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Are genetic variants in the Platelet Derived Growth Factor- β (PDGF- β) gene associated with chronic pancreatitis?

Venkata Muddana, MD¹, James Park, MD¹, Janette Lamb, PhD¹, Georgios I Papachristou, MD¹, Robert H. Hawes, MD³, Randall Brand, MD⁴, Adam Slivka, MD, PhD¹, and David C Whitcomb, MD, PhD^{1,2}

¹Department of Medicine: Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center

²Department of Human Genetics, University of Pittsburgh

³Digestive Disorder Center, Medical University of South Carolina

⁴Department of medicine, Northwestern University

Abstract

Objectives—Platelet derived growth factor β (PDGF- β) is a major signal in proliferation and matrix synthesis through activated PSCs, leading to fibrosis of the pancreas. Recurrent acute pancreatitis (RAP) appears to predispose to chronic pancreatitis (CP) in some patients but not others. We tested the hypothesis that two known PDGF- β polymorphisms are associated with progression from RAP to CP. We also tested the hypothesis that PDGF- β polymorphisms in combination with environmental risk factors such as alcohol and smoking are associated with CP.

Methods—382 patients with CP (n=176) and RAP (n=206) and 251 controls were evaluated. PDGF- β polymorphisms +286 A/G (rs#1800818) seen in 5'-UTR and +1135 A/C (rs#1800817) in first intron were genotyped using single-nucleotide polymorphism polymerase chain reaction (SNP PCR) approach and confirmed by DNA sequencing.

Results—The genotypic frequencies for PDGF- β polymorphisms in position +286 and +1135 were found to be similar in controls and patients with RAP and CP. There was no difference in genotypic frequencies among RAP, CP and controls in subjects in the alcohol and smoking subgroups.

Conclusions—Known variations in the PDGF- β gene do not have a significant effect on promoting or preventing fibrogenesis in pancreatitis. Further evaluation of this important pathway is warranted.

Keywords

PDGF; Growth factors; Polymorphisms; Recurrent acute pancreatitis; Chronic pancreatitis

Introduction

Chronic pancreatitis (CP) is a progressive, inflammatory syndrome of the pancreas with many etiologies and characterized by organ inflammation, dysfunction and progressive replacement of the parenchyma by sclerosis and fibrosis¹. Growing evidence suggests that

Corresponding Author: David C Whitcomb MD, PhD, University of Pittsburgh Medical Center, Mezzanine Level 2, C Wing, 200 Lothrop Street, Pittsburgh, PA 15213, (412) 648-9604, (412) 383-7236 (fax), whitcomb@pitt.edu.

*Currently at University of Pittsburgh Medical Center.

CP begins with recurrent acute pancreatitis (RAP), either through a “necrosis-fibrosis sequence”^{2,3}, or through an immune system activating process as hypothesized in the Sentinel Acute Pancreatitis Event (SAPE) model⁴. However, the factors responsible for the transition from RAP to CP in some patients, but not others are largely unknown. Recent research on the pancreatic stellate cells (PSCs) has provided additional insight into the mechanism of fibrosis in CP^{5,6}. PSCs transition from quiescent to active state following pancreatic injury and thereby proliferate and transform into a myofibroblast-like phenotype with active synthesis and secretion of extracellular matrix proteins into the interstitial space of the pancreas⁷. Active PSCs are further stimulated by alcohol and its metabolite acetaldehyde⁸, oxidant stress, and at least two major growth factors: transforming growth factor β (TGF- β) and platelet derived growth factor β (PDGF- β)⁹. If PDGF- β is an essential or major growth factor for sustained activity of PSCs then major functional variants in PDGF- β or the gene encoding its receptor could affect PSCs activity levels and thereby affect the rate of fibrosis.

We hypothesized that the genetic mutations in PDGF- β could alter the transition from RAP to CP by interfering with normal PDGF- β signaling in the PSCs. The evidence of this effect would be a significant increase in loss-of-function mutations in RAP group, and “normal” frequency in the control group, and a significant decrease in the CP group. We began testing this hypothesis using two single nucleotide polymorphisms (SNPs) in PDGF- β previously reported to be associated with disease^{10,11} and that could potentially modify the function of PSCs and therefore the progression from RAP to CP. We used the North American Pancreatic Study2 (NAPS2) cohort by comparing the frequency of polymorphisms in participants classified as RAP, CP and controls. We also evaluated whether the effect of these PDGF- β SNPs modified the effect of alcohol and smoking in causing CP.

Materials and Methods

Study Population

Subjects were recruited from the NAPS2 cohort. The NAPS2 study is a multicenter, molecular epidemiology study designed to evaluate the genetic and environmental factors predisposing to RAP and CP. A detailed study protocol and methods for the NAPS2 study have been published¹². RAP was defined by the presence of two or more attacks of documented acute pancreatitis (AP) but no imaging evidence of CP. Chronic pancreatitis was diagnosed by the presence of changes primarily on imaging studies or histologic specimens.

All subjects were screened for two known *PDGF- β* polymorphisms which included +286 A/G (rs#1800818) seen in 5'-UTR and +1135 A/C (rs#1800817) seen in first intron^{10,11}. Subjects were stratified into alcohol categories based on the self-reported average number of drinks consumed per week during the period of heaviest lifetime drinking.¹² For analysis, alcohol drinking categories were combined into 3 groups based on their risk for causing CP: 1.) Low risk (Abstainers: No alcohol use or < 20 drinks in lifetime and light drinkers: \leq 3 drinks/week), 2.) Moderate risk (moderate: 4–7 drinks/week for females; 4–14 drinks/week for males and heavy drinkers: 8–34 drinks/week for females; 15–34 drinks/week for males, and 3.) very heavy drinkers: \geq 35 drinks/week.

Smoking status was classified based on number of pack-years. The number of pack-years of smoking was calculated from self-reported amount of smoking (average number of cigarettes smoked per day and the duration of smoking) and stratified as less than 12, 12 to 35, and more than 35 pack-years¹³.

DNA Preparation

An EDTA sample of peripheral blood was also drawn from all the subjects upon enrollment. DNA was extracted using the Flexigene blood system (Qiagen, CA, USA) according to the manufacturer instructions and amplified for direct sequence analysis¹².

Mutation Analysis

PCR primers are designed for both known mutations. +286 A/G (rs#1800818) has forward primer 5'-AGG GTG GCA ACT TCT CC -3' and reverse primer 5' - AGG GAG AGG TGC AAA CT - 3'. +1135 A/C (rs#1800817) has forward primers 5' - CGC TCT TCC TGT CTC TCT GC - 3' and reverse primer 5' - TGT TCT CGG GTT CCC AAA GG - 3'.

PCR amplification was performed in a total volume of 50µl under the following concentrations; for +286 SNP –80 nmol of forward and reverse primer, 2 mmol of magnesium, 20 µmol of dNTP and 10 × PCR Buffer II (ABI, CA) with 20ng of DNA and for +1135 SNP – 200 nmol of forward and reverse primer, 2.5 mmol of magnesium, 200 µmol of dNTP and 10 × PCR Buffer II (ABI, CA) with 20ng of DNA Amplification settings were 95°C for 12 min × 1cycle, 95°C for 30 sec, annealing temperature 68°C × 20 sec and 72°C × 20 sec for 35 cycles and 72°C for 2 min × 1cycle.

A single base extension reaction was performed using another internal extension primer with 3' end annealing adjacent to the polymorphic base pair. The internal primer sequence was as follows: 5' CGC CCA GAG CGG CGA GC 3' for +286 SNP, 5' CAT TCA TTA CCT TCG CCC CC 3' for +1135 SNP. An extension reaction was prepared using thermosequanase buffer (Amersham Pharmacia Biotech), 1 µmol/l internal extension primer, 0.4 U thermosequanase (Amersham Pharmacia Biotech) and 125 nmol/l each dNTP in a 1:16 dilution of dye-labeled dNTP to unlabelled dNTP using the dyes TAMRA C and R 110U/ Perkin Elmer Wellesley, MA, USA) in a 10 µl solution volume. The extension reaction solution was added to the 20 µl purified product and the mixture was incubated at 94°C for 60s, followed by 35 cycles of 94°C for 10s and 55°C for 30s.

The final assay products were read on an LJJ Analyst fluorescence reader (Molecular Devices, Palo Alto, CA, USA). Selected samples were confirmed with DNA sequencing. Sequence products were run on an ABI Prism 3730 Genetic Analyzer and sequence data was analyzed using Sequencher 4.7 (ABI, Foster City, CA)^{14, 15}.

Statistical Analysis

Genotype frequencies were tested for adherence to the Hardy- Weinberg equilibrium using a χ^2 test with one degree of freedom. As a descriptive measure of association between genotypes and outcomes, Odds ratios (ORs) and 95 percent confidence intervals (95% CI) were calculated using χ^2 and Fisher's exact tests. All continuous variables were expressed as mean ± standard deviation (SD). All p-values were calculated using two-sided hypotheses; a p-value of < 0.05 was considered to be statistically significant.

Results

382 patients were selected of which 206 had RAP and 176 had CP. 251 healthy controls were identified who were either spouses of patients or unrelated individuals without a history of pancreatic disease. Patient and control characteristics, alcohol drinking patterns and smoking status are shown in the table 1.

Genotype frequencies did not vary significantly from the values predicted by the Hardy-Weinberg equilibrium (p>0.05). PDGF-β + 286 A/G and +1135 A/C polymorphisms allele

and genotype frequencies were compared among RAP patients, CP patients and controls. Polymorphism +286 G allele frequencies in RAP (38%) and CP (41%) were not significantly different in comparison to controls (38%). Polymorphism +1135 C allele frequency in all three groups was similar (30%). There was no significant difference in allele distribution for PDGF- β polymorphisms +286 A/G and +1135 A/C when compared among the three groups ($p > 0.05$). There was no significant difference in genotype frequencies for polymorphism +286 A/G or +1135 A/C among the three groups. Genotype frequencies of both polymorphisms are shown in figure 1.

To determine if the risk was modified with alcohol and smoking use we compared CP, RAP and controls in different alcohol drinking and smoking subgroups as described above. On performing the analysis in alcohol and smoking categories there appeared to be no significant difference in +286 A/G and +1135 A/C genotype frequencies among three groups. The genotype frequencies with their corresponding p-values are shown in tables 2 and 3.

We have analyzed the data by comparing patients with early stage CP (n=82) vs. advanced stage CP (n=94) using the presence of exocrine and/or endocrine insufficiency as a marker of advanced disease. The analysis did not show any differences in the distribution of +286A/G and +1135 A/C genotype frequencies between early and advanced stage CP.

Discussion

While PDGF- β signaling clearly plays an important role in PSC biology and the deposition of extracellular matrix proteins, the possibility that polymorphisms in the PDGF- β gene are associated with phenotypic transition from RAP to CP has not been explored. We tested this hypothesis by genotyping two PDGF- β polymorphisms at positions +286 (A/G) and +1135 (A/C) that were previously reported to be associated with disease traits, including hepatic fibrogenesis¹⁰. However, no significant difference in the genotype or allele frequencies among patients in our study populations with RAP, CP and controls were identified.

Activation and proliferation of PSCs with deposition of matrix proteins is initiated and driven by several inflammatory cytokines and growth factors including TGF- β 1, PDGF- β ^{9, 16, 17}. PDGF- β represents the most potent mitogen for PSCs, both PDGF- β and the PDGF- β receptor have been shown to be over expressed in human CP¹⁸. However, we are unaware of any studies that investigated the role of the PDGF- β variants in CP. In this study we investigated two PDGF- β SNPs as potential fibrosis modulators especially since these SNPs have already been reported to play role in liver fibrosis¹⁰. We predicted that PDGF- β gain-of-function variants would be enriched in CP and those with loss-of-function would be enriched in RAP, since there would be a blockade in the progression to CP. However, we did not detect any changes in RAP and CP compared to each other or the control group. Of note, four previous studies have investigated TGF- β 1 polymorphisms and their possible association with CP, with two showing statistical association and two showing no statistical association^{19–22}, illustrating the difficulty of drawing conclusions about complex genetics using simple association studies. CP is a complex disorder, with a number of gene \times gene, and gene \times environmental interactions. Excessive alcohol consumption and smoking has been identified as the etiologic risk factor for CP^{13, 23}. However fewer than 5% of heavy drinkers develop CP. Cigarette smoking has been reported to be an independent risk factor for developing CP and increasing its rate of progression^{24, 25}. It has been demonstrated that chronic alcohol consumption and smoking may be sole risk factors only in specific patient subsets whereas in others it is mainly due to interaction with several co-factors. We recently demonstrated the interaction between the calcium sensing receptor gene polymorphisms and alcohol in patients with CP²⁶. However, there seems to be no significant interaction

between alcohol and smoking risk factors and the PDGF- β gene polymorphisms in the development of RAP and CP.

This study has a number of important limitations. First, the evidence for an effect of the two SNPs that we investigated are indirect, and the studies that reported them are subject to false positive associations due to chance, followed by reporting bias. Secondly, our study is only powered to detect large effects because of the relatively small sample size and the imprecise phenotyping – including uncertainty as to which etiologies of RAP actually progress to CP and through which mechanisms. The latter concern was addressed by looking at subsets of patients classified by drinking or smoking, but this further reduces the number of patients in each group, and no trend was seen. The classification system is relatively crude, since NAPS2 is a cross-sectional study. A better phenotype would be to determine the rate of progression in patients with RAP and/or early CP. However, this approach requires a major, long term prospective study since the biomarkers of fibrosis in the pancreas are insensitive and of unknown accuracy. Third, we only investigated two SNPs. A more exhaustive investigation of both PDGF- β and the PDGF- β receptor are required to fully investigate these candidate genes. Fourth, we only investigated one population, even though it represents a cross-section of subjects throughout the United States. The possibility that these SNPs may be important in other populations (e.g. tropical pancreatitis) cannot be excluded. Thus, this study does not diminish the importance of the PDGF- β pathway in CP, but only demonstrates that the effect of the +286 A/G and +1135 A/C variants on the classification of pancreatitis patients is not strong.

In conclusion, CP remains a complex disorder with multiple contributing factors. Illucidation of the complex mechanism requires continued research in the assessment of candidate genes, development of better biomarkers of disease activity and progression, and the cooperative effort of large clinical-translational study groups. Despite the findings reported here, the PDGF- β remain an important candidate system, and further investigation is warranted.

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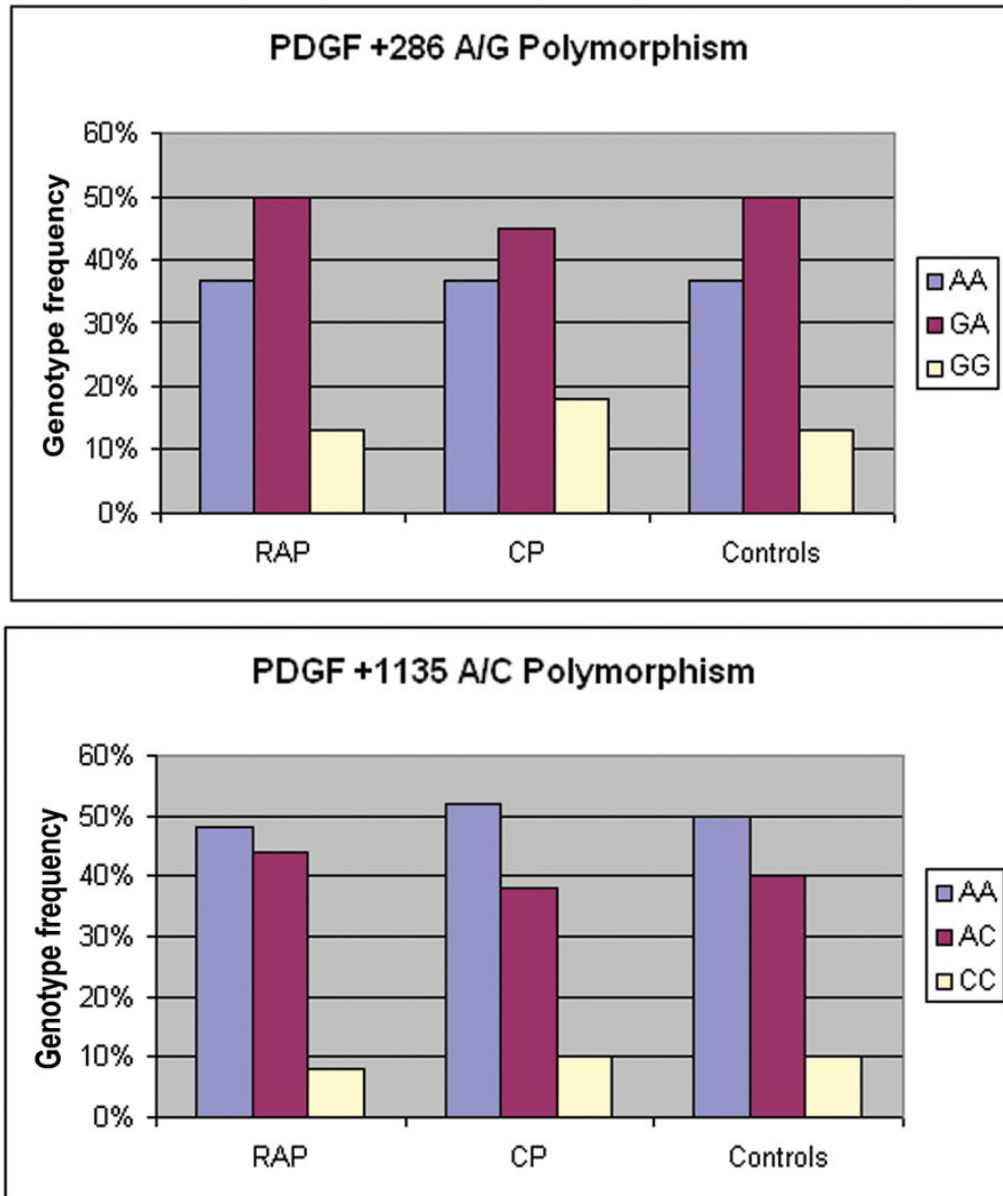


Figure 1.

Figure 1a: Genotype distribution +286 A/G polymorphism among RAP and CP patients and controls

RAP, Recurrent acute pancreatitis. CP, Chronic pancreatitis.

Figure 1b: Genotype distribution +1135 A/C polymorphism among RAP and CP patients and controls

RAP, Recurrent acute pancreatitis. CP, Chronic pancreatitis.

Table 1

Baseline characteristics of the study population

Characteristics	RAP (n=206)	CP (n=176)	Controls (n=251)
Age, Mean (Std.Dev)	44.6(16.4)	48.1(15)	56.7(12.8)
Sex, F/M	109/93	85/91	172/79
Race, White (%)	93%	86%	94%
Alcohol Drinking Categories			
Low risk	113(55%)	78(44%)	158(63%)
Moderate risk	70(34%)	62(35%)	74(29%)
High risk	22(11%)	36(20%)	9(4%)
Smoking Status			
Never	83(41%)	54(31%)	125(51%)
Past	65(32%)	39(22%)	84(34%)
Current	55(27%)	81(47%)	38(15%)

RAP, Recurrent acute pancreatitis; CP, Chronic pancreatitis.

Table 2

Genotype distribution of PDGF gene polymorphisms in RAP, CP and Controls based on alcohol drinking categories

Polymorphism + 286 A/G				
Drinking Category	RAP	CP	Controls	P Value^I
Mild				
AA	41(36%)	30(38%)	58(37%)	0.69*
AG	57(51%)	35(45%)	81(51%)	0.96**
GG	15(13%)	13(17%)	19(12%)	0.51***
Moderate				
AA	26(37%)	26(42%)	27(37%)	0.8*
AG	32(46%)	25(40%)	35(47%)	0.72**
GG	12(17%)	11(18%)	12(16%)	1***
High				
AA	9(41%)	9(25%)	2(22%)	0.14*
AG	12(54%)	19(53%)	7(78%)	0.26**
GG	1(5%)	8(22%)	0	0.58***
Polymorphism + 1135 A/C				
Low				
AA	54(48%)	42(54%)	83(52%)	0.06*
AC	52(46%)	25(32%)	61(39%)	0.37**
CC	7(6%)	11(14%)	14(9%)	0.41***
Moderate				
AA	35(50%)	29(46%)	33(45%)	0.13*
AC	25(36%)	30(48%)	33(45%)	0.51**
CC	10(14%)	3(5%)	8(10%)	0.5***
High				
AA	9(41%)	19(53%)	5(56%)	0.36*
AC	12(54%)	13(36%)	4(44%)	0.86**
CC	1(5%)	4(11%)	0	0.78***

* RAP vs CP

** RAP vs Controls

*** CP vs Controls

RAP, Recurrent acute pancreatitis; CP, Chronic pancreatitis;

^I Fisher exact test

Table 3

Genotype distribution of PDGF gene polymorphisms in RAP, CP and Controls based on smoking categories.

Polymorphism +286 A/G				
Smoking in Pack Years	RAP	CP	Controls	P Value^I
<12				
AA	14(39%)	12(32%)	11(33%)	0.52*
AG	18(50%)	17(46%)	14(42%)	0.41**
GG	4(11%)	8(22%)	8(24%)	0.95***
12 to 35				
AA	9(24%)	10(29%)	18(43%)	0.95*
AG	20(54%)	17(48%)	20(48%)	0.14**
GG	8(22%)	8(23%)	4(9%)	0.21***
>35				
AA	15(50%)	16(43%)	5(26%)	0.75*
GA	12(40%)	15(41%)	12(63%)	0.27**
GG	3(10%)	6(16%)	2(11%)	0.29***
Polymorphism +1135 A/C				
<12				
AA	22(61%)	13(35%)	16(48%)	0.1*
AC	12(33%)	21(57%)	10(30%)	0.11**
CC	2(6%)	3(8%)	7(21%)	0.06***
12 to 35				
AA	13(35%)	17(49%)	23(55%)	0.49*
AC	20(54%)	14(40%)	16(38%)	0.24**
CC	4(11%)	4(11%)	3(7%)	0.79***
>35				
AA	18(60%)	23(62%)	9(47%)	1*
AC	9(30%)	11(30%)	9(47%)	0.54**
CC	3(10%)	3(8%)	1(6%)	0.45***

* RAP vs CP

** RAP vs Controls

*** CP vs Controls

RAP, Recurrent acute pancreatitis; CP, Chronic pancreatitis;

^I Fisher exact test