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The -251 A/T Polymorphism in the *IL8* Promoter is a Risk Factor for Acute Pancreatitis

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Abstract

Objectives—Inflammation in the setting of acute pancreatitis is partially driven by pathogen recognition receptors that recognize damage-associated molecular patterns. IL-8 is a chemotactic factor produced by pathogen recognition receptor expressing cells. A single-nucleotide polymorphism in IL8 promoter region (-251 A/T) has been implicated in inflammatory diseases. We examined whether this IL-8 polymorphism confers susceptibility to AP.

Methods—Patients with AP (n = 357) were prospectively recruited. Clinical data and blood were collected in subjects and controls (n = 347). Severity was defined following the Revised Atlanta Classification. Genotypes were assessed by quantitative PCR using TaqMan probes.

Results—Patients and controls had similar demographics had no difference in Hardy-Weinberg (patients $P = 0.29$, controls $P = 0.66$). Twenty-five percent of patients developed severe AP. Compared to controls, the A/A genotype was more common in AP ($P = 0.041$; odds ratio, 1.42; 95% confidence interval, 1-1.99). Obese patients with the A/A genotype were more likely to develop mild acute pancreatitis ($P = 0.047$).

Conclusions—The -251 polymorphism confer susceptibility to AP and disease severity in obese patients. However, its effect is moderate. One potential mechanism for this susceptibility is via increased IL-8 production by innate cells, with subsequent enhanced neutrophil influx and pancreatic injury.

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Introduction

Acute pancreatitis (AP) is a common and potentially life-threatening condition resulting from pancreatic parenchymal inflammation. In the United States, AP is the leading cause of gastrointestinal related hospital admissions.¹ The high incidence of AP underlies the economic burden in terms of high cost of care and lost productivity. Most cases of AP are mild, characterized histologically by glandular edema with neutrophil infiltrates, and are associated with rapid clinical recovery. A significant fraction of patients, however, suffers a severe course associated with systemic inflammatory response (SIR) and organ failure with high morbidity and mortality.²

AP is instigated by pancreatic injury and architectural distortion, resulting in the release of sequestered zymogens into interstitial spaces. The injury induces a poorly understood inflammatory cascade with endothelial injury, vascular leak, and bacterial translocation.³ This can manifest with severe complications including pancreatic necrosis, systemic inflammation, and organ failure with their attendant mortality.

Immune cells are critical components of inflammation in AP. Innate cells, such as neutrophils, express pathogen recognition receptors (PRRs) that recognize pathogen associated-molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). Upon ligand binding, PRRs activate cascades culminating in the production of prototypical inflammatory cytokines such as IL-1 β , IL-6, and TNF α .⁴⁻⁶ Bacterial translocation and parenchymal injury in AP result in exposure of both PAMPs and DAMPs, resulting in a perpetual cycle of injury.

In this regard, interleukin-8 (IL-8) is a powerful chemotactic factor for neutrophils, and is produced by PRR expressing cells. Consistent with the role of IL-8 in inflammation, a functional single-nucleotide polymorphism (SNP) (rs4073) in the IL-8 promoter (-251 A/T) has been implicated in inflammatory conditions including cancer, periodontitis, and *Helicobacter pylori* gastritis.⁷⁻⁹

The burden of AP is well-known; however, the mechanisms that perpetuate AP are incompletely understood. There are, to-date, a very limited number of studies that have reproducibly identified risk alleles for AP. The published studies have suffered from low patient numbers, they are typically retrospective, and generally do not contain detailed data regarding clinical outcomes. Therefore, the limited available data has been mixed and poorly reproducible.

The cohort of patients with acute pancreatitis at the University of Pittsburgh Medical Center is well characterized clinically and has been extensively published. This cohort is ongoing, and is prospectively enrolled with associated serum samples to obtain biochemical data. Given the paucity of genetic studies in AP, we utilized this clinically well characterized cohort to perform a candidate gene study to determine whether this SNP confers susceptibility to, or modulate the severity of, AP. We additionally assessed the gene-environment interactions by examining the role of obesity, a known important risk factor for severe pancreatitis, in risk and severity in the setting of AP in the setting of IL-8 polymorphisms.^{10,11}

Materials and Methods

Subjects

Prospectively recruited patients and control subjects provided written informed consent prior to study enrollment. The institutional review board of the University of Pittsburgh approved this study. The diagnosis of AP was based on the presence of at least two of three characteristic clinical, biochemical, and/or radiographic criteria, and was in accordance with the standard clinical definition. Inclusion criteria were (1) any patient admitted to the University of Pittsburgh Medical Center (UPMC) with the above criteria older than 18 years, (2) ability to give informed written consent and admitted within 7 days of onset of pain. Patients were excluded if (1) they presented greater than 7 days from the onset of pain, (2) active malignancy and (3) inability to provide written informed consent. We restricted included subjects to the 7-day cutoff as we wanted to capture 'early' rather than the 'late' phase of AP. At the end of their hospitalization, patients were classified clinically as having either mild (MAP), moderate (MoAP), or severe AP (SAP). The definitions were based in the Revised Atlanta Classification,¹² with SAP defined as development of persistent organ failure (OF). Organ failure was defined by the modified Marshall score involving the cardiovascular, respiratory, and/or renal organ systems. Patients were considered to have persistent single OF if they met one of the latter criteria for ≥ 48 hrs, and multi-OF if they met ≥ 2 criteria. Control subjects were recruited from the hospital and clinics at UPMC and included spouses and unrelated healthy subjects. Control subjects were excluded if they were (1) < 18 yrs old, (2) could not provide informed written consent, had either (3) active malignancy, (4) infection or (5) autoimmune conditions.

DNA Isolation and Genotyping Protocols

Whole blood was obtained at enrollment. Samples were placed in EDTA-tubes, and underwent diffusion centrifugation to isolate lymphocytes. Cells were isolated from the appropriate layer of the gradient centrifugation and then lysed for DNA extraction. DNA was isolated and processed using the FlexiGene DNA kit (Qiagen, Valencia, Calif) per kit instructions. The DNA was quantitated using a spectrophotometer (Nanodrop, Thermo Scientific, Waltham, Mass). The amount of DNA varied but was generally between 12-15 ng and was within range (1-20 ng) specified by the SNP genotyping assay kit. Primer pair was designed to amplify a 275 bp region of IL-8 promoter containing -251 A/T polymorphism (TTATCTAGAAATAAAAAAGCATACA[A/T] TTGATAATTCACCAAAT – TGTGGAGCTTATCTAGAAATAAAAAA). Polymerase chain reaction (PCR) amplification was performed per SNP Genotyping Assay kit instructions (Life Technologies, Grand Island, NY). The samples were then processed using the Taqman assay (primer C_11748116_10) (Life Technologies). Fluorescence was measured using a commercially available PCR system (AB 7900HT, Applied Biosystems, Life Technologies).

Statistical Analysis

Genotype frequencies were calculated and tested for adherence to Hardy-Weinberg equilibrium using a chi-square test with one degree of freedom. Comparison between genotype frequencies was made using Armitage trend tests. The chi-square test was applied to compare genotype frequencies, and determine odds ratios (ORs) and 95% confidence

intervals (CIs) between groups. The alpha of 0.05 was considered statistically significant ($P < 0.05$). Continuous variables were analyzed using Student's *t*-test or ANOVA (in instances where there were more than two groups to be analyzed). All statistical analyses were performed using prism (GraphPad, San Diego, Calif).

Results

Patient Demographics and Outcomes

Three hundred fifty-seven patients were recruited between 2003 and 2014 (mean age 52 years, 50% females, mean body mass index [BMI] 29 kg/m²). In addition, blood from 347 healthy control subjects without a history of AP was also analyzed (mean age 54 years, 61% females, mean BMI 28 kg/m²). When comparing patients and controls, age and BMI were similar; however, controls had more females ($P < 0.0001$).

The majority of patients had MAP ($n = 192$, 54%), followed by MoAP ($n = 77$, 21.5%), and SAP ($n = 88$, 24.5%) (Table 1). Patients with SAP were more likely to be male ($P < 0.0001$) and have a higher BMI ($P = 0.002$), which is consistent with prior publications. Biliary pancreatitis was the most common etiology among all patients (40%), followed by idiopathic (19%) (Table 1). Patients with SAP were enriched for biliary or hypertriglyceride-induced AP ($P < 0.003$), while MAP was associated with idiopathic and post-ERCP AP (Table 1).

As expected, patients with SAP had worse outcome compared to those with MoAP and MAP (Table 2). Length of stay was longer in patients with SAP compared to those with MoAP and MAP (37, 17, and 6 days, respectively; $P < 0.0001$), as was the likelihood of ICU admission (92%, 26%, 6%; $P < 0.0001$). Patients with SAP were more likely to have multi-organ failure (MOF) than single organ failure (SOF) (59% vs. 41%). Finally, SAP course was fatal in 17% (Table 2).

IL-8 Genotypes

The frequencies of IL-8 A/A and T/T homozygotes, and A/T heterozygotes were determined in controls and patients with AP (Table 3). There were no significant differences in the Hardy-Weinberg equilibrium in patients ($P = 0.29$) or controls ($P = 0.66$).

Overall, the IL-8 genotype was found to affect susceptibility to AP. Patients with AP exhibited a significantly higher proportion of the A/A genotype compared to controls (30 vs. 23%; OR, 1.42; 95% CI, 1.00-1.99; $P = 0.041$) (Table 3). We found no statistically significant difference in the frequency of the A/A genotype compared to the T-allele between groups when stratified by severity (MAP: 31% vs 69%; MoAP: 29% vs 71%; SAP: 30% vs 70%; $P = 0.58$; OR, 1.19; 95% CI, 0.68-2.06). Collectively, this data demonstrates that the A/A genotype enhances susceptibility to AP, but does not impact severity of disease.

Impact of BMI

Obesity is an important modifier of the severity of AP.¹⁰ In order to examine the interaction between BMI and genotype (gene-environment interactions), we examined the allele frequency as a function of BMI (BMI ≥ 30 kg/m² vs. <30). Overall, 40% of patients were

obese (BMI ≥ 30) compared to 30% of controls ($P = 0.004$). Consistent with published data, 61% of patients with SAP were obese (vs. 32% with MAP and 36% with MoAP). Conversely, 38% of obese patients had SAP (Table 4). The A/A genotype was more common in obese patients compared to obese controls (37% vs. 28%), but this trend did not reach significance ($P = 0.14$). However, we found a higher proportion of obese patients with A/A genotype developed MAP ($P = 0.047$) (Table 4).

Discussion

Our study demonstrates that the -251 A/A genotype (rs 4072) in the *IL8* promoter predicts susceptibility to AP, and is less common in severe disease in obese patients. Similar to risk alleles in inflammatory bowel disease (IBD),¹³ the effect of -251 A/A genotype was only moderate (OR, 1.42). Collectively, these data imply that IL-8 may be involved in the pathogenic mechanism of AP; however, as with genetically complex traits, it does not carry all the risk.

The cytokine IL-8, also known as neutrophil chemotactic factor, is produced by PRR expressing cells including immune cells, mucosal epithelial cells (intestinal and airway cells), and endothelial cells. IL-8 is typically produced in sparing quantities at baseline, but is rapidly up-regulated by sentinel cells upon PRR ligand binding.¹⁴ Functionally, IL-8 potently induces the migration of immune cells including neutrophils, macrophages, and T-cells. In the context of host immunity, the physiologic role of IL-8 is therefore to promote chemotaxis of effector immune cells and mediate pathogen clearance. The -251 A/A SNP is likely a gain-of-function genotype. Macrophages from patients bearing this genotype exhibit enhanced IL-8 to PRR stimulation *in vitro* suggesting that the A/A genotype lowers the threshold for IL-8 synthesis.¹⁵

Collectively, the *in vivo* function of IL-8 coupled with the *in vitro* data on patients with the -251 A/A genotype permit a testable hypothesis for AP susceptibility. Namely, pathogen- and damage-associated molecular patterns exposed by pancreatic inflammation result in enhanced IL-8 cytokine release by PRR expressing cells, culminating in excessive inflammatory cell infiltrates and pro-inflammatory cytokine production. Thus, patients harboring the A/A genotype are more susceptible to AP, whereas its absence permits more rapid homeostasis after injury. This hypothesis is further supported by the results of studies documenting elevated serum IL-8 in patients with AP.^{16,17} Most importantly, this putative mechanism is testable in animal models, which could provide insight into disease mechanism, and ultimately, therapeutics.

The modern era of genetic analysis has yielded extensive insight into the genetics and mechanism of GI conditions, including IBD, liver disease, and hereditary and chronic pancreatitis.^{13,18,19} In contrast, the data in AP is both limited, and suffers from considerable variability, precluding uniform interpretations.^{20,21} In this regard, SNPs in *IL8* have been assessed in AP in two prior publications^{22,23}. In contrast to our findings, there was no association between the -251 A/A genotype and susceptibility in either study, although one study did find it affecting severity.²² However, those studies collectively recruited fewer patients (n=100 and 91, respectively) than presented here (n = 357).

Thus far, excepting our data, the *MYO9B* gene has recently been identified as a risk for AP in a large study. *MYO9B* encodes an actin-based motor heavy chain termed myosin IXB.²⁴ Myosin IXB protein is involved in regulation of tight junctions. Susceptibility to AP due to *MYO9B* is therefore presumably a consequence of abnormal intestinal and/or pancreatic epithelial cell permeability. Two genes, *MCP1* and *TNFA*, have been variably associated with AP severity depending on the population under-study.^{11,23,24} Collectively, larger scale studies are likely needed to clarify whether the variability in the data reflects true population heterogeneity, or is a consequence of underpowered data sets.

Gene-environment interactions are critically important in the manifestation of genetically complex traits. In contrast to high penetrance alleles, disease expression in complex traits is a consequence of exposure to environmental risk factor(s) in genetically predisposed individual(s). The rise of GWAS studies has accelerated the appreciation for gene-environment interactions, as the risk conferred by genetic susceptibility is generally low.¹³ We attempted to combine a known environmental risk (obesity) with this SNP and assess whether obesity potentiates the -251 A/A effect. However, we found that both obese patients and controls had a higher proportion of the A/A genotype compared to non-obese counterparts. The lack of significance in the obese group is most likely to be a consequence of the increased incidence of the A/A genotype in obese controls compared to non-obese controls (28% vs. 21%).

Acute pancreatitis continues to have a large economic burden. Despite recent advances in clinical management of the complications of AP, significant proportions of patients progress to organ failure with high morbidity and mortality. Genetic studies offer the possibility of identifying pathophysiologic pathways that may be targets of medical therapy. Collectively, our study, which is strengthened by the large number of patients, demonstrates that the -251 A/A allele confer susceptibility to AP. Furthermore, it is consistent with prior publications associating the -251 A/A genotype with inflammatory conditions.

Our data combined with the *in vivo* biology of IL-8 suggest that the moderate susceptibility conferred by the A/A genotype may be mediated by enhanced effector cell infiltration and pro-inflammatory cytokine production, and cumulatively provides the clinical basis to investigate this mechanism in translational studies in humans and murine models.

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Table 1
Patient Demographics and Clinical Outcome

	Mild AP (n = 192)	Moderate AP (n = 77)	Severe AP (n = 88)	All AP (n = 357)
Age, mean (SD), y	50 (19)	52 (20)	55 (17)	52 (19)
Sex, male, %	42	51	67 [*]	50
BMI, mean (SD), kg/m ²	28 (7)	29 (7)	32 (6) [†]	29 (7)
Etiology, n (%)				
Biliary	76 (40)	28 (36)	39 (44)	143 (40)
Alcohol	16 (8)	14 (18)	15 (17)	45 (13)
Idiopathic	39 (20)	15 (19)	13 (15)	67 (19)
Post-ERCP	38 (20)	10 (13)	4 (5)	52 (15)
Elevated triglycerides	6 (3)	7 (9)	10 (11)	23 (6)
Other	17 (9)	3 (4)	7 (8)	27 (8)

^{*} $P < 0.0001$ comparisons are between severe AP and all AP (excluding severe AP patients).

[†] $P = 0.002$ comparisons are between severe AP and all AP (excluding severe AP patients).

BMI indicates body mass index; ERCP, endoscopic retrograde cholangio-pancreatography.

Table 2
Outcome of AP

	Mild AP	Moderate AP	Severe AP	All AP
Outcome				
LOS, mean (SD), d	6 (3)	17 (18)	37 (26) *	15 (19)
ICU Admission, n (%)	12 (6)	20 (26)	81 (92) *	113 (32)
Necrotizing pancreatitis, n (%)	0 (0)	65 (84)	67 (76)	312 (37)
Organ Failure				
Transient OF, n (%)	0 (0)	9 (12)	0 (0)	9 (3)
Persistent OF, n (%)	0 (0)	0 (0)	88 (100)	88 (25)
SOF, n (%)	0 (0)	9 (12)	36 (41)	45 (13)
MOF, n (%)	0 (0)	0 (0)	52 (59)	54 (15)
Death, n (%)	0 (0)	0 (0)	15 (17)	15 (4)

* $P < 0.0001$; comparisons are between severe AP and all AP (excluding severe AP).

SOF indicates single organ failure; MOF, multisystem organ failure; ICU intensive care unit; LOS, length of stay; necrotizing pancreatitis is defined as pancreatic and/or peri-pancreatic necrosis

Table 3**Genotypes of AP Patients vs. Controls**

Genotypes	Mild AP	Moderate AP	Severe AP	AP (n = 357)	Controls (n = 347)
A/A	60	22	26	108 (30%)*	81 (23%)
A/T	82	39	41	162 (45%)	169 (49%)
T/T	50	16	21	87 (25%)	97 (28%)
A allele	142	61	67	270 (75%)	250 (72%)
T allele	132	55	62	249 (70%)*	266 (77%)

* $P=0.041$ for AA vs. T allele

Table 4

Genotypes Stratified by BMI

BMI < 30					
Genotype	MAP (n = 131)	MoAP (n = 49)	SAP (n = 34)	AP (n = 214)*	Controls (n = 244)*
A/A	32 (24)	14 (29)	9 (26)	55 (26) [†]	52 (21)
A/T	60 (46)	24 (29)	19 (56)	103 (48)	124 (51)
T/T	39 (30)	11 (22)	6 (18)	56 (26)	68 (28)
A allele	92 (70)	38 (78)	28 (82)	158 (74)	176 (72)
T allele	99 (76)	35 (71)	25 (74)	159 (74) [†]	192 (79)
BMI 30					
Genotype	MAP (n = 61)	MoAP (n = 28)	SAP (n = 54)	AP (n = 143)*	Controls (n = 103)*
A/A	28 (46)	8 (29)	17 (31)	53 (37) [‡]	29 (28)
A/T	22 (36)	15 (54)	22 (41)	59 (41)	45 (44)
T/T	11 (18)	5 (18)	15 (28)	31 (22)	29 (28)
A allele	50 (82)	23 (82)	39 (72)	112 (78)	74 (72)
T allele	33 (54)	54 (71)	37 (69)	90 (63) [‡]	74 (72)

* $P = 0.004$; comparisons are between the ratio of obese- to non-obese subjects within AP and control (chi-square test).

[†] $P = 0.004$; comparisons are between AP and controls

[‡] $P = 0.047$; comparisons are between AP and controls

Data expressed as n (%).