

Tumor necrosis factor α and interferon γ genes polymorphisms and serum levels in pancreatic adenocarcinoma

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Several biochemical pathways can lead to cancer cachexia, one of which involves the elevation of the cytokines, such as tumor necrosis factor α (TNF- α) and interferon γ (INF- γ). It was suggested that TNF- α and INF- γ genes polymorphisms may influence these cytokines serum levels, but published data are still controversial. The aim of our study was to assess the clinical significance of -308G/A TNF- α and +874A/T INF- γ genes polymorphisms as well as TNF- α and INF- γ serum levels in pancreatic adenocarcinoma (PA) and chronic pancreatitis (CP) as regards to healthy volunteers.

We studied 41 patients with pancreatic adenocarcinoma, 56 with chronic pancreatitis and 50 healthy volunteers. Peripheral venous blood samples were obtained from all patients for TNF- α and INF- γ serum concentrations measurement. After DNA isolation TNF- α and INF- γ genes polymorphisms have been studied using restriction fragment length polymorphism (RFLP) analysis. Plasma levels of TNF- α were significantly higher in PA patients (32.7 pg/ml) compared with CP patients (3.2 pg/ml) and control group (<1.6 pg/ml; $p < 0.01$). Similarly, plasma levels of INF- γ in PA patients (12.7 pg/ml) were higher from those in CP and control group (<7.1 pg/ml; $p < 0.01$). In contrast, there were no differences between CP patients and healthy volunteers in INF- γ levels. We observed a trend toward a correlation between weight loss in PA patients and TNF- α serum level ($p = 0.02$). The TNF- α and INF- γ genotype distribution were similar in patients with PA, CP and control group. We have not observed any association between TNF- α and INF- γ serum levels and their genes polymorphisms.

Our results suggest that elevated TNF- α serum level may have clinical significance in the development of cachexia in PA patients. -308G/A TNF- α and +874A/T INF- γ genes polymorphisms probably do not play important role in pancreatic diseases.

Keys words: pancreatic adenocarcinoma, tumor necrosis factor α , interferon γ , cytokines, polymorphism

Progressive weight loss is a common feature of many types of cancer and is responsible not only for a poor quality of life and poor response to chemotherapy, but also a shorter survival time than in patients with comparable tumors without weight loss [1]. Cachexia in cancer diseases is characterized by weight loss involving depletion of host adipose tissue and skeletal muscle mass. These changes cannot be fully explained by the accompanying anorexia and adequate nutritional supplementation alone is unable to reverse the wasting process.

There are several biochemical pathways that can lead to cachexia, one of which involves elevation of cytokines, such

as tumor necrosis factor α (TNF- α) and interferon γ (INF- γ). TNF- α is a proinflammatory cytokine, mainly produced by monocytes, macrophages and by T and B lymphocytes [1, 2]. Serum level of TNF- α is generally undetectable in healthy volunteers, but may increase in some acute and chronic diseases as well as after surgical trauma [3]. Elevated serum levels of TNF- α were found in patients with prostate and colorectal cancers [4, 5]. Earlier, increased TNF- α levels were being associated with weight loss in patients with pancreatic adenocarcinoma and gastrointestinal cancer [6, 7].

It has been suggested that various polymorphisms at the TNF- α gene may control this cytokine production level. Among them, the single base pair polymorphism affecting TNF- α transcription has been identified in the promoter re-

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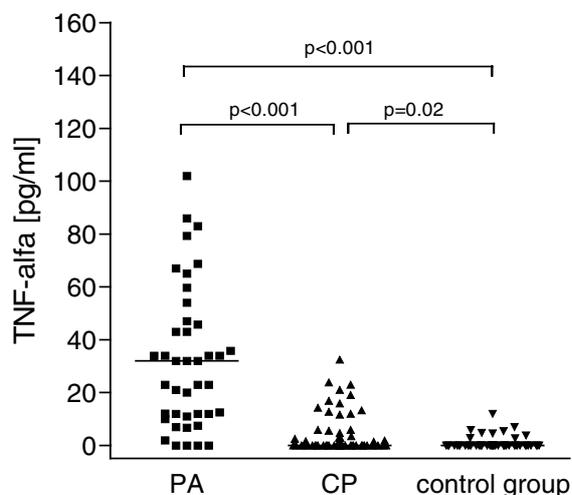


Figure 1. Analysis of TNF- α serum levels in patients with pancreatic adenocarcinoma (PA), chronic pancreatitis (CP) and the control group.

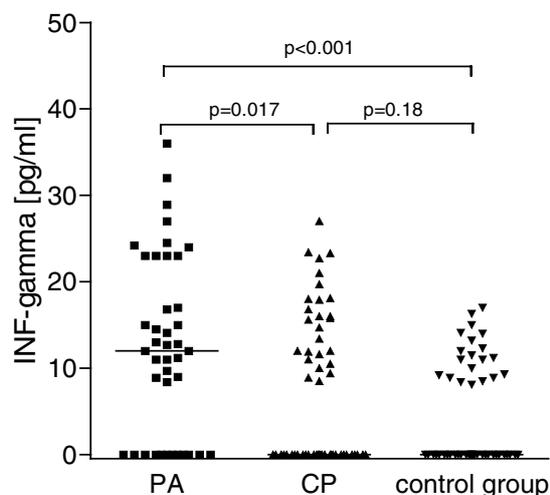


Figure 2. Analysis of INF- γ serum levels in patients with pancreatic adenocarcinoma (PA), chronic pancreatitis (CP) and the control group.

gion of the gene, involving guanine to adenosine at position -308 replacement. The allele A of -308 G/A TNF- α gene polymorphism may be associated with higher TNF- α expression *in vitro* and *in vivo* [8].

INF- γ , the second cytokine involved in cachexia, is a multifunctional cytokine demonstrating an antiproliferative and antifibrotic capacity, which may modulate local anti-tumor immune response [9, 10]. The role of IFN- γ in the pathogenesis of cancer was first suggested as a result of observations of its similarity with TNF- α properties with respect to fat metabolism. Weight loss in mice with lung cancer was associated with INF- γ production and administration of an anti-INF- γ antibody reduced the depletion of body fat, with no effect to total body protein [11]. Among numerous intronic polymorphisms in the INF- γ gene, a polymorphism in the intron 1 at position +874 A/T from the translation start has been identified, with allele T probably associated with high INF- γ synthesis [12].

The aim of our study was to assess the clinical significance of -308G/A TNF- α and +874A/T INF- γ genes polymorphisms as well as TNF- α and INF- γ serum levels in patients with pancreatic adenocarcinoma (PA) and chronic pancreatitis (CP) compared to healthy volunteers.

Patients and Methods

Patients. We studied 147 Caucasian patients: 41 with pancreatic adenocarcinoma (19 men and 22 women, aged 47-84 years), 56 with chronic pancreatitis (36 men and 20 women, aged 21-73 years) and 50 gender- and age-matched healthy volunteers. Analysed patients were hospitalized in Department of Digestive Tract Diseases, Medical University of Lodz Hospital between 2003 and 2006. The study protocol was approved by the ethical committee of Lodz Medical University.

Only patients with confirmed pathology diagnosis of ductal pancreatic adenocarcinoma were included into the study. Nine patients (21,9%) with PA underwent Whipple resection or distal pancreatectomy, 10 patients (24,4%) – palliative surgery and 22 patients (53,6%) – palliative chemotherapy and/or endoscopic treatment (stent insertion). CP diagnosis was established based on pathologic confirmation after surgery – in 17 patients (30,3%) – or based on a typical clinical history and characteristic findings in pancreatic imaging. Indications for surgical intervention in chronic pancreatitis were intractable pain (9 patients), suspicion of pancreatic cancer (6 patients) and pancreatic pseudocyst (2 patients).

The associations of the analysed genotypes, TNF- α and INF- γ serum levels and clinical data at diagnosis have been evaluated. The following demographic and clinical data have been analysed: age, tumor size, lymph node involvement, histological grade, distant metastases, history of smoking, weight loss > 10% as well as selected laboratory parameters: Ca 19-9, total bilirubin, albumin, transaminases and amylase levels.

Blood analysis. Peripheral venous blood samples were obtained from all analysed patients at the time of hospital admission. Sera were separated by centrifugation at 3000 rpm and stored at -80°C until the levels of analysed cytokine were assessed. The serum concentrations of TNF- α and INF- γ have been measured with an enzyme-linked immunoassay (R&D Systems, USA). The limit of detection of TNF- α and INF- γ was determined to be respectively 1.6 and 7.1 pg/ml.

Genotyping. -308G/A TNF- α and +874A/T INF- γ genes polymorphisms have been studied in DNA isolated from blood samples. The primers 5'-GAG GCA ATA GGT TTT GAG GGC CAT-3' (in the forward direction) and 5'-T ACA CAC AAG CAT CAA G-3' (in the reverse direction) were used to amplify the region containing the TNF- α variant. PCR am-

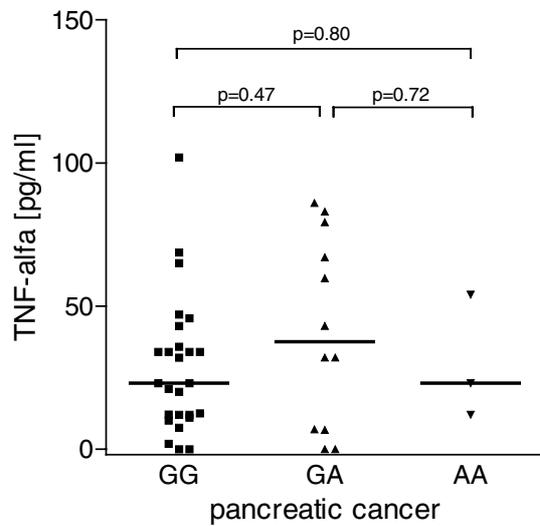


Figure 3. Correlation between TNF- α serum levels and -308G/A TNF- α gene polymorphism (genotype GG, GA, AA) in patients with pancreatic adenocarcinoma.

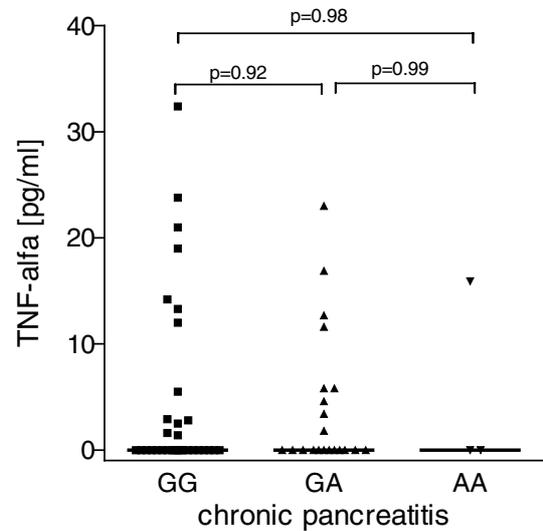


Figure 4. Correlation between TNF- α serum levels and -308G/A TNF- α gene polymorphism (genotype GG, GA, AA) in patients with chronic pancreatitis.

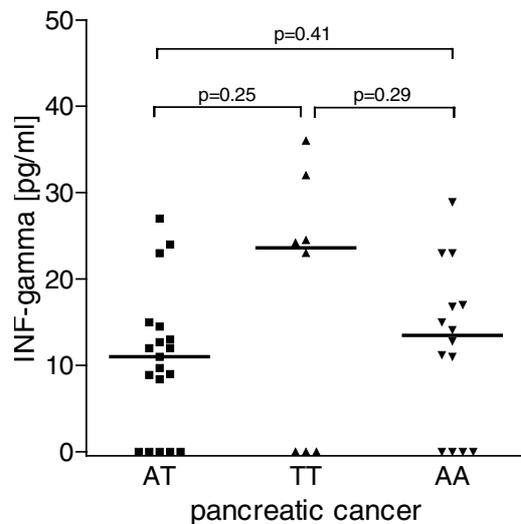


Figure 5. Correlation between INF- γ serum levels and +874A/T INF- γ gene polymorphism (genotype AT, TT, AA) in patients with pancreatic adenocarcinoma.

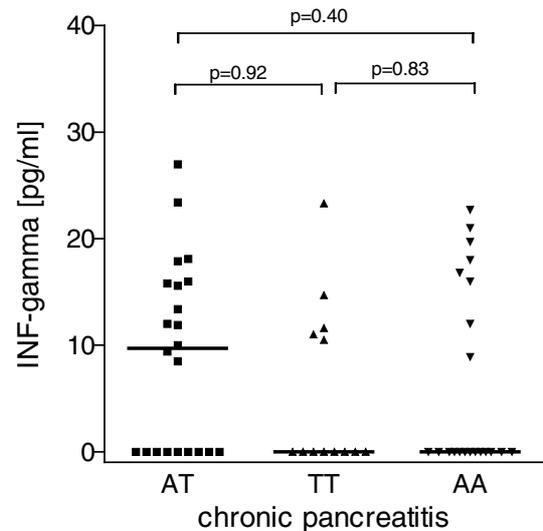


Figure 6. Correlation between INF- γ serum levels and +874A/T INF- γ gene polymorphism (genotype AT, TT, AA) in patients with chronic pancreatitis.

plication was performed in a final volume of 25 μ l containing 80 ng of DNA, 1.5 mM of $MgCl_2$, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of dNTP, each primer at 1.0 μ M and 1.0 unit of Taq polymerase (Takara, Japan) in a 2400 Perkin-Elmer Thermocycler.

10 μ l of the PCR product was digested with 2 units of *Nco*I using the manufacturer's recommended protocol. PCR products were visualised on 3% agarose gels with 10% ethidium bromide. PCR products for the TNF- α variants were

analysed by restriction fragment length polymorphism (RFLP) analysis.

The +874A/T INF- γ single nucleotide polymorphism was genotyped by allelic discriminating TaqMan PCR, using the following primers and probes: forward primer 5'- ATT CAG ACA TTC ACA ATT GAT TTT ATT CTT AC - 3' and reverse primer 5' ACT GTG CCT TCC TGT AGG GTA TTA TT 3'; probe 1: 5'-AAT CAA ATC TCA CAC ACA C-3' and probe 2: 5'-ATC A TCA CAC ACA CAC-3'. PCR

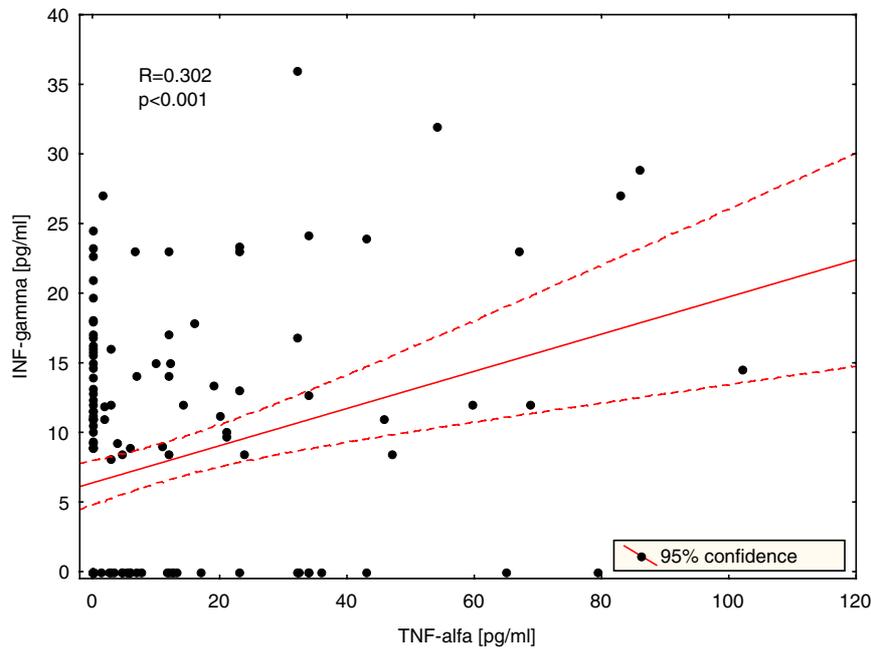


Fig. 7. Correlation between TNF- α and INF- γ in patients with detectable analyzed cytokines serum levels.

and end point analysis was performed in a volume of 25 μ l containing 200 ng of DNA, 1.5 mM of MgCl₂, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of dNTP, 0,05% Tween20, 0,05% Nonidet-P40, each primer at 1.0 μ M and 1.0 unit of Taq polymerase (Takara, Japan) in a 2400 Perkin-Elmer Thermocycler.

Statistics. The results were analyzed according to statistical methods by using StatSoft Statistica for Windows, release 6.0 (StatSoft, Inc., Tulsa, USA). To determine differences between groups Mann-Whitney t-test was used. Association between continues variables was analyzed with Pearson's correlation test. Differences with p-values < 0.05 were considered to be significant.

Results

TNF- α serum levels were detected in 37 patients with pancreatic adenocarcinoma (90,2%), 19 with chronic pancreatitis (33,9%) and in 9 of 50 healthy adults from control groups (18%). INF- γ levels were detectable in 30 PA patients (73,2%), 27 CP patients (48,2%) and 20 healthy controls (40%). Plasma levels of TNF- α were significantly higher in PA patients (mean cytokine level: 32.7 pg/ml; range <1.6-104 pg/ml) compared with CP patients (3.2 pg/ml; range <1.6-34 pg/ml) and control group (<1.6 pg/ml; range <1.6-17 pg/ml; p<0,01). Plasma levels of INF- γ in PA patients (mean cytokine level: 12.7 pg/ml; range <7.1-37 pg/ml) significantly differed from those in the CP (<7.1 pg/ml; range <8.0-27.9 pg/ml). In contrast, there was no differences between CP patients and healthy volunteers in INF- γ serum

levels (<7.1 pg/ml; range <7.1-17.2 pg/ml). Our data also showed that high levels of TNF- α were correlated significantly with INF- γ levels (p<0.001; Fig. 7).

In PA patients, the tumor size ranged from 2 to 6 cm (mean 3,6 \pm 1,2 cm). In 14 patients (34,1%) tumor smaller than 3,5 cm have been detected. Local lymph node metastases were observed in 19 patients with PA (46,3%) and liver metastases in 8 patients (19,5%). Twenty patients (48,8%) presented weight loss >10% with the mean weight loss 8.3 \pm 0,8 kg during 6 months.

We observed a trend toward a correlation between weight loss in PA patients and TNF- α serum level (p=0.02; Fig. 8). Mean TNF- α level in patients with weight loss >10% was 56,3 pg/ml (range 14,5-104 pg/ml) compared to 19,2 pg/ml (range <1.6-69 pg/ml) in noncachectic patients. The albumin levels in patients with cachectic and noncachectic advanced PA were not significantly different (p=0.15). There was no other statistically significant association between TNF- α serum level and tumor size, histological grade, regional or distant metastases, laboratory findings or patients sex and age. INF- γ was also no correlated with any clinicopathological data of analysed group of patients.

The TNF- α and INF- γ genotype distribution were similar in patients with pancreatic adenocarcinoma, chronic pancreatitis and control group (p>0.05; Table 1). We have not observed any association between TNF- α and INF- γ serum levels and genes polymorphisms (Fig. 3-6). Polymorphisms of analysed genes were unrelated to the tumor size, histologic grade, regional or distant metastases, laboratory findings or patients sex and age (data not shown).

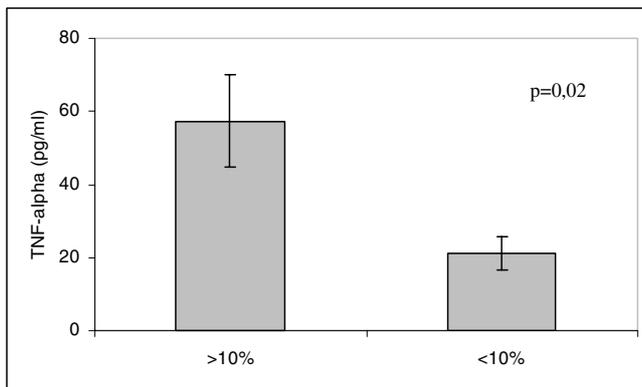


Fig. 8. Correlation between TNF- α serum levels and weight loss of more than 10% in patients with pancreatic adenocarcinoma.

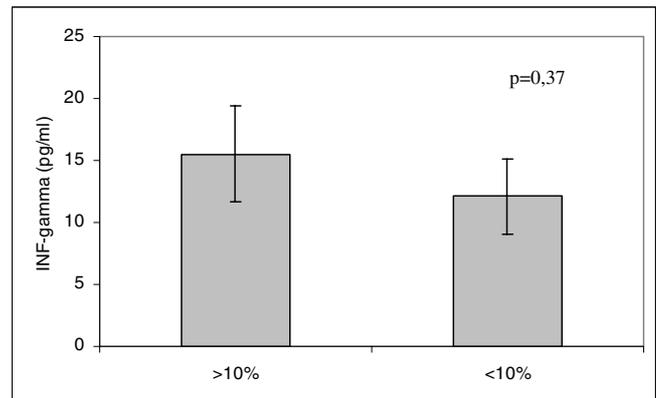


Fig. 9. Correlation between INF- γ serum levels and weight loss of more than 10% in patients with pancreatic adenocarcinoma.

Discussion

Several studies have investigated the possible role of cytokine serum levels and their gene polymorphisms in neoplastic diseases. This impairment of host factors might result in susceptibility or resistance to tumour progression. In our study, plasma levels of TNF- α were significantly higher in patients with pancreatic adenocarcinoma compared with chronic pancreatitis and control group. Our results are in agreement with other studies, which reported elevated TNF- α serum levels in patients with colorectal and nonsmall cell lung cancer compared with normal subjects [4, 13]. Elevated concentration of TNF- α has been also found in patients with B-cell chronic lymphocytic leukemia and lymphoma with particularly poor performance status [14, 15]. Patients with higher TNF- α level had also shorter period free from progression

Table 1. G-308A TNF- α genotypes distributions in patients with pancreatic adenocarcinoma (PA), chronic pancreatitis (CP) and control group

	PA		CP		Control group	
	N	%	N	%	N	%
GG	26,0	63,4	32,0	57,1	31,0	62,0
GA	12,0	29,3	21,0	37,5	17,0	34,0
AA	3,0	7,3	3,0	5,4	2,0	4,0

p>0.05

Table 2. A+874T INF- γ genotypes distributions in patients with pancreatic adenocarcinoma (PA), chronic pancreatitis (CP) and control group

	PA		CP		Control group	
	N	%	N	%	N	%
AA	14	34,1	21	37,5	19	38,8
AT	19	46,3	22	39,3	18	36,7
TT	8	19,5	13	23,2	12	24,5

p>0.01

and shorter overall survival [15]. In contrast, Ebrahimi et al. reported no difference in TNF- α serum levels between PA patients and healthy volunteers group [3]. These contrary findings may be partially attributable to the diversity of patients populations and different protocol applied (plasma stored in liquid nitrogen or frozen at -80°C before analysis).

We observed higher TNF- α serum level in patients with weight loss >10% compared to noncachectic patients. In the study of Karayiannakis et al. increased TNF- α levels were also associated with weight loss in patients with pancreatic adenocarcinoma [6]. Similarly, TNF- α levels were higher in cachectic patients with advanced cancer of prostate compared to noncachectic patients with this disease [5]. Earlier, in experimental studies, TNF- α has been shown to activate muscle protein degradation. Eight-day TNF- α administration to healthy (cancer-free) rats brought about an enhanced rate of degradation of skeletal muscle protein, even though body weight loss was not apparent in the animals [16]. In another study administration of anti-murine TNF- α immunoglobulin to rats bearing the Yoshida AH-130 ascites hepatoma led to decreases in the rates of protein degradation in the skeletal muscle, heart, and liver tissues but it had no effect on weight loss in the animals [17].

However, several studies have failed to detect elevated circulating levels of TNF- α in cachectic cancer patients or have failed to associate the elevation with the development of cachexia [3, 13, 18]. In the study of Kayacan et al. serum TNF- α levels did not differ between cachectic and noncachectic patients with newly diagnosed nonsmall cell lung cancer [13]. The inability to associate serum levels of TNF- α with the development of cachexia may be due to the very rapid blood transit of cytokines, so that they can be transported from the sites of production to the target tissue without causing an elevated serum concentration.

The cachexia development requires multiple cytokines interacting with or augmenting each other effect. TNF- α is believed to act in concert with various cytokines, including

INF- γ and interleukin 1. We observed statistically significant positive correlation between higher TNF- α and INF- γ serum levels. Koerner et al. have found that lipopolysaccharide-stimulated peritoneal macrophages and accumulated more mRNA for TNF- α when treated with INF- γ , suggesting that TNF- α and INF- γ interact at the level of transcription [19]. In experimental study, in rats subjected to transplants of the sarcoma cells, anti-INF- γ antibody reduced weight loss and increased survival. Passive immunization against INF- γ allowed cachectic tumor-bearing rats to eat more food, have a lesser decline in body weight and tolerate larger tumors than similar rats given non-specific control antibody [20]. In our study, plasma levels of INF- γ in patients with pancreatic adenocarcinoma were significantly higher than those in chronic pancreatitis, with no differences between CP patients and healthy volunteers. To the best of our knowledge, this is the first study investigating INF- γ levels in pancreatic cancer, so there are no comparative data. However, INF- γ serum levels were also found to be raised in patients with breast cancer and multiple myeloma, however no association between this level and clinical parameters of the disease have been found [21, 22].

Our study, in a relatively limited number of patients, failed to reveal a significant association between TNF- α serum levels and genes polymorphisms. Similar results were observed in other studies, including patients with *Helicobacter pylori* infection and healthy volunteers [23, 24]. In contrast, in the study of Wilson et al. the allele A of -308 G/A TNF- α gene polymorphism was associated with higher TNF- α expression *in vitro* and *in vivo* [8]. The hypothesized relationship between high TNF- α production and the GA/AA genotype was also found in a study involving lipopolysaccharide-stimulated whole blood samples obtained from healthy volunteers [25]. It is also known, that among numerous intronic polymorphisms in the INF- γ gene, allele T of 874A/T INF- γ polymorphism probably is associated with high INF- γ synthesis [12, 24]. However, we failed to confirm this relationship. The possible explanation of this difference may be the heterogeneity of the patient population tested with different ethnic background. Larger patients study-population may aid in the more accurate evaluation of clinical significance of examined INF- γ polymorphism.

In the current study, the TNF- α and INF- γ genotype distribution were similar in patients with pancreatic adenocarcinoma, chronic pancreatitis and control group (Table 1). Our results do not differ from those of other authors [26–30]. Bendicho et al. did not find statistically significant differences of the allele and genotypic frequencies of the genes coding TNF- α and INF- γ between the control group and patients with chronic pancreatitis [26]. Similarly, in the German patients -308 G/A TNF- α polymorphism was not associated with alcoholic chronic pancreatitis, idiopathic or hereditary pancreatitis [28]. In contrast, in the population-based case-control study among patients with pancreatic cancer, pancreatitis was significantly associated with allele A of -308 G/A TNF- α polymorphism [31].

According to our data, the analysed polymorphisms are also unrelated to the tumor size, histologic grade, regional or distant metastases, laboratory findings or patients sex and age. Similarly in other studies TNF- α -308G/A polymorphism showed no significant associations with clinical findings in patients with prostate and breast cancers [32, 33]. There was also no association between TNF- α genotype and survival in advanced pancreatic cancer (30). On the other hand, Halma et al. observed that other polymorphism of INF- γ gene (a variable length CA-repeat sequence) was consistently associated with increased duration of survival in patients after confirmation of nonresectable pancreatic adenocarcinoma [34].

In conclusion, we suggest that -308G/A TNF- α and +874A/T INF- γ genes polymorphisms probably do not play important role in pancreatic diseases. On the other hand, elevated TNF- α serum level may have clinical significance in the development of cachexia in PA patients, however our results have to be confirmed in larger studies.

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