Relationship between nucleotide-binding oligomerization domain-containing protein 2 variants and severity of acute pancreatitis

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Abstract

Background and aim : **Intestinal barrier dysfunction has been implicated in the development of infectious complications of acute pancreatitis. Nucleotide-Binding Oligomerization Domain-Containing Protein 2 (NOD2) plays an important role in the proper functioning of intestinal defense mechanisms. Here, we investigated the frequency of NOD2 variants in patients with mild and severe acute pancreatitis.**

Materials and Methods : **Groups 1, 2 and 3 comprised healthy participants and patients with mild and severe pancreatitis, respectively. Four NOD2 variants and serum interleukin-6 (IL-6), Tumor Necrosis Factor-a (TNF-a) and lipopolysaccharide-binding protein (LBP) levels were analyzed.**

Results : **Three patients (3/32, 9.4%) in the severe pancreatitis group were positive for the p.R702W variant. This variant was negative in other groups. One, three and three patients in the healthy (1/27, 3.7%), mild (3/36, 8.3%) and severe pancreatitis (3/32, 9.4%) groups tested positive for the 1007fs variant, respectively. No significant differences in the frequencies of NOD2 variants were evident among the groups. Serum IL-6, TNF-a and LBP levels were markedly higher in the severe pancreatitis than the healthy and mild pancreatitis groups (all p<0.001). We observed no significant correlation between cytokine levels and NOD2 variants.**

Conclusion : **Our results support an association between the presence of the p.R702W variant and severe pancreatitis.** (Acta gastroenterol. belg., **2019**, 82, **285-290**).

Keywords : Mucosal immunity, inflammation, cytokines, acute pancreatitis.

Introduction

Despite significant improvements in treatment options, acute pancreatitis (AP) remains an important medical problem owing to high morbidity and mortality. Infectious complications are the main cause of mortality in severe acute pancreatitis (SAP) (1). The majority of infections in AP are of intestinal origin (2). High endotoxin levels derived from gram-negative enteric bacteria have been detected in patients with severe acute pancreatitis, even on the day of admission to hospital. In addition, a significant correlation is reported between endotoxemia and increased mortality rate in these patients (3), suggesting that bacterial translocation plays a role in the development of infectious complications in AP.

Increased intestinal permeability, motility and host immunity are major factors associated with the development of bacterial translocation (BT) in AP (4-6). Nucleotide oligomerization domain 2 (NOD2) is an important intracellular receptor involved in host defense (7). NOD2 is a NOD-like receptor family member that senses and responds to bacterial wall peptides (8). The protein facilitates balance between the host immune system and intestinal flora elements (9), regulates the secretion of several anti-bacterial compounds, augments intracellular bacterial killing (10), and induces a variety of cytokines and chemokines by activating NF-κB and mitogen-activate protein kinases (MAPK) (11). Today, multipl severity scoring systems (Ranson score, APACHE II, and etc.) and serum markers (C-reactive protein, procalcitonin, interleukins, and etc.) have been used to help clinicians in triaging AP patients and predicting their prognosis (12). However, these scoring systems and serum markers cannot sufficiently predict the prognosis or severity of AP. Recent reports indicate that NOD2 variants present a genetic risk factor for infectious complications in cirrhotic patients (13, 14). At the present time, although NOD2 is not used to predict the severity and prognosis of AP, limited studies have focused on the effects of NOD2 variants on the course of acute pancreatitis in humans. Guenther *et al*. (15) reported that a NOD2 variant (p.R702W) is associated with multiple organ failure and mortality in patients with acute pancreatitis.

In the current study, we investigated the potential correlation between NOD2 variants and severity of acute pancreatitis.

Materials and methods

Patients

Patients with acute pancreatitis admitted to the Gastroenterology department of Turgut Ozal Medical Center between May 2015 and August 2016 were included in the study, irrespective of etiology. Acute pancreatitis was defined based on a combination of

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clinical, biochemical and radiological findings. Adult patients over 18 years of age were included. The ethics committee of Inonu University Medical Faculty approved our study.

Determination of patient numbers in groups

Prior to commencement of the study, we established the patient numbers for groups based on the frequencies of NOD2 risk variants reported previously. In the power analysis, alpha=0.05 1-beta (power) 0.80 was applied based on a 20% change in NOD2 frequency in patients with acute pancreatitis. Accordingly, we calculated that each group should contain at least 26 subjects. Experiments were performed on three groups : Group 1 (n=27) containing healthy participants, Groups 2 (n=36) and 3 (n=32) with mild and severe pancreatitis patients according to the Atlanta 2012 classification (16). Patients with severe pancreatitis had persistent single or multiple organ failure (>48 hours). Patients with moderately severe pancreatitis were not included in the study, as they might make it difficult to interpret the results of our work. In total, 95 individuals were included. All patients provided informed consent to participate in the study.

Sample Collection

In our study, blood samples from patients with mild acute pancreatitis were obtained between 0 and 3 days after the onset of illness. Blood of patients with severe acute pancreatitis was taken between 3 and 10 days after the onset of illness. Serum amylase, lipase, creatinin, bilirubin, and liver function data were obtained via routine laboratory analyses. Venous blood samples were collected in bottles containing 9 mL EDTA for analysis of NOD2 risk variants. For cytokine analysis, blood samples were centrifuged (3500 *g* for 10 min), and the supernatant fractions (serum) collected and aliquoted. Samples were subjected to the relevant biochemical analyses or stored at -70°C until required. In all groups, four NOD2 risk variants and serum IL-6, TNF-a and LBP levels were examined.

Molecular Methods

Detection of the NOD2 Gene Mutations

A real-time PCR method developed by Appenrodt et al. (13) was employed to analyze the most frequently detected NOD2 variants, p.R702W, p.G908R, and c.3020insC, in the samples. In addition, 1007fs variant used by Bruns et al. (17) was included for analysis. The primers and probes used to detect NOD2 gene mutations are presented in the Table 1.

The Quantitect Probe RT-PCR kit (Lot no. 154027285, Qiagen, Hilden, Germany) was used for real-time PCR analysis. Each reaction contained the appropriate primer (0.4 µM) and probe (0.2 µM). Amplification was performed in a Rotor-Gene Q Real device (Qiagen, Hilden, Germany) under the following conditions : initial denaturation at 95^{0}C for 15 min followed by denaturation at 94 ^{0}C for 15 sec for 40 cycles and annealing (fluorescent measurements) at 60^{0}C for 1 min as two steps. Mutations in the samples positive for NOD2 variant(s) were confirmed with DNA sequencing with ABI PRISM 310 device (Applied Biosystems, USA).

Sequencing

Primers targeting the mutated regions were generated. Mutations were confirmed by sequencing of amplified DNA regions via PCR using the BigDye Terminator 3.1

Table 1. — **Primer and probe sequences for detection of NOD2 variants**

Gene polymorphism	Primer and probe sequences		Reference
p.R702W	F	5'-CTGAGTGCCAGACATCTGAGAAG-3'	Appenrodt et al [12]
	R	5'-GCTGCGGGCCAGACA-3'	
	P1	VIC-CCTGCTCTGGCGCC-3'	
	P2	FAM-CTGCTCCGGCGCC-3'	
p.G908R	F	5'-TGATCACCCAAGGCTTCAGC-3'	Appenrodt et al [12]
	R	5'-GAACACATATCAGGTACTCA CTGACAC-3'	
	P1	VIC-ACTCTGTTGCGCCAGA	
	P2	FAM-CTGTTGCCCCAGAAT	
c.3020insC	F	5'-CCAGGTTGTCCAATAACTGCATC-3'	Appenrodt et al [12]
	R	5'-RCCTTACCAGACTTCCA GGATGGT-3'	
	P1	VIC-TGCAGGCCCCTTG	
	P2	FAM-CTGCAGGCCCTTG	
1007fs	F	5'-GTCCAA TAACTGCATCACCTACCTAG-3'	Bruns et al [16]
	R	5'-CTTAC CAGACTTCCAGGATGGTGT-3'	
	P1	FAM-CCCTCCTGCAGGCCCTTGAAAT	
	P2	VIC-CCTC CTGCAGGCCCCTTGAAA	

kit (Applied Biosystems, USA) and the ABI PRISM 310 (Applied Biosystems, USA) automatic base sequencing device.

Serum interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and lipopolysaccharide-binding protein (LBP) levels

Serum levels of LBP, IL-6, and TNF-α were studied using ELISA in a Basic RADIM Immunoassay Operator (BRIO ; Radim spa, Pomezia, Italy) device. The following ELISA kits used were : LPB : Catalog Number 201-12-0361 ; Sunred Biological Technology, Shanghai, China ; sensitivity : 0.135 ng/mL, normal range 0.2-60 µg/mL, IL-6 : Catalog Number 201-12-0091 ; Sunred Biological Technology, Shanghai, China ; sensitivity : 2.112 ng/mL, normal range 3-600 ng/mL, TNF-α : Catalog Number 201.12.2047 ; Sunred Biological Technology, Shanghai, China ; sensitivity : 0.153 ng/mL, normal range 0.2-60 ng/mL.

Statistical Analysis

We employed Fisher's Exact and One-Way Anova tests for comparison between groups. Tukey's test was used for post-hoc analysis. In all evaluations, $p<0.05$ was considered statistically significant.

Table 2. — **Gender frequencies and median age according to group**

Groups	Male (%)	Median age (± SD)
Healthy (n=27)	40	34.59 ± 8.23
Mild AP (n=36)	36	61.42±16.73
Severe AP (n=32)	56	59.58±21.06
Total (n=95)	44	53.11±20.19

AP : Acute pancreatitis.

Results

Patient Characteristics

The gender frequencies and mean ages of patients according to groups are presented in Table 2.

NOD2 Risk Variants

Two mutations (p.G908R and c.3020insC) were absent in all three groups. Table 3 shows the frequencies of other NOD2 risk variants. We detected the p.R702W variant in three patients (3/32, 9.4%) from the severe pancreatitis group only. The 1007fs variant was present in one, three and three patients from the healthy control (1/27, 3.7%), mild (3/36, 8.3%) and severe pancreatitis (3/32, 9.4%) groups, respectively. We observed no significant differences in the frequency of p.R702W and 1007fs variants between the mild and severe pancreatitis groups.

Serum IL-6, TNF-a and LBP levels

Serum IL-6, TNF-a and LBP levels were significantly higher in the severe pancreatitis than the healthy and mild pancreatitis groups ($p<0.001$) (Table 4). Our data revealed no significant differences in serum IL-6, TNF-α and LBP levels between patients with and without NOD2 variants.

Discussion

In this study, we observed no statistically significant differences in the frequency of NOD2 variants between groups. However, the frequency of the p.R702W variant in patients with severe pancreatitis was higher than that in patients with mild pancreatitis. Moreover, serum IL-6, TNF-α and LBP levels in patients with severe pancreatitis

Table 3. — **Frequencies of NOD2 risk variants**

Groups	Number of p.R702W variants (%)	Number of 1007fs variants (%)	Number of all examined NOD2 variants (%)
Healthy (n=27)	0 (0%)	1 (3.7%)	1 (3.7%)
Mild AP (n=36)	0 (0%)	3 (8.3%)	3 (8.3%)
Severe AP (n=32)	3 (9.4%)	3 (9.4%)	5* (15.6%)
Total (n=95)	3 (3.2%)	7 (7.4%)	9* (9.5%)

* One patient had both p.R702W and 1007fs variants. AP : Acute pancreatitis.

Table 4. — **Mean of serum IL-6, TNF-a and LBP levels stratified according to group**

Groups	IL-6 (ng/mL)	TNF-a (ng/mL)	LBP (µg/mL)
Healthy (n=27)	3.17±2.22	7.16 ±5.18	8.43±6.37
Mild AP (n=36)	3.64±2.58	8.46±5.91	9.64±7.19
Severe AP (n=32)	6.61±2.57[a, b]	15.17±5.99[a, b]	18.85±8.83[a,b]
Lacking NOD2 variants (n=86)	4.33±2.85	9.87±6.64	11.84±8.70
p.R702W and 1007fs variants (n=9)	2.85±1.87	7.12±4.14	7.73±4.00

[a] $p<0.001$ compared to control, [b] $p<0.001$ compared to the mild pancreatitis group. AP: Acute pancreatitis

were significantly higher than those in patients with mild pancreatitis.

While the mortality rate associated with mild acute pancreatitis is nearly 0%, this can increase up to 80% in cases of severe pancreatitis despite intensive treatment (16). At present, no reliable markers are available that can be effectively used to estimate cases that potentially progress to severe pancreatitis. The existence of a reliable predictor may aid in the identification of severe acute pancreatitis cases and consequently reduce mortality rates by preventing the development of serious complications with the use of appropriate measures. Bacterial translocation plays a major role in the development of infectious complications in AP (2). The mechanisms underlying bacterial translocation in severe AP are also common in critical illnesses, such as sepsis, liver diseases, and burns (14, 18, 19), including failure of the intestinal barrier, together with bacterial overgrowth due to intestinal motility changes and impaired immunity (20). NOD2 is a cytoplasmic protein that binds muramyl dipeptide (MDP), a common peptidoglycan motif of gram-positive and -negative bacteria, leading to NF-κB activation and cytokine (TNF-alpha and interleukin-6) induction (21). NOD2 has been shown to play an important role in maintaining gut homeostasis (22). Variants of this protein may present a genetic risk factor in the development of infectious complications in cirrhotic patients (13,14). Similarly, NOD2 variants have been shown to confer susceptibility to several chronic inflammatory disorders, such as Crohn's disease, Blau syndrome and early-onset sarcoidosis (23), suggesting a key role in the pathogenesis of bacterial translocation. In this context, we aimed to determine whether NOD variants function in the pathogenesis of severe acute pancreatitis. We examined four NOD2 variants (p. R702W, p.G908R, c.3020insC and 1007fs) in healthy subjects and patients with pancreatitis. Two of the variants (p.G908R and c.3020insC) were not observed in any of the groups while the p.R702W variant was detected in three patients (3/32, 9.4%) from the severe pancreatitis group only. Interestingly, the 1007fs variant was detected in three, three and one patients in mild (3/36, 8.3%) and severe pancreatitis (3/32, 9.4%) and healthy groups (1/27, 3.7%), respectively. p.R702W and 1007fs variants were associated with severe pancreatitis (both identified in one patient). No significant differences in p.R702W and 1007fs variants were evident between the mild and severe pancreatitis groups. Although the frequency of NOD variants was not markedly different between groups, the incidence in patients with severe pancreatitis (5/32, 15.6%) was higher than that in patients with mild pancreatitis (3/36, 8.3%) and healthy subjects (1/27, 3.7%). Guenther et al. (15) reported that the p.R702W mutation is associated with multiple organ failure and mortality in large patient populations from Europe and USA with acute pancreatitis. While we observed no significant differences between groups, the frequency of this variant in the severe pancreatitis group was higher than that in patients with mild pancreatitis (9.4% versus 0%). This finding supports a strong relationship between the presence of the p.R702W variant and severe pancreatitis, consistent with the data of Guenther.

Several NOD2 variants have been identified to date. Large geographic fluctuations of NOD2 variants have also been observed in different populations (24, 25), with differing frequencies among countries (26, 27). To our knowledge, no studies have focused on the frequency of NOD2 variants in patients with acute pancreatitis in Turkey. Therefore, ethnicity may have an effect on our results.

Local recruitment and activation of inflammatory cells in acute pancreatitis lead to release of proinflammatory cytokines, including interleukin-6 and TNF-α, that play a pivotal role in the pathogenesis of SAP and acute pancreatitis-related distant organ failure (28-30). In our study, serum IL-6 and TNF-α levels in patients with severe AP were significantly higher than those in patients with mild AP. Earlier, Malstrom et al. (28) reported higher IL-6 and TNF-α levels in patients who developed renal, respiratory, and circulatory failure in acute pancreatitis. Our results support previous findings on elevated cytokine levels (IL-6 and TNF-α) in severe pancreatitis. NOD2 is a cytoplasmic protein that promotes nuclear factor (NF)-kB activation and cytokine induction (31). We observed no significant differences in IL-6 and TNF-α levels between patients with and without NOD2 variants. Qian et al. (7) investigated NOD2 expression in a rat model of acute pancreatitis. They reported that NOD2 expression in acute pancreatitis is correlated negatively with that of TNF-α. NOD2 variants have been shown to affect the production of TNF-α and interleukin proteins in Crohn's disease and cirrhosis (14, 32-35). The relationship between cytokine levels and NOD2 variants in patients with acute pancreatitis has not been documented in the literature. Although our findings suggest no significant relationship between these cytokines and NOD2 variants, it is difficult to reach an informed conclusion solely based on these data, owing to several study limitations. Firstly, the number of patients was relatively small. Secondly, we could not detect a significant association between NOD2 variants and severe pancreatitis, except for the p.R702W variant. Thirdly, at least 30 NOD2 risk variants have been identified in different ethnicities (24), among which we only focused on four well-known variants. Further studies on a larger selection of NOD2 variants in patients with acute pancreatitis are warranted to investigate the potential relationship with inflammatory cytokines.

Lipopolysaccharide-binding protein is an acute-phase protein synthesized predominantly by the liver (35). High LBP levels are utilized as a marker in severe inflammatory conditions, and detected in patients with sepsis, SIRS and acute pancreatitis (35-38). In our investigation, higher LBP levels were distinguished in patients with severe AP. However, we were unable to show a relationship between LBP levels and NOD2 variants. Limited studies have focused on the association between LBP and NOD2

variants in various diseases. Reiberger et al. showed higher LBP levels in cirrhotic patients with NOD2 variants (34) while Lakatos and colleagues reported no relationship between LBP and NOD2 variants in patients with Crohn's disease (39). The relationship between LBP and NOD2 variants in patients with acute pancreatitis needs to be established in future studies.

In conclusion, the frequency of the p.R702W variant in patients with severe pancreatitis was higher than that in patients with mild pancreatitis in our study group, indicative of a strong relationship between the presence of this variant and severe pancreatitis. However, we observed no correlation between inflammatory cytokines and NOD2 variants, which requires further investigation.

Conflict of interest

All authors have no conflict of interest.

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