Weak cation exchange magnetic beads coupled with matrix-assisted laser desorption ionization-time of flight-mass spectrometry in screening serum protein markers in osteopenia

Wei-Tao He1†, Bo-Cheng Liang1†, Zhen-Yu Shi1, Xu-Yun Li1, Chun-Wen Li2* and Xiao-Lin Shi3*

Abstract
The present study aimed at investigating the weak cation magnetic separation technology and matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) in screening serum protein markers of osteopenia from ten postmenopausal women and ten postmenopausal women without osteopenia as control group, to find a new method for screening biomarkers and establishing a diagnostic model for primary type I osteoporosis. Serum samples were collected from postmenopausal women with osteopenia and postmenopausal women with normal bone mass. Proteins were extracted from serum samples by weak cation exchange magnetic beads technology, and mass spectra acquisition was done by MALDI-TOF-MS. The visualization and comparison of data sets, statistical peak evaluation, model recognition, and discovery of biomarker candidates were handled by the proteinchip data analysis system software(ZJU-PDAS). The diagnostic models were established using genetic arithmetic based support vector machine (SVM). The SVM result with the highest Youden Index was selected as the model. Combinatorial Peaks having the highest accuracy in distinguishing different samples were selected as potential biomarker. From the two group serum samples, a total of 133 differential features were selected. Ten features with significant intensity differences were screened. In the pair-wise comparisons, processing of MALDI-TOF spectra resulted in the identification of ten differential features between postmenopausal women with osteopenia and postmenopausal women with normal bone mass. The difference of features by Youden index showed that the highest features had a mass to charge ratio of 1699 and 3038 Da. A diagnosis model was established with these two peaks as the candidate marker, and the specificity of the model is 100 %, the sensitivity was 90 % by leave-one-out cross validation test. The two groups of specimens in SVM results on the scatter plot could be clearly distinguished. The peak with m/z 3038 in the SVM model was suggested as Secretin by TagIdent tool. To provide further validation, the secretin levels in serum were analyzed using enzyme-linked immunosorbent assays that is a competitive inhibition enzyme immunoassay technique for the in vitro quantitative measurement of secretin in human serum.

Keywords: Osteopenia, Weak cation exchange magnetic beads, Proteomics, Biomarkers

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Background
Osteoporosis is a skeletal disorder characterized by low bone mass and bone microstructure degeneration. With the advent of the aging society, the incidence rate of osteoporosis is rising quickly, osteoporosis has become a serious threat to the health of the elderly (Consensus NIH 2001; Czerwiński et al. 2006).

Before people develop osteoporosis, they have a condition called osteopenia. Osteopenia was firstly defined by the World Health Organization (WHO) in June 1992(Knapp et al. 2004). Osteopenia usually doesn’t cause any symptoms. Losing bone mass is not painful. Broken bones or fractures can occur, but these problems tend to happen after osteoporosis has developed. Osteopenia is not a disease, but if you meet the criteria for osteopenia, you are at higher risk for developing osteoporosis. Early diagnosis and treatment can prevent osteopenia from becoming osteoporosis.

The bone mineral density (BMD) measurement is useful in clinical application on the diagnosis of osteoporosis and the prediction of fracture in elderly women, but it is a lagging-not an early-indicator. Dual-energy X-ray absorptiometry (DXA) is currently recognized as gold standard of osteopenia and osteoporosis diagnosis (Kanis et al. 2013). Although DXA measurement is the gold standard, but early symptom of osteopenia or osteoporosis is not easy to detect, so that many patients with osteopenia or osteoporosis are often found after the first fracture. BMD does not reflect the change of bone matrix, bone turnover and bone strength. BMD could not accurately predict the risk of bone fractures (Oei et al. 2013).

With the completion of human genome project, the post genomic era is coming. We can rapidly screen the specific biomarkers of the disease by proteomics technologies (Ray et al. 2011), clarify the pathogenesis of the disease, and explore new methods of preventing and treatment.

Magnetic beads with large separation capacity has been widely used in cancer research using serum samples and beads based technology enrichment are high-throughput, simple and quick to operate (Wu et al. 2012; Fan et al. 2012).

Matrix-assisted laser desorption/ionization-time of light-mass spectrometry (MALDI-TOF-MS) is a recently emerged proteomics method, with the potential to detect various clinical samples, such as serum, urine, pleural effusion, ascites and a number of secretions (Zhang et al. 2012; Zheng et al. 2011). Analysis of the serum samples proteome may detect changes that reflect bone issues with pathological processes in the bone turnover. Furthermore, serum samples are simply and non-invasively obtained. Thus, proteomic analysis of serum samples may be a useful tool in the prediction, early diagnosis, treatment monitoring, and prognostic assessment of osteopenia patients. Weak cation exchange magnetic beads (MB-WCX) combined with MALDI-TOF-MS is one such approach that offers a unique tool for profiling of proteins, but this approach has not been used in the osteopenia area.

In this study, we introduced a developed optimal method of mass spectrometry-based technology, weak cation magnetic separation technology combined with MALDI-TOF-MS, searching for efficient serum protein biomarkers, attempting to predict Molecular mechanisms of osteopenia, thus reducing the uncertainties and potential risks in the primary type I osteoporosis patients. In this study, we developed a strategy for screening serum proteins <20 kDa to analyze serum profiles and find potential biomarkers for the osteopenia.

Results
Statistical analysis of baseline data
There are no statistically significant difference (P > 0.05) between comparisons of subjects of age, weight, height, and duration of menopause and it’s comparable. The results were shown in Table 1.

Detection of differential features
By application of WCX combined with MALDI-TOF MS, we analyzed the proteomic profiles between two groups. The serum samples contain a high diversity of proteins. A total of 133 peaks in the molecular weight range of 1000–20,000 Da were identified between the two groups in this study. The results are shown in Fig. 1.

Discriminative ability of representative differential features
Genetic algorithm (GA) and support vector machine (SVM) embedded in ZJU-PDAS software (Qiu et al. 2009) were used to establish cross-validated classification model for distinguishing osteopenia group from normal bone mass group. Although there were 10 peaks showing different peaks between two groups (P < 0.05), only two (1699 and 3038 Da) of them showed statistically significant differences in further analysis by Youden Index (Fluss et al. 2005; Bantis et al. 2014). The peak mass, P values, average peak intensity, and standard deviation (SD) of the four peaks were shown in Table 2. The peaks analyzed to generate a classification model were based on the two peaks with m/z of 1699 and 3038 Da in Fig. 2.

In the optimal SVM model, the two peaks with molecular weight of 1699 Da were upregulated in patients with osteopenia and 3038 Da were downregulated.

Development of diagnostic models and evaluation of their diagnostic accuracy
The top peaks of 1699 and 3038 Da could distinguish serum samples between osteopenia group from normal
bone mass group effectively. The results are shown in Table 3. The serum proteomic model, based on a group of 2 peaks, accurately recognized osteopenia group from normal bone mass group. A diagnosis model was established with these two peaks as the candidate marker. Two groups of specimens could be clearly distinguished in the SVM result scatter plot. The result is shown in Fig. 3.

Validation of the candidate biomarker secretin using ELISA

The peak with m/z 3038 in the SVM model was suggested as Secretin by TagIdent tool. Enzyme-linked immuno-sorbent assays (ELISA) is commonly used in clinical settings, for the direct validation and quantification of the identified Secretin, the ELISA technique was used.

The 80 serums for ELISA validation from 40 osteopenia patients and 40 postmenopausal women with normal bone mass, specifically (Table 4). Statistical analysis showed that there is no bias for age, weight, height, and duration of menopause between the osteopenia group and healthy control.

The secretin concentrations of postmenopausal women with normal bone mass and osteopenia patients differed significantly (P < 0.05). Result showed that the normal bone mass group had significantly higher secretin signals

Table 1 Comparisons of clinical features of patients

| Groups                     | Age (years) | Weight (kg) | Height (cm) | Duration of menopause (years) |
|----------------------------|-------------|-------------|-------------|-----------------------------|
| Osteopenia (n = 10)        | 56.32 ± 3.61| 53.16 ± 5.36| 160.50 ± 10.53| 5.26 ± 2.61               |
| Normal bone mass (n = 10)  | 55.00 ± 3.48| 52.24 ± 3.97| 161.48 ± 11.00| 4.76 ± 1.57               |
| t                          | 0.83        | 0.44        | 0.20        | 0.52                       |
| P values                   | >0.05       | >0.05       | >0.05       | >0.05                      |

Data shown are mean ± standard deviation

Table 2 Statistics of significantly different expressed peak intensities to distinguish patients from controls

| m/z  | Osteopenia group | Normal bone mass group | P values | Expression change |
|------|------------------|------------------------|----------|------------------|
| 1699 | 299.04 ± 140.72  | 164.32 ± 105.04        | 0.037    | ↑                |
| 3038 | 383.78 ± 332.46  | 649.97 ± 236.04        | 0.014    | ↓                |

Data shown are mean ± standard deviation
Fig. 2 Discriminative ability of representative differential features. The top 2 discriminating peaks of 1699 and 3038 Da could distinguish serum samples between osteopenia and postmenopausal women with normal bone mass effectively. a Displayed the peaks of 3038 Da. b Displayed the peaks of 1699 Da.
than that of osteopenia group. The result is shown in Table 5. As expected, the results of ELISA validation is consistent with the MALDI-TOF MS results.

Discussion

Although some progress has been made in the analysis of osteopenia disease, and to date there was no previous study to describe the application of MB-WCX combined with MALDI-TOF-MS technology in serum samples in osteopenia. In the present study, our results demonstrated that a diagnostic model was constructed successfully, which had discriminated postmenopausal women with osteopenia and postmenopausal women with normal bone mass.

In recent years with the development of proteomics technology, a variety of biological markers in clinical been widely applied (Siew et al. 2011; Hampel et al. 2014; Rousseau and Garnero 2012). Biological markers play an important role in disease diagnosis. Using serum proteomics technology (Ray et al. 2011) (Nanjappa et al. 2013) to find important molecular markers provides an effective method for the clinical diagnosis of the disease. Currently, the commonly used serum bone markers, such as total Procollagen I N Terminal Peptide (PINP) (Pollmann et al. 2007), the carboxy-terminal cross-linking telopeptide of type I collagen (β-CrossLaps) (Chubb et al. 2015), N-terminal osteocalcin and 25-hydroxyvitamin D3 reflect the changes of bone metabolism. However, some bone turnover markers have a low sensitivity

| Groups       | Predicted osteopenia | Predicted normal bone mass | Sum Accuracy (%) | Error (%) |
|--------------|----------------------|-----------------------------|------------------|----------|
| Osteopenia   | 9                    | 1                           | 10 90            | 10       |
| Normal bone mass | 0              | 10                          | 10 100           | 0        |

The specificity of the model is 100 %, the sensitivity was 90 % by leave-one-out cross validation test.

Table 3 Diagnostic accuracy of different classification models in the training set

Table 4 Comparisons of clinical features of patients in the ELISA validation

| Groups          | Age (years)   | Weight (kg)   | Height (cm)   | Duration of menopause (years) |
|-----------------|---------------|---------------|---------------|------------------------------|
| Osteopenia (n = 40) | 56.56 ± 3.78  | 54.09 ± 4.98  | 161.43 ± 10.29 | 5.34 ± 2.56 |
| Normal bone mass (n = 40) | 56.10 ± 3.50  | 53.29 ± 3.85  | 162.52 ± 10.58 | 4.95 ± 1.63 |
| t               | 0.56          | 0.80          | 0.47          | 0.81                         |
| P values        | >0.05         | >0.05         | >0.05         | >0.05                       |

Data shown are mean ± standard deviation
Table 5 Comparisons of secretin concentrations in the ELISA validation

| Groups               | Secretin concentration (pg/mL) | t values | P values |
|----------------------|-------------------------------|----------|----------|
| Normal bone mass (n = 40) | 307.48 ± 74.68               | t = 8.523| <0.05    |
| Osteopenia (n = 40)   | 157.41 ± 82.62                |          |          |

Data shown are mean ± standard deviation

and specificity in the early diagnosis of osteopenia that did not meet requirements of non-invasive and specific early diagnosis of osteopenia in the clinical practice (Bhattacharyya et al. 2008).

In this study, we screened 2 discriminating peaks of 1699 and 3038 Da which had distinguished serum samples between postmenopausal women with osteopenia and postmenopausal women with normal bone mass effectively. The serum proteomic model, based on a group of 2 peaks, accurately recognized postmenopausal women with osteopenia and postmenopausal women with normal bone mass with a sensitivity of 90% and a specificity of 100% by Leave-one-out test verification. The peak with m/z 3038 in the SVM model was suggested as Secretin by TagIdent tool. Results also showed that the normal bone mass group had significantly higher secretin signals than that of osteopenia group by ELISA. Secretin was known to be produced in response to low pH in the duodenum or passage of food into the duodenum in an effort to buffer the gastric acids. Secretin was also structurally closely related to calcitonin (Kopic and Geibel 2013). Perhaps the lower secretin in the osteopenia implied less buffering of gastric acid, which could thereby inhibit calcium absorption.

For screening biomarkers, a variety of proteomic approaches have recently been performed to determine the protein composition in clinic. Most of these proteomic technologies used mass spectrometric (MS) techniques combined with several analytical techniques such as two-dimensional gel electrophoresis (2D-GE) or liquid chromatography (LC). While these approaches provided a large amount of data, they are generally very time-consuming and hence restrictive in the number of comparative samples that can be analyzed.

MB-WCX with MALDI-TOF-MS technology is an approach that offers a unique platform for high-throughput protein profiling of complex biological samples such as serum samples (Pusch and Kostrzewa 2005) (Cho et al. 2012). Thus, magnetic beads-based enrichment approaches have the potential to capture and enrich low abundance, low molecular weight species (Whiteaker et al. 2007).

In addition, the advantage of MALDI-TOF-MS is the direct use of crude sample, large-scale, high-throughput, automated, and minimal sample requirements. MALDI-TOF-MS not only can find a protein or biological markers, it also detected the combination of proteins existing in different forms (Gould et al. 2004).

In this study, all aspects of data processing such as sample inclusion and exclusion, serum collection, processing and storage of serum, protein separation and purification, sample point target, MS acquisition, experimental methods and parameter settings were optimized. Discovery researches in the clinical samples is better than in vitro and in vivo experiments.

In the aspect of data processing, data analysis software system was used to analysis data. The noise analysis was removed using undecimated discrete wavelet transform (UDWT) analysis (Aiazzi et al. 2002; Fowler 2005). The model was established by SVM method. SVM were used to align and integrate hundreds of mass data points from large numbers of samples, and could be used to process many samples in parallel. This approach is sensitive and fast, these were features essential for clinical use. SVM exhibited many unique advantages in solving small sample, nonlinear and high dimensional pattern recognition problems and achieve the statistical theory of structural risk minimization principle. The application of these techniques obtained serum protein diagnostic model with high credibility.

Conclusions

This study provides a new serological method for discovering serum protein markers to screen and diagnose osteopenia. This will be helpful in preventing and treating primary type I osteoporosis. Therefore, WCX magnetic beads and MALDI-TOF-MS can be used as a fast and cost-effective approach for serum samples discovery of predictive biomarkers of disease in osteopenia.

We speculate that the Secretin (SECR_HUMAN, P09683, Chain: 28–54, pl: 9.46, Mw: 3040) would be potential biomarker for forecasting osteopenia in postmenopausal women.

However, there is still a gap between our findings and their application in clinical practice. Future controlled studies with larger sample sizes are required to determine the sensitivity and specificity of secretin in the diagnosis of osteoporosis. The further validation, function, interaction, and metabolic pathways of these proteins will be the future research. Moreover, Regarding to the MALDI-TOF-MS technology, for this technology does...
not identify protein or peptides sequence, the identities of peaks by Tagldent may not be the indeed protein or peptides. And therefore, the purification and identify of indeed protein or peptides were needed based on the standard sequencing approaches in the further.

**Methods**

**Subjects and samples collection**

Serum samples were collected from consenting patients \((n = 20, 10 \text{ from postmenopausal women with osteopenia} + 10 \text{ from postmenopausal women with normal bone mass})\) enrolled from department of orthopedics, the Second Affiliated Hospital of Zhejiang Chinese Medicine University, from June 2013 and January 2014.

**Diagnostic criteria**

The diagnosis of osteopenia was based on the following recommended criteria by the WHO: Survey the lumbar vertebra normal position bone density by using dual energy X-ray absorptiometry (Unnanuntana et al. 2010; Frost et al. 2012), \(T\) score \(-1\) to \(-2.5\) could be diagnosed as osteoporosis \([T = \text{the standard deviation of (measured value-peak bone mass)/(normal adult bone density)}]\).

**Inclusion criteria**

Patients were included in the study based on the following criteria: (1) they conform to the diagnostic criteria of osteoporosis; (2) they were postmenopausal women; (3) they were from 50 to 60 years old.

**Exclusion criteria**

Patients were excluded from the study based on the following criteria: (1) those that also had diseases that severely affect the metabolism of bone or calcium, such as diabetes, Cushing’s syndrome, function changing of the thyroid or parathyroid, osteomalacia, rheumatoid arthritis, multiple myeloma, bone tumor, osteoarthritis, Paget’s disease, and osteogenesis imperfecta; (2) those that also had severe primary cardiac diseases, or diseases of the cerebral vessels or hematopoietic system; (3) those that also had severe liver function or renal insufficiencies; (4) those taking drugs within the past 6 months that affect bone metabolism, such as estrogen, steroid hormones, calcitonin, parathyroid hormones, bisphosphonates, fluoride, vitamin D, anticonvulsant drugs, and diuretics; (5) those who had a medical history of mental illness; and (6) patients with Alzheimer’s disease.

**Ethical review**

This study was approved by the local Ethics Committee of The Second Affiliated Hospital of Zhejiang Chinese Medicine University. The patients and volunteers provided written informed consent for their participation before they enrolled in this study.

**Sample collection and preparation**

The blood samples \((5 \text{ ml})\) were collected in the morning and allowed to clot at room temperature for up to 1–2 h. The samples were then centrifuged at \(4^\circ\)C for ten min at 3000 rpm \((\text{in } 943 \times g)\). The serum were frozen and stored at \(-80^\circ\)C for future analysis. The length of cryopreservation period for each serum sample was less than 6 months.

**Weak cation magnetic separation technology analysis**

Magnetic beads-based weak cation exchange chromatography \((\text{MB-WCX})\) (Bruker Daltonics, Germany) was used for peptidome separation of samples. We prepared a 200-\(\mu\)L sample tube of thoroughly mixed weak cation magnetic suspension by adding \(10 \mu\)L magnetic beads binding buffer, \(10 \mu\)L magnetic beads suspension, and \(5 \mu\)L serum, mixing at least five times using the sampling gun, and standing at room temperature for 5 min. We put the sample tube into the magnetic separator, maintained the magnetic field for 1 min, used the sampling gun to absorb the liquid after separation of the magnetic beads and liquid, and added to the sample tube \(100 \mu\)L magnetic beads cleaning buffer. We then moved the sample tube ten times between the two adjacent holes of the magnetic separator, left to stand, and used the sample gun to absorb the supernatant again after magnetic beads adherence. We repeated the cleaning process twice. We then took down the sample tube from the magnetic separator, added \(5 \mu\)L magnetic beads elution buffer to the sample tube, and repeated the pipetting. We put the sample tube into the magnetic separator and let it stand for 2 min, transferred the supernatant to a clean 0.5-\(\mu\)L sample tube when the magnetic beads were completely adhered, then added \(5 \mu\)L magnetic beads stabilizing buffer. The eluate was then ready for spotting onto MALDI-TOF MS targets and measured.

**Anchor chip spotting**

1 \(\mu\)L eluted sample was spotted onto a MALDI-TOF AnchorChipTM target \((600 \mu\text{m anchor diameter})\) and air-drying at room temperature, then 1 \(\mu\)L matrix \((0.3 \text{ mg/mL HCCA, } 50 \%\text{ACN, } 2 \%\text{TFA})\) was spotted onto MALDI-TOF AnchorChipTM target.

**MALDI-TOF MS**

AnchorChipTM target was placed into the Microflex mass spectrometer (Bruker Daltonics). Samples were detected after calibration of the instrument by ClinProt standard (Bruker Daltonics). MALDI-TOF MS parameters are detailed in Additional file 1.
**Detection data analysis**

The Zhejiang University-ProteinChip data analysis system (ZJU-PDAS) software, designed at Zhejiang University Cancer Institute, was used to analyze the raw data (Shi et al. 2014). The process was as follows. (1) The original spectrum was uploaded to the server to map data for protein analysis, and to process homogeneous data. (2) We removed the mass-to-charge ratio (m/z) of raw data below peak 2000, used the UDWT analysis method to remove the noise caused by the mass spectrometer itself, and used the amended data after the removal of the baseline noise spectrum and correction of the molecular weight values map. (3) We identified the protein charge ratio peaks with local minima, and filtration peak signal-to-noise ratios of less than 3. The differences in m/z between each sample of <0.3 % of the peak were clustered together. After clustering, peaks appeared only in the sample in less than 10 % removed. (4) To find the peak intensity in the respective sample treated as uniform. (5) After pretreatment, we filtered out protein charge ratio peaks using the Wilcoxon rank sum test, and screened differential protein peaks (P < 0.05). (6) We screened a random combination of different proteins, and used a genetic arithmetic model and the support vector machine (SVM) computing model to establish serum protein fingerprints.

For the SVM, we used the radial basis function, a gamma value of 0.6, and the penalty function (C) was set to 19. The highest elected combinations were used to build the SVM model to predict the Youden index. Because the final candidate signs matter, we used the leave-one-assessment model to predict the effect of an established law and the final results were cross-validated.

ZJU-PDAS specific parameters of analysis were detailed in Additional file 2.

**Identities of the two differential peaks suggested by TagIdent**

Using TagIdent tool (Washam et al. 2013; Liu et al. 2012) network software (http://web.expasy.org/tagident/), molecular weight (Mw) range was set to 0.3 %, Isoelectric point (Pl) range value was set to min = 4, max = 14. Checked for protein sequences with cysteines oxidized (−SS−); Organism name: *homo sapiens*; Tagging: Display only the sequences matching the tag, Displayed the predicted N-terminal sequence, Databases on UniProtKB/Swiss-Pro. Proteins as candidate were selected by the most similar molecular weight.

**Immunoassay for quantification of the candidate biomarker secretin**

After the identity was suggested by TagIdent, ELISA was used to detect the levels in sera. Secretin levels were measured in the serum samples using an ELISA 96-well plate for secretin (CEB075Hu, Cloud-Clone Corp., Houston, USA). The serum samples were diluted 1:1 with a 0.01 mol/L PBS (PH 7.0–7.2) according to the manufacturer’s instructions and analyzed on a RT-2100C micro plate reader (Rayto, USA) at a wavelength of 450 nm according to the manufacturer’s instructions.

**Statistical analysis**

All MALDI-TOF-MS spectrum were analyzed by ZJU-PDAS software to detect the peak intensities of interest, and to compile the peaks across the spectrum obtained from serum samples. All statistical comparisons were done by SPSS software version 13.0 (SPSS Inc., USA). The comparison of the age, weight, height, duration of menopause, and comparisons of secretin concentrations was done by t test, test level of α = 0.05. The Wilcoxon rank sum test (test level of α = 0.05) was used to compare features. P values <0.05 were considered statistically significant. Genetic algorithm (GA) (Deb et al. 2002), Support vector machine (SVM) (Weston et al. 2000), and Youden Index (Schisterman et al. 2005) methods were used to establish the diagnosis model and predict the candidate markers. The model was verified by leaving-one-out cross validation test (Wuebker et al. 2010).

**Additional files**

- **Additional file 1.** Detection parameters of Microflex MALDI-TOF-MS instrument.
- **Additional file 2.** Specific parameters of Zhejiang University-ProteinChip data analysis system (ZJU-PDAS).

**Abbreviations**

GA: genetic algorithm; MB-WCX: weak cation exchange magnetic beads; MALDI-TOF-MS: matrix-assisted laser desorption ionization-time of flight-mass spectrometry; MS/MS: tandem mass spectrometry; DXA: dual-energy X-ray; BMD: bone mineral density; UDWT: undecimated discrete wavelet transform; SVM: support vector machine.

**Authors’ contributions**

XLS made substantial contributions to the conception and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; CWL carried out the WCX magnetic bead and MALDI-TOF MS analyses. WTH and BCL participated in the design of the study and performed the statistical analysis. SZY and XYL participated in the design and coordination of the study, and assisted with drafting the manuscript. All authors read and approved the final manuscript.

**Author details**

1 The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou 310005, China. 2 Department of Diagnostics of Traditional Chinese Medicine, College of Basic Medical Science, Zhejiang Chinese Medical University, Hangzhou 310005, China. 3 Department of Osteology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China.

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Competing interests
The authors declare that they have no competing interests.

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