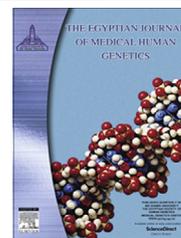




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ORIGINAL ARTICLE

# *MTRR* gene variants may predispose to the risk of Congenital Heart Disease in Down syndrome patients of Indian origin



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## KEYWORDS

Down syndrome;  
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**Abstract** *Background:* Down syndrome (DS), also called as trisomy 21, is one of the most leading cause of intellectual disability. DS is associated with a number of phenotypes including Congenital Heart Disease (CHD), Leukemia, Alzheimer's disease, Hirschsprung's disease and others. DS affects about 1 in 700 live births.

*Objectives:* The study aims to investigate the association of *MTRR* (*Methionine synthase reductase*) gene polymorphisms (C524T and A66G) with the risk of CHD in DS patients.

*Methods:* PCR and PCR-RFLP methods were used for the genotyping of study samples and results were validated using Sanger's sequencing.

*Results:* *MTRR* C524T and A66G were significantly associated with the increased risk of CHD in DS. We have also reported two novel polymorphisms, T19775C and 19778\_19778delG, in DS with CHD cases with a frequency of 93% and 40%, respectively. These two polymorphisms were not found among DS without CHD group.

*Conclusion:* Results from this study indicate that the *MTRR* C524T and A66G polymorphisms influence the risk of the occurrence of CHD in DS patients of Indian Origin. This is the first report from India highlighting the potential association of *MTRR* C524T and A66G polymorphisms with CHD in DS. We are also the first one to report two novel polymorphisms, T19775C and 19778\_19778delG in DS with CHD group. Hence these four polymorphisms can be used to evaluate the risk of CHD in DS patients.

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## 1. Introduction

Down syndrome (DS), also referred to as trisomy 21, is associated with various birth defects like congenital mental disability, hypotonia, Congenital Heart Disease (CHD), Hirschsprung's disease and others. The frequency and severity of these morphological defects vary significantly among affected individuals. DS affects about 1 in 700 live births [1,2].

CHD is a common disorder among newborn infants [3] which can be caused by environmental or genetic factors. About 50% of babies with DS are born with CHD, which is a much higher percentage compared to 1% of children born with CHD without DS. The most common CHD seen in DS patients is atrioventricular septal defect (AVSD). Other common heart defects include ventricular septal defects (VSD), atrial septal defects (ASD), tetralogy of fallot (TOF) and patent ductus arteriosus (PDA). However, the exact etiology of CHD in DS remains poorly understood.

Recently, several studies indicated that a gene involved in homocysteine metabolism, *methylenetetrahydrofolate reductase (MTHFR)*, is an important candidate gene for influencing the risk of CHD in DS [4–9,14]. However, only a few studies are reported indicating the role of another gene involved in homocysteine metabolism, *Methionine synthase reductase (MTRR)*, in context with CHD in DS [5,9–14]. No such studies have been published from India highlighting the importance of *MTRR* gene in the occurrence of CHD in DS patients.

*MTRR* is a key enzyme in folate dependent homocysteine metabolism and its gene is located on chromosome 5p15.2–15.3 [15]. *MTRR* catalyzes the conversion of the inactive form of methionine synthase (MTR) into its active form, by the regeneration of methyl (III) cobalamin, the cofactor of MTR. The disturbances in its catalytic activity lead to a higher level of homocysteine. The most common polymorphism in *MTRR* gene is A66G (rs1801394) substitution [16], leading to a change of isoleucine to methionine in amino acid 22 (I22M). The I22M variant is located in the putative FMN-binding domain of the *MTRR* enzyme that is suggested to interact with MTR. Substitution in this part of the enzyme thus disrupted the binding of *MTRR* to the MTR-cobalamin-complex, thereby decreasing the rate of homocysteine remethylation [16,17]. Another polymorphism C524T (rs1532268) leads to an amino acid change from serine to leucine (S175L). Since no results are available from India, we investigated the association of the two polymorphisms of *MTRR* gene with the occurrence of CHD in DS.

## 2. Subjects and methods

### 2.1. Subjects

Sixty DS with CHD patients and sixty patients DS without CHD were recruited in this study from the Department of Advanced Pediatric Center, Post Graduate Institute of Medical Education and Research, Chandigarh and Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow. Exclusion criteria were race other than Indian. The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki) for experiments in humans.

### 2.2. Genotyping of *MTRR* gene

Peripheral venous blood was taken in EDTA coated vial and the genomic DNA was isolated using standard Phenol Chloroform method. Predesigned primers were used to amplify *MTRR* gene for both the variants using PCR (conditions given in Table 1). The temperature profile used in PCR for both the polymorphisms were performed using initial denaturation at 95 °C for 5 min, followed by 35 cycle at 95 °C for 30 s, at 59 °C for 30 s, at 72 °C for 30 s and final extension at 72 °C for 10 min. The PCR amplified products were digested using restrictions enzymes (*Xho I* for C524T and *Nde I* for A66G) and was kept at 37 °C overnight followed by the electrophoresis on a 3.5% agarose gel. In order to verify the genotypes by PCR-RFLP, the purified PCR products of random samples selected were sequenced directly. (AB Sequence Detection System 310 software package; Applied Biosystems).

### 2.3. Statistical analyses

Genotype and allele frequency distributions of two polymorphisms in the *MTRR* gene were compared between patients of DS with CHD and DS without CHD, using the chi-square test. The odds ratios (OR) were calculated with the 95% confidence intervals (95%CI). All analyses were performed using SPSS for Windows, version 16.0. The *p*-values were considered to be significant at 0.05.

## 3. Results

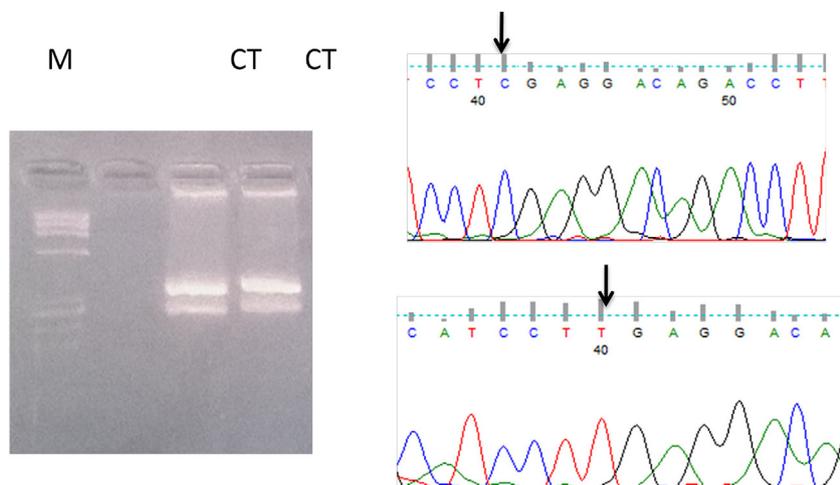
### 3.1. Frequency and genotyping of the *MTRR* C524T polymorphism

Through PCR-RFLP and Sanger's sequencing, *MTRR* C524T polymorphism was evaluated using *Xho I* restriction enzyme which digest the PCR amplified products, thereby dividing into three genotypes, *CC* (247 bp and 62 bp), *CT* (309 bp, 247 bp and 62 bp) and *TT* (309 bp) (Fig. 1). The allelic and genotype frequencies are summarized in Tables 2a and 2b. The frequency of *T* allele was slightly higher in DS with CHD (*C* = 51%; *T* = 33%) patients when compared to DS with CHD patients (*C* = 54%; *T* = 30%; *p* = 0.66). The genotype frequencies of DS with CHD and DS without CHD were *CC* = 44%, *CT* = 36%, *TT* = 20% and *CC* = 59%, *CT* = 6% and *TT* = 31%, respectively (*p*-value = 0.002).

### 3.2. Frequency and genotyping of the *MTRR* A66G polymorphism

Similarly, through PCR-RFLP and Sanger's sequencing, *MTRR* A66G polymorphism was evaluated using *Nde I* restriction enzyme which digests the PCR amplified products, thereby dividing into three genotypes- *AA* (126 bp, 25 bp), *AG* (151 bp, 126 bp, 25 bp) *GG* (151 bp, Fig. 2). The allelic and genotype frequencies are summarized in Tables 2a and 2b. The frequency of *A* allele was slightly higher in DS with CHD (*A* = 53%; *G* = 31%) patients when compared to DS without CHD patients (*A* = 45%; *G* = 38%; *p* < 0.0001). The genotype frequencies of DS with CHD and DS without CHD patients were *AA* = 43%, *AG* = 24%,

Variants	Primers	Annealing temp (°C)	Amplification fragments (bp)	Restriction enzymes	Genotypes
<i>MTRR</i> c. 524 <i>C &gt; T</i>	F-5'-TCAAGCAGAGGACAAGAG-3' R-5'AGAGACTCCTGCAGATGTAC-3'	59	309	<i>Xho I</i>	<i>CC</i> -247 bp, 62 bp <i>CT</i> -309 bp, 247 bp, 62 bp <i>TT</i> -309 bp;
<i>MTRR</i> c. 66 <i>A &gt; G</i>	F-5'-GGCAAAGGCCATCGCAGAAGACAT-3' R-5'CACTCCCAACCAAAATTCTTCAAAG-3'	59	151	<i>Nde I</i>	<i>AA</i> -126 bp, 25 bp <i>AG</i> - 151 bp, 126 bp, 25 bp <i>GG</i> -151 bp



**Figure 1** (A) shows the PCR digest fragments using *Xho I* enzymes separated on agarose gel showing *CT* genotype of *MTRR* c. 524*C > T* variant of band size of 309 bp and 247 bp. Lane M shows molecular marker. (B) and (C) shows the confirmation by Sanger's sequencing showing *CC* and *TT* genotype, respectively.

Groups	Genotypic frequency % (n)				<i>p</i>	Allelic frequency % (n)		
	<i>CC</i>	<i>CT</i>	<i>TT</i>	<i>p</i>		<i>C</i>	<i>T</i>	<i>p</i>
DS with CHD ( <i>N</i> = 60)	44 (26)	36 (22)	20 (12)	0.002	51 (61)	33 (39)	0.66	
DS without CHD ( <i>N</i> = 60)	59 (35)	10 (6)	31 (19)		54 (64)	30 (36)		

*p* < 0.001 is considered as significant.

Groups	Genotypic frequency % (n)				<i>p</i>	Allelic frequency % (n)		
	<i>AA</i>	<i>AG</i>	<i>GG</i>	<i>p</i>		<i>A</i>	<i>G</i>	<i>p</i>
DS with CHD ( <i>N</i> = 60)	43 (26)	40 (24)	16 (10)	< 0.0001	53 (64)	31 (37)	<i>p</i> < 0.001	
DS without CHD ( <i>N</i> = 60)	53 (32)	2 (1)	45 (27)		45 (54)	38 (46)		

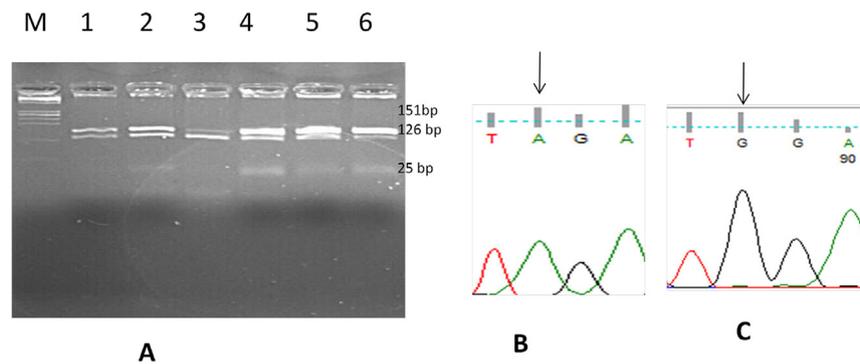
*p* < 0.001 is considered as significant.

*GG* = 10% and *AA* = 53%, *AG* = 2% and *GG* = 45%, respectively (*p*-value < 0.001).

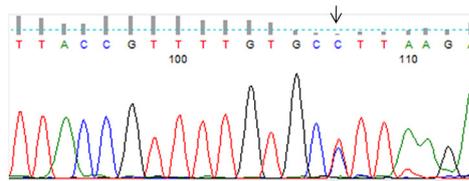
### 3.3. Novel polymorphism in *MTRR* gene

Sanger's sequencing revealed two novel *MTRR* gene polymorphisms in DS with CHD cases. The first polymorphism is

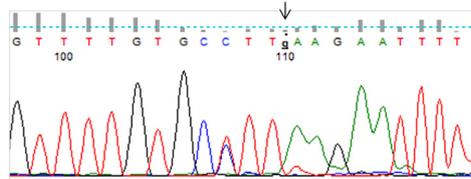
C19775T which was found at exon intron junction in *MTRR* gene with the frequency of 93% (*n* = 56) in DS with CHD cases. The second polymorphism is a deletion g.19778\_19778delG which was also found at exon intron junction in *MTRR* gene with the frequency of 63% (*n* = 40) in DS with CHD cases. Both of the polymorphisms were predicted as disease by MutationTaster software. These polymorphisms



**Figure 2** (A) shows the PCR digest fragments using *Nde I* enzymes separated on agarose gel showing *AG* genotype of *MTRR C*. *66A > G* variant of band size of 151 bp and 126 bp. Lane M shows molecular marker. (B) and (C) shows the confirmation by Sanger's sequencing showing *AA* and *TT* genotype, respectively.



Sanger's sequencing revealed a novel intronic g.19775T>C polymorphism in *MTRR* gene in DS with AVSD cases



Sanger's sequencing revealed a novel intronic deletion g.19778\_19778delG in *MTRR* gene in DS with AVSD cases

**Figure 3** Shows the Sanger's sequencing result of novel polymorphisms.

were not found in DS without CHD group. The sequence obtained was thoroughly scanned and compared against the published sequence of *MTRR*. (Table 4 and Fig. 3).

### 3.4. Association of risk of *MTRR* polymorphisms with the risk of CHD in DS

The association of the *MTRR C524T* polymorphism with the risk of the occurrence of CHD in DS is shown in Table 3a. Our data suggested that this polymorphism was associated with the increased risk of CHD with DS in a heterozygote comparison (*CT Vs CC*; *OR* = 4.9 (95% *CI*: 1.75–13.9;  $\lambda^2 = 9.9$ ;  $p = 0.001$ ) and dominant model (*CC Vs CT + TT*; *OR* = 1.9 (95% *CI*: 0.95–2.01;  $\lambda^2 = 3$ ;  $p = 0.08$ ), while models like allele contrast (*C Vs T*; *OR* = 3.37 (95% *CI*: 1.71–6.65,  $\lambda^2 = 13$ ,  $p = 0.0003$ ) and homozygote comparison (*CC Vs TT*; *OR* = 0.85 (95% *CI*: 0.35–2.05,  $\lambda^2 = 0.12$ ,  $p = 0.7$ ) do not shown much association.

Similarly, the association of the *MTRR A66G* polymorphisms with the risk of CHD in DS cases is shown in Table 3b. Our data suggested that this polymorphism was

**Table 3a** Association of *MTRR C524T* polymorphisms with the risk of CHD in DS.

Comparisons	Tests of associations		
	<i>OR</i> (95% <i>CI</i> )	$\chi^2$	<i>p</i>
Homozygote comparison ( <i>CC Vs TT</i> )	0.85 (0.35–2.05)	0.12	0.70
Heterozygote comparison ( <i>CC Vs CT</i> )	4.94 (1.75–13.91)	9.98	$p < 0.01$
Dominant model ( <i>CC Vs CT + TT</i> )	1.9 (0.95–2.01)	3.0	0.08
Recessive model ( <i>CC + CT Vs TT</i> )	0.53 (0.23–1.24)	2.13	0.14
Allele contrast ( <i>C Vs T</i> )	1.13 (1.71–6.65)	0.19	0.0003

**Table 3b** Association of *MTRR A66G* polymorphism with the risk of CHD in DS.

Comparisons	Tests of associations		
	<i>OR</i> (95% <i>CI</i> )	$\chi^2$	<i>p</i>
Homozygote comparison ( <i>AA Vs GG</i> )	0.45 (0.18–1.11)	3.04	0.08
Heterozygote comparison ( <i>AA Vs AG</i> )	13.33 (1.59–111.3)	8.4	$< 0.0001$
Dominant model ( <i>AA Vs AG + GG</i> )	1.49 (0.73–3.07)	1.20	0.20
Recessive model ( <i>AA + AG Vs GG</i> )	0.24 (0.10–0.57)	11.2	$< 0.0001$
Allele contrast ( <i>A Vs G</i> )	0.66 (0.35–1.19)	0.81	0.2

associated with the increased risk of CHD in DS cases in a heterozygote comparison (*AG Vs AA*; *OR* = 13.3 (95% *CI*: 1.59–111;  $\lambda^2 = 8.4$ ;  $p < 0.001$ ) and in dominant model (*AG + GG Vs AA*; *OR* = 1.49 (95% *CI*: 0.73–3.07,  $\lambda^2 = 1.2$ ,  $p = 0.20$ ), while models like homozygote (*AA Vs GG*; *OR* = 0.45 (95% *CI*: 0.18–1.11  $\lambda^2 = 3.04$ ,  $p$  value = 0.08); recessive model (*GG Vs AG + AA*; *OR* = 0.24 (95% *CI*: 0.10–0.57,  $\lambda^2 = 11.2$ ,  $p = 0.0008$ ), dominant, and allele contrast (*A Vs G*; *OR* = 0.6 (95% *CI*: 0.385–1.19,  $\lambda^2 = 0.81$ ,  $p = 0.2$ ) do not show much association.

**Table 4** showing novel polymorphisms detected in *MTRR* gene in DS with CHD.

SNP	Nature of mutation	MutationTaster software	Study group frequency (n%); N = 60
g.19775T > C	Intron exon Junction	Disease causing	DS with CHD = 56/93%; DS without CHD = 0
g.19778_19778delG	Deletion of G	Disease causing	DS with CHD = 40/67%; DS without CHD = 0

#### 4. Discussion

The association between DS and CHD has been well established since 1950, when the incidence of CHD present in DS patients was thoroughly described [18]. Since then several cohort studies [19,20] have significantly contributed to the close relationship between DS and CHD. The same studies have also demonstrated that the types of CHD most commonly associated with trisomy 21 are AVSD and VSD, which together account for 76% of all CHD seen in DS patients [20]. Only some DS patients have other defects such as ventricular septal defects (VSD), atrial septal defects (ASD), tetralogy of fallot (TOF) and patent ductus arteriosus (PDA).

CHD in DS is a complex interaction between environmental and genetic factors. Furthermore, growing evidence indicated that the genetic factors play an important role in the development of CHD in DS. One of the examples of genetic factor like MTHFR has been studied and reported that MTHFR, which is involved in the homocysteine pathway, is one of the most important candidate genes for influencing the susceptibility to CHD in DS. However, only a few studies have been reported emphasizing the role of *MTRR* which is another important gene involved in homocysteine pathway [21–24].

In present study, firstly we investigated the distribution of *MTRR* C524T polymorphisms by PCR-RFLP and evaluated the influence of these two polymorphisms on the risk of occurrence of CHD in DS patients of the Indian population through the association of DS without CHD patients. Our findings suggested that the results were significant for allelic and genotype frequencies for CHD in DS cases and non CHD controls (Tables 2a, 2b, 3a and 3b).

In conclusion, to our knowledge, we are the first to investigate the potential association of *MTRR* C524T and *MTRR* A66G polymorphisms with the risk of the occurrence of CHD in DS patients of Indian origin. Further Sanger's sequencing was also performed to validate the results. The results from this study suggests that *MTRR* C524T and A66G polymorphisms are associated with the increased risk of CHD in DS patients of Indian origin and could be used as a genetic marker for evaluating the occurrence of CHD in DS. Further studies with larger different ethnic populations are needed to confirm same results in their ethnicity.

We have also identified two novel polymorphisms T19775C and 19778\_19778delG, in DS with CHD patients with a high frequency. These two polymorphisms were not present in DS without CHD patients which suggest the role of these polymorphisms in the occurrence of CHD in DS patients of Indian origin. Hence these two polymorphism along with A66G and C524T should be considered as markers for CHD in DS.

#### Competing interest

The authors declare that there is no competing interest.

#### Authors' contribution

A.A. has participated in collection of blood, standardization of techniques, data interpretation, documentation and initial drafting of the manuscript. I.P. has evaluated the patients clinically and further referred us for molecular analysis. S.A. had taken a principal role in the conception of ideas, analysis of results and final editing of the manuscript. All authors read and approved the final manuscript.

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