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A known functional polymorphism (Ile120Val) of the human *PCMT1* gene and risk of spina bifida

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Abstract

Folate binding protein 1 (*Folr1*) knockout mice with low maternal folate concentrations have been shown to be excellent animal models for human folate-responsive neural tube defects (NTDs). Previous studies using the *Folr1* knockout mice revealed that maternal folate supplementation up-regulates the expression of the *PCMT1* gene in *Folr1* nullizygous neural tube tissue during neural tube closure. *PCMT1* encodes the protein repair enzyme L-isoaspartate (D-aspartate) O-methyltransferase (PIMT) that converts abnormal D-aspartyl and L-isoaspartyl residues to the normal L-aspartyl form. PIMT is known to protect certain neural cells from *Bax*-induced apoptosis. *Pcmt1*-deficient mice present with abnormal AdoMet/AdoHcy homeostasis. We hypothesized that a known functional polymorphism (Ile120Val) in the human *PCMT1* gene is associated with an increased risk of folate-responsive human NTDs. A case-control study was conducted to investigate a possible association between this polymorphism and risk of spina bifida. Compared to the Ile/Ile and Ile/Val genotypes, the homozygous Val/Val genotype showed decreased risk for spina bifida (adjusted odds ratio = 0.6, 95% confidence interval: 0.4–0.9). Our results showed that the Ile120Val polymorphism of *PCMT1* gene is a genetic modifier for the risk of spina bifida. Val/Val genotype was associated with a reduction in risk for spina bifida.

Keywords

L-Isoaspartate (D-aspartate) O-methyltransferase (PIMT); *PCMT1* gene; Polymorphism; Neural tube defects; Association study

Introduction

Neural tube defects (NTDs) are a group of severe congenital malformations characterized by a failure of neural tube closure during early embryonic development. Periconceptional folic acid supplementation can prevent 50–70% NTDs [1–3]. Recent studies have focused on possible genetic mechanisms underlying the protective effects of folic acid. The *Folr1* (folate binding protein 1) knockout mouse is a well-established animal model for folate-responsive

NTDs. Mice lacking *Folr1* gene die prematurely in utero around gestational day 10 (E10) [4, 5]. The null embryos can be partially rescued by supplementing the pregnant dams with 25 mg/kg/day 5-folinic acid, although a reduced number of nullizygous pups will still present with exencephaly [5]. A recent microarray study using this mouse model provided intriguing evidence that *Pcmt1*, the gene encoding methyl-transferase, L-isoaspartate (D-aspartate) O-methyltransferase (PIMT, E.C. 2.1.1.77), was highly expressed in the developing neural tube, and was up-regulated in these tissues when the normal phenotype was rescued by folate supplementation [6]. These observations indicate a potentially important contribution by the *Pcmt1* gene product in the developing neural tube, and its possible involvement in *Folr1* knockout phenotypes which can be rescued by folate supplementation [6].

PIMT is a cytosolic protein repair enzyme that initiates the conversion of L-isoaspartyl and D-aspartyl residues to normal L-aspartyl residues. PIMT “repair” reactions help to maintain proper protein conformation by preventing the accumulation of damaged proteins containing abnormal amino acid residues. This process helps prevent cells from premature aging. When this gene is ablated in gene targeting studies, nullizygous *Pcmt1* knockout mice suffer from epileptic seizures that result in the animal’s death at an average of 42 days of age [7]. Farrar and Clarke [8] measured metabolites, including AdoMet and AdoHcy in the brains of *Pcmt1* nullizygotes, *Pcmt1* heterozygotes, and wildtype mice. Highest levels of AdoMet and lower levels of AdoHcy were found in the brains of *Pcmt1* null mice, and to a lesser extent, in the heterozygous mice, when compared with wildtype mice. These observations suggested that the reaction catalyzed by PIMT contributes significantly to cellular methyl group homeostasis. Although the up-regulation of the *Pcmt1* gene was observed in folate supplemented *Folr1*^{-/-} mice in genetic microarray studies, to date there is no direct evidence ascribing a functional role for the *PCMT1* gene in neural tube closure.

The human *PCMT1* gene maps to chromosome 6q24–25, which is the syntenic region of the mouse *Pcmt1* gene located on chromosome 10, and encodes a 24.5-kDa protein [9,10]. Two transcript variants have been found [11] containing seven and eight exons, respectively. The 120th amino acid of the PIMT protein was found to be polymorphic (Ile120Val, *rs4816*, originally named as Ile119Val, alternative dbSNP entry: *rs11155687*). This amino acid is highly conserved across species (from *Escherichia coli* to *Homo sapiens*), and is located in the SAM binding motif (*InterPro*: *IPR000682*). Analyses of the enzymatic activities of the Ile₁₂₀ and Val₁₂₀ variants in red blood cell lysates showed that Ile₁₂₀ isoform has higher thermo-stability and a higher specific activity, while Val₁₂₀ has greater methyl-accepting substrate affinity. The higher specific activity and thermo-stability of the Ile₁₂₀ isoform is thought to be balanced by the potentially compensating higher substrate affinity of the Val₁₂₀ isoform. This polymorphism was found to be involved in the repair process of age-damaged proteins [12,13].

Considering the potential role of *PCMT1* in embryonic development, as well as its possible contributing role to the process by which folate rescues the abnormal *Folr1*^{-/-} phenotype, we hypothesized that the functional polymorphism Ile120Val in human *PCMT1* may modify spina bifida risk. The purpose of our study is to investigate a possible association between this polymorphism and the risk of spina bifida in a cohort of California infants.

Materials and methods

Subjects

Data were derived from the California Birth Defects Monitoring Program, a population-based active surveillance system for collecting information on infants and fetuses with congenital malformations [14]. Program staff collected diagnostic and demographic information from multiple sources of medical records for all live-born, still-born fetuses (defined as >20 weeks

gestation), and pregnancies electively or spontaneously terminated. Nearly all structural anomalies diagnosed within one year of delivery were ascertained. Overall ascertainment has been estimated, as 97% complete [15]. Included for study were 152 infants with spina bifida (cases) and 423 nonmalformed infants (controls). Case and control infants derived from two different datasets. One dataset included 48 infants with spina bifida (cases) and 85 infants without structural malformations (controls). These cases and controls were randomly selected from among all cases and nonmalformed control infants ascertained by the CBDMP corresponding to birth years 1983–1986. The other dataset included 104 infants with spina bifida (cases) and 338 infants without structural malformations (controls). These case and controls derived from a previously described case-control study [16]. The first dataset was limited to information only about race/ethnic background. The second dataset included detailed maternal interview information on important covariates such as maternal periconceptional vitamin use. Each case and control infant was linked to its newborn bloodspot, which served as the source of DNA in our genotyping analyses. All samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects.

DNA extraction and genotyping

Genomic DNA was extracted from dried newborn screening blood spots using the Puregene DNA Extraction Kit (Gentra, Minneapolis, MN). SNPs rs4816 (Ile120Val) and rs4552 (located at 3'-UTR) were detected using TaqMan SNP genotyping assay (Assay-on-Demand, C__11415888_10 and C__1803682_10) (Applied Biosystems, Foster City, CA) on an ABI PRISM 7900 Sequence Detection System following the manufacturer's instructions. Genotyping was performed by lab technicians who were blinded to the case-control status of the samples and all genotyping assays were repeated to ensure the accuracy of results. Only genotypes with consistent results were considered successfully completed.

Statistical analysis

Deviation from Hardy–Weinberg equilibrium among control infants was evaluated by a χ^2 test. Logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) utilizing SAS software (version 9.1). Some models were adjusted for race/ethnicity (defined as nonHispanic white, Hispanic white, or other) to reduce potential confounding due to population stratification. Breslow–Day's test was used to assess for evidence of statistical heterogeneity between the two primary race/ethnic groups in our study population, nonHispanic white and Hispanic white. We further investigated a potential interaction between maternal periconceptional intake of vitamin supplements containing folic acid and infant *PCMT1* polymorphisms on spina bifida risk.

Results

Distributions of maternal race/ethnicity and maternal periconceptional folic acid use between cases and controls are displayed in Table 1. Among case mothers, 42.1% were nonHispanic whites and 46.7% were Hispanic whites, whereas among control mothers, 56.2% were nonHispanic whites and 32.2% were Hispanic whites. Hispanic mothers were nearly twice as likely to deliver infants with spina bifida (OR = 1.9, 95% CI: 1.3–2.9). Women who took folic acid during periconceptional period had substantially reduced risk of spina bifida comparing to those who did not take folic acid, reflected by an OR of 0.3 (0.2–0.4). Therefore maternal ethnicity and folic acid use were considered as major confounding factors in our study population and their confounding effect were evaluated.

148 (97.4%) spina bifida cases and 419 (99.1%) control infants were successfully genotyped for the SNPs rs4816 and rs4552. These two SNPs were in complete linkage disequilibrium

(LD); therefore, only two haplotypes (A-T and G-A) were considered analytically. No deviation from Hardy–Weinberg equilibrium was found among control infants. We focused on analyzing the functional polymorphism Ile120Val (rs4816). The OR associated with heterozygotes Ile/Val was 1.0 (0.6–1.5) comparing to Ile/Ile homozygotes. The OR associated with Val/Val homozygotes was 0.6 (0.3–1.0) comparing to homozygotes Ile/Ile. Since the heterozygotes exhibited OR around 1.0 in the total population as well as in each subgroups, we combined Ile/Val and Ile/Ile as reference group in all further analyses. Therefore, the OR associated with Val/Val homozygotes was 0.6 (0.4–0.9) (Table 2), compared to Ile/Val heterozygotes and Ile/Ile homozygotes. The ORs for Val/Val genotype in nonHispanic whites was 0.5 (0.3–1.0) and in Hispanic whites was also 0.5 (0.2–1.3) (Breslow–Day test: $\chi^2 = 0.01$, $P > 0.05$).

We also investigated whether maternal vitamin use modified the risk associated with the variant genotype. The subset of cases and controls whose mother were interviewed were used in the analysis. The OR calculated from this restricted dataset (OR = 0.5, 0.3–1.0) was comparable with that of the overall dataset. Some evidence for statistical heterogeneity was observed between nonHispanic women who used folic acid and who did not use folic acid (Breslow–Day test: $\chi^2 = 5.05$, $P < 0.05$). However, the sample sizes were too small to draw definitive conclusion. No heterogeneity was observed between Hispanic white women who used or did not use folic acid (Breslow–Day test: $\chi^2 = 0.51$, $P = 0.48$) Table 3.

Discussion

This is the first report of a modifying effect of the *PCMT1* Ile120Val polymorphism on human spina bifida risk. Infants with the Val/Val genotype, which encodes the protein isoform with both lower enzymatic activity and thermo-stability but higher affinity to the substrate AdoMet, appeared to have lower spina bifida risk in the overall population. This was also observed in the two major ethnicity groups, nonHispanic whites and Hispanic whites. These observed lowered risks suggest that the affinity for AdoMet by PIMT may play a more important role during neural tube closure in embryonic development than what is inferred simply by its specific activity. In vitro studies showed that the Ile/Ile genotype had 20% higher specific activity but 30% lower substrate affinity than Val/Val [12]. Our findings were consistent with DeVry's hypothesis that a 30% increase in substrate affinity in Val/Val genotype could account for a substantial decrease in damaged protein accumulation.

These results are intriguing and suggestive of one possible mechanism underlying the preventive effect of folic acid on spina bifida. We have previously reported that *Folr1*^{−/−} mice have extensive levels of apoptosis within the developing neural tube [17]. In embryos of the same genotype, folate supplementation up-regulates the expression of *Pcmt1* gene; which may help repair damaged proteins and inhibit apoptosis. Similar effects were observed by Huebscher and co-workers [18], who found that the anti-aging drug R(−)-deprenyl, up-regulates *Pcmt1* expression at the mRNA level. They also found that PIMT is indeed able to provide protection from Bax-induced apoptosis through a SAM-binding motif dependent effect [18].

The strengths of this study include its population-based ascertainment of cases and controls and its evaluation of race/ethnicity and folic acid use as potentially important modifiers of risk in the presence of variant genotypes. Maternal folic acid use is a known protective factor for spina bifida. In our study, we observed possible interaction between folic acid use and genotype effect, but the sample sizes were too small and limited the statistical power.

Population variation needs to be evaluated in population-based case control study of genotype-risk association. A complete linkage disequilibrium (LD) was observed between the two SNPs

(rs4816 and rs4552) in both subpopulations (nonHispanic White and Hispanic white) in our study. This finding is in line with previously published work [12]. We further interrogated available SNP genotyping data in *PCMT1* gene from www.hapmap.org [19], using Haploview 3.2 (<http://www.broad.mit.edu/mpg/haploview>) [20]. Twenty SNPs spanning a 58 kb region (chr6:150101955–150163546) have been genotyped from this source to date. Eighteen of the SNPs appeared to be bi-allelic. According to HapMap project data, all SNPs in this chromosomal region form one single haplotype block in the CEPH population (Utah residents with ancestry from northern and western Europe, CEU), Han Chinese in Beijing, China (HCB), and Japanese in Tokyo, Japan (JPT), suggesting that the *PCMT1* gene is located in a recombination “cold spot.” Two haplotype blocks exist in Yoruba in Ibadan, Nigeria (YRI). We also observed significant differences in genotype frequencies between the two major ethnic groups in our study population, nonHispanic whites and Hispanic whites. Population variation of *PCMT1* polymorphism was first reported by DeVry and colleagues. The frequencies of the alleles encoding the Ile₁₂₀ and Val₁₂₀ variants are similar in Caucasian populations, but there is a noted predominance of the Ile₁₂₀ allele present in Asian and African populations [12]. In our study, population variation did not appear to be a significant confounding factor. Decreased risk of the Val/Val homozygotes was observed consistently in both ethnicity groups as well as the overall population, suggesting a fairly strong genotype-risk association regardless of the maternal ethnicity.

Our study provided preliminary data suggesting an association between *PCMT1* gene variants and spina bifida risk. Even though this variation which changes amino acid coding in human *PCMT1* gene has not been reported in other species such as mouse and rat, it will be interesting to study variation of AdoMet affinity and enzyme activity in experimental animals and acquire in-depth view of the mechanism of our observation from current study.

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Table 1

Comparison of maternal race/ethnicity and periconceptional folic acid use in cases and controls

	Case (%)	Control (%)	Odds ratio	95% CI
<i>Maternal race/ethnicity</i>				
NonHispanic white	64 (42.1)	237 (56.2)	Reference	—
Hispanic white	71 (46.7)	136 (32.2)	1.9	1.3, 2.9
Others	17 (11.2)	49 (11.6)	1.3	0.7, 2.4
Total	152 (100.0)	422 (100.0)		
<i>Periconceptional folic acid use^a</i>				
No use	62 (59.6)	91 (27.5)	Reference	—
Use	42 (40.4)	240 (72.5)	0.3	0.2, 0.4
Total	104 (100.0)	331 (100.0)		

^aFolic acid use data is only available in a subset of samples.

Table 2

PCMT1 Ile120Val genotype frequencies in spina bifida cases and controls

	Case (%)	Control (%)	Odds ratio (95% CI)
<i>Overall</i>			
I/I + I/V	122 (82.4)	304 (72.6)	Reference
V/V	26 (17.6)	115 (27.4)	0.6 (0.4, 0.9)
<i>NonHispanic white^a</i>			
I/I + I/V	47 (77.1)	148 (63.0)	Reference
V/V	14 (22.9)	87 (37.0)	0.5 (0.3, 1.0)
<i>Hispanic white^a</i>			
I/I + I/V	64 (90.2)	112 (83.0)	Reference
V/V	7 (9.8)	23 (17.0)	0.5 (0.2, 1.3)

^aBreslow–Day test, $\chi^2_1=0.01$, $P=0.93$.

Table 3Gene-environment interaction-maternal folic acid use and PCMT1 Ile120Val genotype^a

Maternal FA use	PCMT1 genotype	Case	Control	Odds ratio (95%CI)
<i>Overall population</i>				
No use	I/I + I/V	52	77	Reference
	V/V	7	13	0.8 (0.2–2.1)
Use	I/I + I/V	32	165	Reference
	V/V	9	74	0.6 (0.3–1.4)
Total	I/I + I/V	84	242	Reference
	V/V	16	87	0.5 (0.3–1.0)
<i>NonHispanic white</i>				
No use	I/I + I/V	9	23	Reference
	V/V	4	4	2.5 (0.5–12.5)
Use	I/I + I/V	20	92	Reference
	V/V	4	59	0.3 (0.1–1.0)
<i>Hispanic white</i>				
No use	I/I + I/V	39	43	Reference
	V/V	3	8	0.4 (0.1–1.7)
Use	I/I + I/V	10	54	Reference
	V/V	2	12	0.9 (0.2–4.7)

Breslow–Day test (FA use vs no use), $\chi^2_{\text{overall}} = 0.14$, $P = 0.71$; $\chi^2_{\text{white}} = 5.05$, $P = 0.02$; $\chi^2_{\text{Hispanic}} = 0.51$, $P = 0.48$.

^aOnly a subset of samples was used for this analysis.