Association of *MTHFD* Gene Polymorphisms and Maternal Smoking With Risk of Congenital Heart Disease: A Hospital-Based Case-Control Study

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Abstract

Background: *MTHFD* may affect the embryonic development by elevated homocysteine levels, DNA synthesis and DNA methylation, but limited number of genetic variants of *MTHFD* was focused on the association with congenital heart disease (CHD). This study examined the role of *MTHFD* and maternal smoking in CHD risk, and investigated their interaction effects in Chinese populations.

Methods: A case-control study of 464 mothers of CHD infants and 504 mothers of health controls was performed. The exposures of interest were maternal tobacco exposure, single nucleotide polymorphisms (SNPs) of maternal *MTHFD* gene. The logistic regression model was used for accessing the strength of association.

Results: Mothers exposed to secondhand smoke during three months before pregnancy (adjusted odds ratio [aOR] = 1.56; 95% confidence interval [CI]: 1.13-2.15) and in the first trimester of pregnancy (aOR = 2.24; 95%CI: 1.57-3.20) were observed an increased risk of CHD. Our study also found that polymorphisms of maternal *MTHFD* gene at rs1950902 (AA *vs.* GG: aOR = 1.73, 95% CI: 1.01-2.97), rs2236222 (GG *vs.* AA: aOR = 2.38, 95% CI: 1.38-4.12), rs1256142 (GA *vs.*GG: aOR = 1.57, 95% CI: 1.01-2.45) and rs11849530 (GG *vs.* AA: aOR = 1.68, 95% CI: 1.02-2.77) were significantly associated with higher risk of CHD. Furthermore, we found the different degrees of interaction effects between polymorphisms of the *MTHFD* gene including rs1950902, rs2236222, rs1256142, rs11849530 and rs2236225, and maternal tobacco exposure.

Conclusions: Maternal polymorphisms of *MTHFD* gene at rs1950902, rs2236222, rs1256142 and rs11849530, maternal tobacco exposure and their interactions are significantly increased the risk of CHD in offspring. However, more studies in different ethnic populations with a larger sample and prospective designs are required to confirm our findings.

Trial registration:

Registration number: ChiCTR1800016635; http://www.chictr.org.cn/edit.aspx?pid=28300&htm=4

Background

Congenital heart defect (CHD) was often defined as a structural or functional abnormality of the heart and/or great vessels that were present at birth[1]. Among all recognized structural birth defects, CHD was the most common and severe, with 4 to 10 cases per 1000 live births, which imposed a huge economic burden on the society and family[2]. Although the past few decades have seen a rapidly growing interest in exploring the etiology of CHD, the pathogenesis of most CHD cases remains unknown[3, 4].

So far, folate supplementation was the most effective intervention for decreasing CHD[5, 6], while the folate-cycle product homocysteine might affect fetal heart development by disruption of gene methylation, increasing oxidative stress and homocysteinylation of key proteins[5, 7], which indicated that

the occurrence of CHD was highly responsive to changes in genes related to maternal folate-homocysteine metabolism. The methylenetetrahydrofolate dehydrogenase (*MTHFD*) gene, located on chromosome 14q24, encoded the trifunctional enzyme MTHFD (5,10-methylenetetrahydrofolate dehydrogenase[8], 5,10-methenyltetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase). This enzyme catalyzed three sequential reactions in the interconversion of tetrahydrofolate (THF) to 5,10-methylenetettrahydrofolate (5,10-methylene THF), the crucial substrate for 5-methyltetrahydrofolate (5-methlyTHF), which was required for DNA synthesis, DNA repair and provide the methyl donor for regeneration of methionine from homocysteine for subsequent methylation reactions[9, 10]. Plausible mechanisms of the *MTHFD* gene in CHD susceptibility might involve restricted DNA synthesis, high levels of homocysteine, and DNA methylation[5, 11–14]. The experimental studies had revealed that the *MTHFD* gene with mutant genotypes expressed less stable in vitro and low active in vivo MTHFD protein[11, 12], which consequently disturbed *de novo* purine synthesis and impacted DNA synthesis. Moreover, the epidemiologic studies suggested that polymorphisms of the *MTHFD* gene such as rs2236225 and rs1950902 were closely related to homocysteine levels[14–17] and DNA methylation[16], and these data published indicated the plausible association between genetic variants of the *MTHFD* gene and the risk of CHD. *MTHFD* R653Q (rs2236225 G→A) and *MTHFD* R134K (rs1950902 G→A) were the two most well-studied polymorphisms, but previous efforts involved in their associations with CHD risk had yielded conflicting or negative results[11, 17, 18], which might result from insufficient statistical power and different methods. Notably, previous studies focused mainly on a small number of functional nonsynonymous single-nucleotide polymorphisms (SNPs) of the *MTHFD* gene with known biochemical phenotypes such as rs1950902 and rs2236225; the other significant variants have been largely ignored; thus this study represents both the first report and replication efforts in Han Chinese populations.

It had been reported that 85% of CHD resulted from a complex interplay of genetic variants and environmental factors[19]. Maternal tobacco exposure in the periconceptional period, defined as 3 months before pregnancy through the first trimester of pregnancy, was one of the most common environmental factors affecting abnormal fetal development[20]. Amounts of epidemiologic studies about the associations of maternal tobacco exposure in the periconceptional period involved in active smoking and passive smoking and CHD risk were showed heterogeneous results, indicating that people had different susceptibility to the effects of tobacco exposure[21–25]. Convincing evidence showed that it was clear that particular genotypes of metabolizing systems and DNA repair pathways might modulate the effect leading to varying susceptibility to the CHD of tobacco exposure[26]. The maternal tobacco exposure was observed associations with substantial reductions of folate levels in plasma [27, 28]as well as in cord blood[29], and red blood cells[30, 31], even after correcting for folate intake[32], which implied the interactive association between maternal tobacco exposure and polymorphisms of the *MTHFD* in CHD susceptibility. In addition, a recent study suggested that maternal folate levels might partly modify the influence of maternal tobacco exposure during pregnancy on the DNA methylation of the newborn epigenome and therefore affected embryonic development[33]. Based on the above, we hypothesized that there existed the interaction effects between maternal tobacco exposure and the *MTHFD* genetic variants, namely that the

two factors jointly caused the CHD. In our study, a hospital-based case-control design based on the Han Chinese population was performed with the following objectives: (i) to assess the association of genetic variants of maternal *MTHFD* gene with risk of CHD in offspring; (ii) to examine whether maternal smoking including active and passive smoking was significantly associated with risk of CHD in offspring; and (iii) to analyze the interaction effects between maternal smoking and the *MTHFD* genetic variants for CHD.

Methods

Study design and recruitment of study participants

For requirements for a specific article type please refer to the Article Types on any Frontiers journal page. The main characteristics of the participants and research procedure had been described by our previous study[34]. Recruitment was conducted by the Hunan Provincial Children's Hospital (Changsha, Hunan Province, China). A total of 464 CHD patients and their mothers were consecutively enrolled from the Department of Cardiothoracic Surgery between November 2017 and December 2019. The non-CHD patients were from the Department of Child Healthcare in the same hospital during the same time period and were matched to the affected individuals by age and sex. The controls included 504 non-CHD patients who were without any congenital malformations after medical examination and their mothers. All CHD cases were diagnosed using echocardiography and confirmed by surgery. The CHD patients with structural malformations involving another organ system or known chromosomal abnormalities were excluded. Considering the homogenous ethnic background may reduce residual confounding factors from genetic and cultural differences, we only included the Han Chinese descent. We further excluded mothers who achieved pregnancy by assisted reproductive technology including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) and reporting. Again, mothers who reported a history of depression or other psychiatric disorders or were diagnosed with depression or a psychiatric illness were also excluded. We classified the 464 CHD cases into 7 broad categories as described previously[35]. In particular, 28 (6.0%) had conotruncal defects, 360 (77.6%) had septation defects, 11 (2.4%) had left ventricular outflow tract obstruction, 17 (3.7%) had right ventricular outflow tract obstruction, 16 (3.4%) had anomalous pulmonary venous return, 20 (4.3%) had complex CHD, and 12 (2.6%) had other CHD defects (Table S1).

Our study was approved by the ethics committee of the Xiangya School of Public Health of Central South University, and written informed consent was obtained from all mothers. Besides, we have registered this study in the Chinese Clinical Trial Registry Center (registration number: ChiCTR1800016635).

Information collection

A self-designed questionnaire was used to collect the corresponding information by specially trained investigators. This questionnaire was developed by experts in the field of CHD research and administered to eligible mothers (test-retest reliability=0.833; Cronbach’s alpha=0.782). In the present study, we

collected maternal sociodemographic characteristics (i.e., age at pregnancy onset (years)、residence location, maternal education level (years) and annual income in the past 1 year (RMB)), history of adverse pregnancy outcomes (i.e., spontaneous abortion, stillbirth, preterm birth, gestational diabetes mellitus, gestational hypertension, and premature rupture of membranes), family history (i.e., consanguineous marriages, and history of congenital heart disease), cold or fever in the periconceptional period, and personal lifestyle and habit in the periconceptional period including drinking alcohol, drinking tea, living near environmental pollution source, dyeing hair or perming, decorating housing and folate use. The mentioned above information was further confirmed by consulting their Maternal and Child Health Manual and medical records. In China, each pregnant woman will be provided with a Maternal and Child Health Manual, which will record their basic demographic characteristics, behavioral habits, illness, and the results of various medical examinations during pregnancy. We also examined the SNPs of the maternal *MTHFD* gene, which were described below.

Sequencing of *MTHFD* gene and genotyping

The *MTHFD* gene was the candidate gene for the present study. When mothers completed the questionnaires mentioned above, they were asked to provide 3 to 5 milliliters of peripheral venous blood for genotyping. Methods for DNA extraction and genotyping have been described previously[34]. The laboratory technician, who performed the genotyping, retyped and double-checked each sample, and recorded the genotype data, was blinded to whether the samples were from cases or controls. SNP markers were selected using the SNPBrowser™ program (version 3.0) provided by AppliedBiosystems Inc. This program allowed the selection of SNP markers from the HapMap database. For each target gene, tagging SNPs were selected based on the pairwise $r^2 \geq 0.8$. However, we excluded these SNPs with minor allele frequencies less than 10% in Caucasians. We imposed a minimum SNP genotyping call rate at the level of 50%, which was applied to ensure data integrity of the individual's genotypes that had been called. And successful rates for SNPlex assays were >96% for 4 SNPs, from 90% to 96% for 1 SNPs. Finally, these genetic loci (rs1950902, rs2236225, rs2236222, rs11849530 and rs1256142) were selected as candidate loci for this study.

Statistical analysis

Statistical analysis was performed using R software, version 3.5.0 (R Foundation for Statistical Computing). All tests were performed significantly for a two-sided *P* value not exceeding 0.05, except where otherwise specified. Additionally, the false discovery rate (FDR) control was used in this study to correct for multiple testing. The statistically significant results were those with the false discovery rate *P* value (FDR_*P*) < 0.1. Qualitative data were described using frequencies and percentages, and quantitative data were described using means and standard deviations (SDs). Hardy-Weinberg equilibrium (HWE) was tested for the control group (significance level at $P<0.01$). We used a two-phase analytical method based on genetic model selection (GMS) to test associations between SNPs and CHD, and the specific calculation process had been described previously[36]. The genetic models contained the dominant model (calculated for mutant type homozygote versus wild type homozygotes and heterozygote), the recessive

model (calculated for heterozygotes and mutant type homozygotes versus wild type homozygotes), and the addictive model (calculated for wild type homozygotes versus heterozygote versus mutant type homozygote). We classified the genetic model into the recessive model if $Z_{HWDTT} > c$, the dominant model if $Z_{HWDTT} < -c$, and in the addictive model if otherwise, where we chose $c = \Phi^{-1}(0.95) = 1.645$. The Pearson χ^2 test was used to compare the differences of nominal variables across groups. And for ordinal categorical variables, Wilcoxon rank sum test was used. Odds ratios (ORs) and their 95% confidence intervals (CIs) were used to show the level of association. Crude ORs were calculated by univariate logistic regression, while adjusted ORs (aORs) were calculated by multivariable logistic regression. We used logistic regression and controlled for potential confounders, to analyze the main effects and interactive effects of the gene-environment interaction of maternal *MTHFD* gene and smoking experiences for risk of CHD in offspring. Of note, in the present study, we focused only on the risk of total CHD associated with maternal smoking and genetic variants of the *MTHFD* gene and did not assess the risk of specific CHD subtypes due to the limited number of sample sizes for these subtypes.

Results

Baseline characteristics of study population

After considering the inclusion criteria, we finally recruited 464 CHD cases and their parents into the case group and 504 health infants and their parents into the control group. Comparisons of baseline characteristics across groups were summarized in Table 1. Our study showed that there were statistically significant differences between two groups for the following characteristics: maternal education level (years), annual income in the past 1 year (RMB), history of adverse pregnancy outcomes, consanguineous marriage, history of congenital malformations in family, cold or fever in the periconceptional period, and personal lifestyle and habit in the periconceptional period including drinking alcohol, drinking tea, living near environmental pollution source, dyeing hair or perming and folate use (all *P* values < 0.05). Thus, these factors were adjusted when accessing the association of maternal tobacco exposure, the genetic variants of the maternal *MTHFD* gene, and their interactions with the risk of CHD in offspring.

Table 1. Baseline characteristics in case and control groups[a]

Baseline characteristics	Control group (*n*=504)	Case group (*n*=464)	Univariate analysis[d]
Age at pregnancy onset (years)			χ^2=0.191; *P* = 0.662
<35	434(86.1%)	404(87.1%)	
≥35	70(13.9%)	60(12.9%)	
Residence location			χ^2 =39.390; *P* = 0.662
Rural areas	276(54.8%)	344(74.1%)	
Urban areas	228(45.2%)	120(25.9%)	
Education level (years)			*Z* =12.306; *P* <0.001[b]
≤9	6(1.2%)	66(14.2%)	
9-12	100(19.8%)	190(40.9%)	
12-16	168(33.3%)	130(28.0%)	
>16	230(45.6%)	78(16.8%)	
Annual income in the past 1 year (RMB)			*Z* = 15.946; *P* <0.001[b]
≤50,000	144(28.6%)	372(80.2%)	
50,000-100,000	216(42.9%)	68(14.7%)	
100,000-150,000	46(9.1%)	10(2.2%)	
>150,000	98(19.4%)	14(3.0%)	
History of adverse pregnancy outcomes			χ^2 = 12.033; *P* = 0.001
No	280(55.6%)	206(44.4%)	
Yes	224(44.4%)	258(55.6%)	
Consanguineous marriage *P*<0.001			χ^2 = 14.480;
No	502(99.6%)	446(96.1%)	
Yes	2(0.4%)	18(3.9%)	
History of congenital heart disease in family			χ^2 = 20.759; *P* <0.001
No	500(99.2%)	436(94.0%)	

Yes	4(0.8%)	28(6.0%)	
Cold or fever[c]			χ^2 = 16.513; *P* <0.001
No	446(88.5%)	366(78.9%)	
Yes	58(11.5%)	98(21.1%)	
Drinking alcohol[c]			χ^2 = 9.060; *P* = 0.003
No	468(92.9%)	404(87.1%)	
Yes	36(7.1%)	60(12.9%)	
Drinking tea[c]			χ^2 = 9.257; *P* = 0.002
No	402(79.8%)	404(87.1%)	
Yes	102(20.2%)	60(12.9%)	
Living near environmental pollution source[c]			χ^2 = 38.443; *P* <0.001
No	470(93.3%)	370(79.7%)	
Yes	34(6.7%)	94(20.3%)	
Dyeing hair or perming[c]			χ^2 = 12.532; *P* <0.001
No	474(94.0%)	406(87.5%)	
Yes	30(6.0%)	58(12.5%)	
Folate[c]			χ^2 = 23.917; *P* <0.001
No	34(6.7%)	78(16.8%)	
Yes	470(93.3%)	386(83.2%)	
Decorating housing[c]			χ^2 = 2.757; *P* = 0.097
No	462(91.7%)	438(94.4%)	
Yes	42(8.3%)	26(5.6%)	

[a]Data presented as number (percentage) unless otherwise indicated.

[b]The Wilcoxon rank-sum test method was used; otherwise, the χ^2 test was used.

[c]The exposure occurred in the periconceptional period.

[d]*P* < 0.05 was considered to indicate a statistically significant difference.

Maternal tobacco exposure and risk of CHD in offspring

Table 2 showed the association between maternal smoking and the risk of CHD in offspring. The prevalence rate of active smoking in 3 months before pregnancy in our controls (2.0%) was lower than the smoking rate among Chinese women in China Adult Tobacco Survey Report in 2015 (2.7%)[37]. None of the mothers in cases and controls reported active smoking in the first trimester of pregnancy. Mothers who reported active smoking in 3 months before pregnancy had an increased risk of CHD in offspring compared with the controls (P <0.001), but this association was not independent of potential confounders (P = 0.052). After adjustment for baseline data, mothers exposed to secondhand smoke in 3 months before pregnancy were observed an increased risk of CHD in offspring (aOR = 1.56; 95% CI: 1.13-2.15). Additionally, the risk of CHD in offspring was significantly higher among mothers who were exposed to secondhand smoke in the first trimester of pregnancy (aOR = 2.24; 95% CI: 1.57-3.20).

Table 2. Maternal smoking and risk of congenital heart defects in offspring[a]

Exposure	Control group (n=504)	Case group (n=464)	Unadjusted OR (95% CI)	P	Adjusted OR (95% CI)[b]	P[c]
Active smoking in 3 months before pregnancy						
No	494 (98.0%)	432 (93.1%)	1.00 (reference)	-	1.00 (reference)	-
Yes	10 (2.0%)	32 (6.9%)	3.66 (1.78-7.53)	<0.001	2.37(0.99-5.65)	0.052
Active smoking in the first trimester						
No	504 (100%)	464 (100%)	-	-	-	-
Yes	0 (0%)	0 (0%)	-	-	-	-
Passive smoking in 3 months before pregnancy						
No	316 (62.7%)	222 (47.8%)	1.00 (reference)	-	1.00 (reference)	-
Yes	188 (37.3%)	242 (52.2%)	1.83 (1.42-2.37)	<0.001	1.56 (1.13-2.15)	0.007
Passive smoking in the first trimester						
No	406 (80.6%)	274 (59.1%)	1.00 (reference)	-	1.00 (reference)	-
Yes	98 (19.4%)	190 (40.9%)	2.87 (2.154-3.83)	<0.001	2.24 (1.57-3.20)	<0.001

Abbreviations: CI = confidence interval.

[a]Data presented as number (percentage) unless otherwise indicated.

[b]Adjusted for maternal education level (years), annual income in the past 1 year (RMB), history of adverse pregnancy outcomes, consanguineous marriage, history of congenital malformations in family, cold or fever in the periconceptional period, and personal lifestyle and habit in the periconceptional period including drinking alcohol, drinking tea, living near environmental pollution source, dyeing hair or perming and folate use.

[c]$P < 0.05$ was considered to indicate a statistically significant difference.

Genotypes Frequencies of SNPs and the results of HWE tests and GMS

The genotype frequencies for each SNP of the maternal *MTHFD* gene and the results of HWE tests were summarized in Table S2. The HWE tests showed that the genotype frequencies of the 5 SNPs of maternal *MTHFD* gene in the control group were all within HWE (all *P* values >0.01). The results of the GMS of each SNP were presented in Table S3. The genetic models of SNPs including rs1950902, rs2236225, rs2236222 and rs11849530 were all classified into the addictive model since $Z_{HWDTT} > -1.645$ and $Z_{HWDTT} < 1.645$. The genetic model of rs1256142 was classified into the dominant model because of $Z_{HWDTT} < -1.645$. We initially ascertained the genetic models of overall SNPs of the maternal *MTHFD* gene, which was used for accessing the association between each SNP and risk of CHD in offspring based on the corresponding genetic model.

Genetic variants of maternal *MTHFD* gene and risk of CHD in offspring

The association between each maternal SNP of the *MTHFD* gene and the risk of CHD in the Han Chinese population was shown in Table 3. The univariate analyses suggested that there were statistically significant differences for the genetic variants at rs1950902 (AA *vs.* GG: $P = 0.006$; the addictive model: $P = 0.002$), rs2236222 (GG *vs.* AA: $P = 0.001$; the addictive model: $P = 0.001$) and rs1256142 (GA *vs.* GG: $P = 0.035$)

Table 3. *MTHFD* genes in mothers and risk of congenital heart disease in offspring

SNPs	Univariate logistic regression		Multivariable logistic regression[c]		
	Unadjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*	FDR_*P*[d]
rs1950902					
G/G	1.00 (reference)	-	1.00 (reference)	-	-
G/A	1.29 (0.85-1.96)	0.224	1.38 (0.80-2.39)	0.247	0.309
A/A	1.80 (1.18-2.73)	0.006	1.73 (1.01-2.97)	0.046	0.090
Additive[a]	1.36 (1.12-1.64)	0.002	1.30 (1.02-1.65)	0.025	0.090
rs2236225					
G/G	1.00 (reference)	-	1.00 (reference)	-	-
G/A	1.21 (0.92-1.59)	0.178	1.16 (0.81-1.65)	0.427	0.493
A/A	1.16 (0.61-2.20)	0.648	1.03 (0.47-2.27)	0.937	0.937
Additive	1.15 (0.92-1.44)	0.211	1.09 (0.82-1.45)	0.546	0.585
rs2236222					
A/A	1.00 (reference)	-	1.00 (reference)	-	-
G/A	1.27 (0.97-1.66)	0.087	1.27 (0.96-1.67)	0.096	0.144
G/G	2.50 (1.46-4.29)	0.001	2.38 (1.38-4.12)	0.002	0.015
Additive	1.42 (1.16-1.75)	0.001	1.40 (1.14-1.73)	0.002	0.015
rs11849530					
A/A	1.00 (reference)	-	1.00 (reference)	-	-
G/A	0.91 (0.69-1.20)	0.498	1.24 (0.87-1.77)	0.243	0.309
G/G	1.13 (0.77-1.65)	0.536	1.68 (1.02-2.77)	0.042	0.090
Additive	1.02 (0.86-1.22)	0.809	1.28 (1.02-1.62)	0.037	0.090
rs1256142					
G/G	1.00 (reference)	-	1.00 (reference)	-	-
G/A	1.44 (1.03-2.02)	0.035	1.57 (1.01-2.45)	0.048	0.090
A/A	1.20 (0.83-1.74)	0.340	1.57 (0.97-2.56)	0.068	0.113
Dominant[b]	0.92 (0.70-1.21)	0.550	1.57 (1.03-2.40)	0.037	0.090

Abbreviations: CI = confidence interval; SNPs = single nucleotide polymorphisms; *MTHFD* = methylenetetrahydrofolate dehydrogenase; FDR_*P* = false discovery rate *P* value.

[a]Addictive means wild type homozygotes vs. heterozygote vs. mutant type homozygote.

[b]Dominant means wild type homozygote vs. mutant type homozygotes and heterozygote.

[c]Adjusted for maternal education level (years), annual income in the past 1 year (RMB), history of adverse pregnancy outcomes, consanguineous marriage, history of congenital malformations in family, cold or fever in the periconceptional period, and personal lifestyle and habit in the periconceptional period including drinking alcohol, drinking tea, living near environmental pollution source, dyeing hair or perming and folate use.

[d]FDR_*P* < 0.1 was considered to indicate a statistically significant difference.

between the case and control groups. We further accessed the potential associations between genetic variants of maternal *MTHFD* gene and risk of CHD in offspring by aORs and their 95% CIs from logistic regression analysis. After adjustment for the potential confounders, the polymorphisms including rs1950902 (AA *vs.* GG: aOR = 1.73, 95% CI: 1.01-2.97; the addictive model: aOR =1.30, 95% CI: 1.02-1.65), rs2236222 (GG *vs.* AA: aOR = 2.38, 95% CI: 1.38-4.12; the addictive model: aOR = 1.40, 95% CI: 1.14-1.73) and rs1256142 (GA *vs.*GG: aOR = 1.57, 95% CI: 1.01-2.45; the dominant model: aOR = 1.57, 95% CI: 1.03-2.40) were still observed an increased risk for CHD, respectively. In addition, rs11849530 (GG *vs.* AA: aOR = 1.68, 95% CI: 1.02-2.77; the addictive model: aOR = 1.28, 95% CI: 1.02-1.62) was also observed a significant association with higher CHD risk.

Table 4. Interactions between SNPs of *MTHFD* gene and maternal smoking detected by logistic regression

SNPs	Active smoking before pregnancy		Passive smoking before pregnancy		Passive smoking in the first trimester	
	aOR (95%CI)[a]	FDR_*P*[b]	aOR (95%CI)	FDR_*P*	aOR (95%CI)	FDR_*P*
rs1950902 (additive)	1.44 (1.02-2.04)	0.075	1.22(1.07-1.40)	0.008	1.38(1.18-1.60)	<0.001
rs2236225 (additive)	2.15 (1.14-4.05)	0.075	1.38(1.12-1.70)	0.008	1.62(1.29-2.04)	<0.001
rs2236222 (additive)	1.71 (0.87-3.36)	0.149	1.17(0.97-1.42)	0.129	1.38(1.11-1.72)	0.004
rs11849530 (additive)	2.10 (1.21-3.64)	0.075	1.35(1.13-1.61)	0.008	1.64(1.34-2.00)	<0.001
rs1256142 (dominant)	1.52 (0.96-2.41)	0.149	1.26(1.06-1.50)	0.023	1.53(1.26-1.86)	<0.001

Abbreviations: aOR = adjusted odds ratio; CI = confidence interval; SNPs = single nucleotide polymorphisms; *MTHFD* = methylenetetrahydrofolate dehydrogenase; FDR_*P* = false discovery rate *P* value.

[a]Adjusted for maternal education level (years), annual income in the past 1 year (RMB), history of adverse pregnancy outcomes, consanguineous marriage, history of congenital malformations in family, cold or fever in the periconceptional period, and personal lifestyle and habit in the periconceptional period including drinking alcohol, drinking tea, living near environmental pollution source, dyeing hair or perming and folate use.

[b]FDR_*P* < 0.1 was considered to indicate a statistically significant difference.

Interactions between maternal smoke exposure and *MTHFD* gene for risk of CHD

We modestly identified the four polymorphisms including rs1950902, rs2236222, rs1256142, and rs11849530 with significant main effects on CHD risk in the Han Chinese population. Moreover, though rs2236225 was not observed a significant main effect on CHD risk, this polymorphism was the most extensively studied one. Thus, we kept the 5 SNPs of the *MTHFD* gene including rs1950902, rs2236222, rs1256142, rs11849530 and rs2236225 for the interactions analysis. Interactions between maternal SNPs of *MTHFD* gene in the corresponding genetic model and maternal smoke exposure on CHD risk were summarized in Table 4. Our results showed there were statistically significant interaction effects between active smoking in 3 months before pregnancy and genetic variants of maternal *MTHFD* gene at rs1950902, rs2236225 and rs11849530. Specifically, mothers with GG/GA genotypes at rs1950902 (the addictive model: aOR = 1.44, 95% CI: 1.02-2.04), AA/GA genotypes at rs2236225 (the addictive model: aOR = 2.15, 95% CI: 1.14-4.05) and GG/GA genotypes at rs11849530 (the addictive model: aOR = 2.10, 95% CI: 1.21-3.64) generated a 1.44-fold, 2.10-fold and 2.15-fold increased CHD risk when they smoked in 3 months before pregnancy, respectively. Additionally, maternal passive smoking was also observed interaction effects with *MTHFD* gene at rs1950902, rs2236225, rs2236222, rs11849530 and rs1256142 in the Han Chinese population**.** To be specific, the mothers with GG/GA genotypes at rs1950902 (the addictive model: aOR = 1.22, 95% CI: 1.07-1.40), AA/GA genotypes at rs2236225 (the addictive model: aOR = 1.38, 95% CI: 1.12-1.70), GG/GA genotypes at rs11849530 (the addictive model: aOR = 1.35, 95% CI: 1.13-1.61) or AA genotype at rs1256142 (the dominant model: aOR = 1.26, 95% CI: 1.06-1.50) had significantly higher CHD risk in offspring when they were exposed to secondhand smoke in 3 months before pregnancy. Moreover, when mothers carried the AA/GA genotypes at rs1950902 (the addictive model: aOR = 1.38, 95% CI: 1.18-1.60), AA/GA genotypes at rs2236225 (the addictive model: aOR = 1.62, 95% CI: 1.29-2.04), GG/GA genotypes at rs2236222 (the addictive model: aOR = 1.38, 95% CI: 1.11-1.72), GG/GA genotypes at rs11849530 (the addictive model: aOR = 1.64, 95% CI: 1.34-2.00) or AA genotype at rs1256142 (the dominant model: aOR = 1.53, 95% CI: 1.26-1.86), exposure to secondhand smoke in the first trimester w**o**uld increase the susceptibility to suffer from a CHD-affected delivery.

Discussion

Convincing evidence implies that periconceptional intake of folic acid leads to a 40% to 60% reduction in the risk of a CHD-affected delivery, which makes investigating the association between genetic polymorphisms in genes related to folate metabolism and CHD risk an attractive pursuit[38]. The *MTHFD* gene plays a key role in the folate-homocysteine metabolism pathway, encoding a single protein with three catalytic properties crucial for DNA synthesis, DNA repair, and methylation reactions. The epidemiologic and experimental studies indicate the plausible association between genetic variants of the *MTHFD* gene and the risk of CHD[11-14, 39], but previous efforts on the association have yielded controversial results[40] and are limited to a small number of functional nonsynonymous polymorphisms. This is therefore the first study to explore the other variants in the coding region of the *MTHFD* gene on CHD risk and represent replication efforts in Han Chinese populations. Furthermore, folate-homocysteine metabolism may partly modulate the effect leading to varying susceptibility to the CHD of tobacco exposure[26, 33]. Thus this study also seeks to examine the interactions of maternal tobacco exposure and polymorphisms of the *MTHFD* gene on CHD risk, which may help to provide new clues for future etiological research and intervention of CHD.

In the present study, four genetic variants of maternal *MTHFD* gene including rs1950902, rs2236222, rs1256142 and rs11849530 were revealed to have significant associations with an increased risk of CHD in case-control studies based on the Han Chinese population (Figure.1 B). The information from the public databases and the literature revealed unequivocally these SNPs were all within the coding region; one was functional nonsynonymous polymorphism with a known biochemical phenotype and the other were synonymous ones[8, 11]. Notably, the well-studied polymorphism rs1950902 G→A (*MTHFD* G401A) leads to an arginine (G allele) to a lysine (A allele) substitution, lying within the dehydrogenase/cyclohydrolase domain of MTHFD protein. The mutant genotypes of the *MTHFD* gene at rs1950902 may influence the stability of the enzyme and change the catalytic activity[17], causing disturbance of the folate-homocysteine metabolism pathway and therefore may alter folate or homocysteine levels. It was reported that rs1950902 was significantly related to elevated plasma homocysteine and reduced folate levels[16, 39]. These observations suggested that *MTHFD* rs1950902 could affect embryonic development employing restricted DNA synthesis and a high level of homocysteine. Considering the results of the present study and the phenotypes related to rs1950902, we postulate that this polymorphism plays a role in fetal heart development. One previous study[41], however, reported negative results that the significant association between *MTHFD* 1950902 and tetralogy of Fallot were not observed. Due to our limited sample size of each subtype of CHD cases, we didn't analyze the association between specific CHD subtypes and genetic variants of maternal *MTHFD* gene. Besides, the present study modestly found the synonymous polymorphisms within the intronic region including rs2236222, rs1256142 and rs11849530 were associated with increased CHD risk, and the plausible mechanism of these polymorphisms increasing CHD susceptibility was likely to affect codon usage and translational efficiency[42].

Particularly, it was worth mentioning that *MTHFD* rs2236225 (*MTHFD* R653Q), the most investigated genetic variant of the *MTHFD* gene, leads to an arginine (G allele) to glutamine (A allele) substitution in

the MTHFD protein. Convincing evidence suggested that mutant type protein of *MTHFD* gene at rs2236225 caused significant DNA synthesis restriction[11] and increased homocysteine levels[14, 16, 39]. In addition, the specific CHD subtypes such as atrial septal defects[13], tetralogy of Fallot [43] and aortic stenosis[11] had been reported significant associations with this polymorphism. In this present study, however, we did not observe the significant association between *MTHFD* rs2236225 and CHD risk, which may partly be explained by the different susceptibility of folate-homocysteine imbalance to every subtype of CHD and the relatively low proportion of the specific high susceptibility to subtypes in our study[5]. Overall, some of the polymorphisms involved in our study had not been confirmed before, and literature involved in the association between genetic variants of maternal *MTHFD* gene and CHD was still lack. It needs further and clearer evidence to figure out the mechanism.

We also examined the association between maternal smoking including active and passive smoking and the risk of CHD in offspring. Findings from the present study suggested that mothers who reported passive smoking at home or in the workplace 3 months before pregnancy and the first trimester of pregnancy were observed a 1.56-fold and 2.24-fold increased CHD risk, respectively, which was basically consistent with a recent meta-analysis [40]. Obviously, the maternal passive tobacco exposure in the first trimester of pregnancy was shown more harmful than in 3 months before pregnancy, which may be partly explained by the fact that the former was the sensitive period for fetal heart development. Additionally, a great number of previous epidemiologic studies supported that periconceptional active smoking was associated with risk of CHD in offspring[40]. However, we modestly did not observe that maternal active smoking in 3 months before pregnancy could increase CHD risk after adjusted potential confounders, and even none of our subjects reported active smoking during pregnancy. The causes for the insignificant association between maternal active smoking in 3 months before pregnancy and CHD risk may be due to our limited sample size and the subjective smoking records. There was a possibility that pregnant smokers underreported their smoking and such potential misclassification might lead to underestimation of the impact of maternal active smoking on CHD. The behavior was attributed to medical and societal pressures that made pregnant women reluctant to report their smoking activities[44].

Our results also showed the different degrees of interaction effects between polymorphisms of *MTHFD* gene including rs1950902, rs2236222, rs1256142, rs11849530 and rs2236225 and maternal tobacco exposure (Figure.1 B). As shown in the figure, active smoking before pregnancy seemed more harmful interacted with the SNPs of the *MTHFD* gene on CHD risk, compared with passive smoking before pregnancy or in the first trimester. Of note, among these polymorphisms, *MTHFD* rs2236225 was relatively the most effective one to modify the association between maternal tobacco exposure and CHD risk (aOR=2.15). However, studies concerning the interactions of SNPs in folate-related genes and maternal tobacco exposure were lack, Hobbs[45] indicated that the combined effect of elevations in maternal homocysteine, smoking, and the *MTHFR* 677C>T polymorphism increased the risk of having a CHD-affected pregnancy (aOR=11.8). Possible mechanisms by which the *MTHFD* gene interacted with maternal tobacco exposure to increase CHD susceptibility include elevated serum homocysteine, increased DNA methylation, and DNA synthesis restriction. It was well-studied that the maternal tobacco

exposure was observed associations with substantial reductions of folate levels[27-29, 32], elevated levels of homocysteine[46] in cord blood, and altered global genomic methylation. The polymorphisms of the *MTHFD* gene also showed a close relation to plasma homocysteine and DNA methylation. Hence, the mutant type of MTHFD protein and periconceptional tobacco exposure jointly caused the plasma homocysteine elevated, and high levels of homocysteine consequently affected fetal heart development by disruption of gene methylation, increasing oxidative stress and homocysteinylation of key proteins (Figure.1 A). Additionally, a recent study suggested that maternal folate-homocysteine metabolism may partly modify the influence of maternal tobacco exposure during pregnancy on the DNA methylation of newborn epigenome and therefore affected embryonic development[33], which provided indirect evidence to support our findings. Nevertheless, specific mechanisms are unclear and need further research.

The limitations of the study need to be addressed. First, based on a case-control study, information on maternal tobacco exposure was collected through self-reported interviews, and therefore recall bias inevitably had to be taken into consideration. To reduce recall bias to some extent, the exposure information was further confirmed by consulting their Maternal and Child Health Manual and medical records. Second, despite adjusting many confounders, potential confounding factors cannot be entirely ruled out. Third, there are so many genes that are also involved in cardiovascular development, but we only focused on the *MTHFD* gene. Fourth, considering population stratification bias in epidemiologic studies, we recruited the participants restricted to the Han Chinese ethnicity, and further work was needed to estimate the effect of the *MTHFD* gene and maternal tobacco exposure in CHD risk within other populations. Fifth, sample size limitations prevented us from examining specific CHD subtypes.

Conclusions

The current findings observed that maternal polymorphisms of the *MTHFD* gene at rs1950902, rs2236222, rs11849530, and rs1256142 were significantly associated with the risk of CHD in offspring. In addition, a positive association between maternal passive smoking in the periconceptional period and risk of CHD was found. Furthermore, our results showed the different degrees of interaction effects between polymorphisms of the *MTHFD* gene including rs1950902, rs2236222, rs1256142, rs11849530 and rs2236225, and maternal tobacco exposure. It seemed that *MTHFD* rs2236225 was relatively the most effective one to modify the association between maternal tobacco exposure and CHD risk among these polymorphisms, and maternal active smoking was more harmful interacted with SNPs of the *MTHFD* gene on CHD risk, compared with passive smoking. However, considering the complexity of the mechanism and the limitation of sample size, more studies in different ethnic populations with a larger sample and prospective designs were required to confirm our findings.

List Of Abbreviations

CHD = congenital heart disease; aOR = adjusted odds ratio; CI = confidence interval; SNPs = single nucleotide polymorphisms; *MTHFD* = methylenetetrahydrofolate dehydrogenase; FDR_*P* = false discovery rate *P* value; GMS = genetic model selection; HWE = Hardy-Weinberg equilibrium

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Xiangya School of Public Health Central South University (No. XYGW-2018-36). Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

QXL, JYD, JQL and YHL performed the experiments. SMZ, TTW and LJZ analyzed the data and statistical analyses. JS, YPL and MTS contributed reagents/material/analysis tools. XLS and JBQ wrote the main manuscript text. PH, LTC, JHW and MTS collected reference and managed data.

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Figures

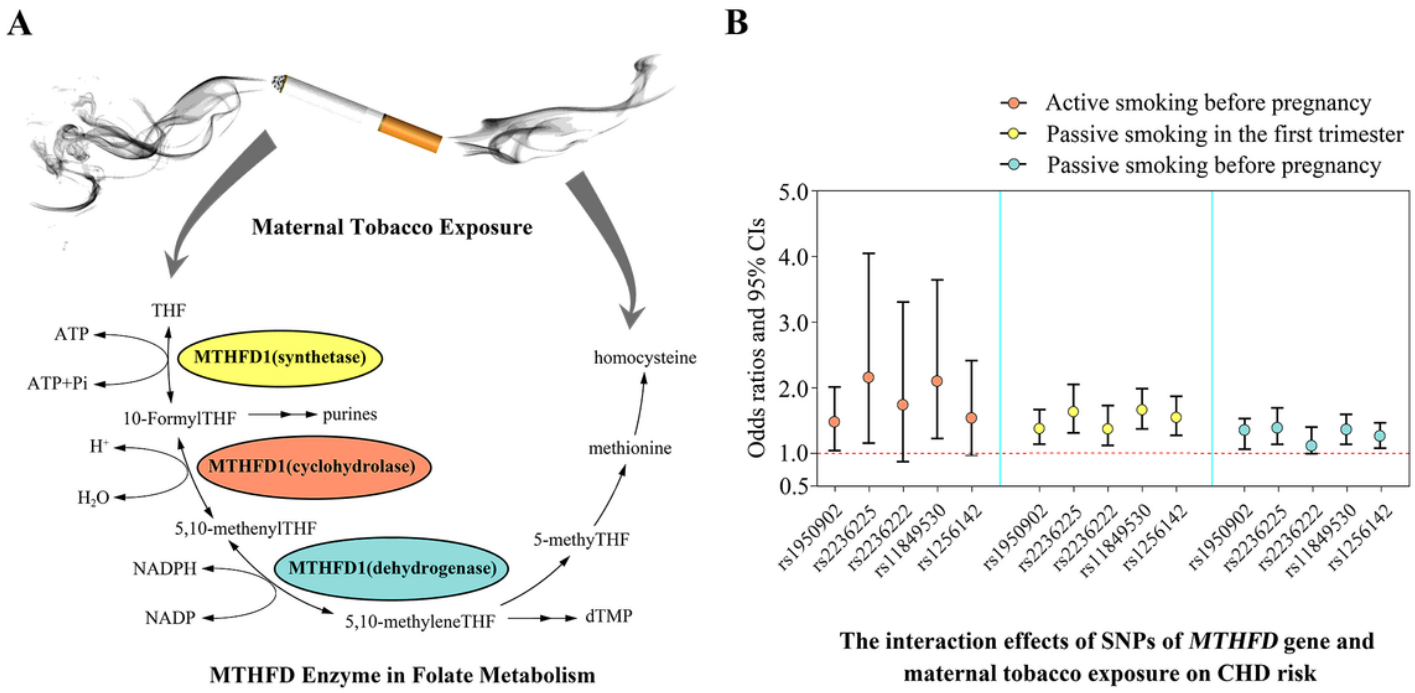

Figure 1

Possible mechanisms and the key findings Abbreviations: MTHFD = methylenetetrahydrofolate dehydrogenase; THF = tetrahydrofolate; CHD = congenital heart disease; SNPs = single nucleotide polymorphisms. A) The possible mechanisms by which genetic variants of MTHFD gene interacted with

maternal tobacco exposure on the risk of CHD. B) The interaction effects of SNPs of the MTHFD gene and maternal tobacco exposure on CHD risk.

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