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Original Article

Relationship of MTHFD1 G1958A and CBS 844ins68 polymorphism with congenital heart defects in North Indian population (Jammu and Kashmir): A case-control study

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ABSTRACT

Objectives: There are many multifactorial causes for Congenital Heart Defects (CHDs) in which both genetic and non-genetic factors play role. MTHFD1 and CBS are two of the key enzymes that plays pivotal role in the metabolic pathway of homocysteine. Most of the studies revealed that genes involved in folate/homocysteine pathways are involved in the occurrence of CHDs. The present study was planned to investigate the role of common polymorphisms in MTHFD1 and CBS gene in children with CHD in Jammu region of Jammu and Kashmir UT.

Material and Methods: A total of 160 (80 CHD patients and 80 controls) children were enrolled for the present case-control study. After extraction of genomic DNA genotyping of SNP MTHFD1 G1958A(rs2236225) was done by PCR-RFLP and CBS 844ins68 polymorphism was done by PCR technique.

Results: Our results show that there is no significant association between MTHFD1G1958A and CBS 844ins68 polymorphism with CHD. In case of SNP MTHFD1 G1958A allele A found to be higher in both patient and control group and inCBS 844ins68 polymorphism frequency of risk allele 'I' found higher in cases (0.06) as compared to controls (0.04). The homozygous genotype for 844ins68 (II) was found absent in both the patients and control group.

Conclusion: We conclude that both MTHFD1 G1958A and CBS 844ins68 polymorphism were not found to be genetic risk factor in the development of CHD in population of Jammu region of Jammu and Kashmir UT.

Keywords: Congenital heart defects, Homocysteine pathways, Polymorphism, Jammu

INTRODUCTION

Congenital heart defects (CHDs) are defined as a birth defect that results due to improper development of cardiac blood vessels during the developmental stage. Congenital heart disease caused 261,247 deaths (95% uncertainty interval 216,567–308,159) globally in 2017, a 34.5% decline from 1990, with 180,624 deaths (146,825–214,178) being among infants.[1] In India, Uttar Pradesh, Bihar, and Assam show the highest prevalence (264.7, 224.37, and 212.71/100,000 individuals per year) and the lowest in Goa and Kerala (140.69 and 142/100,000). The etiology of CHD is not clear; it is generally agreed that the development of CHD is likely to be caused by both genetic and non-genetic risk factors.[2,3] Genetic alterations such as chromosomal aberrations, single-gene disorders, and variation in the common gene with small effects seem to be the major causes of CHD.[4]

Many studies revealed that folate consumption during early pregnancy and the whole gestational period could decrease the chances of CHD.[5,6] Therefore, deficiency of any folate metabolic enzymes due to any gene alterations results in an inborn error in the metabolism of homocysteine which is an independent risk factor for CHD and various cardiovascular diseases.[7,8] Several investigations involving different populations have been done to recognize the association of different candidate genes of homocysteine and folate pathway enzymes such as MTHFR, MTR/MS, MTRR, cystathionine beta-synthase (CBS),[7] methylenetetrahydrofolate dehydrogenase (MTHFD1),[9] TYMS,[10] MTHFD1, and

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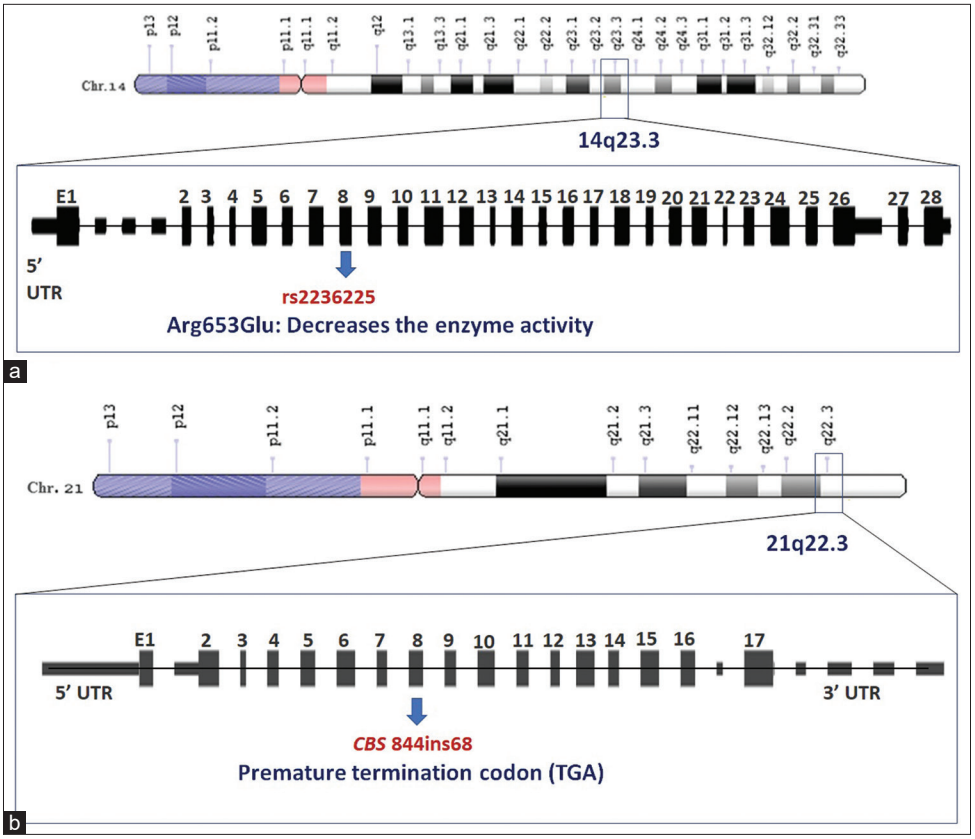

Figure 1: (a) Cytogenetic location, structure, and SNP G1958A (rs2236225) of methylenetetrahydrofolate dehydrogenase 1. (b) Cytogenetic location, structure, and 844ins68 polymorphism of cystathionine beta-synthase.

CBS[11] gene polymorphism with CHD. Single-gene polymorphisms in genes encoding these enzymes nowadays could be research hotspots of genetic susceptible factors for CHD.[11] In the present study, we selected two folate-dependent enzymes which have an important role in folate and homocysteine metabolism and are attractive candidate genes in CHD which are MTHFD1 and CBS.

MTHFD1 gene is located on the long arm of chromosome 14 (14q23.3) consists of 28 exons [Figure 1a] and encodes cytoplasmic NADP-dependent enzyme which is trifunctional and has a key role in folate metabolism. The MTHFD1 enzyme has two independent domains: At N-terminal, the dehydrogenase-cyclohydrolase domain and C-terminal, the synthetase domain (GeneCards-Human Genes). *MTHFD1* G1958A variant presents on exon 21 and synthetase domain of the enzyme, it results in the replacement of amino acid arginine to glutamate at 653 position and, hence, decreases the enzyme activity.[11]

CBS gene is located on the long arm of chromosome 21 (21q22.3) spans over 30 kb and consists of 23 exons[12] [Figure 1b]. The *CBS* gene encodes the enzyme cystathionine β-synthase involved in the folate pathway. This enzyme has a key role in the transsulfuration pathway and can use Vitamin B6 as a cofactor. Polymorphism 844ins68 is a 68 base pairs insertion that occurs in exon 8 in the *CBS* gene between nucleotide 844 and 845 and results in interruption of the normal sequence of the codon 282 (proline, at nt 844), this insertion encodes 11 amino acids followed by a premature termination codon (TGA) in the *CBS* mRNA.[21] It catalyzes the first step of the transsulfuration pathway which is a conversion of homocysteine and serine to cystathionine that acts as a substrate for amino acid cysteine synthesis, as shown in [Figure 2]. The present study was undertaken to assess the MTHFD1 G1958A polymorphism and CBS 844ins68 gene polymorphism in CHD patients and controls in a population of the Jammu region of J&K UT.

MATERIAL AND METHODS

Study population and area

The present study was carried out on 80 confirmed cases of CHD and 80 unrelated healthy controls belonging to different areas of the Jammu region of Jammu and Kashmir UT. These were enrolled in the outpatient and inpatient Department of Pediatrics, SMGS Hospital, Government Medical College, Jammu. We included that cases with moderate-to-severe

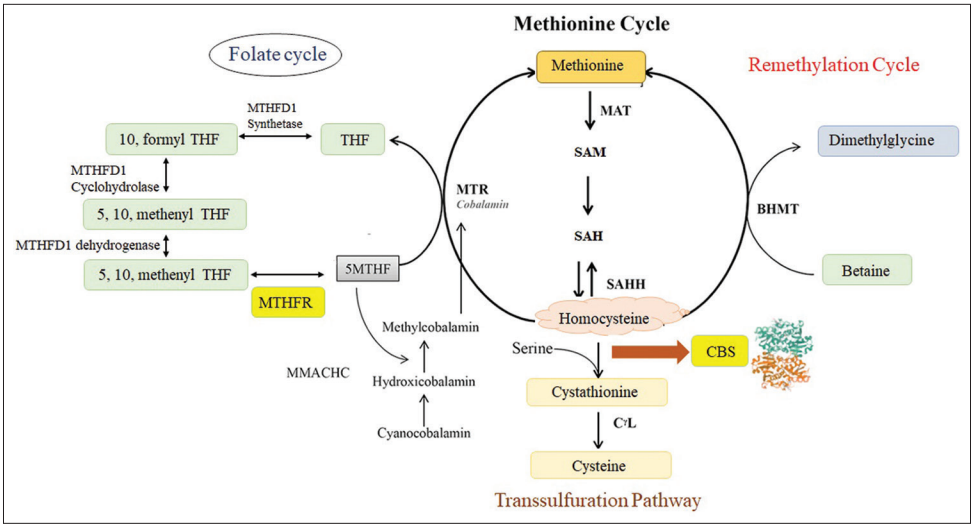

Figure 2: The homocysteine and folate metabolic pathway: Role of different enzymes (MTHFD1 and CBS enzyme) in homocysteine metabolism. (THF: Tetrahydrofolate, MTHFD1: Methylenetetrahydrofolate dehydrogenase 1, CBS: Cystathionine beta-synthase, MTHFR: Methylenetetrahydrofolate reductase, MTR: Methionine synthase, and MTRR: Methionine synthase reductase).

CHD phenotypes confirmed by echocardiography and cases with chromosomal anomalies or genetic syndromes or with any other birth defect in addition to CHD were excluded from the study. According to the Global Burden Disorder-2019, prevalence estimates of CHD in India are 0.2% (GBD Compare|IHME Viz Hub (healthdata.org), and thus, the sample size was calculated using the prevalence of the disease with a 95% confidence interval and 5% margin of error and found the required sample size (Sample Size Calculator).

Ethical approval and collection of data and blood sample

The present study design was duly approved by the Institutional Ethical Committee, University of Jammu (RA/19/3119). For the data collection, a detailed pre-designed health questionnaire including parameters such as age, gender, dwelling, educational status, clinical profile, and a detailed family history of the patient was taken by interviewing the parents/guardian of the patient. Each study participant was made aware of the nature of the study. Blood collection was done in EDTA coated vial after having informed written consent from each study participant/attendant or guardian of the patient after that blood samples were stored at −20°C until DNA was isolated at Central Facility Laboratories of Department of Zoology and Major Lab of Institute of Human Genetics, University of Jammu.

Extraction of genomic DNA and genotyping

Extraction of genomic DNA was done by phenol-chloroform method and qualitative and quantitative analysis of extracted DNA was done by 0.8% of agarose gel electrophoresis and spectrophotometry.

Genotyping of MTHFD1 gene

The determination of MTHFD1 gene polymorphism was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping technique. The target DNA amplification of MTHFD1 gene polymorphism was detected using forward primer 5'-CCTGGTTTCCACAGGGCACTC-3' and reverse primer 5'-CCACGTGGGGGCAGAGGCCGGAATACCGG-3'.[13] PCR components include 5 µl ×5 PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 0.5 µl dNTP mix (10 mM), 0.5 µl of each primer (100 pmol/µl), 2 µl DNA (100 ng/µl), 0.2µl *Taq* Polymerase (5U/µl), and 14.8 µl of DH_2O forming final 25 µl of total reaction mixture.

PCR thermal cycler conditions: It includes 36 cycles involving initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. The amplified product size obtained for MTHFD1 gene was 310 bp.

RFLP conditions: The 310 bp PCR product thus obtained was given restriction digestion with *MspI* restriction endonuclease and the components used were 2 µl restriction buffer, 0.2 µl *Msp I* enzyme, 10 µl PCR product, and 7.8 µl double-distilled water forming total 20 µl volume and then kept overnight at 37°C in a water bath. The results of RFLP were then visualized by 3.5% of agarose gel electrophoresis.

Genotyping of CBS gene

This *CBS* 844ins68 polymorphism was done by PCR using forward primer 5'-CGGCGGTATTGGCCACTC-3' and reverse primer 5'-GGCCGGGCTCTGGACTC-3' (Boyles *et al*., 2006). The PCR reaction mixture includes 5 µl ×5 PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 0.5 µl dNTP mix (10 mM), 0.5 µl of each primer (100 pmol/µl), 2 µl DNA (100 ng/µl), and 0.4 µl *Taq* polymerase (5U/µl) forming 25 µl total reaction volume.

PCR conditions include initial denaturation at 95°C for 5 min, denaturation at 94°C for 50 s, annealing at 61°C for 1 min, extension at 72°C for 45 s, and final extension at 72°C for 7 min. Then, the PCR product thus obtained was analyzed using 3% agarose gel electrophoresis.

Statistical analysis

The descriptive analysis was done using mean ± standard deviation and frequency distribution. Genotypic and allelic frequency was calculated by the gene counting method. Hardy–Weinberg equilibrium (HWE) analysis and the differences in genotypic frequencies between two study groups were examined using Pearson's goodness of Chi-square test. To access the association of CHD risk by risk variants, odds ratios (ORs) with a 95% confidence interval (CI) were calculated under different genetic models using the Statistical Package for the Social Sciences (SPSS-version 20) software and using MedCalc software (https://www.medcalc.org/calc/odds_ratio.php). $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Demographics features

A total of 160 individuals 80 (50%) CHD cases and 80 (50%) controls were included in the study. There were 49 (61.25%) male and 31 (38.75%) female patients and the male-to-female ratio was approximately 5:3. About 53.75% of patients were under the age of 3 years, 31.25% were between 4 and 6 years, 10% were between 7 and 9 years, and the rest 5% were above 10 years of age. With respect to controls, the frequency of males was 60% and that of females was 40%.

Regarding the clinical presentation, different clinical symptoms observed in CHD patients were heart murmur in 83.75% of cases, breathing difficulty in 65%, poor weight gain (42.5%), feeding difficulty (40%), tachycardia (36.25%), pyrexia (33.75%), respiratory distress (28.75%), anemia (23.75%), cyanosis (20), and history of seizures (11.25%) [Figure 3a]. These clinical complications were found to be due to abnormal heart functioning due to a defect in the structure of the heart.

We have also observed that about 22.5% of babies were prematurely delivered and the rest 77.5% were full-term babies. Furthermore, delayed crying after birth was observed in 20% of cases. About 32% of babies were found to be delivered through cesarean mode; the rest 48% were delivered normally. Birth order Ist was found to be in 37.5% of cases, followed by 3rd birth order in 35% of cases, 2nd birth order in 15% of cases, and the rest 10% of cases of birth order 4th [Figure 3b].

Genotyping

MTHFD1 G1958A

Genotyping of MTHFD1 G1958A gene polymorphism

The G to A substitution at nucleotide 1958 of *MTHFD1* presents on exon 21, results in the replacement of amino acid arginine to glutamate at 653 positions, and, hence, creates a *MspI* restriction enzyme site. "G" is the ancestral allele and marked the presence of a restriction site for the *MspI* enzyme whereas the absence of a restriction site confirms minor or rare allele "A." However, the genotyping results of the present study have revealed that "A" allele is behaving as the major allele in our population and "G" allele is the minor or risk allele for the disease undertaken in the present study. The genotypes of all individuals were obtained as GG – 196 bp, 86 bp, and 28 bp (wild), AG – 282 bp, 196 bp, 86 bp, and 28 bp (heterozygous), and AA – 282 bp and 28 bp (variant) [Figure 4].

Both the study groups were in the range of HWE. The allocation of genotypes and alleles of study individuals along with Chi-square values for HWE is shown in [Table 1]. The association of *MTHFD1 G1958A* polymorphism with the risk of CHD can be obtained by calculating the OR through logistic regression analysis [Table 2]. Here, we have taken G-allele as a risk allele for CHD. No significant association was found under any of the tested genes. In general, we can conclude that *MTHFD1* G1958A polymorphism is a weak marker for CHD risk in a population of Jammu.

Genotypic and Allelic frequencies of CBS 844ins68 gene polymorphism

After genotyping 160 individuals (80 CHD cases and 80 controls) [Figure 5], it was observed that out of 80 CHD patients, 71 (88.75%) found to have homozygous wild-type genotype WW, 9 (11.25%) have heterozygous genotype IW, and 0 (0%) have homozygous variant type genotype II. In case of 80 control individuals in 92.5% (n = 74) of cases were found with WW genotype, 7.5% (n = 6)of cases with heterozygous genotype and none have been found with homozygous variant type genotype (II).

As for allelic frequency was concerned, the frequency of a wild allele (W) in CHD patients was found 0.94 whereas in controls was 0.96, respectively, the frequency of the risk (I) allele in CHD cases was found to be 0.06 which is slightly higher as compared to controls, that is, 0.04. The variant

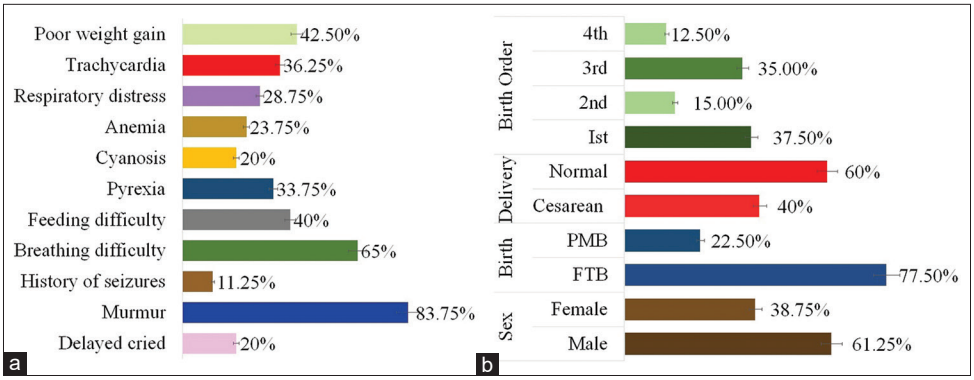

Figure 3: (a) Clinical features variations among patients. (b) Features variation among cases.

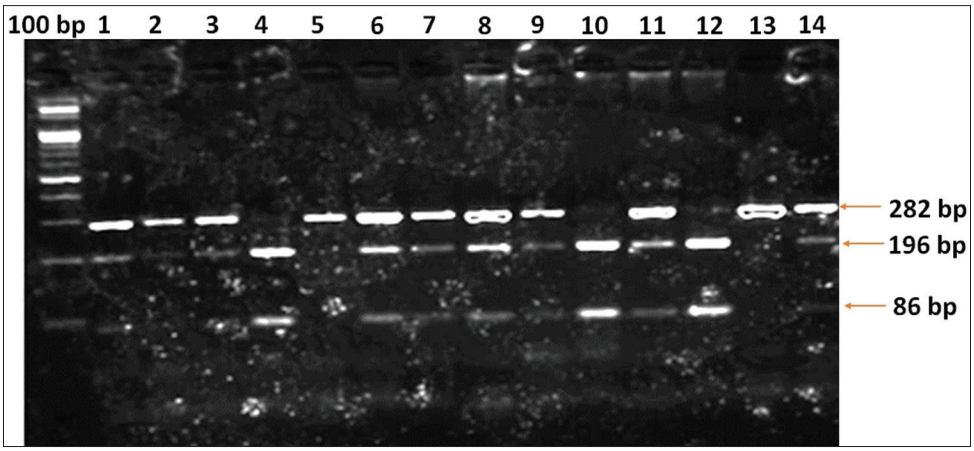

Figure 4: The restriction digestion of MTHFD1 PCR product with Msp I enzyme. Lanes no. 1–100 bp ladder, lanes 2, 4, 7, 8, 9, 10, 12, and 15 – heterozygous (GA), lanes 3, 6, and 14 – homozygous wild (AA), and lanes 5, 11, and 13 – homozygous variant (GG).

Table 1: Genotypic and allelic frequency distribution in CHD patients and controls for *MTHFD1*G1958A polymorphism.

Category	Genotypes/alleles (%)					χ^2	*P*-value
	AA (Wild)	AG (Hetero)	GG (Variant)	A (Wild)	G (Risk)		
CHD cases (*n*=80)	26 (32.5%)	44 (55%)	10 (12.5%)	0.6	0.4	1.7	>0.05
Controls (*n*=80)	21 (26.25%)	43 (53.75%)	16 (20%)	0.53	0.47	0.5	>0.05

Table 2: Association of MTHFD1 polymorphism with CHD.

Genotypes/ alleles	Cases (*n*=80)	Controls (*n*=80)	OR (C.I.=95%)	*P*-value
AA	26	21	1 (Reference)	-
AG	44	43	0.83 (0.41–1.68)	0.6
GG	10	16	0.51 (0.19–1.34)	0.2
AG+GG	54	59	0.74 (0.37–1.46)	0.4
A	96	85	1 (Reference)	-
G	64	75	0.76 (0.45–1.18)	0.2

genotype "II" was absent in both cases and controls. The genotypic and allelic frequencies along with Chi-square values for Hardy–Weinberg calculations for the study participants are shown in [Table 3].

DISCUSSION

Variation in the genes involved in the folate pathway plays an important role in the etiology of congenital anomalies, including CHD.[14] Folate is essential for the proper development of the embryo and the fetus, it is very important during embryogenesis because it can help prevent some major birth defects like congenital heart defects and neural tube defects. It has been hypothesized that genetic polymorphisms in folate-metabolizing enzymes affect global

Table 3: Genotypic and allelic distribution of CBS polymorphism among cases and controls along with χ^2 and *P* values for Hardy–Weinberg equilibrium calculations.

Category	Genotypes/alleles (%)					χ^2	*P*-value
	WW (Wild)	1W (Hetero)	II (Variant)	W (Wild)	I (Risk)		
CHD cases (n=80)	71 (88.75%)	9 (11.25%)	0 (0%)	0.94	0.06	0.28	0.59
Controls (n=80)	74 (92.5%)	6 (7.5%)	0 (0%)	0.96	0.04	0.12	0.72

*We were not able to calculate odds ratio for *CBS* due to a lack of II genotypes in both CHD cases and control participants

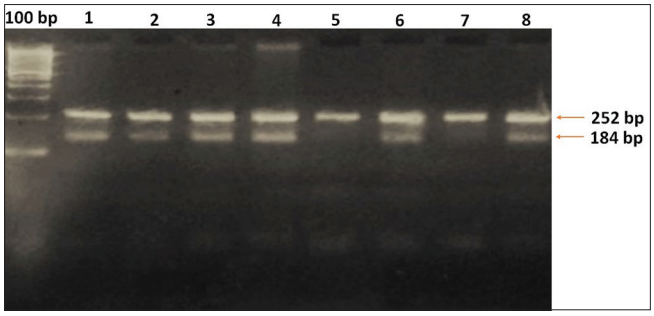

Figure 5: PCR products of CBS 844ins68 polymorphism. Lanes 1–100 bp ladder, lanes 2, 3, 4, 5, 7, and 9 – heterozygous type (IW), and lanes 6 and 8 – homozygous wild-type genotype (WW).

DNA methylation as well as changes in the availability of nucleotides for the synthesis and repair of DNA.[15]

MTHFD1 gene encodes a trifunctional NADP-dependent cytoplasmic enzyme (5, 10 MTHFD, 5, 10-methenyltetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase) and is one of the enzymes involved in folate and homocysteine metabolism. The three activities of the *MTHFD1* enzyme catalyze one of three sequential reactions in the interconversion of 1-carbon derivatives of tetrahydrofolate, which are substrates for methionine, and *de novo* purine and pyrimidine synthesis. *MTHFD1* G1958A variant presents in the synthetase domain of the enzyme, results in the replacement of arginine by glutamate, and, hence, reduces the enzymatic activity. This substitution reduces the *de novo* synthesis of methionine and hence alters DNA methylation reactions which are an essential phenomenon for embryogenesis.[11] Several studies suggested the association of *MTHFD1* G1958A polymorphism with CHD.[9,16,11] However, various other studies showed a null association between *MTHFD1* G1958A polymorphism and CHD.[17,18]

In the present study, we did not observe any significant difference between the genotypic frequency of *MTHFD1* G1958A polymorphism in CHD cases and controls. In both CHD patients as well as in controls, the genotypic frequency was in agreement with HWE. In our study from the Jammu region, we reported a lack of association of *MTHFD1* G1958A polymorphism with the risk of CHD. Likewise, Wang and the group also failed to proclaim a significant relation of *MTHFD1* A1958G polymorphism with the risk of CHD under any of the genetic models.[17] No significant association was observed between the CHD patients and controls in both genotypic and allelic distributions of *MTHFD1* A1958G gene polymorphism in the Chinese population.[18] On the other hand, Shaw and group have found a significant association of *MTHFD1* G1958A variant with conotruncal heart defects with OR 1.7 (1.1–2.7) in the population of California,[16] another study by Christensen *et al*., these workers also reported MTHFD1 A1958G polymorphism associated with increased risk of CHD (OR 2.11 95% CI [1.01–4.42]) in the Canadian population.[9]

To the best of our knowledge, there is no previous report from India and we are the first to analyze this MTHFD1 G1958A variant from North India concerning CHD. In our investigation in both patient and control groups, the frequency of "AA" genotype was found to be more compared to GG genotype so "A" allele was found to be a major allele, and the G allele act as a minor or risk allele in the present study, and also, this is compatible with the genotypic frequency as reported by Jaiswal *et al.*[19] (in Down syndrome patients' mothers and control mothers) in North Indian.

The *CBS* gene encodes the enzyme cystathionine β-synthase which converts homocysteine to cystathionine which is the first irreversible step in the transsulfuration pathway and hence maintained homocysteine concentration in blood. The 68 bp insertion in the *CBS* gene at exon 8 between base 844 and 845 (844ins68) has induced a TGA and, hence, altered gene expression.[20] This insertion has been associated with elevated homocysteine and homocystinuria level which, in turn, is associated with many congenital malformations and CHD.[21] In the fetus, *CBS* expression is confined to only neural tube and heart tissues (in endocardium cells) that can specify its potential function in the cardiac development of the embryo.[8] *CBS* 8444iss68 was previously studied to be associated with many diseases such as premature occlusive arterial disease,[22] congenital heart disease,[23] and cleft lip and palate.[24] Furthermore, *CBS* 844ins68 polymorphism has shown a null association with diseases such as vascular thromboembolic disease,[25] cardiovascular diseases, and mentally retarded (MR) children.[26]

In our study, we did not find any significant difference in genotypic frequency of *CBS* 844ins68 polymorphism in cases and controls. Likewise, a previous study carried out by Li *et al*., 2005, also reported heterozygous condition

for *CBS* 844ins68 (12.57%) more in CHD patients than in controls (2.97%), and also, they did not report any individual with homozygous mutant genotype (II) but they found a significant association of *CBS* 844ins68 with CHD and reported *CBS* 844ins68 as a risk factor for CHD. A study from India on the non-syndromic cleft lip with or without cleft palate (NSCLP) patients by Murthy *et al*., 2015, found a significant association of *CBS* 844ins68 polymorphism with NSCLP and reported no homozygous condition for mutant genotype (II) in any individual and frequency of heterozygous IW genotype in the patient group observed to be 5.9% and in controls 10.4%.

In the present study, we did not observe any study on *CBS* 844ins68 polymorphism in association with CHD. Another study conducted by Dutta *et al*., 2005, from India on MR children found a lack of association of *CBS* 844ins68 polymorphism with MR children, genotypic frequency observed in MR children was WW (93.7%), IW (6.31%), and II (0).

Enclosing the section, there are hardly a few studies dealing with the genetic background of CHD from India, this is the first-ever report on the genetic susceptibility of CHD with *MTHFD1*A1958G and *CBS* 844ins68 polymorphisms in the population of Jammu. Furthermore, as we did not find any previous report on the role of said polymorphisms with CHD. Result disparity might be due to diverse reasons, first might be due to the variation in the ethnicity, second might be the different sample size, third might be the technique used for the genotyping, and many other factors could play a role. Hence, to confirm the current findings, larger studies with more rigorous study designs that include other ethnic populations are necessary and this might be accomplished by meta-analysis.

CONCLUSION

In the present work, genetic analysis of *MTHFD1*G1958A and *CBS* 844ins68 polymorphism was not found associated with CHD in our population. No significant differences were observed in the genotypic frequency of both *MTHFD1*G1958A and *CBS* 844ins68 polymorphism between patients and controls. In general, we can conclude that both *MTHFD1* A1958G and *CBS* 844ins68 polymorphism were weak markers for CHD risk in our population.

Statement of ethics

The present study design was duly approved by the Institutional Ethics Committee, the University of Jammu vide notification number (**RA/19/3119**).

Consent to participate

Data were done after having informed written consent from each study participant.

Consent for publication

Not applicable.

Data availability statement

The data from the present study are unavailable to the public due to restrictions. Interested researchers should contact Dr. Parvinder Kumar (Parvinderkb2003@gmail.com) who oversaw the data collection.

Authors' contributions

Ankush Bala and Dr. Jyotdeep Kour Raina conducted the experiment, Ankush Bala drafted the manuscript, Amrit Sudershan edited the manuscript, figures, and table, Dr. Sanjeev Digra assisted in the diagnosis of the suspected subject, Manoj K Dhar and Rakesh K Panjalia edited the manuscript, and whole research work was conducted under the guidance of Dr. Parvinder Kumar.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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