TLR10 and NFKBIA contributed to the risk of hip osteoarthritis: systematic evaluation based on Han Chinese population

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Multiple lines of evidence have confirmed the importance of genetic factors for hip osteoarthritis (HOA). Our study aimed to investigate the associations of TLR10 and NFKBIA with respect to the HOA risk in Han Chinese individuals. A total of 1,043 HOA patients and 2,664 controls were recruited. Then, 23 tag single-nucleotide polymorphisms (SNPs) in the TLR10 and NFKBIA genes were selected for genotyping. Genetic association analyses were conducted in both single-marker and haplotype-based ways. Gene by gene, two-way interactions were analysed using a case-only method. Multiple bioinformatics tools were utilised to examine the potential functional significance of the SNPs. Two significant SNPs, rs11096957 (OR = 1.26, P = 1.35 × 10−5) and rs2273650 (OR = 1.2, P = 1.57 × 10−3), were significantly associated with HOA risk. Rs11096957 was also associated with the severity of the HOA. Bioinformatics analysis indicated that the allele T of rs2273650 would create new miRNA/SNP target duplexes, which suggests that rs2273650 could alter the NFKBIA expression by affecting the miRNA/SNP target duplexes. Our study identified significant association signals from NFKBIA with HOA for the first time, and it also confirmed the contribution of TLR10 to the HOA risk. These findings would provide clues for identifying individuals at high risk of HOA.
several studies have demonstrated that the TLR family mRNA transcription and protein expression level is much higher in OA synovial and cartilage than in normal cartilage. For animal experiments, the knockout of TLR-4 resulted in a less severe phenotype in a mouse model of arthritis, which indicates that the overexpression of the TLR-4 gene could produce an excessive inflammatory response and result in OA. Therefore, the TLR family could be involved in the occurrence and development of OA. In fact, TLR activation causes increased expression of pro-inflammatory cytokines that are regulated by signalling pathways that involve nuclear factor kB (NF-kB) transcription factors, which have been identified to play a significant role in OA. However, among the TLR family, TLR-10 is clustered together with TLR-1 and TLR-6 in the region of 4p14 and is the only member of the TLR family that has an anti-inflammatory effect by inhibiting NFkB signalling. NF-kB is held in the cytoplasm in an inactive state complexed with IkBa, an inhibitory protein that is encoded by the NFKBIA gene in the genomic region of 14q13.2 in resting cells. IkBa is phosphorylated by inducible expression of IkB kinases (IKKs), which mediates IkBa degradation to result in activated NF-kB translocation to the nucleus to initiate inflammation-related gene transcription. Thus, it is reasonable to hypothesise that common variants of NFKBIA could potentially regulate NF-kB signalling and alter cytokine profiles, which would lead to inflammatory responses in some susceptible individuals.

Recently, a study in the Croatian population that involved 500 OA patients and 397 controls reported that a single-nucleotide polymorphism (SNP) rs11096957 in TLR-10 is significantly associated with predisposition to HOA ($P = 0.04$, OR $= 1.41$, 95% CI $= 1.02–1.94$). Although the relationship of the NFKBIA gene with KOA was evaluated in European populations that consisted of 189 KOA cases and 197 healthy controls, the SNP rs8904 was identified to be marginally associated with KOA only in females ($P = 0.02$). Until now, they are the only two studies that report an association between TLR-10 and NFKBIA correlated with OA in European populations, and these results suggest that TLR-10 and NFKBIA could be the key genes in the pathogenesis of HOA. However, given that different ethnic populations could exhibit genetic heterogeneity of OA, the replications of the study using large-scale samples from other different populations are needed to confirm these results. Although several previous published GWASs have included both TLR10 and NFKBIA, none SNPs of the two loci have been reported to achieve genome-wide significance. In addition, there is no research that regards TLR-10 and NFKBIA in terms of the risk of HOA in the Han Chinese population. Thus, the present case-control study aims to investigate the associations of TLR-10 and NFKBIA with HOA risk in Han Chinese individuals, which would potentially shed light on the underlying pathological mechanisms of OA.

Methods

Study subjects. In our study, a total of 3,707 study subjects comprised of 1,043 HOA patients and 2,664 controls were recruited from Luoyang Orthopedic Hospital of Henan Province. All of the subjects were random unrelated Han Chinese individuals. The diagnosis of HOA was based on the criteria of the American College of Rheumatology, and all of the controls had no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness, or movement restriction). The HOA patients were confirmed by clinical examination and radiographic inspection. A questionnaire was used to collect demographic characteristics from subjects with regard to general information, smoking, drinking, occupations, sports activities, previous hip injuries and family history of OA and other diseases (Table 1). Only patients with a score of 2 or more (based on the Kellgren-Lawrence (K-L) grading standard) were included in the present study. The healthy control subjects had no symptoms of arthritis or any other joint-related disorders and had no family history of OA or other rheumatic diseases. Study subjects were excluded if they had inflammatory arthritis (rheumatoid, polyarthritic or autoimmune disease), post-traumatic or post-septic arthritis, and skeletal or developmental dysplasia. Further, all of the subjects in both groups were free of systemic or organic diseases. This study was performed according to the ethical guidelines of the Declaration of Helsinki (version 2002) and was approved by the Luoyang Orthopedic Hospital of Henan Province. Informed consent forms were obtained from all subjects.

SNP Selection and Genotyping. Tag SNPs covered the gene regions of TLR10 and NFKBIA and were selected for genotyping based on the 1,000 genome data of Chinese Han populations. Minor allele frequency (MAF) $> 0.01$ and $r^2 > 0.6$ were utilised as criteria for tagging. A total of 23 tag SNPs, 14 from TLR10 and 9 from NFKBIA, were selected for genotyping. Genomic DNA was extracted from peripheral blood leukocytes according to the manufacturer's protocol (Genomic DNA kit, Axygen Scientific Inc., California, USA). Genotyping was performed for all SNPs using the Sequenom Mass ARRAY RS1000 system (Sequenom, San Diego, California, USA). The results were processed using Typer Analyzer software (Sequenom), and the genotype data were generated from the samples. To ensure the accuracy of the genotyping, we have randomly chosen 5% of our study subjects and repeated the genotyping process for them. The concordance rate of this process was 100%, which indicates that the genotyping results of our study were reliable.

Statistical Analyses. MAFs were calculated, and Hardy-Weinberg equilibrium was tested for all of the genotyped SNPs. Logistic models were fitted for each SNP with age and BMI added as covariates to investigate the potential genetic associations. Haplotype association analyses were also performed. The analyses above were performed by the genetic analysis software Plink. Genomic control was conducted to detect and correct the potential influence of inflation significance caused by underlying population stratification, and a null distribution of the inflation factor $\lambda$ was created by 10,000 bootstrapping. Multiple comparisons were corrected by Bonferroni corrections, and thus, the threshold of the $P$ values used for single marker-based association analyses were 0.05/23 $\approx$ 0.002. The associations of targeted SNPs with disease severity measurements of K-L grade scale were evaluated by $\chi^2$ tests. In addition, because of the potential biological connections between TLR10 and NFKBIA, there could be a potential effect of gene-by-gene interactions on the risk of HOA from both genes. We have tested this two-locus interaction by conducting case-only tests for all SNP pairs between TLR10 and NFKBIA using Plink.
Bioinformatics Analyses. Bioinformatics analyses were performed to investigate the potential functional significance of the targeted SNPs, and two types of tools were utilised. For non-synonymous SNPs, we examined their functional consequences on the gene products using Polyphen2 and SIFT. In addition, RegulomeDB, which evaluated the evidence for the functional significance of SNPs using ENCODE data, was also utilised. RegulomeDB assigned a score that ranged from 1 to 7 for each SNP to indicate its functional significance. In addition, we examined the effect of targeted SNPs on miRNA-mediated gene repression using PolymiRTS.

Expression quantitative trait loci (eQTL) analyses. We extracted eQTL data for ~40 human tissues of targeted SNPs from the GTEx database. Data on the gene expression for different genotypes were extracted and compared. Significant SNPs with eQTL effects were reported.

Results

Genetic associations of TLR10 and NFKBIA with HOA. As shown in Table 1, the age and BMI were distributed differently between the cases and control. No significant differences could be identified for the other individuals and environmental factors, including the gender, smoking status, alcohol drinking, occupation type, and activity. Two significant SNPs, rs11096957 (OR = 1.26, \( P = 7.41 \times 10^{-5} \)) and rs2273650 (OR = 1.2, \( P = 1.23 \times 10^{-12} \)), were identified to be significantly associated with the disease status of HOA after adjusting for the effects of age and BMI (Table 2). The SNP rs11096957 is a non-synonymous SNP that is located in the exonic region of the gene TLR10. The SNP rs2273650 is located at the 3′ untranslated region (UTR) of the gene NFKBIA. The mean statistics for the 21 non-significance tests was 0.47 with a 95% confidence interval [0.37, 0.57] (Supplemental Fig. S1). This finding indicated that no significant inflation of the test statistics could be obtained from our single-marker-based association analyses. Therefore, there were very limited effects of population stratification for this study. This significant hit of the gene NFKBIA was validated and replicated in haplotypic association analyses. Linkage disequilibrium (LD) blocks were constructed for both genes separately, and the haplotypes were tested for their associations with HOA (Supplemental Figs S2 and S3). A two-SNP haplotype, rs2273650–rs8904, of gene NFKBIA was identified to be significantly associated with HOA (Table 3).

Associations of rs11096957 with the severity of HOA. Both significant SNPs identified in the single-marker-based association analyses, rs11096957 and rs2273650, were tested for their associations with the severity of HOA in the patient samples. Rs11096957 from gene TLR10 was found to be significantly associated with the severity of HOA as measured by KL grade scaling (Table 4). The T allele of rs11096957 was a significant indicator of the severity of HOA in our samples, and the pattern was very clear. All of the KL-4 grade HOA patients had a genotype of TT for rs11096957, while no patients of HOA with the KL-2 grade had a genotype of TT.

| HOA patients (N = 1,043) | controls (N = 2,664) | statistics | \( P \) |
|-------------------------|----------------------|------------|--------|
| Age, mean ± sd | 61.8 ± 8.0 | 60.7 ± 8.1 | \( t = 3.97 \) | \( P = 7.41 \times 10^{-5} \) |
| BMI, mean ± sd | 26.2 ± 1.5 | 25.8 ± 1.5 | \( t = 7.15 \) | \( P = 1.23 \times 10^{-12} \) |
| Gender (%) | | | | |
| Male | 497 (48) | 1287 (48) | | |
| Female | 546 (52) | 1377 (52) | \( \chi^2 = 0.11 \) | 0.7452 |
| Smoking (%) | | | | |
| Yes | 248 (24) | 618 (23) | | |
| No | 795 (76) | 2046 (77) | \( \chi^2 = 0.11 \) | 0.7401 |
| Drinking alcohol (%) | | | | |
| Yes | 307 (29) | 815 (31) | | |
| No | 736 (71) | 1849 (69) | \( \chi^2 = 0.42 \) | 0.5152 |
| Occupation (%) | | | | |
| Managerial | 446 (43) | 1162 (44) | | |
| Non-managerial | 597 (57) | 1502 (56) | \( \chi^2 = 0.19 \) | 0.6623 |
| Activity (%) | | | | |
| Inactive | 400 (38) | 1042 (39) | | |
| Less active | 366 (35) | 981 (37) | | |
| More active | 277 (27) | 641 (24) | \( \chi^2 = 2.62 \) | 0.2701 |
| KL grading scale (%) | | | | |
| KL-2 | 510 (49) | | | |
| KL-3 | 388 (37) | | | |
| KL-4 | 145 (14) | | | |

Table 1. Characteristic information of study subjects.
Epistasis analyses. Case-only tests were performed for all SNP pairs between TLR10 and NFKBIA. A total of 126 tests were conducted (14 × 9). No significant SNP pairs were identified after applying the Bonferroni correction (the threshold of the P value is 0.05/126 ≈ 4 × 10⁻⁵). The most significant SNP pair was rs11096957 (TLR10) and rs8904 (NFKBIA) with P = 0.0165 (Supplemental Table S1).

Functional consequences of the selected SNPs. We obtained the functional consequences for the significant non-synonymous SNP rs11096957 of TLR10 (Table 5). For rs11096957, both Polyphen2 and SIFT have
classified it as an SNP with a functional consequence ("possibly damaging" for Polyphen2 and "damaging" for SIFT) for the protein encoded by TLR10. In addition, the minor allele of the significant SNP from NFKBIA was found to create new binding sites for multiple miRNAs, which could mediate the down-regulation of NFKBIA expression. The RegulomeDB scores for rs11096957 and rs2273650 were 7 and 4, respectively, which indicates that the evidence of functional significance for both SNPs was very limited.

**Analyses of eQTL.** We extracted eQTL data of 44 human tissues from GTEx for rs1109695 (no data can be obtained for rs2273650 due to its low MAF in Europeans). A significant signal for eQTL was observed only from cells of EBV-transformed lymphocytes (Fig. 1).

**Discussion**

In this study, we gained evidence that supports the roles that TLR10 plays in the onset and development of HOA. We identified a significant non-synonymous SNP, rs11096957, which was significantly associated with the disease status of HOA. Although a previous candidate gene-based study obtained similar results with a sample from European populations, our findings can serve as a successful replication of this previous study in the Chinese Han population. Compared to the previous study, which focused only on the association with the disease status of OA, we also examined the association between the severity of HOA and rs11096957. Our findings showed that rs11096957 could serve as a significant indicator for the severity of HOA, when measured by the KL grade scale. A dosage-dependent pattern for the T allele of rs11096957 can be found to be associated with the severity of HOA. In addition, according to the results of bioinformatics analyses, the minor allele of rs11096957 can significantly alter the structure of the protein encoded by TLR10 and in turn affect its function fundamentally. On the other hand, eQTL analyses offered us only limited evidence for the role of rs11096957 on the gene expression level of TLR10 (1 significant hit out of 45 human tissues). Combined with all of this evidence from different aspects, we believe that the SNP rs11096957 was susceptible to HOA with a true functional effect by disrupting the structure of the protein encoded by TLR10. The association signal identified from our study could be more than solely a statistical association but could also have a functional effect and a representation of biological and pathological mechanisms.

Another significant hit was obtained for the gene NFKBIA. The SNP rs2273650 is located at the 3’ untranslated region of NFKBIA, and thus, unlike exonic variants, it has nothing to do with the protein structure. We determined that the T allele of this SNP could increase the risk of HOA by approximately 20%, although no significant association was identified between this SNP and the severity of HOA in our case samples. To the best of our knowledge, our study was the first study to identify a significant association signal for the SNP rs2273650. A

| Chr | Position | SNP       | RegSc | Polyphen2  | SIFT     | PolymiRTS |
|-----|----------|-----------|-------|------------|----------|-----------|
| 4   | 38776490 | rs11096957| 7     | possibly damaging | DAMAGING | —         |
| 14  | 35870797 | rs2273650 | 4     | —          | —        | —         |

| The derived allele creates new miRNA site: hsa-miR-4266/ hsa-miR-4695-5p/ hsa-miR-4729/ hsa-miR-4779 |

Table 5. Functional consequences for candidate SNPs based on bioinformatics analyses. RegSc: Score from RegulomeDB.

![Figure 1. The eQTL pattern of SNP rs11096957 for TLR10 in 45 human tissues. The P value threshold is indicated by the red dotted line.](image)
previous study that was based on European women determined that another SNP of *NFKBIA*, rs8904, is associated with OA. The SNP rs2273650 was not genotyped in that study because of its low MAF in the European population (MAF = 0.005 in the European population based on data from the 1000 genomes project). Interestingly, both rs2273650 and rs8904 were genotyped in our study, and the two SNPs were in strong LD. Given that it is not sufficient to draw conclusions from limited SNPs analyses, we performed haplotype analyses, which indicated a similar pattern with single marker-based associations. The haplotype formed by these two SNPs was significantly associated with HOA disease status. However, unlike rs2273650, there is very limited evidence to indicate the functional significance of rs8904, and it is probably only a surrogate of some underlying SNP that has a functional effect. Functional analyses have shown th...
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Author Contributions

Tang H. and Liu H. conceived and designed the study. Cheng Z. and Ma W. carried out candidate SNPs selection and statistical analyses. Tang H., Ma W., Liu Y. and Tong Z. conducted subject screening. Tang H., Ma W., Tong Z., and Sun R. contributed to the collection and preparation of control DNA samples. Tang H. wrote the paper.

Additional Information

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