The **BDKRB2** +9/-9 Polymorphisms Influence Pro-Inflammatory Cytokine Levels in Knee Osteoarthritis by Altering TLR-2 Expression: Clinical and *in Vitro* Studies

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**Key Words**
Bradykinin B2 receptor (BDKRB2) • Gene polymorphism • Toll-like receptor (TLR)-2 • Osteoarthritis (OA) • Synoviocytes

**Abstract**
*Background/Aims:* The bradykinin B2 receptor (BDKRB2) +9/-9 gene polymorphisms have been shown to be associated with the susceptibility and severity of osteoarthritis (OA); however, the underlying mechanisms are unclear. In this study, we investigated the correlation between the *BDKRB2* +9/-9 polymorphisms and pro-inflammatory cytokine levels in OA and the molecular mechanisms involved. *Methods:* A total of 156 patients with primary knee OA and 121 healthy controls were enrolled. The *BDKRB2* +9/-9 polymorphisms were genotyped. The tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 levels were determined using Enzyme-linked immunosorbent assay (ELISA). The toll-like receptor (TLR)-2 and TLR-4 mRNA levels were determined by quantitative real-time PCR. The basal and bradykinin-stimulated pro-inflammatory cytokine secretion in human OA synoviocytes and the involvement of TLR-2 and mitogen-activated protein kinases (MAPKs) were investigated. *Results:* The presence of -9 bp genotype is associated with higher TNF-α, IL-6, and IL-8 levels and higher TLR-2 expression in OA patients. The basal and bradykinin-induced TLR-2 expressions in human OA synoviocytes were significantly reduced by specific inhibitors of p38, JNK1/2, and ERK1/2. Both the B2 receptor antagonist MEN16132 and TLR-2 silencing inhibited IL-6 and IL-8 secretion in human OA synoviocytes. *Conclusion:* The data suggested that the *BDKRB2* +9/-9 polymorphisms influence pro-inflammatory cytokine levels in knee osteoarthritis by altering TLR-2 expression.
Introduction

Osteoarthritis (OA) is a primary joint disease that causes deformity and joint disability in millions of patients worldwide [1]. The main features of OA are degeneration and loss of articular cartilage, but all the joint components, including the bone, synovium, menisci, and ligaments are affected [2]. Although the pathogenesis of OA is not fully understood, current knowledge indicates that synovial inflammation, which generates inflammatory mediators implicated in the OA pain and catabolic cartilage degradation, plays a substantial role in the OA process [3]. These inflammatory mediators include inflammatory cytokines, metalloproteinases, prostaglandin E2, and nitric oxide [4-6]. Interleukin (IL)-1β and tumor necrosis factor (TNF)-α contribute to the degeneration of articular cartilage. IL-1β is widely used as a chondrocyte apoptosis-inducing agent [7], and accelerate catabolism of rat chondrocytes and cartilage explants induced by Osthole [8]. Other cytokines including IL-6 and IL-8 have also been implicated in OA [9]. Additionally, inflammatory pathways mediated by Toll-like receptor (TLR)-2 and TLR-4 may play a central role in OA pathophysiology [10]. TLRs are innate pattern-recognition receptors that are activated by not only pathogens but also endogenous damage-associated molecular patterns (DAMPs). Activation of these receptors leads to production of inflammatory cytokines through nuclear factor (NF)-κB pathway [11]. In OA joints, TLRs are likely activated by DAMPs derived from damaged extracellular matrices of articular joint tissues [10]. TLR-2 and TLR-4 are upregulated in lesion areas of OA cartilage [12]. Many proteases and cytokines that promote cartilage catabolism are dependent on NFκB, which is central to all TLR signaling [13]. NFκB knockdown minimizes synovitis and cartilage damage of OA in rats [13, 14]. Collectively, these results highlight the involvement of TLR-mediated innate immune response in the OA process.

Evidence also indicates that bradykinin and B₂-bradykinin receptors (BDKRB2, Class I G protein-coupled receptors) are involved in the pathophysiology of OA [15]. Bradykinin, a vasodilator and inflammatory nonapeptide, has been detected in the synovial fluid of OA patients and its levels correlate with the degree of synovitis [16, 17]. BDKRB2 is present in human synovial tissues and in cultured human synoviocytes [18-20]. Long-term bradykinin stimulation of human synoviocytes leads to increased release of IL-6 and IL-8 [21]. Administration of a specific B2 receptor antagonist has been reported to reduce inflammatory pain in rats [22]. These findings support the therapeutic potential of BDKRB2 blockade in OA treatment.

Our previously research reported that BDKRB2 +9/-9 polymorphisms are associated with the susceptibility and severity of OA [23]; however, the association between BDKRB2 +9/-9 polymorphisms and inflammatory processes in OA remains unclear. In this study, we investigated the relationship between BDKRB2 +9/-9 polymorphisms and pro-inflammatory cytokine levels in patients with primary knee OA. Additionally, we studied the molecular mechanisms involved using isolated human OA synoviocytes. We found that TNF-α, IL-6 and IL-8 levels were associated with BDKRB2 +9/-9 polymorphisms in these OA patients. Our in vitro studies indicated that these effects of BDKRB2 +9/-9 polymorphisms were mediated by p38, JNK1/2, and ERK1/2-dependent alterations in TLR-2 expression.

Materials and Methods

Patients

A total of 156 patients diagnosed with primary OA of the knee based upon the American College of Rheumatology criteria [24] were enrolled in this study. The severity of OA was classified with the Kellgren-Lawrence (K&L) grade. A total of 121 healthy volunteers were enrolled as controls. Demographic data were collected through subject interviews. Data on established risk factors of OA were obtained from medical records and subject interviews. OA cases caused by inflammatory arthritis (rheumatoid, polyarthritic, or autoimmune disease), post-traumatic or post-septic arthritis, skeletal dysplasia or developmental dysplasia were excluded. All control subjects had no prior history of arthritis or symptoms of joint diseases (pain,
swelling, tenderness or restriction of movement). The clinical characteristics of all study subjects including age, sex, body mass index (BMI), smoking status, knee activity, and regular exercise was recorded in Table 1. Obesity was defined as BMI >30 kg/m$^2$. This study was approved by the Ethics Review Committee of Tangdu Hospital. All participants gave written informed consent. The blood and synovial fluid samples were collected and immediately centrifuged at 3,000 rpm for 10 min. The supernatants were stored at -70°C until analysis.

**BDKRB2 genotyping**

Genomic DNA was extracted from whole blood using TIANamp Blood DNA Kit (TianGen Biotech, Beijing, China) following manufacturer’s instructions. The samples were genotyped for the BDKRB2 +9/-9 polymorphisms within exon 1 of the BDKRB2 gene using a polymerase chain reaction (PCR) as previously described [23]. Briefly, the 80 bp (-9) and/or 89 bp (+9) fragments of the gene were amplified by PCR using the forward primer 5'-TCCAGCTCTGGCTTCTGG-3' and the reverse primer 5'-AGTCGCTCCCTGGTACTGC-3'. The amplified DNA fragments were separated by 8% polyacrylamide gel electrophoresis and visualized with ethidium bromide and ultraviolet light.

**Isolation, culture, and treatment of human OA synoviocytes**

Human OA synovial membranes were collected from OA patients (15 females, 5 males, 70 ± 5 years of age) who have undergone total knee joint replacement surgery. Synoviocytes were isolated and cultured as described in previous reports [25]. Briefly, synovial tissues were finely minced and digested with collagenase type 1A (Sigma-Aldrich, St Louis, MO, USA) at 37°C in 5% CO$_2$ atmosphere for 16 h. The digested tissue was filtered through a 70-mm nylon mesh and washed, and then cells were collected after centrifugation and cultured in Dulbecco’s minimum essential medium (DMEM)/HAM F12 (Sigma-Aldrich) containing penicillin (100 U/ml) and streptomycin (100 mg/ml), supplemented with 10% fetal bovine serum (Sigma-Aldrich) in 5% CO$_2$ at 37°C. All experiments were performed using cells in the third passage. Non-transfected cells or siRNA-transfected cells were grown to near confluence and incubated with or without bradykinin (1 μM) for 24 h in the presence or absence of the specific B2 receptor antagonist MEN16132 (1 μM, Sigma-Aldrich) or icatibant (1 μM, Sigma-Aldrich), or the specific p38 inhibitor SB203580 (10 μM, Sigma-Aldrich), the specific JNK1/2 inhibitor SP600125 (30 μM, Sigma-Aldrich), or the specific ERK1/2 inhibitor PD98059 (30 μM, Sigma-Aldrich). The inhibitor concentrations in this study were selected according to the previous reports [21, 26]. In experiments using transfected cells, cells were incubated with a final concentration of 100 nM TLR-2 siRNA or scrambled siRNA (Scr. siRNA) for 1 h prior to bradykinin treatment. TLR-2 siRNA and scrambled siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Enzyme-linked immunosorbent assay (ELISA)**

IL-1β, TNF-α, IL-6, and IL-8 levels in the serum and synovial fluid of study subjects and the culture medium of human OA synoviocytes were determined using ELISA kits from R&D Systems (Minneapolis, MN, USA) following manufacturer’s instructions. Absorbance at 450 nm was recorded on a microplate.

### Table 1. Demographic and clinical characteristics of all subjects in the study. OA = osteoarthritis; KL = Kallgren-Lawrence; ns = not significant; * = statistically significant (vs. Control)

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<th>Variables</th>
<th>Cases (n=156)</th>
<th>Control (n=121)</th>
<th>P</th>
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<td>Female, n (%)</td>
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<td>67 (55.4%)</td>
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<td>36 (29.8%)</td>
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<tr>
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<td>16 (13.2%)</td>
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Quantitative real-time PCR

Total RNA was extracted from synovial tissues using TRIzol reagent from Invitrogen (Grand Island, NY, USA). Then, equal amounts (5μl) of total RNA were reverse-transcribed and cDNA was synthesized using the SuperScript III cDNA synthesis kit from Invitrogen. PCR reactions were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Bio-Rad Laboratories, Richmond, CA, USA) following manufacturer’s instructions. Housekeeping genes GAPDH and HPRT1 were used as internal controls. All primer sequences are presented in Table 2. The specific primers were diluted to a final concentration of 1μM in final volume of 10 μl in the PCR amplification reaction: 5 μl of 2 x SYBR Green PCR master mix, 0.2 μl of each specific forward and reverse primer, 3.6 μl of DNase-free water and 1 μl of cDNA template. After an initial denaturing at 96°C for 10 min, the amplifications were performed with 40 cycles of 95°C for 5 s and 60°C for 20 s. The relative expression levels of TLR-2 and TLR-4 mRNA were calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis

Cell lysates were centrifuged for 10 min at 14,000 × g at 4°C. The supernatants were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking in 1% BSA in TBS (20 mM Tris, 150 mM NaCl, pH 7.5) for 1 h at room temperature, the membranes were incubated with primary antibody for TLR-2 (1:200), p-p38, p-38 (1:250), p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2 (1:500) (Santa Cruz Biotechnology) for 2 h at room temperature. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and detected using ECL Plus Western Blot Detection Reagents (Amersham, USA). The membranes were stripped and reprobed with mouse anti-GAPDH antibody (Santa Cruz Biotechnology) to confirm equal loading and the integrity of the samples.

Statistical analyses

Data analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 19.0, SPSS Inc, Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). Each experiment was performed in duplicate. Differences between groups were interpreted using the Student t test or analysis of variance (ANOVA) followed by Bonferroni post hoc test in case of significant F value. Differences with a P value < 0.05 were considered statistically significant.

Results

Pro-inflammatory cytokine levels in the serum and synovial fluid of OA patients are significantly associated with the BDKRB2 +9/-9 polymorphisms

Our ELISA results showed that these four pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-8, were present at significantly higher levels in both the serum and synovial fluid of OA patients compared with healthy controls (Fig. 1). We subsequently analyzed the relationship between levels of these cytokines and the BDKRB2 +9/-9 polymorphisms in OA patients and healthy controls. In OA patients, significantly higher TNF-α and IL-8 levels were...
detected in the serum and synovial fluid of the BDKRB2 +9/-9 genotype carriers compared with the +9/+9 carriers (Fig. 2A, 2E, 2D, 2H). Additionally, IL-6 levels were significantly higher in the serum and synovial fluid of the BDKRB2 -9/-9 and +9/-9 genotype carriers compared with the +9/+9 carriers (Fig. 2C, 2G). However, IL-1β levels were not significantly different between the three genotypes in OA patients. In healthy controls, no significant differences in any of these four cytokines were detected in the serum or synovial fluid between the three genotypes (Fig. 2A-H).

**TLR-2 expression in OA synovial tissues is significantly associated with the BDKRB2 +9/-9 polymorphisms**

Our data showed that TLR-2 and TLR-4 were significantly upregulated in OA synovial tissues compared with healthy controls (Fig. 3A, P < 0.01). To explore whether alterations in TLR-2 and/or TLR-4 signaling contribute to cytokine level changes induced by BDKRB2 +9/-9 polymorphisms, we investigated the relationship between synovial mRNA levels of TLR-2 and TLR-4 and the BDKRB2 +9/-9 polymorphisms in OA patients and healthy controls.
In OA patients, significantly higher TLR-2 mRNA levels were detected in the +9/-9 and -9/-9 carriers compared with the +9/+9 carriers (Fig. 3B, \( P < 0.01 \)), while no significant differences in TLR-4 were found between the three genotypes (Fig. 3C, \( P > 0.05 \)). In healthy controls, no significant differences in TLR-2 or TLR-4 were detected between the three genotypes (Fig. 3B, 3C). These data suggested that alterations in TLR-2 signaling might be responsible for cytokine level changes induced by BDKRB2 +9/-9 polymorphism in OA patients.

**BDKRB2 upregulates TLR-2 expression in human OA synoviocytes via MAPK pathways**

The significant association between TLR-2 expression in OA synovial tissues and the BDKRB2 +9/-9 polymorphisms suggested that BDKRB2 regulates TLR-2 expression in OA.
Consistent with this hypothesis, incubation of human OA synoviocytes with bradykinin (1 μM, 24 h) dramatically increased TLR-2 mRNA and protein expression in these cells, and these effects of bradykinin were blocked by the specific B2 receptor antagonists MEN16132 and icatibant (Fig. 4A, 4B). Interestingly, MEN16132 and icatibant also inhibited basal TLR-2 expression in human OA synoviocytes (Fig. 4A, 4B). To investigate the molecular mechanisms involved in BDKRB2-mediated TLR-2 expression, we examined the possible role of mitogen-activated protein kinases (MAPKs), kinases known to be activated by bradykinin [27]. Incubation with MEN16132 or icatibant inhibited both basal and bradykinin-stimulated phosphorylation of p38, JNK1/2, and ERK1/2 in human OA synoviocytes (Fig. 4C), indicating BDKRB2-mediated functional activation of these MAPKs in these cells. Cell treatment with the specific p38 inhibitor SB202190, the specific JNK1/2 inhibitor SP600125, or the specific ERK1/2 inhibitor PD98059 significantly reduced both the basal and bradykinin-induced mRNA and protein expression of TLR-2 (Fig. 4D, 4E), indicating that BDKRB2 upregulates TLR-2 expression in human OA synoviocytes through the p38, JNK1/2, and ERK1/2 pathways.

**BDKRB2 upregulates pro-inflammatory cytokines IL-6 and IL-8 secretion in human OA synoviocytes via TLR-2**

It has been reported that the 9 bp deletion (-9) genotype of BDKRB2 is associated with higher receptor expression [28], therefore, enhanced BDKRB2 signaling is likely responsible for the higher pro-inflammatory cytokine levels in the BDKRB2 (-9) carriers in OA patients. Consistent with this hypothesis, incubation of human OA synoviocytes with bradykinin led to significantly increased IL-6 and IL-8 secretion, and these effects of bradykinin were blocked by the specific B2 receptor antagonist MEN16132 (Fig. 5B, 5C). MEN16132 also inhibited basal IL-6 and IL-8 secretion in these cells (Fig. 5B, 5C), indicating the role of B2 receptor in
basal cytokine secretion. Similar to MEN16132, TLR-2 silencing in human OA synoviocytes by siRNA transfection significantly inhibited both basal and bradykinin-stimulated IL-6 and IL-8 secretion (Fig. 5B, 5C), suggesting that BDKRB2-mediated signaling upregulates these two pro-inflammatory cytokines in human OA synoviocytes via the TLR-2 pathway. However, TNF-α secretion was not affected by bradykinin, MEN16132, or TLR-2 silencing (Fig. 5A).

**Discussion**

Since the **BDKRB2** +9/-9 polymorphisms and synovial inflammation plays a significant role in OA development, we investigated the relationship between the **BDKRB2** +9/-9 polymorphisms and pro-inflammatory cytokine levels in OA patients and healthy controls. We found that, among OA patients, the -9/-9 genotype carriers had significantly higher serum and synovial fluid levels of TNF-α, IL-6, and IL-8 compared with the +9/+9 carriers. Besides, the +9/-9 carriers had significantly higher IL-6 levels than the +9/+9 carriers. In subsequent studies, we found that the **BDKRB2** +9/-9 polymorphisms were also significantly associated
with TLR-2 expression in OA synovial tissues. In human OA synoviocytes, BDKRB2 regulates both basal and bradykinin-stimulated TLR-2 expression via MAPKs including p38, JNK1/2, and ERK12. Blocking B2 receptor with MEN16132 or TLR-2 silencing by siRNA both inhibited basal and bradykinin-stimulated IL-6 and IL-8 secretion in human OA synoviocytes. Collectively, these results indicated that BDKRB2 upregulates pro-inflammatory cytokine levels in OA by inducing TLR-2 expression. Therefore, increased cytokine levels associated with the -9 genotype are likely responsible for the increased susceptibility and severity of OA in -9 carriers.

Bradykinin and its receptors participate in many acute and chronic inflammatory conditions. B2 receptor antagonists have been synthesized to treat diseases such as rheumatoid arthritis, inflammatory bowel diseases, inflammatory skin disorders, asthma, allergy, pain, and coronary heart diseases [29]. The two main actions of bradykinin in OA are its algesiogenic effect via activating the nociceptors and its inflammatory effect that promotes synovitis, both mediated by BDKRB2 [15]. Intra-articular administration of the specific B2 receptor antagonist icatibant produces an analgesic effect in OA patients [30], supporting the therapeutic potential of B2 receptor blockade in OA treatment. B2 receptor is a G protein-coupled receptor that activates the phospholipase C (PLC) and MAPK pathways. In human synoviocytes, bradykinin stimulates IL-6 and IL-8 secretion via pathways involving the activation of PLC, p38, JNK, ERK1/2 MAPKs, and NFκB [21]; however, how bradykinin, by binding to B2 receptor, activates NFκB in human synoviocytes is unclear. Considering that NFκB is central to all TLR pathways, we speculated that alterations in TLR-2 and/or TLR-4 signaling might contribute to cytokine level changes induced by BDKRB2 +9/-9 polymorphisms. Previous studies reported that bradykinin up-regulates the expression of TLR-2 and TLR-4 and promote inflammatory processes in human gingival fibroblasts [31, 32]. In this study, we demonstrated that the BDKRB2 +9/-9 polymorphism mediates both basal and bradykinin-stimulated IL-6 and IL-8 secretion in human OA synoviocytes. To the best of our knowledge, this is the first report on the crosstalk between bradykinin and TLR-2 signaling in the context of pro-inflammatory cytokine production in OA.

**Fig. 5.** TLR-2 silencing inhibits basal and bradykinin-stimulated pro-inflammatory cytokine secretion in human OA synoviocytes. Human OA synoviocytes were incubated with TLR-2 siRNA or scrambled siRNA (Scr. siRNA) for 1 h in the presence or absence of bradykinin (1 μM). After 24 h, TNF-α (A), IL-6 (B), and IL-8 (C) levels in the culture medium were determined by ELISA. Samples from 6 patients were used. Each experiment was performed in duplicate. *P < 0.05, **P < 0.01 vs. control; ***P < 0.01 vs. control with bradykinin, ns = no significant differences.
Previous studies have shown that bradykinin alone can induce B2 receptor-dependent PGE(2) production and COX-2 gene expression in human synoviocytes [33]. In addition, BK can potentiate IL-1β-induced COX-2 gene expression and consequent PGE(2) production through B2 receptor and the p38, JNK, ERK1/2, and NFκB pathways. Thus, B2 receptor blockade can potentially inhibit OA progression by disrupting multiple inflammatory signals.

**Conclusion**

The data demonstrated that the *BDKRB2* +9/-9 polymorphisms influence pro-inflammatory cytokine levels in knee osteoarthritis by altering TLR2 expression. The molecular mechanisms involved are illustrated in Fig. 6. Our findings highlight the critical role of the bradykinin/*BDKRB2* and TLR-2 pathways, and their crosstalk in OA inflammation, providing further evidence that targeting these two pathways might be an effective strategy to treat OA.

**Acknowledgements**

The study was supported by the grant from the National Natural Science Foundation of China (Grant NO. 81301582), Postdoctoral Science Foundation of Jiangsu Province (Grant No. 1402003B) and China Postdoctoral Science Foundation funded project (Grant No. 2015M572812).

**Disclosure Statement**

None.

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