Effects of receptor activator nuclear factor κB gene polymorphisms on the susceptibility to knee osteoarthritis

A case–control study

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Abstract

The present study aimed to explore genetic association of receptor activator nuclear factor κB (RANK) polymorphisms with individual susceptibility to knee osteoarthritis (OA) in different Kellgren–Lawrence (KL) grades.

This case–control study included 138 knee OA patients and 145 healthy individuals. RANK rs1805034 and rs8086340 polymorphisms were genotyped through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The effects of RANK polymorphisms on knee OA risk were analyzed via χ² test or Fisher exact test, and the results were expressed using odds ratios (ORs) with corresponding 95% confidence intervals (CIs).

The C allele of rs1805034 polymorphism had significantly higher frequency in knee OA patients than in controls (P = .044), indicating that this allele could increase the risk of knee OA (OR = 1.424, 95% CI = 1.010–2.008). Besides, the CC genotype and C allele of the rs1805034 polymorphism were significantly associated with elevated risk of knee OA in moderate grade (CC vs TT: P = .018, OR = 3.071, 95% CI = 1.187–7.941; C vs T: P = .012, OR = 1.787, 95% CI = 1.131–2.823). However, rs8086340 polymorphism had no significant association with knee OA risk.

The C allele of RANK rs1805034 polymorphism is closely correlated with increased risk of knee OA, especially for moderate grade.

Abbreviations: BMI = body mass index, CI = confidence interval, HWE = Hardy–Weinberg equilibrium, IRB = institutional review board, KL = Kellgren–Lawrence, MRI = magnetic resonance imaging, OA = osteoarthritis, OPG = osteoprotegerin, OR = odds ratio, RA = rheumatoid arthritis, RANK = receptor activator nuclear factor κB, RANKL = RANK-ligand, SNP = single nucleotide polymorphism.

Keywords: classification, knee osteoarthritis, polymorphisms, RANK

1. Introduction

Osteoarthritis (OA) is one of common chronic degenerative joint diseases, prevalent in middle, and older people.[1] Joint pain and stiffness are main symptoms of this disease.[2,3] In early stage, OA symptoms may present only during exercise; with its progression, the disease will show constant symptoms. OA reduces the life quality of the patients, representing a main cause of disability in the elderly.[4] In recent years, OA sees an increasing morbidity rate worldwide.[5] Thus, it is necessary to explore the etiology for this disorder. A variety of plausible factors have been confirmed to be involved in this disease.[6–9] The interactions between these factors play a crucial role in the initiation and development of OA.[10,11] However, not all individuals exposing to similar risk factors will eventually suffer from OA, and genetic factors would be responsible for tremendous differences in individual susceptibility. It is well known that bone lesions are main pathological change in OA.[12] Thus, we hypothesized that genes associated with bone metabolism might affect OA risk.

Receptor activator nuclear factor κB (RANK), also known as TNF receptor superfamily member 11a (TNFRSF11A), is involved in RANK—RANK-ligand (RANKL) —osteoprotegerin (OPG) signaling pathway which participates in the regulation of bone metabolism, including the development and maturity of osteoclasts.[13,14] RANK is a type I transmembrane protein, usually abounding in osteoclasts and cartilage cells.[15,16] It has been reported that RANK was highly expressed in OA patients at advanced stages, especially in cartilage cells.[17] Human RANK gene is located at chromosome 18q21.33, and its genetic mutations may alter the protein functions.

Gene polymorphisms refer to the existence of 2 or more discontinuous variants, genotypes or alleles in biological genomes. Single nucleotide polymorphism (SNP) is a common type of gene polymorphisms and is abundant in human genome. Some SNPs have the capability to influence the gene functions, thus resulting in changes in biological traits, even diseases. Genetic association of SNPs with individual susceptibility to diseases has become a research hot in recent years. According to the 1000 genomes projects, minor allele frequencies of RANK
gene polymorphisms rs1805034 and rs8086340 were more than 0.1 in Chinese Han in Beijing (CHB) population. Besides, rs1805034 and rs8086340 SNPs have already been explored in rheumatoid arthritis (RA).\textsuperscript{19,20} The aim of the present study was to explore the associations of RANK gene rs1805034 and rs8086340 SNPs with OA risk in a Chinese Han population.

2. Materials and methods

2.1. Participants

This case-control study was approved by the institutional review board (IRB) of Affiliated Zhongshan Hospital of Dalian University (IRB number: DLU124-201411). Written informed consent was signed by every participant. Study process conformed to the Declaration of Helsinki.

The participants were recruited from Affiliated Zhongshan Hospital of Dalian University between January 2015 and December 2016. Knee OA patients were diagnosed via physical examinations and magnetic resonance imaging (MRI), according to corresponding criteria.\textsuperscript{20} The patients with Kellgren–Lawrence (KL) score ≥2 were included in the case group.\textsuperscript{21} KL classification was performed by experienced radiologist through MRI examinations. Healthy individuals had no signs of knee OA or the histories of other arthritis or joint diseases. Age and gender distributions in the controls were matched with those in the cases. Besides, individuals with inflammatory arthritis, severe trauma of knee joint or skeletal dysplasia were excluded from this study. Participants lacking essential data were also excluded from this study. During sample collection period, only 138 knee OA patients and 145 healthy controls met the included criteria. The statistical power based on these included subjects was 0.49, less than 0.7, and this condition might reduce the accountability of our results.

2.2. Sample collection and genotyping method

After 12 hours of fasting, peripheral blood was collected from the elbow vein of each participant and put into EDTA tub. Genomic DNA was extracted from the blood samples using TIANamp Blood DNA Kit (TIANGEN, Beijing), and the procedures were carried out according to the introduction of the manufacturer. RANK gene rs1805034 SNP was amplified via polymerase chain reaction (PCR) and digested by restriction enzyme, according to the steps in previous study.\textsuperscript{22} Primer sequences for RANK gene rs8086340 SNP was designed by Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR reaction was performed in a system of 2.5 μL, including 2μL template DNA, 2.5 μL 10× buffer, 0.3 μL DNA Taq polymerase, 0.5 μL dNTP, each 0.7 μL of forward and reverse primers, and ddH₂O. PCR reaction was carried out abiding by the following procedures: 94°C initial denaturation for 10 minutes; 40 cycles of 94°C denaturation for 50 seconds, 61°C annealing for 40 seconds and 72°C extension for 1 minute; and 72°C final extension for 10 minutes. Then these PCR products were digested using restriction enzymes at 37°C overnight (Table 1).

2.3. Statistical analysis

PASW18.0 was used for data calculations. Significant level was set at 0.05 (2 side). Continuous variables were compared between OA patients and controls adopting t test or Mann–Whitney U test. Genotype distributions of our studied polymorphisms both in case and control groups were examined for their conformity to Hardy–Weinberg equilibrium (HWE). Genotype and allele frequencies were obtained via direct counting. Association of RANK gene SNPs with knee OA susceptibility was assessed through χ² test or Fisher exact test. The effects of RANK SNPs on the susceptibility to knee OA in different KL grades were also detected employing χ² test or Fisher exact test. Association strength was appraised applying odds ratios (ORs) with 95% confidence intervals (CIs). Furthermore, the results were adjusted for age, gender, body mass index (BMI), smoking, and drinking using logistic regression analysis.

3. Results

3.1. Characteristics of the participants

Knee OA patients included 57 males and 81 females with a mean age of 53.80 ± 11.46 years. While the control group was constituted by 60 males and 85 females, and their mean age was 56.58 ± 11.56 years. No significant difference existed in age

| Table 1 | Primer sequences of RANK SNPs. |
|---|---|
| SNP | Position | Primer sequences | Annealing temperature, °C | Fragments length, bp | Restriction enzyme |
| rs1805034 | Exon6 | F: 5′-TGTGTCTTCTCTCGTGGS-3′ | 61 | 323 | Sall |
| | | R: 5′-AGGAATCTAAACACGCTGAC-3′ | | | |
| rs8086340 | Intron1 | F: 5′-AGTGAAGATGCCCGACAGTGTGATG-3′ | 61 | 210 | TaqI |
| | | R: 5′-ACGTGTCGGCGGAAAGGCT-3′ | | | |

RANK = receptor activator nuclear factor κB, SNP = single nucleotide polymorphism.

| Table 2 | Basic characteristics of participants. |
|---|---|
| Characteristics | Knee OA n =138, % | Control n =145, % | P |
| Age | 55.80 ± 11.46 | 56.58 ± 11.56 | .667 |
| Gender | | | |
| male | 57 (41.30) | 60 (41.38) | .990 |
| female | 81 (60.70) | 85 (58.62) | |
| BMI, Kg/m² | 24.93 ± 4.28 | 23.67 ± 4.55 | .072 |
| Smoking | | | |
| no | 97 (70.29) | 105 (72.41) | .693 |
| yes | 41 (29.71) | 40 (27.59) | |
| Drinking | | | |
| no | 98 (71.01) | 108 (74.48) | .512 |
| yes | 40 (28.99) | 37 (25.52) | |
| KL classification | | | |
| Mild (K-L score =2) | 55 (39.86) | – | |
| Moderate (K-L score =3) | 52 (37.68) | – | |
| Severe (K-L score =4) | 31 (22.46) | – | |

BMI = body mass index, KL = Kellgren–Lawrence, OA = osteoarthritis.
or gender distribution between case and control groups (Table 2, \( P > .05 \)), suggesting that the cases and controls were well matched. Smokers and drinkers were more common in case group than in control group, though the difference had no significant difference. According to disease severity, the patients were divided into mild (KL score=2), moderate (KL score=3), and severe (KL score=4) subgroups and the number of the patients in these 3 subgroups were 55 (39.86%), 52 (37.68%), and 46.21%, 42.76%, 11.03% in control group.

### 3.2. Association of RANK SNPs with knee OA susceptibility

Genotype and allele distributions of RANK rs1805034 and rs8086340 SNPs accorded with HWE in both case and control groups (Table 3, \( P > .05 \)), indicating that the participants could represent general population.

The frequencies of the TT, TC, and CC genotypes of the polymorphism rs1805034 were 35.51%, 47.83%, 16.67% in case group, and 47.83%, 39.31%, 13.89% in control group. Although the TC and CC genotypes had higher frequencies in OA patients than in healthy individuals, the differences had no statistical significance (Table 3, \( P > .05 \)). After adjustment for age, gender, BMI, smoking, and drinking, rs1805034 polymorphism showed no significant association with knee OA susceptibility either. Meanwhile, the C allele of the polymorphism rs1805034 was more frequent in OA patients than in controls (\( P = .044 \)), indicating that the C allele was associated with increased susceptibility to knee OA (OR=1.424, 95% CI=1.010–2.008).

The GC and CC genotypes of the rs8086340 SNP had higher frequencies in OA patients. Besides the C allele of this polymorphism was more frequent in cases than in controls. However, we failed to find any significant association between rs8086340 and OA susceptibility (\( P > .05 \)).

### 3.3. Association of RANK SNPs with knee OA susceptibility in different KL grades

Genotype distributions of RANK rs1805034 and rs8086340 SNPs in OA cases of different grades are shown in Table 4. We evaluated the association of RANK SNPs with OA risk in different grades. RANK rs1805034 SNP was slightly correlated with increased risk of knee OA at both mild and severe grades. The CC genotype and C allele of the rs1805034 SNP were significantly correlated with increased susceptibility to moderate OA (CC vs TT; \( P = .018 \), OR=3.071, 95% CI=1.187–7.941; C vs T; \( P = .012 \), OR=1.787, 95% CI=1.131–2.823). Additionally, the polymorphism rs8086340 had no distinct association with knee OA risk at any grades (Table 5).

### 4. Discussion

Growing evidence have demonstrated that RANK/RANKL/OPG signaling pathway might be involved in OA development.[23–25] Polymorphisms in RANK gene might alter the signaling way, thus contributing to disorders related to bone metabolism. In this case-control study, we investigated the genetic association of RANK rs1805034 and rs8086340 polymorphisms with individual susceptibility to knee OA. We found that the C allele of rs1805034 polymorphism might increase the disease risk, especially for moderate grade.

In the present study, the frequency of the C allele of the polymorphism rs1805034 was significantly higher in knee OA patients than in controls, increasing the disease risk by 1.424

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**Table 3**

Association of RANK SNPs with knee OA susceptibility.

| Genotype/allele | OA n=138, % | Control n=145, % | \( P \) | OR (95% CI) | \( P \) | OR (95% CI) |
|-----------------|------------|-----------------|-------|-------------|-------|-------------|
| rs1805034       |            |                 |       |             |       |             |
| TT              | 49 (35.51) | 67 (46.21)      | –     | –           | –     | –           |
| TC              | 66 (47.83) | 62 (42.76)      | .145  | 1.456 (0.878–2.414) | .875  | 1.056 (0.538–2.072) |
| CC              | 23 (16.67) | 16 (11.03)      | .070  | 1.066 (0.941–4.106) | .223  | 1.821 (0.694–4.781) |
| T               | 164 (59.42)| 196 (67.59)     | –     | –           | –     | –           |
| C               | 112 (40.58)| 94 (32.41)      | .044  | 1.424 (1.010–2.008) | .685  | 1.217 (0.471–3.146) |
| \( P_{\text{HWE}} \) | .923       | .772            |       |             |       |             |

**Table 4**

Genotype distributions of RANK SNPs in different KL grades of OA.

| Genotypes/alleles | Mild | Moderate | Severe |
|-------------------|------|----------|--------|
| rs1805034          |      |          |        |
| TT                | 24 (43.64)| 15 (28.84)| 10 (32.26)|
| TC                | 24 (43.64)| 26 (50.00)| 16 (51.61)|
| CC                | 24 (43.64)| 26 (50.00)| 16 (51.61)|
| T                 | 7 (12.72) | 11 (21.15)| 5 (16.13)|
| C                 | 38 (64.55)| 48 (86.15)| 36 (58.00)|
| \( P_{\text{HWE}} \) | .543 | .969      |        |

**Table 5**

Knee OA patients

| Genotypes/alleles | Mild | Moderate | Severe |
|-------------------|------|----------|--------|
| rs8086340          |      |          |        |
| GG                | 47 (34.06)| 57 (39.31)|        |
| GC                | 70 (50.72)| 68 (46.90)| 14 (47.45)|
| CC                | 21 (15.22)| 20 (13.79)| 4 (12.90)|
| G                 | 164 (59.42)| 164 (59.42)|        |
| C                 | 112 (40.58)| 108 (37.24)| 38 (61.52)|
| \( P_{\text{HWE}} \) | .923 | .969      |        |
times. Gianfrancesco et al found that the C allele was positively correlated with the severity of Paget’s disease of bone (PDB).\[^{1,2}\] Meanwhile, the CC genotype of the rs1805034 SNP might act as a risk factor for osteoporosis in women with RA.\[^{1,19}\] However, Assmann and co-workers reported that the rs1805034 polymorphism had no significant association with psoriatic arthritis.\[^{1,24}\] Besides, they observed no significant correlation between this SNP and RA either.\[^{1,27}\] These studies could testify our results indirectly.

The GC and CC genotypes of the rs8086340 SNP exhibited higher frequencies in case group than in control group, but these differences were not significant, so this polymorphism had no significant association with knee OA risk. While previous study demonstrated that the rs8086340 SNP was closely related to the presence of anticitrullinated peptide antibodies (ACPA) but not erosion in RA patients.\[^{1,19}\]

Knee OA patients in our research were divided into 3 grades according to their severity, so we further analyzed the association of our interested polymorphisms with the disease in different grade groups. However, no significant association was discovered between the rs1805034 SNP and OA risk in either mild or severe grade. Whereas, the CC genotype of the rs1805034 SNP had significantly higher frequency in moderate OA patients than in controls, indicating a promoting effect of the CC genotype on knee OA. Meanwhile, the C allele of the polymorphism rs1805034 was also more frequent in moderate OA, demonstrating its enhancing effect on moderate OA. However, we failed to find any significant association between the rs8086340 SNP and knee OA susceptibility in either mild moderate or severe grade group. This was the first study on the relationship of RANK polymorphisms with knee OA severity in Chinese Han population.

Limitations in the present study should be noticed. First of all, the sample size was not large enough, so the statistical power was restricted, particularly in stratified analysis. However, our study adopted strict sample collection, experimental procedures, and scientific statistical analyses, so our results had certain representativeness. Second, study participants from only 1 region could not represent general Chinese Han population. Third, OA is a complex disease affected by the interactions between genetic and environmental factors. However, such interactions were not considered in this study. Besides, confounding factors were not adjusted either, which might influence the accuracy of our findings. Finally, causal relationship between RANK polymorphisms and OA risk was not certified in the present study. Therefore, well-designed studies with larger sample size based on multiple centers should be performed in the future. In vivo and in vitro researches should be designed to explore functional roles of RANK gene in OA etiology and potentially related molecular mechanisms. Besides, polymorphism distributions vary dramatically between different populations and the association of RANK polymorphisms with OA risk observed in this study might be not applicable in other population.

In conclusion, the C allele of rs1805034 polymorphism may be a potential risk factor for knee OA, especially for moderate grade. These results may help us to understand the mechanism of knee OA.

### Author contributions

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