Endocr (2010) 38:83–86
DOI 10.1007/s12020-010-9353-1

ORIGINAL ARTICLE

Genetic variant in the aquaporin 9 gene is associated with bone mineral density in postmenopausal women

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Received: 13 October 2009 / Accepted: 1 June 2010 / Published online: 18 June 2010

Abstract This study investigated whether single nucleotide polymorphisms (SNP) in the aquaporin 9 (AQP9) gene is associated with bone mineral density (BMD) in Thai postmenopausal women, after an initial genome-wide screening using high-throughput SNP genotyping in pooled DNA samples. Subjects consisted of 516 postmenopausal women aged 50 or more. High-throughput SNP screening was performed by comparing the estimated allele frequency derived from hybridization signal intensities of pooled DNA samples on the Affymetrix 500 K SNP genotyping chip set. The SNP was then genotyped for each subject individually. Data were expressed as mean ± SEM. Pooled DNA SNP screening revealed the allele frequency of an intronic A/T SNP rs2414539 in the AQP9 gene as being different between subjects with femoral neck BMD in tertiles 1 and 3. Individual genotyping in all subjects revealed that femoral neck BMD in subjects with TT, TA, and AA genotypes were 0.79 ± 0.06 ($n = 3$), 0.75 ± 0.01 ($n = 98$), and 0.71 ± 0.01 g/cm^2 ($n = 415$), respectively. The presence of the T allele in rs2414539 was associated with femoral neck BMD ($r = 0.11$, $P < 0.05$) but not with lumbar spine BMD. The relationship was still significant after controlling for body weight and age ($P < 0.05$). Genetic variation in the AQP9 gene is associated with femoral neck BMD in postmenopausal women, and may represent one of the susceptibility genes for phenotypes related to bone mass.

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Keywords Aquaporin 9 (AQP9) · Bone mineral density (BMD) · Postmenopausal women

Introduction

Bone mass is determined by both genetic and non-genetic factors, probably through their influences on bone turnover. Imbalance between bone formation and bone resorption can lead to abnormal bone mass. For example, postmenopausal osteoporosis is characterized by excessive bone resorption. The condition can be ameliorated by antiresorptive agents such as estrogen and bisphosphonates. Bone turnover is controlled by a number of factors including calcium and vitamin D status, certain cytokines, and the RANK/RANKL/OPG system [1, 2]. Candidate genes involved in these pathways have been studied to elucidate the genetic susceptibility for postmenopausal osteoporosis. The results, however, are conflicting, suggesting the genetic heterogeneity of osteoporosis.

Recently, aquaporin 9 (AQP9) was found to represent a novel factor affecting bone resorption. Of all the aquaporins studied, only AQP9 is expressed in osteoclast lineage cells and AQP9 inhibitor impairs the differentiation of RANKL-stimulated osteoclast precursors [3]. However, AQP9 does not appear essential for the normal physiology of osteoclasts, as demonstrated by the normal bone phenotype in AQP9-null mice [4]. Studies with regard to AQP9 and bone metabolism have so far been limited to experimental animals. The role and significance of AQP9 in humans are currently unknown. It is the purpose of the present study to explore the potential role of AQP9 in human bone metabolism by investigating genetic variants in AQP9 which may be associated with bone mineral density (BMD) in postmenopausal women, after an initial

screening using high-throughput single nucleotide polymorphism (SNP) genotyping in pooled DNA samples.

Results

When comparing the estimated allele frequencies of SNPs along the AQP9 gene represented in the 500 K genotyping microarray between postmenopausal women with femoral neck BMD in the lower and the higher tertiles, it was found that rs2414539 A/T SNP had the highest estimated odds ratio (Fig. 1). Subjects in all tertiles were then individually genotyped for rs2414539 using real-time PCR.

The genotype distribution of rs2414539 was 415 (80.4%) AA, 98 (19.0%) AT, and 3 (0.6%) TT. This distribution did not deviate from the Hardy–Weinberg equilibrium.

Because of the rarity of the TT genotype, AT and TT genotypes were combined for further analyses. There was no difference in age or body weight with regard to the presence of the T allele. Femoral neck BMD in subjects with the T allele was significantly higher. However, no difference in lumbar BMD was found (Table 1). Multiple linear regression analysis showed that the presence of the T allele in rs2414539 was significantly associated with femoral neck BMD after controlling for age and body weight (Table 2).

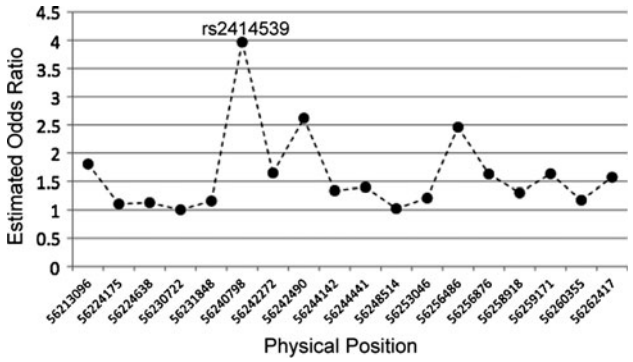

Fig. 1 Estimated odds ratio of having BMD in the lower tertile of intragenic SNPs along the AQP9 gene

Table 2 The association of AQP9 (presence or absence of the T allele in rs2414539) with femoral neck BMD after controlling for body weight and age

	Standardized coefficient	*P*-value
Femoral neck BMD	0.08	<0.05
Body weight	0.24	<0.001
Age	−0.42	<0.001

Discussion

In this study, we utilized screening of pooled DNA samples on SNP genotyping microarrays, and identified the association of femoral neck BMD with an intragenic SNP in the AQP9 gene. Susceptibility to osteoporosis and skeletal responsiveness to therapeutic agents are genetically determined [5, 6]. To date, a number of candidate genetic variants have been identified as potentially associated with osteoporosis. The validity of some of the reported variants, however, is in question due to the inherently high rate of non-reproducibility across populations. On the other hand, genome-wide association studies have been utilized without a priori assumptions to identify, in an unbiased manner, genetic variations associated with osteoporosis phenotypes [7, 8]. This approach, although fruitful, is associated with high expenses which can be prohibitive in situations with limited financial resources. To circumvent the issues related to cost, a number of investigators have resorted to DNA pooling, and have performed hybridization on many fewer SNP genotyping microarray chips [9, 10]. The approach has been demonstrated to have acceptable validity [11]. However, the signal-to-noise ratio is obviously higher compared to individual genotyping, making the approach suitable for screening purposes rather than for definitely pinpointing the susceptibility to SNPs [12].

AQP9 is one of the 13 AQPs discovered to date. AQP9 is expressed in many tissues [13] including osteoclasts. Recently, AQP9 expression in osteoclasts has been demonstrated during osteoclast differentiation, and may mediate the increase in cytoplasm after cell fusion [3]. In contrast, AQP9-null mice have a normal bone phenotype

Table 1 The association of BMD between the rs2414539 SNP with and without T allele groups

	AA ($n = 415$)	AT or TT ($n = 101$)	*P*-value
Femoral neck BMD (g/cm^2)	0.71 ± 0.01	0.75 ± 0.01	<0.05
Lumbar spine BMD (g/cm^2)	0.89 ± 0.01	0.92 ± 0.02	NS
Age (year)	66.9 ± 0.3	66.1 ± 0.6	NS
Body weight (kg)	55.3 ± 0.9	56.3 ± 1.0	NS
BMI (kg/m^2)	24.16 ± 0.65	24.09 ± 0.38	NS

[4], which puts the physiological role of AQP9 into question. In this study, we demonstrated the association between a SNP in intron 1 of AQP9 and femoral neck BMD in postmenopausal women. This raises the possibility of a role of AQP9 in human bone metabolism, despite previous controversial results in experimental animals in vivo and in vitro. The discrepancy between the previous two studies may be due to the presence of stimulation by RANKL in the study where a positive result was shown. Our study was performed in postmenopausal women; and it is well known that estrogen deficiency increases bone resorption, partly through an increase in the RANKL to osteoprotegerin ratio [14]. Our result is in line with the previous study which showed that the AQP9 inhibitor inhibits RANKL-stimulated differentiation of osteoclasts. Nevertheless, our findings should be considered preliminary, and further validation needs to be performed because the SNP associated with bone mass in AQP9 is located in the intronic region, with unclear functional significance.

AQP9 is unique compared to other AQPs in that it mediates transport of other small molecules besides water. AQP9—as well as AQP3, AQP7, and AQP10—also plays a role in glycerol trafficking through cell membranes [13, 15]. This implies that AQPs may influence functions related to glycerol transport in adipocytes, and hence affect the propensity to gain or lose fat mass. Indeed, AQP7 has been shown to be severely underexpressed in the adipose tissue of obese individuals [16]. Moreover, SNPs in AQP7 are associated with type 2 diabetes and adiposity as assessed by body mass index [17]. AQP9 is less expressed in adipose tissue, and the role of AQP9 in fat accumulation is less clear. However, AQP9 is believed to be the only AQP expressed in liver cells [18]. It is well known that body weight and bone mass are correlated, which may be due to the effect of higher mechanical load, higher muscle mass, or a greater amount of adipose tissue in subjects with higher body weight. Given the relationship between AQPs and glycerol metabolism in both adipose tissue and the liver, it is likely that the association between AQP9 variation and bone mass demonstrated in this study may also be mediated through the effect of AQP9 on lipid metabolism and body weight. However, we were not able to demonstrate a relationship between the studied AQP9 variation and body weight or adiposity, as reflected by body mass index, in this study. The influence of AQP9 on adiposity, however, cannot be entirely ruled out, since pooled DNA screening for SNPs likely to be associated with adiposity was not examined in this study.

In conclusion, a genetic variation in the AQP9 gene is associated with femoral neck BMD in postmenopausal women, and may represent one of the susceptibility genes for phenotypes related to bone mass.

Materials and methods

Subjects consisted of 516 postmenopausal women recruited by advertisement to participate in BMD screening. All were ambulatory and were not taking medication that might have altered bone metabolism. The study was approved by the institutional review board, and volunteers signed informed consent prior to the study.

BMD measurement

BMD and body composition were measured by dual-energy X-ray absorptiometry (Lunar Prodigy, Lunar Corp., USA) at the anteroposterior lumbar spine (L2–L4) and femoral neck in each subject. Daily calibration and quality control were performed regularly according to manufacturer's instructions. In vivo coefficients of variance for the anteroposterior lumbar spine (L2–L4) and femoral neck were 1.2 and 1.6%, respectively.

DNA pooling preparation

DNA was extracted from peripheral blood samples by conventional phenol–chloroform method. DNA samples were diluted in TE buffer (0.1 mM EDTA, 10 mM Tris–HCl, pH 8.0) and quantified using fluorometry (Hoefer® DyNA Quant 200, Hoechst dye) to a target concentration of 100 ng/μl and then to 50 ng/μl. DNA pools were constructed separately for subjects with femoral neck BMD in tertiles 1 and 3 by combining the 50 ng/μl DNA in equal volume. Each pool was purified, and again quantified exactly to 50 ng/μl.

Allele frequency estimation from DNA pools using SNP genotyping microarray

Femoral neck BMD was divided into tertiles. Three pooled DNA samples—each consisting of DNA from 50 individuals with femoral neck BMD in the lower tertile, together with another three pooled DNA samples from subjects with femoral neck BMD in the higher tertile—were formed. Pooled DNA samples were then genotyped using Affymetrix GeneChip® Mapping 500K arrays, following manufacturer's protocol. In brief, 250 ng of DNA sample from each pool was restricted by Nsp I or Sty I restriction endonuclease before ligation. The diluted ligated DNA was amplified by PCR. Purified PCR products were fragmented, labeled, and then hybridized. Estimated allele frequencies and odds ratios for each SNP were calculated from averaging hybridization intensity signals from the arrays. SNP rs2414539 possessed the highest estimated odds ratio, and was selected for individual genotyping.

Individual SNP genotyping

Data from pooled DNA SNP screening revealed that allele frequency of an intronic A/T SNP rs2414539 in the AQP9 gene is different between subjects with femoral neck BMD in tertiles 1 and 3 (Fig. 1). Individual genotyping of all subjects was performed using real-time PCR (TaqMan® MGB probes): 10 ng of DNA was added into the PCR reaction, consisting of TaqMan® Universal Master Mix (1×), and TaqMan® MGB probes for intronic A/T SNP rs2414539 (1×) in a total volume of 10 μl. The real-time PCR reaction protocol was 10 min at 95°C, 40 cycles of 15 s at 92°C, and 1 min at 60°C using 7500 Real-Time PCR System (Applied Biosystems, USA).

Statistical analysis

Data were expressed as mean ± SEM. The differences of variables were determined by the Student's *t*-test. Independent effect of AQP9 on BMD was determined by multiple linear regression analysis. Statistical significance was assigned at $P < 0.05$.

Acknowledgment This study was supported by the National Science and Technology Development Agency of Thailand.

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