Abstract

Objectives: To evaluate the association between the genetic polymorphism of the tumor necrosis factor-alpha (TNF-α) gene and the development of sepsis and septic shock in Thai pediatric patients and to investigate the clinical impacts of TNF-α polymorphisms in this population.

Methods: To perform this genetic association study, a prospective analysis of pediatric patients (age < 18 years) with clinical sepsis/septic shock was conducted. All clinical data were collected by pediatric intensive care experts, and genetic analyses were performed at a central laboratory. A single nucleotide polymorphism (SNP) located in the 5’-promoter region at position -308 was genotyped and the results were associated with clinical phenotypes.

Results: A total of 167 Thai individuals were investigated, 66 of which were pediatric patients with sepsis/septic shock and 101 were healthy controls. Interestingly, we could not identify an association between sepsis and -308 (G/A) polymorphism, which have previously been demonstrated to be a major SNP associated with sepsis in several Caucasian populations, since there was no frequency difference between cases and controls.

Conclusions: In this report, the major TNF-α polymorphism (-308) was not associated with clinical sepsis/septic shock in Thais. This information will be important for future analyses to identify the role of TNF-α as a genetic risk for the development of immunopathology underlying several diseases in Asia.
given pathogens among different individuals play a critical role in the development of clinical sepsis/septic shock. The role of genetic influence on disease-associated mortality in particular infectious diseases has long been observed since the 1980s. Sørensen et al. reported that the death of biological parents due to infection conferred a greater risk for children of dying from the same cause, whereas the death of adoptive parents did not. In the past 10 years, after the completion of the human genome project, several studies, mainly in Caucasian populations, have shown a significant genetic variation in several common human diseases. Many studies on the matter indicate a strong genetic influence on susceptibility to sepsis/septic shock and might be used to predict clinical outcome. In addition, several infections were also found to be affected by genetic variation, including malaria, tuberculosis, leprosy, and infections by helicobacter pylori, HIV, and hepatitis B, among others.

Acute systemic response which produces excessive pro-inflammatory cytokines with inadequately counterbalanced production of anti-inflammatory mediators is the major pathogenesis of sepsis. Tumor necrosis factor-alpha (TNF-α), one of the most critical pro-inflammatory cytokines, plays a crucial role in the pathogenesis of this acute inflammatory response (acute phase reaction) and is involved in systematic inflammation. The TNF-α gene locates at chromosome 6p21.3 spanning approximately 3 kb and contains four exons to produce a 212 amino acid-long type II transmembrane protein arranged in stable homotrimers. Moreover, TNF-α has several roles on human immunopathology, from generating inflammation, cellular proliferation and differentiation, tumorigenesis, and viral replication to inducing apoptotic cell death. In clinical models of sepsis, the administration of TNF-α leads to hypotension, activation of the coagulation cascade, and organ dysfunction, supporting its role as an acute phase mediator. At the in vivo level, increased TNF-α levels have been observed after endotoxin challenge in healthy volunteers and also in patients with septic shock due to both gram positive and gram negative bacteremia. To this regard, it was not surprising that genetic association studies of the TNF-α gene in patients with sepsis/septic shock were one of the early genetic association studies in humans, considering the fact that understanding such genetic risk may confer a better understanding and improving management of lethal conditions such as sepsis. Through these analyses, mainly in Caucasian populations, the major genetic polymorphism within the regulatory regions of the gene coding for TNF-α gene has been identified: -308 (G→A). This transition of guanine to adenine located at the -308 base pair from the transcriptional start site was observed in several in vitro studies, demonstrating that this polymorphic change is associated with increased secretion of TNF-α from macrophages after lipopolysaccharide stimulation and that this potential increment might confer a similar response leading to clinical sepsis in vivo. Notwithstanding, there have been some conflicting data even in the same ethnic population, as a positive correlation between -308 polymorphism and clinical sepsis/septic shock was not always reproduced in other studies. However, considering that it is widely accepted that genetic polymorphism varies between ethnic groups, any comparative studies should be executed within the same genetic background. Nevertheless, it remains important to revisit all major reported polymorphisms in any population to demonstrate whether it is possible to utilize these genetic markers as a universal genetic risk for any given clinical condition. In this sense, we conducted the first prospective study in a Southeast Asian population to evaluate whether there is any association between the TNF -308A polymorphism and the development of sepsis and septic shock in Thai pediatric patients.

Methods

Patients and controls

A prospective study in eligible subjects including all patients with sepsis or septic shock who were Thai in origin and admitted to the pediatric intensive care unit (PICU) was conducted at Siriraj Hospital and Vajira Hospital from November 2007 to April 2009. The sepsis group was defined by the criteria of the International pediatric sepsis consensus conference: (1) evidence of SIRS as manifested by at least two out of four of the following criteria: core temperature > 38.5 °C or < 36 °C, tachycardia (heart rate > two standard deviations [SDs] above normal for age) or bradycardia in children < 1 year old (heart rate < 10th percentile for age), tachypnea (mean respiratory rate > two SDs above normal for age) or need for mechanical ventilation, leukocyte count elevated or depressed for age or > 10% immature neutrophils; (2) a suspected or proven infection. The septic shock group had to meet the following criteria: (1) evidence of sepsis; (2) cardiovascular dysfunction defined by hypotension (blood pressure [BP] < 5th percentile for age or systolic BP < two SDs below normal for age), or need for mechanical ventilation, leukocyte count elevated or depressed for age or > 10% immature neutrophils; (3) inappropriate antibiotic administration within 7 days prior to diagnosis of sepsis or septic shock.
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as determined by the investigators. The control group consisted of 101 healthy unrelated Thai medical students. Clinical demographic data – including underlying diseases, source of infection, responsible pathogens, clinical severity evaluation by the Acute Physiology and Chronic Health Evaluation (APACHE) score,²¹ and management outcomes (survival or death) – were collected and directly evaluated by two PICU experts (S.P. and M.M.). This study was approved by the internal review board and ethics committees at Siriraj Hospital and Vajira Hospital, Bangkok, Thailand. Informed consents were obtained from case and control subjects and/or their parents or guardians.

**TNF -308 genotyping**

Genomic DNA was extracted from 5-mL samples of EDTA anti-coagulated blood using the phenol-chloroform method. Genotyping for polymorphisms of the TNF-α -308 genes was done using the polymerase chain-restriction fragment length polymorphism method. The TNF-α gene promoter was amplified by using a modified protocol previously described²²: TNF-α forward primer (5'- AGG CAA TAG GTT TTG AGG GCC AT - 3') and TNF-α reverse primer (5'- ACA AGC ATC AAG GAT ACC CCT - 3'). The volume for the PCR mixture was 25 µL, containing 0.5 mmol/L of each primer, 1 X PCR buffer, 1.5 mmol/L of MgCl₂, 0.2 mmol/L of each nitrogenated base, 1.25 U of Taq DNA polymerase, 50 ng of DNA, and sterile water. PCR was performed on a MJ mini thermal cycler (Bio-Rad, USA) according to the following program sequence: 95 °C for 5 min, followed by 31 cycles consisting of 95 °C for 30 s, 54.3 °C for 35 s, and 72 °C for 1 min, with a final extension at 72 °C for 15 min. The size of the amplified PCR product was 139 bp and it was subsequently digested overnight with NcoI (recognition site: 5'-CATGG-3') at 37 °C to detect G to A transition at the -308 promoter site. Digested PCR fragments were analyzed by electrophoresis in a 3% agarose gel, visualized by ethidium bromide staining, and documented on Gel Doc System (DNR Imaging System®, Israel). A transition of G→A generates a new digested site resulting in two digested fragments of 119 and 20 bp, while the wide type (G) remains unchanged.

**Statistic analysis**

Descriptive data were expressed as frequency (%) or as mean ± standard deviation. A two-tailed Student t test was used for the comparison of continuous variables with normal distribution and the Mann-Whitney U test was used for continuous variables without normal distribution. Association studies between different groups were analyzed using the chi-square test. A p-value of less than 0.05 was considered statistically significant.

**Results**

Sixty-six Thai patients meeting all the above mentioned criteria of sepsis/septic shock were enrolled, 43 of which were patients with sepsis and 23 with septic shock. Demographic characteristics of patients with sepsis and septic shock are summarized in Table 1. We calculated the values of APACHE score based on underlying conditions and the severity of selected patients. Overall, the PICU mortality rate of 19.7% was in agreement with the predicted risk of death based on the APACHE score. Only two baseline clinical characteristics showed significant differences between survivors and non-survivors (deaths): age at diagnosis (31.2 vs. 75.2 months, respectively) and APACHE score (9.0 vs. 15.1, respectively), as shown in Table 1.

We successfully developed a simple genotype analysis to discriminate between G and A allele (AA) of the TNF-α -308 polymorphism. This PCR-based assay was confirmed by direct genomic sequencing of the TNF promoter region (data not shown). All cases and controls enrolled were studied for this major single nucleotide polymorphism. The genotype distribution of G and A (rare allele) was normally distributed based on Hardy Weinberg disequilibrium in all populations (healthy control and sepsis/septic shock patients) as shown in Table 2. The frequency of AA did not differ among the different groups and none of the patients in the septic group had homozygosity of AA. In a sub-group analysis of the septic shock group, 13 out of 23 subjects included in the group were survivors. Again, the distribution of G and AA was not significantly different between patients who survived and those who died (data not shown).

**Discussion**

This study examines, for the first time in Southeast Asia, the association between the TNF2 allele (which comprises a G-308A transition within the promoter of the TNF-α gene) and susceptibility to sepsis or septic shock, as well as mortality related to these events in our intensive care units. Increasingly, genetic variation in human population was evidenced and several genetic polymorphisms were associated with predisposition to the development of sepsis or septic shock and with poorer outcome. For example, Nadel et al. demonstrated a higher frequency of the TNF-α -308A allele in children with meningococcal disease who died compared with those who survived in the British population. In a French study by Mira et al., the authors reported that the frequency of the AA at the TNF-α -308 again was higher in adult patients with septic shock when compared to French healthy controls. Moreover, among French patients with septic shock, the frequency of the AA was also significantly higher in those
Table 1 - Clinical characteristics of 66 Thai patients with sepsis/septic shock

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sepsis/septic shock (n = 66)</th>
<th>Survivors (n = 53)</th>
<th>Non-survivors (n = 13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months, mean (SD)</td>
<td>39.8 (51.1)</td>
<td>31.2 (45.2)</td>
<td>75.2 (60.1)</td>
<td>0.025</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>32 (48.5)</td>
<td>25 (47.2)</td>
<td>7 (53.8)</td>
<td>NS</td>
</tr>
<tr>
<td>APACHE score*, mean (SD)</td>
<td>10.2 (7.6)</td>
<td>9.0 (7.2)</td>
<td>15.1 (7.4)</td>
<td>0.008</td>
</tr>
<tr>
<td>Primary site of infection, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>40 (60.6)</td>
<td>34 (64.2)</td>
<td>6 (46.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>5 (7.6)</td>
<td>4 (7.5)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Cutaneous/soft tissue</td>
<td>4 (6.1)</td>
<td>3 (5.7)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>2 (3.0)</td>
<td>1 (1.9)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>5 (7.6)</td>
<td>5 (9.4)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Hematogenous</td>
<td>5 (7.6)</td>
<td>3 (5.7)</td>
<td>2 (15.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Others</td>
<td>5 (7.6)</td>
<td>3 (5.7)</td>
<td>2 (15.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Microorganisms, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram-negative</td>
<td>25 (37.9)</td>
<td>21 (39.6)</td>
<td>4 (30.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Gram-positive</td>
<td>6 (9.1)</td>
<td>5 (9.4)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Virus</td>
<td>6 (9.1)</td>
<td>5 (9.4)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Fungus</td>
<td>4 (6.1)</td>
<td>2 (3.8)</td>
<td>2 (15.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Mixed</td>
<td>7 (10.6)</td>
<td>6 (11.3)</td>
<td>1 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Others</td>
<td>18 (27.3)</td>
<td>14 (26.4)</td>
<td>4 (30.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

APACHE = Acute Physiology and Chronic Health Evaluation; NS = not significant; SD = standard deviation.
* This score evaluation system was applied within 24 h of admission of the patient to the intensive care unit; higher scores correspond to more severe disease and a higher risk of death.

who died. Although some evidence appears to support this notion, several other studies could not replicate such association. In a study conducted by Stuber et al.,26 no difference was observed between healthy controls and adults admitted to the surgical intensive care unit (ICU) for severe postoperative sepsis or between survivors and non-survivors (deaths) in the subgroup of patients with severe sepsis. The strength of this study is that cases and controls were well matched regarding disease severity and were ethnically homogeneous. All cases and controls were Caucasian (German) and originated from the same geographical region. In addition, no increased risk of septic shock or mortality was observed in postoperative surgical patients with TNF-α -308A allele among a Chinese adult population from Taiwan, according to the report by Tang et al.27 However, within the septic shock group, patients who carried the TNF-α -308A allele had a greater mortality rate than those who did not.

Table 2 - Distribution of TNF -308 genotypes and allele frequencies in control and sepsis groups

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Control group (n = 101)</th>
<th>Sepsis/septic shock group (n = 66)</th>
<th>p</th>
<th>Survivors (n = 53)</th>
<th>Non-survivors (n = 13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>-308 GG, n (%)</td>
<td>86 (85.1)</td>
<td>58 (87.9)</td>
<td>NS</td>
<td>48 (90)</td>
<td>10 (76.9)</td>
<td>NS</td>
</tr>
<tr>
<td>-308 GA, n (%)</td>
<td>13 (12.9)</td>
<td>8 (12.1)</td>
<td>NS</td>
<td>5 (10)</td>
<td>3 (23.1)</td>
<td>NS</td>
</tr>
<tr>
<td>-308 AA, n (%)</td>
<td>2 (2.0)</td>
<td>0</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>A allele frequency, %</td>
<td>6.4</td>
<td>6.1</td>
<td>NS</td>
<td>4.7</td>
<td>11.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

A = rare allele; G = wide type; NS = not significant.
Herein, our study demonstrates that no difference in the frequency of TNF2 allele was found between control group and sepsis/septic shock group and no increased mortality was reported in association with TNF2 allele. Besides that, in a subgroup analysis of patients with septic shock, the frequency of TNF2 allele in non-survivors was not different from that observed in survivors. In our study, all patients and healthy controls were ethnic Thai who lived mainly in Bangkok and surroundings, which made our study population appear to be homogeneous and might have reduced the confounding effect of population admixture and migration that might be evident in other studies. Several explanations may be considered for our negative findings in this study. Firstly, many different genetic variations might play roles on the development of sepsis or septic shock, and some of them might operate on the TNF and other system (or pathway) for the development of sepsis or septic shock, and some of them might influence the TNF production,17,28 other studies showed contrary results.29,30 It could be possible that TNF-α -308 allele, per se, does not directly influence TNF-α production, but merely works as a genetic marker linked to other regulatory element(s) located on chromosome 6 or to the HLA clusters that might affect straight to the TNF production. This link-marker association might be represented in one ethnic group due to a founder effect, but might not necessarily be reproducible in others genetic groups which have a different ancestral chromosome and evolved through a genetic drift and a bottleneck effect. Finally, the pathophysiology of sepsis or septic shock is a complex and multi-factorial process; a large number of factors affect these conditions other than genetic polymorphism, including the virulence of the etiologic organisms, the length of time between onset of disease and initiation of treatment, or the appropriate monitoring and management of disease or underlying condition. The environmental factors found in Thailand, including type and source of pathogens in our country (which is in the tropics) and appropriateness of clinical management before the development of sepsis and underlying diseases, might be different from those observed in other regions. These factors definitely confound genetic association, if there is any, when one tries to compare and reproduce findings from one population to another.

In addition, there were also some limitations in this study. The first one is the fact that TNF-α levels could be not obtained in this study due to technical difficulties; therefore, we could not demonstrate a correlation between different -308 genotypes and TNF production as shown previously.17,18 The second is a relatively small population of patient subjects, in particular of septic shock patients, which might have some effect on the power of our analysis. Finally, we could only compare septic cases with healthy controls; however, the ideal should be compare individuals who were exposed to the same type and magnitude of infectious pathogens but did not develop clinical sepsis/septic shock. Nonetheless, such controls seem to be difficult to identify, which might hamper the feasibility of the study.

In summary, this study has failed to show an association between TNF-α -308 polymorphism and the risk and the severity of sepsis or septic shock in the Thai population. Further studies including a larger population, with additional polymorphisms or haplotype analysis of TNF-α, should be warranted.

Acknowledgements

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References


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