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Original Research Article

Angiotensin-converting enzyme insertion/deletion polymorphism as a potential risk factor of congenital heart disease: insights from a tertiary pediatric cardiac care centre from North India

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ABSTRACT

Background: Our study aims to elucidate the genetic influence of angiotensin-converting enzyme insertion/deletion (ACE I/D) polymorphism on congenital heart disease (CHD) in a north Indian cohort.
Methods: 667 CHD cases, including 433 individuals with parental data and 104 controls were enrolled and genotyped by polymerase chain reaction. Case-control association, parental transmission test, and association of patients' and parents' clinical parameters with ACE I/D were explored.
Results: Our findings highlight significant associations, notably the increased CHD risk conferred by the DD genotype in females (p=0.036; OR=1.68), its correlation with abnormal hemoglobin levels (p=0.049; OR=1.68), and its impact on primigravida (p=0.05). Conversely, the II genotype was found to significantly elevate the risk of CHD in offspring of tobacco-consuming fathers by 2.5-fold (p=0.029). Notably, cyanotic cases exhibited a heightened prevalence of ACE I/D mutations (p=0.059), with tetralogy of Fallot (TOF) showing the strongest association (p=0.024). Additionally, the DD genotype's involvement in conditions such as stenosis (p=0.026) and pulmonary artery hypertension (PAH) (p=0.05) underscores its clinical relevance. The parent of origin test showed maternal transmission of the D allele in combined (p=0.037) and acyanotic cases (p=0.039) and paternal transmission in ventricular septal defect (p=0.021).
Conclusions: This is the first study from India and possibly the only study globally that reports a significant association between ACE I/D and CHD, highlighting the importance of genetic factors in CHD susceptibility.

Keywords: ACE insertion/deletion, CHD, Association, Parental transmission, North India

INTRODUCTION

Cardiovascular diseases (CVDs) represent a significant global health burden, accounting for approximately 17.9 million premature non-communicable disease deaths annually, with a pronounced impact in regions of low to medium socio-demographic indexes, including India.[1] The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in the pathogenesis of several CVDs and hypertension, with the ACE I/D polymorphism being extensively studied in this context.[2] The ACE gene encodes an enzyme that catalyzes the conversion of ACE 1 to ACE 2 and inactivates bradykinin (a vasodilator protein). ACE 2, a bioactive vasodilator peptide,

regulates blood pressure and aldosterone secretion and is a well-known candidate gene for the study of COVID-19.[3] ACE I/D polymorphism, located at the 16th intron on chromosome 17q23 of the ACE 1 gene, induces alternative splicing of 287 bp Alu elements and is associated with various CVDs including premature atherosclerosis, idiopathic dilated cardiomyopathy, myocardial infarction (MI), ischemic heart disease, left ventricular hypertrophy, as well as the coronary artery disease.[4]

CHD, a prevalent cardiac birth defect, accounts for ~28% of all congenital anomalies and is responsible for high mortality and morbidity of neonates and fetuses worldwide.[5,6] Prevalence rates vary across regions, with global estimates at 4-50 per 1000 live births, 9.3 in Asia, and 1.3-9.2 in India.[5,7] While the ACE I/D variant's association with various CVDs has been extensively documented, few studies have explored its correlation with CHD.[4] Notably, only three studies have been conducted in Saudi Arabia, China, and Egypt, however, none of them could find such an association.[8-10] Despite research from the Indian subcontinent investigating ACE I/D in various conditions like cardio-metabolic risk factors, cardiomyopathy, coronary heart disease, hypertension, chronic obstructive pulmonary disease, type 2 diabetes, breast cancer, infertility and associated pregnancy complications, hemorrhagic stroke, COVID-19 and chronic kidney disorders etc., there remains a notable gap concerning CHD.[11-23] To date, no literature or studies have reported on the association of ACE I/D polymorphism with CHD from India, making our forthcoming study potentially the first to explore this relationship comprehensively. This study aims to fill this critical gap in understanding, potentially shedding new light on genetic predispositions to CHD and contributing to broader insights into cardiovascular genetics and disease pathogenesis.

METHODS

Study samples

The study was conducted after the institutional ethics committee's (IEC) approval. It employed a case-control and trio-based study design, recruiting samples were from patients and their parents who underwent cardiac surgery or CATH intervention from 2018 to 2021 at Sri Sathya Sai Sanjeevani international centre for child heart care and research (SSSSICHC and R), Palwal, Haryana (India), a totally free of cost tertiary pediatric cardiac care center. The study samples were collected at DSIR-SIRO certified Sri Sathya Sai Sanjeevani research foundation (SSSSRF). Additionally, patient information was collected in CHD-specific questionnaire developed at SSSSRF on research electronic data capture (REDCap) web application.[24] Patients showing extracardiac anomalies, clinically identified syndromic features, and any other neurodevelopmental and chronic disorders were excluded. In total, n=667 CHD cases, containing 433 patients having one/both parent samples and the rest 234 with only the patient's sample, after clinical and laboratory examinations, echocardiography confirmation, and written informed consent/assent, were enrolled in this study. Among these cases, 446 (66.8%) were acyanotic and 221 (33.2%) were cyanotic, further categorized into sub-phenotypes, including atrial septal defects (ASD; n=87), ventricular septal defects (VSD; n=292), TOF; n=148), transposition of great arteries (TGA; n=12), single ventricle (SV; n=15), atrio-ventricular canal (AV canal; n=17), total anomalous pulmonary venous connection (TAPVC; n=32) and miscellaneous (n=64). An additional classification included n=268 and n=83 having CHD with stenosis {either pulmonary stenosis (PS) or Aortic Stenosis (AS)} and CHD with PAH, respectively. N=104 healthy controls of the same ethnicity, after echocardiography confirmation and written consent, were included to explore association. Since the genotypic profile do not change after birth i.e. aren't influenced by age-related changes as the defect is formed during the development of the fetus and our study was not related to functional analysis, taking adult controls may not significantly hamper the association study.[25]

DNA extraction and ACE I/D genotyping assay

Genomic DNA was extracted from approximately 2 ml of whole peripheral blood using a non-enzymatic salting out method.[26] DNA quality and quantity were assessed with NanoDrop™ UV Visible spectrophotometry (Thermo Fisher Scientific, United States), aiming for an OD (260/280 nm) ratio of 1.7-1.9. The genotyping was performed by polymerase chain reaction (PCR) using Veriti™ 96-well fast thermal cycler (Thermo Fisher Scientific, United States). The ACE I/D polymorphisms were determined by specific primers (forward primer: 5'-CTGGAGACCACTCCCATCCTTTCT-3'; reverse primer: 5'-GATGTGGCCATCACATTCGTCAGAT-3'), as mentioned by Masud and Qureshi.[27] The PCR mix for 10 µl reaction volume (1x) contained 1 µl of 10x Taq reaction buffer, 0.3 µl of $MgCl_2$, 1.2 µl of 2.5 mM dNTPS, 0.375 µl of 10 pmol forward and reverse primers each, 0.1 µl of Taq Polymerase enzyme, 2 µl of 50-100 ng of template DNA and rest 4.65 µl of injection water. Each PCR also contained a positive and negative control. The PCR cycle followed: initial denaturation at 95^0C for 10 min, followed by 35 cycles of denaturation at 95^0C for 40 sec, annealing at 63^0C for 45 sec, and extension at 72^0C for 1 min, with final extension at 72^0C for 10 min. PCR products were visualized on 2% agarose gel stained with ethidium bromide and imaged under the ChemiDoc XRS gel imaging system (Bio-Rad, United States). The insertion (II: presence of I allele), deletion (DD: presence of D allele), and heterozygous samples (ID) were identified by the presence of 490 bp, 190 bp, and both (490 bp and 190 bp) simultaneously PCR products, respectively (Figure 1).

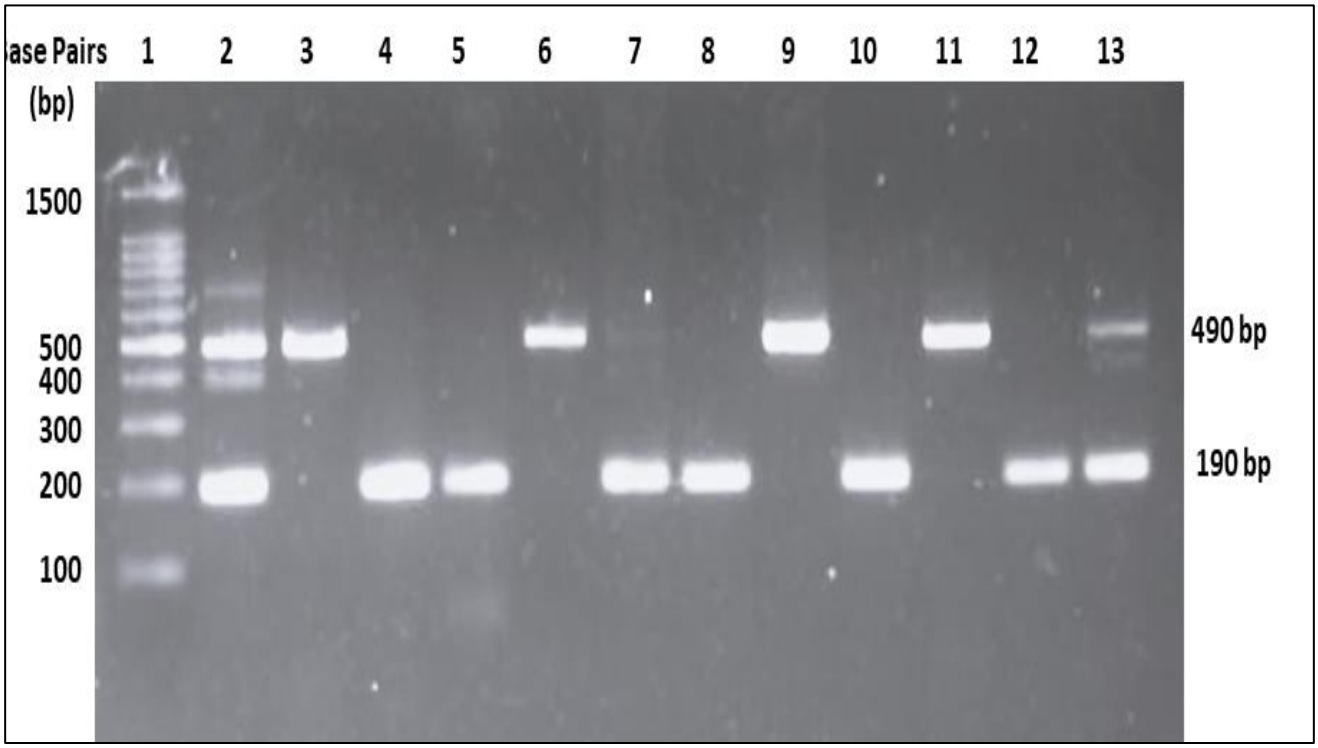

Figure 1: Agarose gel electrophoresis picture of ACE I/D polymorphism.
Lane 1:100bp DNA ladder; lanes 2 and 13; heterozygous I/D; lanes 3, 6, 9 and 11; heterozygous I/I; lanes 4, 5, 7, 8, 10 and 12; heterozygous D/D.

Statistical analysis

The data analysis was performed using the Plink 1.9 beta software.[28] The normally distributed continuous data are presented as mean±standard deviation and non-normal distributed data are presented as median (range) and frequency. A chi-square ($\chi 2$) test and Fisher's exact test (if the count was<5) were performed for association measures including different genetic models (Genotypic, allelic, dominant, and recessive) of categorical datasets. A family-based association (parenTDT) and parent-of-origin (poo) analysis were used to test disease traits by incorporating parental phenotype and transmission of an allele from parent to affected offspring. A p value below 0.05 was considered statistically significant, and p values between 0.05-0.15 were considered to show a trend of association.[29] Odd ratios (OR), and 95% confidence intervals (CIs) were also calculated. Both case-control analysis and analysis of clinical and other parameters within cases were investigated in the study.

RESULTS

Characteristics of the subjects

A total of n=667 CHD cases (denoted as combined cases, i.e. acyanotic and cyanotic both) and n=104 healthy controls were recruited. The cases included 431 males (64.6 %) and 236 females (35.4%), while in the control group, 70 (67.3 %) were males and 34 (32.7 %) were females and didn't show significant differences (p=0.59). The median age of cases and controls was 1.9 years (0.01-32) and 23 years (1.9-68), respectively.

Case-control association

The polymorphism was in Hardy-Weinberg equilibrium (stringent p>0.001), ensuring the reliability of the genetic data. Cases and controls were divided into II, ID, and DD based on genotypes. The genotypic distributions among cases (DD: 0.23, ID: 0.47, II: 0.30) and controls (DD: 0.26, ID: 0.36, II: 0.38) were documented, with comprehensive results presented in the Tables 1 and 2 below. CHD sub-phenotypes where allele counts were less than 10 were clubbed into the miscellaneous category.

Genotypic model (DD Vs ID Vs II): Marginal associations were observed in the combined (p=0.07) and cyanotic groups (p=0.064). Gender-based analysis revealed marginal significance in females (p=0.057). Significant associations were identified in TOF (p=0.024), miscellaneous group (p=0.018), and CHD with PS or AS (p=0.026).

Allelic model (D Vs I allele): No allelic associations were observed in acyanotic, cyanotic, or combined cases, or in gender-specific analyses. However, a strong association was found in TAPVC in CHD sub-phenotype analysis

[p=0.026; OR (95 % CI)=0.5 (0.37-0.93)].

Dominant model [(DD + ID) Vs II]: Significant differences were noted in female cases [p=0.036; OR (95% CI)=1.68 (1.03-2.74)], with marginal associations in cyanotic [p=0.059; OR (95% CI)=1.61 (0.98-2.62)] and combined cases [p=0.07; OR (95% CI)=1.48 (0.96-2.27)].

Sub-phenotypic analysis showed significant associations in TOF [p=0.022; OR (95% CI)=1.86 (1.1-3.23)] and CHD with PS or AS [p=0.028; OR (95% CI)=1.81 (1.06-3.09)], with marginal association in TAPVC [p=0.075; OR (95% CI)=0.48 (0.22-1.08)].

Recessive model [DD Vs (II + ID)]: No associations were obtained in any category. Trends were observed in TAPVC [p=0.11; OR (95% CI)=0.41 (0.13-1.27)] and miscellaneous group [p=0.12; OR (95% CI)=0.53 (0.24-1.18)] in sub-phenotypic analysis.

Family-based association and parent-of-origin study

Family-based association test (parenTDT) with 433 CHD families revealed a significant association with the D allele in TAPVC (p=0.046) and a trend in ASD (p=0.096). Parent-of-origin (poo) test highlighted strong parental transmission in combined (p=0.037) and acyanotic cases (p=0.038), and in both cases, the maternal transmission was prominent with p values of 0.022 and 0.039, respectively. In acyanotic category, only VSD showed significant D allele transmission (p=0.021), possibly from paternal origin (p=0.056) (Table 3).

ACE I/D polymorphism association with clinical data of patient and parents

Clinical and family history data captured on the REDCap application were rigorously analyzed with control and within cases, wherever needed (Table 4). CHD with stenosis and with PAH were compared within cases, depending on presence or absence, and the former found no association in any model, while the later showed genotypic association (p=0.05) along with a trend in the recessive model (p=0.10).

The patient's hemoglobin (HGB) was categorized into four groups: A is for cases with normal HGB, B is for the control group with normal HGB, C is for cases that have HGB <11.5, and D is for cases with HGB>16.5. C and (C+D) showed significant association when compared with the B group in the dominant model, with p=0.05 [OR (95% CI)=1.71 (1.04-2.79)] and 0.049 [OR (95% CI)=1.68 (1.04-2.69)] respectively. C group also showed association w.r.t. A in the recessive model [p=0.045; OR (95 % CI)=1.54 (1.01-2.35)].

A trend of associations was observed in C vs A (p=0.13) and C vs B (p=0.13) in genotypic, and C vs A (p=0.12) and C vs B (p=0.12) in the allelic model. No association was observed in the D group. (C+D) group demonstrated a genotypic trend of association (p=0.11) with the B group. A trend of association was found in early diagnosis (<1 year vs >1 year) in the recessive model (p=0.12).

The heartbeat of the patient was also marginally associated with the D allele in the genotypic (p=0.07) and dominant model (p=0.098). Pneumonia in cyanotic and acyanotic patients was compared and described as a marginal association in the dominant model (p=0.13). Other parameters such as BMI, delivery type, gestational age, birth weight, oxygen saturation (SpO_2) level, and complications in patients e.g. breathlessness, suck rest suck cycle, diarrhea, pneumonia, recurrent cough cold, and cyanosis showed no significant associations with angiotensin-converting enzyme insertion/deletion polymorphism.

The parameters related to maternal history, complications, and nutritional intakes during pregnancy were also investigated. Only primi gravida showed association in the dominant model [p=0.05; OR (95% CI)=0.71 (0.49-0.98)] with a trend observed in the genotypic model (p=0.083). The anemic status of the mother during pregnancy was found marginally associated in the dominant model [p=0.06; OR (95% CI)=0.69 (0.48-1.01)], with a trend in the allelic model (p=0.097). The mother's abnormal menstrual cycle, young (<18 years) or late (>35 years) gravida, bad obstetric history (OH), hypertension, and nutrient intake were independent of angiotensin-converting enzyme insertion/deletion polymorphism.

Addictions such as alcohol and tobacco consumption and smoking were observed separately in both parents, and only paternal tobacco consumption was found related in recessive [p=0.029; OR (95% CI)=2.59 (1.07-6.27)], genotypic (p=0.09) and allelic (p=0.09) models.

The environmental factors within a 500 m radius of the patient's residential area like the presence of any pollution source, the presence of a cell tower, and the quality of water (soft and hard water) were examined and a trend of association was observed for the presence of cell tower only in the allelic model (p=0.104), rest were found unlinked with this polymorphism (Table 4).

These investigations provide significant insights into potential risk factors associated with angiotensin-converting enzyme insertion/deletion polymorphism, underscoring its relevance in understanding disease susceptibility and progression.

Table 1: Case-control association in different models.

Test of association	Counts (frequency)						χ^2; p value						
	Controls (n=104)	Combined (n=667)	Acyanotic (n=446)	Cyanotic (n=221)	Male cases (n=431)	Female cases (n=236)	Combined vs controls	Acyanotic vs controls	Cyanotic vs controls	Acyanotic vs cyanotic	Male cases vs controls	Female cases vs controls	Male vs female cases
Genotypic model													
DD	27 (0.26)	154 (0.23)	103 (0.23)	51 (0.23)	96 (0.22)	58 (0.25)	5.24; 0.07#	4.19; 0.12#	5.49; 0.064#	0.48; 0.78	4.12; 0.12#	5.73; 0.057#	1.25; 0.53
ID	37 (0.36)	315 (0.47)	207 (0.46)	108 (0.49)	201 (0.47)	114 (0.48)							
II	40 (0.38)	198 (0.30)	136 (0.31)	62 (0.28)	134 (0.31)	64 (0.27)							
Allelic model													
DD	91 (0.44)	623 (0.47)	413 (0.46)	210 (0.47)	393 (0.46)	230 (0.49)	0.63; 0.43	0.44; 0.51	0.81; 0.37	0.17; 0.68	0.23; 0.63	1.44; 0.23	1.21; 0.27
II	117 (0.56)	711 (0.53)	479 (0.54)	232 (0.53)	469 (0.54)	242 (0.51)							
Dominant model													
DD + ID	64 (0.62)	469 (0.70)	310 (0.69)	159 (0.72)	297 (0.69)	172 (0.73)	3.25; 0.07#	2.46; 0.12#	3.56; 0.059#	0.42; 0.52	2.04; 0.15#	4.37; 0.036*	1.15; 0.28
II	40 (0.38)	198 (0.30)	136 (0.31)	62 (0.28)	134 (0.31)	64 (0.27)							
Recessive model													
DD	27 (0.26)	154 (0.23)	103 (0.23)	51 (0.23)	96 (0.22)	58 (0.25)	0.41; 0.52	0.38; 0.54	0.32; 0.57	<0.001; 0.99	0.64; 0.42	0.07; 0.79	0.45; 0.49
II + ID	77 (0.74)	513 (0.77)	343 (0.77)	170 (0.77)	335 (0.78)	178 (0.75)							

*D: deletion; I: insertion, *Significant p values. #p values showing trend of association.

Table 2: CHD sub-phenotypes based association in different models.

Test of association and categories	Genotypic model		Allelic model		Dominant model		Recessive model	
	Counts	X^2; p value	Counts	X^2; p value	Counts	X^2; p value	Counts	X^2; p value
Controls	27/37/40	(ref)	91/117	(ref)	64/40	(ref)	27/77	(ref)
ASD	24/38/25	2.16; 0.34	86/88	1.23; 0.27	62/25	1.99; 0.16	24/63	0.06; 0.80
VSD	68/135/89	3.73; 0.15	271/313	0.46; 0.51	203/89	2.23; 0.14#	68/224	0.30; 0.58
TOF	34/77/37	7.50; 0.024*	145/151	1.35; 0.25	111/37	5.22; 0.022*	34/114	0.29; 0.59
TGA	5/4/3	1.50; 0.47	14/10	1.44; 0.23	9/3	0.86; 0.36	2/7	0.06; 0.81
SV	4/7/4	0.93; 0.63	15/15	0.33; 0.55	11/4	0.78; 0.38	4/11	0.003; 0.95
AV Canal	5/7/5	0.51; 0.77	17/17	0.37; 0.55	12/5	0.51; 0.47	5/12	0.09; 0.77
TAPVC	4/10/18	3.89; 0.14#	18/46	4.98; 0.026*	14/18	3.17; 0.075#	4/28	2.52; 0.11#
Misc.	10/37/17	8.02; 0.018*	57/71	0.02; 0.89	47/17	2.50; 0.11#	10/54	2.47; 0.12#
CHD phenotypes with stenosis (PS or AS)	35/81/40	7.33; 0.026*	151/161	1.08; 0.29	116/40	4.82; 0.028*	35/121	0.43; 0.51
CHD phenotypes with PAH	25/29/29	0.45; 0.79	79/87	0.55; 0.46	54/29	0.25; 0.62	25/58	0.39; 0.53

*ref: reference; ASD: atrial septal defect; VSD: ventricular septal defect; TOF: tetralogy of Fallot; TGA: transposition of great arteries; SV: single ventricle; AV canal: atrio-ventricular canal; TAPVC: total anomalous pulmonary venous connection; Misc.: miscellaneous; PS: pulmonary stenosis; AS: aortic stenosis; PAH: pulmonary artery hypertension, *Significant p values. #p values showing trend of association.

Table 3: Family-based association and parent-of-origin test.

Test of association and ccategories	Family-based association (parenTDT)			Parent-of-origin analysis (POO)					
	T	U	χ^2; p value	Paternal		Maternal		Z_POO	P value (POO test)
				T:U	X^2; p value	T:U	X^2; p value		
All cases	145	165	1.29; 0.26	84:76	0.4; 0.53	61:89	5.23; 0.022*	2.08	0.037*
Acyanotic cases	103	115	0.66; 0.42	61.5:52.5	0.71; 0.39	41.5:62.5	4.24; 0.039*	2.07	0.039*
Cyanotic cases	50	42	0.69; 0.40	23.5:22.5	0.02; 0.88	26.5:19.5	1.07; 0.30	-0.63	0.53
ASD	23	13	2.78; 0.096#	11:7	0.89; 0.35	12:6	2; 0.16	-0.35	0.73
VSD	80	75	0.16; 0.69	48:31	3.66; 0.056#	32:44	1.90; 0.17	2.31	0.021*
TOF	32	32	0; 1	15.5:16.5	0.03; 0.86	16.5:15.5	0.03; 0.86	-0.25	0.80
TAPVC	4	12	4; 0.046*	2:6	2; 0.16	2:6	2; 0.16	0	1
TGA+SV+AV canal+Misc. group	16	22	0.95; 0.33	10.5:12.5	0.17; 0.68	5.5:9.5	1.07; 0.30	0.55	0.58

Abbreviations: T: Transmitted minor allele count; U: Un-transmitted allele count; Z_POO: Z score for difference in paternal Vs maternal odd ratios, *Significant p values. #p values showing trend of association.

Table 4: Association of patient and parents related parameters and environmental factors with ACE I/D polymorphism.

Test of association and categories within CHD cases		Genotypic model		Allelic model		Dominant model		Recessive model	
		Counts	χ^2; p value	Counts	χ^2; p value	Counts	χ^2; p value	Counts	χ^2; p value
CHD phenotypes	With PAH Without PAH	25/29/29 129/286/169	5.94; 0.05*	79/87 544/624	0.06; 0.81	54/29 415/169	1.25; 0.26	25/58 129/455	2.64; 0.10#
Patient characteristics & complications	Normal HGB within cases (A) HGB of control group (B) Abnormal HGB (<11.5) (C) Abnormal HGB (>16.5) (D) Abnormal HGB (C + D)	49/120/72 27/37/40 66/103/65 12/31/18 78/134/83	- - 4.06; 0.13# (w.r.t. A) 4.02; 0.13# (w.r.t. B) 0.02; 0.98 (w.r.t. A) 3.69; 0.16 (w.r.t. B) 4.41; 0.11# (w.r.t. B)	218/264 91/117 235/233 55/67 290/300	- - 2.37; 0.12# (w.r.t. A) 2.41; 0.12# (w.r.t. B) <0.001; 0.98 (w.r.t. A) 0.06; 0.83 (w.r.t. B) 1.79; 0.18 (w.r.t. B)	169/72 61/40 169/65 43/18 212/83	- - 0.26; 0.61 (w.r.t. A) 3.84; 0.05* (w.r.t. B) 0.003; 0.95 (w.r.t. A) 1.35; 0.25 (w.r.t. B) 3.85; 0.049* (w.r.t. B)	49/192 27/77 66/168 12/49 78/217	- - 4.01; 0.045* (w.r.t. A) 0.18; 0.67 (w.r.t. B) 0.01; 0.91 (w.r.t. A) 0.84; 0.36 (w.r.t. B) 0.009; 0.92 (w.r.t. B)
	Age of diagnosis <1 year Age of diagnosis >1 year	92/213/131 55/92/59	2.53; 0.28	397/475 202/210	1.38; 0.24	305/131 147/59	0.13; 0.72	92/344 55/151	2.48; 0.12#
	Fast heart beat Normal heart beat	88/156/115 65/158/79	5.28; 0.07#	332/386 288/316	0.27; 0.60	244/115 223/79	2.73; 0.098#	88/271 65/237	0.82; 0.36
	Pneumonia (in cyanotic cases) Pneumonia (in acyanotic cases)	12/33/14 26/58/45	2.61; 0.27	57/61 110/148	1.03; 0.31	45/14 84/45	2.34; 0.13#	12/47 26/103	<0.001; 0.98
Maternal history	Primi-gravida Multi-gravida	47/81/69 107/234/129	4.97; 0.083#	175/219 448/492	1.17; 0.28	128/69 341/129	3.82; 0.05*	47/150 107/363	0.09; 0.76
Maternal complications during pregnancy	Anemic Non-anemic	34/71/57 119/243/137	3.53; 0.17	139/185 481/517	2.75; 0.097#	105/57 362/137	3.52; 0.06#	34/128 119/380	0.56; 0.45
Paternal addiction	Tobacco consumption No tobacco consumption	9/7/5 145/308/193	4.81; 0.09#	25/17 598/694	2.86; 0.091#	16/5 453/193	0.36; 0.55	9/12 145/501	4.77; 0.029*
Environmental factors (within 500 m)	Presence of mobile tower No mobile tower	44/72/42 74/156/107	2.53; 0.28	160/156 304/370	2.64; 0.104#	116/42 230/107	1.36; 0.24	44/114 74/263	2.06; 0.152

Abbreviations: PAH: Pulmonary artery hypertension; HGB: Hemoglobin, *Significant p values. #p values showing trend of association.

DISCUSSION

Our study comprehensively explores the relationship of ACE insertion/deletion polymorphism with CHD, covering case-control association, CHD sub-phenotypes, gender-based models, parental transmission tests, and clinical parameter analyses along with environmental components within cases. All patients showed comparatively low BMI (p<0.0001). Case-control test of association showed that the DD genotype and D allele may significantly increase the risk of CHD in combined and cyanotic cases. Strong associations were observed in TOF in genotypic (p=0.024) and dominant (p=0.022; OR=1.86) models, TAPVC in the allelic model (p=0.026), and the miscellaneous category (p=0.018). Kumari et al showed a correlation between the DD genotype of ACE with CVD among the rural Indian population and found elevated levels of total cholesterol and triglyceride and low levels of non-high-density lipoprotein (p<0.05).[11] Fajar et al performed a meta-analysis study that revealed D allele association with a high risk of left ventricular hypertrophy in Indonesia.[30] Females exhibit higher risk with DD genotype (p=0.057) and D allele (p=0.036). CHD phenotypes with stenosis features have a higher risk (OR=1.81 in the dominant model) as they demonstrated scarcity of oxygen in the blood due to restricted flow through a narrow passage of aortic and pulmonary valves that may increase blood pressure. As long as the D allele is non-expressive (recessive), the risk of CHD prognosis is low. A recent study reported an association of the D allele and DD genotype with New York Heart Association (NYHA) class IV heart failure (p=0.039 and p=0.049 respectively).[31] Taha et al advocated the risk of MI and unstable angina (UA) in the Egyptian population by epistasis and haplotype profiling of ACE I/D with methylenetetrahydrofolate reductase variants (rs1801133 and rs1801131), apolipoprotein B (R3500Q) and apolipoprotein E (Ɛ4) and concluded that ACE I/D and R3500Q interaction decrease the risk of UA while haplotype association all five variants increase the risk of MI.[32]

The family-based association and parent-of-origin test revealed maternal transmission in combined (p=0.037) and acyanotic (p=0.039) cases and our gender-based association has validated a higher risk of CHD in females with DD genotype and D allele. No study has been conducted so far to explore such investigations as per the literature. VSD, a major acyanotic defect comprising approximately 35.6% of all CHD, showed paternal transmission (p=0.021).[33] Interestingly, TOF which was found to be associated with the case-control test didn't show any clue in the parenTDT test. Among the cyanotic group, only TAPVC demonstrated significant D allele transmission (p=0.046) but was unable to yield the origin of transmission (p=1) because of the higher frequency of the I allele (0.56). Parental transmission patterns in specific CHD subtypes provide further insights into familial inheritance mechanisms impacting disease susceptibility.

A significant difference was obtained in cases with CHD (±) pulmonary arterial hypertension linked with DD genotype (p=0.05). D allele may also play a susceptible role in hemoglobin homeostasis among patients as its dominant expressiveness can decrease the level of hemoglobin by ~1.7 times. D allele if recessively expressed may delay early diagnosis of CHD. DD genotype may be one of the causal factors of a patient's fast heart rate (p=0.07) and comparatively less frequent in primi-gravida cases in the dominant model (p=0.05; OR=0.71). D allele association with high serum ACE increases the risk of thrombophilia and is linked with infertility and recurrent miscarriages however, in the present study, no association was seen for abnormal menstrual cycle and bad obstetric history of the mother.[20,34,35] D allele and DD genotype (dominant model) may reduce the chances of anemia in mothers during pregnancy by around 31% (p=0.06; OR=0.69). Parental addictions such as smoking, tobacco consumption, and alcohol intake can cause chronic obstructive pulmonary disease (COPD) and the DD genotype was found associated with a high risk of COPD among north Indians (p=0.006; OR=2.14).[17] Another study in the Northeast India cohort showed II genotype linkage with tobacco consumption with a ~ 4-fold risk of having lung cancer (p=0.005; OR=4.09).[36] The fathers who were consuming tobacco, since/before marriage till the birth of the child, have the II genotype with 2.5 fold more risk of having a CHD child (p=0.029; OR=2.59). The presence of cell towers may be involved in CHD prognosis with the D allele (p=0.10) but this can't be said confidently until further dedicated study.

Preterm birth happens because of the abnormal placenta, cervix, and uterus, bleeding during gestation, and certain inflammatory actions, and is also correlated with gestational unbalanced blood pressure, including the RAAS system, and thus suggests the role of the ACE I/D variant.[36] Neither D or I alleles nor DD or ID or II genotypes were found associated with preterm birth, caesarian delivery, low birth weight, low SpO_2, Breathlessness, diarrhea, pneumonia, frequent cough and cold among patients, and abnormal BMI, hypertension, nutritional intake during pregnancy among mothers. Other studies highlighted the increased risk of gestational preeclampsia with DD genotype and D allele in recessive (p=0.007; OR=2.09) and allelic (p=0.012; OR=1.75) models although we didn't investigate such analysis in our CHD cohort.[37] Parental smoking and other addictions along with the presence of pollution sources in nearby areas and water quality (soft and hard water) were also not to be linked with ACE I/D in our study population.

To date, only three studies from China (p=0.39; OR=1.13), Saudi Arabia (p=0.2; OR=1.9), and Egypt (p=0.3, OR=1.3) attempted to rule out a relationship of ACE I/D with CHD but unfortunately, none of them were able to give any clue (Table 5).[8-10] The acute-phase reactants e.g. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were present excessively (p=0.02), possibly due to the high frequency of the D

allele in rheumatic heart disease patients.[38] A study highlighted the role of ACE I/D in minimizing the risk of tachyarrhythmias (p=0.01; OR=0.32) and higher the risk of junctional ectopic tachycardia (p=0.04; OR=2.4) in pediatric CHD patients after intervention and therefore, further research is required to find the relationship of ACE I/D with hematological parameters of CHD patients can also enlight the better understanding of the mechanism behind the prognosis of the disease, as these parameters showed promising role in disease diagnosis.[39]

Table 5: Studies on the association of ACE I/D polymorphism with CHD (globally till date).

Year	Population	Sample size		D- allele frequency		P value; OR (95% CI) in different models				Finding	Reference
		Control	Cases	Control	Cases	Genotypic	Allelic	Dominant	Recessive		
2020	Egyptian	70	70	0.60	0.66	1; 1.1 (0.3-3.8)	0.3; 1.3 (0.8-2.1)	1; 1.2 (0.3-2.9)	0.2; 0.6 (0.3-1.2)	No association	Saleh et al[10]
2016	Qinghai Han Chinese	193	59	0.35	0.31	0.66	0.39; 1.2 (0.8-1.9)	0.55; 0.8 (0.47-1.5)	0.4; 0.65 (0.24-1.8)	No association	Jing et al[9]
2015	Saudi Arabian	145	96	0.66	0.67	0.3	0.8; 1.1 (0.7-1.6)	0.2; 1.9 (0.8-4.7)	0.8; 0.9 (0.5-1.5)	No association	Alazhary et al[8]

CONCLUSION

Our study provides robust evidence of ACE I/D polymorphism's role in CHD susceptibility, particularly in specific sub-phenotypes and parental transmission patterns, and highlights the importance of this polymorphism in the stratification of congenital heart anomalies in the context of North India. These findings enhance our understanding of genetic influences and clinical outcomes in CHD, informing future studies on personalized treatment strategies. Given the scarcity of data from other regions of India, further explorations in a large study cohort and different populations across India and other countries are necessary to validate this association and uncover the underlying mechanism of disease biology. This comprehensive analysis contributes valuable insights into the multifaceted role of ACE I/D polymorphism in CHD pathogenesis, guiding advancements in clinical management and genetic research, and will shed some insight on how the ACE I/D polymorphism can be utilized as a biomarker for CHD.

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