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ORIGINAL ARTICLE

TNF-α, IL-6, and IL-8 Cytokines and Their Association with TNF-α-308 G/A Polymorphism and Postoperative Sepsis

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Abstract
Introduction Early prediction of postoperative sepsis remains an enormous clinical challenge. Association of TNF-α-308 G/A polymorphism with sepsis remains controversial. We, therefore, investigated this polymorphism with serum levels of cytokines TNF-α, IL-6, and IL-8 in relation to development of sepsis following major gastrointestinal surgery.
Methods Two hundred and thirty-nine patients undergoing major gastrointestinal surgery were enrolled. Polymorphism was studied through the analysis of restriction fragments of Nco1-digested DNA with the polymerase chain reaction. All patients were followed for 1 month following surgery for evidence of sepsis. Levels of serum cytokines TNF-α, IL-6, and IL-8 were measured preoperatively and postoperatively by enzyme-linked immunosorbent assay (ELISA).
Results Forty-seven (19.66 %) patients developed postoperative sepsis. Patients with postoperative sepsis were significantly (p=0.002) more likely to possess AA homozygous genotype with higher capacity to produce cytokines TNF-α (p<0.0001), IL-6 (p<0.0001), and IL-8 (p<0.0001) as compared to other genotypes. When compared with patients carrying at least one G allele, the AA genotype was associated with a significantly higher probability (odds ratio (OR)=4.17; p=0.003; 95 % confidence interval (CI)=1.5–11.48) of developing sepsis. Compared with the GG genotype, AA was associated with a significantly higher probability (OR=5.18; p=0.0008; 95 % CI=1.82–14.76) of sepsis development.
Conclusion TNF-α-308 G/A polymorphism is significantly associated with the development of postoperative sepsis and with increased expression of cytokines TNF-α, IL-6, and IL-8.

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Keywords Cytokines · Genetic polymorphism · Restriction fragment length polymorphism · Sepsis · Tumor necrosis factor

Introduction

Postoperative sepsis is one of the main complications of major surgery. Recent advances in postoperative care have enabled clinicians to reduce early postoperative mortality and morbidity. Despite the advancement in postoperative care, patients remain at high risk for infection and the associated complications following surgery.[1]

Cytokine networks and tumor necrosis factor (TNF), in particular, appear to play a crucial role in the pathogenesis of infectious diseases.[2] The pro-inflammatory cytokine, TNF-α, is an essential component of the host immune response to infection[3] and is responsible for the release of other pro- and anti-inflammatory mediators. TNF-α also plays a major role in the clinical manifestations of septic shock,[4] and TNF-α serum levels inversely correlate with survival from severe sepsis.[5] Furthermore, inherited variability of cytokine production and genetic predisposition for fatal infectious diseases have been suggested.[6] Several polymorphisms have been identified in the TNF-α promoter,[7] among which the G/A polymorphism at nucleotide position −308 has been shown to directly affect TNF-α expression.[8] In sepsis, interest has particularly focused on the promoter TNF-308 G/A single-nucleotide polymorphism. Although the association of A allele with susceptibility to septic shock has been reported by various studies, the findings have been inconsistent.[9–13] The guanine-to-adenine transition at position −308 in the TNF promoter has been reported to influence TNF promoter activity and is associated with enhanced TNF-α production;[14–16] however, other studies could not confirm this association.[11,17]

In the present study, we hypothesize that −308 G/A polymorphism of TNF-α is associated with TNF-α transcriptional activity and affects the production of inflammatory cytokines, which may play an important role in the development of postoperative sepsis. Thus, our study investigated the association of TNF-α-308 promoter polymorphism with the development of sepsis postsurgery.

Material and Methods

Patient Characteristics and Study Protocol The institutional ethics committee of King George's Medical University, Lucknow, India, approved the study. All participants signed an informed consent. Two hundred and thirty-nine patients who underwent major surgery from October 2010 to January 2013 at the university hospital were included. Of the recruited subjects, 143 (59.83 %) were female and 96 (40.16 %) were male. The mean age was 36.14±11.12 years (range 18–50 years). Elective major surgery was defined as planned surgery requiring more than 1 hour of surgical time and requiring anesthesia and or respiratory assistance. The inclusion criteria were as follows: age 18–50 years, absence of preexisting infection, absence of rheumatoid arthritis or a seronegative inflammatory arthropathy, absence of malignancy, absence of diabetes, no steroid medication, and no acquired or inherited immunodeficiencies. To minimize operation-to-operation variability, patients were operated in the same theater and as the first case of the day. The amount of blood loss and duration of surgery (incision to stitching time) were recorded. The patient characteristics are shown in Table 1. All patients were kept on non-steroidal anti-inflammatory drugs for the management of postoperative pain and received broad-spectrum single-dose intravenous antibiotic (third-generation cephalosporin) for surgical infection prophylaxis. The study team followed all patients for 1 month following surgery for any evidence of sepsis.

Sepsis was defined according to Bone et al.[18]: systemic response to infection, manifested by two or more of the following conditions as a result of infection: body temperature >38.5 or <35.6 °C, tachycardia >90/min, tachypnea >20/min, leucocytosis >12,000/μl, or leucopenia <4,000/μl.

Immunologic Assays of Cytokines Serum was collected preoperatively and postoperatively on the first and fourth day after surgery from all the study patients. Commercially available highly sensitive AviBion human TNF-α, IL-6, and IL-8 enzyme-linked immunosorbent assay (ELISA) kits (Orgenium Laboratories Business Unit, Finland) were used for determinations of serum concentration as per manufacturer's instructions.

Genotyping for TNF-α-308 G/A Polymorphism The genotype of TNF-α-308 gene polymorphism was determined by polymerase chain reaction (PCR) amplification and enzymatic digestion of the products with Nco1 (Fermentas, USA). Briefly, 3 ml of venous blood samples was drawn in ethylenediamine-tetra acid (EDTA) vacutainer tubes, and genomic DNA was extracted using a commercially available DNA isolation kit (QIAmp blood kit, Qiagen, Germany), according to the manufacturer's instructions. A 134-bp PCR product of TNF-α gene was amplified by PCR using the following pair of primers: forward 5′ AGG CAA TAG GTT TTG AGG GCC AT 3′ and reverse 5′ CAT CAA GGA TAC CCC TCA CAC TC 3′.[19] Reaction was performed with 50–200 ng of genomic DNA, 0.2 μmol of each primer, 200 μmol of dNTP (Fermentas, USA), 1.2 mmol of $MgCl_2$, and 1 unit of Taq polymerase (Fermentas, USA) in a total volume of 20 μl. Initial denaturation was performed at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at

Table 1 Characteristics of patients with and without postoperative sepsis

		Patients with postoperative sepsis (n=47)	Patients without postoperative sepsis (n=192)	p value
Age (years)		36.49±11.93	36.05±10.94	0.81
Gender (female/male)		27/20	116/76	0.71
Length of surgery (h)		2.69±0.59	2.12±1.55	0.01*
Blood loss (ml)		126.81±78.77	40.13±52.67	<0.0001*
Hb level (g/dl)	Preoperative	11.09±1.47	11.38±1.74	0.30
	Postoperative	10.52±0.68	11.28±1.14	<0.0001*
No. of WBC/μl	Preoperative	7,285.53±1,830.17	7,210.00±1,668.53	0.78
	Postoperative	10,204.89±6,478.55	7,169.83±1,521.49	<0.0001*
TNF-α level (pg/ml)	Preoperative	52.20±39.80	54.99±47.34	0.71
	1st Postoperative day	118.27±84.51	66.00±48.25	<0.0001*
	4th Postoperative day	211.76±158.10	79.78±60.07	<0.0001*
IL-6 level (pg/ml)	Preoperative	69.92±66.79	63.40±72.16	0.57
	1st Postoperative day	103.12±88.79	77.11±75.30	0.05
	4th Postoperative day	155.16±150.14	74.59±74.15	<0.0001*
IL-8 level (pg/ml)	Preoperative	28.24±26.17	27.43±20.94	0.82
	1st Postoperative day	39.59±26.31	33.07±35.58	0.25
	4th Postoperative day	86.85±86.39	40.49±58.11	<0.0001*

Values are given as numbers or mean±SD. Differences were tested by chi-square and Fisher's exact test for frequencies and Student's t test for mean±SD

SD standard deviation, *TNF* tumor necrosis factor

*p value <0.05 considered statistically significant

56 °C for 1 min, and extension at 72 °C for 1.30 min. This was followed by final extension at 72 °C for 10 min. A total of 10 μl of the resulting PCR product was digested with Ncol for 10 min at 37 °C, and the resulting fragments were analyzed on 3 % agarose gel.

Statistical Analysis Data were expressed as proportion or mean±standard deviation, as appropriate. The test on the proportions between groups was performed using chi-square test or Fisher's exact test. Odds ratios (OR) as an estimation of the relative risk were calculated with 95 % confidence intervals (CI). For comparison between groups, Student's unpaired or paired t test and one-way analysis of variance (ANOVA) was performed. Pearson correlation test was performed for correlation analysis. The power of the study was calculated using G* power calculator version 3.1.7. A p value less than 0.05 is considered as statistically significant.

Results

Postoperative sepsis was diagnosed in 47 (19.66 %) patients, whereas 192 (80.33 %) patients showed an uncomplicated postoperative recovery. The patients with and without postoperative sepsis were comparable in terms of age, gender, blood loss, WBC count, mortality, and duration of surgery (Table 1). The duration of surgery and blood loss were observed to be significantly associated with the development of postoperative sepsis. A significant difference in the WBC count was also observed in patients with and without postoperative sepsis. Sepsis was diagnosed in three patients 2 days after surgery, in five patients 4 days after surgery, in two patients 5 days after surgery, and in the remaining patients 9 days or later after surgery. The most common postoperative complication observed in the sepsis group was surgical site infection followed by anastomotic leak, pancreatic leak, intraabdominal abscess, pneumonia, and multiple organ dysfunctions. Postoperative sepsis and its relation to the site of surgery are shown in Fig. 1.

Allele Frequency and Genotype Distribution

The overall allele frequency was 0.81 for G and 0.19 for A. The allele frequencies of G and A were 0.67 and 0.33 in patients with postoperative sepsis and 0.84 and 0.16 in patients without postoperative sepsis, respectively. In the TNF-α genotype group, 68.62 % (n=164) of patients were homozygous dominant GG, 24.27 % (n=58) were heterozygous GA, and 7.11 % (n=17) were homozygous recessive AA. Patients in the genotype subgroups were matched for age, gender, mortality, blood loss, and duration of surgery (Table 2). Postoperative sepsis was observed in 24/164 (14.63 %), 15/58 (25.86 %), and 8/17 (47.05 %) patients for genotype GG homozygous, GA heterozygous, and AA homozygous, respectively.

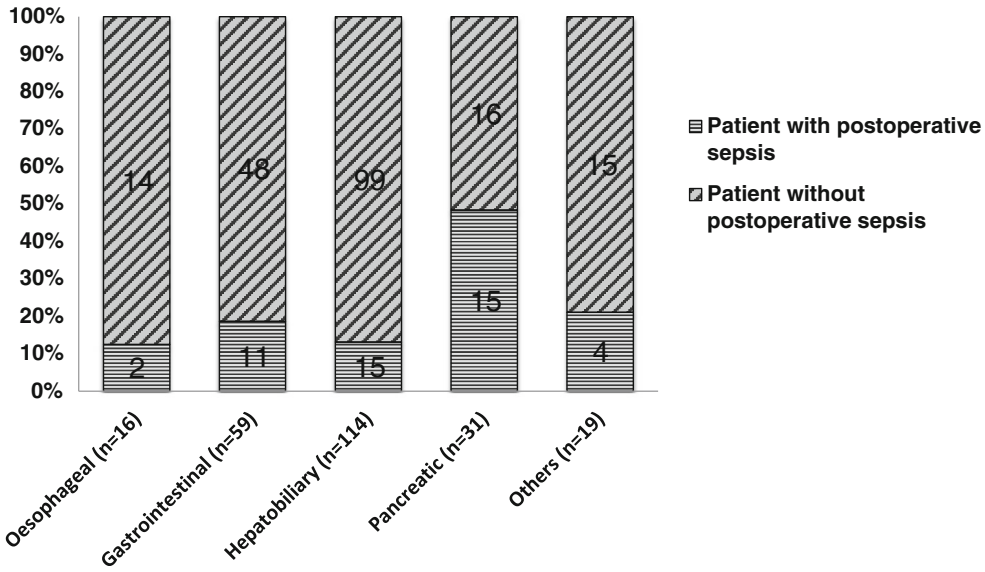

Fig. 1 Postoperative sepsis related to site of surgery. Values are given as *numbers*

Genotype distribution in patients with an uncomplicated postoperative course was significantly different from that in patients with postoperative sepsis. Development of postoperative sepsis was significantly (p=0.002) higher in patients homozygous for the allele AA as compared to other genotypes (Table 2). When compared with patients carrying at least one G allele (GG homozygous and GA heterozygous genotype), the AA homozygous genotype was significantly associated with the development of sepsis (OR=4.17; p=0.003; 95 % CI=1.5 to 11.48). Compared with the homozygous GG genotype, the OR for the homozygous AA genotype was 5.18 (p=0.0008; 95 % CI=1.82 to 14.76). When both the alleles were compared, allele A was found to be positively associated with the development of postoperative sepsis (OR=1.91; p=0.037).

Along with TNF-α-308 G/A polymorphism (p=0.002), blood loss (p<0.0001) and duration of surgery (p=0.01) were also found to be significantly associated with postoperative sepsis (Tables 1 and 2). To study the individual effect of TNF-α-308 G/A polymorphism, multivariate analysis was preformed, which revealed significant association of AA genotype (p=0.001) and blood loss (p<0.0001) with postoperative sepsis. In addition, correlation analysis showed a positive correlation of blood loss with duration of surgery (r=0.512; p<0.0001) in patients who developed postoperative sepsis.

The overall mortality was 3.347 %; 8 of 239 patients expired during postoperative hospital stay. In all eight patients, no statistically significant difference (p=0.827) was observed in the genotype subgroups.

Table 2 Genotypic distribution and characteristics of patients

Genotype		GG (n=164)	GA (n=58)	AA (n=17)	p value
Age (years)		36.08±10.68	36.53±12.41	35.35±11.32	0.922
Gender (female/male)		95/69	38/20	10/7	0.672
Blood loss (ml)		52.27±66.43	70.69±73.69	60.59±70.93	0.206
Length of surgery (h)		2.12±0.63	2.51±2.68	2.38±0.65	0.174
Hb level (g/dl)	Preoperative	11.40±1.73	11.01±1.57	11.62±1.71	0.236
	Postoperative	11.21±1.16	10.92±0.98	11.07±0.90	0.218
No. of WBC/μl	Preoperative	7,137.68±1,693.88	7,335.86±1,728.03	7,687.85±1,630.82	0.381
	Postoperative	7,533.63±3,287.94	7,842.72±2,904.34	10,088.23±5,454.91	0.015*
Mortality		5 (3.05 %)	2 (3.45 %)	1 (5.88 %)	0.827
Sepsis		24 (14.63 %)	15 (25.86 %)	8 (47.05 %)	0.002*

Values are given as numbers (%) or mean±SD. Differences were tested by chi-square test for frequencies and ANOVA for mean±SD

SD standard deviation, *ANOVA* analysis of variance

*p value <0.05 considered statistically significant

Preoperative and Postoperative Changes in Cytokine Levels

Cytokine levels of preoperatively and postoperatively drawn whole-blood samples from patients with and without postoperative sepsis are shown in Table 1. In patients who developed postoperative sepsis, significantly higher serum levels of cytokine TNF-α ($p<0.0001$) was observed at the first postoperative day, whereas at the fourth postoperative day, all the studied cytokines were significantly higher (TNF-α $p<0.0001$; IL-6 $p<0.0001$; IL-8 $p<0.0001$) as compared to cytokine levels in patients without sepsis (Table 1).

TNF-α, IL-6, and IL-8 Cytokine Levels in Relation to Different Genotypes of TNF-α-308 G/A Polymorphism

To study the functional significance of TNF-α-308 G/A polymorphism, we grouped the patients with and without postoperative sepsis according to their genotypes and compared TNF-α, IL-6, and IL-8 cytokine levels.

In AA homozygous genotype patients who developed postoperative sepsis, compared to patients with other genotypes who developed postoperative sepsis, a significant increase in the level of TNF-α was observed at both preoperative and postoperative days (preoperative $p=0.03$; first postoperative day $p=0.001$; fourth postoperative day $p<0.0001$) and a significant increase in IL-6 was observed on both the first and fourth postoperative days ($p<0.0001$), whereas IL-8 increased only on the fourth postoperative day ($p<0.0001$) (Fig. 2).

In patients who did not develop postoperative sepsis, preoperative and postoperative cytokine levels did not vary by genotype (Fig. 3).

Correlation of TNF-α, IL-6, and IL-8 Cytokines

To study the influence of TNF-α production on IL-6 and IL-8 cytokine levels, Pearson correlation test was performed in patients with and without postoperative sepsis. Correlation analysis revealed that in patients who developed sepsis, TNF-α level was positively correlated with IL-6 in both preoperative and postoperative samples (preoperative ($r=0.354$; $p=0.015$); first day postoperative ($r=0.568$; $p<0.0001$); fourth day postoperative ($r=0.580$; $p<0.0001$)). No correlation was observed in the levels of IL-8 and TNF-α

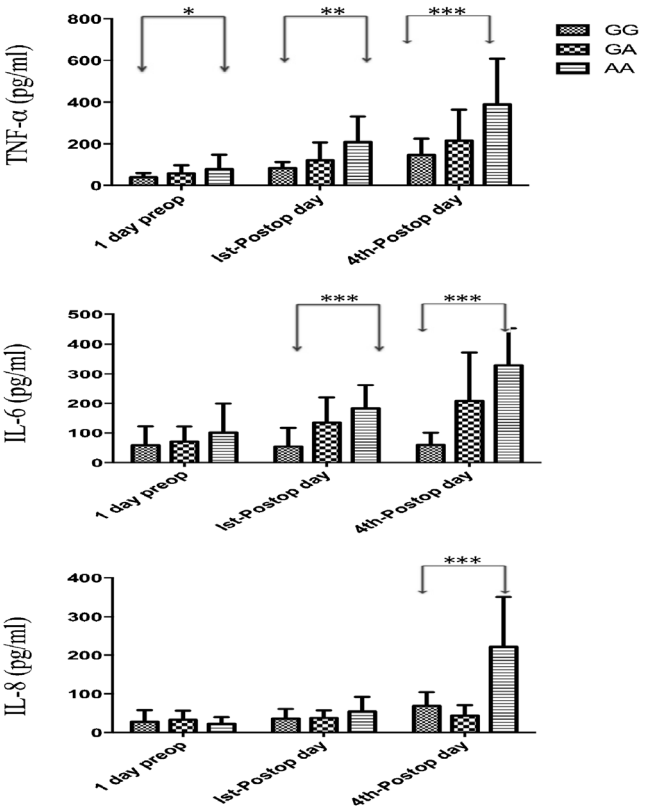

Fig. 2 Preoperative and postoperative TNF-α, IL-6, and IL-8 cytokines in genotype subgroups in postoperative septic patients. Statistical analysis carried out through one-way analysis of variance. Values are means± standard deviation. *TNF* tumor necrosis factor, *IL* interleukin. *$p<0.05$; **$p<0.01$; ***$p<0.0001$

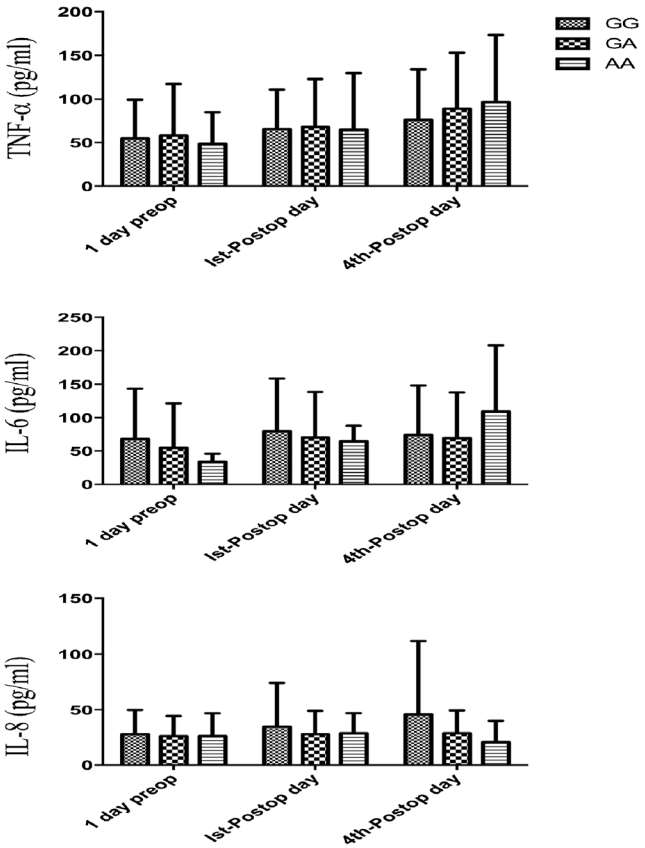

Fig. 3 Preoperative and postoperative TNF-α, IL-6, and IL-8 cytokines in genotype subgroups in patients without sepsis. Statistical analysis using one-way ANOVA indicates insignificant difference in TNF-α, IL-6, and IL-8 cytokine level preoperatively and postoperatively in different genotype subgroups. Values are means±standard deviation. *TNF* tumor necrosis factor, *IL* interleukin, *ANOVA* analysis of variance

preoperatively and on the first postoperative day, whereas on the fourth postoperative day, a significant positive correlation was observed with both TNF-α (r=0.537; p<0.0001) and IL-6 (r=512; p<0.0001) (Table 3).

In patients who did not develop postoperative sepsis, TNF-α level was significantly correlated with IL-6 before surgery (r=0.182; p=0.011), whereas after surgery, no such correlation was observed. In relation to IL-8 cytokine, no correlation was observed with TNF-α and IL-6 in both preoperative and postoperative phases (Table 3).

Discussion

Variation in the immune response among individuals is a well-known fact. Cytokine gene polymorphism can influence not only cytokine gene expression but also susceptibility to disease and disease pathogenesis. Numerous polymorphic modifications in the TNF gene have been linked to TNF production, but the specific location of the genetic elements governing TNF response remains unclear, and its gene expression has been shown to be regulated only in part by transcriptional events.[20] In sepsis patients and in severely injured patients, TNF (TNF-α and TNF-β) gene polymorphisms have been described in various populations. Some investigators have reported the association between TNF-α-308 gene polymorphism and increased susceptibility to severe sepsis,[9,12,21,22] whereas others failed to confirm this.[13,23,24] This lack of consensus on the association of TNF-α-308 gene polymorphism with increased risk of sepsis encouraged us to examine the distribution of the TNF-α genotype in patients who were undergoing elective gastrointestinal surgeries and to follow the incidence of postoperative sepsis. Our study design—evaluating patients for TNF-α-308 gene polymorphism before surgery and correlating this to postsurgery sepsis—is in contrast to the previous studies,[9,13,16] which were limited to patients who already had sepsis. To the best of our knowledge, this is the first study in a unique cohort that evaluated the association of TNF-α-308 gene polymorphism and alteration in cytokine levels with risk of sepsis following major gastrointestinal surgery.

In contrast to certain studies,[13,23,24] our study shows that AA genotype individuals are 4.17 times more likely to develop postoperative sepsis as compared with other genotypes. This is in agreement with the previous studies[9,10,21,22] showing a significantly higher distribution of TNF-α-308 AA genotype in a cohort of patients that developed sepsis following elective major surgery. The study thus supports the hypothesis that interindividual variation in the TNF-α gene may impact on the risk of sepsis following elective major surgery.

The incidence of postoperative sepsis (19.66 %) in the current study was influenced by the AA homozygous genotype distribution, which is in agreement with previous observations in trauma patients.[21] Although the number of septic patients in the present study (n=47) is low, with the cohort of

Table 3 Correlation of TNF-α, IL-6, and IL-8 cytokines in preoperative and postoperative phases

		Preoperative			First postoperative			Fourth postoperative		
		TNF-α	IL-6	IL-8	TNF-α	IL-6	IL-8	TNF-α	IL-6	IL-8
Patients with postoperative sepsis										
TNF-α	*r* value	1	0.354[a]	0.051	1	0.568[b]	0.219	1	0.580[b]	0.537[b]
	p value		0.015	0.736		0.000	0.143		0.000	0.000
IL-6	*r* value		1	−0.140		1	0.176		1	0.512[b]
	p value			0.347			0.241			0.000
IL-8	*r* value			1			1			1
	p value									
Patients without postoperative sepsis										
TNF-α	*r* value	1	0.182[a]	−0.012	1	0.046	−0.006	1	0.056	0.035
	p value		0.011	0.866		0.563	0.937		0.465	0.646
IL-6	*r* value		1	0.023		1	0.032		1	0.015
	p value			0.985			0.691			0.842
IL-8	*r* value			1			1			1
	p value									

r Pearson correlation coefficient, *TNF* tumor necrosis factor, *IL* interleukin

[a] Correlation is significant at the 0.05 level (two-tailed)

[b] Correlation is significant at the 0.01 level (two-tailed)

239 patients, our study yields >80 % power to detect significant influence of this polymorphism in the septic and non-septic group ($p<0.05$).

In patients undergoing elective major surgery, cytokine profiles have previously been proposed as prognostic factors.[25] TNF-α secretions show a high degree of interindividual variability, which is at least partly genetically determined.[26] This study has focused on the relationship between TNF-α genotype; the preoperative and postoperative expression of TNF-α, IL-6, and IL-8; and development of postoperative sepsis.

In contrast to other studies,[11,13] our data showed significantly higher TNF-α whole-blood cytokine response in postoperative septic patients with homozygous AA genotype. Similar results were obtained in the Chinese Han population.[21] Increased level of TNF-α in septic patients as observed in our study has been confirmed by several studies.[14–16] These findings suggest the crucial role of TNF-α in mediating inflammatory response. In addition to TNF-α, IL-6 and IL-8 are also promising biomarkers of sepsis;[27–29] however, one study[30] reported IL-6 as an insensitive marker of sepsis. In our study, we observed higher levels of IL-6 and IL-8 after surgery in postoperative septic patients. In contrast to a previous report,[23] our results indicate that AA homozygous genotype is associated with higher IL-6 cytokine production after surgical trauma. Similar association was observed with IL-8 cytokine in septic patients, suggesting the potential role of this genotype in exacerbating inflammation and susceptibility to sepsis. Increased concentration of TNF-α, IL-6, and IL-8 cytokines and its association with TNF-α-308 polymorphism in early postoperative phase are of great clinical significance in forecasting the outcome of septic complications.

Though increased cytokine response may be due to surgical stress, Majetschak et al.[31] reported that the surgery-associated stress is transient (24 h) and does not contribute significantly to the increased cytokine response. In our study, in patients without postoperative sepsis, we found that the degree of TNF-α production in whole blood was not related to the TNF-α polymorphism. The significant associations of TNF-α-308 polymorphism with postoperative sepsis and postoperative TNF-α cytokine expression suggest an influence of this polymorphism on monocyte reactivity to inflammatory stimuli. However, this association cannot explain the significant difference in the preoperative cytokine levels of TNF-α-308 genotype subgroups in patients with postoperative sepsis.

Analysis of individual data in septic patients showed that preoperative and postoperative alterations of TNF-α and IL-6 cytokine levels were significantly linked with each other. Significantly positive correlation of TNF-α and IL-6 cytokine levels before surgery in both septic and non-septic groups indicates preexisting difference in leucocyte function, which augments specific alteration patterns after postoperative inflammatory stimuli. Further, a significant positive correlation of IL-8 cytokine with TNF-α and IL-6 in the postoperative period of septic patients indicates its association with severity of inflammation.

A possibility that major postoperative complications might be related to surgery or the patient cannot be ruled out. In the current study, to minimize surgeon-related variability, surgeons with similar proficiency operated the patients and as the first case of elective operating room. In agreement with other investigators,[32–34] we also found significant association of the length of surgery and the amount of blood loss with the development of postoperative septic complications (Table 1). Other patient factors such as age and gender had no influence on the development of postoperative septic complications. Though various studies indicate that preexisting patient-related factors (e.g., advanced age, male gender) may influence the development of postoperative complications,[32–38] these findings are inconsistent. Vogel et al.[39] described the rates of sepsis for a variety of hospital-based surgical procedures. Gastrointestinal, cardiovascular, or thoracic procedures accounted for nearly 50 % of cases, and the rates of postoperative sepsis were highest for esophageal, pancreatic, gastric, small bowel, hepatic, and biliary procedures. Hensler et al.[40] reported that the majority of postoperative sepsis occurs after esophagectomy. In our study, among all major elective gastrointestinal procedures, pancreatic surgeries accounted for majority (29.78 %) of the postoperative sepsis.

A limitation of the current study is that the influence of the type of surgical procedure with TNF-α-308 G/A polymorphism genotypes could not be established due to the small number of patients undergoing each type of surgery. However, our study included a selected cohort of patients who underwent gastrointestinal surgeries, and, in this group, patients with TNF-α AA homozygous genotypes when compared with patients with heterozygous GA and homozygous GG were found to be at high risk for postoperative sepsis.

Conclusion

Taking together, our results indicate that TNF-α-308 polymorphism is significantly associated with increased expression of TNF-α, IL-6, and IL-8 serum cytokines and with the development of postoperative sepsis.

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Conflict of Interest The authors declare that they have no competing interests.

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