# Abnormal Variations in the Expressions of LRP5, Runx2, Osterix and RANKL in Bone Tissues Associated With Postmenopausal Osteoporotic Fractures

**Bin Wang**
sanshui district hospital https://orcid.org/0000-0002-7886-8307
**Yanming Cao**
Guangzhou Medical University
**Caiyuan Mai**
Women's and Children's Hospital
**Ram Ishwar Yadav**
Guangzhou Medical University
**Jianliang Gao**
Guangzhou Medical University
**Lei Pan** ( woshi19800101@163.com )
sanshui district hospital

**Research**

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# Abstract

**Objective:** To investigate the variations in the expressions of LRP5, Runx2, Osterix, and RANKL factors in bone tissues associated with postmenopausal osteoporotic fractures (PMOPF).

**Method:** Postmenopausal patients with femur fractures were initially divided into control (31 cases) and PMOPF groups (83 cases). All control group patients were operated within 1 day after injury. The patients with PMOPF were operated based on the time after fracture in the respective groups (patients were divided into groups A, B, and C based on the time after fracture). Samples were collected from femurs at fracture sites during the operation. The expression level of each factor in bone tissues was detected using RT-qPCR, and the bone mass samples were decalcified and then histologically analyzed by immunohistochemistry. We subsequently analyzed significant differences in the expressions of factors (LRP5, Runx2, Osterix, and RANKL) between PMOPF and control groups.

**Results: (1)** LRP5, β-catenin, Runx2, and Osterix were under-expressed in patients with PMOPF relative to the controls ($P<0.05$). In contrast, RANKL was over-expressed in the PMOPF group when compared to the control group ($P<0.05$); **(2)** the expressions of LRP5 and Runx2 were lowest in Group A patients (1–3 days after fracture). Osterix expression was lowest in Group C patients (8–14 days after fracture). Conversely, RANKL expression was highest in Group B patients (4–7 days after fracture).

**Conclusion:** The inhibition or reduction in the expressions of osteogenic factors including LRP5, Runx2, and Osterix of the Wnt/β-catenin and BMP-2/Runx2/Osterix signaling pathways are associated with PMOPF incidence. Specifically, upregulation of RANKL in the RANKL/RANK signaling pathway is associated with the incidence of PMOPF. LRP5 and Runx2 expressions decreased considerably within 1-3 days after fracture; Osterix expression decreased considerably within 8-14 days after fracture; RANKL expression was highest within 4-7 days after fracture, which could be associated with bone repair in PMOPF. The expression level of the aforementioned factors affects the development and progression of PMOPF.


# Introduction

Osteoporosis is a metabolic disorder associated with systemic bone aging and degradation. Osteoporosis is characterized by decreased bone mass, structural degradation, increased brittleness, and susceptibility to fractures.[1] According to reports, 87% of people over 65 years old develop an osteoporotic fracture (OPF) when they accidentally fall.[2] Postmenopausal osteoporosis (PMOP) is a type of osteoporosis that occurs in women after menopause due to estrogen deficiency, resulting in bone loss and bone structure changes. The incidence of osteoporosis in postmenopausal women is 2–3-fold that in non-menopausal women.[3] PMOPF is a severe consequence of PMOP, which can significantly increase disability rates, mortality rates, and result in huge family and socio-economic burdens.[4, 5] The process of osteoporosis development is very complex, and involves osteoclast mediated bone resorption and osteoblast mediated bone formation, which maintain the bone in a state of continuous remodeling

consistent with the normal bone structure and function. Under physiological conditions, bone absorption and formation remain stable; however, when the balance is disrupted, osteoporosis develops.[6, 7]

Osteogenesis and osteoclastogenesis have been the subjects of interest in the prevention and treatment of osteoporosis. The classical Wnt/β-catenin, BMP-2/Osterix, and RANKL/RANK signaling pathways are key factors influencing the regulation of osteogenesis and osteoclastogenesis. LRP5, Runx2, and Osterix are key osteogenic factors associated with the Wnt/β-catenin and BMP-2/Osterix signaling pathway, while RANKL is a key osteoclast factor associated with the RANKL/RANK signaling pathway.[8, 9] However, the variations in the expressions of these factors in PMOPF remain unclear. Therefore, the overall aim of the present study was to investigate factors associated with the development and healing stages of PMOPF based on histological and molecular analyses of fracture healing stages. Specifically, the study aimed to investigate variations in the expressions of LRP5, Runx2, Osterix, and RANKL in bone tissues or bone mass of patients with PMOPF to elucidate their roles in the pathogenesis of PMOPF. RT-qPCR and immunohistochemistry were used to compare variations in the expressions of LRP5, Runx2, Osterix, and RANKL in bone tissues of patients with PMOPF.

# Materials And Methods

### Subjects

In accordance with the Helsinki Declaration, the present study was approved by the Ethics Committee of the People's Hospital of Sanshui and the Second Affiliated Hospital of Guangzhou Medical University. All participants signed informed consent forms. A total of 114 postmenopausal patients with femur fractures were recruited at the People's Hospital of Sanshui and the Second Affiliated Hospital of Guangzhou Medical University from July 2018 to February 2021. Bone mineral density (BMD) of the lumbar was measured using dual-energy X-ray absorptiometry. BMD, height, and body weight values were recorded. Participants were divided into two groups: PMOPF group with 83 cases and the control group, which comprised 31 postmenopausal cases (Non-osteoporotic participants) and other types of fractures. PMOPF group participants were further randomly divided into groups A, B, and C based on the time frame of bone tissue collection after fracture: Group A (30 cases, 1–3 days after fracture), Group B (27 cases, 4–7 days after fracture), and Group C (26 cases, 8–14 days after fracture).

All patients were operated and specimens collected from the fracture sites. The cases included hip arthroplasty (71 cases), limited open restoration and internal fixation (nail fixation; 22 cases), open restoration and internal fixation (plate fixation; 11 cases), and thigh amputation (10 cases). All stratified PMOPF group patients were operated within 1–3 or 4–7 or 8–14 days after injury. All control group patients were operated within 1 day after injury. Patients with secondary osteoporosis, hip osteoarthritis, and pathological fractures that were not osteoporotic-related were excluded. Specimens (the curetted bone mass is equal to or more than 200 mg, and intercepted bone mass greater than 0.5 cm × 0.5 cm × 0.2 cm in volume) were collected from the fracture sites, and we ensured that operation and follow-up

were not compromised. Bone tissue or bone mass samples were collected during surgery and stored immediately in liquid nitrogen.

## RT-qPCR

Bone tissues (100 mg) were ground using liquid nitrogen. Total RNA was extracted from bone tissues using RNAiso Plus (TaKaRa, Dalian, China), and cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Dalian, China). Real-time PCR was performed using primers synthesized by Thermo Fisher Scientific (Waltham, MA, USA) and SYBR Premix kit (TaKaRa, Dalian, China). The sequences of primers used are presented in Table 1. PCR conditions were as follows: denaturation at 94℃ for 5 minutes; 30 cycles of denaturation at 94℃ for 30 seconds; annealing at 58℃ for 30 seconds and extension at 72℃ for 40 seconds; extension at 72℃ for 10 minutes. GAPDH was selected as the internal reference. Data were expressed using the comparative CT ($2^{-\Delta\Delta CT}$) method and normalized to GAPDH.

Table 1
Primer sequence

| Product name | Sequence (5'→3') |
|---|---|
| LRP5 Forward | CGTGTCCAGCGAGATCCT |
| LRP5 Reverse | CCAAGCGAGCCTTTCTACAC |
| Runx2 Forward | CTCCTACCTGAGCCAGATGACG |
| Runx2 Reverse | GTGTAAGTAAAGGTGGCTGGATAGT |
| Osterix Forward | CCAAGTGGGTGGTATAGAG |
| Osterix Reverse | GGGATGGTGGGTGTAAGA |
| RANKL Forward | ATGTGCTGTGATCCAACGAT |
| RANKL Reverse | TGAGACTCCATGAAAATGCAGA |
| GAPDH Forward | GGCATGGACTGTGGTCATGAG |
| GAPDH Reverse | TGCACCACCAACTGCTTAGC |

## Immunohistochemical analysis

Intercepted bone mass was thawed, fixed in 10% neutral formalin for 48 hours, and embedded in paraffin after decalcification in 10% EDTA solution (Zhongshan Jinqiao, Beijing, China). The tissues were then cut into 5-μm thick sections and treated with 3% hydrogen peroxide for 10 minutes. Afterward, the sections were rinsed with phosphate-buffered saline, incubated with primary and secondary antibodies (Bio-Rad, Hercules, USA) sequentially, and exposed to DAB (TaKaRa, Dalian, China). The sections were counterstained with hematoxylin solution (TaKaRa, Dalian, China). Ten visual fields were randomly

selected and observed under a high magnification microscope (400×) (Leica DM500, Solms,germany), and the number of positively stained cells was calculated.

### Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, USA). All experiments were performed at least three times. All data were expressed as means ± data deviation ($\bar{x} \pm s$). Nonparametric test (Mann–Whitney U test) was performed using SPSS Statistics (version 18.0; SPSS Inc., Chicago, USA). A p-value < 0.05 was considered statistically significant.

## Results

### General characteristics of participants in PMOPF and control groups

A total of 114 postmenopausal patients with fractures were included in the present study, and all were treated by surgery based on time frames of the groups. The control group had 31 cases while the PMOPF group had 83 cases. A significant difference was observed in lumbar BMD (T value) between the two groups; however, no significant differences were observed in age, height, and weight between the two groups (P > 0.05, Table 2).

Table 2
Comparison of general characteristics of participants between control and PMOPF groups

| groups | Age (years) | Height (cm) | Body weight (kg) | BMD(T value) |
|---|---|---|---|---|
| control group | 55.97 ± 7.01 | 173.01 ± 7.39 | 64.53 ± 9.99 | 0.11 ± 0.06 |
| PMOPF group | 57.13 ± 5.88 | 169.13 ± 8.66 | 59.86 ± 8.87 | -2.94 ± 0.44 |
| P value | 0.06 | 0.08 | 0.07 | / |

### Expressions of LRP5, Runx2, Osterix, and RANKL in PMOPF (Groups A, B, and C) and control groups

RT-qPCR results revealed that LRP5, Runx2, and Osterix levels in bone tissues were lower in the PMOPF group than in the control group (P < 0.05), while RANKL mRNA level was higher in the PMOPF group than in the control group (P < 0.05). Stratified expression of factors associated with PMOPF revealed that LRP5 and Runx2 expressions were lowest in group A patients (1–3 days after fracture), and the lowest expression of Osterix was observed in group C patients (8–14 days after fracture), RANKL expression was highest in group B patients (4–7 days after fracture). LRP5 and Runx2 levels between the control group and group A exhibited a highly significant difference, although the differences between the other groups were not significant. A highly significant difference was observed in Osterix levels between the control group and group A, between group B and C, however, the differences in Osterix levels between groups A and B were not significant. RANKL levels differed significantly between the control group and

group A, and between groups A and B, however, the difference in RANKL levels between control group B and group C was not significant (Fig. 1).

### Immunohistochemical staining of LRP5, Runx2, Osterix, and RANKL proteins

The immunohistochemical staining results revealed that LRP5, Runx2, and Osterix levels in the bone mass were lower in the PMOPF group than in the control group ($P < 0.05$), while RANKL mRNA level was higher in the PMOPF group than in the control group ($P < 0.05$). Stratified expression of factors associated with PMOPF revealed that LRP5 and Runx2 expressions were lowest in group A patients (1–3 days after fracture) and the lowest expression of Osterix was observed in group C patients (8–14 days after fracture); RANKL expression was highest in group B patients (4–7 days after fracture). Significant differences were observed in LRP5 and Runx2 expression levels between the control group and group A; however, the differences between the expression levels of the factors in other groups were not significant. Differences in Osterix expression levels between the control group and group A, and between groups B and C were highly significant, whereas the difference in Osterix expression levels between group groups A and group B was not significant. The levels of RANKL expression levels between the control group and group A, and between groups A and B exhibited significant differences, whereas RANKL expression levels between control group B and group C were not significantly different (Fig. 2, Fig. 3).

## Discussion

Patients with osteoporosis are prone to developing OPF.[10] There was 20 percent chance of death with complications, and 20 percent or so of patients with recurrent fractures at the proximal femur of OPF patients.[11] PMOP results from a rapid decline in estrogen levels in women after menopause, in turn, resulting in a substantial increase in bone resorption that is mediated by osteoclasts, with a non-corresponding increase in osteoblasts. Therefore, osteoporosis develops when bone resorption is greater than bone formation.[12] Osteoporosis and PMOPF have become critical public health problems globally. The present study investigated the relationships among variations in mRNA and protein expressions of LRP5, Runx2, Osterix, and RANKL in bone specimens collected from patients with PMOPF.

Osteogenesis and osteoclastogenesis are regulated by the Wnt/β-catenin, BMP-2/Runx2/Osterix, and RANKL/RANK signaling pathways.[8–9, 13–17] Based on histological and molecular analyses, the early stage of fracture healing can be divided into: early inflammatory response stage (1 day after fracture), non-specific bone tissue anabolic stage (3 days after fracture), nonspecific catabolic stage (3 days to 1 week after fracture), and specific bone anabolic stage (more than 1 week after fracture). The entire fracture healing phase can be divided into three stages: hematoma organization, original callus formation, and callus reconstruction molding stages. Hematoma organization stage is typically completed within 2–3 weeks after fracture.[18–19] Consequently, we stratified patients with PMOPF into several groups to reflect the healing stages: Group A (1–3 days after fracture), Group B (4–7 days after fracture), and Group C (8–14 days after fracture).

Subsequently, we assessed the correlations among the variations in the expression of several factors in bones of patients and PMOPF development. No statistically significant difference was observed between patients with PMOPF and the controls with regard to age, height, and weight.

In the Wnt/β-catenin signaling pathway, the Wnt, LRP5/6 and FZD complex recruits Dvl and degradative complexes, which inhibit the phosphorylation of β-catenin by GSK-3β. Accumulation of non-phosphorylated β-catenin in the nucleus activates downstream Runx2 and other factors, which result in osteogenesis.[8, 20] LRP5 exists on the surface membranes of numerous cells.[21] Inhibition of LRP5 impairs proliferation and functioning of osteoblasts, in turn, affecting bone formation.[22] Glinka et al.[23] revealed that LGR5 regulated embryonic patterns and proliferation of stem cells through Wnt/β-catenin mediated agonist of R-cavernous signaling. In the present study, we observed substantial under-expression of LRP5 in patients with PMOPF, which impaired osteogenesis and could explain the observed osteoporosis in patients with PMOPF resulting from disrupted osteogenesis. Runx2 is a highly specific biomarker for osteogenesis. Expression of Runx2 gene is essential in bone formation and development. In particular, Runx2 up-regulates transcription of genes for several mineralization-related proteins in osteoblasts.[24, 25] The Wnt/β-catenin pathway directly regulates Runx2, strengthens osteogenic differentiation, and accelerates fracture healing.[27] In the present study, we observed significant under-expression of Runx2 in patients with PMOPF.

LRP5 regulates osteoblastosis and bone formation by activating the expression of Runx2.[26] LRP5 and Runx2 expression levels decreased considerably within 1–3 days after bone fracture but increased thereafter, which suggests that variations in the expressions of LRP5 and Runx2 were highly correlated with osteogenic stage.

BMP-2 modulates the transcription of BMP-2/Runx2/Osterix pathway-related osteogenic genes by activating the expression of Smads.[27] Smads relays TGF-β signals to the nucleus, regulates transcription of target factors, and induces Runx2 expression. Osterix is a key osteogenic factor downstream of Runx2,[28] and Runx2 up-regulates Osterix expression.[29] Osterix was under-expressed in patients with PMOPF, suggesting that the protein is a critical downstream osteogenic factor that influences PMOPF development.

A study by Kaback et al.,[30] which used mice models revealed that cartilage and tissue formation commences 7 days after fracture, and is sustained until day 10. Osterix was generally expressed after 14 days of fracture in the osteoblasts adjacent to the injury site, which promoted hardening of cartilage at the injured site. Numerous studies have demonstrated that BMP exerts a unique osteogenic effect, which fixes the fiber junction within 2 weeks after fracture.[8, 9] In the present study, Osterix expression was lowest in Group C patients (8–14 days after fracture), which was consistent with the findings.

OPG/RANKL/RANK pathway is essential in regulating differentiation of osteoclasts, including the expression of RANKL and RANK on respective cell membranes and pseudoreceptors of OPG. OPG can competitively inhibit the interaction between RANKL and RANK due to the high affinity between OPG and

RANKL, further disrupting the differentiation of osteoclasts, which, in turn, induces bone resorption and apoptosis.[10] Differentiation and maturation of osteoclasts is exclusively stimulated by RANKL, which also inhibits apoptosis in PMOPF.[31] We observed that RANKL was over-expressed in bone specimens obtained from patients with PMOPF, which was consistent with previous findings.[32] In contrast, the highest increase in RANKL expression level was observed within 4–7 days after fracture, which decreased thereafter, reflecting the role of RANKL in osteoclasts and PMOPF healing process.

Although we did not investigate the mechanisms underlying abnormal variations in the expressions of LRP5, Runx2, Osterix, and RANKL in bone specimens associated with PMOPF, our findings provide strong evidence that the Wnt/β-catenin, BMP-2/Runx2/Osterix, and OPG/RANKL/RANK pathways regulate osteogenesis and osteoclastogenesis in patients with PMOPF. The expression of the four factors varied with time after fracture, which could be associated with the various stages of bone repair. The characteristic variations in the expression of the factors can inform on ideal interventions effective in the prevention of PMOP and management of PMOPF.

# Abbreviations

PMOPF: Postmenopausal osteoporotic fracture; PMOP: Postmenopausal osteoporosis; OPF: Osteoporotic fracture; BMD: Bone Mineral Density.

# Declarations

**Availability of data and materials:** The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests** All authors declare no conflicts of interest.

**Consent for publication:** Not applicable.

**Ethics approval and consent to participate:** Ethics committee approval was received for this study from the Ethics Committee of People's Hospital of Sanshui (Medical Research in Guangdong Province 2019003) and the Ethics Committee of The Second Affiliated Hospital of Guangzhou Medical University (NSFC 81574002).

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**Authors' contributions** Bin Wang, Yanming Cao, Caiyuan Mai designed the study. Ram Ishwar Yadav, Jianliang Gao collected and analyzed the samples. Lei Pan performed statistical analysis. All authors wrote and approved the manuscript.

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**Data availability** All data are available upon request

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# Figures

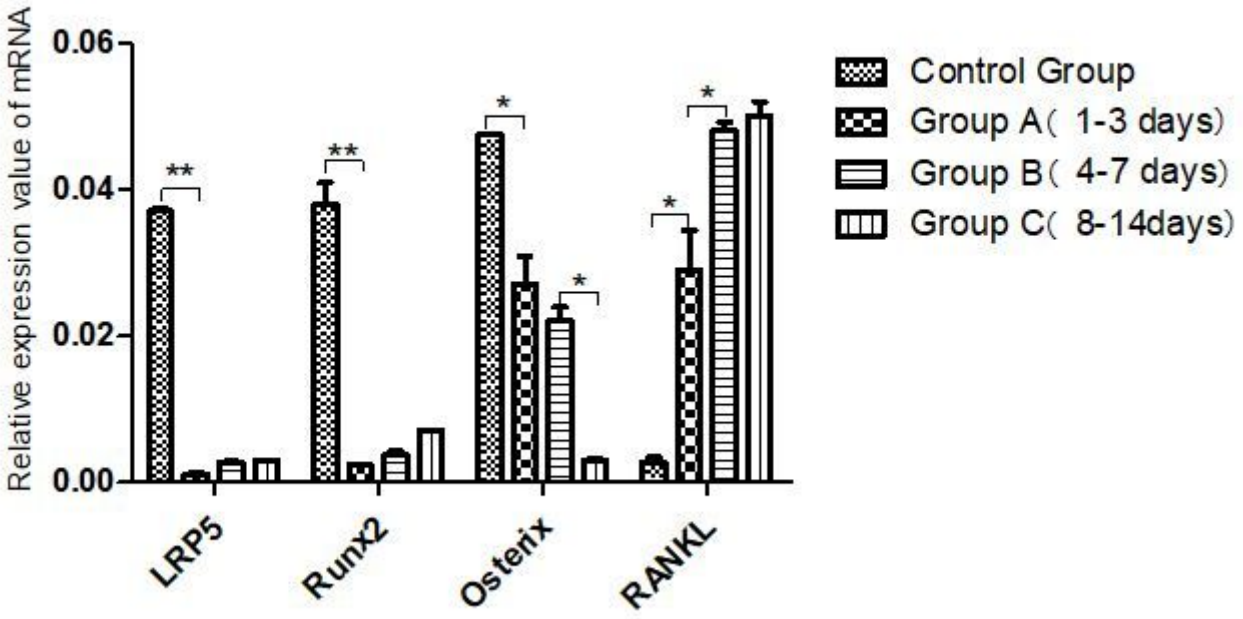
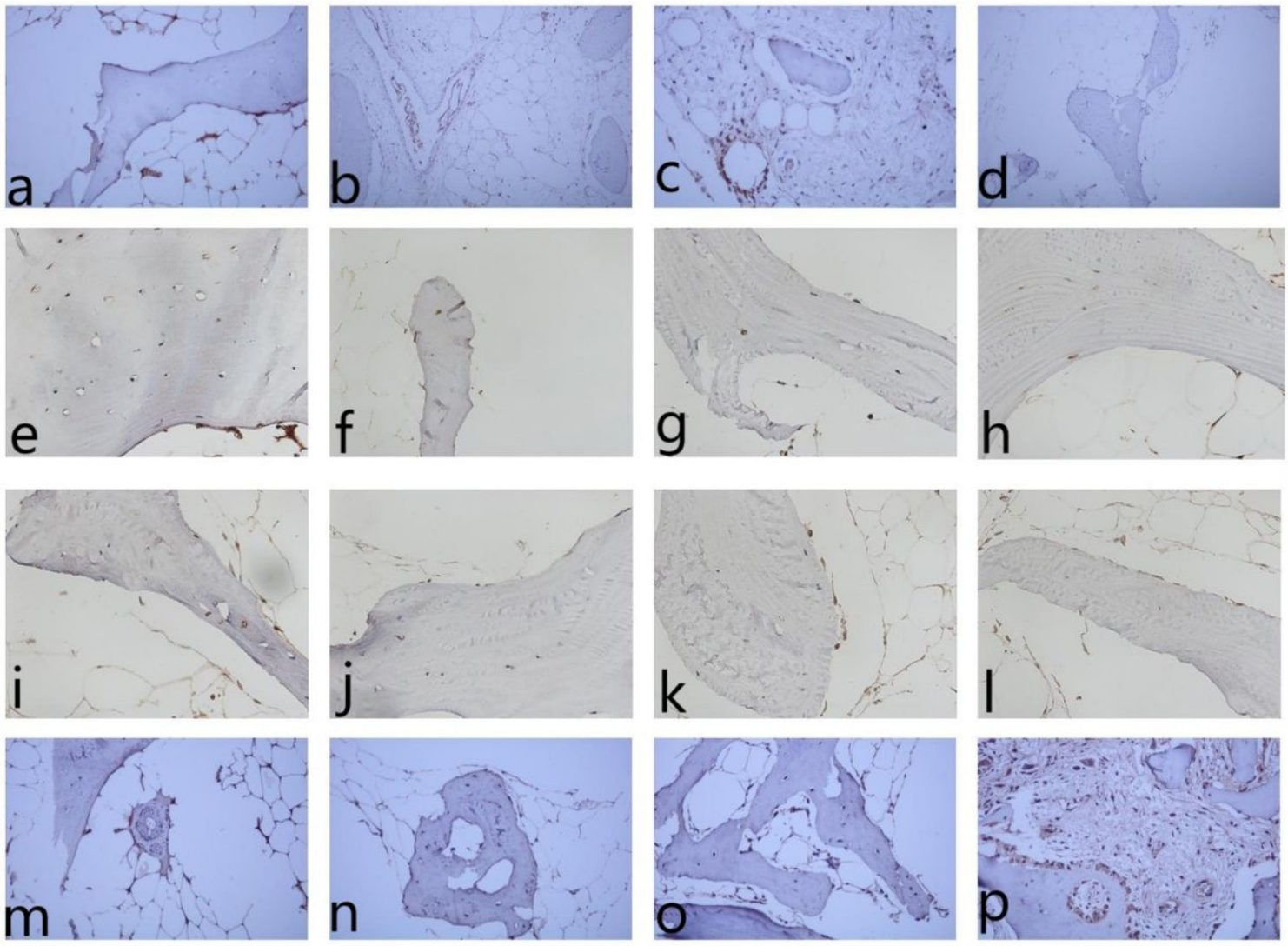



**Figure 1**

Relative mRNA expressions of LRP5, Runx2, Osterix, and RANKL in bone tissues of control and PMOPF groups (including stratified groups A, B, and C). The figure represents RT-qPCR analyses of all bone tissues.




## Figure 2

Immunohistochemical staining of LRP5, Runx2, Osterix, and RANKL in bone mass of participants in the control group and stratified PMOPF group (Groups A, B, and C) (400×). a, e, i, m-LRP5, Runx2, Osterix, and RANKL of the control group. b, f, j, n-LRP5, Runx2, Osterix, and RANKL of Group A. c, g, k, o-LRP5, Runx2, Osterix, and RANKL of Group B. d, h, l, p-LRP5, Runx2, Osterix, and RANKL of Group C.

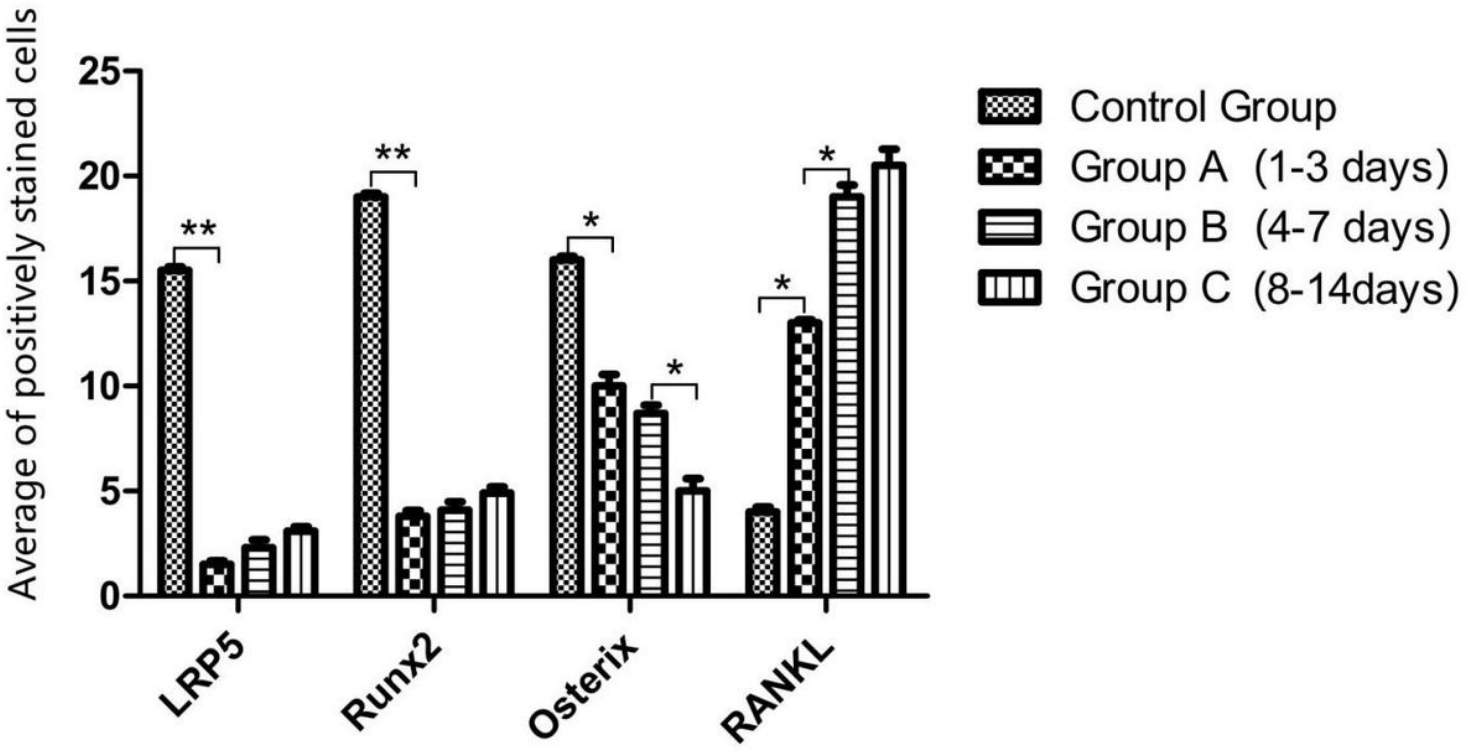



**Figure 3**

A histogram representing quantitative analyses results of immunohistochemical staining in LRP5, Runx2, Osterix, and RANKL in bone specimens.