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A clinical association between Toll-like receptor 2 Arg753Gln polymorphism with recurrent cystic echinococcosis in postsurgery patients: A case control study

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ABSTRACT

Recurrence of hydatid cysts in cystectomy patients has dramatically remained a serious concern within the surgical community. Predisposing factors for recurrence of hydatid cysts remained to be identified. Toll-like receptor (TLR) plays a pivotal role in bridging between acquired and innate immunity in cystic echinococcosis (CE) infection. 117 CE patients including 66 acute hydatidosis (AH; primary infection) and 51 recurrent hydatidosis (RH; chronic infection), and 117 ethnically matched healthy control (HC) were investigated from endemic regions of Iran in the period of 2015–2018. CE patients were definitely confirmed using histopathological and immunological assays. Genotyping of TLR2 Arg753Gln was carried out by restriction fragment length polymorphism and sequencing. The homozygous mutant-type TLR2 Gln/Gln (A/A) was represented to be associated with the occurrence of RH ($P = 0.04$) and conferred a 9 fold risk for susceptibility, while the heterozygous mutant-type TLR2 Arg/Gln (G/A) indicated a tendency to be associated with the occurrence of RH ($P = 0.07$). There was no discrepancy in the frequency of TLR2 Arg753Gln haplotypes between AH patients and HC individuals ($P = 0.09$). The mutant allele A was observed to be a risk factor for susceptibility to RH patients. Our results point to a clinical association between TLR2 Arg753Gln haplotypes with RH in postoperative patients. It can be inferred that allele G may lead to protection against the CE, while mutant allele A may be a diagnostic hallmark in the screening of RH susceptibility. Nevertheless, further studies with a larger sample size of different ethnic populations are required to authenticate this association.

1. Introduction

Echinococcus granulosus sensu lato is the causative agent of life-threatening cystic echinococcosis (CE)/hydatidosis, which lead to serious public health concern particularly amongst the at risk individuals [1,2]. Currently, recurrence of (relapsed) hydatid cysts in post cystectomy patients has dramatically remained a serious problem within the surgical community with respect to follow-up, surveillance, monitoring, and therapeutic programs [3–6]. An unsuccessful operation and spontaneous rupture of hydatid cysts can cause fatal anaphylactic shock during surgery [4,5]. The pathogenicity and susceptibility to hydatidosis can potentially depend on host genetic factors (e.g., Toll-like receptors (TLR), parasite strain, and host physiology) [2,4]. During the chronic hydatidosis infection in consequence of recurrent cases, the hydatid cysts are well tolerated and non-affected by the host immune response [7]. Recurrence of hydatid cysts regularly observes from 3 months to 20 years after unsuccessful post-operative treatment [8]. However, other predisposing factors for recurrence of hydatid cysts remained to be identified. TLRs play a pivotal role in bridging between acquired and innate immunity and serving as detectors of infectious pathogens [9]. As a result of the extracellular nature of *E. granulosus,* TLR2 has been implicated in recognizing surface hydatid components such as lipopolysaccharide and lipoproteins [7]. It has been shown that mRNA expression level of TLR2 is overexpressed in peripheral blood mononuclear cells of patients with chronic hydatidosis [10]. Recently, our previous findings have been shown that the TLR4 Asp299Gly mutant (A/G) and mutant allele G can be a tendency to be associated with recurrent CE in postoperative patients in endemic regions of Iran [4];

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however the potential impact of TLR2 codon/haplotypes has not yet been sufficiently understood for association with acute hydatidosis (AH; primary infection due to egg ingestion) and recurrent hydatidosis (RH; chronic infection due to dissemination of protoscoleces during surgery). A recent study demonstrated that the over expression of TLR2 in patients with hepatic cystic and alveolar echinococcosis might play a possible role in modulating tissue infiltrative growth of the parasite and its persistence in the human host [10]. The TLR2 polymorphism and its association with susceptibility to parasitic diseases have been shown to vary in different ethnic populations [11–14]. This discrepancy with regard to the functional importance of TLR2 may be attributable to genetic variations in TLR responsible for modulating the host immune response (T cell polarization). In the present study, we evaluated the probable role of TLR2 Arg753Gln polymorphism and its association with the susceptibility to RH and AH in Iranian CE populations.

2. Materials and methods

2.1. Ethical approval, Study population (s) and serological assay

All patients were fulfilled an informed consent form to participate in the study. The study protocol was approved by the Ethical Review Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1397.631). In this case-control study, 117 CE patients from general hospitals of Iran (East Azerbaijan, West Azerbaijan, Guilan, and Ardabil Provinces), including 66 cases of acute hydatidosis (AH; primary infection due to egg ingestion) and 51 cases of recurrent hydatidosis (RH; chronic infection due to dissemination of protoscoleces during surgery), and 117 ethnically matched healthy control (HC) were investigated over the last four years (February 2015 to September 2018). The majority of the hydatidosis patients had pulmonary and hepatic hydatid cysts. CE patients were confirmed using radiological, immunological, and histopathological findings. Patients with RH were those who had undergone more than one surgery in their clinical history, while AH patients had no earlier symptoms of CE. Patients with hepatic carcinoma, chronic inflammatory disease, respiratory disorders, and cardiovascular disease were excluded from the examined cases. All collected sera samples were subjected to detect the IgG antibodies against CE by using the Echino ELISA (enzyme-linked immunosorbent assay) Kit (catalog no. 50304096; Pishtaz Teb, Tehran, Iran). Samples with an optical density of 10% higher than the specified cutoff points were considered as positive sera.

2.2. Blood sample collection and DNA isolation

All peripheral blood samples (5 mL) were collected in EDTA (Ethylenediaminetetraacetic acid,) vials and transported to the Central laboratory in TBZMED. DNA was extracted from whole blood using the DNG-plus DNA extraction Kit (Sinaclon, Karaj, Iran). The DNA concentration of each extraction was calculated using a Nano Drop (Thermo Scientific Inc).

2.3. Genotyping of TLR2 Arg753Gln haplotypes by restriction fragment length polymorphism

Single-round polymerase chain reaction (PCR) was done to amplify TLR2 Arg753Gln haplotypes. PCR products were subjected to electrophoresis on 1.5% agarose gel. The primer set and PCR thermal cycling conditions are given in Table 1. The PCR reaction was done in a total volume of 20 μL including; 2.5 μL 10 × PCR buffer, 1 μL each primer (15 pM), 10 μL double-distilled water (D.D.W), 1.5 μL MgCl2 (25 mM), 0.2 μL Taq polymerase (5 U/μL) and 0.8 μL dNTP mixture (10 mM) and 3 μL target DNA. PCR products (10 μL) were subjected to restriction fragment length polymorphism (RFLP) using *AciI* restriction endonuclease (1 μL; with cut site C^CGC, blunt end) (Fermentas, Thermo Fisher, USA) for TLR2 Arg753Gln containing 9 μL double-distilled water

Table 1
PCR conditions, primers, and RFLP method of TLR2 Arg753Gln polymorphism.

Gene	Codon	Primer sequence, 5′–3′	PCR condition Temperature °C/time (sec)	Amplicon Size, base-pairs	Restriction enzyme	Length of the Restriction fragments
TLR2	Arg753Gln	F 5′- GGGACTTCATTCCTGGCAAGT-3′ R 5′- GGCCACTCCAGGTAGGTCTT-3′	35 cycles: 94 °C 30 s, 65 °C 30 s, 72 °C 60s	264bp	*AciI* (Fermentas)	Wild type = 264bp Homozygous mutant type = 234 + 30bp Heterozygous mutant type = 264 + 234 + 30bp

Table 2
Demographic data of examined patients with AH and recurrent CE patients in endemic regions of Iran.

Parameters			Hydatidosis patients (n = 117)	AH patients (n = 66)	Recurrent CE patients (n = 51)
Sex		Female	48	24	24
		Male	69	42	31
Residence		Urban	50	25	25
		Rural	67	42	25
Iran	**Province**	East Azerbaijan	71	40	31
		Ardabil	17	9	8
		West Azerbaijan	12	6	6
		Guilan	17	11	6

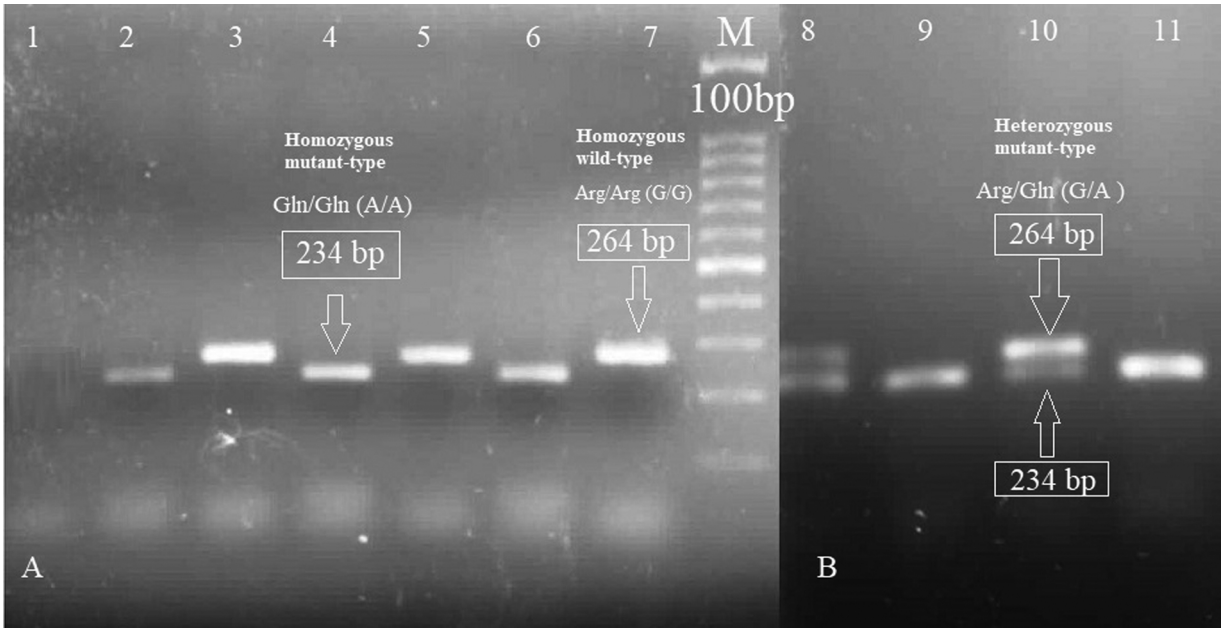

Fig. 1. A and B: Agarose gel electrophoresis of the TLR2 Arg753Gln polymorphism: Lane 1: negative control, lanes 2, 4, 6, 9 and 11: homozygous mutant-type (A/A), and lanes 3, 5, and 7: homozygous wild-type (G/G), lanes 8 and 10: heterozygous mutant-type (G/A), M = 100bp DNA ladder.

and 5 μL 10x enzyme buffer overnight at 37 °C. The digested fragments were observed on a 3% agarose gel and stained with SYBR safe stain. The obtained fragments corresponded to homozygous wild-type (264bp), heterozygous mutant-type (264 + 234 + 30bp), and homozygous mutant-type (234 + 30bp).

2.4. DNA sequencing, phylogenetic analysis, and statistical analysis

Amplicons belonging to AH isolates (n = 10), RH isolates (n = 10), and HC individuals (n = 10) were directly sequenced (Puya Gostar gene, Iran) to confirm TLR2 Arg753Gln codon/haplotypes. The multiple sequences were edited and compared to RefSeq (Accession number: XM_023227837) using the Sequencher software (Tmv.4.1.4). The multiple sequence alignment was performed by BioEdit software based on ClustalW method (7.2.5). To authenticate the genetic variability of TLR2 Arg753Gln, haplotype diversity (Hd) was computed using DnaSP software version 5.10. The $\chi 2$ test was used to compare between the CE patients and HC individuals. The logistic regression analysis was used to assess the association of TLR2 haplotypes with CE susceptibility. The independent Student's *t*-test was used to compare the demographic data of hydatidosis patients.

3. Results

In this investigation, 117 CE patients (48 women; mean age 39.3 ± 13.7 years and 69 men; mean age 45.2 ± 17.3 years) and 117 age-and-sex-matched healthy individuals were included from hyperendemic provinces of Iran (Table 2). Of the 117 CE patients, 51 RH persons had undergone cystectomy within the last four years. The mean diameters of the hydatid cysts were 12.51 ± 2.12 cm. Between hydatidosis patients and HC group, there were no statistically significant differences in sex, age, or residency ($P > 0.05$). Fragment of 264bp (homozygous wild-type) was successfully amplified by targeting the TLR2 gene (Fig. 1A). To investigate whether there was any association between TLR2 mutants and susceptibility to RH and AH, the TLR2 gene was directly genotyped using the PCR–RFLP method. The obtained genotypes of TLR2 Arg753Gln are shown in Fig. 1A and 1B. The homozygous mutant-type TLR2 Gln/Gln (A/A) haplotype was represented to be associated with the occurrence of RH ($P = 0.04$; odds ratio: 2.2; 95% confidence interval: 1.3–17.8) and conferred a 9 fold risk for susceptibility, while the heterozygous mutant-type TLR2 Arg/Gln (G/A) indicated a tendency to be associated with the occurrence of RH ($P = 0.07$ OR: 1.72, 95% CI: 0.33–29.4) (Table 3). There was no discrepancy in the frequency of TLR2 Arg753Gln haplotypes between AH patients and HC individuals ($P = 0.09$; OR: 4.11; 95% CI: 0.61–15.2). The frequency (%) of TLR2 Arg753Gln genotypes identified in AH, RH, and HC individuals is given Table 3. Interestingly, the majority of homozygous/heterozygous mutants (A/A and G/A) were detected in the period of 21–24 months after postoperative, but the difference was not meaningful ($P > 0.05$). A greater haplotype diversity of TLR2 Arg753Gln was found in RH patients (Hd: 0.7) than AH patients (Hd: 0.2) and HC individuals (Hd: 0.0). The distribution of TLR2

Table 3
Genotype distribution of TLR-2 Arg753Gln (CGG→CAG) in AH, recurrent CE patients, and healthy control individuals.

Gene	Codon	Genotype (Haplotype)	Allele	Healthy Group n = 117 (%)	Total CE n = 117 (%)	Acute CE patients n = 66 (%)	Recurrent CE patients n = 51 (%)	CE patients and control subjects			Acute CE patients and control subjects			Recurrent CE patients and control subjects		
								p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI
TLR2	TLR-2 Arg753Gln (CGG→CAG	Arg/Arg (G/G)	G (homozygous wild-type)	117 (100)	97 (82.9)	59 (89.4)	38 (74.5)	Reference group			Reference group			Reference group		
		Arg/Gln (G/A)	A (heterozygous mutant-type)	0 (0)	6 (5.1)	2 (3)	4 (7.8)	0.07	3.2	0.33-10.1	0.1	2.82	0.1-13.7	0.070	1.72	0.33-29.4
		Gln/Gln (A/A)	A (homozygous mutant-type)	0 (0)	14 (12)	5 (7.5)	9 (17.6)	0.03	2.1	0.51-12.3	0.09	4.11	0.61-15.2	0.04	2.2	1.3-17.8
		Allele frequency	G Allele (Arg)	234	200	120	80	Reference group			Reference group			Reference group		
			A Allele (Gln)	0	34	12	22	0.02	0.22	0.451-8.9	0.88	6.6	0.66-19.8	0.045	7.08	0.49-23.5

Note; NC, not calculated, CI; confidence interval, OR; odds ratio.

Arg753Gln homozygous/heterozygous mutants (G/A and A/A) in patients with RH (n = 4/9) was more than AH patients (n = 2/5), but the difference was not significant ($P > 0.05$) (Table 3). The mutant allele A was observed to be a risk factor for susceptibility to RH patients ($P = 0.045$; OR: 7.08; 95% CI: 0.49-23.5%) (Table 3). The number of homozygous/heterozygous mutants of TLR2 Arg753Gln based on cystectomy organs (Hepatic cyst, pulmonary cyst, and other) did not show a significant association between mutant types and cyst location ($P > 0.05$). Based on sequencing and multiple alignment analyses, the non-synonymous substitutions of TLR2 Arg753Gln (Glutamine: Q replaced by Arginine: R) were observed at codons 57 in RH and AH patients, while no amino acid substitution was identified in the healthy group (Fig. 2).

4. Discussion

Host genetic background towards the alteration of innate immune markers (e.g., TLR2 and TLR4 genes) can potentially influence the outcome of parasitic infections [15–18]. In chronic CE, the host–parasite interactions and resulting innate immune responses to recurrent hydatid cysts have not fully understood [4]. This case-control study provides the clinical evidence that would support the possible role of TLR2 Arg753Gln haplotypes in susceptibility and recurrence of hydatid cyst in AH and RH patients in Iranian population. We found an association between the A/A genotype/mutant allele A and susceptibility to symptomatic RH in this studied population, while the dominant G/G genotype and the allele G appear to hypo-responsiveness against hydatidosis infection. On the other side, our earlier findings have shown that the A/G genotype and mutant allele G of TLR4 Asp299Gly have a tendency to be associated with the occurrence of RH and conferred a 3-fold risk for susceptibility. This may be due to haplotype diversity in the TLR2/TLR4 genes in patients with symptomatic RH resulting in defective TLR2/TLR4 signaling cascades, which modulates the immune response system of the host [19]. Furthermore, no significant correlation was observed between the occurrence of the TLR2 Arg753Gl mutants and susceptibility to AH. This notion also supports by our previous findings in which TLR4 Asp299Gly genotypes had not shown to be a considerable association with AH susceptibility [4]. This congruency is posed by the fact that the extracellular domains and adaptors of TLR2 and TLR4 may not be attributed to strong genetic variations, by skewing the immune system towards a Th2 response in AH patients. A study has shown that the soluble egg antigens of *Schistosoma mansoni* can enhance the production of TGF-beta and IL-10 in the multiple sclerosis cells (Dendritic cells and B cells) through TLR2-dependent mechanisms and subsequently promoting toward Th2 responses [20]. To our knowledge, most studies associated with TLR2 polymorphism have been explored on protozoan infections [11–14]. In a study conducted by Ejghal et al. [14] it was reported that the polymorphism of the Arg753Gln TLR2 gene leads to increased susceptibility to visceral leishmaniasis in the Moroccan patients [14]. By contrast, no association was observed in the plausible role of TLR2 Arg753Gln polymorphism on the Chagas' disease (caused by *Trypanosoma cruzi*) in the Colombian population [13]. Moreover, no strong association was observed in a study analyzing the role of Arg753Gln TLR2 polymorphism on the cutaneous leishmaniasis caused by *Leishmania major* [11]. Interestingly, another study has been clarified the TLR2Δ22 polymorphism is associated with protection from cerebral malaria due to *Plasmodium falciparum* in Uganda population [12].

A recent study conducted by Guimarães et al. [21] indicated an absence of association of P631 H/R753Q TLR2 codons and malaria complications in Southern Brazilian population [21]. In reality, these inconsistent associations may differ from one population to another as a result of the parasite strain, host genetic background, bottlenecks of TLR2, the gene–gene, and the gene-environment interactions [14]. On the one hand, the overall heterogeneous distribution of TLR2 polymorphism amongst studied populations (East Azerbaijan, Ardabil, West

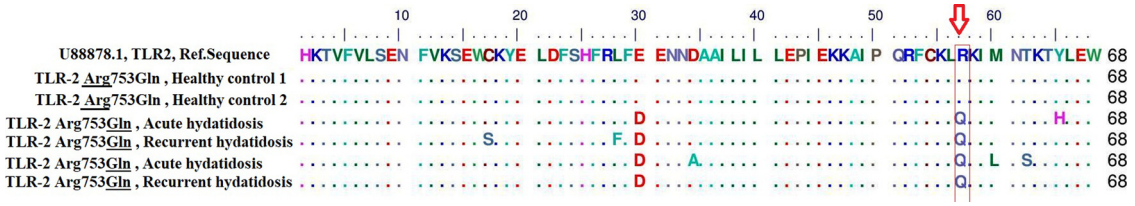

Fig. 2. Multiple alignments of the TLR2 Arg753Gln sequences in recurrent hydatidosiss, acute hydatidosis, and healthy control individuals. The non-synonymous substitution (Glutamine: Q replaced by Arginine: R) occurred at codon 57.

Azerbaijan, and Guilan) can be the result of population expansion and gene migration (genetic drift). It seems likely that the occurrence of mutant allele A may be a diagnostic hallmark for the screening of RH susceptibility in postsurgery patients. However, this preliminary evidence warrants further investigations with a larger sample size of different ethnic populations in order to validate this assumption. Functional mechanism of the TLR2 Arg753Gln polymorphism (Arginine by Glutamine substitution) shows that Arg753Gln provides polar contacts with chains of the DD loop which attenuates the number of polar contacts and would possibility resulted in disruption of the DD loop structure. Dimerization of the TIR TLR2-domain due to the structural integrity of the BB and DD loops is crucial to affirm downstream signaling [22]. In this study, a greater haplotype diversity of TLR2 Arg753Gln was found in RH patients than AH patients and HC individuals. This depicts that the occurring of synonymous haplotypes may probably lead to emergent codons related to RH susceptibility during chronic hydatidosis. It is suggested that the contribution of genetic variations in innate immune response components such as rare TLR2 Arg677Trp codon, TLR1, TLR6 and TLR9 polymorphisms in the susceptibility of AH and RH should be assessed to get a clear picture of this association.

In conclusion, the clinical implication of our observations supports the plausible role of TLR2 Arg753Gln haplotypes in the recurrence of hydatidosis in postoperative Iranian patients. However, more studies with a larger sample size and ethnically diverse population, other susceptibility factors along with marginal statistical associations would be essential to gather more conclusive evidence to authenticate this assumption.

Authors' contributions

AS & MMO: carried out the molecular genetic studies and have been involved in drafting the manuscript. MAM & ZA& JN: participated in the design of the study, contributed to data collection and helped to draft the manuscript. EA & AS: performed the statistical analysis and have been involved in critically revising the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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