Genetic effects of BDKRB2 and KNG1 on deep venous thrombosis after orthopedic surgery and the potential mediator

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Deep venous thrombosis (DVT) is a common complication of orthopedic surgery. Genetic risk factors and high heritability carried a substantial risk of DVT. In this study, we aimed to investigate the potential association in the Han Chinese population between the polymorphisms of BDKRB2 and KNG1 and DVT after orthopedic surgery (DVTAOS). A total of 3,010 study subjects comprising 892 DVT cases and 2,118 controls were included in the study, and 39 single nucleotide polymorphisms (SNPs) in total (30 for BDKRB2 and 9 for KNG1) were chosen for genotyping. Two SNPs, rs710446 (OR = 1.27, \( P = 0.00016 \)) and rs2069588 (OR = 1.29, \( P = 0.00056 \)), were identified as significantly associated with DVTAOS. After adjusting for BMI, the significance of rs2069588 decreased (\( P = 0.0013 \)). Haplotype analyses showed that an LD block containing rs2069588 significantly correlated with the DVTAOS risk. Moreover, bioinformatics analysis indicated that hsa-miR-758-5p and BDKRB2 formed miRNA/SNP target duplexes if the rs2069588 allele was in the T form, suggesting that rs2069588 may alter BDKRB2 expression by affecting hsa-miR-758-5p/single-nucleotide polymorphism target duplexes. Our results demonstrate additional evidence supporting that there is an important role for the KNG1 and BDKRB2 genes in the increased susceptibility of DVTAOS.

Deep venous thrombosis (DVT) is a common disorder, in which is a blood clot occurs inside a vein; DVT has several risk factors, including acquired, inherited and mixed. Major surgery and orthopedic surgery, as acquired risk factors, carry a substantial risk of DVT. Clinically, 40% to 60% of patients acquired DVT after orthopedic surgery; 4% to 10% of those patients developed pulmonary embolism (PE), of which 5% die1. Therefore, identifying patients with a higher risk of DVT after orthopedic surgery (DVTAOS) is essential in guiding diagnosis and reducing the death rate. A growing body of literature has indicated that all strong, moderate, and weak genetic risk factors with high heritability carry a substantial risk of DVT2. Tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) are important biochemical constituents of the fibrinolytic system, which affects DVT3. This study found evidence that the polymorphisms in the fibrinolytic system might influence PAI-1 and t-PA3. With hundreds of mutations responsible for defective genes, deficiencies of antithrombin could lead to a high risk of DVT3.

A previous study has shown that Kng1-deficiency is associated with a decrease in thrombosis4. With the development of high-throughput DNA sequencing techniques, genome-wide association studies (GWASs) have provided supportive evidence for the polygenic nature of many complex diseases susceptibility3–11 and have identified some SNPs that contribute to the risk of DVT, such as Kininogen-1(KNG1)12. However, these results cannot explain the genetic portion of DVT. So far, the molecular mechanisms of DVT remain unknown. The KNG1
gene, which is found on human chromosome 3, also known as alpha-2-thiol proteinase inhibitor, encodes a high-molecular-weight kininogen (HMWK) and low-molecular-weight kininogen (LMWK). KNG1 is a constituent of the blood coagulation system, as is the HMWK protein. KNG1 is associated with the genetic factors of activated partial thromboplastin time (aPTT), which is a risk marker of DVT\(^1\). In addition, Hu had proven that variants of KNG1 genes potentiate the risk of thrombosis in ischemic stroke in the Chinese population\(^1\). The association between KNG1 and DVTAOS is largely unknown, so it is urgent to study this mechanism. In addition, the protected mechanism for the thrombosis of Bradykinin receptor B2 knockout (BKB2R\(^-/-\)) in mice via the plasma kallikrein/kinin and renin angiotensin systems also provides new genic factor insights for DVT\(^1\). The \(BDKRB2\) gene in humans encodes a G-protein coupled receptor for bradykinin called BKB2R; this receptor elicits many responses, including vasodilation, edema, smooth muscle spasm and pain fiber stimulation. The dysfunction of \(BDKRB2\) plays an essential role in some physiological and pathological processes, such as kidney development\(^6\), the inflammatory process in osteoarthritis\(^7\) and hepatocellular carcinoma progression\(^8\). BK, which is antithrombotic for endothelium binding to BKB2R in the intravascular system, promotes blood flow through NO, prostacyclin formation and tPA liberation\(^9,10\). Interestingly, BDKRB2 knockout mice are protected from thrombosis by increased nitric oxide and prostacyclin\(^10\). Considering the potential relationship between \(BDKRB2\) and thrombosis, the underlying association and mechanism between \(BDKRB2\) variants and DVT are worth investigation.

Considering the genetic heterogeneity of disease occurrence and the different etiology of DVT, the exploration of possible associations between KNG1/BDKRB2 and DVT among the Han Chinese population may shed light on the underlying mechanisms of DVT. Above all, we reasoned that alleles of KNG1 and BDKRB2 might be associated with an increased risk of DVTAOS. To test the above hypothesis, we aimed to investigate whether common variants in BDKRB2 and KNG1 have interactive effects with DVTAOS. Providing optimal thromboprophylaxis to a patient who is at DVT risk will ensure the best reductions in DVT-related morbidity and mortality from a genetic perspective via this research.

**Methods**

**Study subjects.** In the current study, we recruited 3,010 subjects undergoing knee or hip orthopedic surgery at Luoyang Orthopedic Hospital of Henan Province (Luoyang, China) from August 2012 to July 2017. Of these, 892 were diagnosed with DVTAOS and were designated as a case group; and 2,118 had no typical DVT symptoms or signs and were designated as a control group. Each subject received anticoagulants routinely within 5 hours of surgery. Two independent sonographers performed preliminary screening using lower-extremity color-Doppler ultrasound, and all subjects were assessed for DVT postoperatively within 6 days. When it was difficult to obtain a definitive diagnosis, venography was used to confirm the diagnosis. Subjects with a history of venous thromboembolism or clinical evidence of venous thromboembolism were excluded from the study. A total number of 211 study subjects were excluded for this criterion. All subjects were unrelated Han Chinese individuals. The clinical data and characteristics of all the subjects were measured or recorded and are summarized in Table 1. Both body
without BMI included as a covariate. After adjusting for BMI, the significance of rs2069588 decreased slightly to
DVTAOS. We performed a mediation analysis to examine the effect of SNP rs2069588 on DVTAOS with or
corrections were applied for multiple comparisons. Plink22 was utilized to perform the genetic association analy-
disequilibrium (LD) blocks for our genetic data and performed haplotype-based association analyses. Bonferroni
of targeted SNPs after adjusting those relevant clinical variables. In addition, we also constructed the linkage
which could serve as potential mediators, and (3) we performed mediation analysis to examine the direct effect
associated with DVTAOS status as shown in Table 1 (BMI and hyperlipidemia status) to obtain the clinical variables
hits; (2) we examined the correlations between these significant SNPs and the clinical variables which were asso-
tigated in a three-step procedure. (1) We fit simple logistic models for each SNP and screened out the significant
Results
Significant SNPs identified from simple models. Two SNPs, rs710446 (OR = 1.27, P = 0.00016) and
rs2069588 (OR = 1.29, P = 0.00056), were identified as significantly associated with DVTAOS status through
simple logistic models (Table 2). SNP rs710446 was a nonsynonymous change of KNG1, which altered the amino
acid from Ile to Thr. SNP rs2069588 was located at the 3’ untranslated region (UTR) of the gene BDKRB2.
Mediation analyses for SNP rs2069588. SNP rs2069588 was found to be significantly correlated with
BMI (P = 0.02, Supplemental Table S2). Combined with the result that BMI was strongly associated with the
disease status of DVTAOS (P = 4.93 × 10⁻¹⁰, Table 1), it might be a potential mediator between rs2069588 and
DVTAOS. We performed a mediation analysis to examine the effect of SNP rs2069588 on DVTAOS with or
without BMI included as a covariate. After adjusting for BMI, the significance of rs2069588 decreased slightly to
P = 0.0013 (Table 3). The significance of BMI also decreased from 10⁻⁹ to the 10⁻⁸ level, which was obtained in a
model with BMI and DVTAOS status only.
Haplotype-based association analyses. A total number of 10 2-SNP LD blocks were constructed for
both KNG1 and BDKRB2 (Supplemental Figure S1 and S2). One LD block, rs2069583-rs2069588 from BDKRB2,
was significantly associated with DVTAOS status (P = 0.0001, Supplemental Table S3). Since rs2069588 was also

| GENE | CHR | SNP | POS | A1 | MAF | OR | SE | L95 | U95 | STAT | P |
|------|-----|-----|-----|----|-----|----|----|-----|-----|------|---|
| KNG1 | 3   | rs710446 | 186459927 | C  | 0.27 | 1.27 | 0.06 | 1.12 | 1.43 | 3.77 | 0.00016 |
| BDKRB2 | 14 | rs2069588 | 96708667 | T  | 0.17 | 1.29 | 0.07 | 1.12 | 1.48 | 3.45 | 0.00056 |

Table 2. Genetic associations between single polymorphisms and DVT after orthopedic surgery. CHR: chromosome; POS: position; A1: tested allele; MAF: minor allele frequency; SE: standard error; L95: lower bound of 95% confidence interval; U95: upper bound of 95% confidence interval; STAT: statistics. Threshold for P values used here was 0.05/39 ≈ 0.001.

| Variables | Model1* | Model2** |
|-----------|---------|----------|
|           | OR | SE | STAT | P    | OR | SE | STAT | P |
| rs2069588 | 1.29 | 0.07 | 3.45 | 5.59 × 10⁻⁴ | 1.27 | 0.07 | 3.21 | 0.0013 |
| BMI       | —   | —   | —    | —    | 1.16 | 0.02 | 5.96 | 2.47 × 10⁻³ |

Table 3. Mediation analysis of rs2069588 and DVT with or without BMI adjusted. *Univariate model with genotypes of rs2069588 only. **Multivariate model including both genotypes of rs2069588 and BMI.

mass index (BMI) and hyperlipidemia status showed significant difference between the DVTAOS case group and
the controls. Written informed consent was obtained from all subjects. This study was performed in accordance
with the ethical guidelines of the Declaration of Helsinki (version 2002) and was approved by the Medical Ethics
Committee of Luoyang Orthopedic Hospital of Henan Province.

SNP selection & Genotyping. Tag SNPs were searched in the study. Tag SNPs of BDKRB2 and KNG1 with
minor allele frequency (MAF) ≥ = 0.1 based on 1000 genome CHB data were selected for genotyping, and the r²
criterion used for tagging was 0.6. In total, 39 SNPs (30 for BDKRB2 and 9 for KNG1) were chosen (Supplemental
Table S1). Genomic DNA was isolated from the peripheral blood using a Tiangen DNA extraction kit (Tiangen
Biotech Co. Ltd, Beijing, China) according to the manufacturer’s protocol. SNP genotyping was performed using
a Sequenom MassARRAY platform with iPLEX GOLD chemistry (Sequenom, San Diego, CA, USA) based on the
manufacturer’s protocols. The results were processed using Sequenom Typer 4.0 software21, and the genotype data
were generated from the samples. Genotyping was conducted by laboratory personnel blinded to case-control
status, and the genotyping results, data entry and statistical analyses were independently reviewed by two authors.
We randomly reperformed the analysis on 5% of the sample, with a concordance of 100%.

Statistical analyses. The genetic association between our selected tag SNPs and DVTAOS status were inves-
tigated in a three-step procedure. (1) We fit simple logistic models for each SNP and screened out the significant
hits; (2) we examined the correlations between these significant SNPs and the clinical variables which were asso-
related with DVTAOS status as shown in Table 1 (BMI and hyperlipidemia status) to obtain the clinical variables
which could serve as potential mediators, and (3) we performed mediation analysis to examine the direct effect
of targeted SNPs after adjusting those relevant clinical variables. In addition, we also constructed the linkage
disequilibrium (LD) blocks for our genetic data and performed haplotype-based association analyses. Bonferroni
corrections were applied for multiple comparisons. Plink22 was utilized to perform the genetic association analy-
ases. Haploview23 was used to make LD plots. Genotypes of SNPs were coded in the additive mode in all statistical
analyses.

Expression quantitative trait loci (eQTL) and bioinformatics analyses. We investigated the eQTL
pattern of the significant SNPs using data extracted from the GTEx database24. The potential functional signifi-
cance of the significant SNPs was examined by SIFT (for nonsynonymous SNPs)25 and the PolymiRTS Database
(Supplemental Table S4). In total, 49 SNPs in 21 candidate genes were included (Table 2). Expression and
expression quantitative trait loci (eQTL) were analyzed using data extracted from the GTEx database24. The
potential functional significance of the significant SNPs was examined by SIFT (for nonsynonymous SNPs)25 and
the PolymiRTS Database (for SNPs located at regulatory region)26.

Results
Significant SNPs identified from simple models. Two SNPs, rs710446 (OR = 1.27, P = 0.00016) and
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P = 0.0013 (Table 3). The significance of BMI also decreased from 10⁻¹⁰ to the 10⁻⁸ level, which was obtained in a
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Haplotype-based association analyses. A total number of 10 2-SNP LD blocks were constructed for
both KNG1 and BDKRB2 (Supplemental Figure S1 and S2). One LD block, rs2069583-rs2069588 from BDKRB2,
was significantly associated with DVTAOS status (P = 0.0001, Supplemental Table S3). Since rs2069588 was also
identified as significant in single marker-based analyses, the significance of the LD block of rs2069583-rs2069588 could be considered a replicate for the results of the single marker-based analyses.

**Figure 1.** eQTL pattern for rs2069588 on the gene BDKRB2 based on data extracted from the GTEx database. Threshold of the P values was 0.05/45≈0.001.

**Figure 2.** The allele of rs2069588 disrupts miRNA/SNP target duplexes. Hsa-mir-758-5p and BDKRB2 produce miRNA/SNP target duplexes if the rs3025039 allele is T.

identified as significant in single marker-based analyses, the significance of the LD block of rs2069583-rs2069588 could be considered a replicate for the results of the single marker-based analyses.

**Significant eQTL and functional significance for rs710446 and rs2069588.** Significant eQTL signals (Fig. 1) were identified for rs2069588 on BDKRB2 from tissue of the cerebellum (NES = −0.95, \( P = 3.20 \times 10^{-14} \)) and cerebellar hemisphere (NES = −0.89, \( P = 9.40 \times 10^{-11} \)). No significant eQTL signal was identified for rs710446 on KNG1. The full results of eQTL analyses are summarized in Supplemental Table S4 and S5. SIFT predicted that rs710446 had very limited functional consequences on the protein encoded by KNG1 (rated “tolerated” for all changes), although it altered one amino acid. According to the genomic database from the University of California, Santa Cruz (http://genome.ucsc.edu/), rs2069588 acts as a 3′ untranslated region (3′ UTR) variant, which may affect microRNA binding. We used a free online tool (http://bioinfo.life.hust.edu.cn) to examine the predicted target gain or loss in microRNA binding and found that the C allele of rs2069588 causes a loss of microRNA/SNP target duplexes of hsa-miR-758-5p and BDKRB2 (Fig. 2). On the other hand, T allele of rs2069588 is a stable microRNA binding site at the 3′ UTR region of BDKRB2.

**Discussion**

In this study, we investigated the genetic association between DVTAOS and two loci: KNG1 and BDKRB2. Significant evidence for both loci was found to establish their association with DVTAOS. Early studies based on European populations have provided supportive evidence for the association between DVT and rs710446. Our results on rs710446 successfully replicated these previous findings in the Han Chinese population despite focusing on DVTAOS, which is a postoperative complication of DVT. The direction of the effect and the OR in the previous study were similar to ours (OR = 1.19 to OR = 1.27). On the other hand, we identified an SNP, rs2069588, located at the 3′ UTR, conferring risk of DVTAOS based on the Han Chinese population. To the best of our knowledge, our study is the first to establish the genetic association between the gene BDKRB2 and DVTAOS.
In addition to exploring the potential genetic effects in the simple model, we also noticed some potential mediators in the effects between SNPs and DVT AoS. In our study, we statistically proved that BMI might partly mediate the effect of rs2069588 to DVT AoS. From the results of our mediation analyses, we can see that when both BMI and rs2069588 were included in the logistic model, the effect size and significance of rs2069588 were reduced. Combined with the other results, which showed that BMI was significantly associated with the disease status of DVT AoS and genotypes of rs2069588, our findings indicate that part of the effect of rs2069588 on DVT AoS was mediated through BMI. In addition, BMI cannot be a confounder because the direction of effect between BMI and rs2069588 should be from rs2069588 to BMI and was unlikely to be reversed. Interestingly, it seems that our findings on the role of the mediator of BMI were not only based on statistical analyses of our data but could be supported by previous publications. BMI and obesity have long been known as risk factors for DVT27,28. On the other hand, although no evidence has been published to establish a direct connection between BDKRB2 and obesity or BMI, BDKRB2 was reported to be significantly associated with other human metabolism-related traits, including body fat modulation29 and diabetes30. In addition, interaction effects have also been identified between the SNPs of BDKRB2 and physical activity and blood pressure39. These previous findings indicated that the effect between rs2069588 and BMI might also be mediated by some other underlying factors in our study. However, the mediation effect of BMI was not identified for rs710446.

Our eQTL analyses based on the data extracted from the GTEx database revealed some significant signals for rs2069588 in BDKRB2. However, it is unclear why these eQTL signals were identified in human brain tissues. We know that eQTLs are very common in the human genome and are far more common than disease association signals. A great many SNPs have “significant” eQTL data in GTEx simply because they represent points along an eQTL association peak. Therefore, we need to be careful to interpret the results of eQTL analyses and more experimental studies are still needed in the future.

The SNP rs2069588 of BDKRB2 was predicted to be a miR-758-5p binding site and its C allele may cause a loss of the original miR-758-5p binding site and its C allele may cause a loss of the original miR-758-5p binding site. We hypothesize that its T allele as a stable miR-758-5p binding site in the 3′ UTR region of BDKRB2, and the T allele down regulates the gene expression of BDKRB2 by binding miR-758-5p compared to the C allele. It is interesting to note that the T allele of rs2069588 is the risk allele for DVT AoS and increased the risk of DVT AoS by approximately 30% in our data. Increasing evidence indicates that bradykinin plays critical roles in coagulation and fibrinolysis15,31. Previous studies revealed that by binding to the constitutive bradykinin B2 receptor in the intravascular compartment, bradykinin promotes prostacyclin and plasmin formation and tissue plasminogen activator (t-PA) liberation30, which can inhibit thrombosis. Evidence indicated that bradykinin binding to a-thrombin would substantially reduce the fibrinogen to fibrin conversion and inhibit clot formation35. Moreover, exogenous bradykinin attenuated deep venous thrombosis via reduced tissue factor (TF) expression at the mRNA and protein level in the mouse model35. Hence, the reduction of bradykinin B2 receptor expression may result in upregulation of TF factor expression, and initiate the physiological coagulation cascade, eventually leading to DVT.

A potential limitation of this study is the potential population stratification (PS) which might cause false positive results as a confounder. As ours was a candidate gene-based study, we cannot perform some statistical methods, such as principal component analysis or genomic control, to properly control PS. However, on the other hand, in order to restrict population stratification we have applied some criteria, such as recruiting samples by hand, in order to restrict population stratification we have applied some criteria, such as recruiting samples by hand. Another potential limitation of this study is the potential population stratification which might cause false positive results as a confounder. As ours was a candidate gene-based study, we cannot perform some statistical methods, such as principal component analysis or genomic control, to properly control PS. However, on the other hand, in order to restrict population stratification we have applied some criteria, such as recruiting samples by hand.

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