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Toll-like receptor 4 D299G polymorphism in metabolic disorders: a meta-analysis

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Abstract The toll-like receptor 4 (TLR4) plays a key role in the activation of innate immune response participating in the recognition of lipopolysaccharides. Changes in the innate immune response are involved in the pathogenesis of some metabolic disorders such as metabolic syndrome and type 2 diabetes mellitus (Met-S and T2DM). It has been recently shown the role of gut microbiota in the perpetuation of both insulin resistance and low-grade chronic inflammation. Some studies have reported that TLR4 D299G polymorphism is associated with metabolic disorders, however results have been inconsistent. Two recent meta-analyses showed that D299G is associated with inflammatory bowel disease and gastrointestinal cancers risk, two pathological states in which the luminal microbial flora-host cells interaction may be implicated. We conducted a systemic review of the published data considering all eligible published studies (six studies with 1696 cases and 3388 controls for D299G) and a meta-analysis was performed to evaluate the association between TLR4 D299G polymorphism and the risk for metabolic disorders. Five studies were identified for T2DM: three corresponding to Caucasian populations and two to mixed populations. The remaining study analyzed Met-S in a Caucasian population. We observed a significant association between D299G polymorphism and metabolic disorders (T2DM and Met-S) risk (OR = 0.566, 95 % CI: 0.347–0.925, $p = 0.023$) particularly in Caucasians. No association was found in mixed population subgroup. Our meta-analysis identified that the AG/GG genotypes of D299G are associated with decreased metabolic disorders risk.

Keywords TLR4 · Meta-analysis · D299G · Polymorphism · Diabetes · Metabolic syndrome

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Introduction

Toll-like receptors (TLRs) are a large family of highly conserved proteins playing a key role in the activation of innate immune response from Drosophila to humans. TLRs are type 1 transmembrane proteins possessing an N-terminal extracytoplasmic leucine rich repeat (LRR) ligand binding domain and an intracytoplasmic conserved Toll/IL1-receptor domain (TIR) responsible for its activity [1]. TIR domains interact with different adaptor proteins such as MyD88, leading to the activation of nuclear factor kB (NF-kB) and the mitogen-activated protein kinase signaling cascade [2]. Additionally, TLRs sense endogenous ligands released from damaged tissue or necrotic cells such

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as heat shock proteins, fibronectin, fibrinogen, minimally modified and oxidized low-density lipoprotein (LDL) and free fatty acids inducing chronic low-grade inflammation and activation of the innate immune system [3, 4].

The toll-like receptor 4 (TLR4), is a member of this family participating in the recognition of conserved pathogen-associated molecular patterns, such as lipopolysaccharides (LPS) [5] found in most gram-negative bacteria. Several studies in animal models as well as in humans have shown that changes in the innate immune response are involved in the pathogenesis of some metabolic disorders such as metabolic syndrome (Met-S) and type 2 diabetes mellitus (T2DM) [6]. It has been recently shown the role of gut microbiota in the perpetuation of both insulin resistance (IR) and low-grade chronic inflammation [7]. The activation of TLR4 signaling induces up-regulation of inflammatory pathways related to IR induction. It has been also demonstrated that a loss-of-function mutation ($Pro^{712}His$) in TLR4 prevents diet-induced obesity and IR in C3H/HeJ mice [8].

It is believed that the ability to respond accurately to different ligands may be impaired by single nucleotide polymorphisms (SNPs) within TLR4 gene [9]. Since two miss-sense variants encoded in the ectodomain of TLR4 gene, D299G and T399I, have been reported to be associated with endotoxin hyporesponsiveness [10], hundred of association studies have been performed with a wide range of pathologies. However, these studies have leaded to contradictions regarding the role of both polymorphisms and their possible effects on inflammatory and metabolic disorders like atherosclerosis, IR, Met-S and T2DM [11, 12]. This could be partially explained considering that associations between this variants and disease predisposition have been shown to depend on ethnic backgrounds and gender [13], in particular may be due to the separate analysis of D299G and T399I SNPs without taking into account that these variants co-segregate in some populations [12]. Furthermore, this inconsistency may be attributed to studies with small sample size, inadequate statistical power or uncorrected multiple hypothesis testing.

However controversial data was reported regarding the impact of these polymorphisms (D299G and T399I) on TLR4 expression and the molecular mechanisms responsible for signaling deficiencies have remained unclear. *Figueroa* et al. [13] have recently demonstrated that D299G-mediated impairment of LPS-induced activation of signal transduction pathways is caused by a deficient recruitment of two adapters MyD88 and TRIF to TLR4.

Finally, two recent meta-analyses showed that D299G is associated with inflammatory bowel disease (Crohn's disease and ulcerative colitis) [14] and gastrointestinal cancers [15] risk, two pathological states in which the luminal microbial flora-host cells interaction may be implicated.

Given the premise of D299G functional commitment and considering previous meta-analyses statistically significant between this polymorphism and inflammatory diseases, we conducted a meta-analysis considering all eligible published studies to evaluate the association between TLR4 D299G polymorphism and the risk for metabolic disorders.

Methods

Study selection

We searched among Pub Med-Medline databases for all publications on the association between TLR4 D299G polymorphism and T2DM and Met-S. The key words were as follows: TLR4 D299G, TLR4 Asp299Gly, metabolic syndrome, insulin resistance, obesity and type 2 diabetes. In addition, we also searched references of retrieved articles (last search was updated 11th September 2012). Studies should meet the following criteria: (a) the publication was a case–control study; (b) association of TLR4 D299G polymorphism and diabetes or Met-S; (c) the study reported OR data for their calculation and (d) the control subjects satisfied the Hardy–Weinberg equilibrium (HWE). Exclusive criteria: no report about the genotype frequency, or insufficient information for data extraction. Finally, we identified 6 studies on the association between D299G polymorphism in TLR4 gene and T2DM.

Data extraction

Two authors extracted data independently and in duplicate, and agreement was reached on all items, including: author's last name, journal and year of publication, country of origin, selection and characteristics of diabetes/Met-S cases and controls, ethnicity of the study population, genotypes and numbers of cases and controls. The results were compared and disagreements were discussed and resolved by consensus. The two authors who conducted the literature search (FSB & APS) also extracted the data from the studies independently. Any disagreement was adjudicated by consensus and by consulting two additional authors (FCL & EP). The following information was recorded for each study: first author, year of publication, region, ethnicity, disease, size of case and control groups, frequencies of G allele in control subjects, and evidence of HWE in control subjects (Table 1).

Statistical analysis

The association between the D299G TLR4 polymorphism and T2DM and Met-S risk was measured by Odds Ratio

Table 1 Characteristics of literature included in the meta-analysis

First author	Years	Country	Ethnicity	Met. disorder	Case	Control	maf	HWE
Manolakis AC	2011	Greece	Caucasian	T2DM	286	413	13.4	Yes
Maldonado-Bernal C	2011	México	Mixed	T2DM	477	538	5.9	Yes
Bagarolli RA	2010	Brazil	Mixed	T2DM	211	200	11	Yes
Penas-Steinhardt A	2010	Argentine	Caucasian	Met-S	172	449	9.7	Yes
Kolek MJ	2004	U.S.	Caucasian	T2DM	333	1561	9.8	Yes
Illig T	2003	Germany	Caucasian	T2DM	217	227	10.7	Yes

(OR) with 95 % confidence intervals (CIs). Statistical heterogeneity between studies was assessed with the $\chi 2$-based Q test [16], heterogeneity was considered significant when $p < 0.05$. The inconsistency index I^2 was also calculated to qualify variation in OR attributable to heterogeneity: higher values of the index indicate the existence of heterogeneity. When heterogeneity was not an issue, fixed effect model with Mantel–Haenszel method was used [17]. Otherwise, a random effect model with inverse variance method was used. In addition, we also performed stratification analyses by ethnicity.

Funnel plots were used to evaluate potential publication bias. The standard error of log OR of each study was plotted against its OR and funnel plot asymmetry was further assessed by Egger's test.

All statistical evaluations were made assuming a two-sided test with a significance level of 0.05. All analyses were performed using Stata version 11.0 software (Stata, College Station, TX, USA).

Results

Study selection

The initial search strategy to identify association studies for T2DM, Met-S and other metabolic disorders (such as obesity, impaired glucose tolerance, etc.) and the D299G polymorphism in TLR4 gene yielded a total of 30 potentially relevant references among all the databases, 14 of which were overlapping. After subsequent analysis, six studies including a total of 1696 cases and 3388 controls were selected as they fulfilled the established inclusion criteria (Table 1) [9, 18–21]. These studies included only associations between T2DM and Met-S with the D299G polymorphism. The distribution of genotypes in controls was consistent with the HWE for all selected studies.

Meta-analysis

Five studies were identified for T2DM: three corresponding to Caucasian populations and two to mixed populations. The remaining study analyzed Met-S in a Caucasian population. For all studies, G allele frequency of TLR4 D299G polymorphism was ranging from 0.059 to 0.134.

As meta-analysis was performed with fixed effect model, which resulted in a significant Q-statistic ($p = 0.001$) indicating heterogeneity across studies, a random effect model was used.

We observed a significant association between D299G polymorphism (AG and GG genotypes combined) and metabolic disorders (T2DM and Met-S) risk based on the six studies published so far (OR = 0.566, 95 % CI: 0.347–0.925, $p = 0.023$) (Fig. 1).

We also performed sub-analysis stratified by ethnicity founding that the presence of D299G polymorphism decreased the metabolic disorders risk in Caucasians OR = 0.496 (95 % CI: 0.276–0.891, $p = 0.019$), however, no association was found in mixed population subgroup, OR = 0.741 (95 % CI: 0.296–1.851, $p = 0.520$) (Fig. 1).

Publication bias

Evaluation of publication bias was assessed through Funnel plot asymmetry (Begg's method). There was no publication bias in our study ($p = 0.628$ for dominant genetic model comparison) (Fig. 2).

Discussion

According to previous evidence, TLR4 activation induces up-regulation of inflammatory pathways related to IR induction [5]. Strikingly TLR4 is expressed not only on innate and adaptive immune cells but also on insulin-responsive cells such as adipose tissue and skeletal muscle. Moreover, recent studies in pancreatic islets demonstrate that LPS inhibit β-cell insulin gene expression in a TLR4-dependent manner, suggesting a novel mechanism by which changes in TLR4 function may impact the metabolic homeostasis [22]. Considering this background we performed a systematic review on the association between TLR4 D299G polymorphism and metabolic disorders risk. Six studies with 1696 cases and 3388 controls information

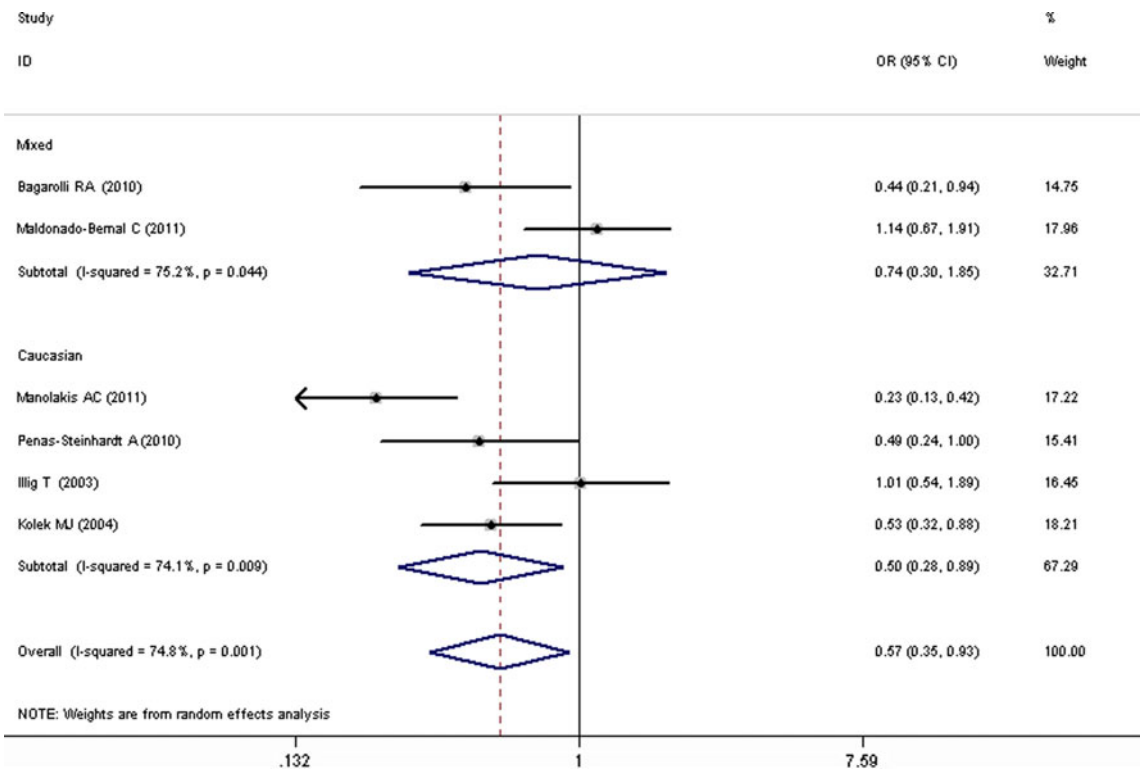

Fig. 1 Meta-analysis results of the association between TLR4 D299G polymorphism with metabolic disorders. Forest plot of odd ratios (ORs) stratified by ethnicity

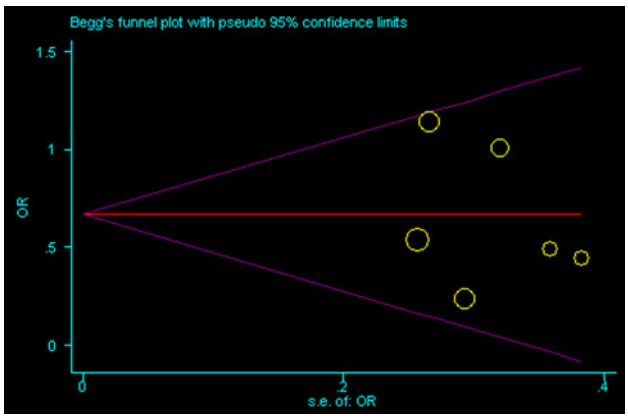

Fig. 2 Begg's funnel plot for publication bias test, plot of standard error by log odds ratio

were available for a dominant genetic model analysis. Our meta-analysis identified that the AG/GG genotypes are associated with decreased metabolic disorders risk by a random effects model.

In order to understand the significance of these findings, additional lines of evidence have to be discussed. *Arbour* et al. [10] showed that D299G TLR4 variant exhibited blunted responses to LPS but the molecular mechanisms responsible for this observed signaling deficiency was unclear and controversial for the last years. *Figueroa* et al. [13] recently described that deficient recruitment of signaling adapters MyD88 and TRIF to TLR4 appears to be the mechanism responsible for D299G-mediated impairment of LPS-induced activation of signal transduction pathways.

Our results suggest that carriers of G allele are protected against metabolic disorders such as DMT2 and Met-S. To deepen our study, population subgroups were analyzed. In the Caucasian subgroup, the AG/GG genotype is associated with significantly decreased T2DM/Met-S risk. The absence of a statistical effect in the mixed subgroup might be explained by the differences in D299G allele frequency among populations. Significant differences were found between D299G prevalence between the major continents. African populations show the higher prevalence of D299G G allele (10–18 %) while 6–14 % of Indo-European individuals were heterozygote for the D299G allele (allele frequency 3–7 %, respectively). In contrast, TLR4 D299G polymorphism in Asian and American populations is practically missing [23]. The lower frequency of the G allele in the Asian population requires larger sample sizes to detect statistically significant associations.

These findings denote that TLR4 gene could be implicated in metabolic disorders development. This is consistent with findings in TLR4 genetically deficient mice which were reported to be an "ideal body type", when fed on

regular chow, having an increased density, size and bone mineral content, as well as decreased body fat. Unlike many laboratory wild-type mice, this strain does not become obese with age [24]. Recently another TLR4 polymorphism rs11536889 was associated with protection against overweight, reinforcing the role of this gene in inflammatory-related metabolic disorders [25]. This polymorphism is located in the 3′ untranslated region (3′UTR) and appears to be responsible for a lower rate of mRNA translation [26].

To conclude, this meta-analysis provides evidence of the association between the TLR4 D299G polymorphism and decreased risk for DMT2 and Met-S. However, further large comprehensive studies among populations, with more detailed individual data are needed to confirm our results. Moreover, future gene–gene and gene–environment interactions should also be analyzed, to have an accurate understanding of the association between TLR4 polymorphisms and metabolic disorders.

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References

1. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 95(2):588–593
2. Barton GM, Medzhitov R (2003) Toll-like receptor signaling pathways. Science 300(5625):1524–1525
3. Pickup JC, Mattock MB, Chusney GD, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 1997;40(11):1286–1292. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9389420. Accessed October 21, 2012
4. Pickup JC, Crook MA (1998) Is type II diabetes mellitus a disease of the innate immune system? Diabetologia 41(10):1241–1248
5. Takeda K, Kaisho T, Akira S. Toll-like receptors. Ann Rev Immunol. 2003;21:335–376. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12524386. Accessed October 13, 2010
6. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860–867. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17167474. Accessed October 4, 2012
7. Petruzzelli M, Moschetta A. Intestinal ecology in the metabolic syndrome. Cell Metab. 2010;11(5):345–346. Available at: http://dx.doi.org/10.1016/j.cmet.2010.04.012
8. Tsukumo D, Carvalho-Filho M, Carvalheira J et al (2007) Loss-of-function mutation in toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes 56:1986–1998
9. Maldonado-Bernal C, Trejo-de la O A, Sánchez-Contreras ME, et al. Low frequency of toll-like receptors 2 and 4 gene polymorphisms in Mexican patients and their association with type 2 diabetes. Int J Immunogenet. 2011;38(6):519–523. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21902816. Accessed October 10, 2012
10. Arbour NC, Lorenz E, Schutte BC, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000;25(2):187–191. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10835634
11. Steinhardt AP, Aranguren F, Tellechea ML, et al. A functional nonsynonymous toll-like receptor 4 gene polymorphism is associated with metabolic syndrome, surrogates of insulin resistance, and syndromes of lipid accumulation. Metabolism. 2009. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19922963
12. Ferwerda B, McCall MBB, Verheijen K, et al. Functional consequences of toll-like receptor 4 polymorphisms. Mol Med. 2008;14(5–6):346. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2215765/. Accessed March 5, 2012
13. Figueroa L, Xiong Y, Song C, et al. The Asp299Gly polymorphism alters TLR4 signaling by interfering with recruitment of MyD88 and TRIF. J Immunol. 2012;188(9):4506–4515. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22474023. Accessed November 12, 2012
14. Shen X, Shi R, Zhang H, et al. The toll-like receptor 4 D299G and T399I polymorphisms are associated with Crohn's disease and ulcerative colitis: a meta-analysis. Receptor. 2010:69–77
15. Jing JJ, Li M, Yuan Y. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in cancer: a meta-analysis. Gene. 2012;499(2):237–242. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22441122. Accessed November 12, 2012
16. Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327(7414):557–560. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=192859&tool=pmcentrez&rendertype=abstract. Accessed October 6, 2012
17. Petitti DB (2000) Meta-analysis, decision analysis, and cost-effectiveness analysis: methods for quantitative synthesis in medicine, 2nd edn. Oxford University Press, New York
18. Thomas I, Friedhelm B, Andreas S, Rolf H, Sylvia M, Wolfgang R, Wolfgang K, Christa M, Wichmann HE, Hubert K, for The KORA Study Group1 2 (2003) The endotoxin receptor TLR4 polymorphism is not associated with diabetes or components of the metabolic syndrome. World Health 52:1–4
19. Kolek MJ, Carlquist JF, Muhlestein JB, et al. Toll-like receptor 4 gene Asp299Gly polymorphism is associated with reductions in vascular inflammation, angiographic coronary artery disease, and clinical diabetes. Am Heart J. 2004;148(6):1034–1040. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15632890. Accessed June 22, 2011
20. Bagarolli R a, Saad MJ a, Saad STO. Toll-like receptor 4 and inducible nitric oxide synthase gene polymorphisms are associated with type 2 diabetes. J Diabetes Complicat. 2009;4
21. Manolakis AC, Kapsoritakis AN, Tiaka EK, et al. TLR4 gene polymorphisms: evidence for protection against type 2 diabetes but not for diabetes-associated ischaemic heart disease. Eur J Endocrinol/Eur Fed Endocri Soc. 2011;165(2):261–267. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21628510. Accessed October 10, 2012
22. Amyot J, Semache M, Ferdaoussi M, Fontés G, Poitout V. Lipopolysaccharides impair insulin gene expression in isolated islets of Langerhans via toll-like receptor-4 and NF-κB signalling. PLoS One. 2012;7(4):e36200. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3338606&tool=pmcentrez&rendertype=abstract. Accessed October 22, 2012
23. Ferwerda B, Mccall MBB, Alonso S et al (2007) TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. PNAS USA 104(42):16645–16650
24. Johnson GB, Riggs BL, Platt JL, Biology T, Clinic M (2004) A genetic basis for the "Adonis" phenotype of low adiposity and strong bones. FASEB J 4:1–15
25. Penas-Steinhardt A, Barcos LS, Belforte FS, de Sereday M, Vilariño J et al (2012) Functional characterization of TLR4 +3725 G/C polymorphism and association with protection

against overweight. PLoS ONE 7(12):e50992. doi:10.1371/journal.pone.0050992
26. Sato K, Yoshimura A, Kaneko T, et al. A single nucleotide polymorphism in 3′-untranslated region contributes to the regulation of toll-like receptor 4 translation. J Biol Chem. 2012; 287(30):25163–25172. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22661708. Accessed November 21, 2012