

*Full length Research Paper*

# Evaluation of genetic heterogeneity in glutamate carboxypeptidase II (H475Y) and reduced folate carrier (SLC19A1) gene variants increased risk factor for the development of neural tube defects in eastern region of India

Ajit Kumar Saxena<sup>1\*</sup>, S. Pandey<sup>1</sup> and L. K. Pandey<sup>2</sup>

<sup>1</sup>Human Molecular Cytogenetic Laboratory, Centre of Experimental Medicine and Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India.

<sup>2</sup>Department of Obstetrics and Gynecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India.

Accepted 3 May, 2012

In humans, neural tube failure to close during the 4th week of gestation leads to the development of severe congenital malformations of the central nervous system because of an error in maternal folate metabolism associated gene variants. The frequency of genotypic variants of GCP II (H475Y) and folate carrier RFC1 (SLC19A1) gene polymorphism (80 G → A) were evaluated as potential candidate gene(s) and also assess their clinical association to increase “risk” in neural tube defects (NTDs). In the present study, blood samples (0.5 ml) were collected from NTD cases, mother and their respective controls and genomic DNA was isolated to evaluate the impact of GCP II and RFC1 genotypic variants as risk factor using polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) analysis. Significant differences ( $p < 0.05$ ) were observed between case mothers and control for GCP II genotype using Fischer’s exact two tailed probability test. The odd ratio was calculated to determine the risk factors at 95% C.I. (1.56-87.60), which seems to be very high, suggesting significant involvement of GCP II gene in the development of NTDs. The significant ( $p = 0.03$ ) risk factor was also calculated (OR=4.85: 95%, C.I. 1.33-17.36) for RFC1 gene between heterozygote (GA) and homozygote (AA) mothers having NTDs child. The present finding strongly suggests that genotype variants of GCP II and RFC1 gene, in heterozygous condition, are responsible for increasing as independent risk factor for the development of NTDs like meningomyelocele (MMC) susceptibility in this region.

**Key words:** Glutamate carboxy peptidase, reduced folate carrier, gene polymorphism, neural tube defects, meningomyelocele.

## INTRODUCTION

Neural tube defects (NTDs) are severe form of congenital malformation of central nervous system including brain and spinal cord where neural tube fails to close in early embryonic development. Such “birth defects” are multifactorial in origin including genetics and environmental

factors (Finnell et al., 2000; Cabrera et al., 2004; Detrait et al., 2005; Beaudin and Stover, 2009; Saxena et al., 2011). The most common form of NTDs is anencephaly and meningomyelocele where the exact cause is still not clearly defined in literature. Certainly, NTDs involve large number of variants across single gene and these variants (genes) differ in population between different ethnic groups. Epidemiological studies reveal that periconceptional folic acid supplementation may reduce

\*Corresponding author. E-mail: [draksaxena1@rediffmail.com](mailto:draksaxena1@rediffmail.com).

risk factor up to 75% (MRC, 1991; Botto and Yang, 2000; Van der Put et al., 1995). Meningomyelocele (MMC), the most severe form of spina bifida and possessor is difficult to survive because of dysplastic spinal cord with lack of neural function. In MMC both meninges and the spinal cord protrude through a gap in the vertebral column and the lesion is not covered by the skin. These anomalies can occur at any point along with the developing neural tube, although, lumbosacral lesions are the most common (Hunter et al., 1996). Most of the children of MMC survived after surgical intervention with lifelong disabilities (Detrait et al., 2005).

Dietary factor such as folate, predominantly exists in the form of polyglutamates and hydrolyzed to monoglutamates before absorption by the enzyme folypoly- $\gamma$  glutamate carboxypeptidase (FGCP) exists in jejunum. The folate monoglutamates are absorbed in proximal part of small intestine by folate carrier (Chandler et al., 1991). Reduced folate carrier (*RFCI*) is an essential cofactor for the synthesis of purines and pyrimidines synthesis for maintaining the genomic instability (Simonet and Sang, 2005). Recently, a polymorphism of glutamate carboxypeptidase II (H475Y) gene encoding enzyme is responsible for decrease folate level and increased total plasma homocysteine (tHcy) level in NTDs cases (Devlin et al, 2000). The GCP-II gene is localized on chromosome 11p11.2 and the gene product consists of 750 amino acid residues, termed folypoly - glutamate carboxypetidase (FGCP) which hydrolyze the terminal glutamate residues before absorption. Thereafter, the monoglutamyl folate derivatives are transported through the membrane via the folate transporter as reduced folate carrier. Similarly, *RFCI* gene is assigned on chromosome 21 (21q22.2-22.3) with number of common variants in the coding sequences and best studied H27R polymorphism of 80G A alleles changing an arginine into histidine (Chango et al., 2000; Marco et al., 2001). *RFCI* (SLC19A1), a cell surface transmembrane protein and is involved in bidirectional movement of folate across the membrane (Matherly et al., 2007; Hou and Matherly, 2009). In human, the exact causes of neural tube defects are still unknown but it seems to be involvement of mutivariants genes differs between different ethnic groups. *RFCI*, an essential molecule to carry folate for the developing embryo through placenta during organogenesis and has been associated with risk factor in NTDs. It has been observed that polymorphism of *GCP-II* (1561CT; H475Y) and *RFCI* (SLC19A1; 80GA) gene regulate the availability of dietary folate and increase susceptibility towards MMC risk in population (Shang et al., 2008; Pei et al., 2009). The rationale behind the selection of this gene is based on polymorphic variation with other genes, epidemiological studies and diversified biological function (Williams et al., 2002; Zhu et al., 2007; Shang et al., 2008; Pei et al., 2009). Linkage studies showing interesting findings but due to lack of reproducibility and

their association with other genes (Greene et al., 2009; Beaudin and Stover, 2009; Copp and Greene, 2010). Hence, logically hypothesize that these gene variants are associated with development of MMC. However, the present study becomes imperative and curiosity has been generated with the aim to evaluate the frequency of polymorphic genetic variants of *GCP-II* and *RFCI* gene to access the "risk factor" in developing NTDs and their clinical association has not been documented earlier in Indian literature.

## MATERIALS AND METHODS

The majority of MMC probands and their parents were enrolled after obtaining written consent from the participant's attendant/guardians. The criteria for inclusion of an individual were based on clinically diagnosed MMC. The level of defect was determined by review of image of radiographs and from medical records. In present study those cases having continues chemotherapy or previous history of genetic disorder other than NTDs were excluded. The project was dually approved by Institutional review and ethical committee.

Blood samples (n=100) from proband, mother and their respective controls were collected from the OPD of the Department of Pediatric Surgery and transfer to Human Molecular Cytogenetic Laboratory of Centre of Experimental Medicine and Surgery, Institute of Medical Sciences for genetic studies in EDTA vials and stored at  $-20^{\circ}\text{C}$  under sterile condition till further analysis. Present study was dually approved by Institute ethical committee and samples were collected after informed written consent from the participant's attendant/guardians.

Genomic DNA was isolated from isolation kit (Bioneer, Korea) for further genetic analysis. Polymerase chain reaction (PCR) was carried out by using *RFCI* specific forward 5'AGT GTC ACC TTC GTC CCC TC3' and reverse 5'CTC CCG CGT GAA GTT CTT 3'primers as reported by Chango et al. (2000), while for GCP-II forward 5'- CAT TCT GGT AGG AAT TTA GCA-3' and reverse 5'-AAA CAC CAC CTA TGT TTA ACA-3' (Devlin et al., 2000) in total volume of 50  $\mu\text{l}$  containing 50-100 ng of genomic DNA, 20 pmole of each primer, 200  $\mu\text{M}$  of each dNTPs mix with Taq buffer (10 mM Tris HCl pH 8.3, 50 mM KCl), 3.0 mM  $\text{MgCl}_2$  and 3 unit of Taq polymerase (New England Biolab). PCR product was separated on agarose gel and RFLP analysis was carried out using *HhaI* & *AccI* restriction enzyme for RFC and GCP-II respectively. The guanine (G) changed into adenine (A) at position 80 in RFCI while for GCP-II cytosine change into thymine at position 1561. The amplified products (6  $\mu\text{l}$ ) were digested at  $37^{\circ}\text{C}$  for 3 h in reaction volume of 25  $\mu\text{l}$  containing 1U of *HhaI* & *AccI* restriction enzyme and NEB buffer (2.5  $\mu\text{l}$ ) (New England, Biolabs). Digested products were separated on 3% agarose gel stained with Et.Br and DNA fragments were visualized on Gel Doc system (SR Biosystem).

## Statistical analysis

Fischer exact two tailed probability test was used to observe the significant differences ( $p < 0.05$ ) between NTDs cases, mothers and their respective controls. The relative risk factor, the odd ratio (O.R) was calculated at 95% confidence interval (C.I.) for combination of different genotype. The Hardy Weinberg Equilibrium was used to determined individual allele frequency.

## RESULTS

GCP II H475Y polymorphism was analyzed on genomic

**Table 1.** Genotype frequency of GCPII (H475Y) gene polymorphism in NTDs cases and their controls.

Cases/Control of NTDs	Genotype frequency (%)			% Allele frequency	
	CC	CT	TT	C	T
NTD cases	8.00	92.00	0	13.5	11.5
Control child	20.00	75.00	0	11.5	7.5
NTD mothers	6.67	93.30	0	8.0	7.0
Control mothers	33.34	53.30	0	9.0	4.0

**Table 2.** GCPII (H475Y) genotypes showing odd ratio (O.R) and C.I at 95% in NTD cases and their respective controls between homozygous and heterozygous condition.

GCPII C → T	O.R	(95% C.I)	p-value
<b>NTDs cases vs. control</b>			
CC/CC	0.35	0.067-1.865	0.03
CT/CT	3.833	0.738-19.288	0.124
<b>NTDs mother vs. control mother</b>			
CC/CC	0.143	0.020-1.129	0.169
CT/CT	12.250	1.563-87.603	0.035*

Statistical analysis shows level of significant\* ( $p < 0.05$ ) differences using Fisher exact probability test between homozygous and heterozygous condition with respect to controls.

DNA of the patients (NTD cases), mothers and their respective controls. The prevalence of this polymorphism shows three types of genotype variants that is, CC (wild type), CT, and TT (rare type) with highest frequency (92%) of CT between NTD cases and controls as summarized in Table 1. Because the genotype distribution did not differ significantly among NTD cases and their respective controls hence, we combined cases and mothers with their respective controls to increase statistical power for examination of possible interaction of gene polymorphism. The odd ratio was calculated at 95% C.I (0.73-19.28) between cases and controls seems to increase three fold (OR: 3.8) but shows lack of significant ( $p > 0.12$ ) differences. Interestingly, the significant differences ( $p = 0.035$ ) were observed between the cases mothers and control in heterozygous condition (Table 2). The individual allele frequency (T allele) was also calculated using Hardy Weinberg equilibrium which reveals highest frequency (11.5 %) in NTD cases when compared with controls (7.5 %).

RFC I (80 GA: R27H) gene polymorphism showing three type of genotypic variants that is, homozygous GG (wild type) and AA (rare type) and heterozygous (GA) condition as documented in Table 3. The highest genotype frequency was observed in homozygous (AA) condition in NTDs cases (20%) when compared with controls (12%), however the highest frequency (48%) was observed in NTDs mother. The individual allele (A) frequency was also calculated in NTDs cases (0.42) and their respective controls (0.28). Statistical analysis was

carried out using Fischer exact two tailed probability test shows significant difference between NTD mothers and control mothers in heterozygous condition ( $p = 0.006$ ) with O.R (odd ratio) was 2.37 at 95% C.I (1.32-4.26) as mention in Table 4.

On the basis of severity of disease in preclinically diagnosed NTDs cases and their mothers, the data was further analyzed to determine the genotype frequency of *RFCI* variants considered as an important candidate gene regulate folate metabolism for the development of NTD as documented in Table 5. The rare genotype frequency (50%) was observed in homozygous (AA) state of thoracomeningomyelocoele (TMMC) cases. Similarly the highest frequency (60%) was also pragmatic in mother of TMMC cases in heterozygous condition.

## DISCUSSION

According to MRC (1991) report, folic acid supplementation to the mother having previous history of having NTDs may reduce the incidence up to 70% for developing risk of congenital malformations associated with central nervous system in population, hence, maternal folate act as modifier for NTD (Botto et al., 2005).

Present study suggested that the significant genotypic variants of GCPII (H475Y) in heterozygous condition in NTDs mother act as risk factor for developing NTDs because such (GCPII) variants are associated with

**Table 3.** Distribution of *RFCI* genotype and their allele frequency between NTDs cases and their respective controls.

Groups	Genotype frequency (%)			Allele frequency (%)	
	GG	GA	AA	G	A
NTD cases	36.00	44.00	20.00	0.58	0.42
Control	52.00	32.00	12.00	0.68	0.28
NTD mothers	36.00	48.00	16.00	0.60	0.40
Control mothers	64.00	28.00	8.00	0.78	0.22

**Table 4.** *RFC I* genotypes showing odd ratio (O.R) and C.I. at 95% in NTD cases and their respective controls between homozygous and heterozygous conditions.

RFCI 80 G → A	O.R	(95% C.I)	p-value
<b>Cases vs. control</b>			
GG/GG	0.52	0.30-0.91	0.03*
GA/GA	1.67	0.94-2.97	0.11
AA/AA	1.83	0.85-3.94	0.18
<b>Case mother vs. control mother</b>			
GG/GG	0.32	0.18-0.56	0.00*
GA/GA	2.37	1.32- 4.26	0.006**
AA/AA	2.19	0.91- 5.26	0.13

Statistical analysis showing significant\*, highly\*\*(p<0.05) differences between cases and controls in homozygous and heterozygous condition after using Fischer exact probability test.

**Table 5.** *RFCI* genotypes showing allele frequency (%) in clinically diagnosed NTD cases.

Mutation	Genotype (%) in NTD case			Genotype (%) in NTD mother		
	LMMC	TMMC	Open NTD	LMMC	TMMC	Open NTD
80 G → A						
GG	41.7	37.5	75.0	38.5	25.0	33.5
GA	33.0	12.5	00.0	30.7	60.0	50.0
AA	33.4	50.0	25.0	38.5	25.0	16.7

LMMC: *Lumbosacral myelomeningocele*, TMMC: *Thoraco myelomeningocele*.

lowering the folate level and higher homocysteine level as reported earlier by Devlin et al. (2000). Alternative mechanism is also quite possible that GCP II polymorphism may also influence FGCP (folylpoly-  $\gamma$ -glutamate carboxypeptidase) activity through altered post translational processing and /or activity due to altered configuration of the catalytic region of the enzyme. The intestinal FGCP cleaves glutamate residues from FGCP play an important regulatory role in intestinal absorption from dietary FGCP (Halsted, 1990). Hence, the polymorphic variants in the present study affecting the activity of FGCP would predictably decrease the intestinal absorption and consequently decrease folate level and increase homocysteine level in the body. Our findings also suggests that GCP II allele variance may also affects folate absorption resulting in lower serum folate levels

and increasing incidence of NTDs in eastern region of the population. Our data agree with those of Brancaccio et al. (2001) findings between control and NTD cases for H475Y of GCP II polymorphism decreases risk for NTD with an OR of 0.35 (0.06-1.86). In the present study we are unable to observed homozygosity (TT rare type) in NTDs cases may be due to small sample size as also observed by earlier study of Afman et al. (2003).

*De novo* synthesis of folate does not take place in mammals and required efficient reduced folate carrier (*RFCI*) for the cell proliferation and tissue regeneration (Matherly and Goldman, 2003). *RFCI* act as an essential cofactor for the synthesis of purines and pyrimidines synthesis for maintaining the genomic instability (Simonett and Sang, 2005).

*RFCI* is an important carrier of folate and across the

placenta easily during the embryonic development (Anthony, 1992). The transport of folate molecules in blood cells occur either through a carrier or as receptor mediated mechanism of 5-methyltetrahydrofolate to maintain adequate intracellular concentration of folate in cytoplasm and prevent the incidence of NTD. Hence, the study of polymorphic variation of *RFCI* gene becomes an imperative for determining "risk factor" in the present case cohort study because of earlier information elucidate the controversial reports regarding distribution of frequency between wild and rare type alleles. Interestingly, present study shows significant ( $p=0.006$ ) difference with 2.3 fold increase of odd ratio at 95% C.I. 1.32 - 4.26 confirming risk for NTDs in heterozygote (GA) state (Table 3), similar findings are also reported by De Marco (2003) and Relton et al. (2003). Although, 80GG genotype in combination with low red blood cell folate levels was associated with a 4.6-fold increase risk in NTDs (Morin et al., 2003). Earlier studies are evident that homocysteine and folate status of the mother has direct impact on NTDs outcome, hence required to further evaluate whether the maternal genotype has a direct impact on development of NTD risk (Kirke, 1993; Molloy et al., 1998). In fact, it is possible that maternal genotype could play an etiopathogenic role in NTDs either due to inadequate supply of folate to the embryo or accumulation of homocysteine with increased concentrations may disrupts the process of neural tube closure. We have observed that the variation in prevalence of TMMC cases were highest among other clinical sub groups may be either due to epigenetic factor or patients belongs to heterogeneous ethnic groups. Environmental factors contributing significant role in the development of NTDs if mothers might have exposed to teratogens acts as carrier mediated interruption transport of folic acid into the cells from out sources as evident from our study. Our findings from homogenous samples reveal significant variation of the G/A allele between subgroups (NTD case, mother and controls). Several earlier studies of *RFCI* 80G allele are contradictory with evidence that either guanine or adenine is associated with increased risk in NTD affected cases and their mothers (Botto and Yang, 2000; Anthony, 1992; Kirke, 1993).

## Conclusion

On the basis of earlier linkage studies have yielded some interesting findings but due to lack of reproducibility and their association with certain genes required are evaluation of polymorphic variation of *GCPII* and *RFCI* as candidate gene (Greene et al., 2009; Beaudin and Stover, 2009; Copp and Greene, 2010). To elucidate the etiology of NTDs whether the genotypic variants of *GCPII* influences the function of *FGCP* enzyme and other studies are also required based on controlled dietary folate supplement levels in individuals having different

genotypes. The variable frequency of genotype in heterozygous state for both the gene *GCPII* H475Y and *RFCI* 80GA (475 CT/80 GA) in NTD mothers may play a significant role in regulation of folate metabolism either together or independently to maintain the adequate supply and absorption of folate through dietary supplement as discuss earlier. Similarly, the phenotypic heterogeneity in NTDs may also help to explain mixed response of *RFCI* gene polymorphism with different degree of severity in NTDs due to an increase frequency of "A" allele may act as an independent risk factor for "Birth Defects". Moreover, the present study is small and having interesting findings reporting first time in eastern region has not been reported earlier. However, further study is required to confirm the hypothesis that the variations in dietary intakes of folate are influenced by genetic variants of H475Y *GCPII* and 80GA *RFCI* polymorphism. Therefore, larger group of populations are necessary to investigate this associations further to reestablish the functional significance in different clinically defined NTDs. Such study are still continue to increase more samples of the same ethnic group to make the study significant in Indian population. Although, the author unable to reach in conclusion that how both the genes are interact (linked) to each other and responsible for increasing either as independent or together as risk factor for the development of NTDs.

## ACKNOWLEDGEMENTS

Ajit Kumar Saxena thankfully acknowledges the Department of Biotechnology, Ministry of Science and Technology, Government of India, grant no. BT/PR/9022/MED/12/331/2007, for providing financial assistance to carry out this research project. The authors are also thankful to Department of Biotechnology for providing the fellowship to the second author J. Gupta.

## REFERENCES

- Afman LA, Frans JM, Trijbels , Blom HJ (2003). The H475Y polymorphism in the glutamate carboxy peptidase II gene increase plasma folate without affecting the risk for Neural tube defects in Humans. *J. Nutr.* 133(1):75-77.
- Anthony AC (1992). The biological chemistry of folate receptors. *Blood* 79:2807-2820.
- Beaudin AE, Stover PJ (2009). Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a minireview. *Birth Defects Res. A Clin. Mol. Teratol.* 85:274-284.
- Botto LD, Lisi A, Robert-Gnansia E, Elisabeth Robert-Gnansia, Erickson JD, Vollset SE, Mastroiacovo P, Botting B, Cocchi G, de Vigan C, de Walle H, Feijoo M, Irgens LM, McDonnell B, Merlob P, Ritvanen A, Scarano G, Siffel C, Metneki J, Stoll C, Smithells R, Goujard J (2005). International retrospective cohort study of neural tube defects in relation to folic acid recommendations: Are the recommendations working? *Br. Med. J.* 330:571.
- Botto LD, Yang Q (2000). 5,10- methylenetetrahydrofolate reductase gene variants and congenital anomalies: a HuGE review. *Am. J. Epidemiol.* 151:862-877.
- Brancaccio R, Passariello A, Buoninconti A (2001). Multiple genotyping

- for genes of the folate/methionine metabolism in Italian NTD patients. Abstract book of 3<sup>rd</sup> international Conference on Homocysteine metabolism (abs).
- Cabrera RM, Hill DS, Etheredge AJ, Finnell RH (2004). Investigations into the etiology of neural tube defects. *Birth Defects Res C Embryo Today* 72:330-344.
- Chandler CJ, Harrison DA, Bufington CA, Santiago NA, Halsted CH (1991). Functional specificity of jejunal brush-border pteroylpolyglutamate hydrolase in pig. *Am. J. Physiol.* 260:G865-G872.
- Chango A, Emery-Fillon N, de Courcy GP, Lambert D, Pwster M, Rosenblatt DS, Nicolas JP (2000). A polymorphism (80G A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. *Mol. Genet. Metab.* 70:310-315.
- Copp AJ, Greene ND (2010). Genetics and development of neural tube defects. *J. Pathol.* 220:217-230.
- De Marco P, Calevo MG, Moroni A, Arata L, Merello E, Cama A, Finnell RH, Andreussi L, Capra V (2001). Polymorphisms in genes involved in folate metabolism as risk factors for NTDs. *Eur. J. Pediatr. Surg.* 11:S14-S17.
- De Marco PMG, Calevo A, Moroni LA, Merello E, Cama A, Finnell RH (2003). Reduced folate carrier polymorphism (80A G) and neural tube defects. *Eur. J. Hum. Genet.* pp. 245-252.
- Deltrait ER, George TM, Etchevers HC (2005). Human neural tube defects: developmental biology, epidemiology, and genetics. *Neurotoxicol. Teratol.* 27:515-524.
- Devlin AM, Ling E, Peerson JM, Fernando S, Clarke R, Smith AD, Halsted CH (2000). Glutamate carboxypeptidase II: a polymorphism associated with lower levels of serum folate and hyperhomocysteinemia. *Hum. Mol. Genet.* 9:2837-2844.
- Finnell RH, Gelineau-Van Waes J, Bennett GD (2000). Genetic basis of susceptibility to environmentally induced neural tube defects. *Ann. NY Acad. Sci.* 919:261-277.
- Greene ND, Stanier P, Copp AJ (2009). Genetics of neural tube defects. *Hum. Mol. Genet.* 18:R113-R129.
- Halsted CH (1990). Intestinal absorption of dietary folates. In Picciano, M.F., Stokstad, E. L.R and Gregory, J. F. (eds), *Folic acid metabolism in health and disease*. Wiley-Liss, New York, NY, pp. 23-45.
- Hou Z, Matherly LH (2009). Oligomeric structure of the human reduced folate carrier: identification of homooligomers and dominant-negative effects on carrier expression and function. *J. Biol. Chem.* 284:3285-3293.
- Hunter AG, Cleveland RH, Blickman JG, Holmes LB (1996). A study of level of lesion, associated malformations and sib occurrence risks in spina bifida. *Teratology* 54:213-218.
- Kirke PN, Molloy AM, Daly LE, Burke H, Weir D G, Scott JM (1993). Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q. J. Med.* 86:703-708.
- Matherly LH, Goldman DI (2003). Membrane transport of folates. *Vitam. Horm.* 66:403-456.
- Matherly LH, Hou Z, Deng Y (2007). Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. *Cancer Metastasis Rev.* 26:111-128.
- Molloy AM, Mills JL, Kirke PN, Ramsbottom D, Mcpartlin JM, Burkett, Conley M, Morin I, A.M. Devlin, D. Leclerc, N. Sabbaghian, Halsted CH, Finell R, Rozen R (1998). Low blood folates in NTD pregnancies are only partly explained by thermolabile 5,10-methylenetetrahydrofolate reductase: low folate status alone may be the critical factor. *Am. J. Med. Genet.* 78:155-159.
- MRC (1991). Vitamin Study Research Group: Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* 338:131-137.
- Pei L, Liu J, Zhang Y (2009). Association of reduced folate carrier gene polymorphism and maternal folic acid use with neural tube defects. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 150B:874-878.
- Relton CL, Wilding CS, Jonas PA, Lynch SA, Tawn EJ, Burn J (2003). Genetic susceptibility to neural tube defect pregnancy varies with offspring phenotype. *Clin. Genet.* pp. 424-428.
- Shang Y, Zhao H, Niu B, Li Wl, Zhou R, Zhang T, Xie J (2008). Correlation of polymorphisms of MTHFRS and RFC-1 genes with neural tube defects in China. *Birth Defects Res. A Clin. Mol. Teratol.* 82:3-7.
- Simonett F, Sang WC (2005). Gene Nutrient Interaction in one carbon susceptibility to neural tube defect pregnancy varies with offspring phenotype. *Clin. Teratol.* 85:274-284.
- Van der Put NMJ, Steegers-Theunissen RPM, Frosst P (1995). Mutated Methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lncet* 346:1070-1071.
- Williams LJ, Mai CT, Edmonds LD, (2002). Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. *Teratology* 66:33-39.
- Zhu H, Cabrera RM, Wlodarczyk BJ, Andreussi L, Cama A, Capra V (2007). Differentially expressed genes in embryonic cardiac tissues of mice lacking *Folr1* gene activity. *BMC Dev. Biol.* 7:128.