RESEARCH ARTICLE

Lack of Associations between TLR9 and MYD88 Gene Polymorphisms and Risk of Chronic Lymphocytic Leukemia

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Abstract

Background: Genetic factors like single nucleotide polymorphisms (SNPs) may play an important role in the etiology of chronic lymphocytic leukemia (CLL). Mutations in Toll like receptor 9 (TLR9) and myeloid differentiation primary response 88 (MYD88) genes may lead to an abnormal immune response that may cause greater cell proliferation and thus alter an individual's susceptibility to haematological malignancies including CLL. **Objective:** This work was designed to study any association of the TLR9 (rs2066807C/G and rs187084T/C) and MYD88 (L265P) single nucleotide polymorphism (SNPs) with risk of CLL in Egyptians. **Materials and methods:** One hundred patients with CLL and 100 healthy controls from the Egyptian population were genotyped by the polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) method. **Results:** With TLR9 rs2066807C/G the CC genotype was more frequent in both control and patient groups while for TLR9 rs187084T/C the TT genotype was most common. There were no significant associations with CLL risk. With MYD88 (L265P) only the TT genotype was detected. **Conclusion:** Our preliminary data suggest that polymorphisms in the TLR9 and MYD88 genes may not contribute to CLL susceptibility. To the best of our knowledge, this study is the first dealing with TLR9 and MYD88 gene polymorphisms in CLL patients. Further studies with larger sample size should be conducted to validate these results in the Egyptian population.

Keywords: CLL- MYD88- TLR9- polymorphism- PCR/RFLP

Introduction

Chronic lymphocytic leukemia (CLL) is the common form of adult leukemia. CLL is characterized by the accumulation of mature B cells in the peripheral blood, bone marrow, and lymphoid tissues (Mertens et al., 2011) CLL affects mainly eldest people. The median age at diagnosis is 72 years. CLL affects men twice as often as women in all populations. In 2015 American Cancer Society reported that CLL affecting about 15,000 new patients each year in the United States. Resulting in almost 5000 cancer related deaths yearly (Siegel et al., 2014).

CLL patients present a highly variable clinical course, some die within a few months of diagnosis (Gianfelici, 2012) whereas others survive prolonged periods without requiring therapy. Till now the exact cause of CLL is unknown and most of the cases are sporadic. Factors associated with CLL development are genetic variations, and disorders of the immune system (Jevtovic-Stoimenov et al., 2017) The immune system plays an important role in the control of cancer development as it can prevent development by eliminating cancer cells before or by inhibiting tumor growth and progression. Several studies suggested that abnormal immune response may cause greater cell proliferation (Smyth et al., 2006; Swann et al., 2007).

Toll-like receptors (TLRs) constitute a family of receptors that play a key role in the realization of innate and adaptive immune response. It recognizes unmethylated CpG motifs that present in bacteria and viruses through the myeloid differentiation primary response (MYD88)-dependent signal transduction pathway and activated nuclear factor Kappa B (NFkB) channels, resulting in inflammatory factors that play an important role in tumor immunity (Takeda et al., 2003; Akira et al., 2001).

TLR9 signaling by CpG-B oligonucleotides may induce apoptosis in CLL (Rozkova et al., 2010; Liang et al., 2010). However, studies showed that CpG immunostimulatory oligonucleotides either alone or in combination with interleukin 2 (IL-2) induce proliferation of CLL cells (Decker et al., 2000; Jahrsdorfer et al., 2001). Several studies suggested that TLR9 can decrease the viability of B-CLL cells and increase its susceptibility to apoptosis in the cell culture (Jahrsdorfer et al., 2006; Jahrsdorfer et al., 2005).

TLR9 polymorphism has been reported to have associations with asthma, systemic lupus erythematosus, atherosclerosis (Lazarus et al., 2003) and various cancers including Hodgkin's lymphoma (HL), acute lymphocytic leukemia, hepatocellular carcinoma and non-Hodgkin's lymphoma (Mollaki et al., 2009; Zhang et al., 2013). Most of the studies focused on three SNP's in TLR9, including

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rs352140(C/T), rs5743836 (T/C) and rs187084(C/T) (Zhang et al., 2013).

Human MYD88 contains 6 non-synonymous SNPs in its coding region, which makes it one of the most polymorphic TLR adapter proteins (Nagpal et al., 2011). Recently, studies reported that mutation in MYD88 (MYD88 L265P) lead to 30% of activated B-cell–like diffuses B-cell lymphomas. MYD88-L265P is a marker highly characteristic of Waldenstrom macroglobulinemia (WM), a low-grade B-cell lymphoplasmacytic lymphoma, while it reaches only up to 2% to 5% in CLL and is strikingly enriched among patients expressing mutated IGHV genes (Jevtovic-Stoimenov et al., 2017).

Mollaki et al., (2009) studied the association of TLR9 and MYD88 polymorphisms with the risk of HL. Data showed that genetic variations of TLR9 only may alter the susceptibility to the disease.

In this study, we hypothesized that TLR9 and MYD88 innate immunity related genes may play an important role in the development and clinical course of CLL and polymorphism in those genes may be a risk for CLL. Thus we evaluated the potential role of genetic variants in rs2066807C/G, rs187084T/C and MYD88 L265P in 100 chronic lymphocytic leukemia patients and 100 healthy controls.

Materials and Methods

Patients and controls

This study was conducted on 100 CLL patients (61 men and 39 women) that recruited consecutively from Department of Hematology at Ein Shams hospital and national cancer institute (Cairo Governorate, Egypt) during 11 months from June 2012 to May 2013. One hundred unrelated healthy blood donors free of any chronic diseases, living in the same geographical area and having the same ethnic origin as patients were recruited as normal healthy controls. Informed consent was obtained from all the study subjects.

All investigations were done in accordance with Ein Shams and Cairo University, Health and Human Ethical Clearance Committee guidelines for Clinical Researches. The local ethics committee approved the study protocol. Detailed clinical characteristics were recorded for each patient. Cytogenetic test, routine laboratory investigations, and Bone marrow biopsy or aspirate that was performed at diagnosis were collected from the patients' records.

DNA isolation

Blood samples were collected on ethylene-diamine-tetra-acetic acid (EDTA) tube by venipuncture from all subjects. Genomic DNA was extracted from whole blood-EDTA samples using (QIAamp DNA Mini Kit) Genomic DNA Purification Kit (QIAGEN, USA) according to the manufacturer's instruction.

Genotyping

TLR9 (rs2066807C/G), (rs187084T/C) and MYD88 (L265p) were analyzed by using polymerase chain reaction-restriction fragment length polymorphisms (PCR- RFLP). PCR of the TLR9 (rs2066807C/G)

and (rs187084T/C) polymorphisms were performed in one tube with 12.5 μl final reaction volume. PCR mixtures consisted of DreamTaq Green PCR Master Mix (2X) (Fermentas, USA), 5 pmol of each primer [Forward: 5'-TGGGCTGTTTCTCCATACAA-3', and Reverse: 5'-AAACCAGCTGAGGGATTCAGT-3' primers for (rs2066807C/G) and Forward: 5'-TTATTCCCCTGCTGGAATGT-3', and Reverse 5'- CCCAGCAGCAACAATTCAT-3' primers for (rs187084T/C)] 2.5 μl of DNA. The PCR cycling conditions were carried out in a Biometra thermal cycler (Biometra GmbH, Germany) as follows: 95°C for 5 minutes (min) and 35 cycles of denaturing at 95°C for 30 second (s), annealing at 56°C for 45 s, extension at 72°C for 30 s and a single final extension at 72°C for 10 min.

The PCR product (344 bp and 202 for TLR9 (rs2066807C/G) and (rs187084T/C); respectively) were discerned by digestion with Tth111I (PsyI) (rs2066807C/G) and Hpy188III (rs187084T/C) restriction enzymes. The digested PCR products were visualized by 4% agarose gel in 0.5X Tris-acetate-EDTA (TAE) buffer with ethidium bromide staining (10 mg/ml). Resulting Products were determined relative to the migration of a 50 bp step ladder (Fermentas, USA) (Figure 2).

PCR of MYD88 (L265p) polymorphism was performed in one tube with 12.5 μl final reaction volume. PCR mixtures consisted of DreamTaq Green PCR Master Mix (2X) (Fermentas, USA), 5 pmol of each forward: 5'-CTGGCAAGAGAATGAGGGAATGT-3' and reverse: 5'-AGGAGGCAGGGCAGAAGTA-3' primers and 2.5 μl of DNA PCR cycling conditions were carried out in a Biometra thermal cycler (Biometra GmbH, Germany) as follows: 94°C for 2 min and 32 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s and a single final extension at 72°C for 5 min. The PCR product (489 bp) was digested by addition of BsiEI restriction enzyme. and visualized by 2% agarose gel electrophoresis in 0.5X TAE buffer with ethidium bromide staining. The relative size of PCR product was determined by comparison of migration of a 100 bp DNA molecular weight ladder (Fermentas, USA). The mutated allele contains a BsiEI site resulting in 289 bp and 200 bp fragments, whereas the wild-type allele does not (Figure 3).

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 19 (LEAD Technology Inc.). Data were presented as means with the corresponding standard deviation (SD). Comparisons among different groups were performed by independent t-test. The online tool SNP stats (http://bioinfo.iconcologia.net/SNPstats) performed the haplotype analyses and calculated the LD parameters (D′ and r^2). The genotype, allele, and haplotype frequencies were compared between cases and controls using a chi-square test ($\times 2$). The odds ratio (OR) and 95% confidence intervals (CI) were calculated to assess the risk associated with particular allele, genotype or haplotype. Correlation between variables was determined using Spearman's correlation test. A p-value of < 0.05 was

considered significant.

Results

Patient's characteristics

A total of 100 cases and 100 controls were analyzed in this study. The CLL group of patients comprised of 61 males and 39 females with mean age of 60.34±11.87. The detailed demographic and hematological characteristics of the patients enrolled in this study are presented in Table 1. A significant reduction in all haematological parameters (hemoglobin, RBCs, platelets, and WBCs) was observed in CLL group as compared to normal controls.

Association between TLR9 and MYD88 genes polymorphisms and CLL

Genotyping of TLR9 (rs2066807C/G) showed that the CC genotype is more frequent in both control and patient groups (Table 2). In contrast, the GG genotype is absent in both groups. While CG genotype is rare in both groups. Genotyping of TLR9 (rs187084T/C) showed that the TT genotype is more frequent in both two groups where it was found more frequent in the patient group than control. TC genotype is the same in both control and patient groups. While the CC genotype is more frequent in control group than the patient one. The frequency of TLR9 (rs2066807C/G and rs187084T/C) haplotypes in CLL patients and healthy controls is shown in Table 2. The CT and CC are the most frequent haplotype in
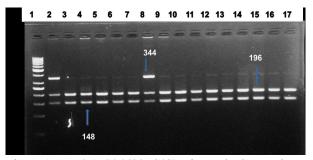

Figure 1. TLR9 (rs2066807C/G) Digested PCR Products of Sixteen Samples. Lane (1) 50 bp ladder, Sample Lanes (2 and 8) are CG Genotype, Sample Lanes (3-7) are CC Genotype. Lanes (9-17) are Control.

Table 1. Demographical and Hematological Characteristics of Control and CLL Patients

Laboratory Investigations	Control group (N=100)	CLL group (N=100)	P	Correlation with the disease
Demographical Data				
Gender (Male ♂/ Female ♀)	34/66	61/39	P<0.01	r =0.260 P<0.01
Haematological Data				
Hemoglobin (g/dl)	11.72 ± 1.77	11.03 ± 2.43	P<0.05	r = - 0.16 P<0.05
RBCs (U/L)	4.19 ± 0.59	3.81 ± 0.92	P<0.01	r = -0.42 P<0.01
PLT* (U/L)	265.79± 82.55	171.62±89.19	P<0.01	r = -0.48 P<0.01
WBCs (U/L)	4.19 ± 0.59	3.81 ± 0.92	P<0.01	r = -0.42 P<0.01

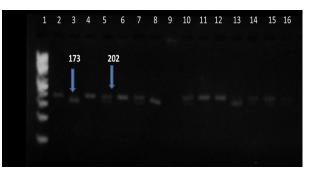All data are presented as mean±SD.*Platelet (PLT). NS: not significant.

Figure 2. TLR9) rs187084T/C (Digested PCR Products of Lane of Fourteen Samples. (1) 50 bp Ladder, Sample Lanes (3, 8 and 13) Showed CG Genotype. While Sample Lanes (4, 6, 11-12) Show the TT Genotype. Sample Lanes (5, 7, 10, 14-16) showed genotype TC. Sample Lane (2) Show Control TT Genotype.

control and patient groups, while GT and GC are rare ones. Insignificant increased in haplotype frequency between the 2 studied groups was reported. The linkage disequilibrium (LD) pattern between the (rs2066807C/G and rs187084T/C) SNPs showed a non-significant LD, with a D' value of 0.18 and r2 value of -0.007.

Genotyping of MYD88 (L265p) showed that both control and patient groups have TT genotype and both TC and CC genotypes are completely disappeared (Table 3).

Analysis of SNPs revealed that there was an insignificant change in the distribution of TLR9 (rs2066807C/G, rs187084T/C) and MYD88 (L265p) genotypes between patients and healthy controls. Although alleles in rs187084T/C are slightly different in CLL patients, it did not reach statistical significance.

Discussion

Genetic landscape of CLL revealed that the CLL genome displays a high degree of heterogeneity between patients and within the same patient (Fabbri and Dalla-Favera, 2016). Genetic variations or polymorphisms in genes especially genes that codes for molecules important in development and progression of diseases are probably important factors in the amplification of intrinsic biological differences, resulting in clinically distinct outcomes and confer genetic susceptibility to cancer (Saborit-Villarroya et al., 2011). TLRs constitute a family of receptors that
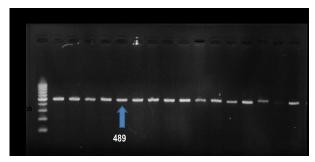

Figure 3. TLR9 MYD88 (L265p) Digested PCR Products of Sixteen Samples. Lane (1) is 100 bp Ladder. All Sample Lanes (2-17) are TT Genotype. Lanes (2-4) are Show Control.

Table 2. Genotype, Allele and Haplotype Frequencies of TLR9 (Rs2066807c/G) and (Rs187084t/C) and Gene Polymorphisms in CLL Patients and Controls

Polymorphism	Control (N=100)(N,%)	Patients (N=100)(N,%)	Odd ratio (95% Confidence Interval)	p-value
TLR9 (rs2066807C/G)				
Allele Frequency				
C	198 (99%)	199 (99.5)	2.02 (0.18-22.64)	NS
G	2 (1%)	1 (0.5%)	0.49 (0.04-5.54)	NS
Genotype Frequency				
CC	98 (98%)	99 (99%)	2.02 (0.18-22.64)	NS
CG	2 (2%)	1 (1%)	0.49 (0.04-5.54)	NS
GG	0	0	-------	-------
TLR9 (rs187084T/C)				
Allele Frequency				
T	158 (79%)	170 (85%)	1.51 (0.89-2.52)	NS
C	42 (21%)	30 (15%)	0.66 (0.39-1.11)	NS
Genotype Frequency				
TT	75 (75%)	81 (81%)	0.60 (0.26-1.36)	NS
TC	8 (8%)	8 (8%)	1.00 (0.36-2.77)	NS
CC	17 (17%)	11 (11%)	0.60 (0.26-1.36)	NS
Haplotype frequency				
CT	0.78%	0.85%	1	-----
CC	0.21%	0.15%	0.79 (0.53-1.17)	NS
GT	0.01%	0.01%	0.48 (0.03-6.69)	NS
GC	0.00%	0	0.48 (0.03-6.69)	NS

Table 3. Genotype and Allele Frequencies of MYD88 (L265p) Gene Polymorphism in CLL Patients and Controls

Polymorphism	Control (N=100) (N,%)	Patients (N=100) (N,%)	Odd ratio (95% Confidence Interval)	p-value
(rs2066807C/G)				
Allele Frequency				
T	200 (100%)	200 (100%)	1.00 (0.02-50.64)	NS
C	0	0	------	-----
Genotype Frequency				
TT	200 (100%)	200 (100%)	1.00 (0.02-50.64)	NS
TC	0	0	------	-----
CC	0	0	-------	-------

play a key role in the realization of innate and adaptive immune response. Also, TLRs play role in the processes of cell proliferation, survival, apoptosis, pyroptosis and angiogenesis (and consequently in tissue remodeling and repair. (Fukata et al., 2006; Brown et al., 2007; Kim et al., 2007; Rakoff-Nahoum, et al., 2008)

CLL is a disease of the elderly and is rarely encountered in individuals under the age of 40 years (Siegel et al., 2014) Mean age of group of patients enrolled in our study is 60.34±11.87 and that is congruent with incidence of age recorded in many publications (Siegel et al., 2014; Fabbri et al., 2016; Redaelli et al., 2004). Gender in our patients showed that percentage of males is around 1.5 fold of females and that is consistent with literature showed that rates of CLL in males are higher than in females (Redaelli et al., 2004)

Polymorphisms in TLR genes ha been reported to be correlated with cancer risk. As it may change the

balance between pro- and anti-inflammatory cytokines, modulating the risk of infection, chronic inflammation, and cancer. TLR9 gene polymorphisms and cancer risk have been published in the past years as it has been reported that there is association between TLR9 polymorphism and HL (Mollaki et al., 2009). Mutation of MYD88-L265P provides useful information in the evaluation of B-cell lymphoproliferative disorder. Additional studies confirmed that MYD88-L265P is a highly characteristic marker for WM, a low-grade B-cell lymphoplasmacytic lymphoma associated with secretion of monoclonal immunoglobulin M (IgM) in the serumas 90% of patients with WM carried the mutation (Treon et al., 2012).

Thus, in this study, we investigated the effect of TLR9 gene polymorphisms and MYD88 on CLL in Egyptian population.

Our results showed that allelic frequencies and

genotypic distributions at the TLR9 (rs2066807C/G) polymorphism did not differ between CLL patients and the control group as previous studies reported by Huang et al. (2012) that was done in TLR9 (rs2066807C/G) polymorphism and SLE risk in Chinese patients.

Although alleles in rs187084T/C are slightly different in CLL patients, it did not reach statistical significance. Similarly, Huang et al., (2012) and Li et al., (2012) studies did not support a genetic association between rs187084 and SLE in the Chinese patients and in Asian population respectively. In contrast to our study, Zhang et al., (2013) observed that no significant associations were found between rs187084 polymorphism and cancer risk.

The same result was found in MYD88-L265P.There were no differences detected in CLL patients and the control group, that similarly with previous studies reported by Argentous et al., (2014) and Ondrejka et al., (2013). But Puente et al., (2011) found that MYD88-L265P mutation was found in 2.9% of CLL cases. These results may suggest that TLR9 (rs2066807C/G), (rs187084T/C) Polymorphisms and MYD88-L265P mutation may not be involved in the pathogenesis of CLL in Egyptian population.

In conclusion, this study observed that there was no significant association between polymorphisms in SNPs of TLR-9 genes, MYD88 and CLL risk in Egyptian population. Therefore, larger prospective studies are needed to confirm our findings especially in Egyptian populations. In the light of these data, further studies concerning other TLR gene polymorphisms will contribute to a better understanding of the pathogenesis of the disease.

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