Turkish Journal of Molecular Biology & Biotechnology
(2017) 2: 63-70

ORIGINAL ARTICLE

Turkish JMBB

Functional Variants in TLR4 and The Risk of Morbid Obesity

Tuğba SOYDAŞ[1], Hülya ARKAN[1], Güven YENMİŞ[1,5], Ahmet DİRİCAN[2], Özcan KARAMAN[3], M. Muzaffer İLHAN[3], Ertuğrul TAŞAN[4], Gönül KANIGÜR SULTUYBEK[6]*

[1]Department of Medical Biology, Istanbul University, Cerrahpasa Medical Faculty, Istanbul, Turkey

[2]Department of Biostatistics and Medical Informatics, Istanbul University, Istanbul Medical Faculty, Istanbul, Turkey

[3]Department of Endocrinology and Metabolism, Umraniye Training and Research Hospital, Istanbul, Turkey

[4]Division of Endocrinology and Metabolism, Department of Internal Medicine, Bezmialem University, Bezmialem Medical Faculty, Istanbul, Turkey

[5]Acibadem Healthcare Services, Acibadem University Kerem Aydinlar Campus, Labgen, Istanbul, Turkey

[6]Department of Medical Biology and Genetics, Istanbul Aydin University, Medical Faculty, Istanbul, Turkey

Keywords
Morbid obesity, single nucleotide polymorphism, TLR4

ABSTRACT • Morbid obesity (MO) is an inflammatory disorder with multifactorial etiology, caused by a combination of genetic, autoimmune and environmental factors. Toll-like receptor 4 (TLR4) has been linked with several autoimmune and inflammatory disorders. We, therefore, investigated the genetic variations between TLR4, and MO susceptibility. *TLR4 asp299Gln* rs4986790, Thr399Ile rs4986791 were genotyped in 213 female patients with MO and 204 female healthy controls using the PCR-RFLP method. The frequency of AA genotype and A allele of rs4986790 were significantly higher in MO patients compared to control group (p=0.0378, p=0.0423; respectively), whereas differences in rs4986791 polymorphism were not statistically significant. TLR4 rs4986790 and rs4986791 SNPs demonstrated statistical significance among the combined genotypes AA+CC genotype (p=0.001) in the patient and control subjects. Our findings prove that both single and combined genotype analysis of rs986790 and rs4986791 polymorphisms indicate that the wild genotypes of AA genotype of rs4986790 provide a risk factor against MO disease and AA+CC combined genotype are strongly associated with enhanced risk of MO in a group of women in a Turkish population.

INTRODUCTION

Morbid obesity (MO), the most severe form of obesity, is defined as body mass index (BMI) >40 kg/m^2 and mainly characterized by low-grade inflammation in both blood and white adipose tissue (WAT). Part of the systemic inflammation associated with obesity in which many inflammatory cells tend to accumulate originates from WAT itself. Therefore Morbid obesity (MO), is characterized by excess ad-

Correspondence: Gönül KANIGÜR SULTUYBEK, Istanbul Aydin University, Medical Faculty, Department of Medical Biology and Genetics, 34295 Sefakoy-Kucukcekmece, Istanbul, Turkey, Email: kanigur@istanbul.edu.tr

ipose tissue mass which is considered as an important participant in the regulation of many pathological processes including inflammation (1).

Toll-like receptors (TLRs), originally characterized in the innate immune system, are pattern recognition receptors that are activated by pathogen-associated molecular patterns, such as bacterial cell wall components and induce inflammatory responses (2). TLRs activate the nuclear factor-kappa B (NF-KB) pathway, which results in the synthesis and release of pro-inflammatory cytokines, thereby augmenting the local inflammatory responses (3,4).

Among the 10 human TLRs identified so far, TLR2 and TLR4 are the most defined members (5). The potential clinical and biological significance of TLR4 receptor has been reported in several infectious, autoimmune and inflammatory disorders (6,7). Two common gene polymorphisms in the extracellular domain of the TLR4 receptor have also been defined. An A/G transition at aminoacid 299 resulting in an asparagine to glycine change (Asp299Gly) and a C/T transition at amino acid 399 resulting in a threonine to isoleucine change (Thr399Ile) which is reported to change receptors' ligand binding site, leading to a reduced capacity to elicit inflammation (8). Asp299Gly and Thr399Ile gene polymorphisms of human TLR4 have been shown to be associated with functional changes that predispose people to be less responsive to lipopolysaccharide (LPS) and have an increased risk of severe infection susceptibility to pathogenic bacterial infections (9-11). It has been suggested that the carriage of these gene polymorphisms is related to a risk of atherosclerosis and other chronic inflammatory diseases (6,7,12) through the activation of the inflammatory cells via the NF-KB pathway (13).

All in all, polymorphisms in TLR4 gene could be attractive candidates for searching morbid obesity risk, opening the question whether individual variations in the expression of these genes might account for different susceptibilities to MO. Therefore, we analyzed the association of two SNPs (*TLR4* Asp299Gly rs4986790 and *TLR4* Thr399IIe rs4986791) alone or combined with susceptibility to MO in a group of women in a Turkish population as a case-control study.

MATERIALS and METHODS

Subjects

The study included 213 female morbid obese patients and 204 female healthy control subjects at the Department of Endocrinology and Metabolism, Bezmialem University Hospital, Istanbul, Turkey. The age distribution was 38.0±5.7 years for patients and 39.0±7.3 for control subjects. The inclusion criteria were BMI > 35 kg/m^2 for morbid obesity and BMI <25 kg/m^2 for healthy control subjects. The exclusion criteria were alcohol abuse, having any systemic disease including chronic kidney, liver or cardiovascular diseases, or malignancies, rheumatologic disorders or any inflammatory diseases. All study subjects have Turkish origin and provided signed informed consent prior to the sample and data collection, and the study protocol was approved by the Institutional Ethical Committee of Bezmialem University (03/06/2015 - 11/10).

DNA Isolation and Polymorphism Analysis

DNA isolation was performed from the collected peripheral blood of samples using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany; Product No. 11796828001) and the DNA samples with OD ratio 1.8±0.1 were included in the study.

For all PCRs described here, a total of 100 to 200 ng of DNA was amplified in a 25 µl PCR reaction mixture. All PCR and digestion procedures were carried out within Veriti DX Thermal Cycler(Applied Biosystems, USA) and the PCR and digestion products were analyzed in ethidium bromide-stained 3.5% metaphor agarose gels.

For TLR4 Asp299Gly, genomic DNA was amplified to have a 468 bp length product. The PCR product was then digested with the restriction enzyme NcoI (New England Biolabs). During digestion, the products were incubated at 37 °C for 15 min and run on an agarose gel for 60 minutes at 75 V, and directly screened under UV light. The heterozygous genotype (AG) gives three bands of 468 bp, 446 bp, and 22 bp; as only one strand of the amplicon is cut. The homozygous mutant genotype (GG) is observed as two bands of 446 and 22 bps whereas homozygote wild genotype (AA) is observed as a single band with 468 bp (Figure 1).

For TLR4 Thr399Ile genomic DNA was amplified to have a 385 bp length product. The PCR product was then digested with the restriction enzyme HinfI (New England Biolabs). RFLP incubation condition was the same as NcoI enzyme used for TLR4 Asp299Gly polymorphism. The heterozygous genotype (CT) gives three bands of 385 bp, 365 bp, and 20 bp, as only one strand of the amplicon is cut. The homozygous mutant genotype (TT) is observed as two bands of 365 and 20 bps, whereas homozygote wild genotype (CC) is observed as a single band with 385 bp (Figure 2).

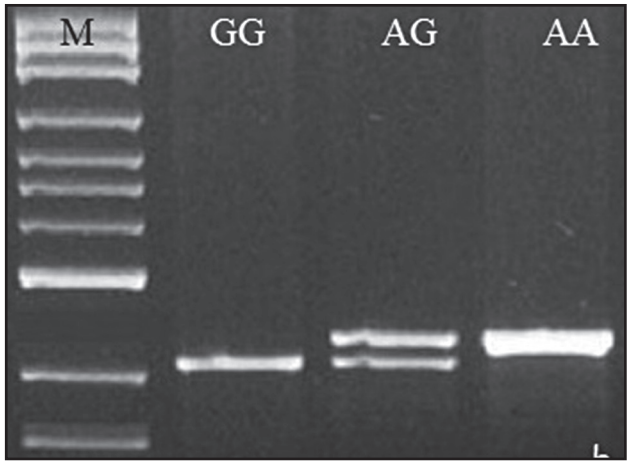

Figure 1 The enzyme digestion patterns of TLR4 rs4986790 polymorphisms.

Genotyping verification for randomly opted PCR products was performed with Sanger sequencing. The reaction mix was prepared with DTCS Mix (Beckman Coulter, USA) and run on GenomeLab GeXP Genetic Analysis System (Beckman Coulter, USA).

Data Processing and Analysis

Sanger sequencing data analysis was carried out with DNAStar Software (Version 14.0.0.86). For single gene only genotype analysis GraphPad Prism (Version 5), for logistic regression analysis SPSS software version 18, for haplotype analysis Haploview (Version 4.2) were used for the patient and control samples' values. Hardy-Weinberg equilibrium (HWE) test and genotype-allele frequencies' comparison between the cases and the controls were performed using chi-square analysis. Odds ratio (OR) and respective 95% confidence intervals (CIs) were reported to evaluate the effects of any difference between allelic and genotype distribution. A two-sided p-value <0.05 was considered statistically significant.

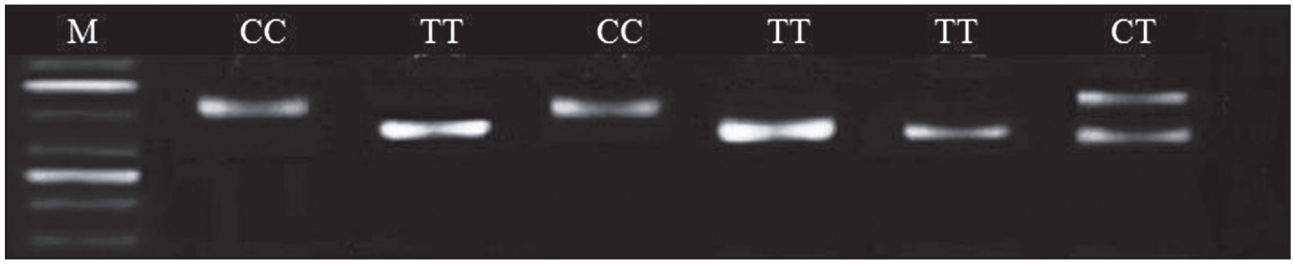

Figure 2 The enzyme digestion patterns of TLR4 rs4986791 polymorphisms.

RESULTS

The distribution of the genotype and allele frequencies of all polymorphisms studied is shown Table 1. 213 patients and 204 control subjects were successfully genotyped for the TLR4 rs4986790 and rs4986791. The distribution of TLR4 rs4986790 but not TLR4 rs4986791 were consistent with HWE (p=0.371, p=0.0803 respectively). The frequency of rs4986790 AA genotype was statistically higher in the patients compared with control group (p=0.0378, OR: 2.128, 95% CI: 1.029-4.401). By comparing the allele frequencies, we found that, having the A allele accelerated the risk for MO by 2.061 times (p=0.0423, OR: 2.061, 95% CI:1.011 - 4.200). According to our results, when we compared MO patients and healthy control group, genotypes and alleles frequencies of rs4986791 polymorhism of TLR4 were not found any significant differences (Table 1).

Combined Effects of TLR4 rs4986790 and rs4986791 Polymorphisms

In addition, the associations of TLR4rs4986790 and rs4986791 polymorphisms were calculated by performing logistic regression analysis. TLR4 rs4986790 and rs4986791 SNPs demonstrated statistical significance among the combined genotypes in the patient and control subjects (Table 2). We observed a significantly increased risk for MO in the case of the combined AA+CC genotype (p=0.001).

Table 1 The distribution of genotype and allele frequencies of rs4986790 and rs4986791 polymorphisms in MO patients and control subjects.

TLR4 299 rs4986790	**Patient n (%)**	**Control n (%)**	**OR (95% CI)**	**p value**
AA	201 (94)	181 (89)	Ref	
AG	12 (6)	23 (11)	2.128 (1.029 - 4.401)	0.0378
A Allele	414 (97)	385 (94)	Ref	
G Allele	12 (3)	23 (6)	2.061 (1.011 - 4.200)	0.0423
TLR4 399 rs4986791	**Patient n (%)**	**Control n (%)**	**OR (95% CI)**	**p value**
CC	195 (92)	191 (93.5)	Ref	
CT	18 (8)	10 (5)	0.5672 (0.2552 - 1.26)	0.1593
TT	0 (0)	3 (1.5)	7.146 (0.3664 - 139.4)	0.0813
C Allele	408 (96)	392 (96)	Ref	
T Allele	18 (4)	16 (4)	0.9252 (0.4651 - 1.84)	0.8245

$\chi 2$: Chi-square; OR: Odds ratio; CI: Confidence interval; Ref: Reference. GG genotype of TLR4 299 was excluded in the table as none observed in the subjects.

Table 2 Determination of the genetic effects of rs4986790 and rs4986791 polymorphisms on MO.

TLR4 299/399	**$\chi 2$**	**df**	**p value**	**OR (95% CI)**
AA+CC	19.081	3	0.02	
AA+CT	0.269	1	0.506	0.746 (0.314 – 1.771)
AG+CC	13.939	1	0.001	0.163 (0.055 – 0.482)
AG+CT	5.329	1	0.065	7.162 (0.887 – 57.853)

$\chi 2$: Chi-square; df: Degrees of freedom; OR: Odds ratio; CI: Confidence interval. AA+TT, AG+TT, GG+CC, GG+CT, and GG+TT combined genotypes were excluded in the table as none observed in the subjects or the size was not enough for the statistical analysis.

Table 3 Haplotypes associations of SNPs rs4986790 and rs4986791.

Haplotype	Frequency	Case, Control Ratios	$\chi 2$	p value
Haplotype Associations				
Block 1 (0.3 kb) (rs4986790, rs4986791)				
AC	0.940	34.333, 9.737	15.474	8.3658E-5
GC	0.041	0.029, 0.057	3.482	0.062
AT	0.018	0, 0.038	15.874	6.7687E-5

Kb: Kilobase; $\chi 2$: Chi-square.

Haplotype Analysis

The linkage disequilibrium (LD) was assessed using Haploview with block graphically identified from the LD intensity expressed in D'. Haplotype associations are shown in Table 3. The AC and AT haplotypes were found to be associated with MO ($\chi 2$=15.474, p=8.3658E-5 and $\chi 2$=15.874, p=6.7687E-5; respectively). The haplotypes of rs4986790 and rs4986791 have the frequencies of 94.0% AC, 1.8 % AT, 0.1 % GT, and 4.1 % GC (D'=0.021, LOD=0.03 and r^2=0.0).

DISCUSSION

Chronic low-grade inflammation represents a bridge in the explanation of the mechanism between obesity and obesity-related disorders (14). The studies in obese patients focused on the inflammation-related genetic structural alterations are very rare, therefore, there is still no certain marker being identified.

TLRs is one of the NF-KB stimulators, that initiates NF-KB cascade (15). *TLR4*, for example, has been implicated in several immune and inflammatory disorders (16). Many studies have reported that the process of inflammation could potentially be influenced by the variations within the *TLR4*genes. In this study, we raised the question whether polymorphisms of *TLR4* gene are associated with the risk of developing MO. *TLR4* rs4986790 but not *TLR4* rs4986791 are found to be highly associated with MO which correlates with the major data previously published by other groups.

Tlr4 loss of function mutation was described as preventing diet-induced obesity and insulin resistance in an animal model study (17). Jermendy has reported that serum cytokine levels change by *TLR4* polymorphisms in obese children. Therefore, changes in the *TLR4* probably lead to alterations in protein functioning that may contribute to the development of MO (18). Recently, two SNPs in the coding region of *TLR4* gene have been identified that encode aspartic acid-to-glycine and threonine-to-isoleucine amino acid substitutions at positions 299 and 399 within the ectodomain; respectively, D299G and T399I (9,19). Asp299Glyn and Thr399Ile polymorphisms can lead to abnormal signaling by altering the ligand binding affinity of *TLR4* and create an imbalance between pro and anti-inflammatory cytokine secretions, resulting in the risk of chronic inflammation (20). Asp299Gly is reported to be a risk factor for Crohn's disease, ulcerative colitis (UC) (21), infection of patients with HIV infection (22), infection of patients with *Streptococcus* pyogenes (23) but has a protective role in atherosclerosis (6), recurrent cystitis (24), obesity-associated hypertension (25) and nonallergic asthma (26). In contrast, some published studies in autoimmune diseases in general (27) and atherothrombosis (28) represent no association of TLRs.

Thr399Ile is reported to be more frequent in Europe whereas Asp299Gly is to be in Africa (29). The Asp299Gly polymorphism but not Thr399Ile, blunts *TLR4* function, as assessed by cytokine production and NF-KB stimulation in response to LPS

(30), whereas the T399I polymorphism has either a significantly reduced or no inhibitory impact on *TLR4* functions (8). By this point of view, it is understandable that Asp299Gly but not Thr399Ile is more common in disease association. We found no associations of *TLR4* rs4986791 with MO in the presence of HWE inconsistency, which is probably due to control subjects being sampled from different ethnic groups or excess of heterozygotes as a result of an unlikely genotyping error. Interestingly, Thr399Ile is reported as a risk factor in UC (31) and end-stage renal disease patients on peritoneal dialysis (32).

Studies mainly deal either with the Asp299Gly or the Thr399Ile polymorphism but neglect the fact that these polymorphisms also exist in a haplotype (Asp299Gly/Thr399Ile) way (33). Individuals having co-segregating polymorphisms of *TLR4* have more susceptible to gram-negative infections as they are less responsive to LPS (9, 34). Ferwerda, in 2007, has reported that Asp299Gly/Thr399Ile haplotype shows selective neutrality (29). Moreover, AC and AT haplotypes are both found to be risk factors for MO. According to our combined genotype analysis of *TLR4* polymorphisms, Asp299Gly/Thr399Ile is highly associated with increased MO risk. 1.949As *TLR4* was determined as a molecular bridge between inflammation and the innate immune system (35), this association may explain a potential pathophysiological link between obesity and inflammation.

All in all, this study shows that *TLR4* rs4986790 is associated with susceptibility to MO, implying that these polymorphisms are possible genetic risk factors for the progression of MO. Moreover, the AC and AT haplotypes were found to be associated with MO. *TLR4* seems to modulate the immune response in MO although how exactly pathway is in the pathological process including inflammation is to be researched.

Major limitations of our study are the small sample size which may have influenced the statistical power of our analysis, HWE inconsistency in one of the investigated polymorphisms, the single-gender preference of both patient and control samples. Different population studies are necessary to understand whether these variants play considerable roles in MO.

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