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Study of the role of tumor necrosis factor-α (–308 G/A) and interleukin-10 (–1082 G/A) polymorphisms as potential risk factors to acute kidney injury in patients with severe sepsis using high-resolution melting curve analysis

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ABSTRACT

Rational: Septic acute kidney injury (AKI) is a prevalent complication in intensive care units with an increased incidence of complications.

Objective: The aim of the present study was to assess the use of high-resolution melting curve (HRM) analysis in investigating whether the genetic polymorphisms; –308 G/A of tumor necrosis factor-α (TNF-α), and –1082 G /A of Interleukin-10 (IL-10) genes may predispose patients diagnosed with severe sepsis to the development of AKI.

Methods: One hundred and fifty patients with severe sepsis participated in the present study; only sixty-six developed AKI. Both polymorphisms were studied using HRM analysis.

Main findings: The low producer genotype of both studied polymorphism of TNF-α and IL-10 genes was associated with AKI. Using logistic regression analysis, the low producer genotypes remained an independent risk factor for AKI. A statistically significant difference was detected between both studied groups as regards the low producer genotype in both TNF-α (–308 G/A) and interleukin-10 (IL-10) (–1082 G/A) polymorphisms being prevalent in patients developing AKI.

Principle conclusions: The low producer genotypes of both TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms could be considered a risk factor for the development of AKI in critically ill patients with severe sepsis, thus management technique implemented for this category should be modulated rescuing this sector of patients from the grave deterioration to acute kidney injury. Using HRM for genotyping proved to be a highly efficient, simple, cost-effective genotyping technique that is most appropriate for the routine study of large-scale samples.

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Introduction

Septic acute kidney injury (AKI) is a prevalent complication in intensive care units (ICU) with an increased incidence of wide spectrum of complications ranging from prolonged hospital stay to augmented incidence of mortality.[1]

Studies have correlated long-term morbidity and mortality in AKI cases to chronic inflammatory conditions created by the action of various inflammatory cytokines that contribute to renal vascular injury with consequent development of AKI.[2]

Inflammatory response is coordinated by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), which stimulates the synthesis of other pro-inflammatory cytokines, adhesion molecules, and anti-inflammatory cytokines, particularly interleukin-10 (IL-10) that inhibits the secretion of IL-1β, TNF-α, and IL-6, thus regulates pro-inflammatory cytokines production. The balance between pro- and anti-inflammatory cytokines affects the clinical outcome of various inflammatory conditions including AKI.[3]

TNF-α gene is located on chromosome 6p21 within the major histocompatibility complex class-III region.[4] The high-producer genotype (G/A or A/A) of TNF-α rs1800629 polymorphism, known as (–308 G/A), is associated with high promotor activity and has been correlated with augmented spontaneous and stimulated TNF-α production both *in vitro* and *in vivo*.[5]

IL-10 gene is located on chromosome 1q31-q32.[6] Studies indicated that the –1082 G allele of IL-10 rs 1800896 polymorphism; known as (–1082 G/ A), is associated with increased IL-10 production, whereas the A

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allele is associated with diminished IL-10 production, so-called low-producer genotype.[7]

Transcriptional activity of TNF-α and IL-10 genes is affected by polymorphisms involving their promoter regions such as –308 G/A and –1082 G/ A, respectively, thus interfering with gene function mainly through the relevant cytokine being produced.[3]

As TNF-α and IL-10 genes are considered inflammatory response modulators, thus the aim of the present study was to assess the use of high resolution melting curve (HRM) analysis in investigating whether the genetic polymorphisms of –308 G/A tumor necrosis factor-α gene, and –1082 G /A of IL-10 gene may predispose ICU patients diagnosed with severe sepsis to the development of AKI or not.

Materials and methods

One hundred and fifty patients with severe sepsis participated in the present study. All patients aged more than 18 years and were recruited from the ICU. Expected ICU length of stay was more than 48 h. Of all sepsis patients, only 66 developed AKI during their ICU stay.

Patients were included in the study if they met the criteria of severe sepsis[8] criteria of systemic inflammatory response syndrome[9] and at least one of the signs of organ dysfunction, with evidence of the source of infection proved either by culture or visual inspection. All patients' data were recorded daily until death, ICU discharge, or day 28 of admission.

AKI patients were excluded from the study if they were presenting to the ICU for the second time, admitted with history of chronic renal disease (defined as a baseline serum creatinine of 1.40 mg/dL or more) or maintained on chronic dialysis before ICU admission.

The Severity of illness was assessed by Acute Physiological and Chronic Health Evaluation-II score (APACHE II)[10] and Sequential Organ Failure Assessment (SOFA) scores.[11]

Patients' data were all registered in detailed sheets including age, gender, date of ICU admission, pre-existing underlying diseases, the duration of being on dialysis and the frequency of dialysis per week.

The study was approved by the ethics committee, Faculty of Medicine, Alexandria University. Each participant signed a written consent to participate in the study.

Renal function tests including urea, creatinine, and uric acid were offered for all patients. In addition, serum albumin and serum C-reactive protein (CRP) were assayed.

Genetic analysis

Extraction of genomic DNA

Genomic DNA was extracted from whole EDTA blood using PureLink® genomic DNA kit (Life Technologies, CA) according to manufacturer instructions. Concentration and purity of DNA were tested using NanoDrop (Thermo Scientific, Waltham, MA) then all samples were stored at −20 °C till further analysis.

Both TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms were studied using high resolution melting curve (HRM) analysis.

Primers sequences

Sequence of used primers for TNF-α (–308 G/A) polymorphism genotyping[12] is:

Forward primer: CCCCAAAAGAAATGGAGGCAATAGG

Reverse primer: GTAGGACCCTGGAGGCTGAAC

Sequence of primers used for IL-10 (–1082 G / A) polymorphism genotyping[13] is:

Forward primer: AATCCAAGACAACACTACTAAGGCTTC

Reverse primer: CTAAAGTTTAAAAGATGGGGT GGA

PCR amplification and high-resolution melting (HRM) genotyping

HRM genotyping was performed on the Rotor-Gene Q platform (Qiagen, Germany) using EvaGreen® HRM fluorescent dye.

Genetic variations for both SNPs were analyzed using amplification with subsequent high-resolution melting curve (HRM) analysis.

Type-it HRM PCR Kit (Qiagen, Germany) was used. The kit contains 2x HRM PCR Master Mix that includes HotStarTaq® Plus DNA Polymerase, Type-it HRM PCR Buffer (with EvaGreen® dye), Q-Solution® and dNTP mix (dATP, dCTP, dGTP, dTTP).

EvaGreen® dye is a dsDNA-binding fluorescent dye suited for HRM analysis that allows a highly efficient, inhibition-free, PCR amplification.[14]

Amplification was carried out in a final volume of 25 μL containing 2X HRM PCR Master Mix, 10 picomole per reaction for each of the forward and reverse primers, and DNA in a fixed concentration of 50 ng per reaction.

PCR cycling conditions included an initial Taq Polymerase activation step at 95 °C for 5 min followed by 40 cycles of denaturation for 10 s at 95 °C and

Table 1. Epidemiological and biochemical criteria of all cases in the study.

	AKI patients ($n = 66$)	Non-AKI patients ($n = 84$)	p
Gender			
Male	34 (51.5%)	41 (48.8%)	.742
Female	32 (48.5%)	43 (51.2%)	
Age	57.61 ± 6.75	56.91 ± 4.64	.514
Urea (mg/dL)	72.72 ± 17.60	39.22 ± 4.64	<.001*
Creatinine (mg/dL)	4.65 ± 1.28	0.89 ± 0.11	<.001*
Albumin (g/dL)	2.89 ± 0.26	2.93 ± 0.25	.319
CRP (mg/dL)	200.0 (110.0–320.0)	190.0 (108.0–330.0)	.694

Qualitative data were described using number and percent and was compared using Chi square test or Fisher Exact test. Normally quantitative data was expressed in (mean ± SD) and was compared using *t*-student test, while abnormally distributed data were expressed as median (min–max) and was compared using Mann Whitney test.
*Statistically significant at $p < .05$.

annealing/extension step for 30 s at 55 °C to allow for fluorescence data acquisition on the green channel.

After PCR amplification, the HRM was carried out over the range of 65–95 °C rising at 0.1 °C increments each cycle.

Control samples for both studied polymorphisms were obtained using 5′ nuclease assay prior to genotyping by HRM.

In each run, no template control (NTC) and a control of known genotype for each tested SNP were included.

The expected genotypes for TNF-α (–308 G/A) SNP are either the low producer phenotype; GG or the high TNF-α producer phenotype; GA or AA.

As for IL-10 (–1082 G/A) SNP, the expected genotypes are either the homozygous mutant genotype; AA, which is an IL-10 low producer phenotype, the heterozygous genotype (GA) which is an intermediate IL-10 producer or the high producer genotype; GG.

Statistical analysis

Data were analyzed using SPSS software package version 20.0 (SPSS, Chicago, IL). Qualitative data were described using number and percent and was compared using Chi square test or Fisher's exact test. Normally distributed quantitative data were expressed as mean ± SD and compared using student's *t*-test. Abnormally distributed data were expressed as median (Min–Max) and compared using Mann–Whitney *U*-test. Multivariate logistic regression analysis was used to assess variables potentially related to AKI occurrence incident to sepsis in ICU patients. Statistical significance was set at $p < .05$.

Results

One hundred and fifty patients with severe sepsis participated in the present study. On following up all cases, only 66 of them developed AKI.

No statistically significant difference was detected between both studied groups as regards age ($p = .514$) or gender ($p = .742$).

All epidemiological and biochemical criteria of those participating in the study are illustrated in Table 1.

AKI patients showed a higher incidence of vasoactive drug use (27.3% vs. 4.8%, $p < .001$). No statistically significant difference was detected between both studied groups and the occurrence of different diseases as heart failure/coronary heart disease, stroke or diabetes mellitus ($p = .405$, .731, and .934, respectively).

As regards the genotype distribution of TNF-α (–308 G/A) single-nucleotide polymorphism, most of the participants of either group (AKI or non-AKI patients) showed a higher percentage of the low producer phenotype GG (78.8% in AKI patients, and 59.5% in non-AKI patients).

In relation to the second studied polymorphism; IL-10 (–1082 G/A), most participants showed a greater percentage of the intermediate producer phenotype; GA in a percentage of 56.1% in AKI patients and 64.3% in patients who did not develop AKI.

A statistically significant difference was detected between both studied groups as regards the low producer genotype in both TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms being prevalent in patients developing AKI ($p = .012$ and .009, respectively)

The low producer allele of both TNF-α (–308 G/A) polymorphism; G allele, and IL-10 (–1082 G/A) polymorphism; A allele, prevailed in the group of patients developing AKI (87.9% and 56.8%, respectively).

In addition, a statistically significant difference was detected between both groups as regards the allele frequency of IL-10 (–1082 G/A) polymorphism ($p = .004$).

Thus, IL-10 (–1082 G/A) polymorphism was associated with the development of AKI in severely ill septic ICU patients.

Using logistic regression analysis, TNF-α (–308 G/A) polymorphism remained an independent risk factor for AKI after adjustment for age, gender, albumin, CRP, and TNF-α (–308 G/A) (OR =2.657, 95% CI: 1.239–5.698; $p = .012$).

In addition, on using logistic regression analysis, IL-10 (–1082 G/A) polymorphism also remained an

independent risk factor for AKI after adjustment for age, gender, albumin, CRP, and TNF-α (–308 G/A) (OR =3.025, 95% CI: 1.248–7.334; $p = .014$).

Discussion

Generally, AKI receives minimal attention by many clinicians due to the reversibility of the condition evidenced by the improvement of serum creatinine in many patients. Some cases of AKI may be at greater risk of long-term sequelae due to the development of permanent renal injury that affects renal microvasculature with subsequent persistent effects on renal structure and function, thus augmenting the risk of chronic kidney disease. In addition, AKI confers prolonged hospital stay and increased mortality by increasing the risk to cardiovascular diseases;[15] therefore, earlier medical intervention should be offered to this segment of patients to avoid numerous grave long-term complications.

Inflammatory conditions lead to secretion of pro-inflammatory cytokines as TNF-α, a key modulator of inflammation. This pro-inflammatory response is followed by increased expression of anti-inflammatory mediators particularly IL-10 that inhibits production of IL-1β, TNF-α, and IL-6, thus regulates the release of pro-inflammatory mediators and controls the inflammatory process.[16]

It was thought for many years that sepsis induced organ damage results from increased pro-inflammatory cytokines (cytokines storm) such as TNF-α and IL-1β released in response to infectious pathogens.[17] Therefore, several trials were done to inhibit TNF-α and IL-1β in order to prevent organ damage and improve survival, but anti-TNF-α failed to show any clinical significance.[18,19] Based on these findings, new concepts regarding the pathogenesis of organ damage in sepsis have been suggested.[20]

Postmortem studies were done to explain why some septic patients developed organ damage and died from sepsis. Boomer et al found a significant reduction of both pro-inflammatory and anti-inflammatory cytokines in splenocytes isolated from patients who died from sepsis, suggesting a defect in immune function in some septic patients.[21]

Based on these postmortem data, Hotchkiss et al. have proposed that the patient's dysregulated inflammatory response to stimuli may be a risk factor for development of organ damage and death in sepsis.[22]

These studies did not evaluate the genetic background of patients, which could explain the immune response defects observed in these patients.

In the present study, HRM technique was used for genotyping of TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms as potential risk factors for septic acute kidney injury. HRM technique is based on analysis of DNA melting from double-stranded DNA to single-stranded DNA with increasing temperature.[13]

The most prevailing genotype in the present study for both studied groups was the wild type (GG) as regards TNF-α (–308 G/A) polymorphism and the heterozygous genotype; GA as regards the IL-10 (–1082 G/A) polymorphism which come in conformity with other studies on Egyptians.[23,24]

A statistically significant difference was detected between both studied groups as regards the low producer genotype of both polymorphisms; TNF-α (–308 G/A) and IL-10 (–1082 G/A) prevailing in the group of patients who developed AKI. In addition, the low producer allele dominates in AKI patients for both studied polymorphisms.

On using logistic regression analysis, each polymorphism was the sole risk factor behind AKI after adjustment for age, gender, albumin, CRP and the other polymorphism.

Our results agree with the new concept of organ damage in sepsis suggesting a genetic predisposition to the impaired immune response in some patients with sepsis. Single-nucleotide polymorphisms that involve the promotor area of TNF-α and IL-10 genes influence the gene transcriptional activity and, therefore, affect the gene function.[25]

In vitro studies demonstrated that the –1082G allele is associated with high IL-10 production, whereas –1082A allele is associated with low IL-10 production.[7] In addition, the –308A allele was reported to enhance TNF-α secretion both *in vitro* and *in vivo*, while the –308G allele diminishes TNF-α production.[5]

TNF-α production is mandatory in regulation of innate and adaptive immune responses,[26] as reduced TNF-α production is associated with diminished expression of adhesion molecules on vascular endothelium and lack of initial immune response due to decreased migration of leucocytes to site of tissue injury with resultant sepsis by the diminished reaction to infectious agents.[27,28]

Dalboni et al[29] did not find significant difference between septic and non-septic AKI as regards TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms. But when they combined both polymorphisms together, they found that prevalence of the low TNF-α plus low IL-10 producer phenotypes was increased in patients with AKI, which supports our results. Using logistic regression analysis, the study proved that low TNF-α producer plus low IL-10 producer phenotype is considered an independent risk factor for AKI on adjustment for age, gender, ethnicity, APACHE II score, sepsis, albumin, and CRP.

The study of Dalboni et al[29] used PCR–sequence-specific primer (PCR-SSP) in genotyping, while in the current study, HRM analysis was used in genotyping of both TNF-α (–308 G/A) and IL-10 (–1082 G / A) polymorphisms.

HRM is a simple, rapid, and cost-effective method for genotyping and mutation screening that does not include the use of a fluorescent probe. Unlike most other mutation detection methods, which require additional detection post-amplification step, HRM is a closed-tube method in which PCR amplification is followed by immediate HRM analysis in a single run. Therefore, HRM is suitable for high-throughput mutation scanning. It also prevents contamination with PCR products that may occur with other mutation detection methods.[30]

Reduced secretion of TNF-α and IL-10 in critically ill patients compared to healthy controls was reported by previous studies.[31]

In another study, Cardinal-Fernández et al[32] studied various genetic polymorphisms including angiotensin-converting enzyme insertion/deletion; TNF-α –376, –308, and –238; IL-8 – 251; vascular endothelial growth factor (VEGF) + 405 and +936; and pre–B-cell colony-enhancing factor –1001 in a cohort of Brazilian severely ill sepsis patients with a group of them developing AKI. Univariate analysis revealed that only the VEGF +936 CC and the pre–B-cell colony-enhancing factor –1001 GG genotypes were associated with AKI. This can be attributed to different ethnic group studied as compared to the present study.

Sabelnikovs et al[33] studied 103 critically ill patients with sepsis. They found that nonsurvivors had significantly increased TNF-α and IL-10. In contrast to the present study, IL-10 –1082G allele was associated with a higher risk of death in severely septic patients, while TNF-α –308 A allele was not associated with adverse outcome.

Consequently, genetically determined altered secretion of pro-inflammatory and anti-inflammatory cytokines might have an association to AKI occurrence.

Conclusions

The low producer genotypes of both TNF-α (–308 G/A) and IL-10 (–1082 G/A) polymorphisms could be considered a risk factor for the development of AKI in critically ill patients with severe sepsis, thus management technique implemented for this category should be modulated rescuing this sector of patients from the grave deterioration to acute kidney injury. Using HRM for genotyping proved to be a highly efficient, simple, cost-effective genotyping technique that is most appropriate for the routine study of large-scale samples.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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