



Published in final edited form as:

*Am J Med Genet A*. 2005 December 15; 139(3): 194–198. doi:10.1002/ajmg.a.31002.

## Promotor Genotype of the Platelet-derived Growth Factor Receptor- $\alpha$ gene Shows Population Stratification but Not Association with Spina Bifida Meningocele

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### Abstract

Neural tube defects (NTDs) constitute a major group of congenital malformations with an overall incidence of approximately 1 to 2 in 1000 live births in the United States. Hispanic Americans have a 2.5 times higher risk than the Caucasian population. Spina bifida meningocele (SBMM) is a major clinical presentation of NTDs resulting from lack of closure of the spinal cord caudal to the head. In a previous study of spina bifida (SB) patients of European Caucasian descent, it was suggested that specific haplotypes of the platelet-derived growth factor receptor- $\alpha$  (*PDGFRA*) gene P1 promoter strongly affected the rate of NTD genesis. In our study, we evaluated the association of *PDGFRA* P1 in a group of 407 parent-child triads (167 Caucasian, 240 Hispanics) and 164 unrelated controls (89 Caucasian, 75 Hispanic). To fully evaluate the association of *PDGFRA* P1, we performed both transmission-disequilibrium test (TDT) and association analyses to test the hypotheses that *PDGFRA* P1 was (1) transmitted preferentially in SBMM affected children and (2) associated with the condition of SBMM comparing affected children to unaffected controls. We did find that there was a different allelic and genotypic distribution of *PDGFRA* P1 when comparing Hispanics and Caucasians. However, neither ethnic group showed strong association between SBMM and the *PDGFRA* P1 region. These findings suggest that *PDGFRA* P1 does not have a major role in the development of SBMM.

## Keywords

spina bifida meningocele (SBMM); population stratification; platelet-derived growth factor receptor- $\alpha$  (PDGFRA) gene P1 promoter; association study; transmission disequilibrium testing (TDT)

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## INTRODUCTION

Neural tube defects (NTDs) constitute a major group of congenital malformations with an overall incidence of approximately 1 to 2 in 1000 live births in the United States [Northrup and Volcik, 2000]. While NTDs involving the lack of closure in the head region are lethal, lack of closure in the spinal cord (spina bifida meningocele) is viable with proper medical treatment. Spina bifida meningocele (SBMM) is a major clinical presentation of NTDs resulting from lack of closure of the spinal cord caudal to the head. Usually, the individual will be handicapped to different degrees and with impaired skills of daily living, depending on the seriousness of defect. While folate insufficiency has been identified as a major environmental risk factor, there is strong evidence to suggest there are additional genetic and environmental factors associated with risk for NTDs.

A number of candidate genes for NTDs have been studied using natural and transgenic mouse models. Double-mutant mice produced by mating *Patch* mice [mice heterozygous for a mutation at the platelet-derived growth factor receptor- $\alpha$  (*Pdgfra*) gene] and *undulated* mice (mice homozygous for mutations at the *Pax1* gene locus) have a high incidence of lumbar spina bifida occulta [Helwig et al., 1995]. In *Patch* mice, those that are homozygous for mutations in *Pdgfra* have neural tube defects as a component of their phenotype as do mice that are targeted with knockout of the *Pdgfra* gene [Soriano, 1997]. In an *in vitro* assay, Joosten et al. [1998] hypothesized activation of *PDGFRA* expression by an SB-associated *PAX1* mutation to be causally related to risk of NTD formation. It is suspected that the PDGF signaling pathway exerts a spatial-temporal effect on the differentiation of the mesodermal cells resulting in the genesis of a neural tube defect in the mouse model [Joosten et al., 1998]. The human homolog, *PDGFRA*, is located on human chromosome 4q11-q13 with several short tandem repeat markers (D4S398, D4S1600 and D4S1541) flanking the gene and many single nucleotide polymorphisms (SNPs) throughout the gene region. Joosten et al. [2001] genotyped SNPs in the *PDGFRA* P1 promoter region of 49 familial (within 3<sup>rd</sup> degree) SB patients, 76 sporadic SB patients and 77 normal controls. Their findings suggested that the heterozygote H1/H2 $\gamma$  genotype (the at-risk genotype) strongly affects NTD genesis in their European Caucasian SB patients. It should be pointed out that the patient population in Joosten et al. [2001] included only SB patients and not other types of NTDs.

Zhu et al. [2004] also examined the relationship between *PDGFRA* P1 and NTD risk by examining the genotypes in both parents and affected children. In this study, they identified 249 mothers and 167 newborn and fetuses from the Hispanic population near the Texas-Mexico border. In contrast with Joosten, Zhu's study included patients with broadly defined NTDs (including terminations, live and still births with a diagnosis of an NTD, defined as spina bifida and anencephaly). They defined the at-risk genotype as 2 copies of the low activity alleles (i.e., H1 $\alpha$ , H1 $\beta$  or H2 $\gamma$ ). They did not find a significant elevation of the at-risk genotype in the affected children. Interestingly, they found that the at-risk genotype of the patient's mother was significantly skewed from what would be expected by random assortment. Thus, they concluded that the at-risk genotype of *PDGFRA* P1 in the mother established an *in utero* environment for the development of an infant with NTD. Zhu's found that the heterozygous genotypes identified as "at risk" in Joosten, et al [2001] (i.e.,

H1/H2 $\gamma$  or low/high activity) did not convey increased risk with an odds ratio (OR) 1.6 (95% CI – 0.8–3.4).

In this paper, we revisit the relationship between the *PDGFRA P1* and the development of SBMM. Using our unique, multi-ethnic population with a precisely defined phenotype of SBMM (which excludes SBO), we were able to examine population structure of this gene in both Caucasians and Hispanics. It is worth noting that the *Pdgfra* mutation in the *Patch* mice appears to be mesodermally derived SB, which would be homologous to SBO in the human [Payne, 1997]. Finally, we tested the associations of at-risk alleles and genotypes of *PDGFRA P1* in both a case-control setting as well as a TDT setting to capitalize on the strengths of both methodologies.

## MATERIALS AND METHODS

Patients with SBMM and their parents were enrolled into the study at the Shriners Hospital at Houston, TX; the Texas Children Hospital, Houston, TX; Hospital for Sick Children, Toronto, Canada; and the Shriners Hospital at Los Angeles, CA. Informed consent was obtained following the guidelines of the host location IRB with the IRB of University of Texas Health Science Center at Houston being the primary IRB. Our affected case population consisted of 224 males (55.0%) and 183 females (45.0%); 167 were Caucasian (41.0%) and 240 were Hispanic (59.0%). Location and size of spinal defect was available for the majority of patients. Thirteen patients (3.2%) had lesions in the T10–T12 region, 98 patients had an L1–L4 lesion (23.9%) and 277 had a lesion at the L5 region and below (67.7%). Twenty-one (5.1%) had lesions that overlapped more than one of the defined regions. SBO patients were excluded from the analysis. Blood samples were obtained from the patients and their parents and genomic DNA was extracted using the Puragen DNA extraction kit (Gentra). Concentration of DNA was calculated from the 260 nm absorbance. Working DNA stocks of 10 ng/ul were prepared for PCR. In addition, anonymous control DNAs from 75 Hispanic and 89 Caucasian individuals without a personal or family history of NTDs were obtained. These individuals were enrolled through the primary research center in Houston, Texas.

### Laboratory Methods

We first re-sequenced, in both directions, the 1.7 Kb P1 promotor of the *PDGFRA* gene in 96 patients to affirm the existence of all alleles reported by Joosten et al. [2001]. For this re-sequencing, we designed four overlapping fragments spanning the 1.7 Kb *PDGFRA* P1 using primer pairs designed from GenBank sequence X80389. Sequences of the PCR primers are: -1600 GCCTTTTATTCCGCACTCTG, -1091 TCACCTTCATCGCTCTCTGA; -1157 GAGAAGGATGAAGGATGACC, -570 TGGGCAAACCTGTTGAGTCCA; -677 CTTTGTACCGTATTCAGACCA, -189 CAGACTTCGACAACAACCTGG; -240 CCATCCCATCTGGTCTGCT, +160 CTTCTCCTCCGATGTTATTCC. Sequencing primers are -1595 TATTCCGCACTCTGATTTTTGG, -1150 ATGAAGGATGACCCCAAC, -662 CAGACCACCCAGTCTTGTACA, and -235 CCCATCTGGTCTGCTTCTC respectively. Products from PCR were examined on a 1.8% Metaphor agarose gel. PCR products were treated with *Exo* I and SAP (1 and 10 units respectively; USB) overnight and heat inactivated at 85°C for 25 minutes. A volume of 2 to 8  $\mu$ l of the treated PCR products were mixed with 4 pmoles of sequencing primers and 1  $\mu$ l of BDT sequencing mix (ABI) in a 20  $\mu$ l reaction. Cycle sequencing reactions was performed according to standard protocol recommended by ABI for 25 cycles. Products of sequencing were purified through a G-50 column, dried at 95°C for 5 min., and re-suspended in 10  $\mu$ l of Hi-Di formamide (ABI). After denaturing the products at 95°C for 3 min and chilling on ice, the sequence products were fractionated on the ABI3100 Genetic Analyzer using POP6 polymer and the results

were analyzed using the DNA Sequences Analysis Software v3.7 (ABI). The genotypes of each individual were identified, independently confirmed by three laboratory personnel and entered into a Microsoft Excel worksheet with the de-identified patient information.

Our re-sequencing data confirmed the presence of *PDGFRA* P1 alleles H1, H2 $\alpha$ , H2 $\beta$ , and H2 $\gamma$ . For simplicity, we refer to H2 $\alpha$  and H2 $\beta$  jointly as H2. We did not find H2 $\delta$  among the 96 patients and no new alleles were identified. We then designed fluorescent labeled allele specific oligos (ASO) primers for genotyping our complete sample set of 407 triads using the SNaPShot protocol (ABI). ASO primers were made by ABI for the eight polymorphic sites reported by Joosten et al. [2001]. They included: -1507 G/A (FAM-TCCTGTTAGCATTCCGAACAA/VIC-CCCTGTTAGCATTCCGAACAG), -1467A/G (FAM-CTCGCTATTACTTCCACATGTT/VIC-TCTCGCTATTACTTCCACATGTC), -1391G/C (FAM-GGGTCGCGTAGAAGAAGACTGC/VIC-GGGTCGCGTAGAAGAAGACTGG), -1074 C/A (FAM-AGGCTTCCCAGCGACAGCT/VIC-GGCTTCCCAGCGACAGCG), -957A/G (FAM-AATTGACAGCTGAGCCCCAAAC/VIC-AATTGACAGCTGAGCCCCAAAT), -909C/A (FAM-AATTTCGTAGGGTTTCGACCCAA/VIC-ATTCGTAGGGTTTCGACCCAC), -794T/G (FAM-GGCGAGGTTGCCCTGGA/VIC-GCGAGGTTGCCCTGGC) and +68/69 +/- GA (FAM-AGCTACAGGGAGAGAAACAG). These primers were used in a SNaPShot assay (ABI) to obtain genotypes through electrophoresis on an ABI3100 Genetic Analyzer. Genotypes obtained were analyzed using the GeneMapper v1.0 (ABI) software, double checked independently by two or more individuals in the laboratory and saved in a Microsoft Excel worksheet for statistical analysis. Haplotypes were assigned as in Joosten et al. [2001] and we did not perform subcloning. For the affected children, we were able to confirm the haplotype by examining the genotypes of their parents. We inferred functionality from the Joosten et al [2001] and Zhu et al [2004] reports.

### Statistical Analysis

We compared the distribution of alleles and Hardy-Weinberg equilibrium in the two ethnic populations using  $\chi^2$  analysis. All analyses were conducted on the entire sample set (Caucasians and Hispanics) and stratified by parent-identified ethnicity of the child. We evaluated alleles (H1, H2 $\gamma$ , H2), genotypes (H1/H1, H1/H2, H2/H2, H1/H2 $\gamma$ , H2/H2 $\gamma$ ) and promoter activity levels (low/low, low/high, high/high) among all tested samples. The H1 and H2 $\gamma$  alleles were classified as low activity and the H2 allele was defined as high activity [Joosten et al., 2001]. Significance level was set at  $p=0.05$ .

To evaluate the question of case-control association of haplotypes/genotypes/promoter function level, we used logistic regression and frequency tables in SAS [Statistical Analysis Software, Cary NC, 2004] using the affected children in the trios compared to the unaffected controls. Case-control analysis can be subject to spurious associations due to underlying population stratification. To address these concerns, we will also be using the Bayesian Genomic Control method. This method, proposed by Devlin and Roeder [1999], does not require information about the genealogy of the population and corrects for population heterogeneity, poor choice of controls, and cryptic relatedness of cases. The method calculates the posterior probability of association between a gene and the disease state. The posterior probability is defined as significant if it is in excess of 0.50.

For trio analysis, we used the TDT feature of GeneHunter [Kryglyak, 1996; Kryglyak, 1998] and RC-TDT [Knapp, 1999a; Knapp, 1999b]. RC-TDT permutes information on missing parents and is considered to be a more robust method for assessing transmission.

## RESULTS

Table I summarizes the allele and genotype frequencies for the complete population in this study. No H2 $\delta$  allele was observed among all samples tested. Homozygous H1 genotypes present in both Hispanic and Caucasian SBMM patients. We found that among the cases, controls and mothers of cases, there were significant differences between the ethnicities for the allelic distributions for *PDGFRA* P1. Among the cases, Hispanics had a higher frequency of the H1 allele, whereas Caucasians had a higher frequency of the H2 allele. Among controls, we see a similar pattern of increased frequency of H1 in the Hispanics and increased frequency of the H2 in Caucasians. We also had no H2 $\gamma$  alleles in the Hispanic population. In the mothers and fathers of the cases, we also found a similar pattern of H1 and H2 between Hispanics and Caucasians, although the fathers were not significantly different ( $p=0.065$ ). The case populations (overall, Caucasian and Hispanic) were in Hardy-Weinberg equilibrium.

The first TDT analysis was performed with GeneHunter and the second was performed with RC-TDT and these findings are summarized in Table II. In addition to investigating the data stratified by ethnicity, we also stratified by lesion level (lumbar and thoracic) and gender of the affected child (male and female). We found no significant results using GeneHunter or RC-TDT, with the exception of Hispanic trios. In the Hispanics, there was over-transmission of the H1 allele when compared to the expected levels based on parental haplotypes.

Using the cases from the trios and the controls, we performed logistic regression for association of (1) the individual alleles in a dose-response fashion for the high activity haplotypes (0, 1 or 2 copies of the high activity haplotype) or (2) the high/low functional genotype. These findings are summarized in Table III. We found no significant association between the haplotypes in the overall group, Caucasian, or Hispanic groups. When we considered the high/low function genotype as the “risk genotype”, we found no association between cases and controls in any of the three groups.

The genomic control analysis was also performed on the overall group, Caucasian and Hispanic groups. These analyses found that the posterior probability of association was not significant. In the overall group, the posterior probability was 0.02. In the Caucasian group, the posterior probability was 0.03 and in the Hispanic group, the posterior probability was 0.01.

## DISCUSSION

This study is the largest to date to assess the association of *PDGFRA* P1 in the development of SBMM. Similar to Zhu et al. [2004], we did not find the H2 $\delta$  genotype in our SBMM triads or control samples. We did find that there were significant differences in the allele frequencies in the two ethnic populations and also differed from the frequencies reported by Joosten et al. (2001). The H2 $\gamma$  allele was very rare and was not detected in the Hispanic control population. The shift in frequency of the H1 and H2 alleles across the ethnicities strongly suggests an important population stratification which must be accounted for in analyses of *PDGFRA* P1 in humans.

An important distinction between our work and previous work is in the definition of the phenotype. The population of Joosten et al. [2001] was not specifically stated to be limited to SBMM, but rather was described with a more general term of spina bifida aperta. The population of Zhu [2004] was very broadly defined to include multiple types of NTDs as well as viable and non-viable pregnancies. The success of identifying modulating risk factors, such as a gene without major effect, is dependent on defining the phenotype in such a manner that the included cases are genetically homogeneous.

Another important distinction is the sample size of our study population. Joosten [2001] had 76 sporadic patients and 49 familial patients for a total of 125 cases. Zhu [2004] performed analyses on 43 cases. Our sample size of 407 cases is significantly larger and permits us to perform stratifications on not only ethnicity, but gender of the child, lesion level and joint stratification between gender and ethnicity (data not shown). The ability to stratify within our study population increases the chance that we will have a genetically more homogeneous group in which to see minor effects.

Joosten et al. [2001] concluded that in combination with other adverse factors, H1/H2 heterozygotes have an increased risk for malformations resulting in either embryonic lethality or NTDs. Our results clearly excluded H1/H2 as a risk factor for SBMM in our Caucasian and Hispanic patients of Mexican descent. We also further excluded low activity *PDGFRA* P1 promoter as a risk factor for SBMM in Hispanic patients of Mexican descent and North American Caucasian patients. The presence of H1 homozygotes in our Hispanic patients and our controls did not support the notion that this haplotype combination selects against SBMM formation. Additional adverse factors are involved other than the H1 haplotype on the *PDGFRA* P1 among our sporadic Hispanic SBMM patients.

The TDT results (Table II) in the Hispanics are intriguing; however, we must take into account the possibility that missing parents, especially fathers, may have yielded a questionable finding. The Hispanic fathers were also not in alignment with ethnic differences observed among the haplotypes. This suggests our assumption that the genotypic distribution among the missing fathers and present fathers was equal is not supported. The follow up case-control study that specifically tests promoter activity level does not show an association. The case-control and genomic control findings further support our conclusion that the Hispanic TDT results are spurious. When comparing the TDT results to those found with the genomic control method, we look to Bacanu, et al. (2000) for a direct comparison of the power of the TDT and Genomic Control methodologies. They found that the GC is more powerful when heritability is low and the TDT is more powerful when there is substantial population substructure. The inclusion of both of these methods will substantially strengthen the analysis of these data. Because of the rarity of SB and the difficulty in recruiting these families, our ability to utilize these two complementary methods assists us in making the conclusion that there is not an association with *Pdgfra* and SB.

The *Patch* and *undulated* double mutant mouse model suggests *Pdgfra* and *Pax1* exert a digenic effect resulting in SB occulta (SBO). Additionally, *Pdgfra* in the homozygous mutant *Patch* mouse do have neural tube defects, as to targeted homozygous knockouts of *Pdgfra*. Our patient population does not have SBO but rather has SBMM. Thus, the lack of effect of H1/H2 contributing to SBMM may represent differential etiologies of these two conditions. However, our laboratory is currently genotyping *PAX1* in this patient population and will explicitly test the interactive effects of *PDGFRA* P1 and *PAX1*.

In conclusion, we do not find compelling evidence that the *PDGFRA* P1 region is associated with SBMM. While it may participate in gene-gene interactions with other cascade genes, it is not sufficient to cause SBMM on its own. There is significant population stratification within this gene, which is an interesting observation which should be accounted in multi-ethnic studies for to prevent bias.

## Acknowledgments

Grant Sponsor: Shriners Hospital for Children, Grant Number: 8580

Grant Sponsor: The National Center for Research Resources, Grant Number: M01- RR 02558

Grant Sponsor: The National Institute of Child Health and Human Development, The National Institutes of Health,  
Grant Number: PO1 HD35946-06A2

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