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Genetic Polymorphism of Toll-Like Receptor 4 Thr399Ile Variant in Iraqi Kurdish Population: Sulaymaniyah Province

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ABSTRACT

Toll-like receptors (TLRs), encoded by innate immune genes, functions to detect microbial ligands. Studies found that polymorphisms in TLR genes among populations are associated with diseases. Among the classes of TLRs, TLR-4 is important for exploring bacterial lipopolysaccharide. TLR-4 Thre399Ile is one of the most important non-synonymous variant which varies among different background populations. Kurds, an ethnic population descended mainly from indigenous inhabitants of Zagros mountain survived for millennia, with nearly 40 million populations, are distributed in Iraq, Turkey, Iran and Syria. There are no studies on genetic variations of TLR4 in this population, particularly in Iraqi Kurdistan. The aim of this study is to find the percentage frequency of TLR4 Thr399Ile variant in Kurdish populations in Iraq, particularly in Sulaymaniyah province. The percentage of single nucleotide polymorphisms (SNPs) of the TLR4 gene was determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The results showed that the heterozygous variant of TLR4 Thr399Ile is 7.1% (n=85) in Sulaymaniyah populations. However, no homozygous mutant variant was found; this suggests that it is either absent or seldom among Kurds in the region. This study emphasises the perceptiveness of the TLR SNP in Sulaymaniyah populations that recommends future study for linking this genetic variation with both infectious and immunological diseases, in addition to historical, anthropological and archaeological studies.

Keywords: Toll- like receptor 4, polymorphism, SNPs, Iraq, Kurdistan, population

1. Introduction

Innate immune genes, for example those encode receptors that sense microbes, are evolutionarily ancient genes which are conserved and varied possibly due to genetic drift or selective pressure for fighting infections such as bacteria (like tuberculosis), fungi, viruses, or parasites like malaria. These pathogen recognition receptors, known as toll like receptors (TLRs), are a class of 10 evolutionarily transmembrane receptors, named TLR1-10. Among the TLRs, TLR4 plays an important role in innate immune response against Gram negative bacteria. The *in vitro* functions and structures of TLR4 has been previously studied that is found on the innate immune cells recognising bacterial lipopolysaccharide (LPS) which can trigger inflammatory cytokines through NF-*k*B pathways [1]. Thus, studying this gene is needed for finding correlations between healthy and diseased populations.

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Human genome project found 2.1 million single nucleotide polymorphisms (SNPs) with 1% of them related to protein functions and revealed that genetic variations are often common among the genes responsible for immunity [2]. Non- synonymous SNPs have been identified in TLR4 gene that modify amino acid sequences in the gene's leucine-rich repeat domain which recognizes microbial ligands. Among these, a SNP (rs4986791), in which an amino acid threonine (Thr399) is substituted by Isoleucine (Ile399), is known as TLR4 Thr399Ile variant (1196C/T). Research has been conducted on TLR SNPs in most ethnic background populations in the world. Nonetheless, investigation of TLRs is lacking in Iraqi Kurdish populations.

Despite of associations of TLR4 Thr399Ile variant with bacterial infections, this variant has also been linked with prognostic chemotherapy and cancer genetic marker [3], cancer genetic risk factor [4], and a damaging impact on functions and structures of the protein [5]. Additionally, this SNP seems to be an important variant for studying ethnic populations since its frequency varies in different populations in different continents including Asia, Africa and Europe. For instance, it is absent or very rare in

Chinese [3] or Korean populations [6], and infrequent in Africans, however, it is significantly prevalent among Caucasian, European and Middle eastern populations [3].

Kurds, the most ancient indigenous people, lived mainly in Zagros mountains and often northern Mesopotamia for thousands years [7]. Approximately, 40 million Kurdish populations are currently distributed in Turkey, Iran, Iraq and Syria, with 100 thousand Kurds have also been refuged all over the world, including European countries. Kurdish people, from prehistoric times until Ottoman period, appear to be homogeneous people as they have been endogamous and tribal nomads inside Kurdistan lands [7]. Kurdistan has also an ancient history of first village around 12,000 years ago [8]. However, research on this area was previously less focused due to wars and internal conflicts. Within last decade, the region has become the most prominent location for archaeological discoveries in the middle east [9]. Kurdistan, as a middle eastern region between Arabia and Eurasia, might be considered as an important site for ancient human migrations because the migrations outside of Africa spread via Arabic Peninsula to Eurasia [10] and possibly through Kurdistan where ancient caves and archaic villages have been found [8,11]. Therefore, the aim of this study is to explore the percentage frequency of TLR4 Thr399Ile variant in Kurdish populations of Iraqi Kurdistan, particularly in Sulaymaniyah province. The hypothesis is whether this polymorphism is either common or rare or absent in Kurdish population.

2. Materials and Methods

2.1. Study area and population

EDTA preserved venous blood were randomly collected from 85 staffs and students in the University of Garmian. The collected population samples represent populations of Sulaymaniyah province, Kurdistan Regional Government (KRG) of Iraq. The sample distributions among Sulaymaniyah province are shown in the Supplementary Table 1. Only Kurdish people, whose family originally descended from Kurdish ancestry, were included in this study. Others with different backgrounds, such as Arabic, were excluded. An informed consent form was filled by each participant and the research was approved by an ethical committee of Department of Biology, College of Education, University of Garmian (Ethical committee No.2: 07/01/2020). The study was conducted in accordance with the Declaration of Helsinki. The collected blood samples were kept in a refrigerator until total genomic DNA were extracted. This study was conducted in April 2019 and its geographical area is shown in Fig. 1.

Figure 1: Map of the study area (Sulaymaniyah Province). The map was designed by Landsatlook viewer (USGS Products, Data available from the U.S. Geological Survey). The black line indicates the geographical area of Sulaymaniyah province

2.2. Genomic DNA extraction:

Total DNA extraction kit (GENET BIO CO., Daejeon, KR) was used by mixing each blood sample (200 µl) with 20 µl proteinase K in a 1.5 ml tube and incubated at 56°C for 10 minutes. Following manufacturer's instructions, purified genomic DNA samples were eluted with 200 µl elution buffer. The extracted genomic DNA samples were kept at -30°C.

2.3. Genotyping of TLR4 Thre399Ile SNP:

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) were used to amplify the single nucleotide polymorphism of TLR4 Thr399Ile variant gene and then digested with *Hinf1* restriction enzymes.

Forward primer, TLR4 Thr399Ile F: GGTTGCTGTTCTCAAAGTGATTTTGGGA**G**AA; and reverse primer, TLR4 Thr399Ile R: ACCTGAAGACTGGAGAGTGAGTTAAATGCT, (Macrogen Co., Seoul, KR) were used for the SNP rs4986791 [12]. The details of the TLR4 Thre399Ile variant gene is described in Fig.2.

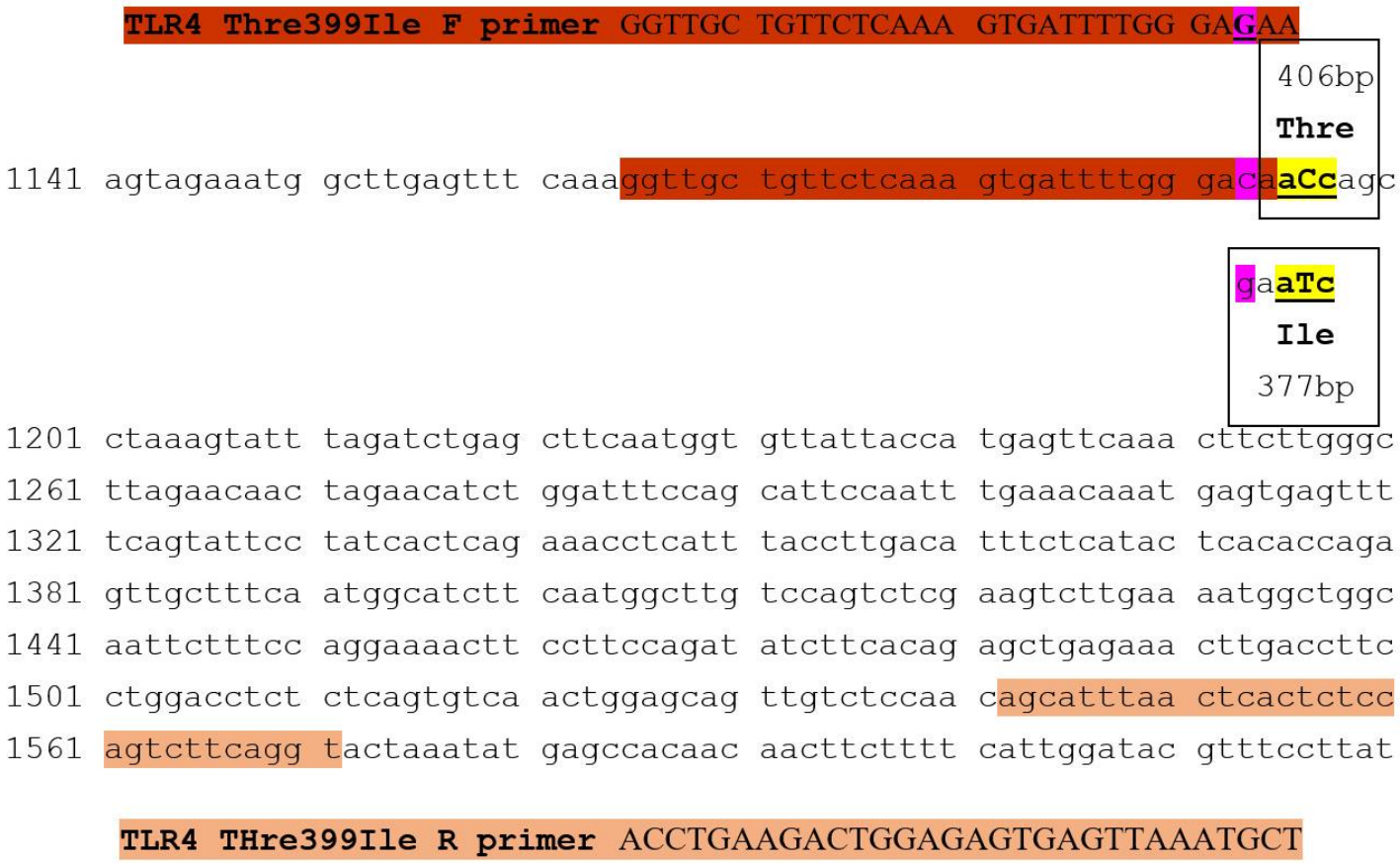

Figure. 2. Homo sapiens TLR4 mRNA for toll-like receptor 4, complete cds: GenBank: AB445638.1. When the primer is designed, the highlighted c (red) is altered to g (red) in the F primer to fit with the restriction site Hinf1 (GANTC)= gaatc. N= any nucleotide. This SNP is known as rs4986791 or 1196 C/T which means the nucleotide cytosine 1196 (C1196) is mutated to threonine 1996 (T1196). The wild type codon (aCc) is Threonine (Thre) and its product size= 406 bp. The SNP codon (aTc) is Isoleucine (Ile) and its product size= 406-29= 377 bp. Thus, the amino acid Thr399 is changed to Ile399. Therefore, the SNP is also called TLR4 Thre399Ieu

PCR reactions were prepared by mixing 10 µl Add start mastermix 2 x (Add Bio Inc, Korea) with 1 µl of each primer (5 pM) and 10 µl genomic DNA. The reactions were run in a thermal cycler (Master cycler nexus, Eppendorf AG, Hamburg, Germany) by initial denaturation at 95°C for 5 minutes; followed by 40 cycles of 95°C for 30 seconds, annealing temperature at 62 for 45 seconds and 72°C for 30 seconds; and finally extended at 72°C for 5 minutes.

Ten (10) µl PCR products were digested by 1 µl of *Hinf1* restriction endonucleases (New England Biolabs, Ipswich, MA, USA) in 4 µl *Hinf1* buffer 4x and 10 µl nuclease free water (Promega) incubated at 37°C for 2 hours. The digested products were mixed with gel loading dye, Purple (6X) (New England Biolabs, Ipswich, MA, USA) and explored on 2% agarose gel, stained with 10 µl Prime safe dye (GENET BIO CO., Daejeon, KR), run with 1x TBE buffer at 100 v, 80 mA for 2 hours. The wild type product size (406 bp) is cleaved with *Hinf1* restriction enzyme to form 377 bp and 29 bp in case of the mutant variant.

To test the Hardy-Weinberg equilibrium, Gene Calculators online program (Chi-square, P>0.05) was used (https://www.genecalculators.net/pq-chwe-check.html).

3. Results and discussion

Current study investigated TLR4 Thr399Ile variant in Kurdish populations in Sulaymaniyah province using PCR-RFLP. The results of this study showed that 6 out of 85 (7.1 %) of the samples have carried the heterozygous mutant alleles of the TLR4

Thr399Ile variant. An example was shown in Supplementary Figure 1.

No homozygous variant was identified and the genotype frequency was not deviated from Hardy-Weinberg equilibrium (Chi-square P=0.735). This variant is infrequent among other populations in middle eastern countries. For example, 5.8 % of TLR4 Thr399Ile heterozygous mutant was determined in healthy Iranian populations and homozygous mutants were only found in chronic cutaneous leishmaniasis cases [12]. Likewise, no homozygous mutants of TLR4 Thr399Ile were found in Turkish populations ,but the heterozygous variants were 9% [13]. A study also suggested weak selective pressure of the TLR4 Thr399Ile variant in fighting infections since the alleles are heterogeneous among Iranian ethnic populations including Iranian Kurds [14]. It has been shown that TLR polymorphisms are associated with both reduced responses to bacterial infections and susceptibility to Gram negative bacterial infections due to LPS signaling impairment [1]. Several studies have focused on laboratory mice, or different ethnic populations including Caucasians and Koreans [1], Iranians [12,14], sub-Saharan Africa, Europe and East Asia [5]. For instance, the TLR4 Thr399Ile SNPs (rs4986791) is only 0.79% in Africans, 8.51% in Europeans, and 0% in East Asians and this SNP is considered as 'possibly damaging' to the function and structure of the protein [5]. Interestingly, in Caucasian populations, TLR4 Thr399Ile is 17.7% heterozygous variant with no any homozygous mutants were found [3].

Up to my best knowledge by the time of writing this manuscript (08/11/2019), no articles were found in Pubmed- NCBI data base when searching for key words as 'TLR4 SNP Iraq'. Although, when searched for 'TLR4 polymorphism Iraq' only one article was published that was about TLR4 Asp299Gly SNP (rs4986790) and the samples were taken from Jordanian populations but one of its authors is from Iraq [15]. Similarly, no data were found for TLR4 SNP in Syria as a country of having Kurdish populations.

In spite of its limitations, this is the first time, TLR4 gene polymorphisms are investigated in Kurdistan Region of Iraq. However, when searching in google for 'TLR SNPs Iraq, in other provinces of Iraq, where the majority of populations are almost Arabs, several articles have been published in several journals which were not cited in this manuscript due to their poor quality. The limitation of the current study is that the other SNP, TLR4 Asp299Gly variant was not investigated. It is worth to mention that this SNP is often co-segregated with the SNP investigated in this study. The co-segregated TLR4 Asp299Gly/Thr399Ile mutants play an important role in inflammatory response to bacterial LPS challenge similar to the wildtype variants known as 'functional neutrality' [16]. Both segregated mutations may have been originated from Africa approximately 60,000 years ago, spreading out through Arabian Peninsula and middle eastern route to Europe as a result of genetic drift [16]. Further researches are required to investigate both SNPs in Kurdish ethnic populations in all Kurdish inhabiting provinces and countries including Iraq, Iran, Turkey and Syria. Particularly, comparing the mutants among the main Kurdish tribes will give an idea about the selective pressure and homogeneity of the TLR4 SNP alleles in Kurdish ethnic populations.

Genetic variations, together with historical, demographic, linguistic, anthropological and archaeological findings, can be exploited as a population marker to study human evolution [17]. Discovery of both Neanderthal and Denisovan like haplotypes of TLR1, 6, and 10 in modern human indicates gene flows among archaic human species [18]. Investigations of gene polymorphisms in Kurdish people might be of archaeological and anthropological interests since the area was a homeland of ancient civilizations and archaic human species. For instance, in Shanidar, (an ancient cave in Zagros mountain belonging to Iraqi Kurdistan), Neanderthal bones were found and dated back to about 40,000-70, 000 years ago [11]. This is apparently the period when both *homo sapiens* and *Homo Neanderthals* encountered and interbred [19,20] Research about ancient DNA has not been recorded yet in human species in the Kurdistan caves. It might be fascinating for both archaeologists and evolutionary geneticists to seek polymorphisms in innate immune genes between the two human species.

4. Conclusion

In conclusions, Iraqi Kurdish populations, lived in Sulaymaniyah province, bear only the heterozygous variant of TLR4 Thr399Ile (7.1%). This suggested the rarity of the homozygous mutant in the province. Further study with large size samples is recommended to confirm this, taking samples from other provinces in Kurdistan region. This also suggests that TLR4 Thr399Ile SNP in Kurdish people is neither very common as in Caucasian nor rare as in East Asian nor uncommon as in African people. This result also suggests that the Kurds has TLR4 Thr399Ile SNP frequency similar to that of Europeans or Middle Eastern nations as in Iran; it supports both Indo-Iranian and Endo-European language families. It seems to be exciting to compare the TLR4 SNPs between modern and ancient human species found in the caves of Kurdistan. Finally, this investigation unlocks a main gate for further research on genetic associations of this innate immune gene with infectious, immunological or non-infectious diseases, and it is also anthropologically and genetically important for studying Kurdish population.

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Supplementary Information

Supplementary information related to this article can be found at: (Supplementary) Niranji, Sh. S. Passer 1 (2020) 32- 36

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