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Risk of Atrioventricular Septal Defects in Down syndrome: Association of MTHFR C677T and RFC1 A80G polymorphisms in Indian Bengali cohort

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Abstract

Background: The etiology of Congenital heart defects (CHD), especially Atrio-Ventricular Septal Defect (AVSD) among individuals with Down syndrome (DS), is enigmatic and may differ across population divides owing to ethnicity and sociocultural differences. The polymorphisms of folate pathway regulators MTHFR and RFC1 as the risk of AVSD among DS individuals from the Indian Bengali cohort have not been explored yet.
Objectives: The aim of the present study is to investigate the association of MTHFR C677T and RFC1 A80G polymorphisms with the incidence of AVSD among individuals with DS in the Indian Bengali cohort.
Methods: Genotyping was done by bi-directional Sanger sequencing of DNA samples from DS with AVSD (N=479; 'DS-AVSD'), DS without AVSD (N=540; 'DS'), karyotypically confirmed euploid with AVSD (N=321; 'Control-AVSD') and euploid without AVSD (N=409; 'Control'). The odds ratio (OR) was calculated to infer the degree of risk imposed by alleles and genotypes. Functional implications of polymorphisms were inferred using the Project HOPE server.
Results: RFC1 A80G polymorphisms was found to be significantly associated with DS-AVSD when compared with control ($p = 0.0001$; $p< 0.0001$), control-AVSD ($p=0.0004$; $p< 0.0001$) and DS ($p< 0.0001$) groups. MTHFR C677T showed a significant association with DS-AVSD compared to the control only ($p=0.0004$; $p< 0.0001$). We also found an elevated risk of AVSD among DS when both polymorphisms are present. In-silico analyses suggest a possible amino acid replacement and subsequent compromised functions of the genes that may result in AVSD.
Conclusion: Our study suggests that the RFC1 A80G polymorphism is a significant risk for developing AVSD among individuals with DS from the Indian Bengali population. The MTHFR C677T polymorphism increases risk when present together with RFC1 A80G polymorphism.

Keywords: Down syndrome, Atrio-ventricular Septal Defect, MTHFR, RFC1, genetic polymorphisms.

1. Background

Down syndrome (DS) is the most prevalent inheritable intellectual disability characterized by the trisomy of chromosome 21 (Hsa21). About 1 in every 700 babies is born with DS (1). Besides intellectual disability, DS is associated with a set of characteristic dysmorphic features such as stunted growth, slanted eyes, flat nasal bridge, protruding tongue, low muscle tone, short neck, etc., and specific health issues like cognitive impairment, respiratory distress, sleep apnoea, compromised immunity and early onset of dementia. Congenital heart defect (CHD) is one of the significant health issues among individuals with DS. The incidence of CHD in DS is about 40-60% of all DS cases, and atrioventricular septal defect (AVSD) contributes ~30% of all the categories. AVSD is responsible for infant mortality before two years of age (2) and manifests as incomplete cardiac septation during embryonic cardiogenesis within the first six weeks of gestation. Imbalances in RNA/protein doses of triplicated genes on Hsa21 have been

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implicated as the primary underlying cause of AVSD. However, the non-occurrence of the defects among many DS individuals has made its etiology intriguing. Cross-talking among the genes on Hsa21 and other chromosomes with their allelic variations may explain this phenotypic variation. Additionally, differences in genetic architecture due to ethnic divides across populations complicate the phenotypes further.

Genes of the folic acid metabolic pathway and their allelic variations have been reported to be associated with AVSD. Their allelic variants are known to modulate gene functions and affect the developmental pathway (3,4). Two polymorphic variants of folate regulator genes Methylenetetrahydrofolate reductase (MTHFR) and Reduced Folate Carrier 1 (RFC1), have been studied for their possible association with AVSD, but the results are inconsistent. MTHFR (chromosome 1; 1p36.3) encodes the enzyme that converts 5,10-methylenetetrahydrofolate to circulating form 5-methyltetrahydrofolate. MTHFR C677T polymorphism (rs1801133) was reported as a risk factor for AVSD among the children with DS from Brazil (5) and Egypt (6) but found non-associated in the DS cohort from Croatia (7). Additionally, this polymorphism did not show any association with AVSD in non-DS Caucasian population (8), though contradictory result was obtained from meta-analysis (9). Non-association of MTHFR C677T with AVSD among non-DS individuals has been identified among children from Brazil (10). A previous study on non-DS children from south India has reported association between the C677T and AVSD (11).

The RFC1 or SLC19A1 (chromosome 21; 21q22.3) encodes a transporter that regulates intracellular folate concentration (12). The RFC1 A80G (rs1051266) polymorphism has been reported to be associated with conotruncal heart defects (CTD) among white and non-white Hispanic non-DS infants (13) and in the Chinese population (14,15).

2. Aim of the Study

The present study is designed to investigate the synergistic association of the MTHFR C677T and RFC1 A80G polymorphisms with the incidence of AVSD among the individuals with DS from the Indian Bengali-speaking cohort, which is one of the very large ethnic and linguistic groups in the globe.

3. Methods

The study was carried out following the ethical guidelines of the Declaration of Helsinki and the research regulations outlined by the Indian Council of Medical Research (ICMR). Methodologies have been reviewed by the ethics committees constituted by the University of Calcutta and IPGME&R, and SSKM hospital, Kolkata, India.

3.1 Study cohort

A total of 1749 subjects were referred randomly from the medical colleges and hospitals of Kolkata and surrounding areas to the Cytogenetics and Genomics laboratory at the University of Calcutta by the clinician collaborators. Cases were defined as individuals having free trisomy 21 (karyotypically confirmed). The subjects were further classified into DS with AVSD (N=479; referred to as 'DS-AVSD') and DS without AVSD (N=540; referred to as 'DS'). Similarly, the age-matched controls (karyotypically confirmed euploid; 2n = 46, XX or 46, XY) were classified into euploid with AVSD (N=321; referred to as 'Control-AVSD') and euploid without AVSD (N=409; referred as 'control'). The families were interviewed personally and in person only after obtaining their written consent. Epidemiological data were recorded through pre-printed questionnaires.

3.2 Tissue collection

About 2ml of peripheral blood samples were obtained from control and case subjects in EDTA-coated vacutainers. Trained physician collaborators collected blood samples at the respective hospitals only after obtaining written consent from the parents or legal guardians. All the samples were kept with a code to maintain the subjects' anonymity.

3.3 Genotyping

Genotyping was performed blindly without knowing the AVSD phenotype of the subjects. Following the manufacturer's protocol, we isolated genomic DNA from whole blood samples using QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany). Approximate 200 µl whole blood samples were treated with 20 µl Protease K enzyme, followed by 200 µl Lysis buffer. It was then incubated at 56 ºC for 10 minutes. We added 200 µl ethanol and mixed the solution well. The mixture was then centrifuged at 8000 rpm for 1 minute using spin columns. We discarded the flow-through, treated the sample with 500 µl wash buffer 1 to remove cellular debris and protein impurities, and centrifuged at 8000 rpm for 1 minute. Next, we discarded the flow-through, treated the sample with 500 µl wash buffer 2 to remove RNA impurities, centrifuged at 14000 rpm for 3 minutes, and discarded the flow-through. In the last step, we treated the sample with 200 µl Elution buffer, incubated the mix at room temperature for 1 minute, then centrifuged at 8000 rpm for 1 minute, and finally collected the flow-through.

Primer3 (v.4.1.0) (www. https://primer3.ut.ee/.com) program was used to design primers, and the OligoAnalyzer tool (https://eu.idtdna.com/) from Integrated DNA Technology was used to test them. The PCR reaction was carried out in 30 µl volume using 50–100 ng of DNA, 1 µl primers (10 mmol/L), 0.2 µl of dNTPs (10 mmol/L; Invitrogen Carlsbad, CA, USA), 1.5 µl MgCl2 (50 mmol/L), 1 x PCR buffer, and 0.8 µl Taq Pol (5 units/1 µl; Invitrogen, California, USA). The used dideoxy sequencing primers sets are provided in Table 1.

We performed Sanger sequencing with Taq Dye Deoxy Terminator sequencing kit (Applied Biosystems, Foster City, USA) and ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, USA). In order to read the chromatogram results, we used FinchTV software. Briefly, the double-stranded DNA becomes single-stranded in an automated sequencer, followed by primer binding and extension with dNTPs. Random incorporation of specific dye-tagged ddNTPs in a specific capillary terminates the reaction, and the signal of specific dye color is read by the reader and interpreted by the program.

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3.4 Functional Prediction of Detected Variants

We conducted the in-silico analysis to predict the imperilments caused by 'risk variants/alleles' at the transcript or protein level of the genes studied. We analyzed the functional implications of both polymorphisms using the 'Project HOPE' server (www3.cmbi.umcn.nl/hope/) and validated the results using the 'Missense 3D Database' (http://missense3d.bc.ic.ac.uk/). While using the Project HOPE server, we provide the amino acid sequence and select the mutation in the corresponding residue. Based on the input, the software predicts the implication of the said mutation. In the case of the Missense 3D server, we have to provide the UniProt ID of the said protein as well as the position and nature of the mutation to be studied. Based on this information, the server predicts the functional effects of the mutation.

3.5 Statistical Analysis

We used Pearson's $\chi 2$ test to measure the differences in the frequencies of alleles and the difference in frequencies of genotype between the groups. Fisher's exact test was performed for comparison between the groups for the synergistic analysis. We tested the Hardy-Weinberg equilibrium of all the genotypes under study. We also performed the Kolmogorov-Smirnov test to confirm the normal distribution of the data. The association of genotypes with AVSD phenotypes was tested by odds ratios (OR) at 95% Confidence Interval (CI). A two-tailed p-value < 0.05 was considered statistically significant for all the analyses. Bonferroni's multiple comparison correction tests were also performed. GraphPad InStat 3 was used to perform Fisher's exact test, OR calculation, and t-test. The SPSS version 23 was used to perform Pearson's $\chi 2$ test and statistical modeling for synergistic effect analyses.

4. Results

4.1 Demographic and epidemiological attributes of the subjects

The demographic and epidemiological data recorded are presented in Table 2 and found to be concordant among the four phenotype groups viz DS-AVSD, DS, control-AVSD, and control. No significant differences were detected concerning the demographic and epidemiological variables, negating any sampling bias.

4.2 Allele frequencies and incidence of AVSD in DS

The frequencies in each of the groups were in Hardy-Weinberg equilibrium. The allele and genotype frequencies of MTHFR C677T and RFC1 A80G variants among the four study groups are represented in Table 3. The frequency of MTHFR C677T variant minor allele 'T' is found to be significantly higher ($p< 0.0001$) in the DS-AVSD group (0.36) when compared to the Control group (0.24) and is associated with 1.75-fold increased odds favoring the DS-AVSD group over the Control group. It is also found to be significantly higher ($p=0.021$) in the Control-AVSD group (0.3) over the control group (0.24) and exhibited 1.325-fold increased odds in favor of the Control-AVSD group over the Control group. The DS-AVSD group (0.36) also showed a significantly higher ($p=0.01$) frequency of the 'T' allele compared to the Control-AVSD group (0.33). It showed 1.321-fold increased odds favoring the DS-AVSD group against the Control-AVSD group. No significant difference was found in the 'T' allele distribution between the DS-AVSD group and the DS group. The RFC1 A80G variant minor allele 'G' showed significant higher frequency in the DS-AVSD group (0.62) when compared with the control group (0.36; $p< 0.0001$), control-AVSD group (0.42; $p< 0.0001$) and DS group (0.51; $p< 0.0001$). The 'G' allele was found to elevate 2.86-folds, 2.244-folds, and 1.562-folds odds in favor of the DS-AVSD group compared to the control group, control-AVSD group, and the DS group, respectively. The 'G' allele was also found to be more frequent ($p=0.0282$) in the Control-AVSD group (0.42) than the control group (0.36), with 1.275-folds increased odds favoring the Control-AVSD group over the Control group.

4.3 Genotype frequencies and incidence of AVSD in Down syndrome

The frequencies of genotypes of MTHFR C677T and RFC1 A80G among the four study groups and the comparison among the groups have been represented in Table 3. The MTHFR 677CT ($p=0.0004$) and TT ($p< 0.0001$) genotypes showed significantly higher frequency in the DS-AVSD group than in the Control group. The CC, CT, and TT genotypes in the DS-AVSD group are 0.41, 0.46, and 0.13, respectively, in contrast to 0.57, 0.38, and 0.05 in the Control group. The CT and TT genotypes are associated with 1.67-fold and 3.418-fold high odds, respectively, in favor of the DS-AVSD group compared to the Control group. The frequencies of the genotypes AA, AG, and GG of RFC1 A80G polymorphism in the DS-AVSD group were recorded as 0.16, 0.43, and 0.41, respectively, compared to 0.38, 0.51, and 0.11 in the control group. Both the 'AG' ($p=0.0001$) and 'GG' ($p< 0.0001$) genotypes showed a significant difference in distribution between these two groups. The 'AG genotype increases 1.946-fold odds in favor of the DS-AVSD group compared to the control group, whereas 'GG' elevates 8.557-fold odds in favor of the same. Comparisons between the control group and the control-AVSD group showed a significantly higher frequency of RFC1 80GG genotype (1.905-fold; $p=0.007$) in the control-AVSD group (Table 3). Comparisons between the DS-AVSD group and the control-AVSD group revealed significantly elevated odds for 'AG' (1.933-fold; $p=0.0004$) and 'GG' (4.492-fold; $p< 0.0001$) genotypes in favor of the DS-AVSD group over the Control-AVSD group. Lastly, when a comparison was drawn between the DS-AVSD and the DS groups, the 'GG' genotype showed higher ($p < 0.0001$) frequency in the DS-AVSD group compared to the DS group, with 2.232-fold elevated odds in favor of the DS-AVSD group. In the DS group, the frequencies of the AA, AG, and GG genotypes were scored as 0.23, 0.52, and 0.25.

4.4 Synergistic effects of the MTHFR C677T and RFC1 A80G polymorphisms

Some subjects were found to have both polymorphisms together. Using a two-by-four table, we examined the possible combinations of mutant genotypes at two loci (taking '0' polymorphic alleles as reference) to assess any potential synergistic impact or gene-gene interaction that would elevate the risk of AVSD among the individuals with DS. As shown in

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Table 4, we found the presence of 4, 5, and 6 variant alleles are associated with 2.138-folds (p=0.0019), 9.8-folds (p< 0.0001), and 10.617-folds (p=0.0005) elevated odds, respectively, favoring the DS-AVSD group over the control group, thus imparting a strong synergistic effect in this regard. Comparison between the control and control-AVSD groups does not show any significant associations. However, we found the presence of 5 and 6 polymorphic alleles to be associated with 4.08-folds (p=0.0054) and 22.983-folds (p=0.0007) increased odds, respectively, favoring the DS-AVSD group over the control-AVSD group. Additionally, we found the presence of 5 and 6 polymorphic alleles to be associated with 3.24-folds (p=0.004) and 5.85-folds (P=0.0037) elevated odds, respectively, in favor of the DS-AVSD group when compared with the DS group.

4.5 Prediction of alteration in proteins due to polymorphic alleles by in-silico programs

In-silico analyses predict the probable impairments conferred by the polymorphisms on the transcripts or protein products of the respective genes. The Project HOPE server results reveal that the MTHFR C677T polymorphism leads to a 'missense' replacement from alanine to valine at the 222nd position (Figure 1a) of the peptide. Valine, a bigger residue than alanine, might create a problem fitting in the protein's core. The altered residue is located in a critical domain needed for the optimum protein function and in contact with residues in another domain. This interaction is probably pivotal for protein functionality. The alteration thus may affect this interaction impairing the protein function.

The presence of the RFC1 A80G polymorphism causes a missense replacement of histidine residue at the 27th position by arginine residue (Figure 1b). The arginine residue is more positively charged than a smaller and neutral histidine. The residue in the protein's transmembrane domain might affect its interactions with the lipid membrane. The alteration introduces a charge, leading to probable repulsion among similarly charged ligands or other residues.

5. Discussion

The genetic etiology of variable incidence of CHD among individuals with DS is probably multifactorial. Trisomic genetic background, along with allelic variants of different candidate genes and their interactions, leads to the manifestation of different types of CHD and even degrees of penetrance of a given CHD type among individuals with DS. The MTHFR 677CT and TT genotypes have been reported to increase the risk of AVSD in DS in a Brazilian population (5), In contrast, only 677CT genotype was reported as a maternal and child risk factor for CHD in the Egyptian population (6). A study conducted in the south Indian population also reported the 677CT genotype as a maternal and child risk factor for AVSD in a non-DS population (11). Our observation is consistent with these previous findings; 677CT and TT genotypes is associated with AVSD among DS individuals. The RFC1 A80G polymorphism has been reported previously to be associated with several congenital disabilities, including heart defects. One study (14) reported an elevated risk of CHD among the offspring carrying the RFC1 80G allele over the individuals without G allele. Similar studies on white and non-white Hispanic non-DS infants, as well as non-DS Chinese infants, have also revealed an association of RFC1 80G allele with heart defects (13,15). As far as published literature is concerned, only one study has predicted the association of RFC1 A80G polymorphism with AVSD in DS (3). So, we decided to check this association among individuals with DS from India. We observed that both RFC1 80AG and GG genotypes exhibited increased odds in favor of the DS-AVSD group over the control and the control-AVSD group. We also observed a significant association of the 80GG genotype with the DS-AVSD group and control-AVSD group compared to the DS group and control group. Our results are concordant with those reported in the previous studies and suggest RFC1 80GG genotype is a risk factor for AVSD development both in disomy and trisomy individuals; but the deleterious effect of the risk genotype became stronger under trisomy 21 genomic background as revealed from more elevated odds in favor of DS-AVSD group (2.23; p< 0.0001) than Control-AVSD group (1.9; p=0.005).

For the first time, we conducted the analyses through statistical modeling to look into the synergistic effects of these two polymorphisms on the risk of AVSD in DS. The synergistic analyses anticipate the statistical probability of increasing risk with the increasing number of risk alleles in the given individual when he carries more than one risk genotype of two different genes. This analysis revealed that the presence of 4, 5, or 6 variant alleles increases the odds in favor of the DS-AVSD group (Table 4). We found out that the MTHFR C677T polymorphism exerted a stronger deleterious effect when it co-occurred with the RFC1 A80G polymorphism than it does alone.

The outcome of in-silico analyses justifies the observed association of a genotype with disease phenotype at the molecular level. It provides the theoretical foundation for future wet lab studies to confirm the notion. Our in-silico results suggest that the MTHFR C677T polymorphism affects the protein function and impairs the enzyme activity, thus contributing to the altered levels of circulating folate. The Project HOPE server predicts that the RFC1 A80G polymorphism probably alters the protein's transmembrane domain, which affects its interaction with the lipid bilayer. Indirect support for this prediction comes from the published literature that reported RFC1 A80G polymorphism causes functional folate deficiency (16). As MTFHR and RFC1 genes are key players in the folate metabolism pathway (17,18), which regulate the global DNA methylation of genes crucial for embryonic cardiac septum development, mutations/polymorphisms of these genes probably impede cellular proliferation during the same. This effect probably gets intensified under the trisomy 21 background when Hsa21 genes with altered doses of their RNA and protein products affect multiples of molecular pathways through bizarre cross-talking. Additionally, it is predictive that confounding effects of interactions among different genetic modifiers generate varying degrees of altered expression of candidate genes involved in cardiac septum development and lead to the differential manifestation of AVSD under trisomy 21 background. Detail molecular study is warranted to establish this notion experimentally.

6. Conclusion

In summary, we, for the first time, demonstrated that MTHFR C677T and RFC1 A80G polymorphisms are risk factors for AVSD among the individuals with DS from Bengali populations, and

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we modeled to infer how these two polymorphic alleles interact with each other when present together to increase the risk of AVSD. This study brings us a significant step closer to understanding the genetic etiology of population-specific variable incidence of AVSD among individuals with DS.

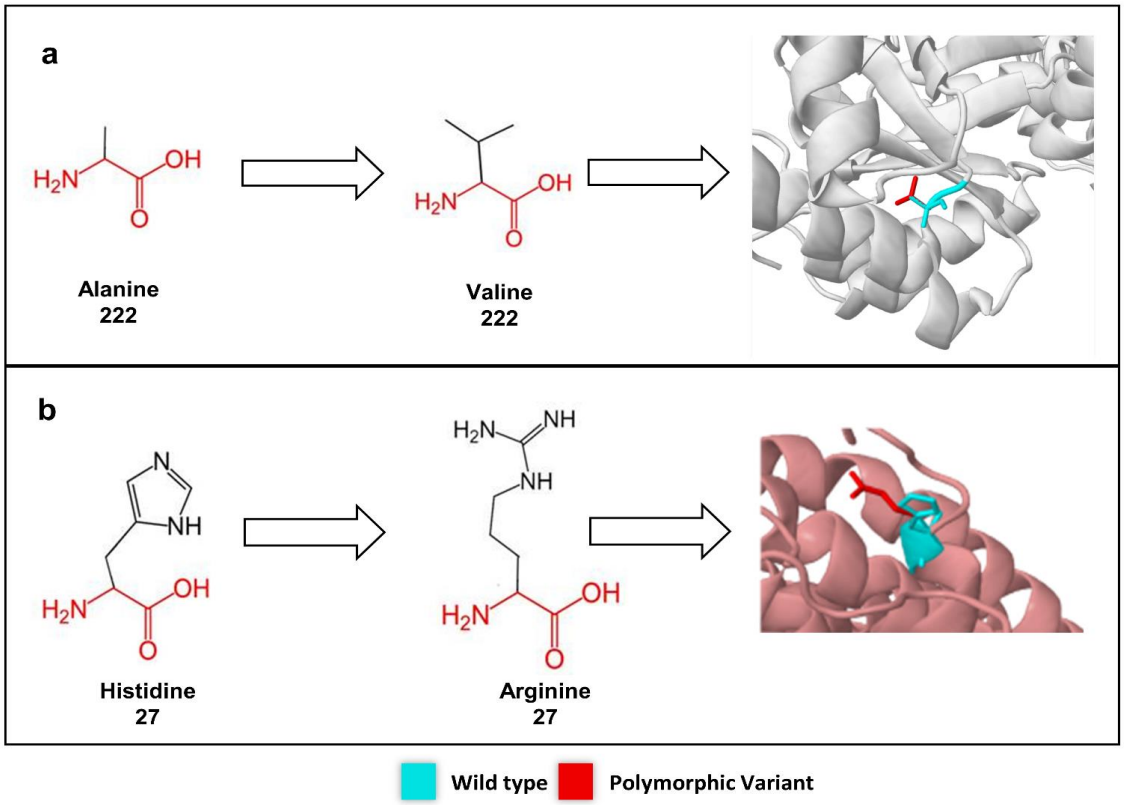

Figure 1. Functional implications of studied polymorphisms obtained from in-silico analyses *for a) MTHFR C677T and b) RFC1 A80G.*

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Table 1. List of forward and reverse primers used for genotyping the variants MTHFR C677T and RFC1 A80G.

Variant	Forward Primer	Reverse Primer
MTHFR C677T (rs1801133)	5′ ACAGTGTGGGAGTTTGGAG 3′	5′ AGTTCTGGACCTGAGAGGAG 3′
RFC1 A80G (rs1051266)	5’ TGGCACTTAAATCACTCCATGT 3'	5’ TGCTCACACATCCAACAGG 3’

Table 2- Epidemiological and demographic attributes of the study cohort. T-tests were used to test for differences between the parameters. P value < 0.05 was considered to be significant (AVSD = Atrio-Ventricular Septal Defect, DS = Down Syndrome, SD = Standard Deviation).

Attributes	Control	Control-AVSD	DS	DS-AVSD
Total Participants	409	321	540	479
Age (Mean ± SD) [in years]	5.6 ± 1.57	5.4 ± 1.54	5.53 ± 1.68	5.01 ± 2.64
Socio Economic Condition				
Low (< INR 30,000/month) [Frequency]	0.57	0.53	0.47	0.74
Medium (INR 30,000 - 50,000/month) [Frequency]	0.31	0.42	0.44	0.23
High (> INR 50,000/month) [Frequency]	0.12	0.05	0.09	0.03
Locality				
Urban [Frequency]	0.31	0.36	0.4	0.36
Semi-urban [Frequency]	0.39	0.3	0.27	0.21
Rural [Frequency]	0.3	0.34	0.33	0.43
Religion				
Hindu [Frequency]	0.89	0.9	0.81	0.85
Islam [Frequency]	0.09	0.07	0.11	0.15
Others [Frequency]	0.02	0.03	0.08	0

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Table 3. Allele and Genotypic frequencies of MTHFR C677T and RFC1 A80G polymorphisms in Control, Control-AVSD, DS, and DS-AVSD groups. * = Significant even after Bonferroni's correction for P value; corrected P value =0.017, CI = Confidence Interval, AVSD = Atrio-Ventricular Septal Defect , DS = Down Syndrome

	Allele	Control (N=409)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	Control (N=409)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
MTHFR C677T	C	0.76	0.64		1	REFERENCE	CC	0.57	0.41		1	REFERENCE
	T	0.24	0.36	27.62	1.75 (1.422-2.154)	< 0.0001*	CT	0.38	0.46	12.506	1.670 (1.263-2.209)	0.0004*
							TT	0.05	0.13	21.184	3.418 (2.010-5.813)	< 0.0001*

	Allele	Control (N=409)	Control-AVSD (N=321)	χ^2	OR (95% CI)	p value	Genotype	Control (N=409)	Control-AVSD (N=321)	χ^2	OR (95% CI)	p value
MTHFR C677T	C	0.76	0.7		1	REFERENCE	CC	0.57	0.51		1	REFERENCE
	T	0.24	0.3	5.33	1.325 (1.049-1.673)	0.021*	CT	0.38	0.39	0.7648	1.162 (0.8533-1.582)	0.3818
							TT	0.05	0.1	6.265	2.178 (1.212-3.913)	0.0123

	Allele	Control-AVSD (N=321)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	Control-AVSD (N=321)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
MTHFR C677T	C	0.7	0.64		1	REFERENCE	CC	0.51	0.41		1	REFERENCE
	T	0.3	0.36	6.182	1.321 (1.065-1.638)	0.0129*	CT	0.39	0.46	5.224	1.437 (1.063-1.943)	0.022
							TT	0.1	0.13	3.059	1.569 (0.9754-2.525)	0.0803

	Allele	DS (N=540)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	DS (N=540)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
MTHFR C677T	C	0.67	0.64		1	REFERENCE	CC	0.45	0.41		1	REFERENCE
	T	0.33	0.36	1.799	1.139 (0.9478-1.368)	0.1798	CT	0.44	0.46	0.6629	1.125 (0.8656-1.462)	0.4155
							TT	0.11	0.13	1.467	1.313 (0.8742-1.973)	0.2258

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	Allele	Control (N=409)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	Control (N=409)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
RFC1 A80G	A	0.64	0.38		1	REFERENCE	AA	0.38	0.16		1	REFERENCE
	G	0.36	0.62	115.36	2.86 (2.358-3.47)	< 0.0001*	AG	0.51	0.43	14.969	1.946 (1.396-2.714)	0.0001*
							GG	0.11	0.41	108.42	8.557 (5.609-13.054)	< 0.0001*

	Allele	Control (N=409)	Control-AVSD (N=321)	χ^2	OR (95% CI)	p value	Genotype	Control (N=409)	Control-AVSD (N=321)	χ^2	OR (95% CI)	p value
RFC1 A80G	A	0.64	0.58		1	REFERENCE	AA	0.38	0.35		1	REFERENCE
	G	0.36	0.42	4.815	1.275 (1.031-1.575)	0.0282*	AG	0.51	0.46	0.0017	1.007 (0.7298-1.389)	0.9673
							GG	0.11	0.19	7.162	1.905 (1.208-3.005)	0.007*

	Allele	Control-AVSD (N=321)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	Control-AVSD (N=321)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
RFC1 A80G	A	0.58	0.38		1	REFERENCE	AA	0.35	0.16		1	REFERENCE
	G	0.42	0.62	60.488	2.244 (1.83-2.752)	< 0.0001*	AG	0.46	0.43	12.569	1.933 (1.352-2.764)	0.0004*
							GG	0.19	0.41	53.633	4.492 (2.989-6.751)	< 0.0001*

	Allele	DS (N=540)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value	Genotype	DS (N=540)	DS-AVSD (N=479)	χ^2	OR (95% CI)	p value
RFC1 A80G	A	0.49	0.38		1	REFERENCE	AA	0.23	0.16		1	REFERENCE
	G	0.51	0.62	24.1	1.562 (1.309-1.864)	< 0.0001*	AG	0.52	0.43	0.4361	1.136 (0.8124-1.588)	0.509
							GG	0.25	0.41	18.909	2.232 (1.561-3.192)	< 0.0001*

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Table 4 . Increasing odds with increase of number of synergistic minor alleles in both tested variants of folate regulator genes. * = Significant even after Bonferroni's correction for P value; corrected P value =0.007, CI = Confidence Interval, AVSD = Atrio-Ventricular Septal Defect, DS = Down Syndrome

Genotype Combinations	Risk Allele	CONTROL (N=409)	DS-AVSD (N=479)	Odds ratio	95% CI	P value
MTHFR 677 CC VS RFC1 80 AA	0	0.24	0.13	1	REFERENCE	
MTHFR 677 CC VS RFC1 80 AA or AG	1	0.21	0.16	1.478	0.9453-2.310	0.0904
MTHFR 677 CC VS RFC1 80 AA or GG	2	0.1	0.08	1.552	0.8969-2.685	0.1243
MTHFR 677 CC VS RFC1 80 AG or GG	3	0.13	0.12	1.818	1.113-2.970	0.0184
MTHFR 677 CC or CT VS RFC1 80 AG or GG	4	0.13	0.15	2.138	1.328-3.442	0.0019*
MTHFR 677 CC or TT VS RFC1 80 AG or GG	5	0.01	0.05	9.8	3.241-29.632	< 0.0001*
MTHFR 677 CT or TT VS RFC1 80 AG or GG	6	0.005	0.03	10.617	2.314-48.705	0.0005*
Genotype Combinations	**Risk Allele**	**CONTROL (N=409)**	**CONTROL-AVSD (N=321)**	**Odds ratio**	**95% CI**	**P value**
MTHFR 677 CC VS RFC1 80 AA	0	0.24	0.16	1	REFERENCE	
MTHFR 677 CC VS RFC1 80 AA or AG	1	0.21	0.21	1.533	0.9612-2.444	0.0773
MTHFR 677 CC VS RFC1 80 AA or GG	2	0.1	0.11	1.729	0.9845-3.038	0.0612
MTHFR 677 CC VS RFC1 80 AG or GG	3	0.13	0.14	1.668	0.9913-2.806	0.0631
MTHFR 677 CC or CT VS RFC1 80 AG or GG	4	0.13	0.14	1.572	0.9352-2.643	0.1108
MTHFR 677 CC or TT VS RFC1 80 AG or GG	5	0.01	0.02	2.402	0.6177-9.34	0.2814
MTHFR 677 CT or TT VS RFC1 80 AG or GG	6	0.005	0	0.3825	0.01801-8.124	0.5497
Genotype Combinations	**Risk Allele**	**CONTROL-AVSD (N=321)**	**DS-AVSD (N=479)**	**Odds ratio**	**95% CI**	**P value**
MTHFR 677 CC VS RFC1 80 AA	0	0.16	0.13	1	REFERENCE	
MTHFR 677 CC VS RFC1 80 AA or AG	1	0.21	0.16	0.9642	0.5864-1.585	0.8997
MTHFR 677 CC VS RFC1 80 AA or GG	2	0.11	0.08	0.8972	0.4977-1.618	0.7647
MTHFR 677 CC VS RFC1 80 AG or GG	3	0.14	0.12	1.09	0.6374-1.865	0.7854
MTHFR 677 CC or CT VS RFC1 80 AG or GG	4	0.14	0.15	1.36	0.8025-2.305	0.284

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MTHFR 677 CC or TT VS RFC1 80 AG or GG	5	0.02	0.05	4.08	1.451-11.469	0.0054*
MTHFR 677 CT or TT VS RFC1 80 AG or GG	6	0	0.03	22.983	1.332-396.43	0.0007*
Genotype Combinations	**Risk Allele**	**DS (N=540)**	**DS-AVSD (N=479)**	**Odds ratio**	**95% CI**	**P value**
MTHFR 677 CC VS RFC1 80 AA	0	0.15	0.13	1	REFERENCE	
MTHFR 677 CC VS RFC1 80 AA or AG	1	0.16	0.16	1.221	0.7742-1.927	0.4177
MTHFR 677 CC VS RFC1 80 AA or GG	2	0.09	0.08	1.006	0.5881-1.720	1
MTHFR 677 CC VS RFC1 80 AG or GG	3	0.13	0.12	1.154	0.7128-1.869	0.6232
MTHFR 677 CC or CT VS RFC1 80 AG or GG	4	0.14	0.15	1.296	0.8141-2.063	0.2889
MTHFR 677 CC or TT VS RFC1 80 AG or GG	5	0.02	0.05	3.24	1.441-7.283	0.004*
MTHFR 677 CT or TT VS RFC1 80 AG or GG	6	0.006	0.03	5.85	1.595-21.451	0.0037*

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

Supplementary material: Proforma of epidemiological data record

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