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Original Article

The rs5743708 gene polymorphism in the TLR2 gene contributes to the risk of tuberculosis disease

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Abstract: Background: It has been reported that one single nucleotide polymorphisms (SNPs) rs5743708 in TLR2 gene might be associated with the susceptibility to tuberculosis disease. Owing to mixed and inconclusive results, we conducted a meta-analysis to systematically summarize and clarify the association between the rs5743708 gene polymorphism in the TLR2 gene and the risk of tuberculosis disease. Methods: A systematic search of studies on the association of the rs5743708 gene polymorphism in the TLR2 gene with susceptibility to tuberculosis disease was conducted in PubMed. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to pool the effect size. Results: A total of nineteen case-control studies from 13 articles on rs2910164 and 3 studies on the rs5743708 gene polymorphism in the TLR2 gene and the risk of tuberculosis disease were included. A significant relationship between the rs5743708 gene polymorphism in the TLR2 gene and tuberculosis disease was discovered in an allelic genetic model (OR: 2.801, 95% CI: 2.130-3.683, P=0.000), a homozygote model (OR: 5.795, 95% CI: 1.982-16.941, P=0.001), a heterozygote model (OR: 2.628, 95% CI: 1.888-3.569, P=0.000), a dominant genetic model (OR: 2.786, 95% CI: 2.003-3.877, P=0.000) and a recessive genetic model OR: (5.568, 95% CI: 1.907-16.255, P=0.002). In sub-group analysis base on ethnicity, significance was observed between the Caucasian group and the Asian group. Conclusions: The rs5743708 gene polymorphism in the TLR2 gene contributes to the risk of tuberculosis disease. Individuals with the rs5743708 gene polymorphism in the TLR2 gene are under a higher risk for tuberculosis disease.

Keywords: TLR2, rs5743708, gene polymorphism, tuberculosis disease

Introduction

Tuberculosis in the past also called phthisis, phthisis pulmonalis, or consumption, is a widespread, and in many cases fatal, infectious disease caused by various strains of mycobacteria, usually Mycobacterium tuberculosis. One-third of the world's population is thought to have been infected with M. tuberculosis, and new infections occur in about 1% of the population each year [1]. A number of factors make people more susceptible to TB infections [2]. The exact reasons as to why only some of the individuals exposed to M. tuberculosis develop uncontrolled disease and others eradicate or limit the disease remains unknown. The evidence suggests that genetic factors may be important determinants of increased susceptibility to progressive disease development [3].

Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells such as macrophages and dendritic cells, that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa, they are recognized by TLRs, which activate immune cell responses. The TLRs include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13.

Toll-like receptor 2 also known as TLR2 is a protein that in humans is encoded by the TLR2 gene. TLR2 has also been designated as CD282 (cluster of differentiation 282). TLR2 is one of the toll-like receptors and plays a role in the

immune system. TLR2 is a membrane protein, a receptor, which is expressed on the surface of certain cells and recognizes foreign substances and passes on appropriate signals to the cells of the immune system [4]. Various single nucleotide polymorphisms (SNPs) of the TLR2 have been identified and for some of them an association with faster progression and a more severe course of sepsis in critically ill patients was reported [5]. The genetic polymorphism of TLR2 (arginine to glutamine substitution at residue 753 (Arg753Gln)) has been associated with a negative influence on TLR2 function.

To date, a number of molecular epidemiological studies have been done to evaluate the association between the rs5743708 gene polymorphism in the TLR2 gene and the risk of tuberculosis disease [6-17]. Ogus et al indicated that the arginine to glutamine substitution at residue 753 polymorphism of the Toll-like receptor 2 gene influences the risk of developing tuberculosis [6]. However, a study done in Indian showed that TLR2 polymorphisms are not responsible for the increased prevalence of TB in the Indian population [10]. Thus, the results were inconsistent or even contradictory, partially because of the possible small effect of the polymorphism on tuberculosis risk and the relatively small sample size in each of published study. Therefore, we performed a comprehensive meta-analysis by including the most recent and relevant articles to identify statistical evidence of the association between the rs5743708 gene polymorphism in the TLR2 gene and risk of tuberculosis that have been investigated. Meta-analysis is an outstanding tool for summarizing the different studies. It can not only overcome the problem of small size and inadequate statistical power of genetic studies of complex traits, but also can provide more reliable results than a single case-control study.

Materials and methods

Publication search

Eligible articles were identified by searching PubMed, Wang Fang (Chinese literature database) and CNKI (China National Knowledge Infrastructure), using combination of the following Medical Subject Headings (MeSH) terms in PubMed database without a language limitation: "Toll-Like Receptor 2", "mycobacterium tuberculosis" and "Polymorphism, Genetic" In addition, we manually checked the reference lists of all eligible articles to identify other eligible studies. If more than one article were published using the same case series, the study with the largest sample size was prior to be selected. The latest research was updated on March 30, 2015, with publication years ranging from 1997 to 2015.

Inclusion criteria and exclusion criteria

To be included in the meta-analysis, the studies must meet the following inclusion criteria: (1) case-control studies comparing tuberculosis cases with healthy or non- tuberculosis controls, (2) evaluation of the association between rs5743708 gene polymorphism in the TLR2 gene and tuberculosis risk, and (3) report of sufficient genotype data of rs5743708 gene polymorphism in the TLR2 gene. Case-only studies, case reports, abstract, reviews, animal studies, or studies containing overlapping data were all excluded. All records were selected by two authors independently according to the inclusion criteria and reached consensus on each record.

Data extraction

The data were manually extracted from each study by two authors independently according to a standard protocol. Studies that did not follow the inclusion criteria, those considered double publications, or those that provided inadequate data were excluded. If the same data appeared in different studies, the data were intended for use only once. The abstracted data comprised the following items: the first author's name, publication year, region, the number of genotypes, genotyping, study design, total number of cases and controls and HWE. Disagreements were resolved by discussion between the two authors. When necessary, another author was consulted to resolve the dispute.

Statistical analysis

The reviewers calculated the risks the rs5743708 gene polymorphism in the TLR2 gene directly from the data given in the eligible studies. The association between the rs5743708 gene polymorphism in the TLR2 gene and

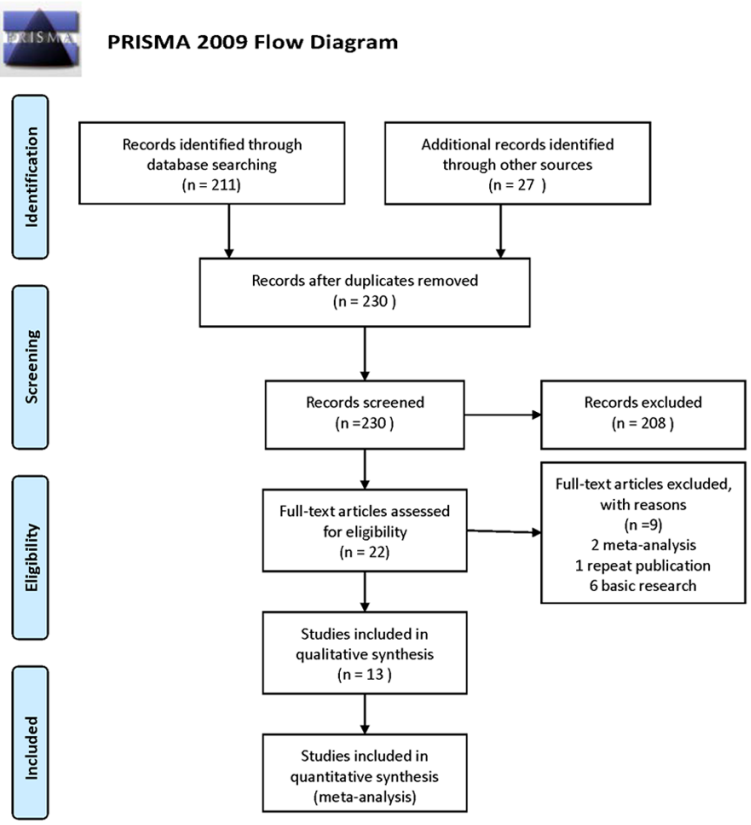

Figure 1. Flow chart of the study selection process.

tuberculosis diseases risk was examined under homozygote model (AA versus GG), dominant model (AA + AG versus GG), recessive model (AA versus AG+GG), and heterozygote model (AG versus GG contrast) [18]. The reviewers also checked the association between allele A of the rs5743708 gene polymorphism in the TLR2 gene and lung cancer risk. Subgroup analyses by ethnicity were performed subsequently. Summary of the OR was obtained using fixed-effect model with the Mantel-Haenszel method. For each meta-analysis performed, a χ^2-based Q-statistic test was employed to assess the between-study heterogeneity, and an I^2 value larger than 50% was defined as heterogeneity. In the case of heterogeneity, random-effects model with the DerSimonian and Laird method was then used to pool the results. The significance of the pooled OR was determined by the Z test with 95 % CI. Sensitivity analysis was implemented by excluding each single study. Funnel plot, together with Egger's regression test, was used to detect any publication bias in the meta-analysis [19].

Results

Study characteristics

A total of 13 publications involving 4970 cases and 4105 controls met the inclusion criteria and were ultimately analyzed (**Figure 1**). **Table 1** presents the main characteristics of these studies. Of the 13 publications, 10 were published in English and only 3 were written in Chinese. The sample sizes ranged from 141 to 1087. There were 9 groups of Asians, 13 groups of Caucasians. All polymorphisms in the control subjects except the paper of Ogus et al in 2004 were in Hardy-Weinberg equilibrium.

Main meta-analysis results

An estimation of the association between the rs5743708 gene polymorphism in the TLR2 gene and the risk of tuberculosis disease was presented in **Table 2**. Besides, **Figure 2** showed the relationship of tuberculosis disease risk with the rs5743708 gene polymorphism in the TLR2 gene in the form of forest plots with allele model. Overall, meta-analyses of total studies showed that the variant allele A of TLR2 Arg753Gln was associated with increased risk of tuberculosis disease when compared with the allele model (OR: 2.801, 95% CI: 2.130-3.683, P=0.000) (**Table 2** and **Figure 2**). The pooled results of total studies suggested that the rs5743708 gene polymorphism in the TLR2 gene was significantly associated with tuberculosis disease susceptibility in five genotypic contrast models.: an allelic genetic model (OR: 2.801, 95% CI: 2.130-3.683, P=0.000) (**Table 2** and **Figure 2**), a homozygote model (OR:5.795, 95% CI: 1.982-16.941, P=0.001), a heterozygote model (OR: 2.628, 95% CI: 1.888-3.569, P=0.000) (**Table 2** and **Figure 3**), a dominant genetic model (OR: 2.786, 95% CI: 2.003-3.877, P=0.000) (**Table 2** and **Figure 4**) and a recessive genetic model OR: (5.568, 95% CI: 1.907-16.255, P=0.002). In sub-group analysis

Table 1. Characteristics of studies of TLR2 rs5743708 gene polymorphism included in this pooled analysis

Author	Year	Region	Ethnicity	Cases			Controls			Genotyping	Study design	Sample size		HWE
				GG	GA	AA	GG	GA	AA			Cases	Controls	
Ogus	2004	Turkey	Caucasian	124	13	14	107	7	2	PCR-RFLP	Case-control	151	116	0.000
Ogus	2004	Turkey	Caucasian	106	12	11	107	7	2	PCR-RFLP	Case-control	129	116	0.000
Jin	2006	China	Asian	99	71	0	168	31	0	PCR-SSP	Case-control	170	199	0.233
Ma	2007	USA	Caucasian	337	2	0	194	0	0	Sequencing	Case-control	339	194	0.986
Ma	2007	USA	Caucasian	171	9	0	105	5	0	Sequencing	Case-control	180	110	0.807
Ma	2007	USA	Caucasian	374	1	0	110	4	0	Sequencing	Case-control	375	114	0.849
Strapagiel	2008	Poland	Caucasian	74	5	0	60	2	0	PCR-RFLP	Case-control	79	62	0.897
Yu	2008	China	Asian	76	1	0	75	0	0	Sequencing	Case-control	77	75	0.977
Biswas	2009	India	Asian	100	0	0	100	0	0	PCR-RFLP	Case-control	100	100	0.980
Xue	2010	China	Asian	204	1	0	202	1	0	Sequencing	Case-control	205	203	0.972
Selvaraj	2010	Indian	Asian	192	1	0	198	1	0	PCR-RFLP	Case-control	193	199	0.971
Ma	2010	China	Asian	543	0	0	544	0	0	Sequencing	Case-control	543	544	0.991
Etokebe	2010	Croatia	Caucasian	102	1	0	104	1	0	Taqman	Case-control	103	105	0.961
Sanchez	2011	Colombia	Caucasian	463	3	0	296	4	0	MALDI-TOF MS	Case-control	466	300	0.684
Dalgic	2011	Turkey	Caucasian	100	38	0	186	14	0	PCR-RFLP	Case-control	138	200	0.608
Dalgic	2011	Turkey	Caucasian	52	8	0	186	14	0	PCR-RFLP	Case-control	60	200	0.608
Dalgic	2011	Turkey	Caucasian	103	35	0	186	14	0	PCR-RFLP	Case-control	138	200	0.608
Dalgic	2011	Turkey	Caucasian	49	11	0	186	14	0	PCR-RFLP	Case-control	60	200	0.608
Dalgic	2011	Turkey	Caucasian	152	46	0	186	14	0	PCR-RFLP	Case-control	198	200	0.608
Wu	2015	China	Asian	216	9	0	418	4	0	PCR–RFLP	Case-control	422	225	0.922
Wu	2015	China	Asian	103	6	0	418	4	0	PCR–RFLP	Case-control	422	109	0.922
Wu	2015	China	Asian	319	15	0	418	4	0	PCR–RFLP	Case-control	422	334	0.922

PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; PCR-LDR: Polymerase Chain Reaction ligase detection; FP: Fluorescence Polarization; HWE: Hardy-Weinberg Equilibrium; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry; BMI: Body Mass Index; NA: Not Applicable.

Table 2. Meta-analysis of the association between TLR2 rs5743708 gene polymorphism and tuberculosis disease risk

Polymorphism	Population	Number of studies	Test of association			Test of heterogeneity		
			OR	95% CI	*P* value	Model	*P* value	I^2
A verse G	Caucasian	13	2.394	1.633-3.511	0.000	REM	0.022	49.5%
	Asian	9	3.422	2.372-4.939	0.000	REM	0.824	0.0%
	Overall	22	2.801	2.130-3.683	0.000	REM	0.096	30.6%
AA versus GG	Caucasian	13	5.795	1.982-16.941	0.001	REM	0.939	0.0%
	Asian	9	NA	NA	NA	NA	NA	NA
	Overall	22	5.795	1.982-16.941	0.001	REM	0.939	0.0%
AG versus GG	Caucasian	13	2.099	1.332-3.309	0.001	REM	0.005	57.2%
	Asian	9	3.985	2.704-5.873	0.000	REM	0.869	0.0%
	Overall	22	2.628	1.888-3.569	0.000	REM	0.022	42.9%
AA+AG versus GG	Caucasian	13	2.225	1.390-3.559	0.001	REM	0.007	57.6%
	Asian	9	3.985	2.704-5.873	0.000	REM	0.869	0.0%
	Overall	22	2.786	2.003-3.877	0.000	REM	0.034	40.8%
AA versus AG+GG	Caucasian	13	5.568	1.907-16.255	0.002	REM	0.933	0.0%
	Asian	9	NA	NA	NA	NA	NA	NA
	Overall	22	5.568	1.907-16.255	0.002	REM	0.933	0.0%

OR odds ratio; CI confidence interval; REM: random effects model; NA: not applicable.

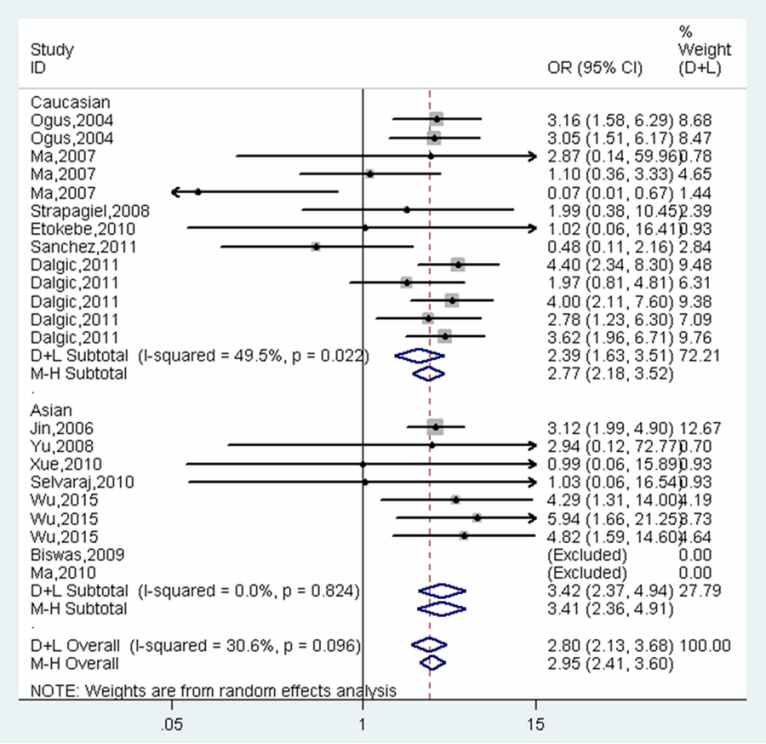

Figure 2. Forest plot of tuberculosis disease associated with TLR2 rs5743708 gene polymorphism under an allele genetic model.

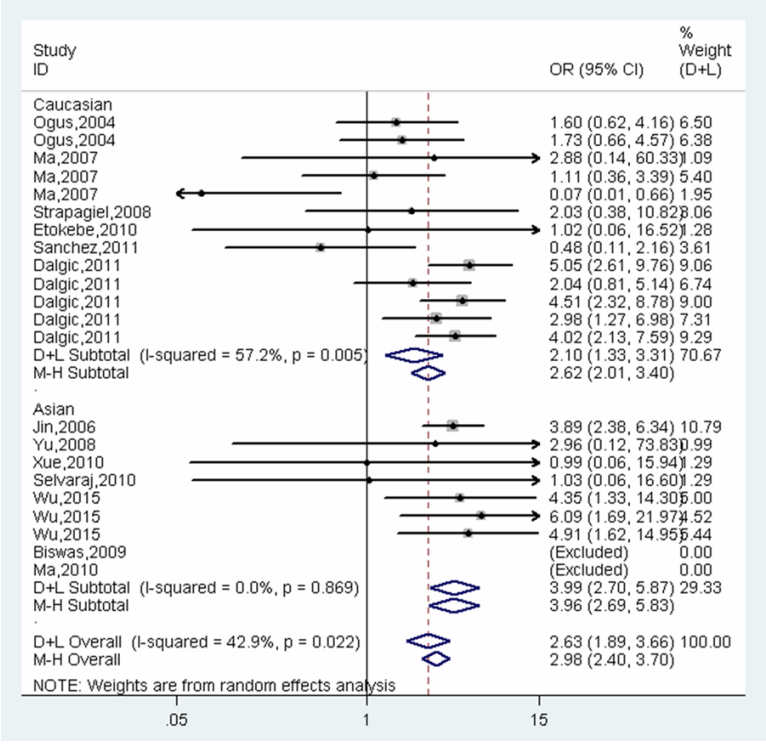

Figure 3. Forest plot of tuberculosis disease associated with TLR2 rs5743708 gene polymorphism under a heterozygote genetic model.

base on ethnicity, significance was observed between the Caucasian group and the Asian group (**Table 2**).

Publication bias

The publication bias of the studies was evaluated using the funnel plot and Egger's test. Publication bias was not seen in the funnel plot (**Figure 5**). No statistically significant difference was discovered in the Egger's test (P=0.097), indicating low publication bias in the current meta-analysis [20]. Furthermore, funnel plots' shape of all comparison models did not reveal any obvious evidence of asymmetry and all *P* values of Egger's tests were more than 0.05, providing statistical evidence of funnel plots' symmetry [21].

Discussion

The present meta-analysis provided robust evidence that the genetic polymorphism of the rs5743708 gene in the TLR2 gene was associated with the risk of tuberculosis disease. Meta-analyses of total studies showed that the genetic polymorphism of the rs5743708 gene polymorphism in the TLR2 gene was associated with increased risk of tuberculosis disease risk in the allelic model, homozygous model, heterozygote model, recessive model, and dominant model.

Moreover, meta-analyses of studies with high quality further identified this association. Subgroup analyses by ethnicity suggested a significant association between the rs5743708 gene polymorphism in the TLR2 gene and

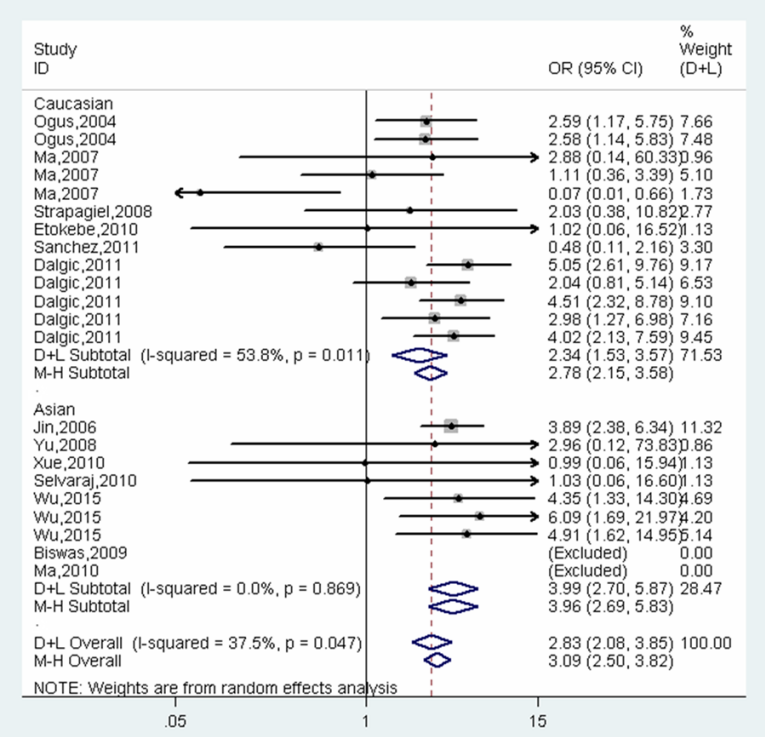

Figure 4. Forest plot of tuberculosis disease associated with TLR2 rs5743708 gene polymorphism under a dominant genetic model.

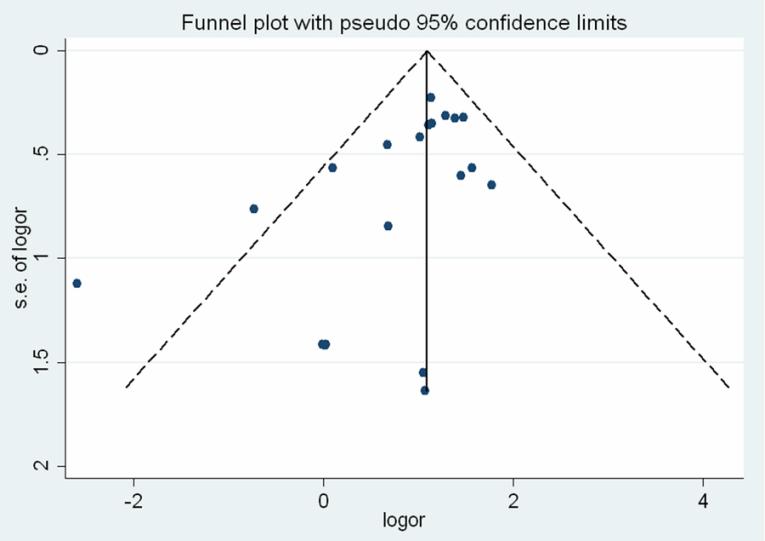

Figure 5. Funnel plot about studies of the association of tuberculosis disease and TLR2 rs5743708 gene polymorphism.

tuberculosis disease susceptibility in Asians and Caucasians. The heterogeneity analysis suggested that ethnicity was one main source of heterogeneity. Thus, the meta-analysis of all available data showed that the rs5743708 gene polymorphism in the TLR2 gene was associated with an increasing risk of tuberculosis disease.

Toll-like receptors (TLRs) represent a class of transmembrane pattern recognition receptors essential for microbial recognition and control of innate immune responses. Commensal bacteria play an important role in maintaining tolerance and active stability of the intestinal epithelial barrier by suppressing intestinal inflammation. TLR2 may provide a target to pharmacologically modulate mucosal injury and intestinal inflammation. Recognition of Mycobacterium tuberculosis by the innate immune system is essential in the development of an adaptive immune response. Mycbacterial cell wall components activate macrophages through Toll-like receptor (TLR) 2, suggesting that this innate immune receptor plays a role in the host response to M. tuberculosis infection. Several studies have found that the TLR2 single nucleotide polymorphisms were associated with susceptibility to tuberculosis disease. Previous studies published to assess the association between the rs5743708 gene polymorphism in the TLR2 gene and tuberculosis disease risks in different ethnic populations have provided contradictory results. Interestingly, we found that The rs5743708 gene polymorphism in the TLR2 gene was significantly associated with tuberculosis disease risk in Asian population and Caucasian population. In our meta-analyses of total studies, the pooled ORs revealed that the rs5743708 gene polymorphism in the TLR2 gene in homozygote model, dominant model, recessive model, and

heterozygote model was associated with increased risk of tuberculosis disease.

The data described above do have limitations. Firstly, almost all the articles were retrospective; it is possible that other unknown confounders may bias the data. Secondly, the derivation of phenotypes from these tests also varies from study to study which could be argued that it is not absolutely appropriate. Furthermore, uncertainty in the clinical evidence is compounded further from heterogeneity across the studies in terms of patient populations, alleles tested, and the manner in which phenotypes are defined. The fourth consideration is that all recruited case-control studies were from Asians and Caucasians; thus, our results may only be suitable for these populations.

In conclusion, in spite of the limitations mentioned above, our meta-analysis supports the growing body of evidence that the rs5743708 gene polymorphism in the TLR2 is emerging as a protective factor for tuberculosis in Asian and Caucasians populations. The importance of stratifying by ethnicity, cancer type, study design, and sample size needs to be standardized in future studies, together with consideration of the association between the TLR2 polymorphism and tuberculosis disease risk. Furthermore, the linkage of the rs5743708 gene polymorphism in the TLR2 with other polymorphisms of TLR2 may help explain the variability in findings association between the rs5743708 gene polymorphism in the TLR2 and tuberculosis disease risk. Furthermore, the linkage of the rs5743708 gene with other polymorphisms of TLR-2 may help explain the variability in findings.

Disclosure of conflict of interest

None.

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References

[1] Huynh GH, Klein DJ, Chin DP, Wagner BG, Eckhoff PA, Liu R and Wang L. Tuberculosis control strategies to reach the 2035 global targets in China: the role of changing demographics and reactivation disease. BMC Med 2015; 13: 88.

[2] Guo XG, Ji TX, Xia Y and Ma YY. Autophagy protects type II alveolar epithelial cells from Mycobacterium tuberculosis infection. Biochem Biophys Res Commun 2013; 432: 308-313.

[3] Hung CL, Chien JY and Ou CY. Associated factors for tuberculosis recurrence in taiwan: a nationwide nested case-control study from 1998 to 2010. PLoS One 2015; 10: e0124822.

[4] Bochud PY, Hawn TR and Aderem A. Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. J Immunol 2003; 170: 3451-3454.

[5] Khalilullah SA, Harapan H, Hasan NA, Winardi W, Ichsan I and Mulyadi M. Host genome polymorphisms and tuberculosis infection: What we have to say? Egyptian Journal of Chest Diseases and Tuberculosis 2014; 63: 173-185.

[6] Ogus AC, Yoldas B, Ozdemir T, Uguz A, Olcen S, Keser I, Coskun M, Cilli A and Yegin O. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J 2004; 23: 219-223.

[7] Lei J, Shi-Ping D, Xiu-Fang Z, Jina-Ping Z and Ji-Cheng L. The Arg753Gln polymorphism of human toll-like receptor-2 gene and its association with tuberculosis disease in Zhejiang Han population. Chinese Journal of Cell Biology 2006; 29: 229-231.

[8] Lei J, Shi-Ping D, Xiu-Fang Z, Jina-Ping Z, and Ji-Cheng L. The Arg753Gln polymorphism of human toll-like receptor-2 gene and its association with tuberculosis disease in Zhejiang Han population. Chinese Journal of Cell Biology 2006; 29: 229-231.

[9] Strapagiel D, Kasztalska K, Druszczynska M, Kowalewicz-Kulbat M, Vrba A, Matusiak A, Chmiela M and Rudnicka W. Monocyte response receptors in BCG driven delayed type hypersensitivity to tuberculin. Folia Histochem Cytobiol 2008; 46: 353-359.

[10] Biswas D, Gupta SK, Sindhwani G and Patras A. TLR2 polymorphisms, Arg753Gln and Arg-677Trp, are not associated with increased burden of tuberculosis in Indian patients. BMC Res Notes 2009; 2: 162.

[11] Etokebe GE, Skjeldal F, Nilsen N, Rodionov D, Knezevic J, Bulat-Kardum L, Espevik T, Bakke O and Dembic Z. Toll-like receptor 2 (P631H) mutant impairs membrane internalization and is a dominant negative allele. Scand J Immunol 2010; 71: 369-381.

[12] Ma MJ, Xie LP, Wu SC, Tang F, Li H, Zhang ZS, Yang H, Chen SI, Liu N, Liu W and Cao WC. Toll-like receptors, tumor necrosis factor-α, and in-

terleukin-10 gene polymorphisms in risk of pulmonary tuberculosis and disease severity. Hum Immunol 2010; 71: 1005-1010.
[13] Selvaraj P, Harishankar M, Singh B, Jawahar MS and Banurekha VV. Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India. Tuberculosis 2010; 90: 306-310.
[14] Xue Y, Zhao ZQ, Wang HJ, Jin L, Liu CP, Wang Y and Li JC. Toll-like receptors 2 and 4 gene polymorphisms in a southeastern Chinese population with tuberculosis. Int J Immunogenet 2010; 37: 135-138.
[15] Yun X. The association analysis of CD14 and TLR2 genes with tuberculosis in the Han Chinese population. Zhejiang University 2010.
[16] Dalgic N, Tekin D, Kayaalti Z, Soylemezoglu T, Cakir E, Kilic B, Kutlubay B, Sancar M and Odabasi M. Arg753Gln polymorphism of the human Toll-like receptor 2 gene from infection to disease in pediatric tuberculosis. Hum Immunol 2011; 72: 440-445.
[17] Sanchez D, Lefebvre C, Rioux J, Garcia LF and Barrera LF. Evaluation of Toll-like receptor and adaptor molecule polymorphisms for susceptibility to tuberculosis in a Colombian population. Int J Immunogenet 2012; 39: 216-223.
[18] Thakkinstian A, McElduff P, D'Este C, Duffy D and Attia J. A method for meta-analysis of molecular association studies. Stat Med 2005; 24: 1291-1306.
[19] Stuck AE, Rubenstein LZ and Wieland D. Bias in meta-analysis detected by a simple, graphical test. Asymmetry detected in funnel plot was probably due to true heterogeneity. BMJ 1998; 316: 469; author reply 470-461.
[20] Egger M, Davey Smith G, Schneider M and Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ 1997; 315: 629-634.
[21] Cochran WG. The effectiveness of adjustment by subclassification in removing bias in observational studies. Biometrics 1968; 24: 295-313.