Life Sciences 216 (2019) 227–232

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Reduced TIPE2 expression is inversely associated with proinflammatory cytokines and positively correlated with bone mineral density in patients with osteoporosis

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ARTICLE INFO

Keywords:
Osteoporosis
TIPE2
Proinflammatory cytokines
BMD

ABSTRACT

Aims: The tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) participates in multiple inflammatory diseases. However, its underlying mechanism in osteoporosis has not been elucidated. The aim of current study is to preliminarily clarify the function of TIPE2 in the pathogenesis of osteoporosis.
Main methods: TIPE2 expression in patients with osteoporosis was measured by Western blot and qRT-PCR methods. Proinflammatory cytokines including TNF-α, IL-1 and IL-6 were assessed via enzyme-linked immunosorbent assay. Serum fasting PINP and β-CTX were measured by the chemiluminescence method. Simple logistic regression analysis was performed for the odds ratio (OR) for TIPE2.
Key findings: TIPE2 expression in patients with osteoporosis was dramatically decreased and negatively correlated with proinflammatory cytokines. Furthermore, TIPE2 level was negatively correlated with fasting β-CTX, but not PINP, indicating that TIPE2 participates in the pathogenesis of osteoporosis dominantly by supression of bone resorption. Interestingly, TIPE2 expression level was positively correlated with bone mineral density (BMD), and its expression level can predict the risk of bone fracture using the simple logistic regression assay.
Significance: Our findings clarify that TIPE2 alleviates the pathogenesis of osteoporosis by suppressing the inflammatory status and the ability of TIPE2 for predicts bone fracture further demonstrated that TIPE2 might serve as a novel diagnostic marker and a therapeutic target for osteoporosis.

1. Introduction

Osteoporosis is a metabolic-related bone disease characterized by low bone mass and increased bone fracture possibility, thereby resulting in a high society burden, substantial morbidity and mortality [1]. Etiology of osteoporosis is normally attributed to abnormal endocrine, metabolic and mechanical factors including insufficient vitamin D and calcium intake, postmenopausal hormonal condition and pregnancy [2]. Growing evidences suggest that chronic inflammation plays a key role in excessive bone loss and destruction of trabecular architecture [3,4]. Importantly, it is evidenced that NF-κB plays an important role in aging-related bone loss or osteoporosis and NF-κB is overactivated in this process [5,6]. Inhibition of NF-κB is essential to prevent osteoporosis or bone loss [7]. Recent researches also demonstrated that TNF-α, a downstream inflammatory factor in the NF-κB signaling pathway, contributes to the pathogenesis of osteoporosis by promoting osteoclast differentiation and inhibiting osteoblast proliferation, thus resulting in an accelerated bone resorption [4,8]. Clinical evidences have shown that osteoporosis is strongly correlated with inflammatory diseases, including rheumatoid arthritis, ankylosing spondylitis and diabetes mellitus [9–11]. Thus, targeting NF-κB might allow inhibition of bone resorption, and it will bridge a novel relationship between osteoporosis and inflammation.

The tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) belongs to the TIPE family. TIPE2 downregulates inflammation and carcinogenesis via diverse mechanisms [12]. It was initially reported as a novel negative regulator for immunity and inflammation [13]. TIPE2-deficient mice would develop multi-organ inflammation and led to premature death. Mechanistically, TIPE2 inhibits the activation of activating protein-1 (AP-1) and NF-κB while promotes Fas

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https://doi.org/10.1016/j.lfs.2018.11.054
Received 1 August 2018; Received in revised form 11 November 2018; Accepted 26 November 2018
Available online 27 November 2018
0024-3205/

Abbreviations

TIPE2	the tumor necrosis factor (TNF)-alpha-induced protein 8-like 2
BMD	bone mineral density
PBMC	peripheral blood mononuclear cells
ROC	receiver operating curve
AUC	area under the curve
PINP	amino-terminal propeptide of type I procollagen
β-CTX	β-isomerized carboxy-terminal telopeptide of type I collagen

induced apoptosis in the process of inflammation [12]. Hitherto, TIPE2 has been investigated in several human chronic inflammatory diseases, such as systemic autoimmunity, hepatitis B and diabetic nephropathy [14–16]. Our previous studies also have suggested that TIPE2 was up-regulated in ankylosing spondylitis and type 2 diabetes mellitus. Its expression level was negatively related with inflammatory cytokines, indicating TIPE2 might serve as a prognostic indicator for assessing the disease severity [17,18]. Interestingly, NF-κB and its downstream factors that play important roles in bone homeostasis are tightly down-regulated by TIPE2 in the inflammatory diseases [6,19,20]. Thus far, however, it remains unclear whether TIPE2 participates in the pathogenesis of osteoporosis.

Here, we examined the TIPE2 expression level in peripheral blood mononuclear cells (PBMCs) in patients with osteoporosis, and analyzed the relationship between TIPE2 and proinflammatory cytokines. Moreover, we measured the concentrations of fasting amino-terminal propeptide of type I procollagen (PINP) and β-isomerized carboxy-terminal telopeptide of type I collagen (β-CTX) in serum, which serves as the bone formation marker and bone resorption marker, respectively. We further analyzed the relationship between TIPE2 expression level and bone mineral density (BMD) to further clarify the function of TIPE2 in the pathogenesis of osteoporosis. In sum, the purpose of this article is to elucidate the underlying effect of TIPE2 on osteoporosis, and to disclose its diagnostic function for bone disorders.

2. Materials and methods

2.1. Human subjects

The study populations (n = 80) were collected from Zibo Central Hospital Affiliated to Shandong University. The exclusion criteria were as followed: patients combined with endocrinopathies (such as diabetes, hyper- or hypoparathyroidism and hyperthyroidism) and metabolic bone, muscle or other skeletal diseases (including osteomalacia, rheumatoid arthritis, and Paget disease); patients who were taking calcium and vitamin D supplements; patients with severe liver or kidney disease; patients with other inflammatory diseases that substantially affect TIPE2 expression, including systemic lupus erythematosus, asthma, myasthenia gravis and chronic hepatitis. Sera were collected and centrifuged from all patients, each patient's BMD at the lumbar spine (L1–L4) and the bilateral femoral neck was measured and fulfilling the osteoporosis criteria. According to the definition of the World Health Organization (WHO), T score less than or equal to −2.5 at any site are diagnosed as osteoporosis. Patients with acute fragile fractures (time from fracture to admission was limited to 1 week) were included as fracture group. Fractures that caused by high-energy injury (such as car crash) were excluded in this study. The age- and sex-matched healthy controls (n = 90) were randomly recruited from Zibo Central Hospital Medical Center and excluded from having any bone disease and metabolic or other inflammatory related diseases. The study was approved by the local ethics committee of Zibo Central Hospital Affiliated to Shandong University. All subjects provided informed consent to join the study. The demographic details for patients and controls are summarized in Table 1.

Table 1
Demographic details for patients and controls.

Subjects	Osteoporosis (n = 80)	Controls (n = 90)
Sex (Male/Female)	13/67	15/75
Mean age (Male, years)	65 ± 12.4	61.5 ± 15
Mean age (Female, years)	63.3 ± 10.0	60.3 ± 18.2
Race	Chinese	Chinese
BMI(kg/m^2)	23.5 ± 2.23	23.0 ± 3.01
Fasting β-CTX (ng/ml)	7.07 ± 5.46	0.62 ± 0.16
Fasting PINP (ng/ml)	37.08 ± 22.37	22.5 ± 10.41
IL-1 (pg/ml)	28.98 ± 14.79	3.24 ± 0.15
IL-6 (pg/ml)	32.43 ± 14.64	2.59 ± 0.24
TNF-a (pg/ml)	32.06 ± 15.23	5.10 ± 0.18
Lumbar spine BMD (g/cm^2)	0.37 ± 0.09	0.95 ± 0.12
Femoral neck BMD (g/cm^2)	0.45 ± 0.11	0.84 ± 0.14

Values are mean ± SD or n, unless stated.

2.2. BMD measurements

BMD of the lumbar spine (L1–L4) and bilateral femoral neck was measured by Dual X-Ray Absorptiometry (Lunar Prodigy, GE Healthcare, London, United Kingdom). All coefficients of variation at the sites were < 1.0%. BMD was calculated as the bone mineral content divided by the surface of the projected bone area (g/cm^2). T-scores were determined according to the following standard formula: T-score = measured value (BMD) - reference value (peak value in a young normal population) / standard deviations.

2.3. Cell culture and vector transfection

Human PBMCs were separated from patients with osteoporosis and healthy controls using Ficoll density centrifugation. The cells were then cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified 5% CO_2 incubator. For RNA or protein determination, cells were seeded in 6 well plates and were treated for 30 min with LPS after transfected with or without TIPE2 lentiviral vector (Genechem Company, Shanghai, China).

2.4. Extraction of RNA and quantitative real-time RT-PCR

PBMCs were isolated by density gradient centrifugation from the peripheral blood anticoagulated with sodium citrate. Cell pellets were lysed with TRIzol reagent (Invitrogen, California, USA) and then total RNA was extracted according to the instructions of manufacturer, quantified by photometrical measurement. One microgram of total RNA was used for cDNA synthesis using UltraSYBR Mixture (CWBIO). 18sRNA is used as an internal reference gene in this study. The sequences of specific primers were designed as follows: TIPE2 forward 5′-ACTGA GTAAGATGGCGGGTCG-3′, and reverse 5′-TTCTGGCGAA AGC GGGTAG-3′; 18S rRNA forward 5′-CGGCTACCAC ATCCAAGGAA-3′; reverse 5′-GCTGGAATTACCGCGGCT-3′.

2.5. Detection of proinflammatory cytokines, fasting PINP and β-CTX

Serum was separated and prepared for the analysis. Commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to measure TNF-α, IL-1, IL-6 (CBA Kit, BD Bioscience, USA) levels according to the manufacturer's instructions. Serum fasting PINP and β-CTX were measured by the chemiluminescence method. Bone turnover markers, including β-CTX and PINP were drawn in the early morning after the examinees fasted overnight. The intra- and inter-assay coefficients of variations (CV) were all < 10% and two duplicate wells were prepared for each sample.

2.6. Western blotting

PBMCs were separated and washed twice with PBS, and then isolated by RIPA lysis buffer containing phenylmethanesulfonyl fluoride. Proteins were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. At the final stage, the membranes were incubated with the following antibodies, respectively: anti-TIPE2 (Santa Cruz Biotechnology, USA), anti-p65 (#8242, CST, USA), anti-p-p65 (#3033, CST, USA).

2.7. Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 17.0 software (SPSS, Chicago, Illinois, USA). The two-tailed non-parametric test is used for measuring differences between two groups, while the Spearman's rank correlation is used to measure the degree of association between two variables. Simple logistic regression analysis was performed for the odds ratio (OR) for TIPE2. ORs were calculated with 95% confidence intervals (CI). The receiver operating curve (ROC) analysis was used to evaluate the predictability of TIPE2. The area under the curve (AUC) and the cut-off value were calculated. A p value < 0.05 was considered statistically significant for all tests.

3. Results

3.1. Decreased TIPE2 expression level in PBMCs from patients with osteoporosis and is negatively correlated with proinflammatory cytokines

As shown in Fig. 1A and B, TIPE2 expression level in patients with osteoporosis (n = 80) was significantly lower than that of healthy controls (n = 90) both in mRNA and protein levels (***$p < 0.001$). Interestingly, the TIPE2 mRNA expression level was inversely correlated with serum IL-1 (Spearman $r = -0.3443$, $p < 0.01$), IL-6 (Spearman $r = -0.3394$, $p < 0.01$) and TNF-α (Spearman $r = -0.5038$, $p < 0.001$) (Fig. 1C, D and **E**; respectively). Those results indicated that TIPE2 may suppress the secretion of inflammatory cytokines, and thus participates in the pathogenesis of osteoporosis.

3.2. TIPE2 expression level is negatively correlated with fasting serum β-CTX, but not PINP

The biochemical markers of bone turnover mainly include two factors: β-CTX which represents bone resorption and PINP which represents bone formation. Consistently, we analyzed the relationship between TIPE2 and β-CTX or PINP. As shown in Fig.2A and B, the results showed that TIPE2 inversely correlated with β-CTX (Fig. 2A, Spearman $r = -0.4339$, $p < 0.001$). However, there was not reflected in an association between PINP and TIPE2 (Fig. 2B, Spearman $r = -0.1743$, $p = 0.14$). The inverse association between β-CTX and TIPE2 suggested that TIPE2 exerts its functions via suppression of

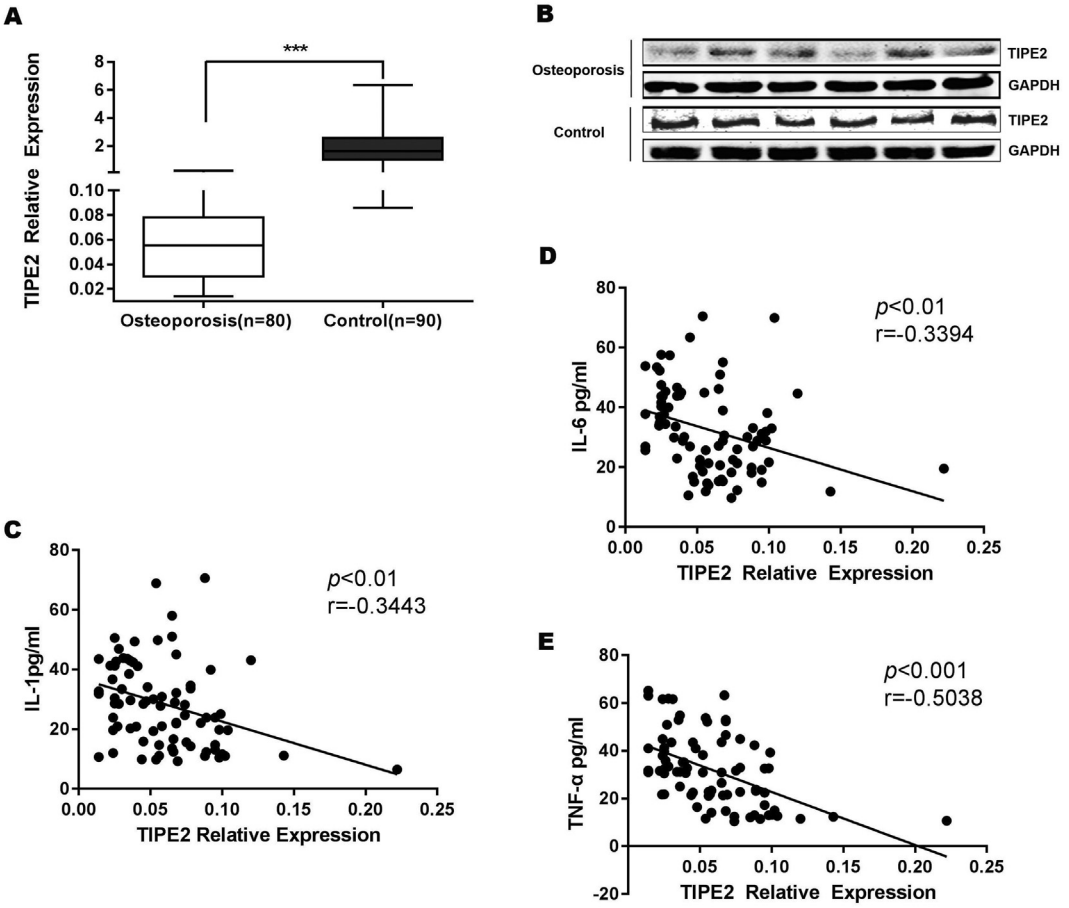

Fig. 1. TIPE2 expression level in PBMCs from patients with osteoporosis and the correlations between TIPE2 and proinflammatory cytokines. The expression level of TIPE2 in PBMCs from patients with osteoporosis (n = 80) was significantly lower than those in healthy controls (n = 90) either in mRNA (Fig. 1A) or protein level (Fig. 1B). As shown in Fig. 1C, D and E, TIPE2 expression level was negatively correlated with IL-1, IL-6 and TNF-α, respectively. Statistical confidences were analyzed by the Mann-Whitney t-test. Values represent means ± SD; for all panels, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

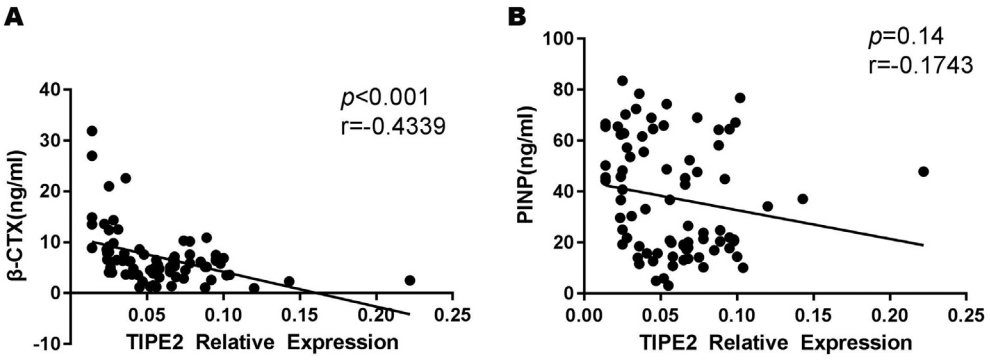

Fig. 2. Correlation analysis of TIPE2 mRNA with β-CTX and PINP. A negative correlation between TIPE2 mRNA and β-CTX was found in patients with osteoporosis (Fig. 2A). However, there was no statistical correlation between TIPE2 level and PINP (Fig. 2B). Statistical confidence was analyzed by Spearman correlation analysis method.

osteoclastogenesis in the pathogenesis of osteoporosis.

3.3. TIPE2 expression level is positively correlated with BMD

Generally, it is well recognized that osteoporosis accompanied with a rise in proinflammatory cytokines [21]. Bone remodeling process is tightly regulated by bone forming osteoblasts and bone resorpting osteoclasts. Perturbation in this process will lead to bone loss and overactivation of an inflammatory signaling cascade [22]. Based on our above results, we further analyzed the relationship between TIPE2 expression level and BMD. There is no doubt that TIPE2 had a significant positive correlation with Femoral neck (FN) (Fig. 3A, Spearman $r = 0.2405$, $p < 0.05$) and Lumbar spine (L1-L4) (Fig. 3B, Spearman $r = 0.2594$, $p < 0.05$) in patients with osteoporosis, suggesting that TIPE2 serves as a protective function involved in the process of osteoporosis.

3.4. TIPE2 can predict the risk of bone fracture

Because of TIPE2 expression level is significantly decreased and is positively correlated with BMD in patients with osteoporosis. Thus, we explored its predictive function for bone fracture using simple logistic regression method. As shown in Fig. 4A, TIPE2 expression level was significantly lower in fracture group (n = 35) than that of control group (n = 45) (*$p < 0.05$). More interestingly, TIPE2 exhibited significant odds ratios to predict bone fracture in patients with osteoporosis. The ROC analysis was performed and AUC value was calculated for the predictive function of TIPE2. As shown in Fig. 4B, the TIPE2 level had a good AUC value (Area 0.6399, 95% CI 0.5174 to 0.7623, $p < 0.05$) to predict bone fractures.

3.5. TIPE2 protects monocytes from being stimulated with LPS in patients with osteoporosis

The clinical investigation has preliminarily demonstrated that TIPE2 serves as a protective factor in the pathogenesis of osteoporosis. In order to further investigate its underlying function in the process of osteoporosis, the monocytes from three patients with osteoporosis were separated and cultured in DMEM, and then stimulated with LPS after transfected with or without TIPE2. The results showed that there had either decreased proinflammatory cytokines in culture supernatant or blunted activity of the NF-κB pathway after TIPE2 transfection, indicating that TIPE2 can weaken the sensitivity of monocytes to LPS stimulation (Fig. 5). Overall, the results further suggested that the reduced TIPE2 expression contributes to an uncontrolled inflammatory condition in the pathogenesis of osteoporosis.

4. Discussion

Hitherto, abnormal TIPE2 expression level has been investigated in several human inflammatory diseases [14,15,23–26]. Evidenced by our group also indicated that increased TIPE2 expression was inversely correlated with disease progression in patients with ankylosing spondylitis and type 2 diabetes mellitus [17,18]. Mechanistically, TIPE2 has been elucidated to inhibit NF-κB activating by negatively regulation of TAK1 signal, and as a negative regulator to downregulate NOD2 inflammatory pathway [12,27,28]. However, the correlation between TIPE2 and osteoporosis, an aging and inflammation related disease, has not been elucidated. In the present study, we revealed that TIPE2 expression level was dramatically decreased. More intestinally, its expression level was inversely correlated with inflammatory cytokines and positively correlated with BMD in patients with osteoporosis.

Decreased expression of TIPE2 gives rise to the elevation of proinflammatory cytokines, which characterize osteoporosis. Emerging clinical observations and molecular evidences reveal that inflammation exerts significant influence on bone remodeling process and osteoporosis [29]. Elevated proinflammatory cytokines combined with the activated immune system have been demonstrated to modulate bone resorption, and as important risk factors contribute to osteoporosis [2]. Aging, and other chronic inflammatory conditions are tightly associated with osteoporosis [6,30]. For example, elevated inflammatory cytokines, which have been demonstrated to regulate bone resorption and bone loss, were observed both in bone marrow and serum in patients with osteoporosis [31,32]. In the present article, we revealed that

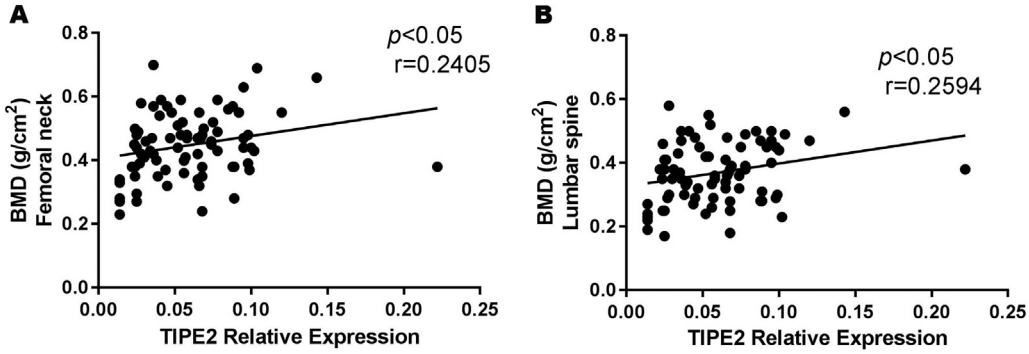

Fig. 3. The relationship between TIPE2 and BMD. The correlation between TIPE2 expression level and BMD was shown in Fig. 3. There had significant positive relationship between TIPE2 level and BMD both in the Femoral neck (FN) (Fig. 3A) or Lumbar spine (L1–L4) (Fig. 3B) in patients with osteoporosis. Statistical confidence was analyzed by Spearman correlation analysis method.

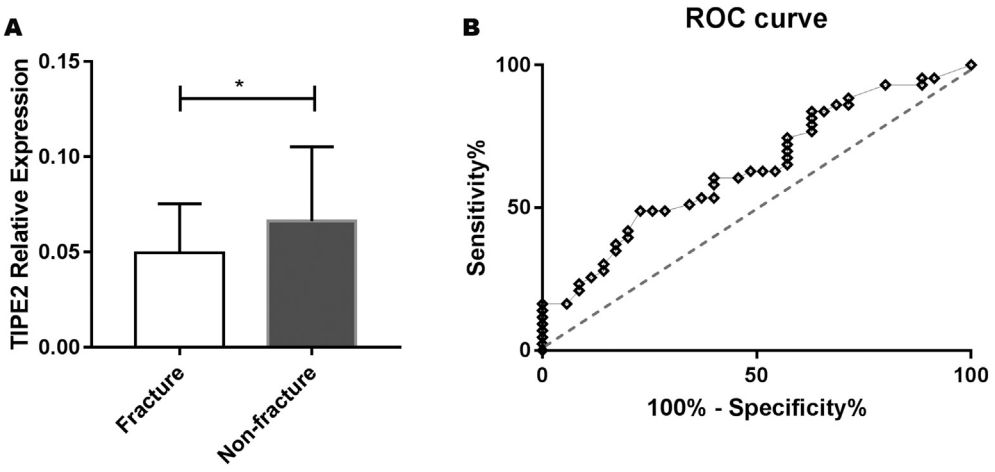

Fig. 4. The ability of TIPE2 for predicting bone fracture. The result showed that TIPE2 level was significantly lower in patients combined with fracture compared to that without fracture (Fig. 4A). Furthermore, the result showed that TIPE2 expression level (OR 3.168, 95% CI 0.5174 to 0.7623, $p < 0.05$) was significantly associated with bone fracture using simple logistic regression method (Fig. 4B).

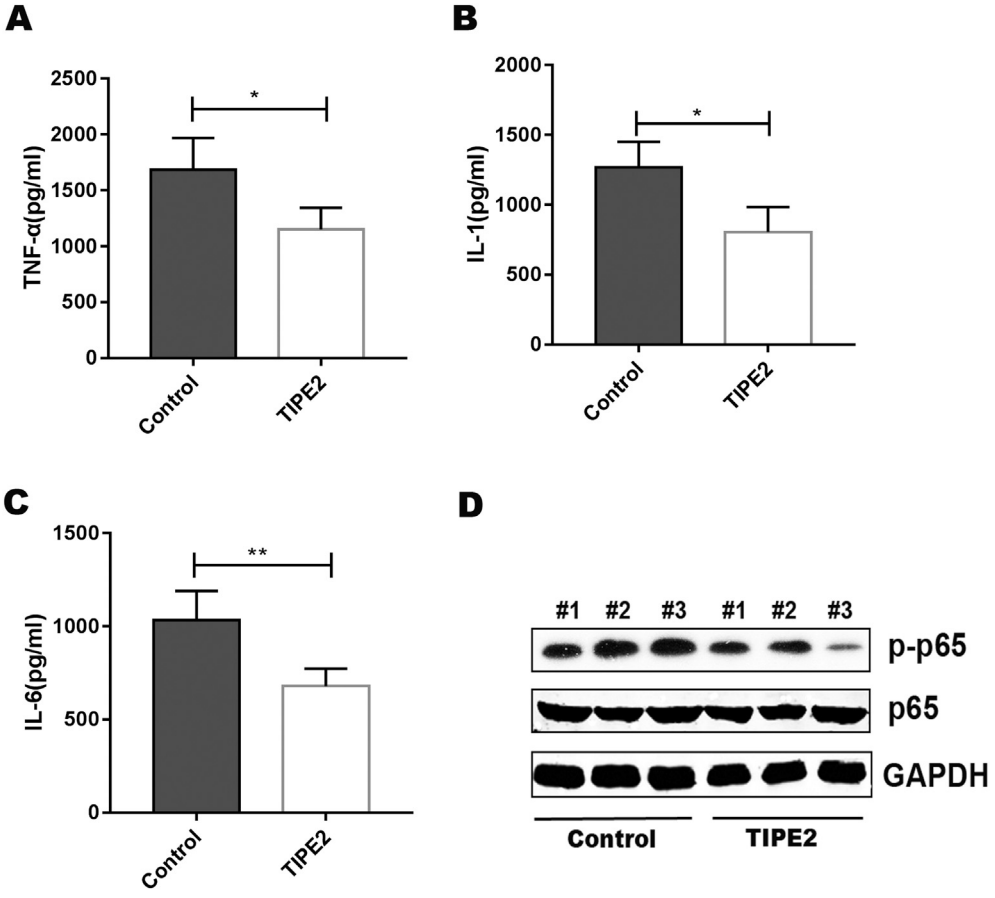

Fig. 5. PBMCs stimulated with LPS after transfection of TIPE2 in patients with osteoporosis. PBMCs were separated from patients with osteoporosis and stimulated with medium containing LPS (Final concentration was 100 ng/ml) after with or without transfection of TIPE2. IL-1, IL-6 and TNF-α levels in supernatant were detected by ELISA (Fig. 5A). The NF-κB pathway was detected using Western blotting method (Fig. 5B). Statistical confidences were analyzed by Mann-Whitney *t*-test. Values represent means ± SD; for all panels, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

dramatically decreased TIPE2 expression level in patients with osteoporosis was inversely correlated with elevated proinflammatory cytokines. Generally, TIPE2 is a negative regulator of inflammation, and it maintains immune homeostasis [28]. Therefore, we hypothesized that the decreased TIPE2 expression level might contribute to an uncontrolled inflammatory condition, thereby resulting in a low bone mass.

The TNF-family molecule RANKL has been specifically implicated in the bone loss in inflammatory disease [33]. Osteoclast differentiation needs M-CSF and RANKL stimulation. Thus, inflammatory cytokines induce pathological osteoclast differentiation, by which causes excessive bone resorption [34]. As a consequence, we observed that TIPE2 level was negatively correlated with the bone resorption marker β-CTX, but not PINP. Given that the β-CTX represents the activation of osteoclast, it is likely demonstrated that TIPE2 negatively regulates osteoclast prior to osteoblast. Furthermore, we observed that decreased TIPE2 expression level was positively correlated with BMD, suggesting that the declined TIPE2 might is a reason for low bone strength. However, its underlying mechanism for osteoporosis needs to be further explored.

TIPE2 plays a central role in inflammation related diseases. In order to further investigate its underlying function in osteoporosis, the monocytes from patients with osteoporosis were obtained and stimulated with LPS after transfection of TIPE2. The results revealed that TIPE2 can blunt the activity of the NF-κB pathway. Interestingly, TIPE2 exhibited significant odds ratio to predict bone fracture, suggesting that TIPE2 might is a strategic factor for diagnosis, treatment and prognosis of osteoporosis. Nevertheless, this case-control study is relative

superficial and lacks mechanism investigation. Thus, the exact role of TIPE2 in the pathogenesis of osteoporosis needs to be further investigated.

In sum, our results showed that TIPE2 expression level was decreased in patients with osteoporosis, and was negatively correlated with IL-1, IL-6 and TNF-α. In addition, TIPE2 was negatively correlated with β-CTX, indicating that TIPE2 may participate in the pathogenesis of osteoporosis by downregulation of osteoclasts. Nevertheless, the positive correlation between TIPE2 and BMD and the ability of TIPE2 for predicts bone fracture further indicated that TIPE2 might serve as a novel diagnostic marker and a therapeutic target for osteoporosis.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Natural Science Foundation of China (Grant Nos. 81600348/81600695), the Natural Science Foundation of Shandong Province (ZR2015HM031/ZR2014HM042/ZR2016HB25).

Author contributions

Conceived and coordinated the research and wrote the paper: J Jiang, X-M Pang, T Li, and P-Q Zhao.

Performed and analyzed the experiments: J Jiang, X-M Pang, X-X Xiang, H Liu, J-G Li, Y Zhang and X-Z Yang.

Contributed to the preparation of samples: H Liu, J-G Li, and X-Z Yang.

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