

# Clinical Relevance of the *TLR4* 11367 Polymorphism in Patients With Major Trauma

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**Objective:** To investigate the clinical relevance of the *TLR4* 11367 polymorphism in patients with major trauma.

**Design:** Genetic functional and association study.

**Setting:** Daping Hospital and Chongqing Emergency Medical Center, Chongqing, China.

**Patients:** A total of 132 patients with major trauma were prospectively recruited.

**Main Outcome Measures:** The *TLR4* 11367 polymorphism was genotyped using single-tube, bidirectional, allele-specific amplification method. Whole peripheral blood samples obtained within 24 hours after admission were stimulated with lipopolysaccharide and then tested for production of tumor necrosis factor  $\alpha$  and interleukin 6. Sepsis morbidity rate and multiple organ dysfunction scores were assessed.

**Results:** The 11367 polymorphism was shown to be strongly associated with less capacity of peripheral leukocytes to produce tumor necrosis factor  $\alpha$  and interleukin 6 in response to ex vivo lipopolysaccharide stimulation in patients with trauma at admission. Results from association study indicated that patients with trauma who carry the 11367C allele were less likely to have sepsis and multiple organ dysfunction.

**Conclusions:** Combined with our previous in vitro functional study, the results suggest that the *TLR4* 11367 polymorphism might be a good predictor of who is more likely to develop complications such as sepsis or multiple organ dysfunction syndrome, depending on genotype.

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**T**RAUMA IS THE FOURTH LEADING cause of death worldwide.<sup>1</sup> One of the most serious complications of major trauma is the sequential dysfunction of vital organs, which is associated with posttraumatic sepsis in

## See Invited Critique at end of article

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most cases.<sup>2,3</sup> Therefore, preventing sepsis and subsequent organ dysfunction is crucial to the treatment of surviving patients with major trauma. Growing evidence suggests that genetic variants, particularly single-nucleotide polymorphisms (SNPs), are critical determinants of both the susceptibility to and outcome after infectious and noninfectious diseases.<sup>4-6</sup> Delineating the variation in genes and associated differences in response to infection may contribute to the development of new

genetically tailored diagnostic and therapeutic interventions that will improve outcomes for patients with major trauma.

Toll-like receptor 4 (TLR4), the crucial member of the type-1 transmembrane receptor family, is essential for initiating the innate response to lipopolysaccharide (LPS) and the structurally similar lipoteichoic acid components of gram-negative and gram-positive bacterial cell walls, respectively.<sup>7</sup> Toll-like receptor 4 has been demonstrated to play a crucial role in the development of sepsis and multiple organ dysfunction syndrome after major trauma and recognized as a key component of the initial injury response.<sup>8-10</sup>

There is growing evidence that genetic variation in the *TLR4* gene is intimately associated with responsiveness to gram-negative bacteria or LPS<sup>11,12</sup> and susceptibility to infectious and noninfectious diseases.<sup>13-25</sup> The *TLR4* G11367C polymorphism is a novel variant that we

previously identified in the 3' untranslated region of the *TLR4* gene in the Chinese Han population.<sup>26</sup> Our study indicated that this polymorphism is a functional SNP, indicating lower expression of *TLR4* and responsiveness of peripheral blood leukocytes in response to ex vivo LPS stimulation. This might be due to its effect on the post-transcriptional regulation of the 3' untranslated region.<sup>27</sup>

In view of the functionality of the *TLR4* 11367 polymorphism and the central role of *TLR4* in the pathogenesis of posttraumatic complications, this study further investigates the clinical relevance of the *TLR4* 11367 polymorphism in patients with major trauma.

## METHODS

### STUDY POPULATION

A total of 132 patients with major trauma were recruited to the study. All of them are Han Chinese and live in Chongqing, China. The 132 patients with trauma (91 men and 41 women) were admitted to the department of trauma surgery in the Daping Hospital and the Chongqing Emergency Medical Center, Chongqing, China, between January 1, 2005, and June 1, 2006. They were enrolled in the study if they met the following criteria: (1) between 18 and 65 years of age, (2) expected Injury Severity Score greater than 16, and (3) probability of survival greater than 1 week. Patients were not eligible if they had penetrating injuries or preexisting cardiovascular, respiratory, renal, hepatic, hematologic, or immunological diseases. The Injury Severity Score was assessed according to the Abbreviated Injury Scale (Update 98) by independent evaluators.<sup>28</sup> All patients requiring surgical intervention received standard surgical care and postoperative treatment in the intensive care unit. The protocol for this study was approved by the Ethical and Protocol Review Committee of the Third Military Medical University, and the informed consent was obtained from the patients or their next of kin. Patient confidentiality was preserved according to the guidelines for studies of human subjects.

### GENOTYPING

The *TLR4*G11367C polymorphism was genotyped using single-tube, bidirectional, allele-specific amplification.<sup>29</sup> Two specific primer pairs were designed: forward outer primer (F1) 5'-GTC ATT CCA AAG TTA TTG CCT ACT AAG-3', reverse outer primer (R1) 5'-GTG ATA TCT CAT TGT GGT TTT TAT TTT C-3', forward inner primer (F1') 5'-TAAACCCGGGGTGACCT-CATGAAATCAG-3', and reverse inner primer (R1') 5'-TCTGAAAAAATAAACTTCTGCTGCTAG-3'. The F1' and R1' primers were used to amplify a 398-base pair (bp) DNA fragment containing the G allele, F1 and R1' to amplify a 257-bp DNA fragment containing C allele, and F1 and R1 to amplify a 599-bp DNA fragment as an internal control. Polymerase chain reaction conditions were as follows: 2 minutes at 95°C followed by 30 cycles of 40 seconds at 95°C, 1 minute at 70°C, and 40 seconds at 72°C. For touchdown reactions, the annealing temperature was 70°C for the first cycle, decreasing by 0.5°C per cycle until the annealing temperature reached to 60°C, then continuing at 60°C in the annealing step of the remaining cycles. Polymerase chain reaction products were electrophoresed in a 2% agarose gel, with subsequent staining by ethidium bromide. The genotype was determined according to electrophoresis bands, which were

further confirmed by DNA sequencing with 10 random samples (Takara Biotech, Dalian, China).

### EX VIVO STIMULATION OF WHOLE BLOOD WITH LPS

A human whole-blood assay was used as described previously.<sup>30</sup> In brief, aliquots of whole blood collected from patients with trauma within 24 hours after admission were mixed 1:1 with RPMI 1640 culture medium (Hyclone, Logan, Utah) and incubated with 100-ng/mL LPS (*Escherichia coli* O26:B6; Difco Laboratories, Detroit, Michigan) in a sample mixer at 37°C for 4 hours.

### TUMOR NECROSIS FACTOR $\alpha$ AND INTERLEUKIN 6 ASSAY

The supernatants were separated from the whole blood after ex vivo LPS stimulation. The levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin (IL-6) in the supernatants were determined with enzyme-linked immunosorbent assay according to the manufacturer's instructions (R & D Systems, Minneapolis, Minnesota).

### CLINICAL EVALUATION

After admission, patients with major trauma were monitored throughout the whole hospital course in the following 6 aspects: respiratory function (PO<sub>2</sub>/fraction of inspired oxygen [FI<sub>O</sub><sub>2</sub>] ratio), renal function (serum creatinine concentration), hepatic function (serum bilirubin concentration), cardiovascular function (pressure-adjusted heart rate), hematologic function (platelet count), and central nervous system function (Glasgow Coma Scale). Multiple organ dysfunction (MOD) was then scored using the method by Marshall et al,<sup>31</sup> and the highest scores were used for association analysis. Sepsis was defined if patients met all of the following criteria: clinical evidence of infection, body temperature greater than 38.5°C or less than 36.5°C, and leukocyte count greater than 10 × 10<sup>9</sup>/L or less than 4 × 10<sup>9</sup>/L. The MOD scores and sepsis status were determined by individuals who did not know the genotypes (W.G. and Q.L.).

### STATISTICAL ANALYSIS

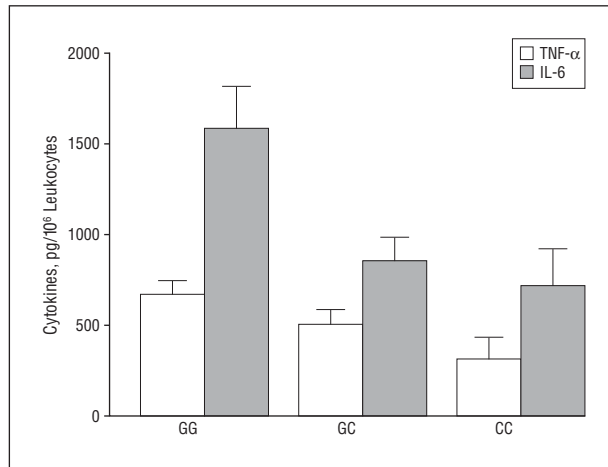
Genotype distribution was tested for departure from Hardy-Weinberg equilibrium using  $\chi^2$  analysis. The association between the G11367C polymorphism and plasma cytokine levels and MOD scores was assessed using analysis of covariance, with age, sex ratio, and injury severity used to adjust for possible confounding effects. Three genetic models such as allele dose, dominant, or recessive model were used. For dominant effect, we compared the C variant allele carriers (heterozygous and homozygous genotypes) with noncarriers (G allele homozygous genotype), whereas for recessive effect, subjects homozygous for the C allele were compared with heterozygous carriers and noncarriers. Allele dose was defined as the number of copies of C allele in the genotype. We performed linear regression analysis to quantify the allele dose effect. The association of genotypes with sepsis morbidity rate was determined by  $\chi^2$  analysis. Odds ratios with 95% confidence intervals were calculated by multiple logistic regression analyses to estimate the relative risk of sepsis. Results were considered to be significant when  $P < .05$ . All statistic analysis was carried out using SPSS Version 11.0 (SPSS Inc, Chicago, Illinois).

**Table. Clinical Relevance of the *TLR4* 11367 Polymorphism in Patients With Major Trauma by Genotype**

Variable	GG	GC	CC	Total	P Value by Association <sup>a</sup>	
					Dominant	Recessive
Sample, No.	90	35	7	132		
Mean (SD) age, y	37.4 (15.0)	38.8 (12.0)	39.3 (11.8)	37.8 (14.1)		
Sex, M:F, No.	59:31	26:9	6:1	91:41		
Mean (SD) ISS	25.6 (6.2)	25.5 (8.6)	25.5 (7.8)	25.5 (8.2)		
Mean (SD) MOD score	2.9 (2.3)	1.2 (1.1)	1.8 (0.4)	2.4 (2.1)	<.001	.47
Patients with sepsis, No.	60 (66.7)	17 (48.6)	2 (28.6)	79 (59.8)	.02	.09

Abbreviations: ISS, Injury Severity Score; MOD, multiple organ dysfunction.

<sup>a</sup>P values for dominant association are for CC + GC vs GG; for recessive association, CC vs GC + GG; P = .001 for allele dose association for MOD score.



**Figure.** Association of the *TLR4* 11367 polymorphism with the capacity of peripheral leukocytes to produce proinflammatory cytokines in patients with trauma at admission. The whole blood samples, collected from patients with trauma within 24 hours of admission, were mixed 1:1 with RPMI 1640 culture medium and incubated with 100 ng/mL of *Escherichia coli* lipopolysaccharide (O26:B6) at 37°C for 4 hours. Tumor necrosis factor (TNF $\alpha$ ) and interleukin 6 (IL-6) levels in the culture supernatants were assayed by a sandwich enzyme-linked immunosorbent assay and expressed as mean (standard error) in picograms per 10<sup>6</sup> leukocytes. For TNF $\alpha$ : P = .04 for dominant association of CC + GC vs GG; P = .16 for recessive association of CC vs GC + GG; and P = .04 for allele dose association. For IL-6: P = .006 for dominant association of CC + GC vs GG; P = .28 for recessive association of CC vs GC + GG; and P = .009 for allele dose association.

## RESULTS

### ALLELE FREQUENCIES AND GENOTYPE DISTRIBUTION

The overall allele frequencies of the *TLR4* G11367C polymorphism in the cohort of the patients with trauma were 81.4% and 18.6%, respectively, which is quite similar to our previous study of a healthy Han Chinese population.<sup>27</sup> The genotype distribution was in Hardy-Weinberg equilibrium (P = .95).

### ASSOCIATION WITH CAPACITY TO PRODUCE PROINFLAMMATORY CYTOKINES

In view of the functional effect of the 11367 SNP, as shown in our previous article,<sup>27</sup> we further investigated the clinical relevance of this SNP in 132 patients with major trauma (mean [SD] Injury Severity Score, 25.5 [8.2]). There were

no statistically significant differences in age, sex ratio, or Injury Severity Scores among patients stratified according to the different genotypes of the 11367 locus (P = .88, P = .42, and P = .93, respectively; **Table**). First, we examined the association of this polymorphism with the capacity of the peripheral leukocytes to produce cytokines in patients with trauma at admission. Our results showed that both TNF $\alpha$  and IL-6 production in response to ex vivo LPS stimulation were strongly associated with the 11367 polymorphism, with a significant difference in the case of dominant effect (P = .04 for TNF $\alpha$ ; P = .006 for IL-6). Data from linear regression analysis further indicated that this association with LPS-induced cytokine production was significantly allele-dose dependent (P = .04 and P = .009, respectively; **Figure**).

### CLINICAL RELEVANCE OF THE *TLR4* 11367 POLYMORPHISM

To examine whether the *TLR4* 11367 polymorphism is associated with the outcome of patients with severe trauma, we further investigated the association of this polymorphism with the development of sepsis and multiple organ dysfunction syndrome. The Table showed that patients with the C variant allele had significantly lower sepsis morbidity than those homozygous for the G allele (P = .02 for CC + CG vs GG). Data from multiple logistical regression analyses indicated that this polymorphism was significantly associated with lower risk of sepsis (odds ratio, 0.428; 95% confidence interval, 0.219-0.838; P = .01). In addition, MOD scores in the patients with trauma who carry the C allele were also significantly lower than those in the patients carrying the G allele, showing a significant difference in case of dominant effect (P < .001). Our data from linear regression analysis indicated that this association with MOD scores was significantly allele-dose dependent (P = .001). The association of the *TLR4* 11367 polymorphism with sepsis morbidity and MOD scores was not influenced by age, sex ratio, or Injury Severity Score.

## COMMENT

Although an increasing number of studies indicate that genetic variation in the *TLR4* gene may be a risk determinant for both infectious and noninfectious diseases,<sup>13-25</sup> most concentrate on the variation at positions

896 and 1196 of *TLR4*.<sup>13-25</sup> Both, leading to amino acid replacement at positions 299 and 399, respectively,<sup>22</sup> have been shown to affect LPS-induced cellular activation<sup>22</sup> and to be associated with increased risk of sepsis in patients with burn injuries.<sup>19</sup> However, these two polymorphisms have not been identified in Chinese<sup>26,27,32</sup> or other Asian populations.<sup>33,34</sup>

The *TLR4* 11367 polymorphism is an SNP we identified in the 3' untranslated region of the *TLR4* gene in the Chinese Han population via direct sequencing.<sup>26</sup> Our previous study indicated that this polymorphism might affect the expression of the target gene and cytokine production of peripheral blood leukocytes by affecting the regulatory effect of the 3' untranslated region of the *TLR4* gene.<sup>27</sup> Given the functionality of the *TLR4* 11367 polymorphism, we further hypothesized that this genetic variation might affect the outcome of patients with major trauma. To avoid confounding association owing to inappropriate patient-control matching, we only selected patients with trauma, and followed them up prospectively to determine whether those who had the genetic variant had better outcome. To elucidate the clinical relevance of this polymorphism, we first investigated its association with the capacity of peripheral leukocytes to produce TNF $\alpha$  and IL-6 in patients with trauma. Identical to our previous report,<sup>27</sup> the capacity of peripheral blood leukocytes to produce TNF $\alpha$  is much lower at admission in patients with the *TLR4* 11367C variant allele than those with the 11367G allele. In addition to TNF $\alpha$ , the production of IL-6 is also shown to be much lower in patients with the *TLR4* 11367C variant allele. These results indicate that the *TLR4* 11367 polymorphism might effect a proinflammatory response in patients with trauma.

Sepsis and multiple organ dysfunction are the 2 major complications that affect the outcome of patients with severe trauma.<sup>35,36</sup> An overwhelming inflammatory immune response has been recognized as central in the pathogenesis of sepsis and multiple organ dysfunction,<sup>2,3</sup> in which *TLR4* plays an important role.<sup>8-10</sup> Given the functional influence of the *TLR4* 11367 polymorphism on the capacity of peripheral leukocytes to produce inflammatory cytokines in patients with trauma, we further hypothesized that this variation might affect the development of sepsis and multiple organ dysfunction syndrome in patients with major trauma. As expected, the *TLR4* 11367 polymorphism appears to be associated with lower risk of sepsis in patients with trauma. This is significantly true in the case of dominant effect ( $P = .02$ ), and approaches significance in the case of recessive effect ( $P = .09$ ). This indicates that patients with trauma who carry the 11367 C variant allele have a decreased risk of sepsis. Sepsis has been recognized as an important cause of multiple organ dysfunction syndrome in patients with trauma.<sup>34</sup> Therefore, the *TLR4* 11367 polymorphism, in addition to association with sepsis, is also shown to be associated with lower risk of multiple organ dysfunction syndrome, showing significant differences in MOD scores in the case of dominant ( $P < .001$ ) and allele-dose ( $P = .001$ ) effects, but not in the case of recessive effect ( $P = .47$ ). Regarding possible reasons for the negative association in the case of recessive

effect, one important reason might be the polygenetic and multifactorial involvement in the pathogenesis of sepsis and multiple organ dysfunction after trauma.<sup>2</sup> In fact, there is increasing evidence that genetic polymorphisms of other genes are associated with the development of posttraumatic complications such as IL-1,<sup>29</sup> TNF  $\alpha$ ,<sup>37</sup> IL-6,<sup>38</sup> IL-10,<sup>39</sup> myeloid differentiation-2,<sup>40</sup> interferon- $\gamma$ ,<sup>41</sup> and IL-18.<sup>42</sup> The susceptibility to sepsis and organ dysfunction in patients with trauma might be the result of a combination of numerous genetic polymorphisms. Another possible reason might be the small sample size, especially in the case of sepsis; only 79 of 132 patients with trauma develop sepsis. The inadequate power may result in false-negative associations. A large sample size will be needed to have adequate power to confirm the clinical relevance of the *TLR4* 11367 polymorphism.

There are some limitations to our study. In addition to the small sample size, the population we studied was only Han Chinese persons who lived in the Chongqing district. As we did not include those who live in other districts in China, our results may not be generalized to other populations. Second, difficulties in obtaining additional blood samples did not allow us to determine plasma cytokine concentrations in patients with major trauma; therefore, the in vivo association between both polymorphisms and plasma cytokine levels could be confirmed in this study.

Despite the limited power of this clinical study, it further demonstrates the clinical relevance of the *TLR4* 11367 polymorphisms in patients with major trauma on the basis of our in vitro functional study. Our data suggest that this SNP might be used as relevant risk estimate for sepsis and multiple organ dysfunction in patients with major trauma.

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**Additional Information:** Both Drs Duan and Gu are principal investigators of this study.



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