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The *PDGF-C* regulatory region SNP rs28999109 decreases promoter transcriptional activity and is associated with CL/P

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Abstract

Human linkage and association studies suggest a gene(s) for nonsyndromic cleft lip with or without cleft palate (CL/P) on chromosome 4q31-q32 at or near the platelet derived growth factor-C (*PDGF-C)* locus. The mouse *pdgfc*$^{-/-}$ knockout demonstrates that PDGF-C is essential for palatogenesis. To evaluate the role of *PDGF-C* in human clefting, we performed sequence analysis and SNP genotyping using 1,048 multiplex CL/P families and 1,000 case-control samples from multiple geographic origins. No coding region mutations were identified, but a novel -986 C>T SNP (rs28999109) was significantly associated with CL/P (p=0.01) in cases from Chinese families yielding evidence of linkage to 4q31-q32. Significant or near significant association was also seen for this and several other *PDGF-C* SNPs in families from the US, Spain, India, Turkey, China, and Colombia, while no association was seen in families from the Philippines, Guatemala, and case-controls from Brazil. The -986T allele abolished six overlapping potential transcription regulatory motifs. Transfection assays of *PDGF-C* promoter reporter constructs demonstrate the -986T allele is associated with a significant decrease (up to 80%) of *PDGF-C* gene promoter activity. This functional polymorphism acting on a susceptible genetic background may represent a component of human CL/P etiology.

Keywords

PDGF-C; SNP; CL/P; promoter activity

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Introduction

Isolated or nonsyndromic cleft lip with or without cleft palate (CL/P) is a common birth defect that affects ~ 1/700 newborns worldwide.[1,2] Although the identification of genes for nonsyndromic CL/P is far from complete, genetic linkage and association studies in humans and animal models have identified at least 16 candidate loci for CL/P.[1,3,4,5] Rare point mutations or significant association has been found between human nonsyndromic CL/P and missense mutations or polymorphic variants in several genes including *PVRL1*, *IRF6*, MSX1, *RUNX2* and *FGF* signaling genes.[1, 3, 4, 6] Only the *IRF6* variant finding has been consistently replicated and recent evidence ascribes its affect to a point mutation in a *TFAP2A* binding site in an enhancer 10Kb upstream of the *IRF6* promoter.[6]

Multiple lines of evidence support the existence of a human CL/P gene on distal 4q including significant associations with deletions of 4q31-qter,[7,8] linkage with D4S192 [9] and significant allelic association for CL/P with D4S192 in Caucasian[10] and Chilean[11] case-control studies The most significant result from multipoint linkage analysis of genome scan markers in Chinese multiplex CL/P families was found in 4q32 with the multipoint linkage peak at 158 Mb (fig. 1).[12] The earlier positive linkage and association findings [9, 10,11] with D4S192 are not inconsistent with the Chinese results because earlier studies did not assess any markers distal to D4S192.

The human *PDGF-C* locus (157.89–158.12 Mb) maps to the linkage peak found in the Chinese families. Mouse knockout studies demonstrate PDGF-C is required for palatogenesis.[13] While human studies support an etiologic role for several genes in CL/P etiology, (*PVRL1*, *IRF6*, and *MSX1*) [14,15,16], expression levels of the mouse homologues of these genes were unaltered in *pdgfc*−/− mutant embryos that develop clefts, suggesting their activity is not related to PDGF-C signaling in palatogenesis, so PDGF-C signaling is a new pathway in palatogenesis, independent of those previously identified.[13]

These findings led us to study the possible role of *PDGF-C* gene variants in human CL/P. Sequence analysis of the gene in Chinese CL/P cases and controls did not identify any coding region mutations in the *PDGF-C* gene. We did identify a novel SNP in the human *PDGF-C* gene promoter region that was associated with CL/P in Chinese families demonstrating linkage to chromosome 4q32.1. Evaluation of the role of this SNP on the transcriptional regulation of human *PDGF-C* gene expression indicates it may have a contributory role in some human CL/P cases. To evaluate the generality of these findings, multiple SNPs within *PDGF-C* were assessed for association with CL/P in 1,048 multiplex CL/P families from Europe, USA, Asia, Central and South America, and 1,000 case-control samples from Brazil.

Materials and Methods

Subjects

Study samples were drawn from 1,048 multiplex families, i.e. those with two or more individuals affected with nonsyndromic CL/P, from the following populations that have been described previously [17,18]: USA and European Caucasians (Iowa; Texas; Pittsburgh; St. Louis; Madrid, Spain; and Turkey), Asia (Shanghai and Beijing, China; West Bengal, India; the Philippines), Central America (Guatemala), and South America (Colombia). An additional set of 500 cases and 500 controls from Brazil was also studied[19]. The families are summarized in Table 1. All studysubjects provided informed consent as approved by institutionalreview boards in boththe United States (University of Pittsburgh, University of Iowa) and each of the other countries involved.

One-hundred and eight affected individuals from the Shanghai, China study population were chosen for complete sequencing of the *PDGF-C* gene (one from each of the 104 families summarized in Table 1 plus an additional 4 cases that were the only participating individuals from their families). 26 cases came from "linked" families, i.e. families that individually had positive LOD scores with anonymous STRP markers in 4q31-q32, and 82 came from "unlinked" families.[12] 113 Chinese controls were also sequenced, 19 from the same Shanghai study population and 94 from the Coriell Han Chinese Human Variation panel (Coriell Cell Repository, Camden, NJ, URL http://ccr.coriell.org/nigms).

Families from Pittsburgh, St. Louis, Spain, India, Turkey, Guatemala, and the Philippines, and the Brazilian case-control samples were utilized for SNP genotyping of the novel SNP identified from sequencing these Chinese cases. All populations summarized in Table 1 except St. Louis, Guatemala and Brazil also had SNP genotyping available for 12 additional SNP's within *PDGF-C* to investigate further the association between CL/P and *PDGF-C* (see Table 3).

DNA sequence analysis of *PDGF-C*

Oligonucleotide primers were designed to amplify 1150 bases of the 5′ regulatory region and all 7 exons of the *PDGF-C* gene, including intron-exon boundaries (primer sequences available on request). Primers were designed using Oligo (Molecular Biology Insights, Cascade, CO, USA) and the *PDGF-C* genomic sequence contained in the chromosome 4 contig (NT_016354). All nucleotide numbering assumes the A of the ATG start codon as nucleotide 1. PCR amplification products were subjected to DNA sequencing using ABI dye-terminator chemistry and analyzed using an ABI 3100 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence results were compared to the reference sequences using the Sequencer Program (Applied Biosystems, Foster City, CA, USA). Identification of transcription factor binding sites was determined computationally using AliBaba2.[20]

Generation of human *PDGF-C* promoter reporter constructs

The human *PDGF-C* cDNA sequence (3 kb) was used as a probe for bioinformatics screening of the *PDGF-C* promoter DNA sequence using the NCBI database NT_016354.18. A 20 kb *PDGF-C* promoter sequence was identified and two *PDGF-C* containing bacterial artificial chromosome (BAC) clones were selected as described by Choi et al. [21] BAC clones (CTD-3161F17, CTD-2510M6, Open Biosystems, Huntsville, AL, USA) containing the *PDGF-C* promoter were subjected to further analysis. Amplified genomic DNA (130 kb to 170 kb insert) from the BAC clones was digested with restriction enzymes HindIII and SstI (New England Biolab, city, state, country) respectively. An 11.4 kb band digested with SstI and an 8.3 kb band digested with HindIII were eluted, subcloned (pBS KSII vector, Stratagene, La Jolla, CA, USA), and sequence verified. 8 kb human PDGF-C promoter DNA double-digested with EcoRV(partial digestion) and SacI and 4 kb human PDGF-C promoter DNA double-digested with EcoRV (from multiple cloning site of pBSKSII vector) and SacI (New England Biolab) were cloned into SmaI and SacI sites of pGL2 Enhnacer vector (Promega, Madison, WI, USA). Point mutagenesis substituting T for C at -986 in the human *PDGF-C* promoter was generated using the QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol, followed by sequence verification. -986 C and -986T constructs were generated for both -1116C and -1116G backgrounds, so that 4 haplotypes were evaluated: -1116G-986C; -1116G-986T; -1116C-986C; and -1116C-986T (see fig 2). We generated two independent PDGF-C promoter reporter constructs (4kb and 8kb) to examine the -986 C>T SNP effects on its transcription activity.

Measurement of -986C and -986T allele human *PDGF-C* promoter activity

-986C and -986T allele human *PDGF-C* promoter Luciferase reporter constructs were transfected into MC3T3, C2C12, Hela, and 293 cells (ATCC, Manassas, VA, USA) using a Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) kit as previously reported.[22] Briefly, MC3T3, C2C12, Hela, and HEK293 cells (5X105/well) in 6-well plates were transfected with human *PDGF-C* promoter Luciferase reporter constructs (2 μg) and TK-Renilla Luciferase construct (0.2 μg). After 72 hours incubation in 5% CO2 at 37° C, clear cell lysates were harvested by microcentrifugation, and Firefly and Renilla Luciferase activity were measured using a dual-Luciferase reporter assay system (Promega, Madison, WI, USA) and the relative activity (ratio between Firefly and Renilla Luciferase activity) was calculated. Results are reported as the mean ± S.E. for three replicate samples using five independent -986C and -986T reporter constructs and were analyzed by Student's t test. Results were considered significantly different for $p < 0.05$.

PDGF-C SNP Genotyping

To assess the generalizability of *PDGF-C* SNP associations in our study populations the novel -986 SNP (rs28999109) identified in the Chinese case-control study was genotyped in families from India, Turkey, Columbia, the Philippines, Pittsburgh, St. Louis, Guatemala, Spain, China, and case-controls from Brazil, utilizing TaqMan® chemistry with the Assay-On-Demand™ C_61773848_10 (Applied Biosystems, Foster City, CA, USA). Additional *PDGF-C* SNPs were analyzed to further investigate *PDGF-C* in CL/P (Table 3). As part of a larger CL/P fine-mapping project (ACL, MLM, JCM, LLF) DNA samples from the kindreds listed in Table 1 were genotyped for multiple SNPs within *PDGF-C* (see Table 3) by the Center for Inherited Disease Research (CIDR) utilizing the Illumina system (http://www.cidr.jhmi.edu/). Three additional SNPs were genotyped in some families using Assay-On-Demand™ probes, Applied Biosystems, Foster City, CA, USA (rs3733486: C_25803666_10; rs1002091: C_7427164_10; and rs13133399: C_9306502_10).

Statistical analysis

Preliminary analyses and case-control comparisons

Each SNP was assessed with PedCheck[23] to test for inconsistencies due to non-paternity or other errors. Standard chi-square tests and Fisher exact tests were used to compare the SNP allele frequencies (from the sequencing studies) between the Chinese cases and controls, and between the Brazilian cases and controls. P-values of 0.05 or less were considered significant.

Allelic Association

Alleles at each *PDGF-C* SNP were tested for association with CL/P using the Family Based Association Test (FBAT). [24] Association was assessed for each SNP in each population, as well as the pooled data from all the populations, plus Caucasian, Asian, and Central/South American subsets. In addition to the individual SNP's, association was also tested using the haplotype version of FBAT (HBAT) [24] in the individual populations and pooled subsets for sliding windows across the *PDGF-C* SNPs.

Results

Sequencing of the human *PDGF-C* gene

Direct DNA sequence analysis of the *PDGF-C* coding regions did not identify any coding region mutations in Chinese cases or controls, but three SNPS were identified. A known C>T SNP (rs3733486), was detected in the noncoding region of exon 1 (-379 from the ATG start codon), in one unaffected and two affected individuals. Two additional SNPs were identified

in the 5′ regulatory domain of the *PDGF-C* gene: a known G>C substitution at -1116 (rs1002091), and a novel C>T substitution at -986 (Submitted to dbSNP; assigned rs28999109), see fig. 2. There was no significant difference in the frequency of the -1116 G>C variant between cases and controls (p = 0.21). Table 2 summarizes the frequency of the C>T SNP variant at -986 in CL/P cases from 4q31-q32 linked and 4q31-q32 unlinked Chinese families, and among Chinese controls. The frequency of the T allele in cases from linked Chinese families was significantly greater than in controls (case allele frequency= 0.15; control= 0.06; p= 0.01), but not for cases from unlinked families (frequency= 0.08; p= 0.34), while the frequency in all cases was borderline significant (frequency= 0.10; p= 0.09

The presence of the wild-type cytosine nucleotide allele at -986 from the ATG start codon (-986C) is associated with 6 overlapping transcription factor binding consensus motifs including, EGR-1, Sp1, WT1, USF, HEB, and ETF (fig 3.). Substitution of thymine at the nucleotide 986 position (-986T) abolishes these cytosine-rich regulatory motifs.

Effects of -986C and -986T SNPs in the human *PDGF-C* promoter on the transcriptional regulation of *PDGF-C* gene

To determine the effects of the -986C/T alleles on *PDGF-C* transcriptional regulation, -986C *PDGF-C* and -986T *PDGF-C* allele Luciferase reporter constructs were co-transfected with the TK-Renilla Luciferase construct into MC3T3, C2C12, and HEK-293 cell lines. The relative activity of 4 kb -986T allele *PDGF-C* promoter was significantly decreased in MC3T3 cell lines (about 80%) (fig. 4a) and in C2C12 (fig. 4c) and HEK293 (fig. 4e) cell lines (about 50%) compared to those of 4 kb -986C *PDGF-C* constructs.

Moreover, relative activities of the 8 kb -986T *PDGF-C* promoter constructs are also significantly decreased compared to those of the 8 kb -986C allele *PDGF-C* promoter (fig. 4b, 4d, 4f) in all three cell lines. Although transcriptional activities of the 4 kb *PDGF-C* 5′ promoter constructs are more than 5 fold greater than all activities associated with the 8 kb promoter constructs, transcriptional activities of all constructs containing the -986T allele were consistently decreased 40% or more compared to -986C constructs (fig. 4), suggesting that this region (from −8 kb to −4 kb) of the human *PDGF-C* promoter may contain tissue specific negative regulatory domain(s). Although there was no statistically significant association with affection status for either allele at -1116, given its close proximity to the -986 SNP within the regulatory region, we measured *PDGF-C* gene promoter activity containing all 4 possible haplotypes of 4 kb reporter constructs (-1116G -986C; -1116G -986T; -1116C -986C; and -1116C -986T) to evaluate the effect of the -1116 G>C SNP on the transcriptional regulation of *PDGF-C* gene expression. As shown in figure 4, -986T allele *PDGF-C* reporter constructs significantly decreased promoter activities compared to those of -986C constructs regardless of -1116G or -1116C in MC3T3 (fig. 5a), HEK-293 (fig. 5b), and C2C12 (fig. 5c) cell lines. In contrast, -986T allele *PDGF-C* reporter constructs did not decrease the promoter activity of human *PDGF-C* gene in Hela cell line suggesting that Hela cells may not have transcriptional regulatory machinery for the human *PDGF-C* gene (fig. 5d).

Allelic association analyses of PDGF-C SNP's and CL/P

The results of the FBAT analyses of multiple *PDGF-C* SNP's are summarized in Table 3. The novel -986 *PDGF-C* SNP (rs28999109) was nearly significantly associated with CL/P in the West Bengal, India families (p-value=0.06), and Spanish families (p-value=0.07) but not in the Turkish, Pittsburgh, St. Louis, Guatemala, nor Filippino families (p-values>0.21). Other *PDGF-C* SNPs were also significantly associated with CL/P in some of the populations, notably within the Colombian, St. Louis, Spanish and Chinese samples (see Table 3). Further, results showed a trend towards significance in additional populations (Iowa, Pittsburgh, Turkey). The results from the Brazilian case-control sample were not significant and are not

shown in detail. Interestingly, case-control analyses from the Chinese families showed significant association with the -986 SNP, and family-based analyses also showed association with an adjacent SNP (rs10020901)_in China (see Table 3, p-value = 0.02), St. Louis (p-value=0.04) and near significant association in PIttsubrgh and Turkey. The pooled FBAT analyses showed a trend towards significance for some of the SNPs in the TOTAL and Caucasian subsets. Haplotype analyses also had population-specific patterns in significance. Table 4 shows the most notable results: a three-SNP haplotype in the Caucasians (comprising SNPs rs983473, rs10517653, rs10517653) was significantly associated with clefting (p-value 0.04), as was a two-SNP haplotype in the Chinese (the -986 SNP rs28999109, and rs1002091; p-value= 0.04)

Discussion

Animal studies demonstrate that PDGF signaling is important in palatal development, with a specific role for PDGF-C.[13, 25] Evidence from genetic linkage, association and cytogenetic deletions provide support for a human CL/P locus in the chromosome 4q31-ter region containing the *PDGF-C* locus.[7–12] These observations led us to evaluate the *PDGF-C* gene for genetic variants that may be etiologic for human CL/P. Sequence analysis identified a novel SNP polymorphism (rs28999109) in the proximal domain of the *PDGF-C* gene in Chinese CL/P families demonstrating maximum linkage to the *PDGF-C* locus.[12] The presence of the less frequent rs288999109 T allele disrupts a highly conserved cytosine-rich regulatory motif in the 5′ proximal regulatory region of *PDGF-C* that has consensus sequence for several DNA binding transcription factors that modulate *PDGF* expression.[26,27] Reporter constructs containing the T allele demonstrate significantly decreased promoter transcription compared to constructs containing the more common C allele ($p < .05$). Decreased PDGF-C expression has been correlated with orofacial clefting in several mouse models.[13, 28, 29, 30] Our finding of a positive association between the rarer rs28999109 T allele in the *PDGF-C* 5′ regulatory region and CL/P in a subset of Chinese families is consistent with a plausible etiologic mechanism for a complex trait such as CL/P. Further evidence for a role of *PDGF-C* in CL/P is provided by our results showing statistically significant association between additional *PDGF-C* SNPs and CL/P in study samples from St. Louis, Spain and Colombia, and a trend near significance in other study populations (Iowa, Pittsburgh, Turkey). Larger study samples will be necessary to confirm the trends towards significance. The results of this study imply population-specific associations with PDGF-C in that one of the largest study samples (the Philippines) showed no evidence of association. Interestingly two of the populations showing associations with the novel -986 SNP (China and India) were both of Asian origin but the remaining Asian population (the Philippines) did not show significant association with any *PDGF-C* SNPs. The populations that showed significant or near-significant association with other *PDGF-C* SNPs were all Caucasian (Spain, Iowa, Pittsburgh, and Turkey) or mixed Caucasian/Native American (Colombia). While results in data pooled across populations was either borderline or not significant, this is not unexpected given the population-specific patterns in the results, which may also contribute to the well-known differences in the epidemiological characteristics of nonsyndromic CL/P.[1,2] We estimated the population attributable risk for the -986 allele in the Chinese to range from 4.2% to 6.4%; therefore one possible explanation for the population-specific differences in the association results may be differences in proportion of risk.

PDGFs play distinct roles at successive stages of mammalian organogenesis.[31] PDGF ligands regulate biological processes by binding to and activating PDGF receptors (PDGFR). This activates the tyrosine kinase domain contained within the intracellular portion of PDGFRs and initiates intracellular signaling events (Erk/MAPK and Akt/PKB) that trigger cellular responses such as proliferation, migration, contraction and cell survival, essential for numerous biological processes.[32] This PDGF signaling specificity is mediated through the activity of

multiple immediate early genes (IEGs).[33] PDGF-dependent tissues include the vasculature, kidney, neural crest-driven skeleton, and thoracic skeleton as well as the branchial arches and craniofacial mesenchyme. Identification and validation of PDGF transcriptional targets have been determined, and the specificity of downstream function is dependent upon PDGF receptor/ligand activation of specific IEGs.[33, 34]

PDGF-C can bind and activate PDGFR -αα and –αβ. PDGFRα is required for neural crest cell development and normal craniofacial development.[35] PDGF-C has been characterized as a key component of PDGFR-α signaling by biochemical analyses [36] and in vivo gene-targeting.[13] Nonsyndromic cleft palate derives from an embryopathy with consequent failure of the palatal shelf fusion.[29] PDGFR-α and PDGF-C are key regulators for embryonic and postnatal development, and are required for normal palatogenesis.[13, 25, 29, 30, 33, 35, 37] Animal models demonstrate that disruption of pdgfr-α signaling through a variety of etiologies including genetic mutation of pdgfr-α [35], microRNA suppression of pdgfr-α translation[38], genetic mutation of PDGF-C ligand[13, 28], suppression of PDGF-C transcription[29] and suppression of PDGF-C protein expression by retinoic acid[29, 30], are all associated with disruption of palatogenesis and the presence of orofacial clefting. While mutations and knockout studies of pdgfr-α and PDGF-C are etiologic for defective palatogenesis and orofacial clefting, knockout studies in mice indicate that other genes etiologic for CL/P (*PVRL1*, *IRF6*, *MSX1*) are not altered, indicating that pdgf-c signaling is a new and independent mechanism that regulates palatogenesis.[1, 13]

The proximal domain of *PDGF* gene promoters can directly modulate gene expression.[39] *PDGF-A* and *PDGF-C* share common gene regulatory mechanisms, and their expression is controlled by the zinc finger transcription factors Sp1 and EGR-1, which have affinity for overlapping nucleotide recognition elements.[26, 27, 40] Additionally, through its ability to repress expression of EGR-1, WT1 may function as a component in a transcription factor complex that regulates *PDGF-C* expression.[41] The presence of the more common cytosine nucleotide in rs28999109 at nucleotide position -986 from the ATG start codon in the *PDGF-C* gene preserves 6 overlapping transcription factor binding consensus motifs (including Sp1, EGR-1 and WT1) within a highly conserved 500 bp interval. Substitution of a thymidine nucleotide at this position in rs28999109 abolishes the consensus sequence for multiple DNA binding transcription factors including Sp1, EGR-1 and WT1. The transcriptional activities of PDGF-C promoter reporter constructs containing the rs28999109 T allele were consistently decreased more than 40% compared to constructs containing the C allele in mesenchymal C2C12 cells and in osteoprogenic MC3T3 cells differentiated from mesenchyme, suggesting that disruption of the consensus sequence may reduce PDGF-C expression in these cells. Decreased *PDGF-C* transcription secondary to the less frequent rs28999109 T allele may act on a susceptible genetic background to impair PDGFRα-PDGF-C signaling, increasing susceptibility to CL/P. Genetic variants in downstream IEGs critical to craniofacial and palatal development could contribute to susceptibility as demonstrated in the mouse model.[33]

Mutations in PDGFR-α [35], as well as mutations of their PDGF ligands[13, 28], including PDGF-C, disrupt craniofacial development and are associated with craniofacial clefting and defective palatogenesis. PDGF signaling specificity is mediated through immediate early genes.[33] Mutations of specific immediate response genes (Arid5b, Tiparp, Sgpl1, BCo55757, Axud1, Mzf6d and Schip1), which are the downstream gene targets controlled by the PDGF pathway[33, 34], are also associated with anomalies of craniofacial development. The associated phenotypes are consistent with the facial clefting seen in pdgfrα−/− mice, but are less severe and less penetrant. Additionally, while pdgfrα−/− mice show severe skeletal defects, loss of one copy of pdgfrα (pdgfrα−/+) increased the severity of skeletal defects (including palatal clefting) in many lines with IEG mutations (Arid5b, Tiparp, BCo55757, BC058969 and Schip1), and created skeletal malformations in one IEG knockout line that had no previous

skeletal defects (Plekha1).[33] These studies demonstrate that mice with mutations in the primary gene targets of PDGF signaling, the immediate early genes (IEGs), show phenotypes in the same structures and cell types as seen in PDGF receptor mutants, supporting the notion that target genes control specific processes downstream of individual receptor tyrosine kinases (RTKs). IEGs are specific for individual RTKs and responsible for particular downstream functions. Additionally, defective craniofacial development was seen as a result of dosage-sensitive genetic interactions with PDGF signaling genes and their downstream targets (IEGs), suggesting that these genes work collectively to implement PDGF function in development.[33]

Nonsyndromic cleft palate derives from an embryopathy with consequent failure of palatal shelf fusion.[29] The etiology of this condition is complex, and multiple genes and environmental factors are likely involved.[3, 4] As a key component of the PDGFR-α signaling pathway, PDGF-C is an important regulator of cell proliferation, survival, migration as well as deposition and maintenance of extracellular matrix.[32] Inactivation of PDGF-C or reduced PDGF-C expression in genetic (Pdgf-c −/−) or teratogen (Retinoic acid) models perturbs regulation of MMPs and TIFs in palatal mesenchyme during branchial arch development.[30, 42, 43] PDGF-C is a potent mitogen, and promotes proliferation of mouse embryonic palatal mesenchymal (MEPM) cells, and is required for branchial arch morphogenesis.[29] PDGF-C expression is also influenced by FGF signaling and small ubiquitin-like modifier modification (SUMO).[44] As alterations of FGF signaling are etiologic for some forms of orofacial clefting[45, 46], SUMO and PDGF-C may also interact with environmental risk factors to influence CL/P susceptibility.

The findings of this study demonstrate the importance of applying both animal and human model approaches in the search for developmental and etiological mechanisms underlying complex traits. Just as mutations in different genes in the PDGF signaling pathway work collectively to implement PDGF function in development[33], mutations and functionally significant genetic polymorphisms in different genes along the pathway (receptor, ligand and IEGs) can collectively result in defective craniofacial development, including craniofacial clefting. Taken together, these findings provide evidence for a role of the rs28999109 *PDGF-C* promoter SNP variant in the etiology of CL/P, and highlight the potential importance of regulatory regions in complex traits.[6, 47] In addition, the associations seen between other *PDGF-C* SNPs and CL/P provide further evidence of a role for *PDGF-C* in some forms of human CL/P.

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Abbreviations

CL/P cleft lip with or without cleft palate

PDGF-C Platelet-derived growth factor C

SNP	single nucleotide polymorphism
Sp1	Specific protein 1
USF	upstream stimulatory factor
WT1	Wilms tumor zinc finger protein 1
EGR-1	early growth response factor 1
HEB	human B-HLH factor
ETF	epidermal growth factor receptor (EGFR)-specific transcription factor

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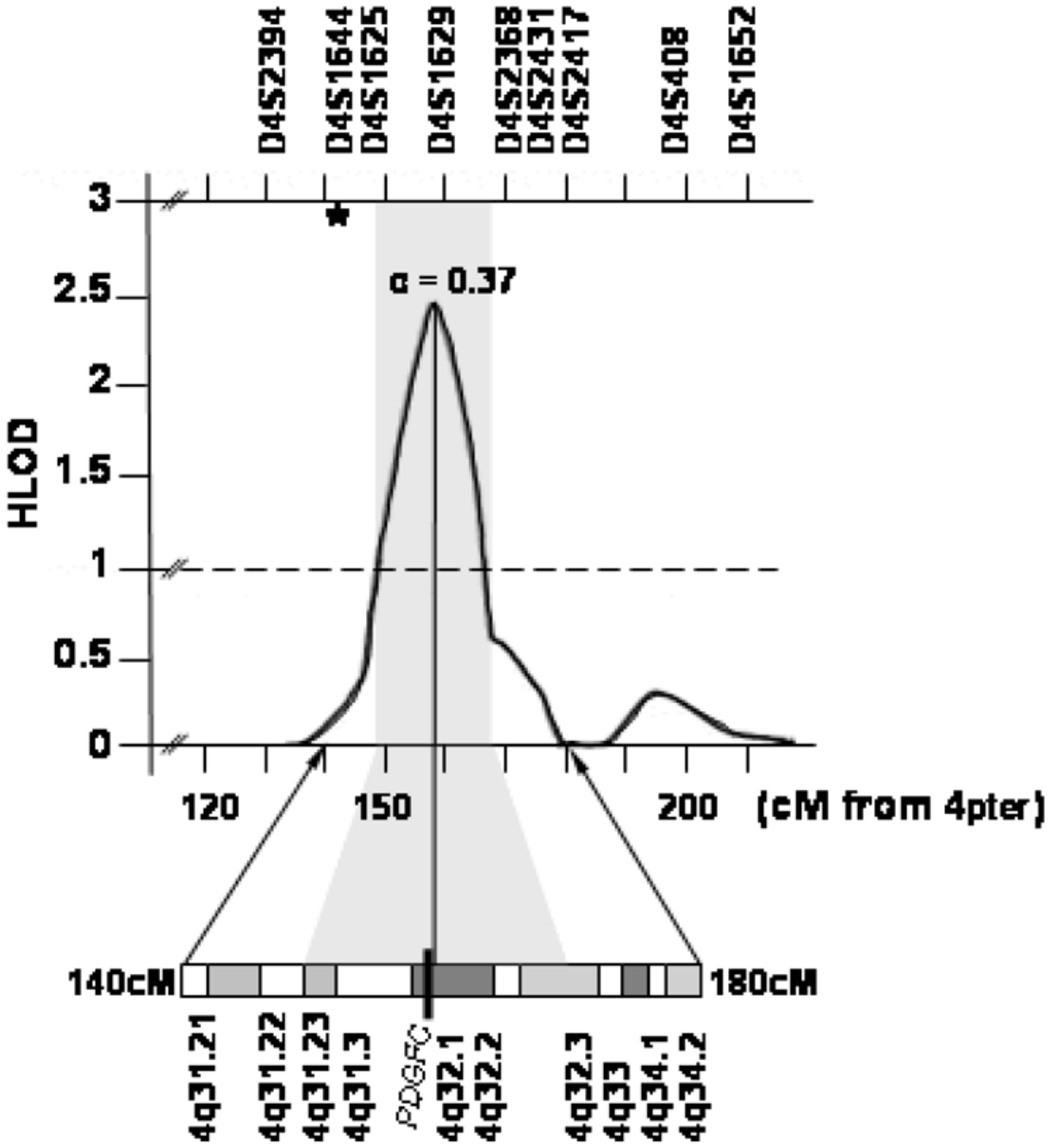

Figure 1.

Multipoint linkage analysis results for chromosome 4q in multiplex Chinese families.[12] Depicted are the multipoint heterogeneity LOD scores. α = the estimated proportion of linked families at the peak. The black bar across cytogenetic band 4q32.1 denotes the map position of the *PDGF-C* locus. The vertical grey-bar denotes the 1-LOD interval. Chromosomal marker locations and cytogenetic correlations are according to the Ensemble Database. The asterisk denotes the location of D4S192 (142 Mb from 4pter) reported to be significant in previous linkage [12] and association [10, 11] reports for CL/P in Caucasian and Chilean populations.

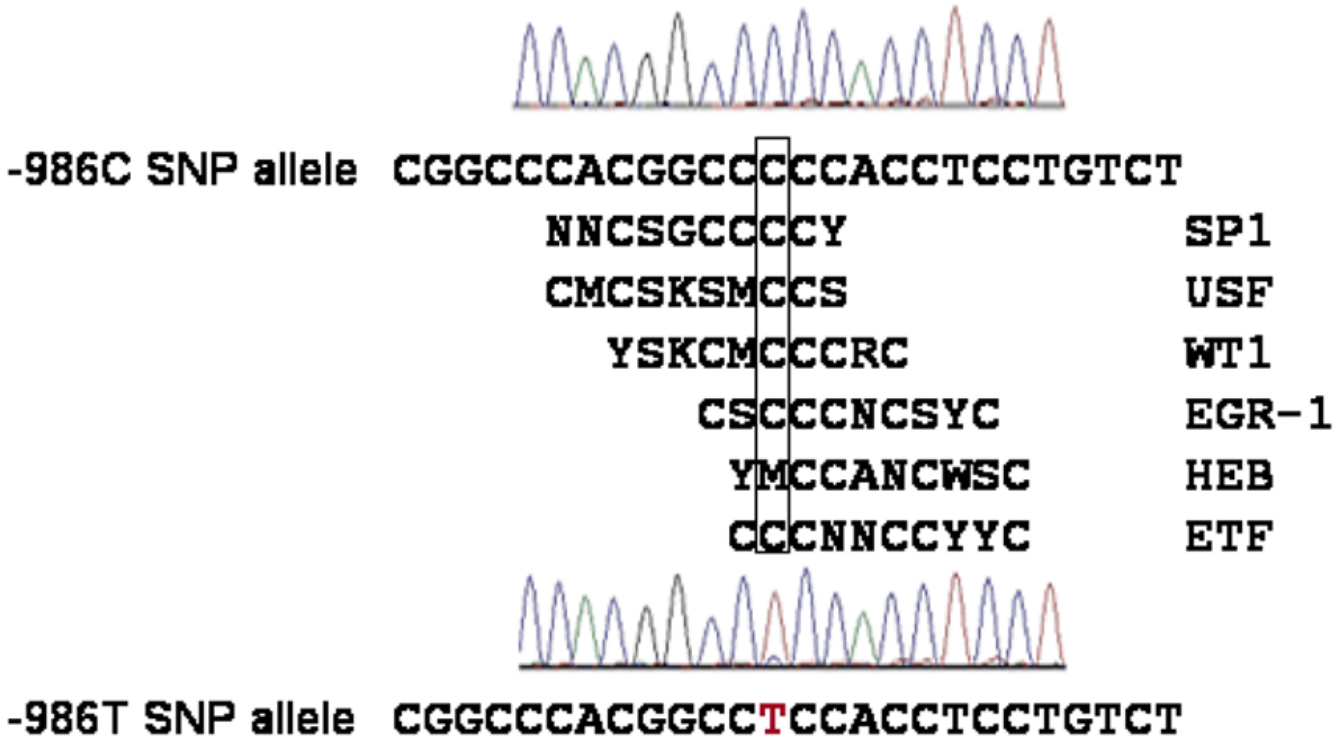

Figure 2.

Genomic structure of human *PDGF-C* promoter and schematic diagrams of -986C and -986T allele human PDGF-C promoter Luciferase reporter constructs. 8 kb human *PDGF-C* promoter DNA double-digested with EcoRV (partial digestion) and SacI and 4 kb human *PDGF-C* promoter DNA double-digested with EcoRV (from the multiple cloning site of pBSKSII vector) and SacI were cloned into SmaI and SacI site of pGL2 enhancer reporter vector.

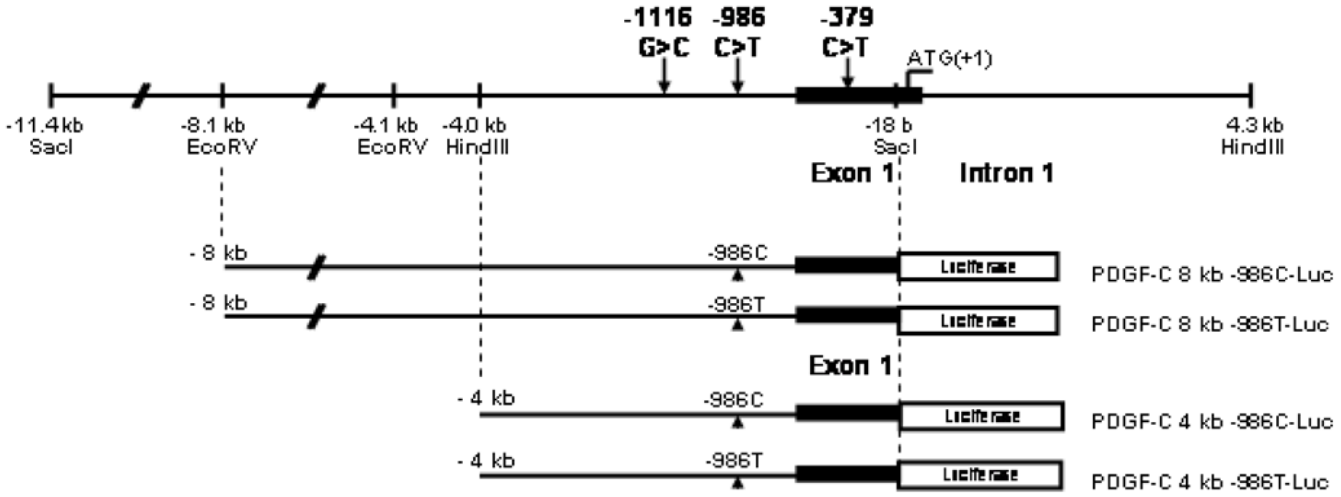

Figure 3.

A C>T SNP (rs28999109) at -986 in the human *PDGF-C* promoter regulatory region abolishes consensus binding motifs for Sp1 (Specific protein 1); USF (upstream stimulatory factor), WT1 (Wilms tumor zinc finger protein-1), EGR-1 (early growth response factor-1), HEB (human B-HLH factor), and ETF (epidermal growth factor receptor (EGFR)-specific transcription factor) (M=A,C; S=C,G; K=G,T; Y=C,T; R=A,G; W=A,T; N=A,C,G,T).

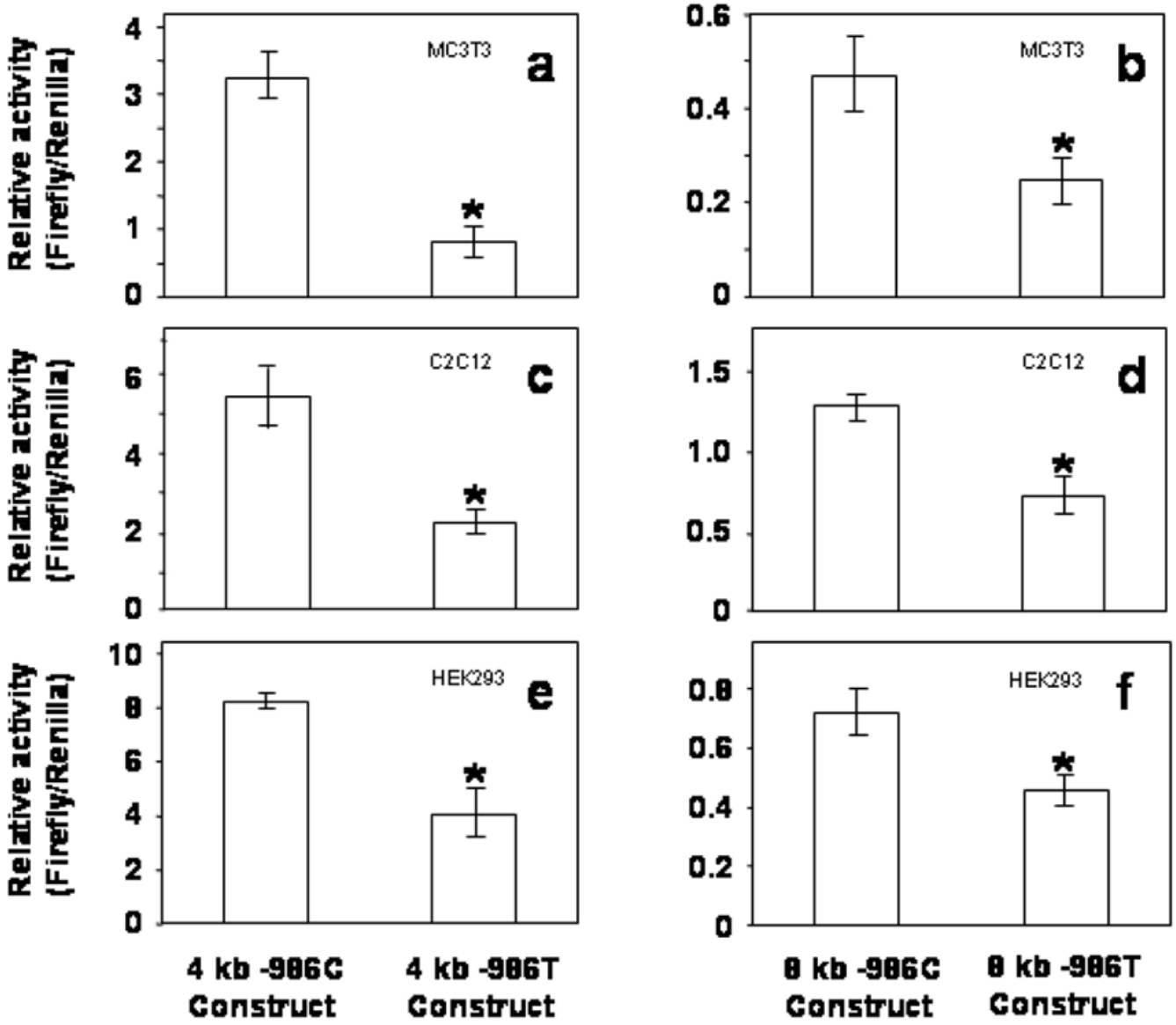

Figure 4.

Presence of the -986T SNP (rs28999109) in the human *PDGF-C* promoter reduces promoter activity. Human PDGF-C Luciferase reporter constructs (2ug) containing either the -986C or the -986T allele were co-transfected with the TK-Renilla Luciferase construct (0.2ug) into mouse osteoblastic precursor cells (MC3T3) (a, b), mouse myoblastic cells (C2C12) (c, d), and human embryonic kidney epithelial cells (HEK293) (e, f) using Lipofectamine Plus kits. Relative activities of the 4 kb -986T allele *PDGF-C* promoter construct are significantly decreased (> 50%) compared to that of the 4 kb -986C allele *PDGF-C* promoter construct in all three cell lines (a, c, e). Relative activities of 8 kb -986T allele *PDGF-C* promoter construct are also significantly decreased (> 40%) compared to those of 8 kb -986C *PDGF-C* promoter construct in these cell lines (b, d, and f).

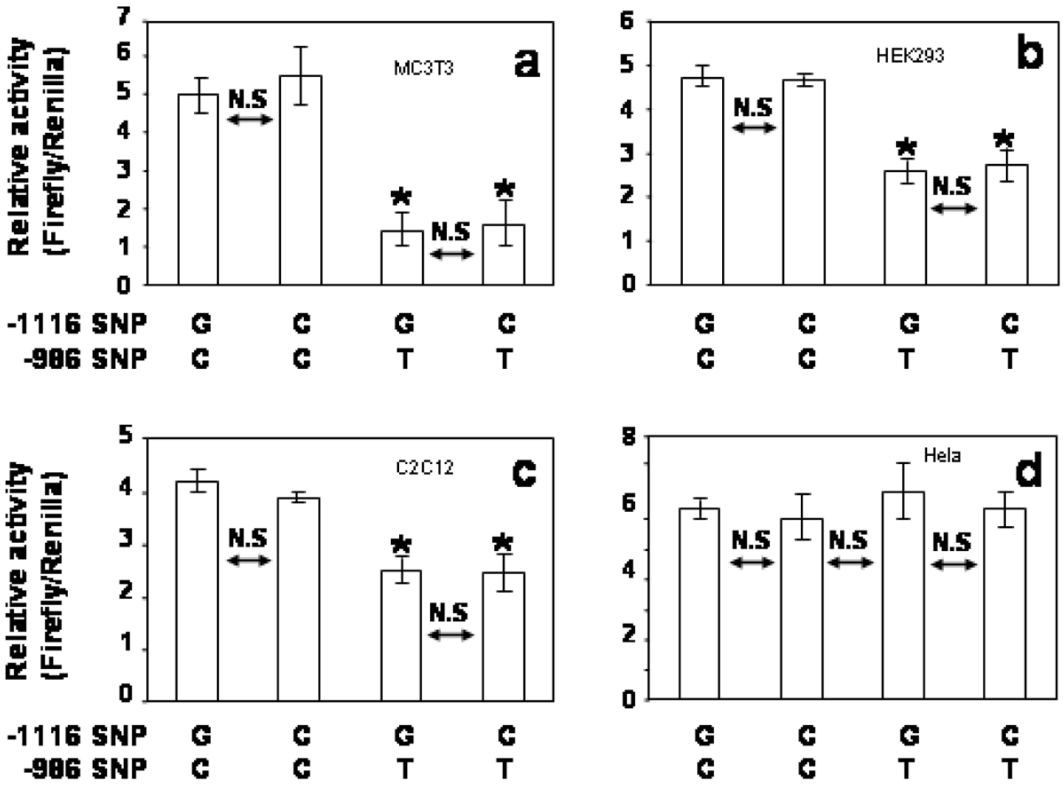

Figure 5.

Effects of -1116 G>C SNP on *PDGF-C* promoter activity. Presence of the -986T SNP in the human *PDGF-C* promoter reduces promoter activity regardless of genotype at the -1116 position. Four haplotypes (-1116G -986C; -1116C -986C; -1116G -986T; -1116C -986T) were generated using the 4kb human PDGF-C promoter reporter construct. The presence of the -986T SNP resulted in a significant decrease the *PDGF-C* promoter activity in MC3T3 cell line (a), 293 cell line (b), and C2C12 cell line (c) regardless of the -1116 genotype. However, the -986 T allele did not affect *PDGF-C* promoter activity in Hela cell line (d).

Table 1

Summary of families and individuals used in these analyses.

		INDIVIDUALS					
POPULATION *	**FAMILIES**	**Genotyped affected**	**Genotyped unaffected**	**Untyped Affected**	**Untyped Unaffected**	**Untyped Unknown**	**TOTAL**
CAUCASIAN							
Iowa	117	126	220	4	9	0	359
Pittsburgh	98	132	292	13	89	1	527
Madrid, Spain	36	43	90	0	3	0	136
Turkey	29	32	55	6	195	0	288
ASIA							
India	53	100	220	37	386	0	743
Philippines	242	664	1337	65	1366	0	3432
China	164	240	552	59	394	38	1283
CENTRAL AMERICA							
Guatemala	77	82	310	11	111	0	514
SOUTH AMERICA							
Colombia	232	349	577	15	368	0	1309
TOTAL--Families	**1048**	**1768**	**3653**	**210**	**2920**	**40**	**8591**
Case-controls (Brazil)	0	500	500	0	0	1000	

[a] "Iowa" includes families from Iowa and Texas; "Pittsburgh" includes families from Pittsburgh, PA and St. Louis, MO; "China" includes families from Shanghai and Beijing, China.

Table 2

Sequencing results for the -986 nucleotide position of the human *PDGF-C* gene promoter in 108 affected individuals from Chinese CL/P families and 113 Chinese controls.

		Genotype				
	N	**C/C**	**C/T**	**T/T**	**χ^2 (1 d.f.)**	**p-value**
CASES—from linked families [a]	26	21	2	3	6.10	0.014
CASES—from unlinked families [a]	82	71	9	2	0.91	0.341
CASES---TOTAL	108	92	11	5	2.84	0.090
CONTROLS—TOTAL	113	102	9	2	--	--

[a] Cases are from multiplex CL/P families from Shanghai, China. “Linked families” are families with evidence of linkage to anonymous STRP marker D4S1629 in 4q31-q32, $HLOD_{MAX}$ is 158 Mb from 4pter.[9]

Table 3

Summary of FBAT analyses of PDGFC SNP's in multiplex CL/P families, by population.

	SNP	MB location	Iowa	Pittsburgh	St. Louis	Spain	Turkey	India	Philippines	China	Colombia	Guatemala	Brazil
rs3815861	T/C	157.903698	--	0.13	--	0.03	--	--	--	--	--	--	--
rs1425486	T/C	157.903135	0.40	0.27	--	0.22	0.20	0.67	0.48	0.26	0.01	--	--
rs983473	T/C	157.914842	0.81	0.13	--	0.02	0.28	0.54	0.49	0.37	0.03	--	--
rs10517653	T/G	157.953440	0.38	0.16	--	0.04	0.32	0.39	0.71	0.39	0.05	--	--
rs2113992	T/C	157.977205	0.20	0.43	--	0.43	0.14	0.81	0.87	0.50	0.02	--	--
rs342318	A/G	158.008405	0.52	0.17	--	0.76	0.32	0.70	0.92	0.33	0.08	--	--
rs894588	A/G	158.036593	0.60	0.70	--	0.61	0.18	0.84	0.43	0.63	0.83	--	--
rs11728198	A/G	158.044847	0.75	0.70	--	0.62	0.11	0.71	0.47	0.42	0.94	--	--
rs6845322	T/C	158.103555	0.42	0.85	--	0.61	0.34	0.43	0.57	0.77	0.78	--	--
rs3733486	G/A	158.111884	--	--	--	--	--	--	0.42	--	--	--	--
rs28999109	C/T	158.124911	--	0.32	0.21	0.07	0.74	0.06	0.39	***	--	0.41	0.70
rs1002091	C/G	158.112621	--	0.19	0.04	0.35	0.09	--	0.83	0.01	--	0.50	0.24
rs13133399	T/G	158.117234	--	0.29	--	0.34	--	--	--	--	--	--	--

Results highlighted in yellow are statistically significant ($p \leq 0.05$), results highlighted in blue exhibit a trend near significance ($0.05 < p \leq 0.20$).

*** see Table 2 for association results with this SNP in the Chinese families.

Table 4

Sliding window haplotype association results for Caucasian and Chinese subsets.

4A. Three-SNP windows in Caucasians

Caucasians										
rs3815861	**rs1425486**	**rs983473**	**rs10517653**	**rs2113992**	**rs342318**	**rs894588**	**rs11728198**	**rs6845322**	**rs28999109**	**rs1002091**	**rs13133399**
0.099775 **											
	0.183495										
		0.036706 *									
			0.294555								
				0.345681							
					0.734077						
						0.297771					
							0.693876				
								0.524789			
									0.632203		

4B. Two-SNP windows in the Chinese

Chinese									
rs1425486	**rs983473**	**rs10517653**	**rs2113992**	**rs342318**	**rs894588**	**rs11728198**	**rs6845322**	**rs28999109**	**rs1002091**
0.522842									
	0.629394								
		0.683704							
			0.577984						
				0.585417					
					0.571157				
						0.840722			
							0.697151		
									0.036375 *

* statistically significant ($p<0.05$)

** borderline significant ($p<0.10$),