CLINICAL STUDY

TLR4 gene polymorphisms: evidence for protection against type 2 diabetes but not for diabetes-associated ischaemic heart disease

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Abstract

Objective: Several factors either predisposing or protecting from the onset of diabetes mellitus type 2 (DM2) have been proposed. Two specific polymorphisms of toll-like receptor 4 (*TLR4*; *Asp299Gly* and *Thr399Ile*) have recently been identified either as candidate protector genes against DM2 and associated neuropathy or risk alleles for the manifestation of diabetic retinopathy. The impact of these alleles on the risk for ischaemic heart disease (IHD) is controversial while their role in diabetes-associated IHD has never been studied.

Design and methods: In order to clarify the potential impact of *TLR4* polymorphisms on the predisposition for DM2 as well as on diabetes-related IHD vulnerability, the distribution of the mutant *TLR4 Asp299Gly* and *Thr399Ile* alleles in 286 DM2 patients and 413 non-DM2 controls with or without IHD, was examined.

Results: Mutant alleles were predominantly detected in 79/413 non-diabetic individuals versus 15/286 DM2 patients ($P < 0.0001$). The rates of positivity for mutant alleles were similar among diabetic patients with or without IHD (7/142 vs 8/144, $P > 0.1$), whereas they proved different among non-diabetic individuals with or without IHD (39/145 vs 40/268, $P = 0.004$). Following multivariate analysis, the difference between diabetic and non-diabetic subjects, with regard to *TLR4* mutations alone, remained significant ($P = 0.04$).

Conclusions: Mutant *TLR4* alleles confer protection against DM2. However, their presence does not seem to play any role, protective or aggravating, in the manifestation of IHD either in diabetic or in non-diabetic individuals.

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Introduction

Among the various endocrine disorders, the biggest challenge is without doubt diabetes mellitus (DM), especially type 2 (DM2; Mendelian Inheritance in Man/MIM ID #125853), a disease of global dissemination and steadily increasing incidence throughout the western societies (1, 2). Several risk factors have been associated with the onset of DM2, including family history of DM, ethnicity, obesity and polycystic ovary disease (2–5). However, only few factors have been found to induce protection against DM and its complications, i.e. nephropathy, retinopathy, and ischaemic heart disease (IHD) (2–6). Among these, specific polymorphisms of toll-like receptor 4 (*TLR4*) were quite recently proposed as being protective against DM2 (6).

Toll (meaning amazing, in German)-like receptors represent an ancient host defence pathway, and human toll, a type 1 transmembrane protein, is an essential immunological component linking innate and acquired immunity, while at the same time playing a crucial role in pathogen recognition. Located on 9q33.1, human *TLR4* (MIM +603030) is a widely studied representative of the TLR family and a key receptor for the recognition of Gram-negative bacteria, fungi, viruses, high-mobility group box-1 (HMGB1) alarmin protein secreted by dying tumour cells and saturated fatty acids (7–10). Being distributed in macrophages, endothelial cells, brain, gut, liver, pancreas, muscle and adipose tissues (10), *TLR4* and its polymorphic alleles have been linked with several disorders, including DM. An up-regulation of *TLR4* levels has been associated with the increased inflammatory response recorded in

DM (11) and has been proposed as the link between inflammation and atherosclerosis in diabetic patients (12). As for the *TLR4*-related genetic variations, it has been proposed that the *TLR4 Asp299Gly* allele confers protection against DM2 (6), as well as diabetic neuropathy (13), while the *TLR4 Thr399Ile* allele against diabetic neuropathy alone (13). On the contrary, the presence of both mutant alleles has been linked to early onset of diabetic retinopathy (14). In view of their impact on IHD, it has been shown that the two alleles may be related to a decreased risk for the onset of IHD, although the results from the available literature are conflicting (15–17).

Methods

Study population

'Mutant' *TLR4 Asp299Gly* or *D299G* (MIM 603030.001) and *Thr399Ile* or *T399I* (MIM 603030.002) polymorphic alleles were genotyped in 699 individuals: 286 with DM2 and 413 without DM2 (controls). Of the 286 diabetic patients, 142 were also diagnosed with IHD, compared with 145 of the 413 controls. All groups were sex, age and smoking frequency matched ($P > 0.1$ in all cases). DM2 patients and IHD controls were recruited and followed at the University Hospital of Larissa, Greece. The remaining group of non-IHD

controls consisted 268 randomly selected healthy blood donors. The demographic and clinical characteristics of study participants are summarised in Table 1. Diagnosis of DM2 was based on the criteria of the American Diabetes Association (18), whereas for IHD participants, diagnosis was established on angiographic documentation, the American College of Cardiology criteria (19) and the Joint European Society of Cardiology and American College of Cardiology Committee consensus (20) (Table 2). Patients with stable angina were not included. All study participants were Caucasians, inhabiting the area of central Greece (Thessaly).

Genotypic analysis

DNA isolation DNA isolation was performed as described previously (21). In brief, genomic DNA was extracted from 200 µl whole blood using an automated DNA extraction device (Magstration System 12GC).

PCR amplification The sequence around the *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms was amplified using a PCR-based protocol. The used primers were designed using Primer 3 software (www.justbio.com). Program-derived pair of forward 5'-TCTAGAGGGCCTG-TGCAATT-3' and reverse 5'-TGAAACTCACTCATTTG-

Table 1 Study population demographics, clinical characteristics and routine examination values. Values are presented as absolute numbers or mean $\pm$ s.d.

	DM2		Controls	
	IHD	Non-IHD	IHD	Non-IHD
Age (years)	63.1 $\pm$ 5.4	62.3 $\pm$ 5.7	62.4 $\pm$ 5.2	62.2 $\pm$ 5.1
Sex				
Male	81	82	92	175
Female	61	62	53	93
Smoking				
Yes	74	70	78	123
No	68	74	67	135
Hypertension				
Yes	86	85	103	187
No	56	59	42	81
BMI (kg/m ²)	26.2 $\pm$ 3.3	25.8 $\pm$ 3.5	25.1 $\pm$ 3	25.2 $\pm$ 3.2
DM duration (years)	12.9 $\pm$ 4.4	12.1 $\pm$ 4.3	–	–
Family history of IHD				
Yes	65	63	53	90
No	77	81	92	178
Cholesterol (mmol/l)	5.18 $\pm$ 0.57	5.1 $\pm$ 0.6	5.21 $\pm$ 0.62	5.13 $\pm$ 0.64
HDL (mmol/l)	0.97 $\pm$ 0.16	1 $\pm$ 0.18	1.06 $\pm$ 0.17	1.08 $\pm$ 0.19
LDL (mmol/l)	2.7 $\pm$ 0.26	2.69 $\pm$ 0.2	2.73 $\pm$ 0.18	2.7 $\pm$ 0.2
Triacylglycerol (mmol/l)	1.9 $\pm$ 0.1	1.89 $\pm$ 0.17	1.85 $\pm$ 0.19	1.84 $\pm$ 0.2
HbA1c*	0.077 $\pm$ 0.006	0.076 $\pm$ 0.007	0.045 $\pm$ 0.012	0.046 $\pm$ 0.014
Glucose (mmol/l)	8.8 $\pm$ 1.1	8.7 $\pm$ 1.1	5 $\pm$ 1	4.8 $\pm$ 1.09
Statin therapy				
Yes	84	48	97	32
No	58	96	48	236

* Proportion of total haemoglobin.

DM2, diabetes mellitus type 2; IHD, ischaemic heart disease; BMI, body mass index.

Table 2 Criteria used for the diagnosis of DM2 and IHD.**DM2***ADA criteria (one of the following)*

- Fasting plasma glucose >7 mmol/l
- Symptoms of hyperglycaemia and random plasma glucose >11.1 mmol/l
- 2-h plasma glucose >11.1 mmol/l during an oral glucose tolerance test

IHD*Angiographic documentation*

- ≥50% narrowing of transluminal diameter of coronary arteries (left anterior descending, left circumflex and right coronary artery)
- Intracoronary thrombi
- Complex lesions

ACC criteria

- Unstable angina
- Angina pectoris or equivalent type of ischaemic discomfort, in the absence of biochemical evidence of myocardial necrosis, accompanied by either
- Angina occurring at rest for >20 min
- New onset angina of at least CCS classification III severity
- Recent acceleration of angina reflected by an increase in severity of at least 1 CCS class to at least CCS class III

*Joint ESC/ACC Committee criteria**Acute, evolving, and recent MI (one of the following)*

- Typical rise and gradual fall (troponin) or more rapid rise and fall (CK-MB) of biochemical markers compatible with myocardial necrosis accompanied by either
- Ischaemic symptoms
- Development of irregular Q waves (ECG)
- ECG changes indicative of ischaemia (ST elevation/depression)
- Coronary artery intervention (i.e. angioplasty)

*Pathological findings of MI**Established MI (one of the following)*

- Development of new irregular Q waves on serial ECGs with or without previous symptoms. Biochemical markers of myocardial necrosis may be within normal range
- Pathologic findings of healed or healing MI

DM2, diabetes mellitus type 2; IHD, ischaemic heart disease; ADA, American Diabetes Association; CCS, Canadian Cardiovascular Society; ESC, European Society of Cardiology; ACC, American College of Cardiology; MI, myocardial infarction; CK-MB, creatine kinase-myocardial band; ECG, electrocardiogram.

TTTCAA-3' primers generated a 438 bp-long fragment. The PCR products were generated using a PTC-200 MJ Research Thermocycler (MJ Research, Inc., Waltham, MA, USA) after 35 cycles of DNA denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The reaction mixture consisted 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM Mg²⁺, 200 µM of each dNTP, 1.5 U Taq DNA polymerase (Invitrogen) and 15 pmol of each primer.

Sequence analysis Purification of the PCR products was performed using the PureLink PCR purification kit (Invitrogen). Automated cycle sequencing for both strands was performed with the ABI analyser using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Products were electrophoresed on the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were aligned using Sequencher PC software (Gene Codes Corp., Ann Arbor, MI, USA) with normal sequences from GenBank (GenBank accession number: NM_138554) and examined for the presence of mutations. All nucleotide numbers refer to the wild-type cDNA.

Routine examinations

HbA1c levels were calculated using the DCA 2000 device (Bayer Corporation). Total cholesterol,

high-density lipoprotein (HDL) and triacylglycerol levels were determined using an enzymatic colorimetric assay and commercially available kits (Olympus Diagnostics, GmbH, Hamburg, Germany), whereas for low-density lipoprotein (LDL), the Friedewald's formula was applied.

Statistical analysis

Statistical analysis included the use of Student's *t*, χ^2 (to exclude deviations from the Hardy-Weinberg equilibrium), Fisher's exact and ANOVA tests. Instead of using the parameter *D*, an estimate of linkage (dis)equilibrium which tends to become affected by allele frequency, an adjusted, for allele frequencies correlation coefficient *r* between the studied loci, was calculated using the equation: $r = D / \sqrt{\text{product of allele frequencies}}$. During comparison, whenever a level of significance $P < 0.05$ was recorded in univariate, multivariate analysis was also performed. Multivariate testing was carried out using multiple logistic regression while considering, *a priori*, age, sex, smoking, family history of IHD, body mass index (BMI), hypertension, HbA1c, lipoprotein and triacylglycerol levels, statin therapy as well as diabetes duration and applying a backward selection process as potential confounding factors. Due to the fact that patients were often under >1 anti-hypertensive/anti-diabetic agent or switched from one agent to another, in order to perform the most

accurate possible analysis, these diverse agents were examined while also taking into account the period or duration of treatment with each drug. However, this led to a great heterogeneity and, during statistical analysis, low overall model performance (or overall model fit, expressed as adjusted R^2 for logistic regression models). Thus, the strongest 'predictors' (or confounding factors) in our statistical analysis, hypertension, not anti-hypertensive therapies, and the presence/absence of DM and DM 'regulation', reflected in HbA1c levels, were ultimately included, yielding the best possible adjusted R^2 . The analyses described above were carried out using the MedCalc 10.2.0.0 statistical software (MedCalc Software bvba, Mariakerke, Belgium). Using the CaTS 0.0.2 power calculator, it was estimated that based on the current size and case-control ratio of our population, the detection of differences, in terms of mutant allele distribution, could be performed with a power exceeding 80% ($\approx 96\%$) at the 0.05 level of significance.

Ethical considerations

The study was approved by the ethics committee of the University of Thessaly Medical School. Informed consent was obtained from all individuals participating in the study.

Results

Mutant *TLR4 Asp299Gly* and *Thr399Ile* alleles were detected in 94/699 individuals (13.4% of study population) and in linkage disequilibrium (100% cosegregation, $r=1$). The studied alleles were found to be in Hardy-Weinberg equilibrium. The relative frequencies of both alleles were 15/286 in DM patients compared to 79/413 in the control group (odds ratio (OR): 0.23, 95% confidence intervals (95% CI): 0.13–0.42, $P<0.0001$; Fig. 1). When the mutant alleles were studied in our population while evaluating only the presence or absence of IHD, a near-trend difference was recorded among subjects with (46/287) or without IHD (48/412) (OR: 1.45, 95% CI: 0.94–2.24, $P=0.1$). The distribution of mutant alleles among DM2 patients with (7/142) or without IHD (8/144) was similar (OR: 0.88, 95% CI: 0.3–2.5, $P>0.1$), while in the control group (without DM2) it was 39/145, in the subgroup with, versus 40/268, in the subgroup without IHD (OR: 2.1, 95% CI: 1.28–3.45, $P=0.004$; Fig. 2). Thus, the recorded difference in mutant allelic frequency between IHD and non-IHD subjects resulted from the control group (non-DM2) exclusively. On the contrary, no statistically significant differences were recorded when the presence of mutant *TLR4* alleles was examined under the light of unstable angina and myocardial infarction, separately or the angiographically documented coronary atheroma burden (number of diseased arteries, occlusion rates, occlusion

Figure 1 Relative distribution of the cosegregated *TLR4 Asp299Gly* and *Thr399Ile* alleles in subjects with (DM=1) or without (DM=0) DM2.

sites – $P>0.1$, in all cases). Similarly, no significant interactions were recorded between *TLR4* and treatment modalities (anti-hypertensive or DM-related medication) or glucose levels ($P>0.1$).

In multivariate analysis, the negative association of *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms with DM2 remained statistically significant after adjusting for confounding factors, although the level of significance decreased (OR: 0.21, 95% CI: 0.07–0.92, $P=0.04$; adjusted $R^2=0.87$). As for the increased presence of the studied genes in the non-DM2 group with IHD, the significance of this finding was completely attenuated after adjustment for age, sex, smoking, family history of IHD, BMI, hypertension, HbA1c, lipoprotein and triacylglycerol levels as well as statin therapy (OR: 0.94, 95% CI: 0.83–1.08, $P>0.1$; adjusted $R^2=0.89$). An overlook of the fluctuations of OR and 95% CIs is presented in Fig. 3.

Discussion

The relative distribution of 'mutant' *TLR4 Asp299Gly* and *Thr399Ile* alleles was assessed in individuals with or without DM2 while at the same time investigating potential implications for the onset of DM-related IHD. In agreement with previous reports (6, 22), our findings suggest that carriers of these mutant alleles are more protected against DM2. As for the impact of the mutant alleles on IHD susceptibility, our data show that compared with carriers of wild-type alleles, they exhibit similar rates of DM-associated IHD. In order to understand the significance of the present finding, additional lines of evidence have to be discussed.

TLR4 Asp299Gly and *Thr399Ile* mutant alleles seem to have originated in Africa in an environment

Figure 2 Comparative distribution of mutant (dark grey) and wild-type (light grey column) alleles in DM2 patients (DM=1) and non-DM2 controls (DM=0) with (IHD=1) or without (IHD=0) ischaemic heart disease (IHD).

where malaria represented a major evolutionary pressure: first the *Asp299Gly* and later on the *Thr399Ile* allele (7, 8). The frequency of these alleles varies from 0% in Polynesian, Southeast-Asian and native South-American populations to 5–9% in Indo-Europeans (7, 8). In a *Plasmodium* spp.-infested environment the *Asp299Gly*, although increasing susceptibility to infections as well as the risk of septic shock, it induced protection against malaria-associated mortality (7, 8). However, the ‘addition’ of the *Thr399Ile* mutant allele, as in Indo-Europeans, reduced the risk for septic shock to rates similar to those observed in carriers of the wild-type alleles (7, 8). This simultaneous presence – linkage disequilibrium – of both mutant alleles was a useful addition, as the presence of the *Asp299Gly* alone would result in greater susceptibility during the plague, typhoid fever and influenza outbreaks in Europe (7, 8). These selective forces acted vigorously in the area of Central Greece, site of origin of our study group, where malaria rates were high until the 1960s, a fact also reflected in the increased frequency of haemoglobinopathies, in our region compared to others, in Greece (23, 24). Thus, as previously shown and confirmed in our study, the percentage of the population carrying the mutant alleles is higher ($\approx 13.5\%$) (25–27) in comparison with other European – except for Dutch, Romanian (14%) and Basque (18%) – or other native Greek populations (6%) (8).

Being in linkage disequilibrium, these mutant alleles are inherited in a cosegregated manner and are present in offspring in the form of a *Asp299Gly/Thr399Ile* haplotype (7, 8). Thus, experts on the subject advise

to address any issues related to these alleles while considering the existence of six distinct haplotypes: wild-type/wild-type, wild-type/*Asp299Gly*, wild-type/*Thr399Ile*, *Asp299Gly/Thr399Ile*, *Asp299Gly/Asp299Gly* and *Thr399Ile/Thr399Ile* (8). The double mutant *Asp299Gly/Thr399Ile* state, although initially considered indistinguishable to the wild-type genotype (7, 8), has been shown to alter the structure of TLR4 itself, leading to a problematic binding of ligands (28). This structural/functional irregularity ultimately seems to be responsible for a more blunt immune response, i.e. a reduced production of IgA against microbial targets, i.e. anti-*Helicobacter pylori* (26), anti-outer membrane porin, anti-chitobioside antibodies (29), compromised recognition of apoptosis signals during anti-cancer therapy (9) or the presence of decreased functional TLR4 levels (30). An activation of functional TLR4 in insulin target tissues, however, by fatty acids – dietary or endogenous – has been shown to exert pro-inflammatory responses, i.e. excessive production of cytokines, reactive oxygen species, eicosanoids and activation of kinases, which in turn lead to insulin desensitisation (10). When bearing in mind these characteristics along with the evidence linking this increased TLR4 functionality with persistent inflammation and subsequent DM2 (11, 31), it is within reason to assume that those carrying a TLR4 suppressive polymorphism, capable of attenuating these phenomena, might eventually be more protected against the disease.

Although, a similar mutant *TLR4*-mediated protective effect against IHD vulnerability would be expected, because a hyporesponsive TLR4 could also suppress the inflammatory processes involved in atherogenesis and plaque destabilisation, this was not verified in our study. This lack of protection, also recorded in other studies, seems to underline the importance of bearing in mind that the altered TLR4 functionality is a ‘double-edged sword’ (16, 17, 32). Therefore, it is possible that the benefits from a less profound inflammatory response within the vascular wall could be compensated by, for instance, an increased susceptibility to infections and subsequently to a

Figure 3 Graph of the odds ratio (OR) and 95% CI of the *TLR4 Asp299Gly/Thr399Ile* for the induction of (a) DM2 and (b) DM2-independent IHD, before (OR) and after (ORAdj) adjustment for confounding.

greater pathogen burden, which has been associated with IHD (7, 8, 33). In addition, any beneficial effect induced by mutant *TLR4* alleles may be outweighed by the presence of well-established risk factors for IHD (smoking, hypertension, DM and so on). Within certain limitations, primarily involving the lower frequency of studied alleles among DM2 patients, our findings do not support an additional role for the *TLR4 Asp299Gly* and *Thr399Ile* genes with respect to DM2-related IHD, because no differences were observed regarding IHD susceptibility in DM2 patients, carrying the mutant or the wild-type alleles. Due to this phenomenon, although our data are not suggestive of any implications of *TLR4* genetic variations for DM-linked IHD, large population studies are required, preferably in populations where these mutations are markedly frequent, i.e. in Basques (8), before this hypothesis can be utterly rejected.

When considering the aforementioned data on the protective potential of *TLR4* mutations, an obvious paradox seems to emerge. Despite the relatively high frequency of protective *TLR4* alleles, in the western populations, an increasing DM2 incidence is being recorded (1, 2). This phenomenon may be due to the impact of lifestyle/environmental factors (fat and calorie-rich, nutrient-poor diet, low levels of exercise and so on), on the development of DM2 (1–3), the contribution of other genetic loci on the diversity of DM2 susceptibility, among ethnicities (4, 34, 35) and the currently 'fixed' mutant *TLR4* genetic background primarily influenced by neutral genetic drift (7, 8), because traditional selective pressures (infections) have been relieved as a result of aggressive anti-microbial practices, i.e. water disinfection, use of antibiotics, etc.

In conclusion, it seems that individuals simultaneously carrying the *TLR4 Asp299Gly* and *Thr399Ile* polymorphic alleles are more protected against the onset of DM2, although they exhibit similar rates of DM-associated IHD compared with those with the wild-type alleles. These results, originating from a study focusing on specific *TLR4* genetic mutations, need to be confirmed by further studies applying next-generation genome wide array techniques, so that the earlier limitations of non-additivity (gene–gene or gene–environment interactions) (35) also influencing the distribution of *TLR4* mutations, even among Indo-Europeans, could be overcome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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