Original Article *Iran J Ped Hematol Oncol. 2023, Vol 13, No 3, 172-181*

Evaluatin of Association of rs4986790 and rs4986791 Single-Nucleotide Polymorphisms in TLR4 with Febrile Neutropenia in Childhood Acute Lymphoblastic Leukemia

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Received: 21 May 2022 **Accepted:** 08 February 2023

Abstract

Background: Infections cause significant complications and, in severe cases, death in patients with childhood Acute Lymphoid Leukemia (ALL). Toll-like receptors (TLRs) play a crucial role in initiating innate immune responses. Previous studies indicate the role of TLR4 gene polymorphisms in the increased risk of infection in adults and children. This study investigated the potential association between Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms in the TLR4 gene with febrile neutropenia, as a hallmark of infection, in children with ALL.

Material and Methods: This cross-sectional study was performed on 51 ALL child patients, with age (mean±s.d.) 5.2 ± 3.4 years. Genotype analysis of rs4986790 and rs4986791 polymorphisms in the TLR4 gene was evaluated by ARMS- PCR and PCR-RFLP, respectively. Statistical analysis was performed using SPSS software. P-values <0.05 were considered significant.

Results: The rs4986790 and rs4986791 polymorphisms were detected in 5.8% and 7.8% of ALL patients, respectively. The mean of recurrence of febrile neutropenia in patients without TLR4 rs4986790 and TLR4 rs4986791 polymorphisms was 3.1 ±2 and 3 ±1.9, respectively, while in patients with TLR4 rs4986790 and TLR4 rs4986791 polymorphisms, they were 4.6 ± 3 and 5.2 ±2.8, respectively (P = 0.09). No association was found between TLR4 rs4986790 and rs4986791 polymorphisms and the number of febrile neutropenia recurrences (P = 0.4).

Conclusion: Although rs4986790 and rs4986791 polymorphisms were detected in ALL patients, these polymorphisms were not associated with febrile neutropenia. It is suggested to investigate other polymorphisms in immune system-related genes and their role in febrile neutropenia.

Keywords: Acute Lymphoid Leukemia, Febrile Neutropenia, Toll-Like Receptor 4

Introduction

The aberrant and altered patterns of gene Acute Lymphoblastic Leukemia (ALL), a major malignancy in children, occurs due to chromosomal abnormality and dysregulation of the cell cycle, which leads to low rates of complete remission after conventional chemotherapy (1, 2). The chemotherapy for ALL patients leads to absolute neutropenia due to wide bone-marrow suppression focused on hematopoietic stem cells, progenitors, and precursors (3). Absolute neutropenia increases the susceptibility of the infection, as a secondary challenge of ALL chemotherapy, because of its major role in innate and humoral immunity (4). Toll-like receptor 4 (TLR4) is a transmembrane protein from the pattern recognition receptor family, also known as cluster of differentiation (CD) 284 (5). TLR4 plays a critical role in neutrophil inducing and stimulating through lipopolysaccharide (LPS)-induced immunogenicity. Also,

[DOI: 10.18502/ijpho.v13i3.13129]

TLR4 controls the lifespan and activation of neutrophils. Activation of TLR4 leads to the inducing of NF-κB intracellular singling pathways, pro-inflammatory and inflammatory cytokine expression, and secretion, which lead to activation of innate immunity (6). So, it has been suggested that the genetics of TLR4 contributes to full activation and chemotherapy-induced neutropenia.

Few studies have been performed on the effect of TLR4 polymorphism on apoptosis and neutrophil lifespan following chemotherapy, and there is controversy about this effect (7). Polymorphisms in TLR4 may reduce the responses to LPS ligands. Also, it may contribute to hyporesponsiveness and inhibit the long-term lifespan of neutrophils (8). The most frequent single-nucleotide polymorphisms (SNPs) of the TLR4 gene are Asp299Gly (rs4986790) and Thr399Ile (rs4986791) which have revealed impairment in ligand and ligand-receptor (LPS and TLR4, respectively) binding (9). In a meta-analysis, Lui et al. showed that rs4986790 or rs4986791 polymorphisms are not associated with sepsis susceptibility (10). But there are some studies regarding the establishment of the association of rs4986790 or rs4986791 polymorphisms with susceptibility to infection with a specific pathogen. He et al. (11) and Meliţ et al. (12) demonstrated that rs4986790 polymorphism increases the risk of infection of H. pylori. Also, a meta-analysis study confirmed that rs4986790 or rs4986791 polymorphisms increase the risk of urinary tract infection (UTI) (13). Also, Kim et al. found a strong association between rs4986790 polymorphism and the risk of infection with human immunodeficiency virus (HIV) (14). Also, Karnaushkina et al. demonstrated that the neutrophilic extracellular trap level in pneumonia patients is significantly lower in patients with rs4986790 polymorphism compared to wild-type TLR4 on the first day of admission (15). In sum, rs4986790 and rs4986791 polymorphisms are associated with neutrophil physio/pathology and infection susceptibility. As mentioned, the risk of infection will increase after chemotherapy of patients with ALL. This study was designed to evaluate the association of "Asp299Gly" and "rs4986791" SNPs in the TLR4 gene and neutropenia-related infection susceptibility in children with ALL.

Materials and Methods

Patients, sample collection, and defining criteria

In this cross-sectional study, 51 samples (47% males and 53% females) of hospitalized children (1 to 13 yr-old; mean 5.2 ± 3.4) with ALL referred to Children's Medical Center, Tehran, Iran, from November 2015 to August 2016) were collected in EDTA (100µl of 10 gr/dl) anti-coagulating tubes. The febrile neutropenia was an inclusion criterion for this study. The samples were stored at 4 °C for up to 5 days. Diagnostic criteria for infection in this study were defined as follows: *Fever*; Body temperature higher than 38.3 °C once or 38 °C within one hour, *Fever recurrence;* Fever events were considered as a recurrence within 72 hours, *Neutropenia*; Absolute neutrophils count less than 500/µl or total WBCs count less than 1000/µl (16).

DNA Extraction and sample preparation

The blood samples were centrifuged at 3000 rpm for 5 mins to separate pellets. Then, 3 ml of lysis buffer (NaCl 150 Mm, Tris 15 Mm, EDTA 10 Mm, pH 7.5), 200 µl SDS 10%, and 1 IU proteinase K were added to samples, respectively, and mixed gently. Then, the samples were incubated at 37 °C for 3 hours for cellular membrane digestion and protein inactivation. After adding 2 ml of saturated salt 6 M, a centrifuge was performed at 2500 rpm for 20 mins. The supernatants were separated and isopropanol was added. The clamped

[DOI: 10.18502/ijpho.v13i3.13129]

DNAs were separated in microtubes and centrifuged at 1300 RPM for 1 min. The clamped DNAs were washed with Ethanol 70% in 1300 for 1 min. 100 µl of Tris-EDTA buffer and overnight room-temperature incubation was used for DNA dehydration. The dehydrated DNAs were stored at 4 °C, after significant quantification by NanoDrop™ 1000 spectrophotometer.

Detection of rs4986791 Polymorphism in *TLR4* by PCR-RFLP

Restriction Fragment Length Polymorphism (RFLP) was used to detect DNA homologous variation of *TLR4* in rs4986791 loci. The polymerase chain reaction (PCR) was performed for amplification of *TLR4* by 12.5 µl of Taq DNA Polymerase 2x Master Mix RED (Ampliqon- Denmark), forward and reverse primers (TableI) [185 bps PCR product size] (0.5 µl of 10mM, for each primer), 100 ng of extracted DNA and dH_2O, up to 25 µl. The detail of the temperature cycles of RFLP-PCR is included in Table II. *Mbo II* restriction enzyme was used to detect rs4986791 SNP in *TLR4*, as a single-site restriction enzyme fragmenting to 23 and 162 bps sequences. For this aim, 27 µl of amplified DNA, 2 µl of 10X Buffer B (Thermo Fisher Scientific- USA), and 5 IU of *Mbo II* (Thermo Fisher Scientific- USA) were incubated at 37 °C for 16 hours. The digested fragments were separated on 3% agar gel electrophoresis in 1x Tris-Boric acid-EDTA buffer.

Genotyping of rs4986790 in *TLR4* by ARMS-PCR

Tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR) was used to detect rs4986790 Polymorphism in *TLR4* with specific primer sets. The detail of the temperature cycles of ARMS-PCR was included in Table III. The digested fragments were separated on 3% agar gel electrophoresis in 1x Tris-Boric acid-EDTA (TBE) buffer.

Statistical Analysis

Descriptive statistics were used to report the frequency of polymorphisms and febrile neutropenia. Also, Fisher's exact test was used to investigate the correlation between polymorphisms and febrile neutropenia. Statistical analysis was performed by SPSS version 21.0. The significance level was considered 5%. The sample size was determined by using the formula: n =Z2P (1-P)/d2 , where n was the sample size, Z was statistics corresponding to level of confidence(=1.96), P was expected prevalence(=0.03) and d was precision(=0.05).

Ethical consideration

This study was approved by the Ethics Committee of the Islamic Azad University-Science and Research Branch (SRBIAU), Tehran, Iran (approval ID: IR.I-AU.SRB.REC.1396.453).

Results

Distribution of Patients

In this cross-sectional experiment, 51 children with ALL were studied, among them 44 patients (86.8%) had Pre-B ALL, 5 patients (9.8%) had B-ALL, and 2 patients(3.9%) had T-ALL. In 49 patients, febrile neutropenia occurred in at least one and eight periods (mean 3.3 ±2). Although febrile neutropenia was more prevalent in female children, this difference was not statistically significant (P = 0.06).

Detected rs4986790 and rs4986791 polymorphisms in *TLR4*

The frequency of rs4986790 and rs4986791 polymorphisms in *TLR4* in 51 children with ALL were evaluated by ARMS-PCR and PCR-RFLP, respectively. Of the 51 patients studied, rs4986790 polymorphism associated with *A>G* replacement was identified in two patients (5.88%). (Figure 1) The rs4986791 polymorphism associated with *C>T*

[DOI: 10.18502/ijpho.v13i3.13129]

replacement was also identified in four patients (7.84%). (Figure 2) All patients had heterozygous polymorphism.

Association of rs4986790 and rs4986791 polymorphism with febrile neutropenia periods

This study investigated the relationship between each polymorphism and the number of neutropenia courses in patients. No association was found between *TLR4* rs4986790 polymorphism and the number of febrile neutropenia recurrences (P = 0.4). The mean recurrence of febrile neutropenia in patients without polymorphism was 3.1 ±2, and in patients with polymorphism was 4.6 ± 3. This difference was not statistically significant. However, in the case of *TLR4* rs4986791, there was a tendency to increase neutropenia in patients. In patients without polymorphism, the number of febrile neutropenia recurrences was 3 ±1.9, and in patients with polymorphism was 5.2 ±2.8. However, it was not statistically significant (P = 0.09). This statistically insignificant result could be due to low polymorphism frequency and low sample size.

Table I: Oligonucleotide sequences applied in the detection of rs4986791 and rs4986790 polymorphisms

Polymorphism	Method	Primer set	Oligonucleotide sequence
rs4986791	RFLP	Forward primer	5'-TGTTATCAAAGTGATTTTGGGACAA-3'
		Reverse primer	5'- AGGTAAATGAGGTTTCTGAGTGATAGG -3'
rs4986790	Tetra-primer ARMS	outer forward primer	5'-TGAACCCTATGAACTTTATCC-3'
		outer reverse primer	5'-GTTAACTAATTCTAAATGTTGCCATC-3'
		Mutant-specific forward primer	5'-GCATACTTAGACTACTACCTCGAAGA-3'
		Wild-type specific reverse primer	5'- CAAACAATTAAATAAGTCAATAATAC-3'

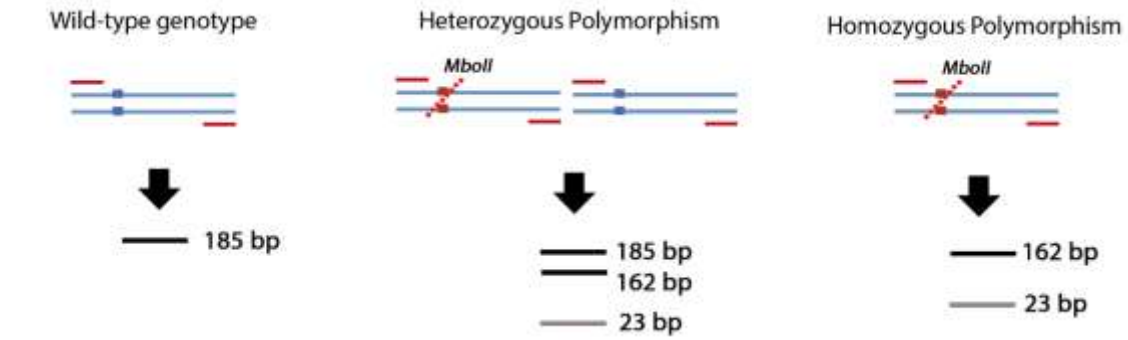
Table II: The temperature, time, and cycles of applied PCR-RFLP for amplification of TLR4.

Step		Temperature	Time	Cycle
First Denaturation		95˚C	3 min	**1**
Amplification Cycles	Denaturation	95˚C	15sec	**35**
	Annealing	62˚C	20sec	
	Extension	72˚C	15sec	
Final Extension		72˚C	5min	**1**

Table III. The temperature, time, and cycles of applied ARMS-PCR for rs4986790 genotyping of TLR4.

Step		Temperature	Time	Cycle
First Denaturation		94˚C	6 min	**1**
Amplification Cycles	Denaturation	94˚C	40sec	**40**
	Annealing	60˚C	45sec	
	Extension	72˚C	45sec	
Final Extension		72˚C	10min	**1**

[DOI: 10.18502/ijpho.v13i3.13129]



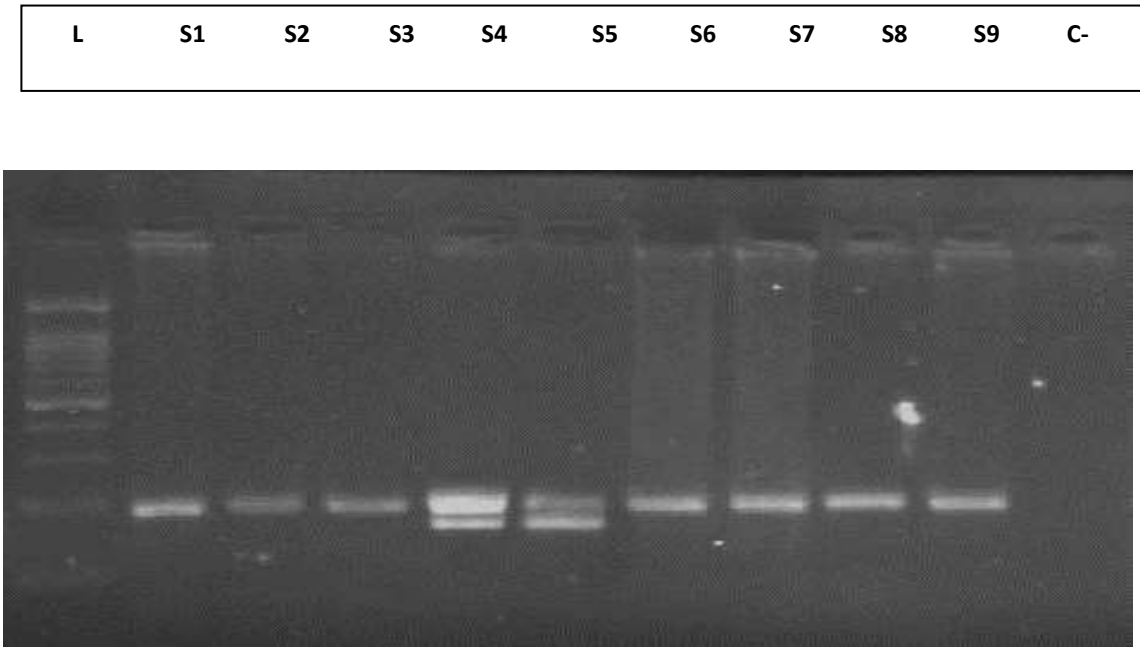


Figure 1. PCR-RFLP for detection of *TLR4* rs4986791 polymorphism. S4 and S5 show heterozygous polymorphism (disgested PCR products with lengths of 185 bp, 162 bp, and 23 bp), while other samples show wild-type genotypes (185 bp). L: 100 bp size marker, Lane C-: negative control.

[DOI: 10.18502/ijpho.v13i3.13129]

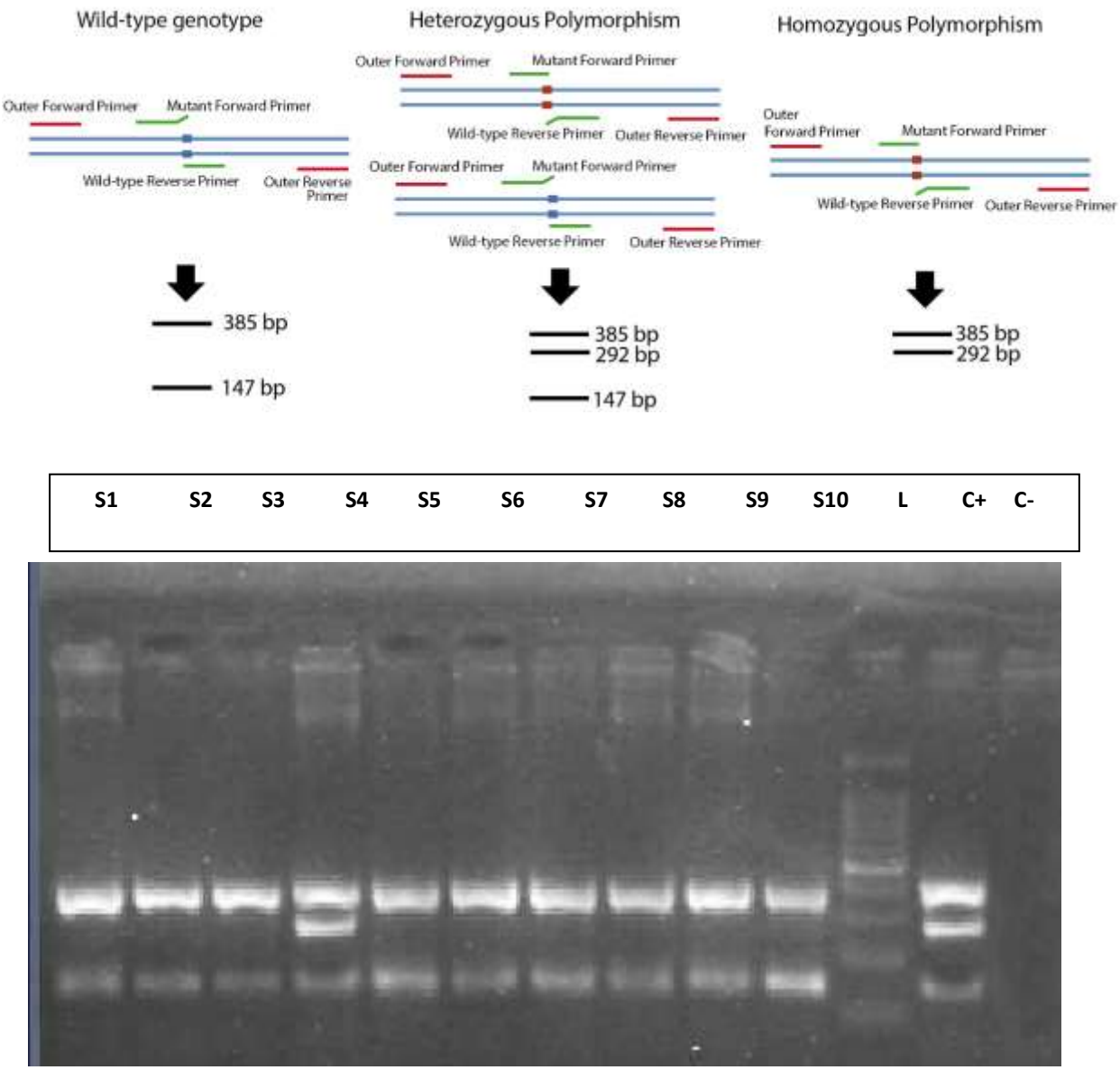


Figure 2. ARMS-PCR for detection of *TLR4* rs4986790 polymorphism. All samples (else S4) show wild-type genotype for *TLR4* rs4986790 polymorphism (PCR products with lengths of 385 bp and 147 bp), while S4 shows heterozygous rs4986790 polymorphism pattern (PCR products with lengths of 385 bp, 292 bp, and 147 bp). Also, positive (heterozygous) and negative controls were used. L: 100 bp size marker, lane C+: positive control, Lane C-: negative control.

[DOI: 10.18502/ijpho.v13i3.13129]

Discussion

Acute lymphoblastic leukemia (ALL) is the most common form of hematopoietic malignancy in children. The complete remission rate of children with ALL has increased significantly (up to 80%) over the past decade due to more effective chemotherapy regimens. Although major advances in this area are due to improvements in the supportive treatment of patients, unfortunately, these chemotherapy regimens have secondary effects on normal cells, especially fast-growing cells, and have effects such as neutropenia (17). Neutropenia increases the risk of severe bacterial infections and is still one of the leading causes of mortality in children with ALL (18).

Although the same chemotherapy regimen is performed in patients, the number, severity, and duration of neutropenia recurrence vary considerably. This variance is partly due to comorbidity, age, pharmacokinetics, and pharmacodynamics of applied chemotherapeutic agents. Genetic differences may also influence the risk of neutropenia during chemotherapy, although few studies have been conducted in this way. TLR4 is a candidate gene that may be associated with the risk of neutropenia following chemotherapy. TLR4 is associated to inhibit neutrophil apoptosis and extend neutrophil functional lifespan during stimulation with lipopolysaccharides via the NF-κB signaling pathway (19).

Specific polymorphisms of TLR4 are associated with decreased response to TLR4 ligands in-vivo and in-vitro, which is related to the pathogenesis of several diseases (7). In this regard, He et al. showed that the increased risk of H. pylori infection is associated with TLR4 rs4986790 polymorphism (11). Also, Meliţ et al. confirmed this finding in children (12). Also, the results of Karnaushkina et al.' study showed the association of a lower rate of neutrophilic extracellular trap level in pneumonia patients with the presence of TLR4 rs4986790 polymorphism on the first day of admission (15). Huang et al. demonstrated that rs4986790 and rs4986791 polymorphisms in TLR4 increase the risk of UTI (13). Also, Kim et al. established that TLR4 rs4986790 polymorphism is more prevalent in HIV-positive patients compared to normal individuals (14). Also, there are some reports to reject the association of rs4986790 and rs4986791 polymorphisms with infection risk. In Lui et al.'s study, it was established that the rs4986790 and rs4986791 polymorphisms of TLR4 are not associated with susceptibility to sepsis (10). In this study, the rs4986790 polymorphism (A>G) was identified in 5.88% of patients, while the rs4986791 polymorphism (C>T) was identified in 7.84% of patients. The results of this study showed no significant correlation between rs4986790 and rs4986791 polymorphism with febrile neutropenia periods, which is a hallmark of infection.

In a case-control study, Rezazadeh et al. Studied 198 patients with brucellosis and 111 healthy controls in terms of rs4986790 polymorphism using the ARMS-PCR. This study showed a higher frequency of the G allele in patients with brucellosis than in healthy controls (33.6% vs. 20.7%) (20). In a 2016 study by Jafari et al., 96 patients with tuberculosis and 122 healthy TLR4 genotype specimens were screened for the rs4986790 and rs4986791 variants by ARMS-PCR. The frequency of the rs4986790 genotype in patients and healthy individuals was 14.6% and 5.7%, respectively. Similarly, the rs4986791 allele in patients and healthy individuals was 4.2% and 0.8%, respectively (21). In Lehrnbecher et al. study on 168 Acute Myeloblastic Leukemia patients, no association was found between TLR4 rs4986790 polymorphism and infection (22). In the study of Yoon et al. on 154 patients with bacteremia and 179 healthy controls, no significant correlation was detected between rs4986790 and rs4986791 polymorphisms and bacteremia

[DOI: 10.18502/ijpho.v13i3.13129]

(23). In our study, allelic frequencies of rs4986790 and rs4986791 TLR4 polymorphisms were detected by PCR-RFLP and ARMS-PCR, respectively, in 5.8% and 7.8% of patients, respectively. None of the above studies found a significant relationship between rs4986790 and/or rs4986791 polymorphism of TLR4 and related disease.

Miedema et al. examined the distribution of SNP genotypes and the frequency of neutropenia recurrence during chemotherapy regimen on 8 SNPs in 194 children with ALL. In this study, 4 polymorphisms of TLR2 and TLR9 (rs10759931 rs11536889, rs1927911, and rs64783) were associated with the risk of neutropenia following chemotherapy. However, no association was found between neutropenia following chemotherapy and rs4986790 TLR4 polymorphism (7). Schnetzke et al. detected rs4986790 and rs4986791 polymorphisms in TLR4 and Arg753Gln in TLR2 in 155 AML patients who received induction therapy. The presence of TLR2 Arg753Gln polymorphism was significantly associated with pneumonia in AML patients. In addition, rs4986790 and rs4986791 polymorphisms act as independent risk factors for sepsis and pneumonia. Based on the results of this study, the presence of TLR2 and TLR4 polymorphisms is a risk factor for infection in AML patients treated with chemotherapy (24).

The different results of studies can be due to different allelic frequencies in different populations, differences in treatment protocols, and differences in treatment cycles. In addition, studies have differed in defining criteria for defining febrile neutropenia(25). It is necessary to perform a study on a larger population of patients, studying different phases of treatment including induction and consolidation therapy, investigating other polymorphisms in TLR2 and TLR9, studying the effect of polymorphism on the maintenance phase of treatment in patients, and the effect of polymorphism on clinical outcome of patients.

Conclusion

Infections cause significant complications and, in severe cases, death in patients with childhood acute lymphoblastic leukemia (ALL). Toll-like receptors play a crucial role in initiating innate immune responses. The results of this study showed that the rs4986790 and rs4986791 polymorphisms in TLR4 are present in 5.8% and 7.8% of ALL patients, respectively, but they are not correlated with febrile neutropenia.

Acknowledgments

This study was approved by the Ethics Committee of the Islamic Azad University-Science and Research Branch (SRBIAU), Tehran, Iran (approval ID: IR.I-AU.SRB.REC.1396.453).

Conflict of interest

The authors declare no conflict of interest.

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[DOI: 10.18502/ijpho.v13i3.13129]