Gene 578 (2016) 32–37

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Genotypic distribution of common variants of endosomal toll like receptors in healthy Spanish women. A comparative study with other populations

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 26 November 2015
Accepted 4 December 2015
Available online 8 December 2015

Keywords:
Toll-like receptors
Genotypic distribution
Single nucleotide substitution
Healthy Spanish women

ABSTRACT

Genetic variants of endosomal toll like receptors (TLR) have been associated with many infectious, autoimmune and inflammatory diseases, but few studies have been reported in the Spanish population. The aim of this study was to describe the allelic and genotypic distributions of some common nucleotide substitutions of endosomal TLRs in healthy Spanish women and to compare them with those already published in other population groups. Nine substitutions were analysed in 150 DNA samples from 150 Spanish, non-related healthy females: TLR3 rs3775291 and rs5743305; TLR7 rs179008 and rs5743781; TLR8 rs3764880 and TLR9 rs187084, rs5743836, rs352139 and rs352140.

Genotyping was carried out by real time PCR and melting curve analysis in a LightCycler 480. A systematic review was performed in order to compare the genotypic distributions in our cohort with those previously published in other population groups. The comparative study was performed with the two tailed Fisher's test or the Yates continuity correction for the Chi-square test when appropriate. No homozygotes for rs5743781 in TLR7 were found, and rs352139 and rs352140 of TLR9 were in strong linkage disequilibrium. Genotype distributions in endosomal TLR are similar to other Spanish series previously reported. As expected, most differences were found when comparing our distributions with Asiatics, but differences were also found with other Caucasian populations. Since there are significant variations in genotypic distributions of TLRs in both interracial groups and within the same ethnic group, to carry out studies of disease susceptibility in more restricted groups is mandatory.

1. Introduction

Toll-like receptors (TLRs) are a family of pathogen recognition receptors which, upon ligand binding, promote innate immunity and establish an adequate immune response. The basis of this activation is the recognition of pathogen-associated molecular patterns, such as nucleic acids and structural components of viruses or bacteria (Pasare and Medzhitov, 2004).

TLRs have a broad and heterogeneous pattern of cellular expression and recognition of ligands. In humans, ten different TLRs (TLR1–10) have been described to date, each having specific ligands and expression profiles, and they can be easily classified into two subgroups according to their location and recognition of molecular patterns. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface where they primarily recognize bacterial structural components, while TLR3, TLR7, TLR8, and TLR9 have their expression restricted to intracellular compartments, mainly on endosomal membranes, where they recognize nucleic acids from viruses and bacteria. TLR3 recognizes the double-stranded RNA of viruses and is expressed mainly in human monocytes and dendritic cells. Both TLR7 and TLR8, which share a great homology, sense single-stranded RNA. TLR7 expression is restricted to dendritic cells and B lymphocytes while TLR8 is primarily expressed on cells of myeloid and NK lineages. TLR9 recognizes compounds rich in CpG and double-stranded DNA (Kawai and Akira, 2010).

TLRs also recognize endogenous ligands which have a great homology with the ligands of pathogens and are expressed inappropriately. Regarding endosomal TLRs, genetic material released into the extracellular environment as a result of apoptosis and necrosis, is phagocytosed inside the cell and processed by endosomes, thereby promoting their activation (Kawai and Akira, 2010).

TLRs activate intracellular signalling pathways that induce the expression of proinflammatory cytokines, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-alpha, directly regulating dendritic cells antigen processing and presentation to activate MHC class II. On the

Abbreviations: TLRs, Toll like receptors; IL, Interleukin; TNF, Tumour necrosis factor; MAF, Minor allele frequency.

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http://dx.doi.org/10.1016/j.gene.2015.12.004
0378-1119/

other hand, endosomal TLRs, in addition to activating the production of proinflammatory cytokines, also promote the production of type I interferons, with a high antiviral activity that stimulates the expression of MHC class I molecules (Kaisho and Akira, 2006).

The ability to respond properly to TLR ligands may be impaired by allelic variants within TLR genes, resulting in an altered susceptibility to infectious, inflammatory or autoimmune diseases (Misch and Hawn, 2008; Sánchez et al., 2004). Many of these allelic variants have an important functional implication as they can change the expression and/or the function of different TLR and therefore modify the immune response. Among all the genotypic distributions reported on endosomal TLRs, only a few relate to Spanish population (Sironi et al., 2012; Sánchez et al., 2009; Soriano-Sarabia et al., 2008; Dominguez-Mozo et al., 2014; Matas-Cobos et al., 2015), where just some of *TLR3*, *TLR7* and *TLR9* variants have been analysed.

Considering their important role in disease susceptibility and/or prognosis, an extensive study including the analysis of the most representative nucleotide substitutions of TLRs in the Spanish population is necessary. In this study, we have focused on endosomal TLRs due to their important role in the development and maintenance of autoimmune diseases and in viral infectious diseases.

2. Materials and methods

2.1. Study population

One hundred and fifty DNA samples from 150 Spanish, adult, unrelated healthy females with a mean age of 28.38 ± 6.84 years, were included. DNA samples were kindly provided by the Spanish National DNA Bank (University of Salamanca, Spain. http://www.bancoadn.org). None of the healthy donors had any cardiovascular, renal, lung, liver or haematological disease nor were they receiving any treatment for any other disease. All of them were seronegative for hepatitis B virus, hepatitis C virus or human immunodeficiency virus. All healthy donors gave their written consent to be included in research studies after approval from the Research and Ethics Committee at the Spanish National DNA Bank. Ethical approval for this study was also obtained from the Institutional Review Board for Human Research (Research Ethics Committee (REC)) at Puerta de Hierro University Hospital, Majadahonda, Madrid, Spain (REC June 27, 2011).

2.2. Allelic variants and genotyping

Nine nucleotide substitutions on the four endosomal TLR were analysed in all DNA samples: *TLR3* rs3775291 (c.1234C > T, p.Leu412Phe) and rs5743305 (c.-1077 T > A, g.4025 T > A); *TLR7* rs179008 (c.32A > T, p.Gln11Leu) and rs5743781 (c.1343C > T, p.Ala448Val); *TLR8* rs3764880 (c.1A > G, p.Met1Val) and *TLR9* rs187084 (c.-1486 T > C), rs5743836 (c.-1237 T > C), rs352139 (c.44A > G, g.1174A > G) and rs352140 (c.1635C > T, p.Pro545Pro).

These SNPs were selected as having a functional role on expression and/or function of TLRs, or have been associated with different diseases. All of them have a minor allele frequency (MAF) of 0.05 in Caucasians, except for *TLR7* Ala448Val, as recorded in the SNPs database (http://www.ncbi.nlm.nhi.gov/SNP).

Genotyping was performed by melting curve analysis with single probes (LightMix, TIB MOLBIOL, GmbH, Berlin, Germany) in the LightCycler 480 real time-PCR system (Roche Diagnostics, GmbH, Mannheim, Germany). PCR was performed in a total volume of 20 μl containing 25 ng of DNA, 1× genotyping Master Mix (Roche Diagnostics) and 1 μl of LightMix. PCR conditions, specified by the manufacturer, consisted of 10 min of initial denaturation at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C and 15 s at 72 °C. Amplification was immediately followed by melting analyses starting with denaturation at 95 °C for 1 min and renaturation at 40 °C for 2 min.

2.3. Comparative study with other populations

A comprehensive search was conducted in the Medline database in order to compare the genotypic distributions in our population with those previously reported in other populations. We selected those studies included in the database before October 2015 which described genotypic distributions in any of the nucleotide substitution analysed in our study in cohorts composed of at least 100 adult individuals, clearly stated as being healthy and not exposed to endemic infectious diseases.

Since *TLR7* and *TLR8* are located in chromosome X, comparative analyses with other populations included only those genotype distributions reported in females. When comparing *TLR3* and *TLR9* polymorphisms, located in chromosome 4 and chromosome 3 respectively, we considered the genotype distributions described in the entire population or only in females when available.

2.4. Statistical analysis

Allelic and genotypic frequencies for each polymorphism were calculated by direct counting. Test for Hardy–Weinberg equilibrium was performed by the Chi-square test online calculator "Simple Hardy–Weinberg calculator-Court lab" (Tufts University, Boston, MA).

Comparative studies of genotypic frequencies with other populations were performed using the Chi Square test or the Fisher's exact test with two tails for 2 × 3 contingency tables. For those genotypes represented in less than 5% of the population, the analyses were performed by the continuity Yates correction of the Chi-square test for 2 × 2 contingency tables comparing the homozygous genotype for the major allele and the carrier status for the minor allele. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Genotypic frequencies of endosomal TLR

Table 1 shows allelic and genotypic frequencies of the nine nucleotide substitutions analysed. Deviation from Hardy–Weinberg equilibrium was not observed for any of them and the MAF was more than 0.1 for all variants, except for rs5743781 of *TLR7*, which was only found in three heterozygote women. Strong linkage disequilibrium between *TLR9* rs352139 and rs352140 was observed as has been also reported in other populations (Hur et al., 2005; Ito et al., 2011). Indeed, both genetic variants cosegregated in all but one female.

3.2. Comparative study with other populations

As shown in Table 2, the genotypic distribution of SNP rs3775291 of *TLR3* was similar to the previously reported in other Spanish (Sironi et al., 2012; Matas-Cobos et al., 2015), German (Gast et al., 2011), Danish (Enevold et al., 2014a) and Serbian (Stanimirovic et al., 2013)

Table 1
Allele and genotype frequencies of nucleotide substitutions of endosomal TLR in healthy Spanish women.

Gene	rs	WT	Minor allele	MAF	HET [n (%)]	HOMO [n (%)]	HWE
TLR3	rs3775291	C	T	0.34	65 (43.3)	19 (12.7)	0.63
	rs5743305	T	A	0.39	70 (46.7)	23 (15.3)	0.84
TLR7	rs179008	A	T	0.18	47 (31.3)	3 (2)	0.34
	rs5743781	C	T	0.01	3 (2)	0	0.90
TLR8	rs3764880	A	G	0.28	60 (40.0)	12 (8)	0.92
TLR9	rs187084	T	C	0.39	71 (47.3)	23 (15.3)	0.94
	rs5743836	T	C	0.11	28 (18.7)	3 (2)	0.38
	rs352139	A	G	0.5	74 (49.3)	38 (25.3)	0.87
	rs352140	C	T	0.5	75 (50)	37 (24.7)	0.87

HET: Heterozygous for the variant allele; HOMO: Homozygous for the variant allele; HWE: Hardy Weinberg equilibrium; MAF: Minor allele frequency; WT: wild type.