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Original Article

Weak cation exchange magnetic beads coupled with MALDI-TOF-MS in screening serum markers in perimenopausal women with osteopenia

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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 10 perimenopausal women with normal bone mass and 10 perimenopausal 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 perimenopausal women with osteopenia and perimenopausal women with normal bone mass. Protein was extracted from serum samples with the sample preparation tool of weak cation exchange magnetic beads (MB-WCX) technology, and mass spectra acquisition was done by MALDI-TOF-MS. The visualization and comparison of large 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 (GA) 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 was selected as potential biomarker. From the two group serum samples, a total of 134 differential protein peaks were selected. 10 protein peaks with significant intensity differences were screened. In the pair-wise comparisons, processing of MALDI-TOF spectra resulted in the identification of 10 differential protein peaks between perimenopausal women with osteopenia and perimenopausal women with normal bone mass. The difference of protein peaks by Youden index showed that the highest protein peaks had a mass to charge ratio of 8162 and 5921 Da. A diagnosis model was established with these two peaks as the candidate marker, and the model specificity of the model is 100%, the sensitivity was 100% by leave-one-out cross validation test. The two groups of specimens in support vector machine results on the scatterplot could be clearly distinguished. The four peaks in the SVM model were identified as Proteinkish-B and C-X-C motif chemokine 6 by TagIdent tool. This study is novel as it provides a new serological method for discovering serum protein markers to screen and diagnose osteopenia. This will help the clinicians preventing and treating primary type I osteoporosis.

Keywords: Osteopenia, weak cation exchange magnetic beads, proteomics, biomarkers

Introduction

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 have become a serious threat to the health of the elderly [1, 2].

Before people develop osteoporosis, they have a condition called osteopenia. Osteopenia was firstly defined by the World Health Organization (WHO) in June 1992 [3]. 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.

Table 1. Comparisons of clinical features of patients

Groups	Age (years)	Weight (kg)	Height (cm)
Osteopenia	53.32±2.61	52.57±5.38	160.67±9.96
Normal bone mass	52.35±1.94	53.24±3.65	161.23±10.53
t	0.94	0.38	0.12
P values	P>0.05	P>0.05	P>0.05

Data shown are mean ± standard deviation.

The BMD measurement is useful in clinical application on the diagnosis of osteoporosis and the prediction of fracture in elderly women, but it is lagging, not early. Dual-energy x-ray absorptiometry (DXA) is currently recognized as gold standard of osteopenia and osteoporosis diagnosis [4]. Although DXA measurement as 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 [5].

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 [6], clarify the pathogenesis of the disease, and explore new methods of preventing and treatment.

Magnetic beads with large separation capacity for sample preparation, and has been widely used in the researches of serum samples in tumor and beads based technology enrichment are high-throughput, simple and quick to operate [7, 8].

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 [9, 10]. 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 identify 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 its comparable. The results were shown in **Table 1**.

Detection of differential protein peaks

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 134 peaks in the molecular weight range of 1000-20000 Da were identified between the two groups in this study. The results were shown in **Figure 1**.

Discriminative ability of representative differential protein peaks

Genetic algorithm (GA) and support vector machine (SVM) embedded in ZJU-PDAS software [11] was 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 (8162 and 5921 Da) of them showed statistically significant differences in further analysis by Youden Index [12, 13]. The peak mass, *P* values, average peak intensity, and standard

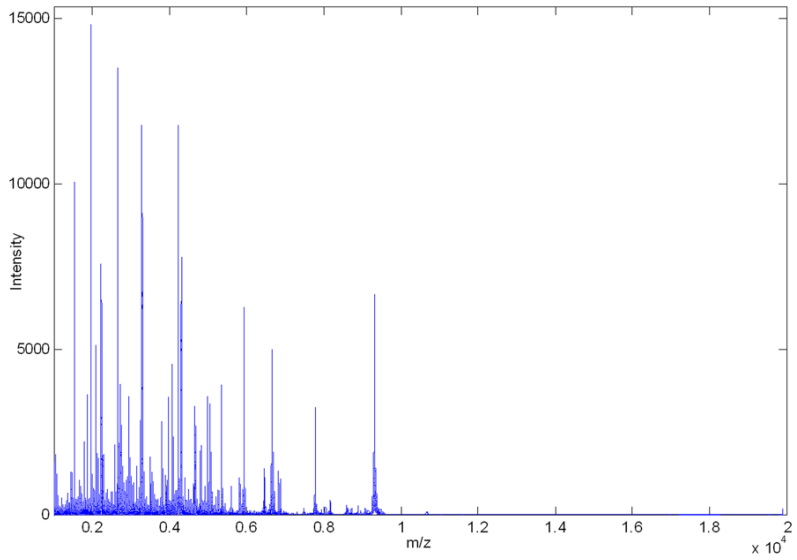

Figure 1. Detection of differential protein peaks.

Table 2. Statistics ofsignificantly different expressed peaks to distinguish patients from controls

m/z	Class 0	Class 1	*P* Values	Expression Change
8162	204.49±125.07	48.76±1601.98	0.002	↑
5921	282.65±379.03	1774.03±264.29	0.014	↓

Data shown are mean ± standard deviation. Class 0 mean perimenopausal women with osteopenia group; Class 1 mean perimenopausal women with normal bone mass group.

deviation (SD) of the four peaks were show in **Table 2**. The peaks analyzed to generate a classification model were based on the two peaks with m/z of 8162 and 5921 Da in **Figure 2**. In the optimal SVM model, the two peaks with molecular weight of 8162 Da were upregulated in patients with osteopenia and 5921 Da were downregulated.

Development of diagnostic models and evaluation of their diagnostic accuracy

The top peaks of 8162 and 5921 Da could distinguish serum samples between osteopenia and normal bone mass group effectively. 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 was shown in **Figure 3**. The specificity of the model is 100%, the sensitivity was 100% by leave-one-out cross validation test. The result was shown in **Table 3**.

Proteins bioinformatics dentification of the two differential peaks

Using TagIdent tool [14, 15] network software (http://web.expasy.org/tagident/), molecular weight (Mw) rang was set to 0.3%, Isoelectric point (PI) rang value was set to min=4, max=14. Checked for protein sequences with cysteine 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. The two peaks in the optimal SVM model were identified as Protein kish-B and C-X-C motif chemokine 6 by TagIdent tool.

Discussion

Although some progresses has been made in the analysis of osteopenia disease in the past several years. 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, whichhad discriminated perimenopausal women with osteopenia and perimenopausal women with normal bone mass.

In recent years with the development of proteomics technology, a variety of biological markers in clinical been widely applied [16-18]. Biological markers play an important role in disease diagnosis. Using serum proteomics technology [6, 19] 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) [20], the carboxy-terminal cross-linking telopeptide of type I collagen (β-Cross-

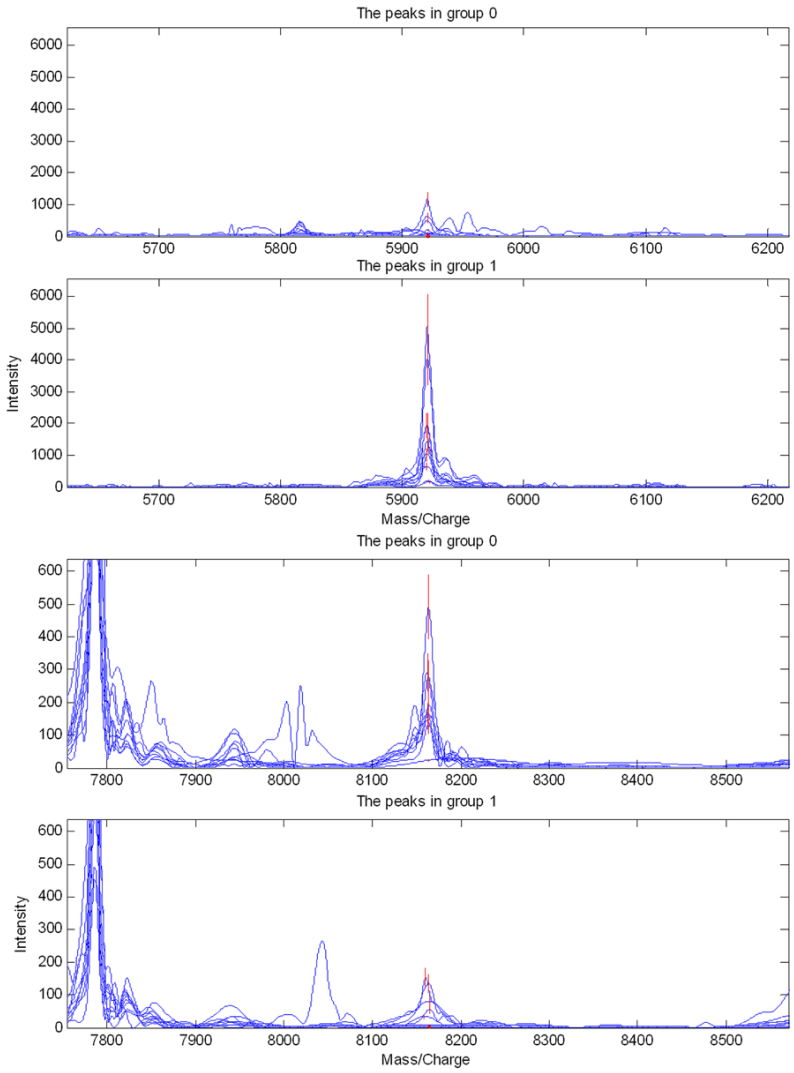

Figure 2. Discriminative ability of representative differential protein peaks. The top 2 discriminating peaks of 8162 and 5921 Da could distinguish serum samples between osteopenia and perimenopausal women with normal bone mass effectively. Group 0 mean group perimenopausal women with osteopenia; group 1 mean group perimenopausal women with normal bone mass.

Laps) [21], N-terminal osteocalcin and 25-hydroxyvitamin D3 reflect the changes of bone metabolism. However, some bone turnover markers have a low sensitivity 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 [22]. In this study, we screened 2 discriminating peaks of 8162 and 5921 Da which had distinguished serum samples between perimenopausal women with osteopenia and perimenopausal women with normal bone mass effectively. The serum proteomic model, based on a group of 2 peaks, accurately recognized perimenopausalwomen with osteopenia and perimenopausal women with normal bone mass with a sensitivity of 100% and a specificity of 100% by Leave-one-out test verification.

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 [23, 24]. Furthermore, the cost of using this technology is low, and further protein identification can be performed from the eluted material without complex purification [25]. Thus, magnetic beads-based enrichment approaches have the potential to capture and enrich low abundance, low molecular weight species [26].

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

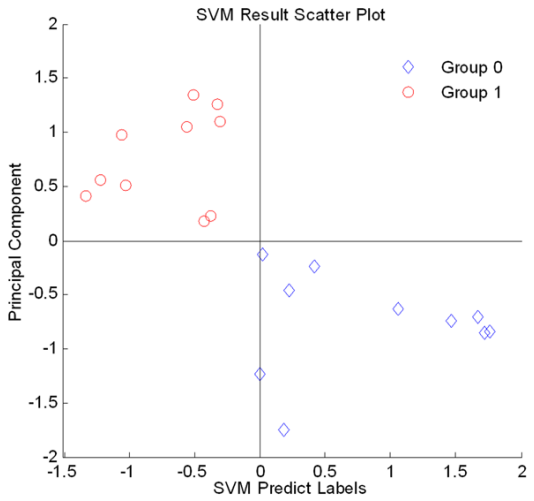

Figure 3. Development of diagnostic models and evaluation of their diagnostic accuracy. SVM result scatter plot, each point represents a sample, x-axis: a main component; y-axis: the predicted value. Mass/Change means M/Z; group 0 mean group perimenopausal women with osteopenia; group 1 mean group perimenopausal women with normal bone mass.

detected the combination of proteins existing in different forms [27].

In this study, all aspects of data processing such as sample inclusion and exclusion, serum collection, processing and storage of serum, serum protein separation and purification, sample point target, MS acquisition, experimental methods and parameter settings were optimized. Discovery researches in the clinical samples are 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 [28, 29]. 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 help the clinicians 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 Protein kish-B (KISHB_HUMAN, Q9NRX6, Chain: 23-74, pI: 10.18, Mw: 5932) and C-X-C motif chemokine 6 (CXCL6_HUMAN, P80162, Chain: 40-114, pI: 9.74, Mw: 8162) would be potential biomarker for forecasting osteopenia in perimenopausal women. However, there is still a gap between our findings and their application in clinical practice. Future blind test, enzyme-linked immunosorbent assay (ELISA) of serum proteins, multiple reaction monitoring (MRM) identification of serum proteins, western blot analysis for validation, and controlled studies with larger sample sizes are required to determine the sensitivity and specificity of the two identified serum biomarkers in the diagnosis of osteopenia. The function, interaction, and metabolic pathways of these proteins will be the future research.

Materials and methods

Subjects and samples collection

Serum samples were collected from consenting patients (n=20, 10 from perimenopausal women with osteopenia plus 10 from perimenopausal 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 [30, 31], T score -1 to -2.5 could be diagnosed as osteopenia [T = the standard deviation of (measured value-peak bone mass)/(normal adult bone density)].

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

Groups	Predicted Class 0	Predicted Class 1	Sum	Accuracy (%)	Error (%)
Class 0	10	0	10	100	0
Class 1	0	10	10	100	0

Class 0 mean perimenopausal women with osteopenia group; Class 1 mean perimenopausal women with normal bone mass group.

Inclusion criteria

Patients were included in the study based on the following criteria: (i) they conform to the diagnostic criteria of osteopenia; (ii) they were perimenopausal women; (iii) they were from 45 to 60 years old.

Exclusion criteria

Patients were excluded from the study based on the following criteria: (i) 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, osteoarthrosis, Paget's disease, and osteogenesis imperfecta; (ii) those that also had severe primary cardiac diseases, or diseases of the cerebral vessels or hematopoietic system; (iii) those that also had severe liver function or renal insufficiencies; (iv) 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; (v) those who had a medical history of mental illness; and (vi) 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 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°C for ten min at 3000 rpm (in 943 ×g). The serum were frozen and stored at -80°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 (MB-WCX) (Bruker Daltonic, Germany) was used for peptidome separation of samples following the standard protocol by the manufacturer: (i) Mix the magnetic beads thoroughly on a vortex device for 1 minute. (ii) Transfer 10 µl BB and 10 µl MB-WCX beads to a standard thin wall PCR-tube and mix by pipetting up and down. (iii) Add 5 µLserum to the solution and mix intensively by pipetting up and down five times. (iv) Wait five minutes for incubation. (v) Place the tube into the magnetic separator and collect the beads at the wall of the tube for 1 minute. The supernatant should be clear. (vi) Remove supernatant carefully by using a pipette. Avoid contact of pipette tips with the beads and take care not to remove beads. (vii) Add 100 µl WB to the tube. (viii) Move the tube back and forth in the magnetic separator ten times and note the movement of the beads. (ix) Collect the beads at the tube wall for 1 minute. (x) Remove supernatant carefully by using a pipette. (xi) Repeat steps 7-10 twice. (xii) Add 5 µl EB and dissolve the beads from the tube wall by pipetting up and down intensively 10 times. Add 5 µl EB and dissolve the beads from the tub wall by pipetting up and down intensively 10 times. (xiii) Collect the beads at the tube wall for 2 minutes. (xiv) Transfer the clear supernatant into a fresh tube. (xv) Add 5 µl SB to the eluate and mix intensively by pipetting up and down. (xvi) The eluate was then ready for spotting onto MALDI-TOF MS targets and measured or stored at -20°C.

Anchor chip spotting

1 µL eluted sample was spotted onto a MALDI-TOF AnchorChipTM target (600 µm anchor diameter) and air-drying at room temperature, then 1 µL matrix (0.3 mg/ml HCCA, 50% ACN, 2% TFA) was spotted onto MALDI-TOF AnchorChipTM target.

MALDI-TOF MS

AnchorChipTM target was placed into the Microflexmass spectrometer (Bruker Daltonics). Samples were detected after calibration of the instrument by ClinProt standard.

MALDI-TOF-MS parameters were detailed in Table S1.

Detection data analysis

ZJU-PDAS (ProteinChip Data Analyze System) software designed by Zhejiang University Cancer Institute was used to analysis the raw data.

ZJU-PDAS specific parameters of analysis were detailed in Table S2.

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 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 protein peaks. *P*-values <0.05 were considered statistically significant. Genetic algorithm (GA) [32], Support vector machine, and Youden Index methods were used to establish the diagnosis model and predict the candidate markers. The model was verified by leaving-one-out cross validation test.

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Disclosure of conflict of interest

None.

Authors' contribution

Xiao-Lin Shi and Chun-Wen Li made substantial contributions to the conception and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; Wei-Tao He, Bo Wang and Min Li carried out the WCX magnetic bead and MALDI-TOF MS analyses. Wei-Tao He and Bo-Cheng Liang participated in the design of the study and performed the statistical analysis. Yu-Zhen Shi and Xu-Yun Li participated in the design and coordination of the study, and assisted with drafting the manuscript. All authors reviewed and approved the final manuscript.

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.

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Table S1. *Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS).* Detection parameters of Microflex MALDI-TOF-MS instrument

Parameters	
N_2 pressure	Approx. 1700-2000 mbar
Laser	First, bombardment with 10-20 shots of high laser energy, then, in the same position, bombardment with 40-50 shots with energy acquisition lower laser energy of 10-20%.
Parameters	Autoflex 1-10 kDa
Shots	8–10 different crystallizations at the same target point, in the 400-500 shots
Spectrometer	
Ion source 1	20 kV
Ion source 2	18.4 kV
Lens	7.5 kV
Pulsed ion extraction	120 ns
Polarity	Positive
Matrix suppression mode	Gating
Gating strength	High/maximum
Suppress up to	Approx. 800 Da
Detection	
Mass range	Low: 900-10,500 Da
Detector gain	1600-1800 V
Sample rate	1.00
Electronic gain	Regular, 100 mV
Real time smooth	High
Laser frequency	25 Hz
Laser attenuator	e.g., 60/30

Table S2. Zhejiang University-ProteinChip data analysis system. (ZJU-PDAS) analysis of specific parameters:

User name		Peaks filtering factor	Wilcoxon
Repeat sample	0	Small size sample	No
Test sample percent	0.3	Batch analyze	1
Wavelet threshold	50	Smoothing window	100
Calibration coefficient (%)	0.03	Minimal m/z (%)	2000
Cluster factor (%)	0.003	Minimal peak threshold (%)	0.1
Excluded spectra threshold (%)	0.2	Minimal signal/noise ratio (%)	3
Minimal intensity	300	*P*-value or number of peaks	22
Algorithm	1	Evaluation	2
GA population	50	GA generation	20

GA: genetic arithmetic; m/z: mass-to-charge ratio.