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A serum metabolomics-based profile in low bone mineral density postmenopausal women

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ABSTRACT

Osteoporosis is characterized as a metabolic disorder of bone tissue, and various metabolic markers are now available to support its diagnosis and evaluate treatment effects. Substances produced as end products of metabolomic activities are the correlated factors to the biological or metabolic status, and thus, metabolites are considered highly sensitive markers of particular pathological states, including osteoporosis. Here we undertook comprehensive serum metabolomics analysis in postmenopausal women with or without low bone mineral density (low BMD vs controls) for the first time using capillary electrophoresis/mass spectrometry. Among the metabolites tested, 57 were detected in sera. Levels of hydroxyproline, Gly-Gly and cystine, differed significantly between groups, with Gly-Gly and cystine significantly lower in the low BMD group and hydroxyproline, a reported marker of osteoporosis, significantly higher. Levels of TRACP5b, a bone resorption marker, were significantly higher in the low BMD group, supporting the study's validity. Taken together, our findings represent novel metabolomic profiling in low BMD in postmenopausal women.

1. Introduction

Osteoporosis is a multifactorial disease caused by various conditions including aging, the menopause, respective inflammatory or metabolic conditions such as rheumatoid arthritis or hyperthyroidism, or steroid use [1]. Osteoporosis is diagnosed based on the presence of bone fragility fracture history, such as hip and vertebral fractures or low bone mineral density (BMD) in lumbar and hip bones [2–4]. The number of patients with the condition is increasing globally owing to increased numbers of elderly people worldwide. A recent epidemiological study revealed that, based on diagnosis of BMD in hips, >60% of women over 80 years in the general population will develop osteoporosis [5]. Since this condition often causes bone fragility fractures in the elderly, diagnosis, clinical intervention and treatment are crucial to prevent injuries.

To date, the gold standard of osteoporosis diagnosis is the presence of bone fragility fractures or low BMD in hip and vertebral bones, as detected by dual energy X ray absorptiometry (DEXA) [3,4]; however, DEXA scans are not commonly used by general practitioners, likely due to their cost. As an alternative, a fracture risk assessment tool (FRAX®) has been developed by the World Health Organization (WHO) to evaluate the risk of fracture risk over a 10-year period [6]. Several bone turnover markers are also now available to support diagnosis and evaluate drug effectiveness in osteoporosis patients [7,8]. Urinary levels of type I collagen cross-linked N-telopeptide (NTX), type I collagen cross-linked C-telopeptide (CTX) plus deoxypyridinoline, and serum tartrate-resistant acid phosphatase 5b (TRACP5b) have been used as markers of bone resorption, while bone alkaline phosphatase (BAP), osteocalcin and procollagen type I N-terminal propeptide (P1NP) can serve as indicators of bone formation. However, specific metabolomic markers useful to diagnose or evaluate drug effects in patients have not been characterized.

A recently introduced '-omics' technology [9], metabolomics can identify end products of metabolism in various body fluids such as plasma, serum and saliva [10,11] and detect changes in metabolic states as a diagnostic tool [12]. Indeed, such changes have been useful in diagnosing diseases such as cancer [13]. Changes in metabolites are also reportedly associated with low bone mineral density in animal models or in humans, and also in osteoclastic cells in vitro [14–17]. However,

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systemic analysis of metabolites indicative of low bone mineral density in postmenopausal women has not been undertaken.

Here, we undertook comprehensive metabolomic analysis of sera obtained from healthy postmenopausal women using capillary electrophoresis/mass spectrometry (CE-MS) analysis for the first time and found significantly lower levels of the dipeptide Gly-Gly, and cystine but significantly higher levels of hydroxyproline in patients showing low versus normal bone mineral density. These findings suggest that metabolic profiling combined with analysis of other bone metabolic markers could predict low bone mineral density in postmenopausal women.

2. Materials and methods

2.1. Subjects

Subjects were all female employees of the Keio University School of Medicine, aged 39–64 years, who had undergone medical examination in September of 2011 [18]. Each completed a self-reported questionnaire regarding menopausal state. This study was approved by an ethics committee at Keio University and carried out in accordance with committee-approved guidelines. Informed consent was obtained from all subjects.

2.2. Measurements

Body height, weight, serum calcium (Ca), inorganic phosphorus (IP), estradiol (E2), TRACP5b, 25(OH)D and intact parathyroid hormone (PTH) levels were assessed in all subjects. Body mass index (BMI) was calculated from body height and weight data. Serum 25(OH)D and intact serum PTH levels were monitored using an 125I RIA kit (DiaSorin, Stillwater, MN, USA) and an ECLIA kit (Cobas, Roche Diagnostics, Basel, Switzerland), respectively. Bone mineral density (BMD) was analyzed using an AOS-100 system (Aloka, Tokyo, Japan).

Statistical analysis was performed using the unpaired two-tailed student's *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS, not significant). All data are shown as means ± S.D.

2.3. Metabolite extraction from serum

Participants' frozen serum samples were thawed and 40 μL aliquots were placed into 360 μL of methanol containing internal standards (20 μmol/L each of methionine sulfone and D-camphor-10-sulfonic acid). Solutions were mixed well and then shaken with 400 μL chloroform and 160 μL Milli-Q water, followed by centrifugation at 10,000 ×*g* for 3 min at 4 °C. The aqueous layer was filtered using a 5-kDa-cutoff filter (Human Metabolome Technologies, Tsuruoka, Japan) to remove protein. The filtrate was dried using a centrifuge concentrator and reconstituted in 50 μL of Milli-Q water containing reference compounds (200 μmol/L each of 3-aminopyrrolidine and trimesic acid) prior to CE-TOFMS analysis.

2.4. CE-TOFMS analysis of metabolome

All CE-TOFMS experiments were performed using an Agilent 1600 Capillary Electrophoresis system (Agilent technologies, Waldbronn, Germany), an Agilent 6220 TOF LC/MS system (Agilent technologies, Santa Clara, CA), an Agilent 1200 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit and a G1607A Agilent CE-electrospray ionization (ESI)-MS sprayer kit. For anionic metabolite analysis, the ESI sprayer was replaced with a platinum rather than a stainless steel needle [19], although other sprayer components remained unchanged. For CE-MS system control and data acquisition, we used an Agilent MassHunter software.

2.5. Cationic metabolome analysis

For cationic analysis, a fused-silica capillary (50 μm i.d. × 100 cm) filled with 1 mol/L formic acid as electrolyte was used [20]. A new capillary was flushed with electrolyte for 20 min and then equilibrated for 4 min by flushing it with electrolyte before each run. The sample solution was injected at 5 kPa for 3 s and a positive voltage of 30 kV was applied. Capillary and sample tray temperatures were maintained at 20 °C and 4 °C, respectively. Methanol/water (50% v/v) containing 0.1 μmol/L hexakis(2,2-difluoroethoxy)phosphazene was delivered as sheath liquid at 10 μL/min. ESI-TOFMS was operated in the positive ion mode, and capillary voltage was set at 4 kV. The flow rate of heated nitrogen gas (heater temperature: 300 °C) was maintained at 10 psig. In TOFMS, fragmentor, skimmer and Oct RF voltages were set at 75, 50 and 125 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference standard masses ([^{13}C isotopic ion of protonated methanol dimer ($^{2}CH_3OH + H$)]$^{+}$, *m/z* 66.06306) and ([hexakis(2,2-difluoroethoxy)phosphazene + H]$^{+}$, *m/z* 622.02896). Exact mass data were acquired at a rate of 1.5 cycles/s over a 50 to 1000 *m/z* range.

2.6. Anionic metabolome analysis

For anionic analysis, a COSMO(+) capillary (50 μm i.d. × 105 cm, Nacalai Tesque, Japan) filled with 50 mmol/L ammonium acetate (pH 8.5) as electrolyte was used [19]. Before the first use, a new capillary was flushed successively with the electrolyte, which was 50 μmol/L acetic acid (pH 3.4), twice for 10 min each. Before each run, the capillary was equilibrated by flushing it with 50 μmol/L acetic acid (pH 3.4) for 2 min and then with the electrolyte for 5 min. The sample was injected at 5 kPa for 30 s and a negative voltage of 30 kV was applied. Capillary and sample tray temperatures were maintained at 20 °C and 4 °C, respectively. Ammonium acetate (5 μmol/L) in 50% (v/v) methanol/water containing 0.1 μmol/L hexakis(2,2-difluoroethoxy)phosphazene was delivered as sheath liquid at 10 μL/min. ESI-TOFMS was operated in the negative ion mode, and the capillary voltage was set at 3.5 kV. The flow rate of heated nitrogen gas (heater temperature: 300 °C) was maintained at 10 psig. In TOFMS, fragmentor, skimmer and Oct RF voltages were set at 100, 50 and 200 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference standard masses ([^{13}C isotopic ion of deprotonated acetate dimer ($^{2}CH_3COOH - H$)]$^{-}$, *m/z* 120.03841) and ([hexakis(2,2-difluoroethoxy)phosphazene + deprotonated acetate($CH_3COOH - H$)]$^{-}$, *m/z* 680.03554). Exact mass data were acquired at a rate of 1.5 cycles/s over a 50 to 1000 *m/z* range.

3. Data analysis

Raw data were processed using our proprietary software (MasterHands) [13,21]. Analysis followed the typical processing steps and detected all possible peaks; subtracted baselines; eliminated redundant features (e.g. isotopic, adduct, and fragment peaks), salt and neutral peaks, and noise peaks (e.g. spike peaks); and generated aligned data matrices [22, 23]. Peaks were identified by matching *m/z* values and normalized migration times of corresponding authentic standard compounds.

Principal component analysis (PCA) was performed using SIMCA-P+ software (version 13.0.0.0, Umetrics, Umeå, Sweden). Statistical analysis was undertaken using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA).

4. Results and discussion

4.1. Study population characteristics

We invited 571 women aged 39 to 64 years who had undergone a medical examination to participate in the study, and informed consent was obtained from 525. To focus on subjects with reduced estradiol