

## Designing of specific primer pairs for quantitative analysis of TLRs (Toll-Like Receptors) genes in patients with celiac disease by polymerase chain reaction technique

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### ABSTRACT

Celiac disease (CD) is an autoimmune enteropathy caused by an abnormal immune response to dietary gluten that occurs in genetically susceptible individuals. Recent studies suggest the crucial role of innate immunity in the pathogenesis of celiac disease. Toll-like receptors (TLRs) are pattern recognition receptors that play a central role in the initiation or maintenance of innate immune responses. Dysregulation of this innate immune receptors results in chronic inflammatory responses. The aim of this study was to investigate the new specific primer pairs for quantitative analysis of human TLR2, TLR4, TLR7 and TLR9 genes in blood samples of patients with celiac disease by polymerase chain reaction technique. At first the DNA of few confirmed patients with celiac disease was extracted directly from blood samples and PCR was carried out using specific primers for human TLR2, TLR4, TLR7 and TLR9 and then amplified PCR products was confirmed by gel electrophoresis.

The result of this study showed that every four TLRs were detected correctly in blood samples of patients with celiac disease with specific size. Therefore we suggest that blood sample of celiac disease patients are alternative source for investigate the TLRs gene expression instead duodenal biopsy specimens.

**Keywords:** Celiac disease, Toll like receptor, polymerase chain reaction.

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### Introduction

Celiac disease (CD) is an autoimmune enteropathy caused by inappropriate reaction of immune system to components of wheat family

cereals in a genetically predisposed individuals that damages the small intestine (1-3).

This disease is characterize by partial to total villous atrophy, crypt hyperplasia, increased

number of intraepithelial lymphocytes and their activation (4). Also the damage of mucosal small intestine resulted in a wide range of clinical manifestations like diarrhea or malabsorption, abdominal pain, fatigue and anemia (5). Population studies showed that around 1–2% of European and US population are infected with this autoimmune disorder (6).

It is well known that human leukocyte antigen (HLA) class II, especially HLA-DQ2 and HLA-DQ8 strongly associated with risk of the CD (7). Many investigations showed that immune system is key actors for response to gluten/gliadin peptide and demonstration of changes in the histology of the small intestinal mucosa (6, 8). In addition, gluten-specific T cell reactivity to gluten is likely to play a central role in the pathogenesis of celiac disease (9).

Recent studies point out the possibility that a group of pattern-recognition receptors (PRRs) which are called Toll-like receptors (TLRs) through the recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) play a role in the onset of response to gluten and activate the innate immune system (10-12).

Currently 10 human and 13 mouse TLRs have been identified. They are transmembrane receptors that some of them are found on the plasma membrane such as TLR1, TLR2, TLR4, TLR5 and TLR6, whereas others are located in the endolysosomal compartment such as TLR3, TLR7, TLR8 and TLR9 (Figure 1)(13). Also these PRR receptors are expressed in intestinal epithelial cells (IECs), enteroendocrine cells, myofibroblasts and on immune cells in the lamina propria such as dendritic cells (DCs) and macrophages (14).

The result of different studies about the role of TLRs in CD pathogenesis showed that some

gliadin-derived peptides such as p31-p43 does not bind to HLA; therefore they are not able to stimulate CD4+ T cells. These peptides through PRRs especially TLRs of intestinal macrophages and dendritic cells leading to increased immune responses against gluten (15). In this process, the toxic gliadin peptide 31–43 via binding to TLRs result in up-regulating the expression of IL-15 from intraepithelial lymphocytes (IELs) and immune cells. On the other hand, this binding could be activate CD83 and CD25 markers in lamina propria cells and induce an innate immune response (15, 16).

In addition, several recent studies showed that one of the key adapter molecule in the TLRs signaling pathways is called myeloid differentiation factor 88 (MyD88) (Figure 1) with releases a paracrine zonulin and opens the intestinal epithelial tight junctions result in gluten peptides passage into the lamina propria and increase immune responses (17, 18). So the aim of this study was to investigate the new specific primer pairs for quantitative analysis of human TLR2, TLR4, TLR7 and TLR9 genes in blood samples of patients with celiac disease by polymerase chain reaction technique.

## **Materials and Methods**

### **Patients**

In this study we used a blood samples of celiac disease patients who was referred to GI clinics of Taleghani hospital (Tehran, Iran), and confirmed their disease by serological tests, endoscopy and followed by pathology. For these patients no other gastrointestinal diseases, autoimmune disorders, cancer or *Helicobacter pylori* infection were reported.

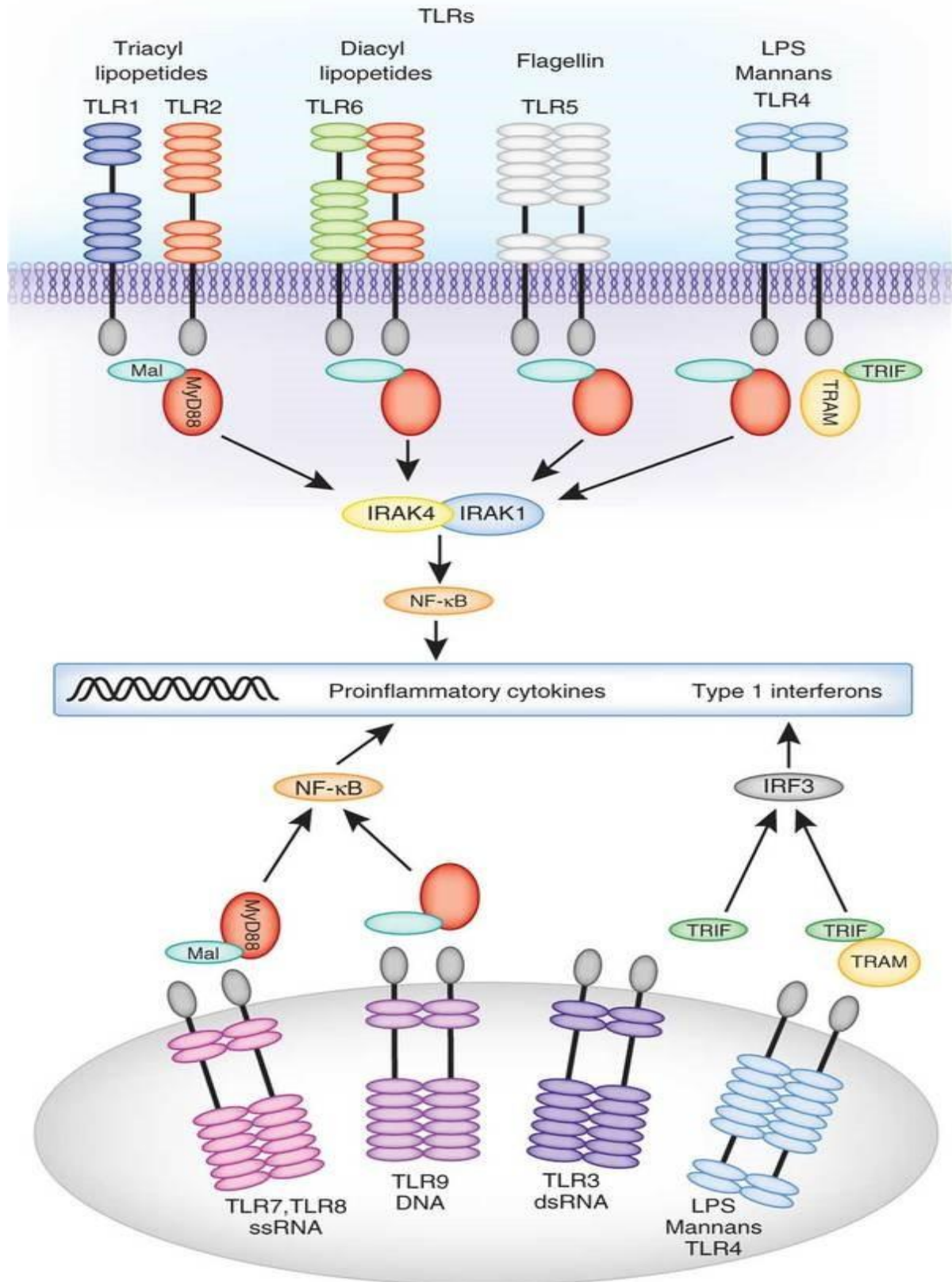


Figure 1. Mammalian TLRs localization and signaling pathways (ref 12).

**RNA extraction and preparation from Human whole blood**

RNA was extracted from buffy coat by commercial kit (YTA Total RNA Purification Mini Kit, Yektatajhez Azma, Tehran, Iran) according to the manufacturer’s instructions. Isolated RNA was eluted to a new RNAase-free tube with 60 mL of RNAase-free water and stored at -70°C.

RNA concentration was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, US). Only high-quality RNA was used in reverse transcription reactions, which were performed using the High Capacity cDNA Reverse Transcription Kit (PrimerScript TMRT Reagent Kit, Takara Bio, Kusatsu, Shiga, Japan) according to the manufacturer’s instructions. The reverse transcription step was carried out using the following program: 25°C for 2 minutes, 42°C for 15 minutes, 85°C for 1 minutes, and 4°C to the end of the run. The concentration of cDNA was calculated on the basis of the concentration of RNA. cDNA samples was stored at -20°C until used as template for PCR.

**Primer designing**

The primer pairs were designed by GenScript Primer Design software (www.genscript.com) (Table 1). In generally, the length of designed primer pairs were around 20-22 bp that the optimal length of PCR primers. Furthermore,

melting temperatures of primers were in the ranges of 56-61oC that produced the best results. Primers with melting temperatures above 65oC had a tendency for secondary annealing. Also two primer pairs had closely matched melting temperatures for maximizing PCR product yield.

The primers had a GC content of around 50-55% to ensure maximum product stability. It was better GC content of forward primers be similar to revers primers. The presence of G or C bases on the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

Lyophilized primers dissolved in a small volume of deionized water or DW to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10 pmol/μl to avoid repeated thawing and freezing. Then all primer solutions were store at -20°C.

**PCR technique**

As presented in table 2 identification of TLRs were performed by polymerase chain reaction technique using commercial Mastermix kit (YTA PCR Mastermix, Yektatajhez Azma, Tehran, Iran). In this process, a total volume of 25 μL containing 2.5 μL of PCR buffer, 1μL magnesium chloride, 0.5 μL dNTP mixture, 0.5μL Taq DNA polymerase, 1μL of template, and 1 μL of each primer.

**Table 1.** Designed primer sequences for TLRs and B2M

	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse	Product length
TLR2	GCATGTGGTGTCTCTGTTC	GAGCTTTCCTTGGCCTCCTT	126 bp
TLR4	TGGACCTGCGATTTAATCCC	GTCTGGATTCAGAGCAGGA	100 bp
TLR7	CACCTGTAGTGCTGTGTCGTT	TCACATCTGAGGGCACCTAAG	77 bp
TLR9	AGGCACCTGTCACTCTTGTACA	GTAGGACAACAGCAGACGATCC	84 bp
B2M	TGCTGTCTCTGAGTTTGATGTATCT	TCTCTGCTCCCCACCTCTATAG	102 bp

**Table 2.** Cycling conditions for amplifying longer PCR products

Gene	Denaturing cycle	Parameters (40 cycles)
TLR2	95°C 5min	94°C 40s, 59.7°C 30s, 72°C 30s, 72°C 10min
TLR4	95°C 5min	94°C 40s, 57.9°C 30s, 72°C 30s, 72°C 10min
TLR7	95°C 5min	94°C 40s, 59.7°C 30s, 72°C 30s, 72°C 10min
TLR9	95°C 5min	94°C 40s, 59.7°C 30s, 72°C 30s, 72°C 10min
B2M	95°C 5min	94°C 40s, 60°C 30s, 72°C 30s, 72°C 10min

PCR products were run on 1.5% agarose gels and stained with ethidium bromide. The specific size of the amplified PCR products was confirmed by gel electrophoresis.

To test the complementary DNAs for representation, PCR with B2-microglobulin (B2M) was performed for the same sample.

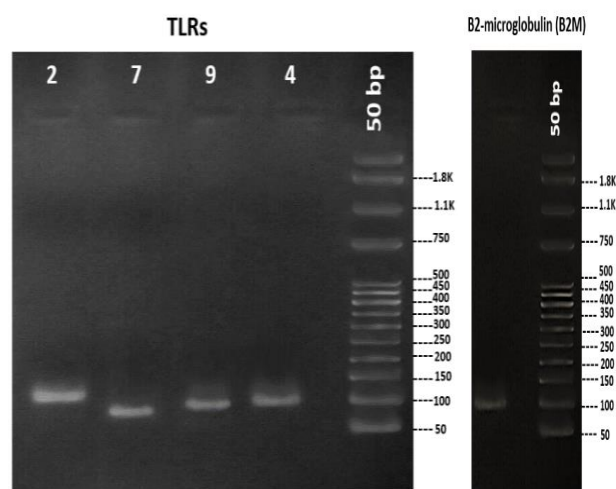
## Results

The molecular analysis of TLRs genes in patients with celiac disease was performed by PCR and specific primer pairs. In order to investigate the specificity of the primers and confirmed the detection of these genes in CD patients, PCR products were run on 1.5% gel by gel electrophoresis technique. Figure 2 show the result of gel electrophoresis. These data were obtained with use of specific primer pairs for human TLR2 (126 bp), TLR4 (100 bp), TLR7 (77 bp), TLR9 (84 bp), B2M (102 bp) and by computerised analysis of PCR products.

It is clear, all of the bands intensity was expressed as absolute integrated optical density; in the other words optical density of the investigated PCR products was corrected for housekeeping gene (B2M). The primers anneal to the template sequences with high specificity, so amplification of nonspecific PCR products and/or primer-dimers was not occur (Figure 2). The reason for this event was well-designed primers and their specificity for template.

The primers and Mg<sup>2+</sup> concentration in the PCR buffer and annealing temperature of the

reaction optimized for efficient PCR processing. Also the figure 2 show that all of the TLRs which investigated in this study, were specifically detected in individuals with celiac disease.



**Figure 2.** Gel electrophoresis of Toll like Receptors (TLRs) and B2-microglobulin (B2M) amplified products. PCR products were run on 1.5% agarose gels and visualized under ultraviolet illumination and stained with ethidium bromide. Each TLR was mentioned by its specific number.

## Discussion

In this study we definitely detected TLR2, TLR4, TLR7 and TLR9 genes as main conductors of innate immune responses, specifically in CD blood samples by specific primer pairs. CD is a complex small intestinal disorder caused by an abnormal immune response (1, 2). This autoimmune enteropathy is due to a

dysregulated immune response to gluten (3). Although both of innate and adaptive immunity are importance for effective response to gluten but components of innate immunity plays a critical role in initiation of this process (6, 8, 19).

There is little available information world-wide regarding the TLRs gene expression in blood samples from patients with celiac disease as more studies performed on duodenal biopsy samples of CD patients. For example, Szebeni et al. (20) have reported a significant upregulation of TLR2 and TLR4 mRNA expression in the duodenal mucosa of children with untreated CD (16 children) and treated CD (9 children) compared with controls. Interestingly, TLR2 and TLR4 mRNA levels were even higher in the duodenal mucosa of children with treated CD compared with untreated CD. TLR2 and TLR4 proteins were easily measureable in the duodenal mucosa of all controls, however they demonstrated increased TLR2 and TLR4 protein levels in children with untreated CD and treated CD. The authors concluded that the alteration of TLR2 and TLR4 expression in the duodenal samples of CD patients confirmed the probable association of innate immune system in the pathogenesis of this disease.

In addition, Kallioma et al. (21) have reported TLRs gene expression profiles in small intestinal biopsies in individuals with celiac disease. They found that the expression of TLR2 was significantly decreased in untreated and treated celiac patients, whereas expression of TLR9 was increased in untreated celiac patients compared with controls.

In study by Eiro et al. (22) was investigated expression TLRs and inflammation mediators in both adult and children with CD. The results of

their study showed high levels of TLR4 expression as well as transcription factors (IRAK4, MyD88, and NF-KB) in CD samples compared to controls. Also no significant differences between this two groups was reported. All of the studies showed the critical role of TLRs in the pathogenesis of celiac but in contrast to our study, genes expression evaluation were performed on duodenal biopsies.

### Conclusion

In conclusion our study revealed that we can use blood sample for gene expression investigations instead of biopsy samples and our designed specific primer pairs specifically detected the TLRs but in order to fully appreciate the role of TLRs in the pathogenesis of celiac disease more studies are needed.

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### Conflict of Interest

The authors declare no conflict of interest.

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