Int J Clin Exp Med 2020;13(10):7979-7985
www.ijcem.com /ISSN:1940-5901/IJCEM0116373

Original Article

Intestinal microecology and expressions of serum fibroblast growth factors in women with postmenopausal osteoporosis

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Received June 16, 2020; Accepted July 19, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Objective: We aimed to investigate the intestinal microecology and fibroblast growth factor (FGF) expression in women with postmenopausal osteoporosis (PMO) and their clinical value in the diagnosis of PMO. Methods: A total of 214 postmenopausal women were analyzed retrospectively. The women were divided into the abnormal group (103 cases) and the normal group (111 cases) according to their bone mineral density (BMD). The levels of intestinal microflora and serum FGF-21, FGF-23, α-klotho, and β-klotho were compared between the two groups, and the correlations of intestinal microflora, FGF-23, α-klotho, and β-klotho with BMD were analyzed. The women in the abnormal group were further divided into the osteoporosis subgroup (59 cases) and the osteopenia subgroup (44 cases) for comparison. Receiver operating characteristic (ROC) curve was plotted to analyze the diagnostic value of intestinal microflora, FGF-23, α-klotho and β-klotho for PMO. Results: Compared with the normal group, the abnormal group had lower levels of bifidobacterium, lactobacillus, α-klotho, and β-klotho and higher levels of enterococcus and FGF-23 (all $P<0.05$). BMD was closely correlated with the levels of intestinal microflora, FGF-23, α-klotho, and β-klotho (all $P<0.05$). Compared with the osteopenia group, the osteoporosis group had lower levels of bifidobacterium, lactobacillus, α-klotho, and β-klotho and higher levels of enterococcus and FGF-23 (all $P<0.05$). ROC analysis revealed the clinical value of lactobacillus, bifidobacterium, enterococcus, FGF-23, α-klotho, and β-klotho in diagnosing PMO (all area under the curve >0.70). Conclusion: The levels of intestinal microflora and serum FGF-23, α-klotho, and β-klotho are closely associated with the development of PMO, and these markers have clinical value in the diagnosis of PMO.

Keywords: Menopause, osteoporosis, intestinal microflora, fibroblast growth factor

Introduction

Menopause is a normal physiological phenomenon usually in women over the age of 45. Due to the degradation of the ovarian function and insufficient estrogen secretion in postmenopausal women, this population can develop postmenopausal osteoporosis (PMO), a systemic metabolic disorder. The disease is characterized by the decrease of bone mass and the abnormality of bone tissue structure and can increase bone fragility and risks of fracture [1-3]. Epidemiological survey revealed that over half of the postmenopausal women experienced osteoporotic fracture, and the incidence rate increases with age [4]. The consequences of osteoporotic fracture are severe. For instance, vertebral fracture is one of the main risk factors for pain and lameness, and the one-year mortality rate after the hip fracture is 24%-30%. Therefore, it is essential to explore the mechanism of PMO for the prevention and treatment of this disease [5].

Intestinal microecology is the largest microecosystem in human body, and its dynamic equilibrium is closely associated with human health. Some studies have reported certain correlations between the dysbiosis of the intestinal microecology and the occurrence and development of various metabolic diseases, which provides the medical researchers with some new insights into the diagnosis and treatment of osteoporosis [6-8]. Fibroblast growth

Table 1. Primer sequences

Primer	Sequence
Bifidobacterium	
Forward primer	GGGTGGTAATGCCCGGATG
Reverse primer	TAAGCCATGGACTTTCACACC
Lactobacillus	
Forward primer	AGCAGTAGGGAATCTTCCA
Reverse primer	ATTTCACCGCTACACATG
Enterobacterium	
Forward primer	GAAGGTCCCCACTTG
Reverse primer	CAATCGGAGTTCTTCGTG
Enterococcus	
Forward primer	AACCTACCCATCAGAGGG
Reverse primer	GACGTTCAGTTACTAACG

factor (FGF) family consists of 23 members. They and their receptors participate in various biological functions, including cell proliferation, differentiation, and migration, tumor development, and tissue repair. At present, most of the studies on the intestinal microflora in osteoporosis patients are fundamental research [9]. There have been few reports on the levels of intestinal microflora in PMO patients, and the relationship between the intestinal microflora and FGF remains unclear. Therefore, we aimed to analyze the intestinal microecology and the expression of FGF in women with PMO in order to provide more guidance for the clinical diagnosis and treatment of this disease.

Materials and methods

Baseline data

A total of 214 postmenopausal women who received bone mineral density (BMD) test in The 910th Hospital of PLA between January 2018 and January 2019 were selected for the study. According to their bone mineral density, the women were divided into the abnormal group (103 cases) and the normal group (111 cases). In the abnormal group, women's age, menopausal period, and body mass index (BMI) were 58.7±5.6 years, 10.2±3.3 years, and 23.21±1.14 kg/m^2, respectively; in the normal group, the age, menopausal period, and BMI were 58.3±5.5 years, 10.4±3.2 years, and 23.38±1.21 kg/m^2, respectively. There were no intergroup differences in age, BMI and other baseline data (all P>0.05), indicating that our study results are comparable between the two groups. Informed consent was obtained from all participants and their family members, and the study was approved by the Ethics Committee of the 910th Hospital of PLA.

Inclusion and exclusion criteria

Inclusion criteria were: 1) women who aged between 45-70 years and their menstrual period had stopped for over one year; 2) women who planned to receive BMD test; 3) women who did not have malignant tumor or autoimmune diseases; 4) women who had no gastrointestinal disease such as constipation or diarrhea and had no history of surgery; 5) women who hadn't taken any antibiotics, gastric motility medication, intestinal probiotics, or other drugs that may influence the study results over the past half-year; 6) women in the abnormal group met the diagnostic criteria of osteoporosis and BMD reduction [10].

Exclusion criteria were: 1) women with osteomalacia; 2) women with diabetes, hyperthyroidism, or other diseases that may affect the bone metabolism rate; 3) women with other diseases that may affect intestinal microflora; 4) women who took drugs that may affect bone metabolism over the past half-year; 5) women with ovary, uterus, or breast-related diseases; 6) women with bone metabolic diseases; 7) women who had poor compliance.

Outcome measures

The BMD values of lumbar vertebrae and proximal femur in all participants were measured with an ultrasonic bone densitometer (AOS-100SA, Aloka, Japan) [10]: if the t value of BMD was over -1.0 standard deviation, the bone density was considered as normal; a t value between -1.0 and -2.5 standard deviation indicated presence of osteopenia; a t value no more than -2.5 standard deviation indicated presence of osteoporosis.

Within 24 hours after grouping, fresh fecal samples were obtained from the participants before the participants received any treatments. The fecal samples were kept in sterile bags and stored at -80°C immediately before they were sent to the laboratory for subpackage and DNA extraction. Levels of bifidobacterium, lactobacillus, enterobacterium, and enterococcus were detected by PCR, and the primer sequences are listed in **Table 1**. RT-PCR was carried out according to the manufactur-

Table 2. Intestinal microflora levels in the two groups

	Bifidobacterium ($*10^4$)	Lactobacillus ($*10^5$)	Enterobacterium ($*10^5$)	Enterococcus ($*10^4$)
Abnormal group (n=103)	6.11±0.78	5.12±0.53	11.98±1.56	7.03±0.87
Normal group (n=111)	9.33±0.92	7.86±0.44	11.69±1.43	6.54±0.91
t	27.511	41.260	1.419	4.020
P	<0.001	<0.001	0.157	<0.001

Table 3. Serum levels of FGF21, FGF23, α-klotho, and β-klotho in the two groups

	FGF21 (pg/mL)	FGF23 (pg/mL)	α-klotho (pg/mL)	β-klotho (pg/mL)
Abnormal group (n=103)	194.76±84.43	247.68±35.33	417.86±55.21	462.93±98.74
Normal group (n=111)	179.54±88.56	194.22±37.65	447.28±57.92	489.51±86.05
t	1.285	10.690	3.797	2.102
P	0.200	<0.001	<0.001	0.037

Note: FGF: fibroblast growth factor.

ers' instructions of the kit (Takara Bio, Japan). The reaction conditions were as follows: predenaturation at 95°C for 30 s, denaturation at 95°C for 5 s, and annealing at 60°C for 30 s. Data were processed with MJ OptionMonitor software version 3.0. The copy number of 16S rDNA of the intestinal microflora in each fecal sample was calculated through the linear relationship between the copy number and C(t).

On the next morning after grouping, 5 mL of venous blood was extracted from each participant on an empty stomach, and the samples were centrifuged at 3,000 rpm for 5 min to separate the serum. The serum levels of FGF-21, FGF-23, α-klotho, and β-klotho were detected by enzyme-linked immunosorbent assay (ELISA, microplate reader: Spectra-Max-Paradigm, Molecular Devices, USA). All test kits were purchased from Beyotime Biotechnology, China.

Based on the BMD values, women in the abnormal group were further divided into the osteoporosis group (59 cases) and the osteopenia group (44 cases). The levels of the intestinal microflora and the serum levels of FGF-21, FGF-23, α-klotho, and β-klotho were compared between these two groups.

Statistical analysis

SPSS 20.0 software was applied for statistical analysis. Count data are presented as number and percentage and were compared by χ^2 test; measurement data are expressed as mean ± sd and were compared by independent samples t-test. Pearson correlation method was used to analyze the associations between BMD and the levels of intestinal microflora, FGF-23, α-klotho, and β-klotho. Receiver operating characteristic curve (ROC) was plotted to analyze the clinical value of intestinal microflora, FGF-23, α-klotho, and β-klotho in the diagnosis of PMO. P<0.05 indicated a statistically significant difference.

Results

Intestinal microflora levels in the two groups

Compared with the normal group, the abnormal group had higher level of enterococcus and lower levels of bifidobacterium and lactobacillus (all P<0.05). There was no difference in the level of enterobacterium between the two groups (P>0.05). See **Table 2**.

Serum levels of FGF-23, FGF-21, α-klotho, and β-klotho in the two groups

Compared with the normal group, the abnormal group had higher level of FGF-23 and lower levels of α-klotho and β-klotho (all P<0.05). There was no intergroup difference in the level of FGF-21 (P>0.05). See **Table 3**.

Correlations between BMD and various markers

BMD was negatively correlated with the levels of enterococcus and FGF-23 and was positively

Table 4. Correlations of BMD with various markers (r)

	Bifidobacterium ($*10^4$)	Lactobacillus ($*10^5$)	Enterococcus ($*10^4$)	FGF23 (pg/mL)	α-klotho (pg/mL)	β-klotho (pg/mL)	BMD
Bifidobacterium ($*10^4$)		0.213	-0.221	-0.227	0.328	0.347	0.335
Lactobacillus ($*10^5$)	0.213		-0.175	-0.246	0.295	0.229	0.317
Enterococcus ($*10^4$)	-0.221	-0.175		0.314	-0.251	-0.223	-0.436
FGF23 (pg/mL)	-0.227	-0.246	0.314		-0.297	-0.313	-0.713
α-klotho (pg/mL)	0.328	0.295	-0.251	-0.297		0.144	0.445
β-klotho (pg/mL)	0.347	0.229	-0.223	-0.313	0.144		0.398
BMD	0.335	0.317	-0.436	-0.713	0.445	0.398	

Note: BMD: bone mass density; FGF: fibroblast growth factor.

Table 5. Intestinal microflora levels in the osteoporosis and the osteopenia groups

	Bifidobacterium ($*10^4$)	Lactobacillus ($*10^5$)	Enterobacterium ($*10^5$)	Enterococcus ($*10^4$)
Osteoporosis group (n=59)	5.22±0.81	4.43±0.56	11.74±1.47	7.47±0.88
Osteopenia group (n=44)	6.85±0.94	5.69±0.51	12.08±1.55	7.02±0.76
t	9.432	11.730	1.134	2.718
P	<0.001	<0.001	0.259	0.008

Table 6. Serum levels of FGF21, FGF23, α-klotho, and β-klotho in the osteoporosis and the osteopenia groups

	FGF21 (pg/mL)	FGF23 (pg/mL)	α-klotho (pg/mL)	β-klotho (pg/mL)
Osteoporosis group (n=59)	201.48±76.65	256.94±26.80	369.59±68.36	400.11±67.77
Osteopenia group (n=44)	187.58±82.34	223.20±30.36	505.80±64.05	547.16±65.24
t	0.882	7.565	10.289	11.068
P	0.380	<0.001	<0.001	<0.001

Note: FGF: fibroblast growth factor.

correlated with the levels of bifidobacterium, lactobacillus, α-klotho, and β-klotho (all P< 0.05). See **Table 4**.

Intestinal microflora level in the osteoporosis and the osteopenia groups

Compared with the osteopenia group, the osteoporosis group had higher level of enterococcus and lower levels of bifidobacterium and lactobacillus (all P<0.05). There was no intergroup difference in the level of enterobacterium (P>0.05). See **Table 5**.

Serum levels of FGF-23, FGF-21, α-klotho, and β-klotho in the osteoporosis and the osteopenia groups

Compared with the osteopenia group, the osteopenia group had higher serum level of FGF-23 and lower levels of α-klotho and β-klotho (all P<0.05). There was no difference in the serum level of FGF-21 between the two groups (P>0.05). See **Table 6**.

ROC curve analysis

The results showed that enterococcus, bifidobacterium, lactobacillus, FGF-23, α-klotho, and β-klotho had clinical value in the diagnosis of PMO (all area under the curve (AUC) >0.70). See **Table 7** and **Figure 1**.

Discussion

Bone homeostasis is a dynamic equilibrium between bone resorption and bone formation [11]. The decrease of estrogen level can cause bone resorption rate to exceed bone formation rate. Therefore, in the early stage of menopause, low estrogen level can result in bone loss in women [12]. Studies have shown that

Table 7. ROC curve results

	Cutoff value	AUC	95% CI	Sensitivity	Specificity	P
Bifidobacterium (*10^4)	6.064	0.874	0.826, 0.922	0.800	0.966	<0.001
Lactobacillus (*10^5)	4.674	0.808	0.737, 0.879	0.832	0.864	<0.001
Enterococcus (*10^4)	6.684	0.727	0.652, 0.802	0.847	0.639	<0.001
FGF23 (pg/mL)	246.962	0.900	0.856, 0.943	0.864	0.871	<0.001
α-klotho (pg/mL)	406.131	0.803	0.741, 0864	0.819	0.712	<0.001
β-klotho (pg/mL)	452.745	0.840	0.784, 0.895	0.787	0.814	<0.001

Note: ROC: receiver operating characteristic; AUC: area under the curve; CI: confidence interval; FGF: fibroblast growth factor.

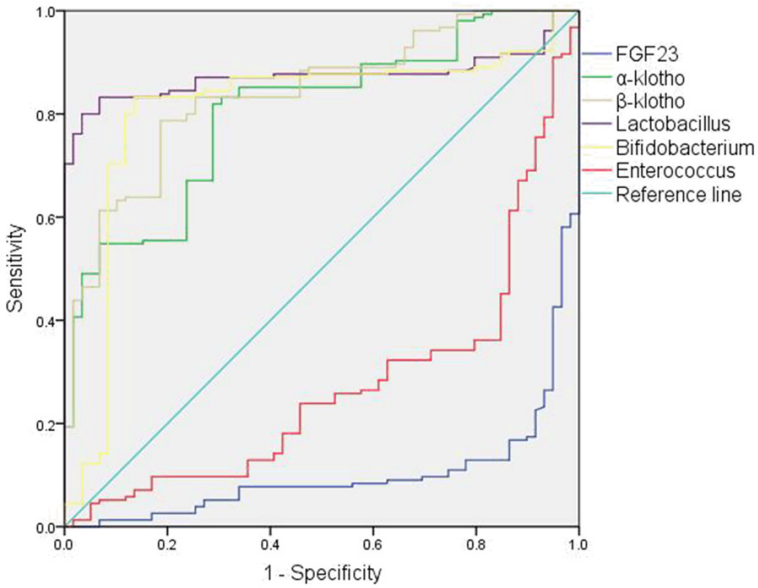

Figure 1. ROC curve results. FGF: fibroblast growth factor; ROC: receiver operating characteristic.

intestinal microflora serves an essential role in sex hormone-associated bone loss [13-16]. One study on germ-free mice reported that even if the level of sex hormone decreased in the mice, the level of osteoclast growth factor did not increase; when the mice with sex hormone deficiency were treated with probiotics, the inflammation in mice' intestinal tract and bone marrow was alleviated and the bone loss was prevented. However, these protective effects could not be achieved when the mice were treated with non-beneficial bacteria [17]. Wang et al. analyzed the bacterial diversity and found that compared with healthy people, specific intestinal microflora was much higher in patients with osteoporosis [18]. In the present study, we found that compared with the normal group, the abnormal group had decreased levels of beneficial bacteria such as lactobacillus and bifidobacterium and increased level of enterococcus. Moreover, a magnitude of these changes were greater in the osteoporotic patients than in the osteopenia group. These results are consistent with the previous study results and demonstrate a close association between the intestinal microflora and the occurrence and development of PMO. Although the detailed molecular mechanism behind this remains unclear, the findings can still provide some new insights into the clinical treatment of PMO. Lactobacillus, bifidobacterium, and enterococcus may have diagnostic value for PMO.

FGF family members have diverse biological functions. In recent years, FGF proteins can be obtained by cloning and purification of FGF in intestinal microflora. The results of our study showed that the levels of FGF-23 and its receptor are closely related to the level of intestinal microflora, which may provide the researchers with some new insights into the interaction between FGF-23 and intestinal microflora. FGF-23 is mainly secreted by osteoblasts and can promote the secretion of urine phosphorus to reduce the reabsorption of phosphate by renal tubules, thereby regulating the levels of calcium and phosphorus in bone metabolism. In recent years, FGF-23 has been widely used in the diagnosis of kidney diseases. A marked increase in the serum level of FGF-23 can indicate the risks of hypercalcemia and hyperphosphatemia. Some studies revealed that FGF-23 level may be closely related to metabolic bone disease [19]. FGF-23 can bind to the co-receptor α-klotho via its c-terminal tail to form FGF23-α-klotho, thus

participating in the calcium and phosphorus metabolism. Some studies have reported that the serum level of klotho decrease in patients with osteoporosis, and the level of soluble klotho are negatively correlated with the FGF-23 level [20]. The results of our study exhibited that compared with the normal group, the abnormal group had increased level of FGF-23 and decreased levels of α-klotho and β-klotho. Moreover, compared with the osteopenia group, the osteoporosis group had higher FGF-23 level and lower α-klotho and β-klotho levels. The correlation analysis showed that BMD was negatively correlated with the FGF-23 serum level and was positively correlated with the levels of α-klotho and β-klotho. These findings suggest that FGF-23 and its receptor are involved in the occurrence and development of PMD. In addition, we found no difference in the FGF-21 level between the healthy women and women with PMD, which may be due to the fact that FGF-21 is mainly involved in vascular sclerosis and lipid metabolism but not in the processes related to calcium and phosphorus metabolism and bone metabolism [21].

Chen et al. documented that FGF-23 has high clinical value in the diagnosis of osteoporosis [9]. In our study, we found that when the serum FGF-23, α-klotho, and β-klotho expression levels reached 246.962 pg/mL, 406.131 pg/mL, and 452.745 pg/mL, respectively, the AUC values were 0.900, 0.803, and 0.840, respectively, and there were high sensitivity and specificity in these markers. This is consistent with previous results which further demonstratest the clinical value of these markers in the diagnosis of PMO.

In conclusion, the number of beneficial bacteria decreases significantly in the intestinal microflora of PMO patients, and the levels of intestinal microflora and serum FGF-23, α-klotho, and β-klotho are closely related to the development of PMO. Lactobacillus, bifidobacterium, enterococcus, FGF-23, α-klotho, and β-klotho show clinical value in the diagnosis of PMO. However, there were some limitations to this study. The sample size was small and dynamic comparison was not conducted for each marker. Therefore, more studies need to be carried out in the future for further verification.

Disclosure of conflict of interest

None.

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