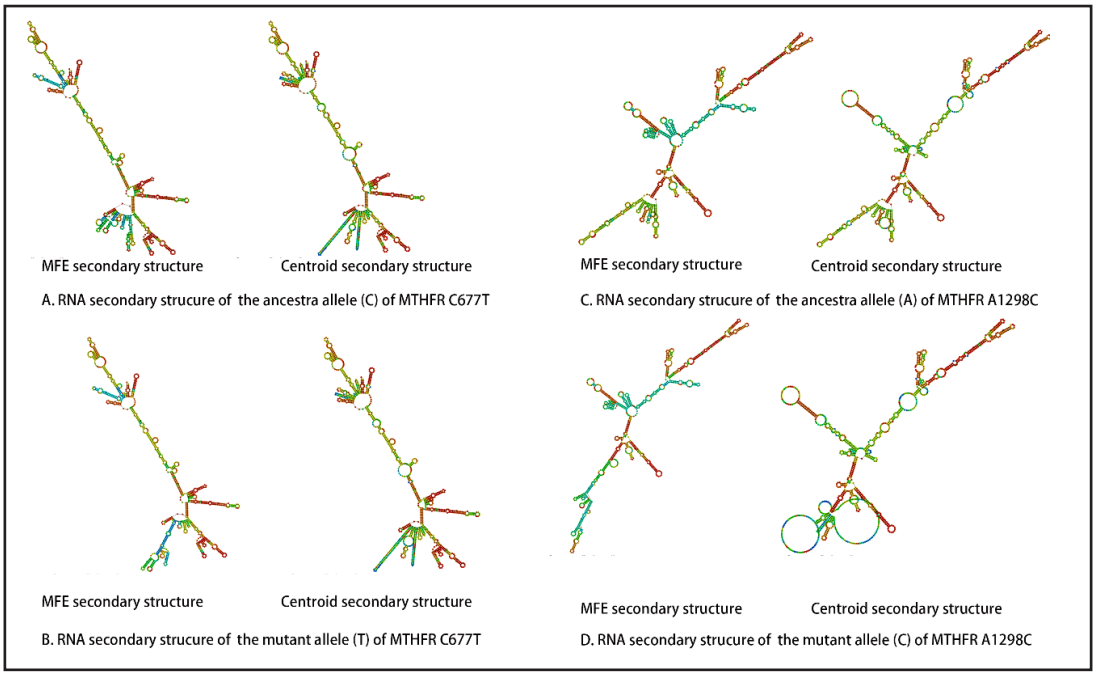

Fig. 7. RNAfold Webserver analysis of C677T and A1298C polymorphism and CHD risk.

(OR=1.77, 95% CI=1.13-2.79, P=0.01), by the PCR-MassArray Assay for the allelic genetic model (OR=2.05, 95% CI=1.02-4.10, P=0.04), dominant genetic model (OR=2.04, 95% CI=1.26-3.32, P=0.004), recessive genetic model (OR=5.26, 95% CI=3.12-8.86, P=0.000), and homozygote genetic model (OR=3.46, 95% CI=1.83-6.55, P=0.0001), by PCR-SNaPShot for the dominant genetic model (OR=2.86, 95% CI=1.83-4.48, P=0.000) and heterozygote genetic model (OR=1.95, 95%CI=1.20-3.15, P=0.04), by PCR-GeXP for the dominant genetic model (OR=3.91, 95% CI=2.06-7.44, P=0.0001), recessive genetic model (OR=11.46, 95% CI=6.91-19.03, P=0.000)). However, for A1298C polymorphism, significant associations were only observed by PCR-TAQMAN (dominant genetic model: OR=0.68, 95% CI=0.51-0.90, P=0.006; heterozygote genetic model: OR=0.64, 95% CI=0.47-0.86, P=0.003) and PCR-GeXP (recessive genetic model: OR=26.22, 95% CI=1.54-445.28, P=0.02).

In the subgroup analysis of the source of controls, for the C677T polymorphism, significant associations were only observed in the hospital based subgroup (Allelic genetic model: OR=1.34, 95% CI=1.12-1.59, P=0.001; dominant genetic model: OR=1.37, 95% CI=1.08-1.73, P=0.009; recessive genetic model: OR=2.03, 95% CI=1.47-2.80, P=0.0001;

homozygote genetic model: OR=1.81, 95% CI=1.29-2.53, P=0.0006). For the A1298C polymorphism, the heterozygote genetic model (OR=0.64, 95% CI=0.47-0.86, P=0.003) in the population based subgroup and the allelic genetic model (OR=1.19, 95% CI=1.01-1.41, P=0.04) and homozygote genetic model (OR=1.50, 95% CI=1.04-2.16, P=0.03) in the hospital based subgroup were associated with CHD risk.

Sensitivity analyses

The sensitivity analysis was conducted by sequentially omitting 1 individual study every time to weigh the influence of each study on the overall meta-analysis. No significant change in the heterogeneity was observed for these two polymorphisms (Fig. 4).

Publication bias

No publication bias was detected among the studies regarding the association between the C677T and A1298C polymorphism and congenital heart defect risk (Fig. 5).

Trial sequential analysis

According to the settings mentioned in the method section, we calculated the required information size for the *MTHFR* C677T and A1298C polymorphisms (Fig. 6). The number of patients included in the meta-analysis exceeded the required information size for the two polymorphisms, which indicated our positive results were confirmed by TSA.

RNA secondary structure analysis

We conducted an RNA secondary structure analysis of the *MTHFR* C677T and A1298C polymorphisms with the RNAfold Webserver. Fig. 7 shows the significant changes in the RNA structure under both the minimum free energy and the centroid secondary structure, which indicated the two variants might affect the stability of the RNA secondary structure.

Discussion

The methylenetetrahydrofolate reductase (MTHFR) is the crucial enzyme concatenating the folate pathway and homocysteine metabolism [46]. Low folate and high homocysteine are a closely related with the occurrence of congenital heart disease [5, 47], which indicates that single nucleotide polymorphisms (SNPs) in the *MTHFR* gene may be genetic risk factors for these disorders. The *MTHFR* C677T and A1298C SNPs are common and functional, with enough data for us to perform a subgroup analysis and a trial sequential analysis. Moreover, the mutant 677T and 1298C alleles are related to the decreased activity of the MTHFR enzyme [48]. Therefore, we chose these two polymorphisms to investigate their associations with CHD risk and significant increased risks were widely observed in both the overall and subgroup analyses.

An increased risk of CHD was detected in the *MTHFR* C677T polymorphism from the overall analysis. The putative risk allele-677T had a 32% increased risk of CHD risk against the C-allele. A 35% increase in CHD risk in the TT+TC genotypes was also detected. The TC and TT genotypes increased CHD risk by 20% and 75% compared to the CC genotype respectively. In addition, a 69% increased risk was also found in the TT genotypes compared to TC+CC genotypes. Moreover, the increased risk of the T allele, TT, TC and TT+TC genotypes was widely observed in the subgroup analysis stratified by region, sample size, genotyping method and source of controls. Furthermore, the TSA test confirmed our positive results. The extensive increased risk of the C677T polymorphism in CHD implied this polymorphism was a strong genetic risk factor for fetal heart defects.

As for the *MTHFR* A1298C polymorphism, the increased risk of the CC genotype was widely detected both from the pooled analysis and the subgroup analysis (West Asian subgroup, no more than 300 subgroup, PCR-GeXP subgroup and hospital-based subgroup), and the positive result was verified by TSA. Interesting results sprouted in the North Europe

and PCR-TAQMAN subgroup; a protective role for the CA+AA and CA genotypes was observed. Several studies also report the protective role of 1298C allele, and Hobbs et al. suggested three possible explanations for the phenomenon, including: (i) an unknown functional polymorphism in linkage disequilibrium with A1298C, (ii) error-free DNA synthesis with abundant purines and pyrimidines caused by the lower activity of the MTHFR enzyme, and (iii) the selective survival of the 1298A allele [49-51]. In summary, the CC genotype of the *MTHFR* A1298C polymorphism had an increased risk of CHD, but the protective role of 1298C allele should be interpreted with caution.

Previous meta-analysises have also drawn a conclusion showing a significant association between the MTHFR polymorphism and CHD [52-57]. The differences between our analysis and the former analyses was the sample size and the exclusion of studies with departure from the Hardy-Weinberg equilibrium. We added new references to expand the sample size for better reaching the significant results. Moreover, studies with departure from the Hardy-Weinberg equilibrium were excluded for homogeneity in the control groups, which would make our results more reliable and stable. Additionally, a trial sequential analysis was conducted to strengthen our positive results. Furthermore, the *MTHFR* C677T polymorphism was highly associated with homocysteine concentrations in the large scale, methodologically independent genome-wide association study [58]. However, no genome-wide association study about the A1298C polymorphism is reported so far.

Reduced MTHFR enzyme activity decreases the synthesis of 5-methyl-tetrahydrofolate (the substrate vital for DNA synthase), interrupts the homocysteine remethylation to methionine (a decreased pool of which may affect DNA methylation), and induces hyperhomocysteinemia [45]. Hyperhomocysteinemia initiates apoptosis in neural crest cells and has embryotoxic effects in heart cells in animal models [59, 60]. Although the *MTHFR* C677T and A1298C polymorphisms both diminish MTHFR enzyme activity, they act in different ways. The 677T variant causes a thermolabile form of the enzyme and is associated with elevated homocysteine levels, but for 1298C, it reduced the enzyme activity by conformational changes of the enzyme that occur after S-adenosyl-methionine regulatory domain binding [61, 62]. Our RNA secondary structure analysis showed that the mutant 677T and 1298C alleles changed the space conformation of the RNA secondary structure of the *MTHFR* gene and influenced the stability of this gene, which may be an explanation for the termolabile form of enzyme caused by the 677T allele and the conformational changes of the enzyme induced by the 1298C allele.

Several limitations were existed in this meta-analysis. First, only English and Chinese databases were searched in our study; a selection of the literature without other language may bias the results. Second, the individual patient heterogeneity and confounding factors might have distorted the analysis. Third, the sample size of the included studies was relatively small in some subgroups, especially for the A1298C polymorphism, which implied that part of our results should be explained with caution. In addition, the potential influence of maternal environment factors on these two polymorphisms is worthy of consideration.

Conclusion

The *MTHFR* C677T polymorphism was associated with a significant increase in congenital heart defect risk in the fetal population based on our analysis. Moreover, the increased risk in the CC genotype of the *MTHFR* A1298C polymorphism was observed, but the protective role of the 1298C allele needs further study.

Disclosure Statement

No conflict of interests exists.

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