Introduction

Tuberculosis (TB) is caused by infection with Mycobacterium tuberculosis (M.tb) and poses substantial morbidity and mortality worldwide. It is however known that TB susceptibility is not only determined by the infection status and environment factors but also by the host genetic components, as proved by epidemiological [1,2,3], twin [4] and adopt studies [5]. Polymorphisms in the HLA haplotype and in the genes for the vitamin D3 receptor (VDR), some carrier family 11 member 1 (SCL11A1), interferon gamma (IFN-γ) promoter, mannose binding lectin (MBL), nitric oxide synthase 2 (NOS2A), and some toll like receptor (TLR) genes have been associated with increased susceptibility to pulmonary tuberculosis ([1,2,3], twin [4] and adopt studies [5]). Polymorphisms in the HLA haplotype and in the genes for the vitamin D3 receptor (VDR), some carrier family 11 member 1 (SCL11A1), interferon gamma (IFN-γ) promoter, mannose binding lectin (MBL), nitric oxide synthase 2 (NOS2A), and some toll like receptor (TLR) genes have been associated with increased susceptibility to pulmonary tuberculosis ([1,2,3], twin [4] and adopt studies [5]). Polymorphisms in the HLA haplotype and in the genes for the vitamin D3 receptor (VDR), some carrier family 11 member 1 (SCL11A1), interferon gamma (IFN-γ) promoter, mannose binding lectin (MBL), nitric oxide synthase 2 (NOS2A), and some toll like receptor (TLR) genes have been associated with increased susceptibility to pulmonary tuberculosis ([1,2,3], twin [4] and adopt studies [5]).

Therefore, the genetic influence is likely to be polygenic in nature. Many of the identified susceptibility alleles act at the level of the macrophage. Mycobacteria are facultative intracellular bacteria that infect and survive in host macrophages, the receptors expressed on the macrophage is initially responsible for detection and recognition of bacilli. Then the activation of receptors causes an immediate signaling transduction resulting in the induction of pro-inflammatory cytokines, which are important for resistant or susceptibility to M.tb infection.

Scavenger receptor (SR) macrophage receptor with a collagenous structure (MARCO) is a class of phagocytic receptors that has been implicated in host defense against bacterial pathogens [7]. Ito et.al found that the MARCO expression was transiently up-regulated on macrophages in response to BCG infection and expressed on macrophages within, and adjacent to, BCG-containing granulomas [8]. MARCO -expressing macrophages
in the splenic marginal zone appear to phagocytose more BCG than neighboring macrophages that do not express MARCO [8]. The following study suggested that the MARCO was expressed constitutively on subsets of macrophages and was up-regulated in response to TLR agonists and whole bacteria [9] but not by pro-inflammatory cytokines [10]. In vivo study, MARCO expression increased on macrophages in response to infection or inflammatory conditions [10,11] and this occurs on macrophages that are directly responsive to the stimuli as well as those distal to the initial infectious stimuli [7,12,13]. Consequently, the increased expression of MARCO may alter the function of MARCO-expressing macrophages by increasing bacterial binding and phagocytic capacity and by altering cytokine production [14]. The evidence from the recent study indicated that the MARCO mediated recognition and presentation of trehalose 6, 6′-dimycolate (TDM, also known as cord factor) and the macrophages expressed MARCO receptor secrete pro-inflammatory cytokines in response to TDM. Macrophages from MARCO deficient mice also produce significantly lower levels of pro-inflammatory cytokines than wildtype macrophages in response to infection with virulent M. tb and identify MARCO might be the additional co-receptors required for TLR-signaling in macrophage response to M. tb and TDM [15]. All of these studies so far provided strong evidence that MARCO might also act as an important mediator in the host immunity against M. tb.

Given the strong functional role of MARCO in host immunity against M. tb infection, the genetic component for MARCO might be conferred resistance or susceptibility to pulmonary TB (pTB). In the present study, we investigated MARCO polymorphisms for association with pTB susceptibility.

Results

Demographic characteristics

All of the subjects were of Chinese Han descent population. The baseline characteristics of the study population, including 923 pTB patients and 1033 NHS, are as shown in table 1.

Analysis of MARCO SNPs in Chinese Han population

A power of detection >90% was achieved for both multiplicative and additive models, assuming an approximative TB prevalence of 0.007 in China, a frequency of 0.1 for high risk alleles and a genotype relative risk of 1.4 (a = 0.05) with our sample size (case–control ratio = 0.894). Fourteen of the 17 SNPs genotyped were in Hardy-Weinberg equilibrium and three SNPs (rs6751745, rs4491733 and rs12987402) showed deviation from HWE (pHardy-Weinberg equilibrium and three SNPs (rs6751745, rs4491733 and rs12987402) showed deviation from HWE (p < 0.05, data not shown). The remaining 14 SNPs were further estimated for their association with pTB using Chi-square test. Single-marker analysis indicated that the minor G allele of rs17009726 was over-represented in case subjects than in controls, after the Bonferroni correction for multiple testing of 14 SNPs (p_{corrected} = 4.93E-5, table 2), thus showing significant association with pTB. Binary logistic regression analysis was used to identify any possible effect of MARCO genotype on pTB, with sex, age, smoking and BCG vaccination status as covariates. Moreover, the AG genotype was significantly associated with an increased risk of pTB by multivariate analysis. This increased risk was present by both additive and dominant model (OR = 1.61, 95%CI = 1.28–2.01, p_{corrected} = 4.96E-4 and OR = 1.65, 95%CI = 1.32–2.05, p_{corrected} = 9.27E-5, respectively, table 3).

The effects of MARCO haplotypes on disease were estimated using four gamete rule as default set and accelerated expectation maximization (EM) algorithm as implemented in the program HaploView v3.2. A total of 4 blocks (figure 1) and 14 Haplotypes with frequencies greater than 1% were identified (Table 4). Of 4 constructed blocks, neither of the haplotypes involved in block 1 (rs6752783 T/G and rs7559955 C/T) had significant association with susceptibility to pTB (table 2). However, both GC haplotype involved in block 2 (rs17009726A/G, rs2278588C/T) and TGCC haplotype in block 3 (rs17795618T/A, rs1371562G/T, rs6761637T/C, rs2011839C/T) were associated with an increased pTB risk after 100000 permutations analysis using haploview (p_{corrected} = 0.0001 and 0.0297, respectively, table 2). No other significant differences were observed in haplotypes frequencies involved in block4 between two groups.

Considering the association with a genetic variant and the pulmonary disease is too uncertain, we further explored the difference between patients with and without G allele of rs17009726 including age, sex, smoking, BCG status and TST, no significant difference was observed (table S1).

Discussion

We have identified genetic variants in the MARCO gene which might be associated with increased susceptibility to pTB. Significant association results were observed for rs17009726 by single point analysis and, 2 SNPs GC haplotype (based on rs17009726 and rs2278588) and 4 SNPs TGCC haplotype (based on rs17795618, rs1371562, rs6761637 and rs2011839). On the
other hand, No significant associations were observed for other infectious disease [17,18,19,20,21], the rs4331426 is within a gene-size (OR = 1.2) [16]. However, unlike the GWAS for other slow. The recently genome-wide association study (GWAS) determination of contributing genetic variants of pTB has been across the different ethnic population, but the progress in case control studies as well as linkage studies were performed [2,4], a serial studies including population based and family based genetics components have contributed to the susceptibility of TB.

| rs#     | Alleles | MA | Case | Control | p-value | \( \beta \)-corrected-value |
|---------|---------|----|------|---------|---------|---------------------------|
| rs17009242 | G/A     | A  | 0.153| 0.151  | 0.842   |                           |
| rs13186645 | C/G     | G  | 0.340| 0.359  | 0.213   |                           |
| rs2077344 | C/T     | T  | 0.350| 0.367  | 0.286   |                           |
| rs6752783 | T/G     | G  | 0.203| 0.210  | 0.593   |                           |
| rs7559955 | C/T     | T  | 0.214| 0.224  | 0.467   |                           |
| rs17009276 | A/G    | G  | 0.139| 0.092  | 3.52E-6 | 4.93E-5                   |
| rs2278588 | C/T     | T  | 0.247| 0.258  | 0.451   |                           |
| rs17795618 | T/A    | A  | 0.199| 0.221  | 0.086   |                           |
| rs1371562 | G/T     | T  | 0.115| 0.131  | 0.134   |                           |
| rs6761637 | T/C     | C  | 0.163| 0.133  | 0.0083  | 0.116                     |
| rs2011839 | C/T     | T  | 0.034| 0.033  | 0.907   |                           |
| rs3731611 | G/T     | T  | 0.045| 0.041  | 0.556   |                           |
| rs4848530 | C/T     | T  | 0.247| 0.264  | 0.181   |                           |
| rs11685286 | T/C   | C  | 0.146| 0.137  | 0.459   |                           |
| rs6751745 | C/T     | T  | 0.279| 0.251  | ND      |                           |
| rs4491733 | A/G     | G  | 0.468| 0.471  | ND      |                           |
| rs12987402 | T/C   | C  | 0.404| 0.414  | ND      |                           |

Data are presented as minor allele frequency, unless otherwise stated. MA: minor allele; HW: Hardy-Weinberg equilibrium; OR: odds ratio; CI: confidence interval; ND: p value was not determined due to the probability for deviation from Hardy-Weinberg equilibrium in controls and cases.

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When the previous studies have indicated that substantial genetics components have contributed to the susceptibility of TB [2,4], a serial studies including population based and family based case control studies as well as linkage studies were performed across the different ethnic population, but the progress in determination of contributing genetic variants of pTB has been slow. The recently genome-wide association study (GWAS) performed in African population identified a pTB related single locus (rs4331426) on chromosome 1q12-13 with a modest effect size (OR = 1.2) [16]. However, unlike the GWAS for other infectious disease [17,18,19,20,21], the rs4331426 is within a gene-desert and its biologic mechanism is unknown. Therefore, identifying the new candidate genes involving M. tuberculosis infection may be helpful for understanding the host susceptibility to pTB.

MARCO gene in human is located on chromosome 2q12-q13. MARCO, as one of class A SRs, has been demonstrated as involving host defense against bacterial pathogens and requiring for TLR-signaling in macrophage response to M. tuberculosis. Given the crucial role of MARCO in host immunity against M. tuberculosis infection, we firstly investigated its SNPs and identified that the SNPs in MARCO were associated with increased risk to pTB in Chinese Han population.

Our data indicated that the G allele of rs17009726 alone or the haplotype GC may confer increased susceptibility to pTB insofar as both allele and haplotype frequencies are higher in pTB case subjects than in control subjects. As for rs6761637, the C allele or CC genotype was not conferred susceptibility to pTB in single-point analysis after multiple testing. When haplotypes were considered, haplotype TGCC containing C allele of rs6761637 were nominal associated with increased susceptibility to pTB and the haplotype frequencies are slightly higher in pTB case subjects than in control subjects. According to our study, the reasonable explanation for the genetic variants of MARCO associated with increased the susceptibility to pTB would be that the genetic variants could affect the expression of MARCO by influencing the mRNA expression or stability, which may alter the function of MARCO -expressing macrophages by decreasing bacterial binding and phagocyte capacity, as well as affecting the cooperation between MARCO and TLR2/CD14 and, less pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) production in response to M. tuberculosis infection.

One important consideration in addressing the significant genetic association is population stratification inherent to case control study, which however, could be minimized in the current research, as little subpopulation structure was observed for genotype distribution within northern Chinese populations [22]. Despite of this main advantage, the current research still lacked the independent replication in other ethnic populations. In addition, SNPs in the study were selected by searching the International HapMap project database, instead of by gene-wide resequencing of MARCO in Chinese Han population. This SNP tagging strategy might miss functional genetic variants, thus only common genetic variants could be implicated in understanding their roles in host susceptibility to or in progression of tuberculosis disease. The SNP identified to be associated with the pTB risk in the current study was located in the intron of the MARCO gene. According to the previous studies, intronic SNP located in the consensus 3’ splice site adjacent to an exon could cause the absence of exon or mutation due to the intron.

| Model | Genotype | Case | Control | OR (95% CI) | \( \beta \)-poste-corrected-value | \( \beta \)-corrected-value |
|-------|----------|------|---------|-------------|---------------------------------|--------------------------|
| Cod   | AA       | 0.743| 0.827  | 1.00        | 3.54E-5                         | 4.96E-4                  |
|       | AG       | 0.235| 0.163  | 1.61(1.28–2.01) | 0.031                           | 0.434                    |
|       | GG       | 0.022| 0.011  | 2.26(1.08–4.76) | 6.62E-6                         | 9.27E-5                  |
| Dom   | AA       | 0.743| 0.827  | 1.00        | 1.57 (1.29–1.92) | 7.86E-6                  |
|       | AG-GG    | 0.257| 0.173  | 1.65(1.32–2.05) | 0.051                           | 0.714                    |
| Rec   | AA-AG    | 0.978| 0.989  | 1.00        |                                |                          |
|       | GG       | 0.022| 0.011  | 2.06(0.98–4.32) |                                |                          |

Data are presented as frequency of genotype, unless otherwise stated; Cod: codominant model; Dom: dominant model; Rec: recessive model; Add: additive model; OR: odds ratio; CI: confidence interval.

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Figure 1. Linkage disequilibrium (LD) map of SNPs. (a) LD map based on D-prime (D'); (b) LD map based on R-squared (R²); (c) haplotypes of SNPs in the “blocks”.

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without splicing, and then affect the disease susceptibility. The bioinformatics analysis predicted no obvious function of this SNP in regulating transcription or translation of MARCO gene (data not shown). Therefore, functional assay of this SNP was not performed in the current stage, although this knowledge might help to get comprehensive understanding of the gene’s role in affecting disease susceptibility.

Another important limit in our study is absence of knowledge about real latent TB infection, or contact with M. tuberculosis of the NHS who is presented as resistant to the disease. Although Chinese adults have been highly exposed to M. tuberculosis, we cannot verify the recent exposure to the bacteria of the NHS individually. This limit might lead to the misclassification of the group, which bias should be addressed. In addition, due to the lack of detailed information of the patients (the disease severity, anti-bacterial effect of the treatment, the TST results, et al), we failed to evaluate the association between the genetic variants and other bacterial effect of the treatment, the TST results, et al), we failed to evaluate the association between the genetic variants and other

### Materials and Methods

#### Ethics Statement

The study was approved by ethics committee of the Hebei Chest Hospital, Shijiazhuang Fifth Hospital and Beijing Electronic Chest Hospital. Written informed consent was obtained from all the participants before inclusion in the study.

#### Study subjects

A total of 923 patients with pTB who were Chinese Han origin were recruited from the Hebei Chest Hospital and Shijiazhuang Fifth Hospital (Hebei, China) during March 2007 to August 2009. pTB patients were recruited by review of medical records and laboratory reports. Patients were diagnosed according to the diagnostic criteria for pulmonary tuberculosis of Ministry of Health, the People’s Republic of China (http://www.moh.gov.cn/publicfiles/business/cmsresources/zxgkdt/cmsarticle/document/doc3242.pdf). The patients were defined as presence of at least one of the following: (1) smear/culture positive for M. tuberculosis (2) culture positive for M. tuberculosis and pathological change of tuberculosis in lung according to chest X-ray (3) pathological change of tuberculosis in the lung according to chest X-ray, typical clinical syndrome (4) pathological change of tuberculosis in the lung, culture positivity of bronchial lavage and/or pleural fluid for M. tuberculosis (5) pathological change in the lung and pathological evidence of TB disease in lung biopsy materials (Lung tissue or tumor location). 1033 normal healthy subjects (NHS) who underwent the physical examination in the Beijing Electronic Hospital (Beijing, China) were recruited during the study period and were matched for age, sex, ethnic and geographical origins to patients. The inclusion criteria for NHS were, absence of clinical signs, symptoms and pulmonary lesions on chest radiographic examination suggesting active tuberculosis. No medical history of TB or other infectious diseases, autoimmune disease, cancer or other disease that affect host immunity.

#### SNPs selection and Genotyping

The SNPs tested were haplotype-tagging SNPs (htSNPs) in the MARCO gene based on the HapMap reference data (www.hapmap.org, the International HapMap Consortium 2003, release 22, on NCBI B36 assembly, dbSNP b126) for Chinese Han populations. SNPs were selected to serve as multimarker tagging algorithm with criteria of $r^2 \geq 0.8$ and for all SNPs with minor allele frequency (MAF) ≥5% using HaploView v3.2 program [23]. The entire MARCO gene was covered. Finally, 17 SNPs, including 14 SNPs in intron region (rs2077344 C/T, rs6752783 T/G, rs4491733 A/G, rs7559555 C/T, rs17009726 A/G, rs2278588 C/T, rs17795618 T/A, rs1371562 G/T, rs6751745 C/T, rs2011839 C/T, rs3731611 G/T, rs2297402 T/C, rs4848530 C/T, rs1165286 T/C), 1 SNP in exon (rs6761637 T/C), as well as 2 SNPs in promoter region (rs17009242 G/A, rs1318645 C/G) were selected (table 2).

Genomic DNA was isolated by Gene Relax DNA extraction kit (TianGen, Beijing, China) according to the manufacturer’s instructions. Genotyping of SNPs was performed using the Sequenom system (SEQUENOM, San Diego, CA 92121-1331, USA) which uses mass spectrometry (MALDI-TOF) to discriminate products by their absolute masses. Primer extension was carried out utilizing a DNA primer adjacent to the SNP, and a specific reaction mix of polymerase, dNTPs and one ddNTP. Samples were analyzed by MALDI-TOF mass spectrometry and the alleles were called by weight (in daltons) of the extension products. Samples from positive and negative control subjects were included on each genotyping plate and checked for consistency. For confirmation the genotyping of variants, re-genotyping was verified by 10% replication of samples and by direct sequencing.

#### Statistical analysis

The homogeneity of baseline characteristics between two groups was tested by Fisher’s exact test or $\chi^2$ test for categorical
variables and by Student’s t-test or Wilcoxon test for continuous variables. Power calculation was performed with the CATS software [available at http://www.sph.umich.edu/csg/abecasis/CATS/]. The tests for deviations from Hardy–Weinberg equilibrium (HWE) were performed for each SNP by HaploView v3.2 program [23]. We used binary logistic regression to estimate the genotype-specific odds ratio (OR) and 95% confidence interval (CI) for pTB risk, after adjusting for sex, age, smoking and BCG vaccination status. An allelic p value of less than 0.05 was considered nominally significant and Bonferroni correction for multiple testing of 17 SNPs was applied to the single-point results of the genetic model analysis of genotype. Haplotype blocks were constructed using four gamete rule as default set and haplotype frequencies were inferred from the genotype data in the combined population (cases and controls) using an accelerated expectation maximization (EM) algorithm as implemented in the program HaploView v3.2. HaploView program was also used to evaluate the statistical significance of haplotype frequency differences between cases and controls with 100,000 permutations for multiple testing. Data were analyzed using Statistical Package for Social Sciences 17.0 (SPSS Inc, Chicago, IL, USA) and Haploview v3.2. In all analysis, statistical tests were based on two-tailed probability.

Supporting Information

Table S1 Characteristic comparison of patients with and without G allele of rs17009726.

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Author Contributions

Conceived and designed the experiments: M-JM WI. W-CC. Performed the experiments: M-JM HL. Analyzed the data: M-JM WL. Contributed reagents/materials/analysis tools: HL J-HY YY H-BW L-PX Y-CQ J-LL M-JC. Wrote the paper: M-JM.

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