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ASSOCIATION OF MTHFR 677CT, 1298AC POLYMORPHISMS AND FOLIC ACID WITH SPINA BIFIDA IN PAKISTANI POPULATION

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Abstract
Spina Bifida (SB) is a neural tube defect that is commonly increasing substantial with genetic component. Complex pattern and various genetic polymorphisms may affect genetic predisposition of the disease that is mostly characterized by folic acid deficiency. Between diverse ethnic populations, the genetic architecture of SB may vary, so it is crucial that such variants are examined in Pakistani population. The present study was specifically targeted to find out the association of blood folate level and methylenetetrahydrofolate (MTHFR) gene polymorphisms with SB in Pakistan. In present study significant p value 0.001 showed the association of blood folate level and SB. Differences of genotypes among MTHFR 1298A→C highly significant between normal groups and SB X^2=13.273(0.001). While 677C→T found to be non-associated X^2= 0.327 (0.827) with SB in population. To conclude, low blood folate level of parents and MTHFR gene polymorphisms 1298A→C can be a cause of inception and progression of SB in local population of Pakistan but relationship between MTHFR genetic polymorphisms to diverse SB complications is yet not obvious and warrants further studies of functional genomics to validate the SB onset and progression with genetic susceptibility of MTHFR gene.

Keywords: MTHFR, spina bifida, neural tube defect, folic acid, HbA1c

INTRODUCTION

Congenital anomalies remain a major source of lifetime morbidity, disability for long-term, economic and psychological setbacks and most of the time mortality worldwide. Spina bifida (SB) is a term that comprises of a group of congenital abnormalities (occulta, meningocele, aperta and myelomeningocele) which are caused by the incomplete spinal column closure that leads to the exposure of meninges or spinal cord or also called herniation. Though comparatively spina bifida showed lower rate of mortality that is 7% than other neural tube defects which is for anencephaly 100%, for encephalocele 46% but it may result in rigorous lifetime morbidity (Alshalan *et al.*, 2018). Pattern of prevalence of spina bifida differs considerably country wise and range of prevalence is from 1 to 5 births per 1000 births. For example: India has about 1.9 cases per 1000 births whereas US has 0.7 births per 1000 births (Bhide *et al*., 2013). According to Behrooz *et al*., 2007 Iran has 3.8 cases per 10.000 births. In the range of NTDs spina bifida is the most frequent nonfatal defect which has generally a frequency of 0.5 per 1,000 births approximately but higher prevalence rates are also observed. While the incidence of spina bifida is 4-5 per 10003 in Pakistan. The incidence of meningocele in Pakistan is 5% while myelomeningocele is 90-95% in all spina bifida cases (Khan *et al.*, 2006).

Prenatal intake of nutrients are highly beneficial and effective on maternal and fetal health but excessive or inadequate nutrients intake can bring epigenetic changes to the fetus as well and these changes can exert worst long standing and short term implications on well-being in the form of pathophysiological changes (Pei *et al.*, 2015). For the controlled cell proliferation and production of DNA and RNA and production of new cells folic acid, a vitamin B group member plays a vital role. To make hemoglobin in the erythrocytes folic acid work with vitamin B12. It supports to lower the risk of fetal birth deficiency like congenital hypertrophic pyloric stenosis, limb deficiencies, obstructive urinary tract anomalies, orofacial clefts and neural tube deficiencies (Kocylowski *et al.*, 2019). To synthesize RNA and DNA and to metabolize amino acids required for cell division, folate is vital for the body to perform all these functions (Marriott *et al*., 2020).

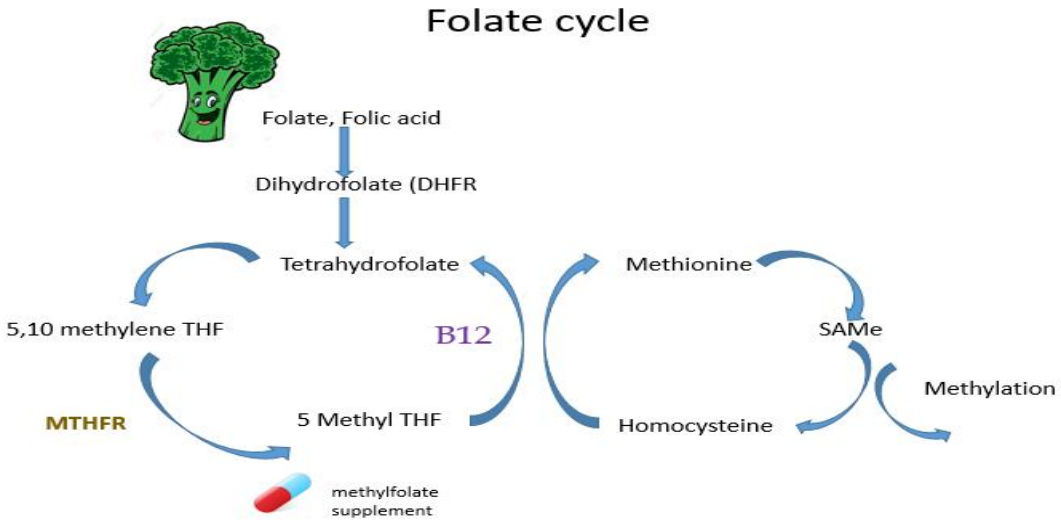

Fig: 1. Folic acid metabolism in relation with MTHFR

During the frequent cell division and growth periods folates needed crucially such as in pregnancy and infancy. Cell division and synthesis of DNA might be obstructed by deficiency of folate in which neoplasms and hemopoietic cells are affected the most due to their higher frequency of cell division (Liu *et al.*, 2020).The liver converts folate into tetrahydrofolate with the help of dihydrofolate reductase therefore biological activity of folate depends on the dihydrofolate action. The conversion of folate is rate limiting action in human body to control the elevated level of nonmetabolized folic acid in the bloods so that uptake of folic acid must not exceed the limit of 1000 µg per day (Bailey and Ayling, 2009).

Several researches have been investigated on the genetic reasons of spina bifida which reported that there is not a single gene involved but various genes are responsible for spina bifida incidence. For genetic factor MTHFR methylenetetrahydrofolatereductase is major gene contribute towards SB and folic acid is contributing as environmental factor. Along with less intake of folic acid by pregnant woman, the genetic polymorphism of one carbon metabolic pathway enzymes may be cause of the spina bifida occurrence (Martinez *et al*., 2009).

Materials and methods

In the current study, researcher investigated some polymorphisms of genes of the folate and homocysteine metabolic pathways including MTHFR 677C→T, MTHFR 1298A→C. To detect MTHFR 677C→T and 1298A→C mutations, PCR amplification and RFLP analysis performed. The experimental work reported in this study carried out in the laboratories at Institute of molecular Biology and Biotechnology (IMBB) and Center for Research In Molecular Medicine (CRIMM) The University of Lahore (UOL), Lahore Pakistan. Current study was cross sectional comparative study. On the basis of verbal inquiry and initial screening of the clinical history and medical reports, number of families fulfilled the selection criteria. Afterwards only twenty four families participated with consent. Control families selected conveniently who visited the hospitals for minor medical issues (sore throat, flu, cough, fever, and pain) and fulfilled the selection criterion. Control families consist of normal child and parents. While case families have one child with any form of spina bifida. The case and control subjects included in this study selected through purposive sampling technique from the different hospitals of Lahore after getting ethical committee approval. Folic acid level estimated using folic acid ELISA kit by cell BiloLabs, Inc (Ashraf *et al*., 2008). Genomic DNA extracted from whole blood by using Vivantis GF-1 blood DNA extraction kit (Ghatak and Muthukumaran, 2013). For PCR the sequences of primers chosen from literature without alteration given in the table. By using particular primers the genomic DNA amplified by following particular (PCR thermocycler T100TM, BioRad) program.

Table.1.MTHFR and MTRR primers

Sr #	Name	Forward/ Reverse	Primer sequence (5'→3')	Base Pair	Reference
1	**MTHFR 677C→T**	Forward Reverse	TGAAGGAGAAGGTGTCTGCGGGA TGAGAGTGGGGTGGAGGGAGCTT	198	Relton *et al.*, 2004
2	**MTHFR 1298A→C**	Forward Reverse	CTTTGGGGAGCTGAAGGACTACTAC CAGGGGATGAACCAGGGTCC	196	Relton *et al.*, 2004

Enzymatic digestion of amplified PCR products

Through polymorphism the amplified fragments of MTHFR then subjected for enzymatic digestion with particular conditions of temperature and time allowing them to verify the individual genotype given in the table. Two enzymes according to their respective gene *HinfI* and *MboII* used for procedure. For genomic DNA (3%), for amplified PCR products and fragments obtained after digestion (2%) agarose gel prepared. Agarose boiled with buffer 1X TBE (Tris-Borate-EDTA) and then stained with Medori Green Safe Buffer (1 µL/mL) (Bulldog Bio, USA). Migration in the gel performed for 120 minutes at 110V. For the comparison of molecular weights of DNA fragments (HyperLadder II, Bioline or VC 100 bp Plus DNA Ladder, Vivantis) a molecular weight marker used. For the visualization of agarose gel UVITEC system (Uvitec Cambridge) using UV light utilized.

Statistical analysis

Obtained results mentioned in the form of mean ± SD (standard deviation) and mean ± SE (standard error). Pearson s' chi-square (X^2) test (statistical approach (Chen *et al* ,. 2017) utilized to compare among the observed data (according to a specific hypothesis) expected to obtain, used to find out the relationship among case and control subjects. Subjects differentiated as case and control groups to investigate the association among biochemical indices and targeted genotypes. Statistically

significant considered when *p*-value < 0.05 using the Statistical Package for Social Sciences (SPSS version 22.0).

Results

Blood folate levels of case and control children showed Std.dev ± 0.67 and ± 1.08 while significant p value 0.032 observed. Case and control fathers showed Std.dev ± 2.35 and ± 1.63 respectively and significant p value was 0.011. Case and control mothers showed Std.dev ± 1.24 and ± 1.54 for blood folate levels. Significant difference shown with p value 0.001.

Table.2. Effect of Blood folate on the incidence of Spina bifida

Variable	Subjects	Group A	Group B	p-value
		Mean±SD	Mean±SD	
Blood folate level	Children	12.28±0.67	12.90±1.08	0.032
	Father	15.45±2.35	17.26±1.63	0.011
	Mother	15.85±1.24	17.51±1.54	0.001

Group A: Case
Group B:Control

Association of MTHFR 677CT polymorphism with spina bifida

The 677CT mutation introduces a *Hinf* I restriction site that results in digestion of a 198-bp PCR amplicon into 175 and 23-bp fragments. Amplification of the (exon 12) part of MTHFR the DNA fragment was achieved using PCR methodology from the blood samples isolated from study subjects. The obtained PCR product was run on the 2 percent agarose gel and the results obtained are presented in Figure.2. All the samples contained 198bp. Restriction digestion of the amplified DNA with *Hinf* l restriction enzyme produced two DNA fragments of 173bp and 25b sizes. The results obtained are presented in Figure.3. 677CC/TT homozygosity was indicated by the presence of (173, 25 bp) two fragments. Statistically no significant difference 677CT X^2= 0.327 (0.827) for distribution of allele found among spina bifida case subjects and control groups (Table.5). Distribution of genotype, allele frequencies and carriage rate of *Hinf* l among case study and control subjects presented in Table .5 .The data revealed that the observed genotypes -/-CC wild type 26 (56.5%), -/+CT heterozygous 20 (62,5%) and +/+TT homozygous 26 (62%) as compared to control subjects -/-CC wild type 20 (43.5%) , -/+CT heterozygous 12 (37.5%, and +/+TT homozygous 16 (38%) respectively

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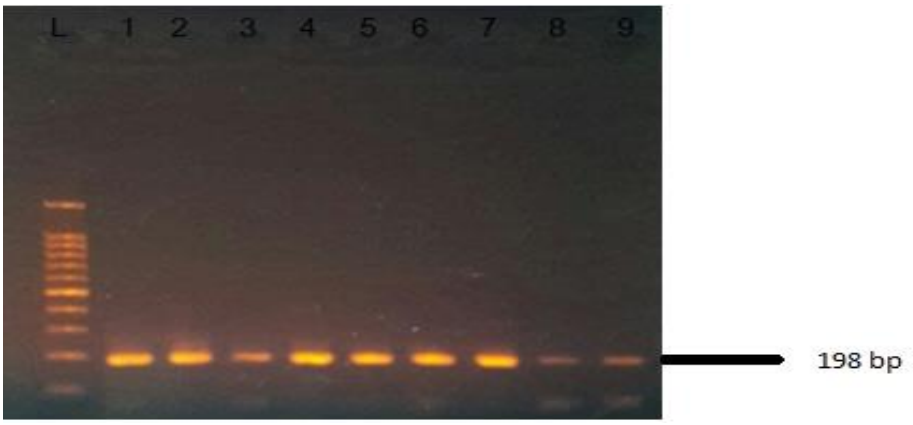

Figure. 2. PCR products of MTHFR gene appearance in the 2% agarose gel. Ladder L. Lane 1-9, 198 bp PCR products.

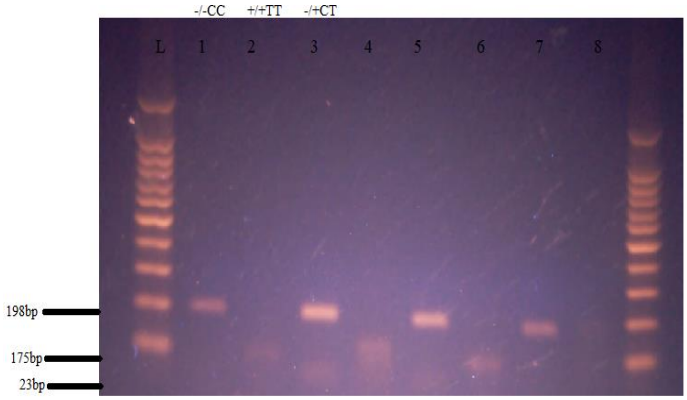

Figure.3. Genotyping of case families revealed by digestion of pcr products by *Hinf* l L, 100bp ladder, lane 1: -/-CC wild type, lane 2: +/+TT homozygous, lane 3:-/+CT heterozygous

Table.5.Distribution of genotype, allele frequencies of *Hinf* l among case and control subjects

Genotype	**Group**		**Total**
	Cases	**Control**	
CC	26 56.5%	20 43.5%	46 38%
CT	20 62.5%	12 37.5%	32 27%
TT	26 62%	16 38%	42 35%
Total	72 100%	48 100%	120 100%

Data expressed as X^2= 0.327 (0.827)

Table.6. Distribution of genotype, allele frequencies of *Hinf* l among case and control subjects separately

Genotype				Total
	CC	CT	TT	
Case child	7 29%	5 21%	12 50%	24 100%
Case father	10 42%	8 33%	6 25%	24 100%
Case mother	9 38%	7 29%	8 33%	24 100%
Control child	7 44%	4 25%	5 31%	16 100%
Control father	8 50%	5 31%	3 19%	16 100%
Control mother	5 31%	3 19%	8 50%	16 100%
Total	46 38%	32 27%	42 35%	120 100%

Data expressed as X^2= 7.329 (0.694)

Association of MTHFR 1298 A→C polymorphism with spina bifida

For detection of MTHFR 1298AC mutations, PCR amplification and RFLP analysis were performed. The 1298AC mutation introduces *Mbo* II restriction site that results in digestion of a 196-bp PCR amplicon into 166, and 30bp fragments. Amplification of the part (exon 12) of MTHFR gene was done through PCR to investigate the polymorphism of 1298 A→C polymorphism in spina bifida

patients, their parents and control group. For assurance the obtained PCR product was run on the agarose gel. The obtained fragment of MTHFR gene shown in Figure.4. Digestive enzymes were further applied to investigate all distinguishing genotypes (Figure.5). Heterozygosity was indicated after the presence of (166 and 30 bp) two fragments when restriction enzyme applied. The figure depicts three (03) types of genotypes. The current study showed the significant differences X^2=13.273(0.001) among spina bifida case subjects and control group (Table:7).Though general dissemination of genotypes between healthy controls and case groups were -/- AA wild type 28.4%, -/+AC heterozygous, 48.3%, and :+/+ CC homozygous 23.3%.

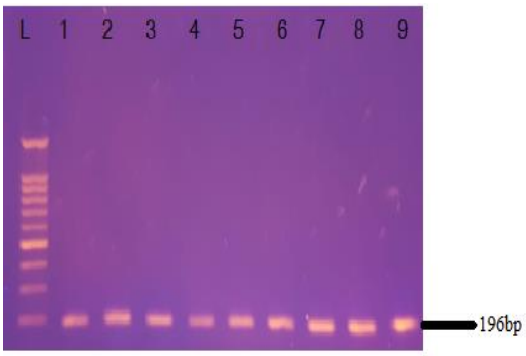

Figure.4. PCR products of MTHFR1298AC gene appearance in the 2% agarose gel, ladder L. Lane 1-9, 196 bp PCR products.

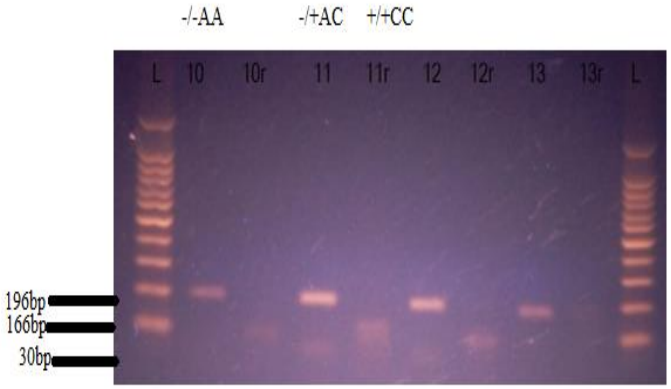

Figure.4.4. Genotyping of MTHFR 1298 A→C PCR products digestion by *Mbo*ll revealed. L, 100bp ladder, lane 10:-/- AA, lane 11: -/+AC heterozygous, lane 11r:+/+ CC homozygous

Table.7.Distribution of genotype, allele frequencies and carriage rate of *Mbo* ll among case and control subjects

Genotype	Group		Total
	Cases	Control	
AA	13 38%	21 62%	34 28.4%
AC	44 76%	14 24%	58 48.3%
CC	15 54%	13 46%	28 23.3%
Total	72 100%	48 100%	120 100%

Data expressed as X^2= 13.273 (0.001)

Table.8.Distribution of genotype, allele frequencies and carriage rate of Mbo ll among case and control subjects separately

Genotype				Total
	AA	AC	CC	
Case child	3 12.5%	19 79%	2 8.5%	24 100%
Case father	6 25%	12 50%	6 25%	24 100%
Case mother	4 17%	13 54%	7 29%	24 100%
Control child	8 50%	5 31%	3 19%	16 100%
Control father	7 44%	5 31%	4 25%	16 100%
Control mother	6 37.5%	4 25%	6 37.5%	16 100%
Total	34 28.4%	58 48.3%	28 23.3%	120 100%

Data expressed as X^2= 20.708 (0.023)

Discussion

Nervous system is effected by multiple chemical macro and micro molecules, and is often characterized as the most complex birth defects. Spina bifida is one of them, it causes lifelong morbidity and sometime mortility. Diagnosis and treatment of spina bifida in infants and adults is complex and multi declaratory processes.

During the pregnancy maternal exposure to the dietry intake influence the phenotype of offspring through genomic programming modification. (Stichelen *et al*., 2019). Folate requires one-carbon metabolism to form the Folic acid which plays key roles in numerous cellular reactions. DNA and RNA synthesis involve amino acid metabolism, biosynthesis of purine and pyrimidine and formation of primary methylating agent S-adenosyl-methionine (SAM) (Liu, *et al*., 2020). Methylenetetrahydrofolate reductase (MTHFR) is key gene in this pathway that is involved in transferring the methyl group to homocysteine (Ferrari *et al*., 2019). The etiology of NTDs has long been genetically associated with the deregulation of the major folate pathway. (Barua *et al*., 2014).

However, conflicting results of MTHFR polymorphisms have been reported in different populations to determine the genetic reasons of spina bifida. Further studies needed to reveal the contribution of these mutations. Present study conducted to find out the association of MTHFR genetic polymorphisms; MTHFR 677CT, 1298AC with blood folate level among spina bifida case and control subjects.

The present study showed a significant p value (0.032) for blood folate level among control and case children. The significant difference indicated the effect of folic acid level on the occurrence of spina bifida. Low folate status found to be associated with neural tube defects and especially with spina bifida particularly in low income regions (Ayaz and Asoglu, 2020). Blood folate level in fathers of case and control subjects showed a significant difference with p value (0.011). A case series described the effect of paternal blood folate level on the offspring DNA methylation. Low blood folate level found to be responsible for hyperhomocysteinemia. This case reports suggested an association between hereditary hyperhomocysteinemia in men and the occurrence of fetal NTDs (Yu *et al.,* 2019). Another study favored the effect of lowered blood folate level on the incidence of spina bifida (Van Der Put *et al*., 2001). Scarce data was available showing the non-association of paternal blood folate level and spina bifida in fetus. In our study mothers of case and control showed significant p value (0.001). The difference revealed the effect of maternal blood folate level and fetal spina bifida. Studies showed the correlation of maternal low plasma folate and spina bifida in offsprings. Experimental

findings have consistently been pointing out correlation between maternal blood folate level and spina bifida incidence (Liu *et al.,* 2020). But many studies indicated the other factors along with maternal blood folate level in the etiology of spina bifida such as genetic predisposition of 5MTHFR gene (Tabatabaei *et al.,* 2020).

Yet further research is required to specify the role of folic acid in spina bifida incidence.

Defects in folate metabolism may arose from a low dietary intake of folates or defect of genes involved in folate metabolism. One of the genes that played an essential role in folate metabolism is the methylenetetrahydrofolate reductase (MTHFR) gene (Zhou-Cun *et al.,* 2007). Enzyme activity that it is associated with hyperhomocysteinaemia can be reduced by MTHFR gene polymorphisms. Other genetic variants, including genes of the folate and the homocysteine metabolic pathways, might be implicated in the pathophysiology of NTDs (Eggink and Steegers-Theunissen, 2020), and in the remethylation pathway of homocysteine, such as methionine synthase (MTR), methionine synthase reductase (MTRR), and glutamate carboxypeptidase II (GCP II]. In the present study two polymorphisms studied and one of them was MTHFR 677CT. Current study confirmed the non-association of MTHFR 677CT polymorphism with spina bifida with X^2= 0.327 (0.827). These results were also supported by Tabatabaei *et al.,* 2022 who also demonstrated the insignificant relationship of MTHFR 677CT polymorphism with the incidence of spina bifida. Likewise Hassan *et al*., 2022 showed same non-association of neural tube defect with MTHFR 677CT polymorphism along with vitamin B12 deficiency. But Weisberg *et al*., 1998 differs from the current results and confirms the association of spina bifida with MTHFR 677CT polymorphism and Jouibari *et al*., 2021 also supported the results of association of neural tube defect with MTHFR 677CT.

The second polymorphism of MTHFR investigated was MTHFR 1298AC and our study showed its association with spina bifida occurrence with X^2= 13.273 (0.001). These results were also supported by Weisberg *et al*., who confirmed the association of spina bifida with MTHFR1298AC polymorphism. Same results were also showed by Van der Put *et al.,* 1995. Unlike the present study results Aranda-Sánchez *et al*., 2021 confirmed the nonassociation of MTHFR1298AC polymorphism and neural tube defects in west Mexican population. Likewise Hassan *et al*., 2022 and Soleimani-Jadidi *et al*., 2022 not confirmed the association of spina bifida and MTHFR1298AC polymorphism. The reduced activity of MTHFR associated with two mutations C677T and A1298C in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. Plasma homocysteine levels elevate and plasma folate levels decrease due to mutant homozygous genotypes of C677T while A1298C mutation changes in neither folate nor homocysteine levels. Present study showed the frequencies of the C677T, A1298C MTHFR mutations for spina bifida (SB) cases, mothers and fathers of SB cases, and controls in Pakistani population. 677CC/TT homozygosity identified by the presence of (175, 23 bp) two fragments but the rate was lower contrary to that of (Volcik *et al*., 2000) but similar to (Goyal *et al*., 2021). Likewise 1298AA/CC heterozygosity showed higher rate after the presence of (166 and 30 bp) two fragments in accordance with (Volcik et al., 2000). Higher rates of 1298AA/CC heterozygosity combining with 677CC/TT homozygosity effects the severity of spina bifida.

Conclusion

Overall, findings showed a significant relationship of blood folate levels with spina bifida (SB). Furthermore, genetic vulnerability for SB and its association with clinical indices were assessed. SB was more prevailing in those subjects who are -/+AC heterozygous 76% had significant differences X^2=13.273(0.001) for MTHFR 1298A→C polymorphism. Though, 677C→T polymorphism was non- significant having X^2= 0.327 (0.827) but still gene polymorphisms of MTHFR can be valuable risk assessment markers for the inception and development of SB.

Recommendations

Functional genomic studies may also be helpful to identify that how these polymorphisms may affect the genetic predisposition to SB. The study can be more significant with large sample size, screening of more than two variants and further procedure of sequencing to identify rare pathogenic variations. It would provide valuable information about spina bifida incidence among the Pakistani population.

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Conflict of interest
There is no conflict of interest.

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