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STUDY LEPTIN AND TOLL-LIKE RECEPTORS GENE POLYMORPHISMS ROLE IN SUSCEPTIBILITY TO AMEBIASIS

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ABSTRACT

Background: Amebiasis, is prevalent worldwide caused by *Enatmoeba histolytica*, Demographic risk factors for this disease are well-illustrated; however, genetic predisposing factors are poorly defined.

Aims: it aims to assess the role of Gln223Arg variants in leptin receptor (*LPTR*) gene and Asp299Gly and Thr399Ile variants in toll-like receptor-4 (*TLR-4*) gene in the susceptibility to amebiasis in Iraqi patients.

Subjects and Methods: a case/control study, included 150 patients with diarrhea were investigated by direct wet mount stained with Lugol's iodine solution for *E. histolytica*. Forty-eight of those gave a positive- results and represented the cases. Other 50, family unrelated, sex-, age-, and geographical residence-matched apparently healthy individuals were chosen to represent the control group. DNA was extracted form blood samples from each participant. *LEPR* and *TLR-4* gene fragments corresponding the three variants were amplified with specific sets of primers. The amplified genes were subjected to direct sequencing.

Results: The variant Gln223Arg was found to be significantly associated with the susceptibility to amebiasis. Both AG and GG genotypes were more frequents in cases than controls with significant differences. This association was further confirmed in genetic model and allele analysis. In recessive model, the prevalence of AG+GG genotypes were significantly higher in cases than controls. Similarly, the minor allele (allele G) was more frequents among cases than controls with significant difference.

Conclusion: These results suggest the role of Gln223Arg variant in *LEPR* gene in the susceptibility to infection with *E. histolytica.*

Keywords: *E. histolytica,* leptin receptor, toll-like receptor-4, gene polymorphism

INTRODUCTION

Amoebic dysentery is a protozoan disease caused by *Entamoeba histolytica*. Worldwide, about 50 million individuals with different ages are affected with this disease annually; 10% of whom can develop invasive amebiasis associated with up to 11000 deaths per annum (Fletcher *et al*., 2012). In Iraq, Al-Taie (2011) ranked this parasite as the most prevalent protozoal infection of gastrointestinal tract. Another recent retrospective large study involved the records of 2761990 individuals reported a 3.78% rate of infection with *E. histolytica* all over the country (Al-Saqur *et al*., 2017).

Factors such as consuming raw vegetables, eating with hands, handling animal excreta in field work, the presence of other members within the family infected with the parasite and close contact with domestic animals are known to significantly increase the risk for having the disease (Anuaradha *et al*., 2012; Duc *et al*., 2011). However, even in population exposed for almost similar environmenttal factors, there is an individual variation in the susceptibility to the disease which indicating a genetic role.

Leptin receptor gene (*LEPR*) is located on short arm of chromosome 1 and consists of 18 exons (Chung *et al*., 1996). These receptors act as ligands for an important hormone, leptein. Although, leptin is commonly linked with obesity, it has wide range of function especially in regulation of body immunity. Leptin takes part in differentiation, proliferation, survival and function of cells involved in innate and adaptive immune response (Fernández-Riejos *et al*., 2008; Lo *et al*., 2009), deviation of the immune response towards Th1 phenotype (De Rosa *et al*., 2007), and modulation of intestinal barrier function by inhibiting apoptosis of intestinal epithelium (Sukhotnik *et al*., 2009). This protein may have a distinctive role against intestinal infections including amebiasis. Certain single nucleotide polymorphisms (SNPs) in *LEPR* gene could encode for aberrant protein with non-optimal function, and accordingly, may be associated with the altered susceptibility to amebiasis. In recent study, we report a significant increase in *E. histolytica* infection in individuals carrying the G allele of the SNP rs1137101 in LEP gene (Duggal *et al*., 2011).

Toll-like receptors (TLRs) are a group of transmembranous glycoproteins that recognize and interact with pathogen associated molecular patterns (PAMPs) (Chang, 2010). Upon interacting with these PAMPs, TLRs initiate inflammation through the activation of the transcription factor NF-κB which up regulate the production of pro-inflamma -tory cytokines, and reactive oxygen and nitrogen species to eliminate the invading micobes (Iwasaki and Medzhitov, 2010). In mammals, 12 TLRs have been characterized, of which TLR2 and 4 are

implicated in most protozoal infections (Ashour, 2015). Locating on chromosome 9, *TLR-4* gene is highly polymorphic with two SNPs (Asp299Gly and Thr399Ile) were thoroughly investigated and found to be associated with different protozoal infections like leishmaniasis (Eighal *et al*., 2016) and malaria (Apinjoh *et al*., 2013).

To the best of my knowledge, there is no previous study in Iraq addressing the impact of LEP and *TLR-4* gene polymorphisms with amebiasis. Therefore, this study aimed to evaluate the role of one SNP in *LEPR* gene (Gln223Arg) and two SNPs in *TLR-4* gene (Asp299Gly and Thr399Ile) in susceptibility to amebiasis among Iraqi patients.

MATERIALS AND METHODS

A total of 150 patients with different ages from both sexes who were suffering diarrhea and attending out clinic of Al-Imamain Al-Kadhumain Medical City, Baghdad, Iraq during the period from September 2016 to May 2017 were investigated for *E. histolytica*.

Stool samples from each subject was collected in a clean, dry tight cover container and examined within a half an hour. The samples were examined for the presence of *E. histolytica.*

Stool sample examination: Macroscopic examination was achieved through observing the consistency of stool, presence of blood and/ or mucous. Microscopic examination involved using one drop of normal saline and small amount of stool from different places of stool by using clean wooden stick, especially where blood or mucous were noticed, then mixed gently with normal saline and covered with cover slip. The prepared slide was stained with Lugol's iodine solution and examined under the low (10x) and high power (40x) of microscope. (Frances *et al*., 2009). The detection of RBC-containing trophozoite is considered diagnostic for the infection with *E. histolytica*. Out of 150 examined patients, 48 patients have confirmed that having infection with *E. histolytica*, and those represented the cases.

Other age, sex, and geographical residence-matched 50 apparently healthy subjects were recruited to represent the control groups. Exclusion criteria for control were the presence of diarrhea at sampling time or any recent history of taking metronidazole. A consent form explaining the purpose and some details about the study was obtained from each participant. Demographic data including age, sex, residence (rural and urban), educational level, socio-economic level, occupation (labor, nonlabor), and marital status were obtained through direct interview with the patients or with his/her either parents in case of children.

DNA Isolation, Gene Amplification and Genotyping: Three ml of venous blood were collected in EDTA tubes from cases and controls. DNA was isolated from leukocytes using a ready kit (Favor prep DNA extraction mini kit/ Favor Gene Biotechnologies/Taiwan) following the manufacturer's protocol. Two set of primers were used for *LEPR* and TLR4 genes amplification. For *LEPR* gene, the forward and reverse primers were: 5'-AATGT CTTGTGCCTTGTGC-3', and 5'-CAGTGTTAA GGCAAAGTGAGATAAGC respectively with an amplicon of 230bp. For *TLR-4* gene forward primer 5'-TCTGGCTGGTTTAGAAGTCCA-3'and reverse primer 5'-AATTGCCAGCCATTTTCAA G-3'were used with an amplicon of 698bp involving the two targeted SNPs in this gene. The reaction tube (Bioneer/Korea) contains 0.4 µmol-l forward primer, 0.4 µmol-l reverse primer, DNA template (2 ng), 4 mmol-l MgCl2, Taq DNA polymerase (0.05 µl), dNTPs (dATP, dCTP, dGTP and dTTP, 0.4 mmol each. The final volume was adjus ted to 50µl by adding ddH2O. PCR conditions for both genes were as previously mentioned (Wang *et al*., 2014). After successful amplification of the targeted regions, 35µl of PCR product along with primers, were sent abroad to Macrogen Company/ Korea for direct sequencing. Alignment was conducted using alignment tool, BLASTn for nucleotide sequence (http://www.ncbi.nlm.nih.gov). Chromas pro software was used to analyze the sequences.

Data Analysis: Data were subjected to statistical analysis using SPSS software (version 20). Continuous variables were expressed as mean± standard deviation (SD) and analyzed with student t test, while binomial variables were expressed as percentages and analyzed with Chi-square test. The association of each SNP with the incidence of amoebiasis was analyzed via calculation the odds ratio (OR) with 95% confidence interval (CI) using logistic regression test. Exact 2-sided significant was depended and a difference with p-value of ≤ 0.05 was considered significance.

RESULTS

Demographic Data: Table 1 shows the baseline characteristics of cases and controls. Although individuals with no education and those of low socio-economic are more frequent among cases, the differences were not significant. Similarly, other factors did not differ significantly between cases and controls.

Table 1: Baseline characteristics of the study population

Variables	Cases 48	Controls 50	P-value
Age (years, mean ± SD)	27.83± 11.7	26.91± 8.64	0.849
Gender			
Male	29 (60.42%)	33 (66%)	0.676
Female	19 (39.58%)	17 (34%)	
Residence			
Urban	22 (45.83%)	23 (46%)	1.00
Rural	26 (54.17%)	27 (54%)	
Educational Level			
No education	7 (14.58%)	4 (8%)	0.301
1-6 years	23 (47.92%)	20 (40%)	0.184
7 years or more	18 (37.5%)	26 (52%)	0.241
Socio-economic Level	17 (35.42%)	10 (20%)	0.190
Low	24 (50%)	28 (56%)	0.085
Medium	7 (14.58%)	12 (24%)	0.485
High			
Occupation			
Laborer	33 (68.75%)	39 (78%)	0.363
Non-laborer	15 (31.25%)	11 (22%)	
Marital status			
Married	30 (62.5%)	34 (78%)	0.672
Unmarried	18 (37.5%)	16 (32%)	

Association of Different Polymorphisms with Amebiasis: Figure 1A and B shows gel electrophoresis of amplified *LPR* and *TLR-4* genes respectively stained with ethidium bromide.

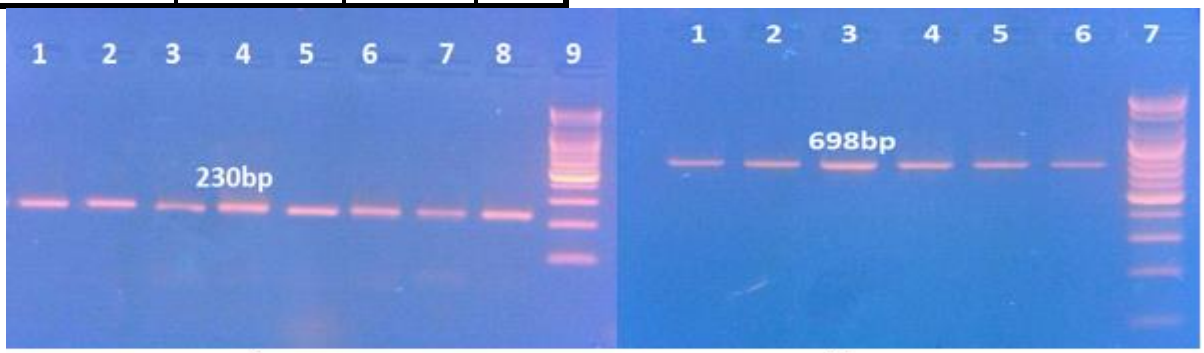

Figure 1: Gel electrophoresis for *LEPR* gene (A) and *TLR-4* gene (B) after amplification with specific primers and staining with ethidium bromide. M: 100bp molecular ladder.

All the studies SNPs were in a good accordance with Hardy Weinberg Equilibrium (HWE). Sequence analysis (table 2) revealed that both Gln223-Arg and Asp299Gly polymorphisms appeared in three genotypes in cases and controls which were AA, AG and GG. For the SNP Q223R, these genotypes accounted for 33.33%, 47.92% and 18.75% respectively in cases compared to 58%, 34% and 8% respectively in controls with significant differrences for both heterozygous genotype, AG (OR= 2.452, 95%CI=1.022-5.882, P= 0.0450, and homozygous mutant genotype, GG (OR=4.078, 95% CI=1.082-15.367, P= 0.038).

Genetic model analysis revealed that this polymorphism exerts its effect through recessive model as the frequency of mutant allele-including genotypes (AG+GG) was far more frequent in cases than controls 66.67% vs 42% with significant difference OR=2.762, 95% CI=1.214-6.283, *P*= 0.016. Furthermore, the rate of mutant allele (G allele) of this SNP in cases (42.71%) was higher than that in controls (25%) with highly significant difference (OR=2.236, 95% CI=1.219-4.103, *P*= 0.01).

For the SNP Asp299Gly, the frequency of the three genotypes among cases and controls were very close and there were no statistically significant differences between the two groups. As well, there were no differences neither in allele frequencies nor in genetic models between cases and controls (table 2).

Table 2: The frequency of different genotypes and allele of *LEPR* and *TLR-4* genes polymorphism in cases and controls.

Variants	Cases (48)	Controls (50)	*P*-value	OR (95%CI)
Gln223Arg Genotypes				
AA	16(33.33%)	29(58%)	0.042	1.0 Reference
AG	23(47.92%)	17(34%)	0.045	2.452(1.022-5.882)
GG	9(18.75%)	4(8%)	0.038	4.078(1.082-15.367)
HWE	0.885	0.945		

Dominant model				
AA + AG	39(81.25%)	46(92%)	0.144	1.0 Reference
GG	9(18.97%)	4(8%)		2.654(0.758-9.288)
Recessive model				
AA	16(33.33%)	29(58%)	0.016	1.0 Reference
AG + GG	32(66.67%)	21(42%)		2.762(1.214-6.283)
Alleles				
A	55(57.29%)	75(75%)	0.01	1.0 Reference
G	41(42.71%)	25(25%)		2.236(1.219-4.103)
Asp299Gly				
Genotypes				
AA	30(62.5%)	37(74%)	0.432	1.0 Reference
AG	14(29.17%)	11(22%)	0.339	1.57(0.622-3.958)
GG	4(8.33%)	2(4%)	0.316	2.467(0.423-14.4)
HWE	0.227	0.332		
Dominant model				
AA + AG	44(91.67%)	48(96%)	0.431	1.0 Reference
GG	4(8.33%)	2(4%)		2.182(0.381-12.5)
Recessive model				
AA	30(62.5%)	37(74%)	0.279	1.0 Reference
AG +GG	18 (37.5%)	13(26%)		1.708(0.722-4.038)
Alleles				
A	74(61.21%)	85(64.29%)	0.201	1.0 Reference
G	22(38.79%)	15(35.71%)		1.185(0.815-3.484)
Thr399Ile				
Genotypes				
CC	29(60.42%)	27(54%)	0.329	1.0 Reference
CT	18(37.5%)	18(36%)	0.867	0.931(0.403-2.151)
TT	1(2.08%)	5(10%)	0.136	0.18(0.02-1.698)
HWE	0.343	0.449		
Dominant model				
CC + CT	47(97.92%)	45(90%)	0.205	1.0 Reference
TT	1(2.08%)	5(10%)		0,191(0.022-1.703)
Recessive model				
CC	29(60.42%)	27(54%)	0.16	1.0 Reference
CT+TT	19(39.58%)	23(46%)		0.769(0.345-1.716)
Alleles				
C	76(79.17%)	72(72%)	0.251	1.0 Reference
T	20(20.83%)	28(28%)		0.677(0.35-1.307)

Similarly, the SNP Thr399Ile had three genotypes (CC, CT and TT) in cases and controls, accounting for 60.42%, 37.5% and 2.08% respectively in cases and 54%, 36% and 10% respectively in controls. Although the mutant homozygous genotype (TT) appeared to have a protective effect, the differences were not enough to rise to significant level (OR= 0.18, 95% CI=0.02-1.698. P= 0.136). This result was further confirmed through the analysis of genetic models and allele frequency, which showed no significant difference in their distribution between cases and controls (OR= 0.769, 95% CI=0.345-1.716, *P*= 0.16 and OR=0.677, 95% CI=0.35-1.307, *P*=0.251 respectively).

DISCUSSION

The current study revealed that *LEPR* but not *TLR-4* gene polymorphisms are significantly associated with the susceptibility to amebiasis. Individuals carrying the heterozygous (AG) or mutant (GG) genotypes of the SNP Gln223Arg in *LEPR* gene are approximately 2.5- and 4-time respectively at increased risk of amebiasis compared to those carrying the wild homozygous genotype (A-A). Moreover, these results suggest a dose-dependent association as carriers of GG are at 1.67-fold increased risk compared to AG carriers. Undoubtedly, this influence is referred to the mutant allele (allele G), and this was conspicuously illustrated via allele frequency analysis.

These results are in accord with a previous work conducted by Duggal *et al.*, (2011) who showed strong association of allele G which encodes for arginine at position 223 of *LEPR* gene with the susceptibility to amebiasis among Bengali population. Interestingly, this association persisted even after adjusting the logistic regression

model for all confounders like age, sex and nutritional baseline. Furthermore, those authors demonstrated that the effect of 223Arg did not only increase the susceptibility to amebiasis, but also it was significantly associated the increase the formation of amoebic liver abscesses.

The significant association of of Gln223Arg with the susceptibility to amebiasis could be explained by the interruption that occurs during the binding of leptin to its receptor. Leptin is a 16 kD glycol-protein hormone which is mainly secreted by adipose tissue (Maruna *et al*., 2001). In fact, leptin exerts its effect through two types of receptors: those that exist in the hypothalamus (long type), which when activated by leptin results in a marked suppression of appetite and increased metabolic activity by influencing thyroid and adrenal hormones (El-Haschimi *et al*., 2000; Dhillon *et al*., 2001). The short types receptors exist in peripheral tissues such as liver and intestine (Correia *et al*., 2002). Upon binding to its receptors (of both kin-ds), leptin initiates a downstream signaling cascade including Janus kinase (JAK2) autophosphorylation with the phosphorylation of three tyrosi-nes in the intracellular domain of the leptin receptor. These events lead to activation of two transcripttional regulators which are signal transducer and activator of transcription 3 and 5 (STAT3 and STAT5) (Banks et al., 2000). Using HEK293T cell lines, Marie et al. (2012) showed that activat-ion of STAT3 but not STA5 was necessary for leptin-mediated protection against amebiasis.

Of the numerous eventual effects of this signaling pathway is anti-apoptotic activity of leptin for many cell types including intestinal epithelial cells (Ogunwobi *et al*., 2007). From pathological point of view, *E. histolytica* induce apoptotic-related alterations in the host cell such as chromatin condensation, DNA laddering and caspase-3 activation (Boettner and Petri, 2005). Thus, it can be hypothesized that, in normal instances, the anti-apoptotic effect of leptin signaling through leptin receptor is responsible for protection effect against *E. histolytica* infection.

The Gln223Arg polymorphism is the result of adenine to guanine substitution in nucleotide 668 of exon 6 in *LEPR* gene. This substitution results in the occurrence of positively charged arginine instead of neutral glutamine at amino acid 223 of in the extracellular domain (the binding site of leptin) of leptin receptor. Thus, it affects the normal function of the receptor and negatively influences the ability of leptin to bind the receptor (Anuradha et al *et al*., 2002). This supposed to influence the normal signaling with subsequent ineffective anti-apoptotic activity.

Undoubtedly, TLRs is important in immune response against *E. histolytica.* Upon binding of the trophozoite to these receptors there will an activetion of the classical signaling pathway which activate nuclear factor (NFκB) and subsequent increase in the secretion of inflammatory cytokines (Chandra and Naik, 2008). Beside these cytokines, recent study showed that this activation induces β-defensin-2 and cationic peptide which are able to destroy the trophozoite. The non-significant association between two SNPs in the *TLR-4* gene and the incidence of amebiasis in the current study be explained from two aspects either of both of which are involved: firstly, many other types of these receptors (like TLR-2) are involved of recognition of the parasite and initiate the inflammatory cascade (Ayala-Sumuana et al., 2013) which can compensate the defect (if any) in TLR-4 and secondly, there are no significant influences of these polymorphisms in the normal function of TLR-4, at least regarding response to infection with *E. histolytica*.

These data strongly suggest the role of G allele of Gln223Arg polymorphism in LEPR gene in the susceptibility to amebiasis among Iraqi population. Further studies are required to confirm this association and to illustrate the mechanisms by which this polymorphism affects the normal function of leptin-leptin receptor binding.

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Conflict of interest: The author declares that he has no competing interests.

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