A *FKBP5* mutation is associated with Paget’s disease of bone and enhances osteoclastogenesis

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Paget’s disease of bone (PDB) is a common metabolic bone disease that is characterized by aberrant focal bone remodeling, which is caused by excessive osteoclastic bone resorption followed by disorganized osteoblastic bone formation. Genetic factors are a critical determinant of PDB pathogenesis, and several susceptibility genes and loci have been reported, including *SQSTM1*, *TNFSF11A*, *TNFRSF11B*, VCP, OPTN, CSF1 and DCSTAMP. Herein, we report a case of Chinese familial PDB without mutations in known genes and identify a novel c.163G>C (p.Val55Leu) mutation in *FKBP5* (encodes FK506-binding protein 51, FKBP51) associated with PDB using whole-exome sequencing. Mutant FKBP51 enhanced the Akt phosphorylation and kinase activity in cells. A study of osteoclast function using FKBP51V55L KI transgenic mice proved that osteoclast precursors from FKBP51V55L mice were hyperresponsive to RANKL, and osteoclasts derived from FKBP51V55L mice displayed more intensive bone resorbing activity than did FKBP51WT controls. The osteoclast-specific molecules tartrate-resistant acid phosphatase, osteoclast-associated receptor and transcription factor NFATC1 were increased in bone marrow-derived monocyte/macrophage cells (BMMs) from FKBP51V55L mice during osteoclast differentiation. However, c-fos expression showed no significant difference in the wild-type and mutant groups. Akt phosphorylation in FKBP51V55L BMMs was elevated in response to RANKL. In contrast, iκB degradation, ERK phosphorylation and LC3II expression showed no difference in wild-type and mutant BMMs. Micro-CT analysis revealed an intensive trabecular bone resorption pattern in FKBP51V55L mice, and suspicious osteolytic bone lesions were noted in three-dimensional reconstruction of distal femurs from mutant mice. These results demonstrate that the mutant FKBP51V55L promotes osteoclastogenesis and function, which could subsequently participate in PDB development.

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**INTRODUCTION**

Paget’s disease of bone (PDB (MIM 167250)) is a common skeletal disorder that is characterized by abnormal focal bone remodeling. It usually affects one or more bones in patients with multiple constitutive symptoms including bone pain, deformity, pathological fractures, deafness and secondary osteoarthritis.1 In European countries, PDB is the second most frequently diagnosed chronic bone disorder after osteoporosis, and it affects ~5% of women and 8% of men in the UK by the age of 80.2 However, PDB is rarely diagnosed in Asian populations; for example, the prevalence of PDB in Japan is only ~0.00028%.3

Although the exact pathogenesis of PDB is still unclear, genetic factors are known to be important in PDB development. Mutations in the *SQSTM1* gene, which encodes p62, have been shown to be widely involved in PDB development and in the severity of the disease phenotype. Among these mutations, p62P392L is the most common; it has been shown to be involved in ~20–50% of cases of familial PDB and in 16% of cases of sporadic PDB.4 In addition to *SQSTM1*, candidate gene approach and genome-wide association studies have identified several other PDB susceptibility genes and loci; these include *TNFRSF11A*, *OPTN*, *CSF1*, *TNFRSF11B*, *VCP* and *DCSTAMP*.5–8 Environmental factors such as virus infection, low-calcium diet, and exposure to pets and toxins have also been proposed to contribute to the etiology of PDB. Although still controversial, some consider paramyxovirus infection, especially measles virus infection, to be a potential risk factor in PDB development.9–11 Recently, a marked decline in the prevalence of PDB in some regions has been
noted, a finding that suggests a role for environmental factors in PDB development.9

Osteoclast dysfunction is the primary defect in PDB.12 Osteoclasts in pagetic bone lesions display increased size, contain multiple nuclei and possess characteristic intranuclear inclusion bodies.13 Osteoclasts derived from the peripheral blood monocytes of patients with PDB are more sensitive than osteoclasts from non-PDB patients to factors that induce bone resorption, including the 1,25-dihydroxyvitamin D3 and receptor activator for nuclear factor κB (RANKL).14 RANKL is a key cytokine that induces osteoclastogenesis.15 The binding of RANKL to its receptor activates signaling pathways including the NF-κB, Akt and MAPK pathways; these pathways ultimately activate NFATC1 and initiate osteoclastogenesis.15 Thus, aberrant regulation of specific signaling pathways is an important component of PDB etiology that may contribute to osteoclast dysfunction and disease development.

In this study, we recruited an extremely rare case of Chinese familial PDB, and genetic disorders in this familial case were studied. No mutations in previously reported genes were found in this family. Instead, a novel missense mutation in \(FKBP5\) was identified using whole-exome sequencing. \(FKBP5\) encodes the FKBP51 protein, which is a regulator of NF-κB activation and Akt phosphorylation.16–18 Given the correlation between the function of FKBP51 and osteoclastogenesis-related signaling pathways, we hypothesized that the \(FKBP5\) mutation might cause PDB by influencing osteoclast formation and biological function.

**MATERIALS AND METHODS**

**Studied family**

Three patients in the studied family were evaluated at the Third Hospital of Jinan, and all patients were clinically diagnosed based on their clinical features and characterized digital radiography (DR) examination findings in bone.1 This study was approved by the ethics committee of Shandong Provincial Hospital Affiliated to Shandong University, and written informed consent was obtained from all participants.

**DNA sequencing**

Genomic DNA was extracted from peripheral blood samples of all family members using a blood DNA extraction kit (Qiagen, Dusseldorf, Germany). To determine whether alterations were present in known PDB susceptibility genes in this family, we sequenced the \(SQSTM1\), \(TNFRSF11A\), \(TNFRSF11B\), \(VCP\), \(OPTN\), \(CSF1\) and \(DCSTAMP\) genes of all family members using the polymerase chain reaction (PCR) followed by Sanger dideoxy sequencing on an ABI 3100 Genetic Analyzer.

**Whole-exome sequencing and candidate gene screening**

We performed whole-exome sequencing of three patients (II3, II6 and II11) and their two healthy siblings (II4, II8). Exome enrichment was achieved using the Agilent Sure-Select Human All Exon Kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on an Illumina instrument using paired-end 101-bp reads at a sequencing depth of \(\times 100\). The sequence reads were mapped to the Human Reference Genome; then, single-nucleotide variants (SNVs) annotation was performed using the Burrows–Wheeler Aligner and Samtools. SNVs with one of the following conditions, a quality score higher than 30, a quality score-to-depth ratio > 2.5, or a map quality score > 25, were chosen for further analysis.

Candidate mutations that co-segregated with PDB were filtered from the set of reversed SNVs according to the following filtering conditions: absence from the Single-Nucleotide Polymorphism Database (dbSNP), non-synonymous mutations located in the gene-coding area, existence in all affected patients, and absence from both unaffected sibling controls.

**FKBP5 gene mutation verification and function prediction**

Five SNVs that co-segregated with PDB were identified using the whole-exome sequencing assay. Of these, the c.163G>C mutation (p.Val55Leu) in exon 4 of \(FKBP5\) was selected as the candidate because \(FKBP5\) encodes FKBP51, which is functionally associated with osteoclast physiology. To verify this mutation in the patients and determine the status of this mutation in the Han Chinese population (that is, to determine whether it is an undefined single-nucleotide polymorphism (SNP)), we sequenced PCR-amplified segments of \(FKBP5\) exon 4 in family members and in 200 Han Chinese controls using standard Sanger sequencing methodology. Function prediction of the mutant \(FKBP5\) was performed using PloyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and PyMol (DeLano Scientific LLC, San Carlos, CA, USA).

**Functional assay of mutant FKBP51 in cell experiments**

The function of the mutant FKBP51\(^{\text{V55L}}\) in the regulation of NF-κB transcriptional activity was measured by luciferase experiments in HEK293 cells. Briefly, pcDNA3.1-FKBP51, pcDNA3.1-FKBP51\(^{\text{V55L}}\) or control plasmid were co-transfected with the PGL4.32[luc2p(NF-kB-RE/Hygro)] luciferase reporter vector and the PRL-TK plasmid into HEK293 cells. Twenty-four hours after transfection, the cells were treated with 20 ng ml\(^{-1}\) tumor necrosis factor-alpha for 4 h and processed for luciferase assays using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

To detect Akt phosphorylation, wild-type or mutant FKBP51-overexpressing HEK293 cells were serum-starved for 12 h and then incubated with lipopolysaccharide (1 μg ml\(^{-1}\), Sigma, St Louis, MO, USA) for 15 min. Total proteins were immediately extracted from the cells, and Akt phosphorylation was detected by western blotting. A rabbit antibody to phospho-Akt (Ser473) and a rabbit antibody to Akt were obtained from Cell Signaling Technology (Danvers, MA, USA). A rabbit antibody to GAPDH and a goat antibody to rabbit IgG were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Monoclonal U937 cell lines expressing wild-type or mutant FKBP51 were serum-starved for 12 h and then activated by RANKL (100 ng ml\(^{-1}\), R&D Systems, Minneapolis, MN, USA) for 15 min. The kinase activity of Akt in these cells was then measured using the KinaseSTAR Akt Activity Assay Kit (BioVision, Milpitas, CA, USA). The kinase activity of Akt was finally determined by examining the amount of exogenous phosphorylated GSK-3 α using western blotting.

**Generation of FKBP51\(^{\text{V55L}}\) KI transgenic mice**

All animal studies were approved by the Institutional Animal Care and Use Committee of Shandong Provincial Hospital affiliated to Shandong University. Animal handling and experiments were performed according to the local legislation titled ‘Regulations for The Administration of Affairs Concerning Experimental Animals.’
To generate KI mice carrying the FKBP51V55L mutation in the endogenous mouse FKBP5 gene (GenBank accession number: NM_010220.4), a target vector containing homology arms, a Neo cassette flanked by LoxP sites, a c.163G>C mutation in exon 3 that results in generation of the V55L mutation in the 3’ homology arm, and a DTA cassette in the 3’ flank was created. The homology arms were generated by PCR using the BAC clones RP23-77G4 and RP23-238B10 from the C57BL/6 library as a template. The c.163G>C mutation was created by site-directed mutagenesis using the TaKaRa MutanBEST Kit (TaKaRa Biotechnology, Dalian, China), and this method was used to insert two AflII and two HindIII restriction enzyme sites, both of which spanned the target sequence, to facilitate the identification of the targeting event. The targeting construct was electroporated into C57BL/6 ES cells, and G418-resistant clones were picked. These clones were further characterized using PCR assays with the primers 3’ PCR_F and 3’ PCR_R, by which positive clones would produce a 3.6-kb fragment. In addition, positive ES clones were identified for correct integration using Southern blotting of AflII- and HindIII-digested genomic DNA, and these positive clones were sequenced for the c.163G>C mutation in exon 3 using a pair of sequencing primers. Cells from the positive clones were injected into recipient blastocysts obtained from pregnant C57BL/6 female mice. The chimeric offspring of the mice were mated with CMV-Cre-recombinant blastocysts obtained from pregnant C57BL/6 female mice, and the offspring of these mice were tested for germline transmission of the targeting sequence and for excision of the neomycin cassette by PCR using a pair of primers that flank the insert site: sense: 5′-CCTCGAGGCATGCGATAATC-3′; antisense: 5′-GGCAATGGCTTCTTCTGTGAT-3′. The mouse colony was maintained by breeding heterozygous; the wild-type and homozygous mice used in this study are littermates.

**Osteoclast formation and TRAP staining**

Osteoclast precursors were prepared as previously described. In brief, mouse bone marrow cells were isolated from the femurs and tibiae of 7-week-old mice. The cells were cultured in α-minimum essential medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum with macrophage colony-stimulating factor (M-CSF) (5 ng ml⁻¹) for 12 h in 100-mm-diameter dishes (Corning, Corning, NY, USA; 1 × 10⁵ cells per 10 ml per dish). After 12 h, nonadherent cells were collected and cultured in 48-well plates (Corning; 1.5 × 10⁵ cells per well) with M-CSF (30 ng ml⁻¹). After 3 days, the floating cells were removed, and the attached cells were used as bone marrow-derived monocyte/macrophage cells (BMMs). The BMMs were cultured in the presence of varying concentrations of RANKL and M-CSF (50 ng ml⁻¹) for 3 days. The cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP), as described in the manual provided by the manufacturer (387A, Sigma-Aldrich, Steinheim, Germany). TRAP-positive giant cells containing more than two nuclei were identified as osteoclasts and counted.

**Pit formation assay**

Bone marrow cells (2 × 10⁵ cells per well) were plated on bovine bone slices (4 mm × 6 mm) that had been placed in 48-well plates. The bone marrow cells were first cultured with M-CSF (30 ng ml⁻¹) for 3 days. The slices were then washed and further cultured with RANKL (100 ng ml⁻¹) and M-CSF (50 ng ml⁻¹) for an additional 7 days, with the medium changed every 3 days. The slices were then cleaned by ultrasonication in 0.25 M NH₄OH buffer and stained with 1% toluidine blue. Resorption pits were photographed under microscopic examination, and the areas of the pits were calculated using Image-Pro Plus (Version X; Media Cybernetics, Silver Springs, MD, USA).

**Analysis of osteoclast-specific gene expression**

BMMs were cultured in six-well plates with RANKL (100 ng ml⁻¹) and M-CSF (50 ng ml⁻¹) for the indicated amounts of time. Then, c-fos and NFATC1 expression was detected using western blotting. To measure signaling pathway activation, BMMs were serum-starved for 4 h before exposure to RANKL for the indicated times; then, the phosphorylation of Akt and Erk, and degradation of IκB were detected by western blotting. For LC3 expression, BMMs were cultured in six-well plates in the presence of RANKL (100 ng ml⁻¹) and M-CSF (50 ng ml⁻¹) for the indicated number of days, and the LC3 expression levels were determined using western blotting. Rabbit antibodies to c-fos, NFATC1 and IκB were obtained from Abcam (Cambridge, MA, USA); rabbit antibodies to pErk, Erk and LC3 were obtained from Cell Signaling Technology.

**Micro-CT analysis**

The hind limbs of 10-month-old FKB51WT and FKB51V55L male littermates (three wild-type and three homozygous littermates) were obtained and fixed in 4% formalin-buffered saline. Micro-CT analysis was performed using a Siemens Inveon micro-CT system (Siemens Medical Solutions, Knoxville, TN, USA). For the assessment of bone morphology, bilateral femurs and tibiae of mice were dissected free of most soft tissues, and the femur and proximal tibia were scanned at an effective pixel size of 9.08 μm (60 kV, 220 μA). Two-dimensional images and three-dimensional (3D) reconstruction of femurs were performed using the Inveon Acquisition Workplace. Trabecular bone parameters were analyzed using the Inveon Research Workplace. Briefly, we measured a stack of slices within a 0.5-mm step length that began at the slice 0.5 mm distal to the growth plate and ended at the slice 1 mm distal to the growth plate. The cortical thickness 1 mm distal to the growth plate was also measured.

**Statistical analysis**

The experimental results were statistically analyzed using Student’s t-test. P < 0.05 was considered statistically significant.

**RESULTS**

**The studied family**

The pedigree of the studied family is shown in Figure 1e. Patient II3, the proband of the family, was diagnosed with...
PDB at 61 years of age when she came to the hospital seeking treatment for lung discomfort. Marked pagetic bone lesions in her humeri were noticed during lung X-ray examination, and subsequent whole body skeletal scanning by DR showed that her skull, humeri, vertebrae, pelvis and femurs were affected by PDB. Typical pagetic bone lesions in the affected bones of this patient are shown in Figures 1a–d. Serum alkaline phosphatase (ALP) (2637 IU l$^{-1}$, normal range: 23–140 IU l$^{-1}$) at the time of diagnosis was remarkably elevated in this patient. Cervical and limb weakness and body height reduction were also noted. No other clinical symptoms or secondary complications such as fracture, osteoarthritis, compression neuropathy or high-output heart failure were noted.

Once the proband was diagnosed, her family members were invited to participate in the study and were screened for PDB. Two siblings (II6 and II11) of this proband were diagnosed with PDB. Patient II6 was 54 years of age when she was diagnosed. DR examination showed that her skull, humeri, vertebrae, pelvis and femurs were affected and that her serum ALP (3337 IU l$^{-1}$) at the time of diagnosis was elevated. This patient’s main clinical symptoms were low back pain, cervical and limb weakness, and slight spinal deformity. The patient claimed that cervical and limb weakness had appeared ~2 years prior to diagnosis. No other complications were found. Patient II11 was 45 years of age when he was diagnosed. DR examination showed that his skull, vertebrae and pelvis were affected and that his serum ALP (1851 IU l$^{-1}$) at the time of diagnosis was elevated. However, sclerosis and bone expansion in this patient were mild compared with those in the other two patients. At the beginning of the study, this patient reported no significant symptoms. The patients and some of the patients’ family members were again invited for further examination and treatment ~2 years ago. DR examination confirmed that there were no new affected bones in the patients. However, the spinal deformity of patient II6 had become more severe. A body height reduction of ~9 cm was noted in patient II11, who complained of cervical weakness. Magnetic resonance examination showed that the first, second and third lumbar vertebrae of this patient were severely compressed and presented typical double-sided depressed deformities (not shown). The serum ALP levels of these patients demonstrated progressive ALP elevation (Table 1). Intravenous infusion of zoledronic acid (Aclasta, Novartis Pharma Schweiz AG, Switzerland) at 5 mg
per year was used for each patient. After 2 years of treatment, the symptoms of patients II3 and III11, including cervical and limb weakness, and body height reduction, were significantly remitted; the serum ALP levels of these two patients also significantly decreased (Table 1), and the calcium and phosphorus homeostasis of the patients was normal. Patient II6 did not return for examination. Five offspring of the patients were also examined, and none of them showed pacetic changes. Detailed information on the patients and their offspring, and also examined, and none of them showed pagetic changes.

Table 1 Clinical features and up-to-date examination results of patients and FKB5 mutation carriers in our pedigree

| Member | Age (years)/sex | Age at diagnosis (years) | Affected skeletal site(s) | Symptoms and signs | Serum ALP (IU l⁻¹) | Calcium (mmol l⁻¹) | Phosphorus (mmol l⁻¹) |
|--------|----------------|--------------------------|--------------------------|-------------------|--------------------|-------------------|---------------------|
|        |                |                          |                          |                   | (normal range: adult: 23–140; child: < 750) | (normal range: 2.2–2.8) | (normal range: 0.85–1.51) |
| Patient II3 | 67/female | 61 | Skull, humeri, vertebrae, pelvis and femurs | Cervical and limb weakness, body height reduction of ~12 cm | First visit: 2637 | 2.39 | 1.09 |
| Patient II6 | 60/female | 54 | Skull, humeri, vertebrae, pelvis and femurs | Bone pain, cervical and limb weakness, vertebral column bowing deformity | First visit: 3337 | 2.26* | 1.27* |
| Patient II11 | 51/male | 45 | Skull, vertebrae and pelvis | Cervical weakness, body height reduction of ~9 cm | First visit: 1851 | 2.14 | 0.90 |
| Carrier III3 | 40/male | NA | NA | NA | 77 | 2.41 | 1.15 |
| Carrier III13 | 39/female | NA | NA | NA | 109 | 2.39 | 1.12 |
| Carrier III15 | 37/female | NA | NA | NA | 74 | 2.44 | 1.04 |
| Carrier III17 | 35/female | NA | NA | NA | 79 | 2.39 | 0.88 |
| Carrier IV2 | 16/male | NA | NA | NA | 279 | 2.55 | 1.53 |

Abbreviation: NA, not available.

*Patient II6 did not respond to the latest invitation. These data represent the serum calcium and phosphorus levels obtained 2 years ago.

No mutations in previously reported disease-related genes were found in the family

Except for the presence of many SNPs, no mutations in SQSTM1, TNFRSF11A, TNFRSF11B, VCP, OPTN, CSF1 and DCSTAMP genes were found in this family. All identified SNPs in these genes are listed in Table 2.

Whole-exome sequencing targeted a missense point mutation in FKB5 gene as candidate

A total of 245 982 SNVs were detected in five samples using WES. Considering the extremely low prevalence of PDB in China, we hypothesized that disease-causing genetic variants would not likely include known SNPs in the general population. Hence, only missense mutations that did not exist in dbSNP and that co-segregated with disease (existed in all patients and were absent from their normal siblings) were considered as candidates. Using this approach, five missense mutations in five genes (FKBP5, ACBD4, MYO7B, AKNA and CCL4L1) were identified (Table 3). The mutations in ACBD4 and MYO7B were previously reported in the Exome Aggregation Consortium (ExAC) database; the mutations in FKB5, AKNA and CCL4L1 show no records in the ExAC database.

The mutation in FKB5 was selected for further investigation because of the important biological functions of FKB5. FKB51 has been shown to regulate the NF-κB and Akt pathways, both of which are involved in osteoclastogenesis.15,16,18 These confirmed functions of FKB51, which may influence osteoclast generation, made the FKB5 mutation a good candidate for a mutation associated with the development of PDB. The other four genes were initially excluded because their previously described functions are unlikely to be associated with this disease.

FKBP5 gene mutation is verified and is predicted to be deleterious to Akt signaling

The identified FKB5 mutation (c.163G>C) is located within exon 4 of the FKB5 gene. None of the 200 Han Chinese individuals examined had this mutation, which confirmed that it is a novel single-nucleotide mutation rather than a polymorphism. Genotyping of the c.163G>C mutation confirmed that all of the patients in the study were GC heterozygotes. Five of the offspring of the patients (II3, III13, III15, III17 and IV2) harbored the same mutation as the patients, and they were GC heterozygotes as well (Figure 2a). Figure 1e shows the offspring of patients who inherited this heterozygous mutation.

FKBP51 has three functional domains: two consecutive FKB12-like domains (the FK1 and FK2 domains) and a three-unit repeat of the tetratricopeptide repeat domain.20 The c.163G>C mutation results in a p.Val55Leu replacement at a highly conserved position within the FK1 domain (Figure 2b). The 3D structure of mutant FKB51V55L showed...
that the V55L mutation lies within the first N-terminal beta strand of FKBP51, precisely on the surface of the FK1 functional pocket (Figure 2c).

Assays showed that the luciferase activity of HEK293 cells transfected with wild-type or mutant FKBP51 did not significantly differ (Figure 2d). However, the Akt phosphorylation level at Ser473 was increased in cells transfected with mutant FKBP51 compared to that in cells transfected with the wild-type control (Figures 2e–g). The phosphorylation level of exogenous GSK-3α, which was phosphorylated by the total Akt extracted from mutant FKBP51-expressing U937 cells, was increased compared with the wild-type group, thus demonstrating that Akt kinase activity is increased in U937 cells expressing the mutant FKBP51 protein (Figure 2h).

Osteoclast formation and bone resorption activity
FKBP51V55L KI transgenic mice were created and used to study osteoclast differentiation and bone resorption activity induced by RANKL in vitro. When the concentration of RANKL was low (30 ng ml⁻¹), osteoclasts derived from wild-type and FKBP51V55L mutant BMMs showed no significant differences. When the concentration of RANKL was increased to 70 or 150 ng ml⁻¹, the number of osteoclasts derived from both FKBP51WT and FKBP51V55L BMMs was increased compared with the low concentration induction groups, and the number of osteoclasts derived from FKBP51V55L BMMs were significantly increased compared with that from FKBP51WT BMMs at the same RANKL induction concentration (Figures 3b and d). The bone resorption function of osteoclasts derived from FKBP51V55L transgenic mice and control mice was also detected by the pit forming assay. We found that the mean area of resorption pits formed from osteoclasts derived from FKBP51V55L transgenic mice was larger than that of resorption pits formed by osteoclasts from control mice (Figures 3c and e).

The NFACT1 messenger RNA expression level was increased in osteoclast precursors from FKBP51V55L mice; in addition, messenger RNAs for the osteoclast-specific molecules TRAP and the osteoclast-associated receptor were increased in the mutant osteoclast precursors in response to RANKL and M-CSF (Figures 4a–d). Western blotting confirmed that the protein expression level of NFATC1 was increased in osteoclast precursors derived from the mutant mice; however, c-fos expression was not significantly different in the wild-type and mutant groups (Figures 4e and f).

Akt phosphorylation is increased during osteoclast differentiation in osteoclast precursors expressing FKBP51V55L
As shown in Figure 5, Akt phosphorylation in response to RANKL was increased in FKBP51V55L BMMs compared with wild-type BMMs, but no differences in the activation of Erk or the degradation of IκB were found in BMMs of these two genotypes (Figure 5a). Because abnormal autophagy has recently been considered a possible mechanism of PDB pathogenesis, we measured LC3 expression in BMMs during osteoclast differentiation induced by RANKL and M-CSF. The results showed that LC3 expression in wild-type and FKBP51V55L BMMs did not significantly differ (Figure 5b).
Intensive trabecular bone resorption phenotype of FKBP51V55L mice

Parametric analysis of the femoral trabecular bone of 10-month-old FKBP51 wild-type and mutant male littermates was performed. This analysis showed that ratio of the bone volume to the total volume of mutant mice was decreased (bone volume ratio FKBP51WT: 0.1401 ± 0.0277; FKBP51V55L: 0.08 ± 0.0219; P = 0.0037; trabecular number FKBP51WT: 3.4393 ± 0.5322; FKBP51V55L: 2.2073 ± 0.3871; unit: mm⁻¹; P = 0.0020; trabecular thickness FKBP51WT: 0.0406 ± 0.0024; FKBP51V55L: 0.0361 ± 0.0036; unit: mm; P = 0.0359). Accordingly, trabecular spacing (FKBP51WT: 0.2561 ± 0.0479, FKBP51V55L: 0.4279 ± 0.0815, unit: mm; P = 0.0018) and trabecular pattern factor (FKBP51WT: 20.60 ± 1.50, FKBP51V55L: 25.50 ± 2.61, unit: mm⁻¹; P = 0.0035) were increased in the mutant mice (Figure 6a). This result proves that Akt kinase activity in mutant FKBP51-transfected U937 cells is higher than in cells expressing the wild-type protein.
were those of the wild-type animals (Figure 6b). No significant cortical thickening or bone expansion was found in the femurs of the mutant animals, and no bending deformity of bone was noted. 3D reconstruction of the distal femurs of two of the three mutant mice showed suspicious osteolytic bone lesions penetrating the cortex; none of the wild-type control animals displayed this phenomenon (Figure 6c).

**DISCUSSION**

In many countries, PDB affects ~1–8% of individuals over 55 years of age.21 Individuals with PDB suffer from various bone problems and complications such as bone pain, deformity, fracture, and hearing loss and other nerve root compression symptoms. Genetic factors are important elements in studies of PDB etiology. In this study, we report an extremely rare case of familial PDB found in a northern Han Chinese family. Genetic studies did not reveal mutations in known disease-related genes in this family, and exome sequencing targeted a novel candidate c.163G>C mutation in *FKBP5* that is associated with PDB through enhanced osteoclastogenesis. The V55L mutation in *FKBP5* was shown to influence the function of FKBP5 in regulating Akt phosphorylation in cells. Osteoclast generation and function studies using FKBP51V55L KI mice showed that the V55L mutation enhanced osteoclastogenesis and osteoclast bone resorption activity in vitro. Akt phosphorylation and NFATC1 expression in BMMs from FKBP51V55L mice were increased compared to normal controls during osteoclast differentiation induced by RANKL. Finally, micro-CT analysis confirmed the presence of intensive trabecular bone resorption in FKBP51V55L.
mice corresponding to the enhanced osteoclastogenesis noted in vitro, and 3D reconstruction of the distal femurs of the mutant mice showed suspicious bone resorption lesions. These studies indicate that the FKBP5 mutation that we identified is associated with PDB through enhancing osteoclastogenesis.

To date, mutations in SQSTM1 have been widely shown to be associated with PDB through regulation of the RANKL and NF-κB signaling pathways.22 Other susceptibility genes such as TNFRSF11A, TNFRSF11B, OPTN, VCP, CSF1 and DCSAMP have also been reported to be related to or linked to PDB. In our study, with the exception of many SNPs, no mutations in these known susceptibility genes were detected. However, PDB is a rare disease in China. To the best of our knowledge, no more than 200 patients have been reported prior to the study in the literature in English and or in the local language.23,24 Hence, because they are common, SNPs were not considered likely to be causal, and mutations in unknown genes were further investigated.

Five genetic mutations that co-segregated with patients were identified using whole-exome sequencing in the studied family. One of these five genes, FKBP5, encodes FKBP51, which is involved in the regulation of a number of fundamental biological processes. It positively regulates canonical NF-κB activation and negatively modulates Akt via protein–protein interactions.17,18,26 Scientists have recently found that FKBP51 also functions in antidepressant therapy by acting as an autophagy inducer.27,28 Two of the processes that are regulated by FKBP51, NF-κB and Akt signaling, are both involved in osteoclastogenesis. Activation of these two pathways promotes osteoclast development, survival, and bone resorption activation, and aberrant NF-κB signaling activation has been confirmed as a critical mechanism in the pathogenesis of
PDB. In addition, insufficient autophagy has been presumed to be correlated with the formation of inclusion bodies in the osteoclasts of patients with PDB, and this may be an additional mechanism involved in PDB development. The correlations between the functions of FKBP51 and PDB pathogenesis made the FKBP5 gene a good candidate for studying the disease causative mutations associated with PDB, whereas none of the other four genes in which mutations were found in the affected individuals are known to have functions related to the biological characteristics of osteoclasts.

Functional assays of the mutant FKBP51 protein in cell experiments demonstrated that the V55L mutation in FKBP51 does not affect its regulatory role in NF-κB activation. In contrast, Akt phosphorylation was elevated in HEK293 cells expressing mutant FKBP51, and the kinase activity of Akt was increased in U937 cells expressing mutant FKBP51. It was previously reported that FKBP51 can enhance pAktSer473 dephosphorylation by acting as a scaffold for Akt and PHLPP, and that deletion of the FK1 domain (residues 1–138) of FKBP51 abolished its ability to enhance Akt-PHLPP interaction.18 We presume that the FKBP51V55L mutation identified in our study decreases the interaction between Akt and PHLPP, consequently reducing the dephosphorylation of pAktSer473 by PHLPP and ultimately resulting in an increase in Akt phosphorylation. These results emphasize an important role of the V55L mutation in increasing Akt activation, a process that may be involved in osteoclast formation.

Mouse models have been used in studies of the etiology of PDB in attempts to determine whether the known mutations in the SQSTM1 gene or viral proteins can elicit Paget’s disease-like disorders in mice.10,35–37 In the FKBP51V55L KI transgenic mouse model we created here, significantly more osteoclasts were formed from BMMs derived from mutant mice that from control BMMs in response to various concentrations of RANKL. TRAP and osteoclast-associated receptor are two osteclast-specific molecules that are expressed in osteoclasts during differentiation.38 In vitro experiments showed that both the TRAP and osteoclast-associated receptor messenger RNA levels were significantly increased in FKBP51V55L mutant BMMs compared with wild-type BMMs after induction by RANKL. Pit formation assays revealed that the mean resorption area formed by osteoclasts generated from FKBP51V55L mice was larger than that of the wild type. These results show that osteoclast precursors from FKBP51V55L mice are more sensitive to RANKL than osteoclast precursors from wild-type mice and that the V55L mutation in FKBP51 promotes osteoclast differentiation. In addition, the V55L mutation enhances bone resorption activation in osteoclasts. Taken together, these results strongly suggest that this mutation is related to PDB development by promoting osteoclast differentiation and activation.

NFATC1 is the key transcription factor in osteoclast differentiation, and its induction depends on both the TRAF6 and the c-fos signaling pathways.39 In this study, we found that c-fos expression levels in wild-type and mutant BMMs did not significantly differ; however, NFATC1 levels in FKBP51V55L BMMs were increased during osteoclast differentiation. This indicated that the V55L mutation in FKBP51 directly influences NFATC1 expression independently of c-fos during osteoclastogenesis. Several signaling pathways that are involved in signal transmission during osteoclast differentiation, including the NF-κB, Akt, ERK, JNK and p38 MAPK pathways, depend on the binding of RANKL to its receptor RANK, and these pathways ultimately influence NFATC1 expression.15 We showed that Akt phosphorylation in FKBP51V55L BMMS increased in response to RANKL and that IkB generation and ERK phosphorylation in mutant and wild-type BMMs were not significantly different. This result confirmed the influence of the FKBP51V55L mutation on Akt phosphorylation in mice BMMs and showed that this mutation increases NFATC1 expression and osteoclast differentiation through enhancing Akt activation without influencing the NF-κB and ERK signaling pathways.

Another possible mechanism associated with FKBP51 function that may be involved in PDB development is autophagy. Insufficient autophagy is considered to be related to the formation of inclusion bodies in Pagetic osteoclasts. In addition, PDB is a typical age-dependent disease that mainly affects people older than 55 years of age, and aberrant autophagy, which has been linked with aging, may partially explain the typically late onset of PDB.40 FKBP51 has previously been shown to enhance the autophagy process. Our results showed that the expression of LC3II, a biomarker...
for autophagy, was not significantly different in mutant and wild-type BMMs during the induction of osteoclast differentiation. This indicated that there is no aberrant autophagy in FKBP51V55L mice during osteoclast differentiation in vitro.

In summary, the results of our experiments prove that the FKBP51V55L mutation increases osteoclast formation by enhancing the activity of the Akt/NFATC1 pathway in a mouse model in vitro.

The pagetic phenotype has previously been reported in P62P394L transgenic mice. In that study, the authors demonstrated that P62P394L mice develop mixed osteolytic and osteosclerotic bone disorders in the distal femur and proximal tibia with increasing age. In our study, the phenotype of the experimental mice was also determined by micro-CT analysis. Excessive trabecular bone resorption was noted in 10-month-old FKBP51V55L mice, corresponding with the enhanced osteoclastogenesis observed in vitro. In addition, resorption bone lesions were detected on a 3D reconstruction of the distal femurs of the mutant mice. The presence of these phenotypes confirmed that the V55L mutation can cause osteolytic bone lesions in vivo. However, osteosclerotic bone disorders involving cortical thickening and expansion were not observed in the mutant mice. This result indicates that the V55L mutation in FKBP51 is insufficient to cause excessive compensatory osteoblastic differentiation in a mouse model. There are two possible reasons for this result: first, the bone micro-environment differs in humans and mice, and these differences may result in different outcomes of osteoblast differentiation in the two species; second, additional gene disorders or environmental factors are needed to trigger aberrant osteoblastic formation in vivo, thereby suggesting an important role of environmental factors in PDB development.

In conclusion, in seeking to identify gene disorders responsible for a Chinese familial PDB, we demonstrated that a novel c.163G>C point mutation in the FKBP51 gene is associated with PDB. The results of our research proved that...
the FKBP5 mutation, which co-segregated with patients, enhances osteoclast differentiation and bone resorption activity, both of which are primary defects in PDB development. These results indicate that the identified FKBP5 mutation is associated with PDB through enhancing osteoclastogenesis and function. Nevertheless, this work also has the limitation that because none of the mutation carriers in the pedigree are yet over 40 years of age and none of them currently show symptoms of Paget bone disorder, the available genetic evidence (for example, generation transmission and linkage studies) regarding this mutation is insufficient. Because PDB usually appears at >40 years of age, the status of these carriers is now considered unknown, and follow-up visits will be conducted in the future to further study the genetic transmission of this mutation.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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1 Takata S, Hashimoto J, Nakatsuka K, Yoshimura N, Yoh K, Ohno I et al. Guidelines for diagnosis and management of Paget’s disease of bone in Japan. J Bone Miner Metab 2006; 24: 359–367.
2 van Staa TP, Selby P, Leufkens HG, Lyles K, Sprafka JM, Cooper C. Incidence and natural history of Paget’s disease of bone in England and Wales. J Bone Miner Res 2002; 17: 465–471.
3 Hashimoto J, Ohno I, Nakatsuka K, Yoshimura N, Takata S, Zamma M et al. Prevalence and clinical features of Paget’s disease of bone in Japan. J Bone Miner Metab 2006; 24: 186–190.
4 Seton M, Hansen M, Solomon DH. The implications of the sequestosome 1 mutation P392L in patients with Paget’s disease of bone. Bone 2001; 28: 104–107.
5 Chung PY, Beyens G, de Freitas F, Boonen S, Geusens P, Vanhoenacker F et al. Evaluation of the role of RANK and OPG genes in Paget’s disease of bone. Calcif Tissue Int 2016; 98: 489–496.
6 Albagha OM, Visconti MR, Alonso N, Langston AL, Cundy T, Dargie R et al. Genome-wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget’s disease of bone. Nat Genet 2010; 42: 520–524.
7 Wuyts W, Van Wesenbeeck L, Morales-Piga A, Ralston S, Hocking L, Vanhoeacker F et al. Evaluation of the role of RANK and OPG genes in Paget’s disease of bone. Bone 2001; 28: 104–107.
8 Chung PY, Beyens G, de Freitas F, Boonen S, Geusens P, Vanhoenacker F et al. Indications for a genetic association of a VCP polymorphism with the pathogenesis of sporadic Paget’s disease of bone, but not for TNFSF11 (RANKL) and IL-6 polymorphisms. Mol Genet Metab 2011; 103: 287–292.
9 Chung PY, Beyens G, Boonen S, Papapoulos S, Geusens P, Karperien M et al. The majority of the genetic risk for Paget’s disease of bone is explained by genetic variants close to the CSF1, OPTN, TM7SF4, and TNFRSF11A genes. Hum Genet 2010; 128: 615–626.
10 Singer FR. Paget’s disease of bone-genetic and environmental factors. Nat Rev Endocrinol 2015; 11: 662–671.
11 Kurihara N, Hiruma Y, Yamana K, Michou L, Rousseau C, Morissette J et al. Contributions of the measles virus nucleocapsid gene and the SQSTM1/p62 (P392L) mutation to Paget’s disease. Cell Metab 2011; 13: 23–34.
12 Teramachi J, Nagata Y, Mohammad K, Inagaki Y, Ohata Y, Guise T et al. Measles virus nucleocapsid protein increases osteoblast differentiation in Paget’s disease. J Clin Invest 2016; 126: 1012–1022.
13 Helfrich MH. Osteoclast diseases. Micrós Res Tech 2003; 61: 514–532.
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36 Hiruma Y, Kurihara N, Subler MA, Zhou H, Boykin CS, Zhang H et al. A SQSTM1/p62 mutation linked to Paget’s disease increases the osteoclastogenic potential of the bone microenvironment. *Hum Mol Genet* 2008; 17: 3708–3719.

37 Daroszewska A, van’t Hof RJ, Rojas JA, Layfield R, Landao-Basonga E, Rose L et al. A point mutation in the ubiquitin-associated domain of SQSTM1 is sufficient to cause a Paget’s disease-like disorder in mice. *Hum Mol Genet* 2011; 20: 2734–2744.

38 Kim K, Kim JH, Lee J, Jin HM, Kook H, Kim KK et al. MafB negatively regulates RANKL-mediated osteoclast differentiation. *Blood* 2007; 109: 3253–3259.

39 Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 2002; 3: 889–901.

40 Chung PY, Van Hul W. Paget’s disease of bone: evidence for complex pathogenetic interactions. *Semin Arthritis Rheum* 2012; 41: 619–641.

41 Klein RM, Norman A. Diagnostic procedures for Paget’s disease. Radiologic, pathologic, and laboratory testing. *Endocrinol Metab Clin North Am* 1995; 24: 437–450.

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