Expression of The Factors Associated With Wnt/β-Catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL Pathways in Postmenopausal Osteoporosis Fracture

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Research article

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Abstract

Objective Detect and analyse the correlation between factors of related pathways (Wnt/β-catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL) and postmenopausal osteoporotic fracture (PMOPF).

Methods The postmenopausal patients with tibial fracture were divided into control group (36 cases) and PMOPF group (36 cases). Using RNAiso Plus method to extract total RNA of bone tissue, RT-qPCR method was used to detect the expression of each factor. Detected the levels of serum of factors by ELISA method in control group. PMOPF group was divided into group A-F according to the blood collection time interval (at different time period), use ELISA method to detect each factor's level. Compared the changes between control group and PMOPF group, and the subdivided groups of PMOPF group.

Results (1) RT-qPCR detected the expressions of LRP5, β-catenin, Runx2, C-myc, Osterix, OPG and LGR4 in PMOPF group were lower than control group ($P<0.05$), but the expression of RANKL was increased ($P<0.05$). (2) ELISA detected the serum levels of LRP5, β-catenin, Runx2, C-myc, Osterix, OPG and LGR4 decreased significantly ($P<0.05$), and RANKL increased significantly ($P<0.05$). LRP5 and Runx2 appeared the lowest in Group B (2-3 days after fracture); β-catenin and C-myc appeared the lowest in Group C (4-7 days after fracture); RANKL appeared the highest in Group C; Osterix appeared the lowest in Group D (8-14 days after fracture); OPG and LGR4 appeared the lowest in Group E (15-28 days after fracture).

Conclusion The related factors of Wnt/β-catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL pathway are closely related to the occurrence of PMOPF. LRP5 and Runx2 decreased to the lowest level within 3 days after fracture, β-catenin and C-myc decreased to the lowest level within 7 days after fracture, the results showed that these changes in Wnt/β-catenin osteogenesis pathway were consistent; Osterix decreased to the lowest level within 14 days after fracture, OPG and LGR4 decreased to the lowest level within 28 days after fracture, which may be related to the difficulty of short-term healing of PMOPF; RANKL increased to the highest level within 7 days after fracture, which may be associated with the increase in bone formation after PMOPF. According to the changes and characteristics of these factors in above pathways, we can regulate or intervene the occurrence and progression of PMOPF.

Introduction

Osteoporosis is a metabolic disorder associated with systemic bone aging and degradation. It is characterized by decreased bone mass, structural degradation, increased brittleness and prone to fracture[1]. There are more than 70 million Osteoporosis patients in China at present, the morbidity rate among wemen over 50 years old in China is 20.7%[2]. The incidence of osteoporosis in postmenopausal women is 2–3 times that of non-menopausal women[3]. Postmenopausal osteoporosis (PMOP) is an osteoporosis in women after menopause due to estrogen deficiency, resulting in bone loss and bone structure changes; PMOPF is a serious consequence of PMOP, which can significantly increase the disability rate, mortality rate and bring great family and socio-economic burden[4]. Therefore, the study of

the pathogenesis, affecting factors, and treatment of osteoporotic fracture (OPF) become the focus of scientific research and clinical practice[5]. Osteogenesis and osteoclastogenesis have been hot spots in the prevention and treatment of osteoporosis. The classical Wnt/β-catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL signaling pathways play important roles in regulating osteogenesis and osteoclastogenesis. LRP5, β-catenin, Runx2, C-myc, Runx2 and Osterix are key osteogenic factors, while OPG, RANKL, LGR4 and RANKL are key related osteoclast factors[6–11]. In this study, RT-qPCR was used to compare the expression of LRP5, β-catenin, Runx2, C-myc, Osterix, OPG, RANKL and LGR4 in bone tissues of PMOPF patients and postmenopausal patients with other types of fracture (control group). ELISA was used to compare the expression of above factors in serum of the two groups. The level of each factor at different time period of PMOPF was further detected by ELISA. Based on histological observation and molecular studies of fracture healing and healing stages, PMOPF group was divided into Group A (1 day after fracture, before operation), Group B (2–3 days after fracture), Group C (4–7 days after fracture), Group D (8–14 days after fracture), Group E (15–28 days after fracture), Group F (29–42 days after fracture). This study aimed to investigate the level of above factors in PMOPF patients in order to understand their expression in the occurrence and development at different time period of PMOPF.

Materials And Methods

Subjects

This study was approved by Ethics Committee of People's Hospital of Sanshui and all participants signed the informed consent. The patients completely consented to this experimental study, signed informed consent and other related matters. Total 72 postmenopausal patients with closed tibial fracture were recruited at the hospital of People's Hospital of Sanshui from January 2018 to November 2020. The Bone Mineral Density (BMD) of lumbar was measured by dual-energy X-ray absorptiometry. BMD, height, and body weight were recorded. Bone turnover markers including β-CTX, PINP, N-MID-OT, 25(OH) D and estradiol were measured. Patients with secondary osteoporosis, osteoarthritis, and pathological fracture due to non-osteoporosis were excluded. They were divided into two groups: PMOPF group (36 cases), and control group (36 cases) described as postmenopausal cases (Non-osteoporotic). Operation of Internal fixation to fractures in all patients were performed within one day of injury. Take the specimens (the curetted bone mass is equal or more than 200 mg) from bone tissue of the fracture end during operation. Bone tissue samples were collected and stored in liquid nitrogen rapidly. Fasting peripheral venous blood was collected within 1 day after injury (before operation) in all cases, After centrifugation, the serum was stored in a deep cryogenic refrigerator. Blood was further continuedly collected in group B-F of PMOPF group as described above (according to the blood collection time interval, at different time period), save the serum as above. Blood collected within 1 day in PMOPF group was belonged to group A.
100 mg bone tissues were grinded in liquid nitrogen. Total RNA was extracted from bone tissue by using RNAiso Plus (Bio-Rad, USA), and cDNA was synthesized by using PrimeScript RT Master Mix (Bio-Rad, USA). Real-time was performed using primers synthesized by Thermo Fisher Scientific (the sequences of the primers were shown in Table 1), and SYBR Premix kit (Bio-Rad, USA). Conditions of PCR were as

follow: 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 against GAPDH.

Table 1
(Primer sequence table)

Product name	Sequence (5'□3')
β-catenin Forward	CCAAGTGGGTGGTATAGAG
β-catenin Reverse	GGGATGGTGGGTGTAAGA
LRP5 Forward	CGTGTCCAGCGAGATCCT
LRP5 Reverse	CCAAGCGAGCCTTTCTACAC
C-myc Forward	CTCCTCCTCGTCGCAGTAGA
C-myc Reverse	GCTGCTTAGACGCTGGATTT
Runx2 Forward	CTCCTACCTGAGCCAGATGACG
Runx2 Reverse	GTGTAAGTAAAGGTGGCTGGATAGT
Osterix Forward	CCAAGTGGGTGGTATAGAG
Osterix Reverse	GGGATGGTGGGTGTAAGA
OPG Forward	TCAAGCAGGAGTGCAATCG
OPG Reverse	AGAATGCCTCCTCACACAGG
LGR4 Forward	CCCGCCAATTGTAGCTCTT
LGR4 Reverse	GTGGACTGCTCCGGGAA
RANKL Forward	ATGTGCTGTGATCCAACGAT
RANKL Reverse	TGAGACTCCATGAAAATGCAGA
GAPDH Forward	GGCATGGACTGTGGTCATGAG
GAPDH Reverse	TGCACCACCAACTGCTTAGC

ELISA

According to kit (TaKaRa, China), Use instructions to prepare standard and reference, draw standard curve, add blood sample and incubate as required, then measure the OD value at 450 nm, calculate the concentration of the detected substance in the sample according to the standard curve.

Data analysis

All experiments were performed at least 3 times. All data were expressed as mean ± standard deviation. The data were analyzed by nonparametric test (Mann-Whitney) using SPSS (version 16.0; Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant. Graphpad Prism 5 software was used for statistical analysis.

Results

The comparison of general data

In this study, 72 cases of fracture were included, and all of them were treated by surgery operation within 1 day. As shown in Table 2, there was significant difference in Lumbar BMD (T value) between two groups, but there were no significant differences in age, height and weight between two groups ($P > 0.05$). In Table 3, the level of β-CTX and PINP in control group was significantly lower than that of PMOPF group ($P < 0.05$), the other parameters including N-MID-OT, 25-(OH)D and estradiol were not statistically significant between two groups ($P > 0.05$).

Table 2
Comparison of general data between control group and PMOPF group (± s)

groups	control group	PMOPF group	P value
Age (years)	53.88 ± 4.02	54.39 ± 5.02	> 0.05
Height (cm)	163.11 ± 11.22	163.61 ± 10.02	> 0.05
Body weight (kg)	53.22 ± 8.90	52.44 ± 7.70	> 0.05
Lumbar BMD(g/□)	1.002 ± 0.055	0.6031 ± 0.022	< 0.05
T value	-1.260 ± 0.039	-3.119 ± 0.399	< 0.05

Table 3
Comparison of bone conversion markers between control group and PMOPF group(± s)

groups	control group	PMOPF group	P value
β-CTX (ng/ml)	0.240 ± 0.019	0.522 ± 0.029	< 0.05
PINP(ng/ml)	44.23 ± 9.16	55.71 ± 6.09	< 0.05
N-MID-OT(ng/ml)	15.09 ± 3.08	15.40 ± 1.18	> 0.05
25(OH)D(ng/ml)	14.19 ± 3.19	14.10 ± 3.05	> 0.05
estradiol(pmol/ml)	40.30 ± 11.28	39.17 ± 9.03	> 0.05

The mRNA expression of factors in control group and PMOPF group in bone tissues

RT-qPCR showed that LRP5, β-catenin, Runx2, C-myc, Osterix, OPG and LGR4 mRNA levels in bone tissues were lower in PMOPF group than in control group ($P < 0.05$), while RANKL mRNA level was higher in PMOPF group than in control group ($P < 0.05$) (Fig. 1).

Figure 1 mRNA expression of factors in bone tissues ($n = 36, P < 0.05$)

The serum levels of each factor in group A-F and control group

The serum LRP5, β-catenin, Runx2, C-myc, Osterix, OPG and LGR4 level of PMOPF group (A, B, C, D, E, F) declined significantly ($P < 0.05$) and RANKL increased significantly ($P < 0.05$). The expression level of each factor at different time period showed that LRP5 and Runx2 appeared the lowest in group B (within 3 days), β-catenin and C-myc appeared lowest in group C (4–7 days), RANKL appeared highest in group C (8–14 days), Osterix appeared lowest in group D (8–14 days), OPG and LGR4 appeared lowest in group E (15–28 days), above can be seen from the broken line diagram. There was no significant difference of each factor among qroups A-F ($P > 0.05$) (Table 4, Fig. 2).

Table 4
Comparison of factors expression levels between control group and PMOPF group (± s)

groups	Control group	Group A	Group B	Group C	Group D	Group E	Group F
LRP5 (pg/ml)	113.20 ± 10.11	95.28 ± 6.19	95.02 ± 5.40	96.01 ± 4.50	96.36 ± 3.19	96.44 ± 4.33	96.60 ± 3.59
β-catenin (ng/ml)	1.680 ± 0.015	1.016 ± 0.008	1.009 ± 0.027	1.005 ± 0.020	1.013 ± 0.004	1.020 ± 0.009	1.017 ± 0.017
Runx2 (ng/ml)	0.391 ± 0.013	0.216 ± 0.009	0.209 ± 0.027	0.222 ± 0.008	0.227 ± 0.004	0.230 ± 0.028	0.231 ± 0.017
C-myc (μg/l)	1.14 ± 0.12	0.77 ± 0.02	0.75 ± 0.06	0.71 ± 0.15	0.72 ± 0.04	0.75 ± 0.06	0.76 ± 0.03
Osterix (ug/ml)	1.30 ± 0.35	0.86 ± 0.08	0.84 ± 0.03	0.84 ± 0.02	0.83 ± 0.03	0.84 ± 0.01	0.85 ± 0.02
OPG (ng/ml)	89.7 ± 1.30	80.5 ± 1.00	80.3 ± 1.31	80.2 ± 1.40	80.1 ± 1.22	80.0 ± 1.29	80.2 ± 0.83
RANKL (ng/ml)	0.22 ± 0.06	0.91 ± 0.11	0.93 ± 0.02	0.94 ± 0.06	0.93 ± 0.11	0.93 ± 0.29	0.90 ± 0.27
LGR4 (ng/ml)	116.22 ± 29.28	82.01 ± 11.31	81.98 ± 17.03	81.03 ± 7.93	81.00 ± 7.78	79.02 ± 10.28	82.37 ± 6.26

Discussion

PMOP is due to the rapid decline in estrogen levels in women after menopause and osteoclast resulting in a significant increase in bone resorption, while osteoblasts did not increase synchronously, resulting in

bone resorption greater than bone formation, it's a metabolic disease[12]. The classical Wnt/β-catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL/RANK signaling pathways play important roles in regulating osteogenesis and osteoclastogenesis[6–11]. Based on histological observation and molecular studies of fracture healing, the early stage of fracture healing is divided into early inflammatory response stage (within 1 days after fracture), non-specific anabolic stage (within 3 days after fracture), non-specific catabolism stage (3 days to 1 week after fracture) and more specific anabolic stage of bone tissue (1 week after fracture); while the typical fracture healing stage was divided into three stages: hematoma organization stage (2–3 weeks after fracture), original callus formation stage (4–6 weeks after fracture), callus reconstruction molding stage (more than 1 years after fracture)[13–14]. Therefore, PMOPF group was further divided into group A (within 1 day after fracture), group B (2–3 days), group C (4–7 days), Group D (8–14 days), Group E (15–28 days) and Group F (29–42 days).

In this study, bone tissue and serum samples from fracture patients were used to explore the correlation between the expression of above factors and PMOPF. We analyzed 72 cases, there were no statistical differences in age, height and weight between the two groups. And there were also no statistical differences in N-MID-OT, 25-(OH)D and estradiol, which was consistent with the changes of bone turnover markers in the two groups. There were statistical differences in β-CTX and PINP between the two groups, which can be illustrated that the bone conversion of PMOPF group was higher than control group.

In the typical Wnt/β-cateinin signaling pathway, Wnt combined with LRP5/6 and FZD to construct complex, recruit Dvl and degradative complex, and inhibit the phosphorylation of β-catenin by GSK-3β. The non-phosphorylated β-catenin will gradually accumulate and enter into nucleus to activate downstream Runx2, C-myc and other factors, resulting in osteogenic differentiation[6, 15].

LRP5 exists on the surface of multiple cell membranes[16]. Some studies have found that the function and proliferation of osteoblast are blocked after LRP5 deletion, which affects bone formation[17]. Glinka's[18] studies suggest that LGR5 can regulate embryonic patterns and the proliferation of stem cell through Wnt/β-catenin mediating agonist R-cavernous signaling. This experiment found that the expression of LRP5 in PMOPF group was significantly decreased, it was related to the limitation of osteogenesis, there may also be many factors such as sclerosis protein binding to LRP5/6, which can inhibited Wnt/β-catenin pathway and caused resistance to bone formation[19]. β-catenin is the most critical factor in Wnt/β-catenin pathway, by activating it's downstream factors, it can affect osteoblast and related expression. Hill TP[20] found that the knockdown of gene β-catenin in mice can increased cartilage production, but osteoblast was decreased, which revealed that β-catenin is necessary for early MSCs to differentiate. The expression of β-catenin decreased in PMOPF group, indicating that the classical Wnt pathway is in an inhibitory state, which could be one of the reasons for osteoporosis, this state reflects the insufficient osteogenesis after PMOPF. Runx2 is an earliest and highly specific marker factor in osteogenesis, it is a key factor that led to bone fragility. It is a necessary gene in bone formation and bone development. Runx2 can up-regulates transcription of various mineralization-related protein genes in osteoblast[21, 22]. Studies have confirmed that the activation of Wnt/β-catenin pathway can

directly regulate Runx2, strengthen osteogenic differentiation and accelerate fracture healing[23]. The expression of Runx2 decreased significantly in PMOPF group, indicating that the low expression of Runx2 may be an important factor affecting PMOPF. C-myc is an important downstream factor of Wnt/β-catenin pathway, Wnt-3a can activate β-catenin signal, thus increase the expression of C-myc[24], C-myc further promote cell cycle from G1 to S, accelerate osteoblast differentiation and proliferation[25]. The expression of C-myc decreased in PMOPF group, which suggest that C-myc as a target factor at the downstream of gene Wnt, can further verified the insufficient osteogenesis at osteoporosis transcription level.

The expression of LRP5, β-catenin, Runx2 and C-myc in PMOPF group was consistently decreased, which indicated that the osteogenesis of the four factors in the Wnt/β-catenin signaling pathway had highly coincident function, and further verified their close interaction of positive correlation and mutual promotion in the pathway. Studies have shown that LRP5 regulate osteoblast development and bone formation by activating Runx2 expression[23]. LRP5 and Runx2 both decreased to the lowest level within 3 days, and then increased, which highly indicated that their changes were consistent at the osteogenic stage. Chen Yan's[26] studies showed that β-catenin have different functions at different time of fracture repair. β-catenin can inhibit its downstream target C-myc by inhibiting glycolysis and glutamine[27]. The concentration of β-catenin in cytoplasm determines whether the expression of C-myc in nucleus can be activated[25]. β-catenin and C-myc all decreased to the lowest within 7 days after fracture and then increased, and the synchronism of the two factors was consistent with the above related studies. The above four factors in the same pathway increased so little (no statistical significance), which may be related to the difficulty healing of PMOPF in the short term.

- In BMP-2/Runx2/Osterix pathway, BMP-2 modulates the transcription of related osteogenic genes by activating Smads signal, thus induce the expression of Runx2[28]. Smads transmit the signal TGF-β to nucleu and regulate the transcription of target factors, and then induce the expression of Runx2, Runx2 can further regulate Osterix[29]. Runx2 is the core factor affecting osteogenesis in Wnt/β-catenin and BMP-2/Runx2/Osterix pathways, so the expression of Runx2 in our study is obvious. In this study, the expression of Runx2 in PMOPF group was decreased, which indicated that Runx2 may affect the bone formation of PMOP by down-regulating the expression of osteoblasts. Osterix is an important osteogenic factor in the downstream of Runx2[30]. The level of Osterix is dependent on the level of Runx2, Osterix only expressed in osteoblastic cells[31]. We found the expression of Osterix in PMOPF group was decreased, which can indicated that Osterix was an important downstream osteogenic factor and it may be one of the factors resulting in PMOP.

Kaback's[32] study showed that after the fracture model in mice was established, cartilage and tissue started to form after 7 days and sustained 10 days, meanwhile the mRNA level of Sox9 increased; Osterix was mainly expressed in the osteoblast near the site of fracture about 14 days later, meanwhile the expression of sox9 decreased, during this time cartilage became hard bone at the injured place. Numerous studies and clinical manifestations confirmed that BMP had unique osteogenic effect, and fiber junction was completed at at 2 weeks' time after fracture[8, 9]. The level of Osterix in BMP-

2/Runx2/Osterix pathway appeared the lowest in group D (8–14 days), which was consistent with above study.

OPG/RANKL/RANK pathway is a very important signaling pathway in osteoclast differentiation, including: RANKL, RANK located on the cell membrane and pseudoreceptor OPG. There is a high affinity between OPG and RANKL, and OPG can competitively inhibits the interaction between RANKL and RANK, further inhibits the differentiation of osteoclast, cause bone resorption and induce apoptosis[10]. The decrease of OPG expression in PMOPF indicated that its function of competitively inhibiting osteoclast is growing weakern and promotes the further occurrence of osteoporosis. RANKL is the only factor to stimulate the differentiation and maturation of osteoclast up to now, and it can prevent apoptosis[33]. This study found that the expression of RANKL increased in PMOPF group, which was consistent with the thoery that RANKL can cause osteoporosis by promoting differentiation of osteoclast. RANKL has strongest expression in bone tissue[34], so our study found that the expression increased more obviously in bone tissue than in serum. The level of OPG decreased to the lowest within 28 days after fracture, which may be related to the difficulty of short-term healing of PMOPF. RANKL increased to the highest within 7 days after fracture, and then a small increase or decrease occurred, reflecting their trend in osteoclast, which may be associated with the increase in bone formation after PMOPF.

LGR4/RANKL/RANK pathway plays an important role in osteoclastogenesis. LGR4 is a receptor of RANKL. LGR4 competes with RANK to combine with RANKL, and inhibit typical RANKL signals during osteoclast differentiation[10]. Binding to LGR4, RANKL activates Gαq and GSK-3β signaling pathway and modulates osteoclast differentiation and bone resorption[11]. We found that the expression of LGR4 was decreased in PMOPF group, which suggested that the fuction of inhibiting osteoclast of LGR4 became weakened, meanwhile RANKL increased in PMOPF group, which was consistent with the theory that the combination of LGR4 competes with RANK and combines with RANKL, then inhibits the osteoclast of RANKL/RANK signaling pathway. LGR4 decreased to the lowest level within 28 days after fracture, reflecting the slow and lasting trend of LGR4, which may be related to the difficulty of short-term healing of PMOPF. Negative regulation to osteoclast of LGR4 can be used to treat osteoporosis and other disease[35]. The whole genome sequencing of human also found that LGR4 has a great function to osteoporosis[36]. Denosumab as an specifical antibody through targeting RANK, can cause osteoclast inactivation by block the combination of RANKL and RANK, but its side effects include calcium homeostasis imbalance and so on[37]. The related studies in mice showed that compare with OPG to RANKL, LGR4-ECD protein has lower affinity with RANKL, and has little negative physiological effect on mice. The above studies show that the advantages of LGR4 and the side effects of antagonistic RANKL have great value in the treatment of osteoporosis[11]. Through the above analysis, There may exist some related relation and mechanism according with the variation tendency of RANKL and LGR4 (RANKL appeared highest during 4–7 days, LGR4 appeared lowest during 15–28 days) in our study. The peak time of RANKL appeared shorter than LGR4, Does it related to the therapeutic effect? We can further study it.

Although this study has limitations that we did not investigate the mechanisms underlying the abnormal expression of factors in bone tissue and serum of PMOPF patients, our results provide strong evidence that the factors related to Wnt/β-catenin, BMP-2/Runx2/Osterix, OPG/RANKL and LGR4/RANKL pathways affected osteogenesis and osteoclastogenesis, and each factor changed at a different subdivided time period in PMOPF group. According to the changes and characteristics of these factors in these pathways, we can regulate or intervene the occurrence and progression of PMOPF.

Abbreviations

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

Declarations

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).

Consent for publication: Not applicable.

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.

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Authors' contributions Bin Wang designed the study and were responsible for writing and review. Caiyuan Mai and Xiping Luo collected, analyzed the samples, Yanming Cao interpreted the data for the work, Zhizhong Wang and Xinxu Li performed statistical analysis. All authors wrote and approved the manuscript.

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Figures

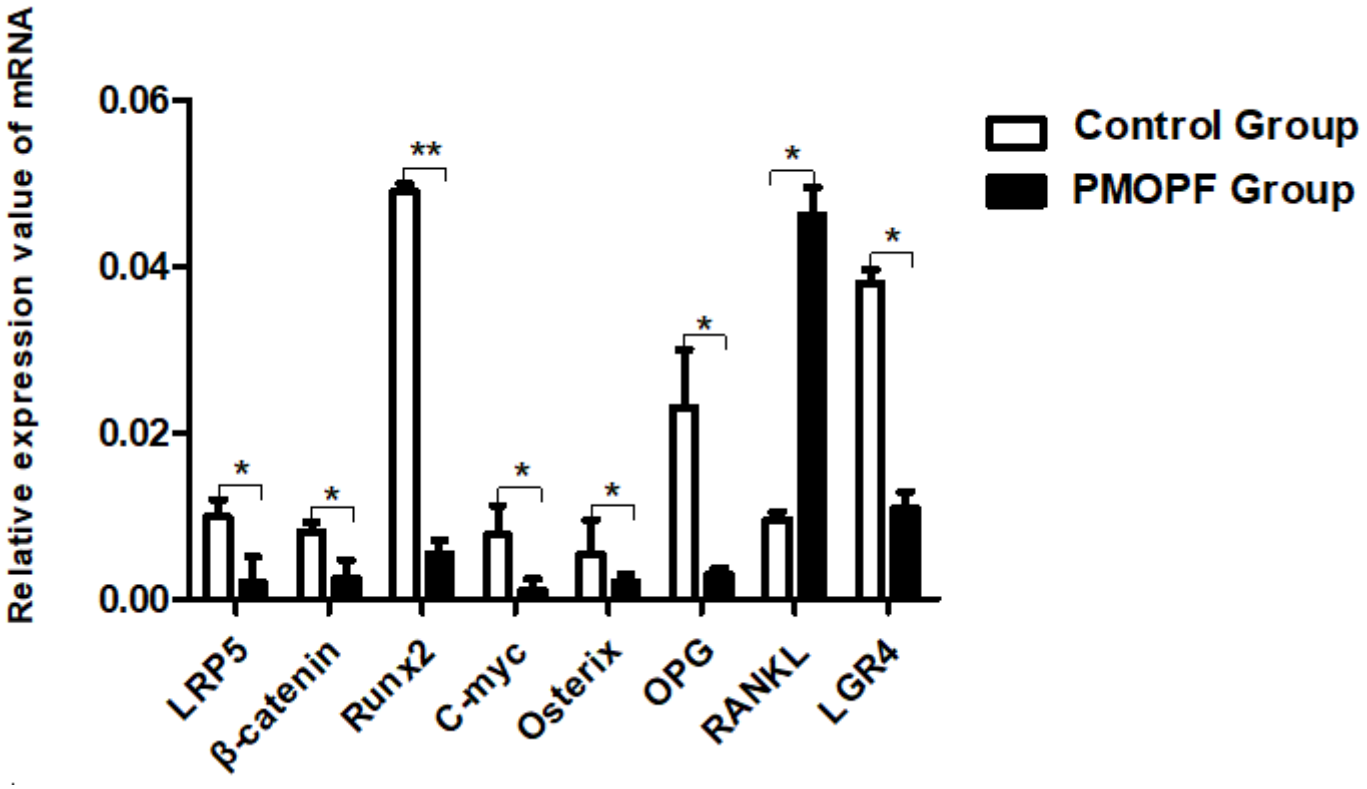

Figure 1

mRNA expression of factors in bone tissues (n=36,P<0.05)

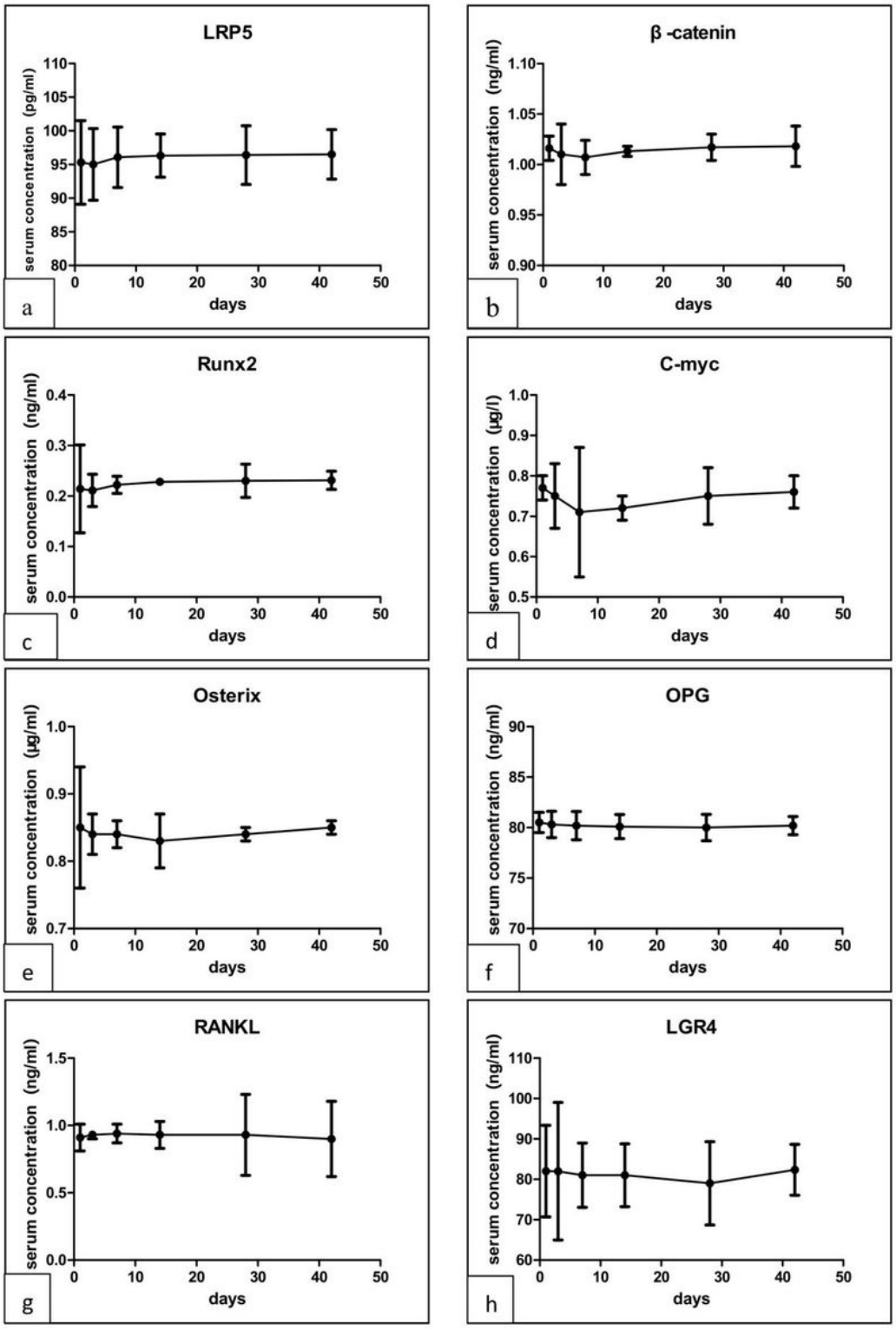

Figure 2

Line diagram of serum levels of each factor in group A-F (n=36)