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Association of *A80G* Polymorphism in the *RFC1* Gene with the Risk for Having Spina Bifida-Affected Offspring in Southeast Mexico and Interaction with *C677T-MTHFR*

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1. Introduction

Spina bifida (SB) is one of the most prevalent congenital anomalies known as neural tube defects (NTD) in Yucatan, at Southeast Mexico. NTD results from failures of normal neural tube closure between the third and fourth week of embryonic development (Chen, 2008; Ramirez-Espitia et al., 2003). The majority of NTD cases can be categorized as either anencephaly with a lack of closure in the region of the head; or spina bifida with a lack of closure below the head (Au et al., 2010). SB refers to defects in the vertebral arches that obligatorily accompany open lesions. When the neural folds remain open, the sclerotome is unable to cover the neuroepithelium and skeletogenesis occurs abnormally, leaving the midline exposed (Greene & Copp, 2009). SB encompasses several subgroups of defects including the protrusion of the nervous tissue and its covering through a defect in the vertebrae named myelomeningocele, meningocele, and lipomeningocele. Myelomeningocele is by far the most common, accounting for greater than 90% of SB cases (Au et al., 2010). Wide variations in SB prevalence based on geography, race/ethnicity, and socioeconomic level suggest that genetic and environmental factors contribute to its etiology (Chen, 2008). Maternal folate status is critical for proper neural tube closure during embryogenesis. However epidemiological studies suggest that factors other than maternal deficiency of folic acid are involved in the etiology of SB. Numerous environmental and genetic influences

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contribute to NTD etiology; accumulating evidence from population-based studies which has demonstrated that folate status is a significant determinant of NTD risk.

Since the observation that periconceptional folic acid supplementation reduces the risk of having a NTD-affected pregnancy by 50–70% (Czeizel & Dudas, 1992; MRC, 1991); and the identification of the C677T SNP in the *MTHFR* gene as the first genetic risk factor of NTD (Whitehead et al, 1995); research has focused on genetic variation in genes encoding for the enzymes involved in the folate cycles and the closely-related homocysteine (Hcy) metabolism. Folate metabolism cross-regulates a complex network of basic biological pathways vital to growth, differentiation, and proliferation of cells. These processes include folate recycling, methionine metabolism, trans-sulfuration, synthesis of purines and pyrimidines, synthesis of serine/glycine, biomolecule methylation, membrane lipid synthesis, and drug metabolism. Neural tube formation involves intricately synchronized cell-cycle activities of the cells composing the neural plate and neighboring tissues. Abnormal activity of genes affecting the balance of the aforementioned biological activities can lead to failure of the neural tube to close appropriately resulting in NTDs. Completion of neural tube closure requires the precise coordination of cell proliferation, survival, differentiation, and migration events; any one of these events could feasibly be disrupted by impairments in folate metabolism (Beudin & Stover, 2007).

Polymorphisms in genes controlling folate–homocysteine metabolism have been suggested as predisposing factors and susceptibility candidates for SB. However, in Yucatan, Mexico, no association between NTD and two common polymorphisms, C677T and A198C of the methylentetrahydrofolate reductase (*MTHFR)* gene was found (Gonzalez-Herrera et al., 2002, 2007). Mutations in the *MTHFR* gene can only partially explain the protective effect of folate against NTD. Therefore, other defects in folate metabolism, such as defective folate receptors and carriers, could also be causes of NTD. Dietary folates mainly exist as polyglutamates. As the uptake and transport of folates in the body occurs as monoglutamates, the dietary polyglutamated folates have to be deconjugated to monoglutamates before absorption. The enzyme responsible for this deconjugation is folylpoly-γ-glutamate carboxypeptidase, which is associated with the intestinal apical brush border and is encoded by the glutamate carboxypeptidase II (*GCPII*) gene. After the deconjugation process the folate monoglutamates are absorbed in the proximal small intestine via a mechanism that involves reduced folate carrier (van der Linden et al., 2006). Ingested folate is hydrolyzed into monoglutamate forms in the intestine by means of a specialized carrier-mediated process, with a protein named the reduced-folate carrier (RFC-1), which only transports the reduced form of folate, including the physiological substrate 5-methyltetrahydrofolate. RFC-1 is a bidirectional anion exchanger that mediates folate delivery into a variety of cells of different origin. A polymorphism, A80G substitution, in exon 2 of the *RFC-1* gene has been identified; that leads to the replacement of a histidine, CAG by an arginine, CGG, (Whetstine et al., 2001; Chango et al., 2000). Mutation of *RFC-1 80* AA to RFC-1 GG could impair folate transport from maternal blood to the fetus. So, the homozygous genotype (GG) could be a biologically plausible risk of NTDs. The A80G polymorphism has been demonstrated as a genetic risk factor for NTDs in both patients and their mothers (De Marco et al., 2003, Shang et al., 2008). Additional studies demonstrated that this variant may interact with

low folate status and *MTHFR* mutations to increase NTDs risk. A study in a Chinese population has reported an increased NTD risk in patients with the GG genotype, especially when their mothers did not take folic acid supplements (Pei et al., 2005). Other studies have failed to find an association between the *RFC-1 A80G* SNP and maternal NTD risk (Relton et al., 2004a, b); although an association between the *RFC-1 GG* genotype and low erythrocyte folate levels has demonstrated (Morin et al., 2003). *RFC-1 A80G* SNP may be a NTD risk factor, especially when maternal folate status is low, suggesting that sufficient folate can attenuate the effect of this polymorphism.

The objective of this study was to analyze the association of A80G polymorphism in the *RFC1* gene with the risk for having spina bifida affected-offspring and its interaction with C*677*T-*MTHFR* polymorphism in mothers and fathers from Southeast, Mexico. This design included only parents, supporting the hypothesis that mutation of *RFC1* gene on the parents side, mainly mother's might alter folate metabolism, since *RFC1* carrier is of particular importance during embryonic development for its role in transporting folate across the placenta. Moreover, metabolic changes, such as limiting the supply of folate to the embryo or facilitating the accumulation and increased transfer of homocysteine, could foster the formation of NTD.

2. Methods

2.1 Population

A case-control association study was performed, including as cases, 183 parents of children with open-dorsolumbar SB (119 mothers and 64 fathers). Case mothers and fathers were unrelated subjects belonging to 108 different families. The affected children were the first occurrence in the nuclear family and they were born consecutively between 1998 and 2004 at the General Hospital of the Secretary of Health of Yucatan, Mexico. Age of cases ranged from 15 to 57 years old and a mean age of 21.6 years. The control group was composed with 195 unrelated volunteer healthy parents (140 mothers and 55 fathers) who have demonstrated having healthy offspring in at least three consecutive children, not having SB or other type of NTD nor delivered an NTD-affected child. The control group was formed with volunteers who attended to Laboratorio de Genética at Universidad Autónoma de Yucatán for other diseases different to congenital malformations. Control subjects had a mean age of 35.3 years and range of 17–60 years. Folate dietary consumption based on a questionnaire of frequency of foods and folate erythrocyte concentrations were previously measured in case and control mothers, and no significant differences were found between them (Duarte-Pinzón et al., 2008). All selected subjects were born and had lived for at least three generations in Yucatan, Mexico. We also used anthropologic and demographic parameters such as language, birth place, surnames, genealogy, history of lifestyle; among others, to mach ethnically control with case subjects, belonging to the same ethnic group of Mestizos, defined as individuals born in the country having a Spanish-derived last name, with family antecedents of Mexican ancestors back at least to the third generation. In addition, we previously determined the absence of substructure or population stratification within the population of Yucatan by using 16 autosomal STR markers (González-Herrera et al., 2010), which may represent a confounder. Subjects with a chronic or degenerative

disease, such as diabetes mellitus type 2, obesity or hypertension were not included. Informed consent was obtained from cases and controls according to the recommendations of Helsinki Declaration. This study was approved by the Bioethics Committee of Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán, México. Confidentiality of participants was strictly maintained.

2.2 Genotyping

DNA was extracted from blood using standard techniques. Genotyping for A80G polymorphism in the *RFC1* gene was performed by polymerase chain reaction amplification and restriction fragment length polymorphism (PCR-RFLP). The SNP A80G was determined with PCR amplification using conditions and primers described by Chango et al., 2000: forward: 5'-AGT GTC ACC TTC GTC CCC TC-3' and reverse 5'- CTC CCG CGT GAA GTT CTT-3'. PCR amplification produced a 230 bp DNA fragment, which was digested with HhaI restriction enzyme. Detection of the fragments was resolved on 4% agarose gel. After restriction enzyme digestion, analysis of *RFC-1* 230 bp fragments showed that the AA genotype had two fragments of 162 and 68 bp, the heterozygous AG genotype had four fragments of 162, 125, 68, and 37 bp, and the homozygous GG genotype had three fragments of 125, 68, and 37 bp. To ensure quality control of genotyping, 10% of samples were randomly selected and genotyped by a second investigator, resulting in 100% concordance. Samples that failed the amplification or digestion as well as doubtful results were repeated to ensure correct identification of variants.

2.3 Statistical analysis

Genotype and allele frequencies in cases and controls were determined by counting method and calculating proportions. The frequencies of genotypes and alleles were used to compare cases and controls by means of a standard X^2 analysis. Exact methods were considered preferable whenever expected values in any cell were < 5. The Hardy-Weinberg equilibrium analysis was calculated both in cases and controls, and was tested using X^2 statistics for goodness of fit (1 df). Association of *A80G-RFC1* genotypes with the risk of having a SB-affected child was estimated considering p values <0.05 as statistically significant and odds ratio (OR) with 95% confidence interval (CI) were calculated using STATA case-control odds ratio program with the Woolf method (STATA version 10.2). Frequencies of *A80G-RFC1* genotypes and alleles were also stratified by gender, in order to determine the maternal or paternal contribution to the risk for having a SB-affected child. Interaction of *A80G-RFC1* polymorphism with *C677T-MTHFR* was assessed using data previously reported (Gonzalez-Herrera et al., 2002).

3. Results

Genotype and allele frequencies for A80G polymorphism of *RFC1* gene for SB parents and their controls, Hardy-Weinberg expectations, as well as the comparison of frequencies between cases and controls on an association analysis are listed in Table 1. Genotype frequencies were distributed according to Hardy-Weinberg expectations (p>0.05) in cases and controls. The heterozygous AG genotype was as frequent as the homozygous GG

genotype in cases, whereas heterozygous AG was the most frequent genotype in controls. Allele G showed a higher frequency than allele A in cases, whereas allele distribution was similar in controls. Comparison of A80G-RFC1 genotype frequency between parents having an SB-affected child and control parents did not show significant differences nor for the AG heterozygous genotype neither for GG genotype, except when compare the GG genotype versus AA+AG (p= 0.009). Distribution of frequency of allele G was also significantly higher in cases than controls (p= 0.04). These findings suggested that both allele G and GG genotype were associated with the parental risk of having an SB-affected child.

Genotypes and alleles	SB Parents N= 183	Control Parents N= 195	Cases vs controls OR (95% CI) p
AA	30 (0.164)	35 (0.180)	Reference
AG	77 (0.421)	104 (0.533)	AA vs AG 0.863 (0.489 – 1.527) 0.662
GG	76 (0.415)	56 (0.287)	AA vs GG 1.583 (0.871 – 2.878) 0.171
Allele A	137 (0.372)	174 (0.446)	Reference
Allele G	229 (0.628)	216 (0.554	A vs G 1.37 (1.01 – 1.801) 0.046**
			GG vs AA+AG 1.76 (1.15 – 2.703) 0.009**
HWE (p)	*(0.609)*	*(0.764)*	

Table 1. Genotype and allele frequencies of *A80G- RFC1* polymorphism in parents of SB children and control parents from Southeast, Mexico. Counts (%). ** Significant

Genotypes and alleles	SB Fathers N= 64	Control Fathers N= 55	SB Mothers N= 119	Control Mothers N= 140	SB fathers vs control fathers OR [95% CI] p	SB Mothers vs control mothers OR [95% CI] p
AA	8 (0.125)	10 (0.182)	22 (0.185)	25 (0.179)	Reference	Reference
AG	26 (0.406)	28 (0.509)	51 (0.429)	76 (0.543)	1.161 [0.397 – 3.391] 0.500	0.763 [0.389 –1.496] 0.490
GG	30 (0.469)	17 (0.309)	46 (0.387)	39 (0.279)	2.206 [0.731 –6.652] 0.173	1.34 [0.656 – 2.738] 0.470
					GG vs AG+AA 1.972 [0.928 –4.191] 0.056	GG vs AG+AA 1.631 [0.968 – 2.751] 0.044**
Allele A	42 (0.328)	48 (0.436)	95 (0.399)	126 (0.450)	Reference	Reference
Allele G	86 (0.672)	64 (0.564)	143 (0.601)	154 (0.550)	1.536 [0.908 – 2.597] 0.070	1.232 [868 – 1.748] 0.248
HWE (p)	0.937	0.999	0. 736	0.710		

Table 2. Genotype and allele frequencies of *A80G- RFC1* polymorphism in parents of SB children and control parents from Southeast, Mexico stratified by gender. Counts (%). ** Significant.

Table 2 shows the genotype and allele frequencies for *A80G-RFC1* gene for SB parents and their controls stratified by gender in order to determine the maternal or paternal contribution to the risk for having a SB-affected child in the studied population. The heterozygous AG genotype was as frequent as the GG genotype in case fathers, whereas for case mothers as well as for control mothers and fathers, the heterozygous AG genotype was the most frequent. Stratification of the polymorphism *A80G-RFC1* frequency by gender did not show significant differences when comparing case fathers versus control fathers for any genotype or allele ($p>0.05$). Comparison of genotype and allele frequencies of polymorphism *A80G-RFC1* between case mothers versus control mothers, showed significant differences for the genotype GG when comparing against AA+AG genotypes, suggesting that the polymorphism *A80G-RFC1* is associated with the risk for having SB-affected offspring only in mothers (OR=1.63, IC 0.968-2.75, p= 0.04). These results demonstrate that the risk for having an SB-affected child is controlled maternally in the population of Southeast, Mexico.

Genotype combinations RFC1/ MTHFR	SB Mothers N=119	Control Mothers N=140	SB mothers vs control mothers OR 95% CI p	SB Fathers N=64	Control Fathers N= 55	SB Fathers vs control Fathers OR 95% CI p
AA/CC	4 (0.034)	4 (0.036)	1.18 [0.29 - 4.83] 0.546	0 (0.0)	2 (0.036)	0.00 [0.00-0.00] 0.211
AG/CC	12 (0.101)	18 (0.129)	0.76 [0.35 -1.65] 0.561	5 (0.078)	10 (0.182)	0.38 [0.12-1.19] 0.077
GG/CC	8 (0.067)	9 (0.064)	1.04 [0.39 - 2.81] 0.559	8 (0.125)	2 (0.036)	3.78 [0.76-18.64] 0.077
AA/CT	12 (0.101)	13 (0.093)	1.09 [0.47 - 2.50] 0.836	7 (0.109)	4 (0.073)	1.68 [0.46-6.09] 0.534
AG/CT	25 (0.210)	44 (0.314)	0.58 [0.32 - 1.02] 0.039**	14 (0.219)	11 (0.200)	1.12 [0.46-2.72] 0.826
GG/CT	25 (0.210)	17 (0.121)	1.92 [0.98 - 3.76] 0.039**	11 (0.172)	12 (0.218)	0.74 [0.29-1.85] 0.643
AA/TT	6 (0.050)	7 (0.050)	1.00 [0.32 - 3.08] 0.603	1 (0.016)	4 (0.073)	0.20 [0.02-1.86] 0.180
AG/TT	14 (0.118)	14 (0.100)	1.27 [0.54 – 2.63] 0.691	7 (0.109)	7 (0.127)	0.84 [0.27-2.57] 0.783
GG/TT	13 (0.109)	13 (0.093)	1.19 [0.53 – 2.69] 0.683	11 (0.172)	3 (0.055)	2.59 [0.94-13.63] 0.043**

Table 3. Interaction of *A80G-RFC1 and C677T-MTHFR* polymorphisms in parents of SB children and control parents stratified by gender. Counts (%). ** Significant

Combined genotypes for both polymorphisms *A80G-RFC1* and *C677T-MTHFR* were determined in order to asses an interaction between these two genes since multiple SNPs have the potential to provide significantly more power for genetic analysis than individual SNPs for increasing the associated risk for having SB-affected offspring. The genotype combination with both heterozygous AG/CT was the most frequent in all studied groups, except for control fathers who showed the highest frequency for the genotype combination GG/CT. Comparison of genotype combination *RFC1/MTHFR* frequencies between SB parents and control parents did not show significant differences ($p>0.05$, data not shown). However, stratification by gender of *RFC1/MTHFR* interaction showed significant differences for the genotype combinations AG/CT and GG/CT in mothers ($p=0.039$), suggesting that AG/CT might be an interaction associated as a genetic protection factor and

that the GG/CT genotype combination might represent a genetic risk factor having an SB-affected child for mothers. In fathers, the genotype combination with both mutants GG/TT was significantly associated with the risk for having SB-affected offspring (p=0.043). Frequencies of *A80G-RFC1/C677T-MTHFR* genotype combinations stratified by gender in the studied population, as well as the comparison of frequencies between cases and controls on an association analysis are listed in Table 3. Results of the interaction of *A80G-RFC1/C677T-MTHFR* suggest that for a genotype combination become a genetic risk factor for having SB-affected offspring in Southeast, Mexico; the homozygous genotype *GG-RFC1* should be present for both mothers and fathers.

4. Discussion

Variants of several folate-related genes have been found to be significantly associated with the risk of NTD, although, many genetic factors are also responsible for NTD due to the numerous candidates and population level differences. Previous investigations of the gene that are specifically involved in folate metabolism, such as 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) have shown that *MTHFR* is associated with an increased risk of NTD (Botto & Yang, 2000). There is a considerable body of data demonstrating that MTHFR gene 677TT homozygosity for the thermolability of the enzyme and *MTHFR A1298C* are genetic factors affecting the susceptibility of the NTD risk. However, in Yucatan, Mexico, no association between NTD and two common polymorphisms, C677T and A1298C of the methylentetrahydrofolate reductase (*MTHFR)* gene was found (Gonzalez-Herrera et al., 2002, 2007). Mutations in the *MTHFR* gene can only partially explain the protective effect of folate against NTD. In this study, we search for another genetic folate risk factor, which might explain the high prevalence of NTD, of SB type in Yucatan, Mexico. Therefore, other defects in folate metabolism, such as defective folate receptors and carriers, could also be causes of NTD. The carrier involved in cellular folate absorbed and transported, such as the reduced folate carrier (RFC), acts together with the folate receptor for folate internalization from tissue into the cytoplasm of the cells. Investigation of folate-related genes is necessary to reveal clues about metabolism underlying the potential embryonic protective effects of folic acid supplementation. Genes involved in the cellular folate transportation, such as *RFC1*, may be primary candidates for folate regulated NTDs (Shaw et al., 2003). Variance or abnormal expression of RFC1 can potentially have a major impact on folate metabolism and represents an excellent candidate for explaining susceptibility to folate-regulated NTDs (Shaw et al., 2002).

The A80G polymorphism has been demonstrated as a genetic risk factor for NTDs in both patients and their mothers (De Marco et al., 2003, Shang et al., 2008). These findings are agreed with our results obtained in the population of Yucatan, where we were able to demonstrate a significant association of both allele and GG genotype of A80G-*RFC1* polymorphism with the risk for having an SB-affected child. Previous familial based-association studies using transmission/disequilibrium test (TDT) have demonstrated the significant preference of the allele G to be transmitted to NTD-affected offspring in affected families compared with control families (Pei et al., 2005). In the population of Yucatan, the risk for having SB-affected offspring associated to A80G-*RFC1* polymorphism was mainly significant in mothers. Because the affected biological mechanism related to A80G-*RFC1*

polymorphism is folate transport; additional folate supplementation is critical to maintain the optimal function of folate carriers and transporters, as well as folate adsorption, mainly in mothers. The folate transport occurs across the placenta in mothers , so maternal control of folate is determinant to maintain optimal folate serum and erythrocyte levels in order to assure an embryonic normal development. RFC-1 is a high-affinity transporter for the folate substrate 5-methyltetrahydrofolate. This carrier is of particular importance during embryonic development for its role in transporting folate across the placenta. The 80A>G mutation affects residue 27 of the protein in which the Arginine (or R; CGG codon) is changed into Histidine (or H; CAG codon). RFC-1 is a protein that requires a functional amino terminus for insertion into the cell membrane for folate transport. Mutation of RFC-1 80 AA to RFC-1 GG could impair folate transport from maternal blood to the fetus. Therefore, this homozygous genotype (GG) could be a biologically plausible risk of NTD (Shang et al., 2008).

As a maternal low folate/high homocysteine phenotype is associated with increased risk of NTD in offspring, women with the GG genotype may have an increased risk of having a child affected by an NTD relative to those with the GA and AA genotypes. In addition, GG homozygous women may be at increased risk of a range of other major pathologies, including cardiovascular disease, in which a low folate/high homocysteine phenotype is a predisposing feature (Stanisławska-Sachadyn, et al 2009). A previous report in California provided evidence for a gene-nutrient interaction between infant *RFC1* G80/G80 genotype and maternal periconceptional intake of vitamins containing folic acid on the risk of spina bifida (Shaw et al., 2002). Based on these studies, we hypothesized that infants with the *RFC1* (A80G) genotype would be at increased risk for NTD secondary to an impaired ability to transport folates to the cytoplasm of a critical cellular population. The metabolic changes, such as limiting the supply of folate to the embryo or facilitating the accumulation and increased transfer of homocysteine, could foster the formation of NTD, so it is the mutation of certain genes on the mother's side that would alter folate metabolism. Significant association between the *RFC-1* A80G polymorphism and NTD risk in Shanxi, China, especially for the occurrence of the upper type of NTD was observed (Shang et al., 2008).

The biologically plausible rationale for exploring genetic variation of RFC1 is based on the knowledge that RFC1 regulates the delivery of 5-methyltetrahydrofolate from the cell's endocytotic vesicle into the cytoplasm (Chango et al., 2000). 5- Methyltetrahydrofolate is required for the remethylation of homocysteine. RFC is also a protein that requires a functional amino terminus for insertion into the cell membrane for folate transport. Thus, increasing maternal serum folate from either supplements or diet could ''correct'' reduced kinetics of transport that result from a variant form of a folate membrane transport protein (Shaw et al., 2003). If a putative genetic defect was severe enough to eliminate RFC1- mediated folate transport through these systems, it is likely that it would be embryolethal. This has been substantiated by recent reports using knock-out mouse models for the folate receptor proteins, as well as for RFC1 (Gelineau-van et al., 2008). Mutation of *RFC1 80AA* to *RFC1 80GG* could impair folate transport from maternal blood to the fetus. Therefore, this homozygous genotype (GG) could be a biologically plausible risk of NTD (Pei et al., 2009)

There is evidence of an interaction between the methylenetetrahydrofolate reductase (*MTHFR*) 1298 A>C and the reduced folate carrier (*RFC1)* 80 G>A polymorphisms with the risk of birth defects in the Italian population (De Marco et al., 2003). Gene–gene interactions between *MTHFR* and *RFC1* gene polymorphisms and the risk of Down syndrome pregnancies in a group of young mothers showed a protective role for the *MTHFR* 1298AA/*RFC1* 80 GA or AA genotype, compared with the 1298AA/80 GG genotype, suggesting that interactions between *MTHFR* and *RFC1* gene polymorphisms could be relevant to the risk for Down syndrome (Coppede et al., 2006, 2007). These findings suggested that there is a significant interaction between maternal or offspring's GG genotype and the deficiency of maternal periconceptional folic acid. Otherwise, a modest gene-nutrient interaction between infant homozygosity for the *RFC1* GG genotype and maternal periconceptional intake of vitamins containing folic acid on the risk of spina bifida has been demonstrated: an increased risk for spina bifida among California newborns with the GG genotype whose mothers had not used vitamins with folic acid in the periconceptional period (Shaw et al., 2002).

Our study contributed with the identification of genotypes at risk for having SB-affected offspring in fathers and mothers from Yucatan, Mexico. Allele G as well as GG genotype of A80G-*RFC1* polymorphism are associated with the risk to have a child with SB, so this polymorphism might be recognized as a genetic marker for susceptibility to SB in the studied population. Results might impact the design of preventive programs in order to decrease the high prevalence of NTD in Yucatán, Mexico.

5. Conclusion

In conclusion, our findings suggest the association of allele G of A80G polymorphism in the RFC1 gene with the genetic risk for having a child affected of spina bifida, which might be controled maternally. Results also suggest that there might be an interaction between A80G-*RFC1* and C677T-*MTHFR,* since the genotype combinations GG/CT in mothers and GG/TT in fathers were associated with the higher risk to have a child with SB in Southeast, Mexico.

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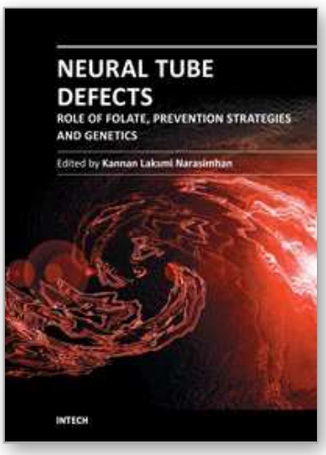

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