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Metabolomics-based profiles predictive of low bone mass in menopausal women

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ABSTRACT

Osteoporosis is a skeletal disorder characterized by compromised bone strength and increased risk of fracture. Low bone mass and/or pre-existing bone fragility fractures serve as diagnostic criteria in deciding when to start medication for osteoporosis. Although osteoporosis is a metabolic disorder, metabolic markers to predict reduced bone mass are unknown. Here, we show serum metabolomics profiles of women grouped as pre-menopausal with normal bone mineral density (BMD) (normal estrogen and normal BMD; NN), post-menopausal with normal BMD (low estrogen and normal BMD; LN) or post-menopausal with low BMD (low estrogen and low BMD; LL) using comprehensive metabolomics analysis. To do so, we enrolled healthy volunteer and osteoporosis patient female subjects, surveyed them with a questionnaire, measured their BMD, and then undertook a comprehensive metabolomics analysis of sera of the three groups named above. We identified 24 metabolites whose levels differed significantly between NN/LN and NN/LL groups, as well as 18 or 10 metabolites whose levels differed significantly between NN/LN and LN/LL, or LN/LL and NN/LN groups, respectively. Our data shows metabolomics changes represent useful markers to predict estrogen deficiency and/or bone loss.

1. Introduction

Osteoporosis is characterized by risk of bone fragility fracture due to several mechanisms, among them, low bone mass owing to aging and hypogonadism (Assessment of fracture risk and its application to screening for postmenopausal osteoporosis, 1994; NIH Consens. Statement, 2000). The number of osteoporosis patients is currently increasing in developed countries worldwide due to aging populations (Reginster and Burlet, 2006). To prevent fragility fractures, patients suspected of having osteoporosis are diagnosed and treated with anti-osteoporosis drugs (Marshall et al., 1996). The criteria for treatment includes bone mineral density (BMD) lower than −2.5SD and fragility fractures in vertebra or hips (Das and Crockett, 2013; Kanis et al., 2013; Siris et al., 2014). Moreover, a fracture risk assessment tool (FRAX) can assess future fragility fracture risks and is now utilized to diagnose patients needing treatment or to avoid treating patients unnecessarily (Kanis et al., 2008). Recent cohort studies reveal that more than half of females over 80 years old in the general population have been diagnosed with osteoporosis based on lower BMD values (Yoshimura et al., 2009), suggesting that more than half of all women become osteoporotic with aging as a natural course of events. Elderly females diagnosed with osteoporosis based on a BMD value lower than −2.5SD are estimated show a bone mass 70% of the young adult mean (YAM); however, increases in BMD following osteoporosis therapy are reportedly limited to 7–10% or 3–5% of baseline BMD in the lumbar spine or femoral neck, respectively (Black et al., 1996; Cummings et al., 2009; Ettinger et al., 1999; Harris et al., 1999). Thus, blocking future loss from the peak bone mass would likely be a more effective way to prevent bone fragility fractures correlated with low bone mass. Doing so requires predictive tools, which are currently unavailable.

Recently, changes in levels of metabolites have been associated with altered bone mineral density in human (You et al., 2014; Qi et al., 2016;

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Miyamoto et al., 2017; Moayyeri et al., 2017) or animal models (Lee et al., 2016; Huang et al., 2016; Ma et al., 2013), or in cultured osteoclastic cells in vitro (Liu et al., 2015). However, comprehensive, systemic metabolomics studies in humans aimed at identifying candidate metabolites associated with low bone mineral density or estrogen deficiency have been limited, requiring further studies to understand menopausal changes in particular. Previously, we used comprehensive metabolomic analysis to show that blood levels of the di-peptides Gly-Gly and Cystine are significantly lower in sera of post-menopausal females exhibiting low relative to normal BMD (Miyamoto et al., 2017). We concluded that both metabolites could be useful markers to predict low BMD without BMD measurements, which require dual energy X ray absorptiometry (DEXA) and are costly (Miyamoto et al., 2017). Here, we searched for additional candidate metabolites useful as markers of low BMD.

Here, we newly recruited female subjects, including pre- and post-menopausal women, and subdivided them into three groups: 1, pre-menopausal and normal BMD (with normal estrogen and normal BMD, NN); 2, post-menopausal and normal BMD (with low estrogen and normal BMD, LN) and 3, post-menopausal and low BMD (with low estrogen and low BMD, LL). We then collected sera for comprehensive metabolomics analysis. Gly-Gly and Cystine levels were significantly lower in LL than in LN groups, confirming our previous study. We also identified 10 metabolites whose levels differed significantly between LN and LL groups. We also identified 24 and 18 metabolites whose levels differed significantly between NN and LN, and LN and LL groups, respectively. Overall, we conclude that our metabolomics profiles could serve as a useful diagnostic tool to monitor the metabolic changes accompanying estrogen deficiency and subsequent bone loss.

2. Materials and methods

2.1. Subjects

Subjects were female employees of the Keio University School of Medicine, aged 39–61 years, who had undergone medical examination in September of 2013 (Miyamoto et al., 2017; Miyamoto et al., 2016) or who had visited our hospital as possible patients. Written informed consent was obtained from all subjects. All subjects were Japanese. Each completed a self-reported questionnaire regarding menopausal status and drug usage. This study was approved by an ethics committee at Keio University School of Medicine and carried out in accordance with guidelines approved by that committee.

2.2. Measurements

All subjects were asked to fast overnight and were assessed for height, body weight, serum calcium (Ca), inorganic phosphorus (IP), 25(OH)D, intact parathyroid hormone (PTH), TRACP5b and estradiol (E2) levels. Body mass index (BMI) was calculated from body weight and height data. Serum 25(OH)D and intact serum PTH levels were analyzed by 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 in all subjects using an AOS-100 system (Aloka, Tokyo, Japan) or dual-energy X-ray absorptiometry (DEXA; GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England).

Statistical analysis was performed using the unpaired two-tailed Student's or Welch'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

Extraction was performed as described (Miyamoto et al., 2017). Briefly, 40 µl aliquots of serum from subjects were mixed with 360 µl methanol containing internal standards (20 µmol/l each of methionine sulfone and D-camphor-10-sulfonic acid). Solutions were shaken with 400 µl chloroform and 160 µl Milli-Q water, and then centrifuged at 10,000 ×*g* for 3 min at 4 °C. The aqueous layer was removed and 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.

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2.4. CE-TOFMS metabolome analysis

All CE-TOFMS analyses were performed using an Agilent 1600 Capillary Electrophoresis system (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 6210 TOF LC/MS system (Agilent Technologies, Santa Clara, CA) as described (Soga et al., 2009). For anionic metabolite analysis, the ESI sprayer was replaced with a platinum rather than a stainless steel needle as described (Soga and Heiger, 2000). Cationic metabolites were separated through a fused-silica capillary (50 µm i.d. × 100 cm) filled with 1 mol/l formic acid as electrolyte (Hirayama et al., 2015), and then a methanol/water (1:1) solution containing 0.1 µmol/l hexakis(2,2-difluoroethoxy) phosphazene was delivered as sheath liquid at a rate of 10 µl/min. The capillary temperature was maintained at 20 °C.

The sample solution was injected at 5 kPa for 3 s, and the separation voltage was set at 30 kV. ESI-TOFMS was conducted in positive ion mode, and capillary, fragmentor, skimmer and Oct RF voltages were set at 4000, 75, 50 and 125 V, respectively. Nebulizer gas pressure was configured at 7 psig, and heated (300 °C) nitrogen gas was supplied at a rate of 10 L/min. Anionic metabolites were separated through a COSMO(+) capillary (50 µm i.d. × 105 cm, Nacalai Tesque, Kyoto, Japan) filled with 50 mmol/l ammonium acetate (pH 8.5) as electrolyte (Soga et al., 2009), and an ammonium acetate (5 mmol/l) in a (1:1) methanol/water solution containing 0.1 µmol/l hexakis(2,2-difluoroethoxy)phosphazene was delivered as sheath liquid at a rate of 10 µl/min. The sample solution was injected at 5 kPa for 30 s. Negative 30 kV was applied for sample separation. ESI-TOFMS was conducted in negative ion mode, and capillary, fragmentor, skimmer and Oct RF voltages were set at 3500, 100, 50 and 200 V, respectively. Other conditions were identical for cationic metabolite analysis.

In both modes, an automatic recalibration function was used to correct for analytical variation of exact masses for each run, as described (Hirayama et al., 2015). Exact mass data were acquired at a rate of 1.5 cycles/s over a 50–1000 *m/z* range.

Raw data were processed using our proprietary automatic integration software (MasterHands) (Sugimoto et al., 2010; Sugimoto et al., 2012). Each peak was identified by matching *m/z* values and normalized migration times of corresponding authentic standard compounds.

Statistical analysis was performed using the Mann-Whitney *U* test (*, $p < 0.06$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant).

3. Results and discussion

3.1. Basic characteristics of study subjects

We invited 597 women aged 39–64 years, who had undergone a medical examination to participate in this study and obtained informed consent from 526. We also obtained informed consent from 325 patients who had visited our osteoporosis out-patient clinic for the first time. Subjects with the following conditions were excluded ($n = 741$) from the study: those who had used medications including but not limited to osteoporosis drugs; those with unstable menstruation status; subjects exhibiting a BMI of < 18.5 or > 30; and individuals with incomplete data sets (Fig. 1). Then, the remaining subjects ($n = 110$) were divided into pre- and post-menopausal groups, and the latter was subdivided into “low BMD” (lower than −1 SD (low estradiol [E2] and