Osteoporos Int. Author manuscript; available in PMC 2013 March 22.

Published in final edited form as:
Osteoporos Int. 2011 June ; 22(6): 1981–1986. doi:10.1007/s00198-010-1405-0.

Homozygous deletion of the *UGT2B17* gene is not associated with osteoporosis risk in elderly Caucasian women

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Abstract

Summary—Previously, homozygous deletion of the *UGT2B17* gene has shown association with hip fracture. Using a high-throughput qRT-PCR assay, we genotyped *UGT2B17* copy number variation (CNV) in 1,347 elderly Caucasian women and examined for effects on bone phenotypes. We found no evidence of association between *UGT2B17* CNV and osteoporosis risk in this population.

Introduction—Genetic studies of osteoporosis commonly examine SNPs in candidate genes or whole genome analyses, but insertions and deletions of DNA, collectively called CNV, also comprise a large amount of the genetic variability between individuals. Previously, homozygous deletion of the *UGT2B17* gene in CNV 4q13.2, which encodes an enzyme that mediates the glucuronidation of steroid hormones, has shown association with the risk of hip fracture.

Methods—We used a quantitative real-time PCR assay for genotyping the *UGT2B17* CNV in a well-characterized population study of 1,347 Caucasian women aged 75.2 ± 2.7 (mean ± SD) years, to assess the effect of the CNV on bone mass density (BMD) at the total hip site and osteoporosis risk.

Results—The *UGT2B17* CNV distribution was consistent with the expected Hardy-Weinberg distribution and not different from frequencies previously reported in a Caucasian population. Data from ANCOVA of age- and weight-adjusted BMD for *UGT2B17* CNV genotype showed no significant difference between genotype groups. Individuals with homozygous or heterozygous deletion of the *UGT2B17* gene showed no increased risk of incident fragility fracture.

Conclusions—These data suggest that quantitative real-time PCR is a rapid and efficient technique for determination of candidate CNVs, including the *UGT2B17* CNV; however, we found no evidence of an effect of *UGT2B17* CNV on osteoporosis risk in elderly Caucasian women.

Introduction

Several large genome-wide association studies have recently shown convincing relationship between single nucleotide polymorphisms (SNPs), bone mineral density (BMD) and fracture risk [1, 2]; however combinations of these SNPs only contribute about 5% of the risk of developing osteoporosis [3]. Alternative structural genomic mechanisms, including copy

All authors state that they have no conflict of interest

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number variations (CNVs), are being investigated as a source of phenotypic variation in diseases such as asthma, autoimmune disease and psychiatric disease [4-12], and so CNVs may also be relevant to osteoporosis. In 2008, Yang et al. conducted a case-control study and detected a CNV including the *UGT2B17* gene [3]. That study found that a *UGT2B17* gene copy number of two was significantly associated with lower BMD, thinner cortical thickness, higher buckling ratio, and increased risk of osteoporotic fracture at the hip [3]. In this study, we examined *UGT2B17* copy number to ascertain the phenotypic effects of *UGT2B17* CNV in a large cohort of postmenopausal women. In the past, CNVs have commonly been detected using either conventional PCR with subsequent analysis of the amplicons by agarose gel [13] or in large-scale analyses by hybridization arrays [14]. Quantitative real-time PCR (qRT-PCR) has been proven to be a powerful and sensitive technology for the quantification of deoxyribonucleic acid (DNA) in various applications including gene expression and regulation [15]. We, and others, have recently developed qRT-PCR methods for efficient genotyping of candidate CNV using either SYBR Green or dual-labeled florescent probe methodologies [16]. This study reports a qRT-PCR method that is a rapid and straightforward technique for determination of candidate CNVs and examines the effect of *UGT2B17* CNV on osteoporosis risk.

Methods

Subjects

The *UGT2B17* CNV was genotyped in 1,347 individuals who were participants of the Calcium Intake Fracture Outcome Study (CAIFOS), a 5-year controlled trial looking into the effects of calcium supplementation on prevention of osteoporotic fractures. The participants in the study were postmenopausal Caucasian women aged between 70 and 85 years and represent a random population sample, as described previously [17]. Subjects were excluded if they were taking medication that were known to affect bone metabolism or were suffering from illness that suggested non-compliance to the trial, and subjects were randomized to 1.2 g of calcium carbonate daily or a matched placebo [18]. All subjects completed a lifestyle questionnaire [19]. Height and weight were measured at baseline. One thousand three hundred eighty-seven women gave consent to have blood samples taken for genetic analyses. Written informed consent was collected from each subject, and the human rights committee of the University of Western Australia approved the study.

BMD and quantitative ultrasound measurements

Dual-energy X-ray absorptiometry (DXA) was undertaken using a Hologic 4500A machine (Hologic, Boston, MA, USA) to measure BMD at the total hip and femoral neck regions at year 1 of the study [18]. The coefficient of variation (CV) at the total hip and femoral neck were 1.2% and 1.4%, respectively [18]. A total of 1,103 subjects had both BMD and genotype data available. At baseline, quantitative ultrasound (QUS) of the left calcaneus was measured twice in all subjects [19].

Measurements were taken using a Lunar Achilles ultrasound machine (Lunar Corp., Madison, WI, USA) employing the manufacturer's quality assurance methods. The average of the two measurements of speed-of-sound (SOS), broadband ultrasound attenuation (BUA) and stiffness were recorded and used in the analyses [19]. Using the manufacturer's standards, the CV for SOS and BUA were 0.43% and 1.59%, respectively [19].

Fracture status

Clinical incident fractures due to a minimal trauma, as defined by falling from a height of less than 1 m, were assessed during the 84-month follow-up from baseline using radiographic reports. Fractures of the face, skull, fingers, and toes were not included.

Biochemistry

Each subject had a blood sample collected after an overnight fast at baseline. The measurement of serum estradiol was carried out using radioimmunoassay (Orion Diagnostica, Espoo, Finland) and this assay had an inter-assay CV of 20% at 18 pmol/L and 6.6% at 100 pmol/L and an intra-assay CV of 5% at 100 pmol/L [20]. Sex hormone-binding globulin (SHBG) was measured using an immunochemiluminometric assay (Imulite Co., LA, USA) and the inter- and intra-assay CVs were 6.8% and 7.1%, respectively at 24 nmol/L [20]. The free estradiol index (FEI) was calculated as the molar ratio of estradiol to SHBG. Genomic DNA was extracted and purified from ethylenediamine tetraacetic acid peripheral blood for genotyping analyses [20].

UGT2B17 CNV genotyping

Primers—For the test primer pair, oligonucleotides were designed to specifically amplify a region within the *UGT2B17* gene. Due to high sequence homology between *UGT2B17* and other *UGT* genes, the *UGT2B17* test primers (Fwd 5′CCAGATGAGTATGGGCACTACA, Rev 5′GGTCTCAGGTAATCCTCCACCT) were designed with the assistance of Primer3 software [21] and the University of California – Santa Cruz human genome browser (NCBI Build 36.1) [22]. Normalization of *UGT2B17* CNV data was made to a reference gene, *neuropeptide FF receptor 2* (*NPFFR2*; Fwd 5′TCATCTGGGTCCTAGCCATC, Rev 5′AGGGGAGCCAGGTAGATGTT), which is co-located on 4q13 and was selected because there have been no previous reports of copy number variation within or around this gene. All primers were synthesized by Sigma GenoSys (Brisbane, Australia) and tested to ensure amplification of single distinct bands with no primer-dimer formation.

Relative real-time quantitative PCR—Relative copy number differences in the *UGT2B17* gene were analyzed with qRT-PCR using the SYBR Green I method. The qRT-PCR was performed in a 96-well plate on an iQ5 Real-Time PCR Detection System (Bio-Rad, USA). Each reaction was run in duplicate and contained 7.5 μL 2 x QuantiFast SYBR Green PCR Master Mix (Qiagen, USA), *UGT2B17* or *NPFFR2* forward and reverse primers at a final concentration of 1 μM and 30 ng of purified genomic DNA in a final reaction volume of 15 μL. Every plate included two no-template control samples and duplicates of two *UGT2B17* CNV = 1 and *UGT2B17* CNV = 0 control samples. The qRT-PCR protocol utilized the following conditions: denaturation (95°C for 5 min), amplification and quantification step (repeated 35 cycles at 95°C for 10 s and 60°C for 30 s with a single fluorescence measurement) with a melting curve analysis (65°C to 95°C with fluorescence measurement taken at every 0.5 °C increment). Melting curve analyses were performed using Bio-Rad iQ5 Optical System Software v2 to ensure only a single product was amplified. To determine the efficiencies of target amplifications, standard curves were created using a tenfold dilution series of a single genomic DNA sample across five orders of magnitude with each dilution amplified in triplicate. *UGT2B17* CNV was determined using the cycle threshold (C_T) values and amplification efficiencies values yielded by the qRT-PCR for both the test (*UGT2B17)* and control (*NPFFR2)* genes, using the Pfaffl method [23]. The *UGT2B17* copy number was validated in 95 samples using a traditional PCR and gel electrophoresis method described by Wilson *et al.* [13] with marker C (5′ CCTGGAAGAGCTTGTTCAGA 3′) located within exon 1 of *UGT2B17* and a deletion marker J (5′ TGCACAGAGTTAAGAAATGGAGAGATGTG 3′) employed for the validation studies.

Data analyses

Statistical analysis was performed using SPSS for Windows v16 (SPSS Inc., Chicago, IL, USA). A chi-square test was used to confirm that the CNV genotype distribution was in

Hardy-Weinberg equilibrium. We first tested whether the following variables: age, height, weight and calcium supplementation were significantly associated with BMD, QUS and biochemical variables using multiple linear regression - significantly associated variables were retained as covariates in subsequent analyses. The differences in phenotypic means for each genotype group were examined using analysis of covariances (ANCOVA), with the covariates age, height and weight incorporated in the analysis of QUS; height and weight in the analysis of biochemical phenotypes; and age and weight included in the model for BMD. Post hoc analyses of significant associations from ANCOVA used Tukey's test. Cox proportional hazard analysis was performed to examine the genotypic effects on incident fracture rates. Using the genetic power calculator [24] and the most conservative effect size reported of 0.67% [3], we calculated 78-85% power ($\alpha = 0.05$) to detect an association with the traits examined in this study. Two-tailed *P* values are reported throughout, with values ≤ 0.05 considered statistically significant.

Results

The demographics, bone density, QUS and biochemistry data for the CAIFOS population are presented in Table 1.

Genotyping and validation

In the study group, we detected 161 subjects (12.0%) with a homozygous deletion, 613 subjects (45.5%) with a heterozygous deletion and 573 (42.5%) subjects with two copies of the *UGT2B17* gene (chi-square test for deviation from the Hardy-Weinberg equilibrium, *P*=0.989). The accuracy rate for the genotyping assay was shown to be 100% as determined by concordance of results from qRT-PCR with conventional PCR and gel electrophoresis in the subset of 95 samples genotyped with both methods. Genotyping success rate in the CAIFOS population for *UGT2B17* was 100%. Regarding efficiency, we noted an approximately twofold greater efficiency in genotyping throughput for 96 samples with the qRT-PCR method versus the conventional PCR and electrophoresis technique.

Association analyses

There was no evidence of association between the *UGT2B17* CNV genotype and BMD at either total hip or femoral neck sites (*P*=0.100 and *P*=0.879, respectively; Table 2). After stratifying for the presence or absence of the *UGT2B17* gene, there also was no evidence of association between BMD at either total hip or femoral neck sites and *UGT2B17* CNV (*P*=0.523 and *P*=0.802, respectively; data not shown). Although qualitative ultrasound parameters were found to be associated with *UGT2B17* CNV as shown in Table 2, post hoc analysis indicated that these significant associations were due to the data for heterozygous individuals and thus did not support our hypothesis of an additive genetic effect. A Cox proportional hazards regression analysis showed no significant difference for incident fracture events between *UGT2B17* CNV groups over 84 months adjusted for age. In relation to the biochemistry parameters, subjects with homozygous deletion of the *UGT2B17* gene had significantly lower serum concentrations of SHBG (*P*=0.024), but neither estradiol nor FEI was significantly associated with *UGT2B17* CNV (*P*=0.974 and *P*=0.333 respectively; Table 2). SHBG correlated significantly with estradiol (r= −0.203, *P*<0.001) and similarly, SHBG correlated significantly with FEI (r=0.653, *P*<0.001).

Discussion

In this study, we investigated a possible effect of the *UGT2B17* CNV in the pathogenesis of osteoporosis. The frequency distribution observed in this study is the same as that reported previously in another mixed male and female group of Caucasians [3]; however, the present

study did not detect any association between the *UGT2B17* CNV and BMD at either the hip or femoral neck sites after correction of the data for age and weight. Yang et al. reported that increasing dosage of the *UGT2B17* CNV yielded an increased risk for osteoporosis, lower BMD, and poorer bone geometry in both their Asian and Caucasian cohorts [3]. One important difference between that study and the current report is that the Caucasian group examined by Yang et al. included men and women. We studied only Caucasian women and found no additive genetic association between BMD, QUS, or fragility fracture and *UGT2B17* CNV, thus one possibility is that the effect of the gene deletion on bone phenotypes is mainly relevant to men. This would be consistent with the role of the protein product of the gene, UDP-glucuronosyltransferase 2B17, in androgen metabolism [25]. There is an apparent specificity of this enzyme for different steroids, with UDP-glucuronosyltransferase 2B17 reported to show higher glucuronidation activity for testosterone and dihydrotestosterone compared to estradiol [25]. Other reports showing association of the *UGT2B17* CNV with prostate cancer [26, 27] and serum testosterone concentration [28] provide further evidence for a role of the gene deletion in androgen metabolism; however, a gender specific effect cannot be proven in the current study, since we did not explore the CNV and phenotype association in men. Indeed, a further difference with the study design of Yang et al. is that the Caucasian individuals in their study had a mean age of 50.23 (standard deviation=18.24) and included pre-, peri-, and post-menopausal women, whereas in this study women were approximately 25 years older and were all postmenopausal, with concomitant lower mean serum sex steroid concentrations. Also, as part of the CAIFOS design, individuals in this study were randomized to treatment with a calcium supplement or placebo, but we have previously shown that this had no effect on BMD, QUS or fracture in these women [18]. Furthermore, in our analyses we considered the possible role of calcium supplementation in explaining the difference in our results to those of Yang et al. by including treatment as a covariate in the analysis, but there was no evidence of any effect. In this study, we observed no significant association between *UGT2B17* gene copy number and estrogen levels or free estradiol index, although we did detect a significant association with SHBG. SHBG is a binding protein with a role in the transport of estradiol in blood and the concentration of this protein is important in determining the FEI - an index of biologically available estradiol in study subjects. We found a positive correlation between estradiol, FEI and SHBG, but the data indicates that the association between *UGT2B17* CNV and SHBG is probably a false positive finding and is unlikely to be physiologically relevant because *UGT2B17* CNV does not seem to affect the levels of estradiol or FEI, which would be expected for a true positive result with a role in modulating the biochemical phenotype.

There have been several reports regarding the conspicuous interethnic differences of the expression of the *UGT2B17* gene [29]. Most African populations display high copy number of the *UGT2B17* gene, followed by Europe and West Asian populations with intermediate frequency and the East Asian population exhibit high frequency of copy number deletion of the *UGT2B17* gene [29]. This marked difference in *UGT2B17* gene expression between diverse ethnic groups could potentially lead to conflicting conclusions in studies not well controlled for population admixture; however our study population is of Caucasian ancestry and genotype frequencies observed were consistent with previous reports. The initial case-control and replication studies performed by Yang et al. were on Chinese subjects in whom the frequency of this CNV deletion is higher than in Caucasians. From that study, the effect of the *UGT2B17* gene copy number in the Caucasian group appears smaller compared to the Chinese subjects, raising the possibility of a potential effect of ethnicity on the association, but we did not study Chinese individuals and so this will require further investigation in other multiethnic studies. We employed a qRT-PCR genotyping technique that facilitates genotyping of large sample sets with high accuracy and sensitivity compared to traditional PCR techniques, gel electrophoresis and the manual calling of genotypes. Certainly, our

experience is that SYBR Green based qRT-PCR is an efficient and rapid method for the genotyping of *UGT2B17* CNV, and by the selection of appropriate primer and control genes this technique can easily be modified for the genotyping of other CNVs. We chose to use only one control gene to provide a two-copy reference signal and found this completely satisfactory, but additional control genes could be included when modifying the method for the analysis of other CNVs if desired. SYBR Green based qRT-PCR methods are simple to establish and require little optimization. Dual-labeled florescent primers could also be used simultaneously in a single reaction for test and control CNV for each sample to improve efficiency of sample throughput; however the optimization for multiplex qRT-PCR assays is more demanding and may be justified only if the assay is to be used routinely or for a very large study population.

In summary, we conclude from the study of a large cohort of postmenopausal Caucasian women, that the *UGT2B17* CNV has no clinically relevant association with any of the bone metabolism-related phenotypes studied and is unlikely to play a major role in the pathogenesis of osteoporosis.

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Table 1

Demographics, bone density, quantitative ultrasound, and bone biochemistry of the CAIFOS population

Demographic Variables (n=1,347)	
Age (yrs)	75.2 ± 2.7
Height (cm)	158.8 ± 6.0
Weight (kg)	68.6 ± 12.3
Incident Total Fracture Number (%) (84 months)	283 (21.0)
Incident Hip Fracture Number (%) (84 months)	38 (2.8)
DXA Bone Density (n=1,104)	
Total Hip BMD (mg/cm^2)	0.812 ± 0.124
Femoral Neck BMD (mg/cm^2)	0.691 ± 0.104
Heel Quantitative Ultrasound (n=1,298)	
Average BUA (dB/MHz)	100.5 ± 8.0
Average SOS (m/s)	1512.9 ±26.0
Average Stiffness (%)	70.6 ± 11.5
Bone Biochemical Traits (n=1,330)	
Estradiol (pmol/L)	27.9 ± 18.2
SHBG (nmol/L)	55.1 ± 25.3
Free Estradiol Index	2.94 ± 3.01

Results are mean±SD or percentage

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Table 2

BMD, QUS and sex hormone biochemistry parameters in relation to the distribution of the *UGT2B17* CNV genotype

	0 copy	1 copy	2 copies	*P* value
Hip DXA bone density (n)	(120)	(513)	(470)	
Total hip BMD (mg/cm^2)	0.818 ± 0.119	0.815 ± 0.127	0.806 ± 0.122	0.100
Femoral neck BMD (mg/cm^2)	0.693 ± 0.107	0.690 ± 0.106	0.692 ± 0.100	0.879
Heel quantitative ultrasound (n)	(154)	(591)	(551)	
Average BUA (Db/MHz)	99.9 ± 7.6	100.9 ± 7.8	100.3 ± 8.2	0.044
Average SOS (m/s)	1512.3 ± 24.5	1514.8 ± 26.2	1510.9 ± 26.0	0.018
Average Stiffness (%)	70.3 ± 10.6	71.4 ± 11.5	69.9 ± 11.8	0.023
Sex hormone biochemistry parameters (n)	(161)	(599)	(568)	
Estradiol (pmol/L)	28.1 ± 16.8	27.8 ± 15.9	28.1 ± 20.8	0.974
SHBG (nmol/L)	52.2 ± 21.7	54.2 ± 26.1	56.8 ± 25.4	0.024
FEI (units)	2.73 ± 2.26	2.91 ± 3.14	3.03 ± 3.06	0.333
Incident osteoporotic confirmed fractures				
Number (%)	44 (27.3)	118 (19.2)	121 (21.1)	
Hazard ratio (95% CI)	1.30 (0.92-1.83)	0.89 (0.69-1.15)	1 (reference)	0.110

Results are given as mean±SD. *P* values were determined using ANCOVA. The hazard ratio for fracture was adjusted for age, height, weight, and calcium supplementation