RESEARCH ARTICLE

MATERNAL GENE POLYMORPHISMS OF VITAMIN B12 METABOLIC PATHWAY AND THEIR ASSOCIATION WITH CONGENITAL HEART DISEASES

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Manuscript Info

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Abstract

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Congenital heart disease (CHD) describes a huge set of structural and functional abnormalities that arises during cardio embryogenesis. Low levels of maternal vitamin B12 can disturb the homeostasis of the fetal folate metabolism pathway. The gene CUBN encodes for the intrinsic factor– cobalamin receptor (cubilin), which acts as a receptor for intrinsic factor to many compounds such as vitamin B12.Main aim of our study was to analyze association of CUBN G253A (rs1801222) variant with maternal serum folate, vitamin B12 and homocysteine (Hcy) levels and thereby its link with congenital heart defects. 110 pregnant women with vitamin B12 deficiency and carrying fetuses with CHD and equal number of age matched healthy women were selected as cases and control subjects respectively. Maternal serum folate, vitamin B12 and homocysteine levels as well as genetic polymorphism CUBN G253 $\rightarrow$ A were assessed. In the present study, it was observed that cases with GA genotype, A allele under the dominant (p=<0.001) and allelic models (p<0.001) respectively and cases with AA genotype under the co-dominant (p<0.01) and recessive models (p<0.001) demonstrated an increased risk of an offspring with CHD. Furthermore, while cases with GA and AA genotypes of CUBN G253A variant showed significantly elevated Homocysteine, it was observed that all the CHD case mothers were vitamin B12 deficient irrespective of the CUBN genotype.

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Introduction:-

The most prevalent congenital human birth defect is congenital heart disease (CHD), which affects 9.1 out of every 1,000 live births worldwide 1.CHD is caused by a combination of hereditary and environmental causes. Studies show that the state of maternal nutrition and the materno-fetal transfer of nutrients influence the fetal metabolism and ultimately fetal outcome 2. It was determined that the imbalanced folate metabolism pathway might lead to CHD 3. Vitamin B12, or cobalamin (Cbl), plays an important role in folate metabolism. Vitamin B12 participates in folate-dependent re-methylation of homocysteine to methionine. It is hence an important regulator of homocysteine metabolism 4. Vitamin B12 deficiency invariably compromises methionine synthase activity resulting in elevated Hcy levels 5. Abnormally high homocysteine levels are associated with CAD 6 and high maternal homocysteine levels have been postulated as potential risk factor for congenital heart defects 7. Hence, low concentrations of folic

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acid and vitamin B12 result of defective utilization of Hcy which leads to its accumulation in the cells. Dietary intake is the main source of Vitamin B12 in the body and vitamin B12 transport machinery plays a crucial role in attaining required circulating levels of the vitamin. The significance of vitamin B12 transport machinery may be evidenced from a condition referred to as Imerslund-Gräsbeck syndrome caused by low levels of vitamin B12 and caused by mutations in the AMN or CUBN gene which are a part of transport machinery for Vitamin B12 8. Therefore, vitamin B12 levels are largely dependent on the proper functioning of the transport machinery GWAS and candidate gene studies determined that vitamin B12 levels could be influenced by genetic variants of the vitamin B12 metabolic pathway.

CUBN and AMN form a complex called cubam that represents part of the IF receptor responsible for intestinal vitamin B12 uptake 4. The CUBN gene is located on chromosome 10 at locus 10p13 and encodes for vitamin B12 binding protein cubilin. It is involved in promoting the entry of vitamin B12 into cells through receptor-mediated endocytosis 4and forms a part of vitamin B12 transport machinery along with Amnion less protein. Amnion less attaches (binds) to cubilin, anchoring cubilin to the cell membrane. Cubilin can interact with molecules and proteins passing through the intestine or kidneys. Several studies have reported an association between variation within the CUBN gene and circulating vitamin B12 concentration [8]9. The present study was carried to test the association between CUBN gene polymorphism and congenital heart defects. This was performed by estimating the allelic and genotype frequencies of the CUBN gene polymorphism with that of concentrations of homocysteine, folic acid and vitamin B12 in pregnant women.

Materials and Methods:-
Subjects:
In the present study two hundred and twenty pregnant women between 16 to 28 weeks of gestation, enrolled between September 2016 to December 2018 at Govt. Modern Maternity Hospital, Care Hospital and Asian Institute of Fetal Medicine, Hyderabad enrolled were included in the study. The study was approved by the institutional ethical committee. Informed consent was obtained from all the participants of this study who were willing to participate. All the subjects were examined clinically and detailed history was recorded with particular reference to the known risk factors for CHD using a standard questionnaire. The pregnant women selected for this study were screened for vitamin B12 deficiency and among those individuals, a total of 110 pregnant women with vitamin B12 deficiency and carrying fetuses with CHD, as diagnosed by 3D/4D ultrasound by fetal medicine specialist and pediatric cardiologist were included as cases**.** An equal number of age- matched healthy women (n= 110) with two live births and normal B12 levels were recruited as controls. Pregnant women with known infections such as Rubella, women habituated to alcohol or tobacco and exposed to teratogenic drugs were excluded from the present study.

Biochemical parameters
Folic acid, vitamin B12 and total homocysteine (tHcy) concentrations were analyzed. Folic acid and vitamin B12 in serum were determined with the solid phase enzyme-linked immunoassay (ELISA) method, using Calbiotech Company test kits. The total homocysteine concentration in plasma was determined by double-antibody sandwich enzyme-linked immunosorbent one-step process assay method using kit manufactured by company Qayee Bio.

DNA isolation and genotyping
3ml of venous/ peripheral blood was collected in EDTA tubes. Genomic DNA was extracted from blood of the mothers using DNA extraction kit from the company Qiagen. SNP G253A of CUBN gene (rs1801222) was genotyped using allele specific polymerase chain reaction (AS-PCR) modified method from Ye, S et al. (1992) 10. The primers: Common Forward primer (CFP): 5'-ATGGAAATGAACGTTAGAGG-3', Wild reverse primer (WRP): 5'-CAGGGCTGTTGGGTGATG-3', Mutant reverse primer (MRP): 5'-CAGGGCTGTTGGGTGATA -3' were used for amplification. The size of the amplified product was 165bp. For each sample, two reaction tubes were setup (A and B), one with CFP and MRP and the other with CFP and WRP primer pairs respectively. The amplified product size was 165 bp using both the primer pairs. Hence, the reason to take them in separate reaction tubes. The results of PCR were determined on 2 % agarose gels as seen in the **Figure: 1**.
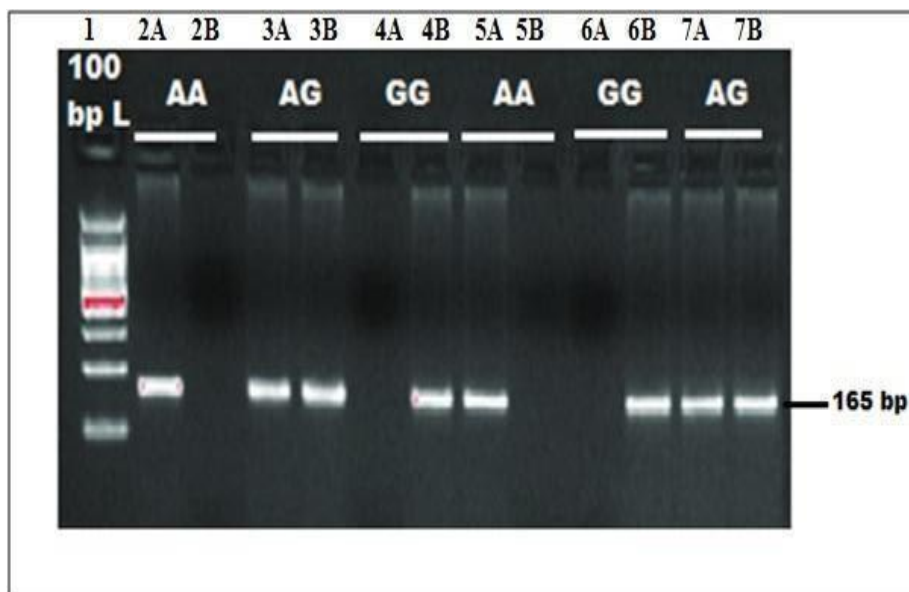
Figure 1:- Agarose gel representation of G253A genotyping. Lane represents the 100bp ladder. Lanes 2A, 2B and 5A, 5B represent samples with homozygous (AA) genotype. Lanes 3A, 3B and 7A, 7B represent a heterozygous (AG) genotype. Lanes 4A, 4B and 6A, 6B represent samples with Homozygous (GG) genotype.

Statistical Analysis:
Hardy-Weinberg equilibrium was tested for polymorphism of CUBN gene in cases and controls. Association between genotypes and CHD was examined by Odds ratio with 95% confidence interval (CI) and Chi Square analysis. Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. Allele and genotype frequencies are calculated using the Hardy Weinberg calculator. SNPstat was used to analyze single SNPs under multiple inheritance models (co-dominant, dominant, recessive and over-dominant). The Folic acid, Homocysteine and vitamin B12 levels in patients and controls were compared by Student's t-test and levels of significance was defined as $P<0.05$. All of the tests were performed using Statistical Package for the Social Sciences (SPSS) version 16 (IBM Corp., Armonk, NY). Independent Sample T-tests was done to match the age, and BMI of each group.

Results:-
In the present study, we have included 110 pregnant women with vitamin B12 deficiency and carrying fetuses with CHD as cases (The cases were screened for the fetal anomalies by a fetal medicine specialist and pediatric cardiologist by 3D/4D ultrasound). Teratogenic effects of certain drugs, smoking and alcoholic habits, family history of the disease (pedigree analysis) and presence of any other diseases were recorded in specially designed structured questionnaire), equal number of age matched healthy women (n= 110) as controls (**Table 1 and 2**). The genotypic distribution and allele frequencies of CUBN gene G253A polymorphism in cases and controls are given in (**Table 3**). The genotype frequencies among both the case and control groups were consistent with Hardy Weinberg Equilibrium (HWE). In the present study, it was observed that cases with GA genotype and A allele under the dominant (GG vs. GA/AA, OR=2.10, p=<0.001) and allelic models (G vs. A, OR=2.40, p<0.001) demonstrated 2-3 folds risk towards CHDs, whereas cases with AA genotype under the co-dominant (GG vs. AA, OR=8.40, p<0.01) and recessive models (GG/GA vs. AA, OR=7.17, p<0.001) demonstrated an increased 7-8 folds risk towards having an offspring with CHD (**Tables 4**). This suggests that CUBN 253 'A' allele may contribute to the susceptibility of CHD. Further, the relation with the maternal serum levels of vitamin B12, folic acid, homocysteine have revealed that, all the CHD case mothers were vitamin B12 deficient irrespective of the CUBN genotype. However, cases with GA and AA genotypes of CUBN G253A variant showed significantly elevated Homocysteine (**Table 5**).

Table 1:- Demographic data of Controls and Cases (Categorized variables).

	Controls	Cases	χ^2	OR	p-value
	N (%)	N (%)		(95% CI)	

Pregnant women	110(100)	110(100)	-	--	--
With Family History	12(10.9)	19(17.3)	1.35	0.58(0,26-1.27)	0.24
Without Family History	98(89.1)	91(82.7)			
With Diabetes Mellitus	7(6.3)	9(8.1)	0.06	0.76(0.26-2.17)	0.7
Without Diabetes Mellitus	103(93.7)	101(91.9)			
With Hypothyroidism	18(16.4)	28(25.4)	2.22	0.57(0.29-1.11)	0.13
Without Hypothyroidism	92(83.6)	82(74.6)			
With Consanguinity	38(34.5)	43(39)	0.31	0.82(0.47-1.42)	0.57
Without Consanguinity	72(65.5)	67(61)			
Vegetarian	18(16.4)	15(16.5)	0.14	1.23(0.58-2.64)	0.7
Non vegetarian	92(83.6)	95(86.4)			
*Significant at p<0.05					

OR, odds ratio; CI, confidence interval; $\chi 2$, Chi Square

Table 2:- Demographic data of Controls and Cases (continuous variables).

	Controls N=110			Cases N=110		
	Mean ± SD	Min	Max	Mean ±SD	Min	Max
Age (years)	24.29±3.8	19	35	25.10±3.7	18	32
BMI (Kg/M2)	25.35± 5.1	17	42	25.41±4.9	16	40

SD, Standard deviation

Table 3:- Allele frequencies in case and control participants.

		Controls N=110 n (%)	Cases N=110 n (%)	$\chi 2$	OR(95% CI)	p-Value
Allele frequency	G A	165(0.75) 55(0.25)	122(0.55) 98(0.45)	17.6	2.40(1.60-3.61)	0.0001*
Minor allele frequency	A	0.25	0.45		- - - -	
HWE (p-value)	- -	0.45	0.02		- - - -	

OR, odds ratio; CI, confidence interval; $\chi 2$, Chi Square

Table 4:- Association between *CUBN* gene variant G253A and CHD in different genetic models.

Model	Genotype	Controls N=110 n (%)	Cases N=110 n (%)	$\chi 2$	Odds ratio (95% CI)	p-Value
Co-dominant	GG	60(54.5)	40(36.4)	0.9	1.00	0.32
	GA	45 (40.9)	42 (38.2)		1.40 (0.78-2.50)	
	AA	5(4.5)	28 (25.4)	11.7	8.40 (2.99-23.58)	**0.0001***
Dominant	GG GA-GG	60(54.5) 50(45.5)	40(36.4) 70 (63.6)	6.6	2.10(1.22-3.60)	**0.006***
Recessive	GG-GA AA	105(95.5) 5(4.5)	82(74.5) 28 (25.4)	17.25	7.17(2.65-19.38)	**0.0001***
Over dominant	GG-AA GA	65(59.1) 45 (40.9)	68(61.8) 42(38.2)	0.07	0.89(0.52-1.53)	0.68
Log-Additive	- - - -	- - - -	- - - -	- - - -	2.24(1.50-3.35)	0.0001*
*Significant at p<0.05 (Odds p-values)						

OR, odds ratio; CI, confidence interval; χ2, Chi Square

Table 5:- Maternal serum vitamin B12, folic acid, homocysteine stratified according to *CUBN* G253A **.**

Genotypes (n =controls/ cases)	Vitamin B12		Folic acid		Homocysteine	
	Controls Mean ±SD	Cases Mean ±SD	Controls Mean ±SD	Cases Mean ±SD	Controls Mean ±SD	Cases Mean ±SD
GG (60/40)	340.7±92	165.9±40.8	3.39±1.93	3±1.8	15.9±8.3	18±10
GA (45/42)	312.9±107.1	182.9±37.5	2.9±1.49	2.9±1.8	18.1±9.2	20±10.4
AA (5/28)	289.6±144.5	161.7±43.8	3.89±2.2	3±1.8	14.9±7.2	21.5±13.2

SD, Standard deviation

Discussion:-
Genetic polymorphisms in the folate metabolizing genes and their association with congenital heart defects has been studied extensively 113. Also, the role of vitamin B12 in homocysteine metabolism and link between hyperhomocysteinemia and disturbance in the folate metabolism pathways has been observed 12. Vitamin B12 transport machinery is complex with the various protein molecules influencing the transport from ingestion to its entry into the blood 13. One such protein is cubilin which along with another protein amnionless forms a receptor complex that is necessary for the uptake of Intrinsic factor-cobalamin (IF-Cbl) complex into ileal cells of the intestine 14. Studies suggest that cubilin mediates IF-Cbl endocytosis at the placental maternal fetal interface level and is expressed by a number of absorptive epithelial elements, which include yolk sac extra embryonic visceral endoderm etc.,15. Limited studies were carried out on the vitamin B12 transport machinery and genes encoding them. The GWAS and candidate gene studies have identified 19 genes and 49SNPs in these genes which play a crucial role in vitamin B12 metabolism 16. One hypothesis states that polymorphisms of CUBN gene can decrease the transportation and the absorption of B12 in the ileum 17. A link between CUBN rs1801222 and reduced vitamin B-12 status was observed in few studies 9. Also, the association of genetic polymorphisms in the vitamin B12 metabolic pathway and circulating maternal vitamin B12 levels with effects on fetal metabolism and congenital heart defects has been a subject of study by many genetic researchers 9. G253A (rs1801222) is a missense mutation in exon 8 that is projected to reduce CUBN functionality, lowering vitamin B-12 absorption. Currently, studies on variants of CUBN gene association with vitamin B12 status have yielded conflicting results. Hazra et al. (2009) was the first to report an association of 'G' allele of rs1801222 (Ser253Phe) SNP with higher levels of vitamin B12 (β = 0.05 pg/ml, P = 2.87×10-9) in 4763 individual subjects of USA 18, which further confirmed by Grarup et al. (2013) on examining 45,571 individuals from Icelandic and Danish (β = 0.10- 0.17 p,mol/L, P = 3.83×10-75) populations 19. While Zinck et al. (2015) have reported the conflicting results on 3114 Canadian individuals (85% Caucasian and 15% non Caucasian) where the 'G' allele was associated with vitamin B12 deficiency with higher risk (OR 1.61 pmol/l, 95% CI 1.24-2.09, p = 3.0×10-4) 9. Hazra et al. (2009) and Grarup et al. (2013) had observed that the MAF 'A' was found to be 0.28 and 0.41 in Caucasians residing in USA and populations of Norway, respectively 18 19.

Conclusion:-
Our study shows a significant association of maternal G253A polymorphism of CUBN gene with congenital heart defects. Also, 'A' of the CUBN G253A variant shows an elevated homocysteine levels. The study pinpoints on the role of the CUBN gene in the expression of biochemical parameters such as vitamin B12 and homocysteine levels in CHD. Identifying the association between the gene and the nutrient interactions is beneficial and is of importance as these come under modifiable risk factors. In a developing country like India, where the burden of nutrient deficiency is like a tip of the iceberg, identifying such associations further benefits those sub populations where the genetic polymorphisms may exacerbate the existing deficiency. However, apart from genetic mechanisms, epistatic, epigenetic and metbolomic mechanisms also play a pivotal role in heart restructure in case of CHD.

Conflicts of Interest
The authors declare no conflict(s) of interest.

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