Clinical Implication of Toll-Like Receptors (TLR2 and TLR4) in Acute Myeloid Leukemia Patients

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Abstract

Backgrounds: Toll-like receptors 2; 4 (TLR2;4) are an essential component of the innate immunity and play an important role in immune-surveillance and immune response to various microorganisms. This study aimed to investigate the association between TLR2 and TLR4 polymorphism and the risk of acquiring severe infections, and impact on AML patient’s outcome. Subjects and methods: Using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP); we analyzed three SNPs in the TLR2 (Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) in 120 AML patients and 100 healthy control subjects. Results: No significant differences in genotype or alleles frequency between healthy controls and AML patients regarding TLR2 Arg753Gln, TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms (P>0.05 for all). Neutropenic fever was detected in 110 out of 120 (91.7%) of the studied AML patients. The sepsis and pneumonia were identified in 20 out of 120 patients (16.7%). The incidence of sepsis was associated with TLR2 Arg753Gln: AG genotypes, A allele and TLR4 Asp299Gly: CT genotype and C allele as compared to other genotypes and alleles. Moreover; TLR2 (Arg753Gln) GG polymorphisms significantly associated with shortest overall survival (OS) and shortest disease-free survival (DFS); while TLR4 polymorphisms affect the DSF only but not OS. In AML patients TLR2 Arg753Gln gene polymorphism is associated with high susceptibility to sepsis and TLR4 (Asp299Gly and Thr399Ile) gene polymorphism is associated with high susceptibility for both pneumonia; and sepsis. Conclusion: TLR2 Arg753Gln (AG, GG genotype) polymorphisms are associated with shortest OS and DFS. Moreover; significant association between TLR2 polymorphisms, TLR4 Arg753Gln polymorphisms and risk of severe infections in AML patients was documented.

Keywords: TLR2- TLR4- polymorphisms- AML

Introduction

Toll-like receptors (TLRs) are a family of transmembrane receptors which play an important role in the host defense against microorganisms. TLRs are mainly expressed in human immune-related cells such as monocytes, neutrophils, macrophages, dendritic cells, T cells, B cells and NK cells (Uematsu et al., 2006; Rybka et al., 2015). TLRs alert the immune system to infection by recognizing pathogen-associated molecular patterns derived from various microorganisms (Beutler et al., 2004a; Beutler et al., 2004b). Furthermore, functional TLRs are expressed not only in immune cells, but also in cancer cells, thus implicating a role of TLRs in tumor biology (Zhu et al., 2013). About eleven human TLRs have been identified up till now and each of them participates in a specific intracellular signaling pathway. TLRs 1, 2, 4, 5 and 6 are typical for bacterial products, TLRs 3, 7 and 8 are characteristic for viral infection and TLR9 is associated with bacterial and viral inflammatory response (Tsujimoto et al., 2008). TLR2 and TLR4 are two of the most studied TLRs to have an important role in the recognition of both bacterial and fungal pathogens (Netea, et al., 2007).

Leukemia is a type of blood cancer that results from an abnormal functioning of the bone marrow, which tends to cause an abnormal proliferation of white or red blood cells. It may occur in acute or chronic form (Yamaguti et al., 2010; Aref et al., 2020). Induction chemotherapy of acute leukemia results in a long period of neutropenia that is associated with a high risk of infectious complications (Syrijälä et al., 2010). Infectious complications continue to be one of the major causes of morbidity and mortality in patients with acute leukemia (Schnetzke et al., 2015). Sepsis and pneumonia represent the most important infectious complications and are associated with an increased mortality after induction chemotherapy (Hamalainen et al., 2008). The occurrence of infectious complications represents a crucial factor affecting the...
prognosis of acute leukemia patients, so prophylaxis and treatment of infections have an important role in the clinical management of these patients (Gupta et al., 2010).

Sepsis is a complex clinical syndrome that results from a systemic inflammatory response to bacteria and/or bacterial products (Uematsu et al., 2006). It remains a leading cause of death in the non-cardiac intensive care unit (ICU) despite improvements in antibiotic therapy and supportive care.

Only a few retrospective analyses investigate whether polymorphisms of innate immunity influence the risk of severe infections in acute leukemia patients. Importantly, these studies do not demonstrate any correlation between different SNPs and important clinical events such as fungal infections or sepsis (Schnetzke et al., 2015).

The Expression of Toll-like Receptors in Patients with Acute Myeloid Leukemia Treated With Induction Chemotherapy have been studied (Ryoka et al., 2015; Ramzi et al., 2018); however the impact of Toll like receptors 2, 4 polymorphism on the AML patients outcome are not previously studied.

In this study, we investigated the impact of TLR2 and TLR4 SNPs on susceptibility of AML patients towards developing sepsis, pneumonia and fever during severe neutropenia following intensive chemotherapy as well as its impact on the patient’s outcome.

Subjects

This is a case control study including 120 patients with newly diagnosed acute myeloid leukemia (AML) (64 male, 56 females; median age 48 years, range 18–77 years) and 100 normal healthy controls (46 males, 54 females; median age 47 years, with age range between 26–62 years). The study was approved by the Institutional Revision Board (IRB) of Mansoura University. Patients with secondary AML, and pediatric AML were excluded from this study.

Exclusion criteria

Patients with secondary AML, and pediatric AML were excluded from this study.

Follow up

The included patients were observed for up to 12 months.

Materials and Methods

Analysis of TLR polymorphisms

Genotyping of TLR2 Arg753Gln (R753Q, rs5743708), TLR4 Asp299Gly (D299G, rs4986790) and TLR4 Thr399Ile (T399I, rs4986791) was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Briefly, genomic DNA was isolated from peripheral blood or bone marrow samples using Gene JETTM Whole Blood Genomic DNA Purification Mini Kits from Thermo Scientific (lot no k0781, Lithuania, EU).

PCR was done by amplification of extracted genomic DNA via thermal cycler (ARKTIK Thermal Cycler, Thermo Scientific Co.). TLR4 Asp299Gly forward primer, 5'- AGCATCTTGAAGTAACTCCATG-3' and reverse primer: 5'- GAGAGATTTGAGTTTCAATGTTGGG-3'; TLR4 Thr399Ile forward primer, 5'-GGTTGCTGTTTCTCAAGTGATTTTTGGGAGAA-3' and reverse primer: 5'-GGAAATCCAGATGTTCTGTTCTAAAGTGATTTTGGGAGAA-3'; TLR2 Arg753Gln forward primer, 5'-TTGACTCCATTGAAAAGAGC-3' and reverse primer, 5'-TAAATATGGGAACCTAGGAC-3'.

Reactions were performed in a 25 μl volume containing 12.5 μL of red master mix, 0.1μL of both forward and reverse primer, 1 μL of DNA, 11.3 μL of distilled water (DW).

One hundred nanograms of genomic DNA was amplified using the following cycling conditions for PCR of TLR4 Asp299Gly: 95°C for 15 min, 35 cycles of 95°C for 30 s, 55°C for 60 s and 72°C for 30 s followed by a final extension of 72°C for 10 min. The PCR protocol for TLR4 Thr399Ile was the same as for TLR4 Asp299Gly except that annealing temperature was 53°C. The PCR protocol for TLR2 Arg753Gln was the same as for TLR4 Asp299Gly except that annealing temperature was 54°C for 30 s and the final extension of 72°C for 5 min.

The PCR products were then digested at 37°C with HinfI for Thr399Ile polymorphism for 5-15 min (New England Biolabs Inc, lot no 0411604), NcoI for Asp299Gly polymorphism for 25 min (New England Biolabs Inc, lot no 0061605), PstI for Arg753Gln polymorphism for 5-15 min (New England Biolabs Inc, lot no 0021504). The digestion of PCR products were confirmed using Geen JETTM Whole Blood Genomic DNA Purification Mini Kits from Thermo Scientific (lot no k0781, Lithuania, EU).

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The PCR products were then digested at 37°C with HinfI for Thr399Ile polymorphism for 5-15 min (New England Biolabs Inc, lot no 0411604), NcoI for Asp299Gly polymorphism for 25 min (New England Biolabs Inc, lot no 0061605), PstI for Arg753Gln polymorphism for 5-15 min (New England Biolabs Inc, lot no 0021504). The digests were run on a 2% agarose gel and visualized under UV light using ethidium bromide.

Digestion of PCR product for TLR4 Asp299Gly using NcoI enzyme produced a fragment of 190 bp for homozygous wild type, produced two fragments of 168 and 20 bp for homozygous mutant type, and produced three fragments of 190, 168 and 20 bp for heterozygous mutant type.

Digestion of PCR product for TLR4 Thr399Ile using HinfI enzyme produced a fragment of 124 bp for homozygous wild type, produced two fragments of 98
and 26 bp for homozygous mutant type, and produced three fragments of 124, 98 and 26 bp for heterozygous mutant type.

Digestion of PCR product for TLR2 Arg753Gln using PstI enzyme produced a fragment of 300 bp for homozygous wild type, produced two fragments of 190 and 110 bp for homozygous mutant type, and produced three fragments of 300, 190 and 110 bp for heterozygous mutant type.

Statistical analysis

The statistical analysis of the data was performed by using excel (Microsoft office 2013) program and SPSS (Statistical Package for Social Science) program (SPSS, Inc, Chicago, IL) version 20. Chi square test was used to compare groups. Quantitative data were presented as median and range, mean and standard deviation. For comparison between two groups Mann-Whitney test (for non-parametric data) was used. For comparison of more than two groups; Kruskal-Wallis (for non-parametric data) was used. Univariate analysis used to evaluate association of polymorphism with occurrence of sepsis, pneumonia and fever. For survival analysis, Kaplan-Meier curves were used and compared by log-rank test. The P value<0.05 was considered statistically significant.

Results

Patients characteristics

The patient’s characteristics are shown in Table 1.

Association between TLR polymorphism and AML

No significant differences in genotype or alleles frequency regarding TLR2 Arg753Gln, TLR4 Asp299Gly and TLR4 Thr399Ile polymorphisms in AML and healthy controls. (P>0.05 for all) (Tables 2-4). All three SNPs were in accordance with Hardy–Weinberg equilibrium (χ² test).

Impact of TLR2 Arg753Gln and TLR4 (Thr399Ile and Asp299Gly) genotype variants on development of sepsis

TLR2 Arg753Gln: AG genotypes and A allele; TLR4 Asp299Gly: CT genotype and C allele; TLR4 Thr399Ile AG genotype and A allele showed significant higher risk to sepsis as compared to other genotypes and alleles (Table 5). Pneumonia was significantly associated with TLR4 Asp299Gly AG genotype and A allele and TLR4 Thr399Ile

Table 1. AML Patients Characteristics

| Parameters                              | AML Patients |
|-----------------------------------------|--------------|
| Age (years) median (range)              | 48 (18-77)   |
| Gender                                  | Male (n=64)  |
|                                        | Female (n=56)|
| Peripheral blood blast (%) median (range)| 24.5 (0 - 90)|
| Bone marrow blast (%) median (range)    | 80.0 (23-95) |
| AML subtypes                            |              |
| AML with minimally differentiated       | (n=6)        |
| AML without maturation                  | (n=24)       |
| AML with maturation                     | (n=32)       |
| AML myelomonocytic                      | (n=40)       |
| AML monoblastic                         | (n=18)       |
| Cytogenetic findings                    |              |
| Good (n=30)                             |              |
| Intermediate (n=55)                     |              |
| Poor (n=35)                             |              |
| Infections status                       |              |
| Neutropenic fever (n=80)                |              |
| patients Sepsis (n=20)                  |              |
| Pneumonia (n=20)                        |              |
| Causes of Infections (n=80)             |              |
| Bacterial (n=60); Fungal (n=15); Viral (n=5)| |
| Sources of infection                    | Respiratory System (n=50); Abdomen (n=20) |
| Urinary tract (n=8); Skin (n=2)         |              |
| Induction remission response            | Responder (n=74) |
| Resistant (n=30)                        |              |
| Partial response                        | (n=16)       |
| Disease Free Survival (DFS)             | Relapse (n=55) |
| No relapse (n=65)                       |              |
| AML patients outcome                    | Alive (n=46) |
|                                          | Dead (n=74)  |

Figure 1. Impact of TLR2 Arg753Gln Genotype Variants on AML Overall Survival (OS) (Months)

Figure 2. Impact of TLR4 Thr399Ile Genotype Variants on AML Overall Survival (OS) (Months)
CT genotype and T allele; but there is no association was found with TLR 2 TLR2 Arg753Gln polymorphism (Table 6).

Survival Analysis
At the end of follow up period (12 months), OS of studied AML patients estimates 49.7% at 6 months interval and 34.9% at 12 months interval (Table 7). The impact of TLR polymorphism on the AML patient’s overall survival was studied by Kaplan Mire curve. AML patients have TLR2 (Arg753Gln) GG genotype had the shortest OS; followed by those have AG and GG genotypes (P=0.001) (Figure 1); while TLR4 (Thr399Ile and Asp299Gly) polymorphism did not affect the OS (P=0.06; 0.104 respectively) (Figures 2 and 3).

Moreover; the studies on the DFS revealed that AML patients have TLR2 polymorphic AA genotype (P=0.002) (Figure 4) and those who have TLR4 Thr399Ile CC genotype have the longest DFS as compared to the other genotypes CT; TT (Figure 5) (P= 0.015); while TLR4 Asp299Gly didn’t have an association with DFS (P=0.48) (Figures 6).

Discussion
Genetic variations of the pathogen recognition system are supposed to be responsible for individual differences concerning the response to infectious stimuli. In detail, a series of SNPs affecting key molecules of innate immunity including surface molecules such as TLR2, TLR4, TLR5 and different variants of the mannose-binding lectin 2 or intracellular downstream molecules as the

| Genotypes | Control (n=100) | AML (n=120) | OR | Relative risk of AML |
|-----------|---------------|-------------|----|----------------------|
| AA        | 78            | 78          | 1  | -                    | R   |
| AG        | 18            | 33          | 1.833 | 0.667 | 5.039   | 0.24 |
| GG        | 4             | 9           | 2.25      | 0.351 | 14.4   | 0.392 |
| A         | 174           | 189         | 1.805     | 0.818 | 3.985   | 0.143 |
| G         | 26            | 51          | -          | -     | -      | -    |

| Genotypes | Control (n=100) | AML (n=120) | OR | Relative risk of AML |
|-----------|---------------|-------------|----|----------------------|
| CC        | 82            | 78          | 1  | -                    | R   |
| CT        | 16            | 36          | 2.365     | 0.852 | 6.564   | 0.098 |
| TT        | 2             | 6           | 3.153      | 0.272 | 36.55   | 0.358 |
| C         | 180           | 192         | 2.25       | 0.959 | 5.27    | 0.062 |
| T         | 20            | 48          | -          | -     | -      | -    |

CI, Confidence interval; OR, odds ratio
IL-1 receptor-associated kinase and the TIR-domain-containing adaptor protein have been attributed with an increased risk of infections (Ahmad-Nejad et al., 2011; Kumpf et al., 2010; Sánchez-Canossa et al., 2016).

Table 4. Distribution of TLR4 Asp299Gly Genotype Variants and Alleles in AML Groups Versus Control

| Genotypes | Control (n=100) | AML (n=120) | OR | 95% CI | P value |
|-----------|----------------|-------------|----|--------|---------|
| AA        | 86             | 84          | 1  | -      | -       |
| AG        | 12             | 30          | 2.559 | 0.836 | 7.832 | 0.099 |
| GG        | 2              | 6           | 3.071 | 0.265 | 35.49 | 0.899 |
| A         | 184            | 198         | 2.439 | 0.967 | 6.148 | 0.058 |
| G         | 16             | 42          |      |        |        |        |

CI, Confidence interval; OR, odds ratio

Table 5. Impact of TLR2 Arg753Gln and TLR4 (Thr399Ile & Asp299Gly) Genotype Variants Regarding Sepsis among AML Patients

| Genotypes | Absence of sepsis (N=100) | Presence of Sepsis (N=20) | OR | 95% CI | P value |
|-----------|---------------------------|---------------------------|----|--------|---------|
| AA        | 90                        | 5                         | 1  |        | -       |
| AG        | 6                         | 9                         | 21.37 | 3.28  | 139.1  | 0.001 |
| GG        | 4                         | 6                         | 30.33 | 1.31  | 697.6  | 0.032 |
| A         | 186                       | 19                        | 17.64 | 3.632 | 85.74  | <0.001 |
| G         | 14                        | 21                        |      |        |         |        |
| CC        | 75                        | 10                        | 1  |        | -       |
| CT        | 20                        | 13                        | 4.57 | 1.02  | 20.34  | 0.046 |
| TT        | 5                         | 2                         | 2.28 | 0.124 | 41.98  | 0.577 |
| C         | 170                       | 33                        | 2.727 | 0.869 | 8.55   | 0.04  |
| T         | 30                        | 17                        |      |        |         |        |
| AA        | 85                        | 11                        | 1  |        | -       |
| AG        | 10                        | 2                         | 10.28 | 1.73  | 60.9   | 0.01  |
| GG        | 5                         | 7                         | 2.57 | 0.14  | 47.01  | 0.524 |
| A         | 180                       | 25                        | 4.318 | 1.209 | 15.42  | 0.024 |
| G         | 20                        | 15                        |      |        |         |        |

CI, Confidence interval; OR, odds ratio
Herein, we investigated the impact of the TLR2 and TLR4 polymorphisms on the susceptibility of AML patients to infection as well as patient’s outcome. We found that AML patients who have TLR2 Arg753Gln: AG genotypes and A allele; TLR4 Asp299Gly: CT genotype and C allele; TLR4 Thr399Ile AG genotype and A allele showed significant higher association to sepsis and pneumonia as compared to other genotypes and alleles. This finding is consistent with that reported by Schnetzke et al., (2015) who reported that the presence of the TLR2 Arg753Gln polymorphism was significantly associated with pneumonia in AML patients and that the cosegregating TLR4 polymorphisms Asp299Gly and Thr399Ile were independent risk factors for the development of both sepsis and pneumonia. Furthermore; TLR2 polymorphism has been demonstrated as a risk factor for development of severe infections in critically ill patients from a surgical intensive care unit (Ahmad-Nejad et al., 2001) and higher rates of infection recurrence and initial septic shock in liver-transplant recipients developing gram-positive infections (Lee et al., 2001). In addition; the results are also supported by that found by Schnetzke et al., (2015); Avdonina et al., (2017) who stated the association TLR4 polymorphisms were independent risk factors for the development of both sepsis and pneumonia in AML patients.

It has been reported that TLR4 polymorphisms are present in 10% of Caucasian populations and are reported to have a positive correlation with susceptibility to several infectious diseases (including gram-negative sepsis), atherosclerosis, asthma, malaria and also Helicobacter pylori-induced gastric cancer (Mockernut et al., 2006; Hold et al., 2007). Two studies (Agnese et al., 2002; Lorenz et al., 2002) revealed that the risk of septic shock due to infection by Gram-negatives is increased in the presence of these SNPs.

The impact of TLR polymorphism on the AML patient’s overall survival was studied by Kaplan Mire curve. AML patients who have TLR2 (Arg753Gln) GG genotype had the shortest OS; followed by those have AG and AA genotypes (P=0.001); while TLR4 (Thr399Ile and Asp299Gly) polymorphism did not affect the OS (P=0.06; 0.104 respectively). These findings are inagreement with that reported a previous study (Schnetzke  et al., 2015).

Moreover; the studies on the DFS revealed that AML patients have TLR2 polymorphic AA genotype(P=0.002) and those who have TLR4 Thr399Ile CC genotype have the longest disease free survival as compared to the other genotypes CT; TT ; while TLR4 Asp299Gly didn’t have an impact on OS . No previous study was evaluated the impact of these polymorphism on the AML patient’s outcome.

In conclusion, TLR2 Arg753Gln (AG; GG genotype) polymorphisms are associated shortest OS and DFS. Moreover; significant association between TLR2 polymorphisms, TLR4 Arg753Gln polymorphisms and risk of infections in AML patients was documented.

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**Conflict of interest**

The authors declare there is no any conflict of interest.
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