THRA and DIO2 mutations are unlikely to be a common cause of increased bone mineral density in euthyroid post-menopausal women

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Abstract

Objective: A new autosomal dominant disorder due to mutation of *THRA*, which encodes thyroid hormone receptor α , is characterised by severely delayed skeletal development but only slightly abnormal thyroid status. Adult mice with disrupted thyroid hormone action in bone due to a mutation of *Thra* or deletion of *Dio2*, encoding the type 2 deiodinase, have high bone mass and mineralisation despite essentially euthyroid status. No individuals with *DIO2* mutations have been described and the adult phenotype of patients with *THRA* mutations is largely unknown. We hypothesised that screening euthyroid adults with high bone mineral density (BMD) could be used to identify individuals with mutations of *THRA* or *DIO2*.

Design: The Osteoporosis and Ultrasound Study (OPUS) is a 6-year prospective study of fracture-related factors from five European centres.

Methods: A cohort of 100 healthy euthyroid post-menopausal women with the highest BMD was selected from the OPUS population. We sequenced the intron–exon boundaries and critical exons of *THRA* and *DIO2* in these subjects. TSH, free 3,5,3'-L-triiodothyronine, free thyroxine, vitamin D, parathyroid hormone and bone turnover marker concentrations, and BMD measurements were available in all OPUS participants.

Results: No coding sequence or splice site mutations affecting *THRA* or *DIO2* were identified.

Conclusions: Mutations affecting *THRA* or *DIO2* are not a common cause of high BMD in healthy euthyroid post-menopausal women.

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Introduction

Thyroid hormones (thyroxine (T₄) and 3,5,3'-L-triiodothyronine (T₃)) are essential for skeletal development, post-natal growth, and bone maturation. In adults, thyroid hormones regulate bone turnover, and normal euthyroid status is required to maintain optimal bone mass and mineralisation (1). Accordingly, thyrotoxicosis is a well-established cause of osteoporosis, and hypothyroidism is associated with an increased risk of fracture (2).

The thyroid gland secretes T₄ and T₃ in a ratio of about 15:1, but T₄ is a pro-hormone and must be converted to T₃ in the peripheral tissues (3). Conversion of T₄ to T₃ in target cells is catalysed by type 2 iodothyronine deiodinase (DIO2), and the activity of this enzyme regulates intracellular availability of T₃. Two nuclear receptors, TR α and TR β , mediate T₃ action in the target cells, and their relative levels of expression vary between tissues.

For example, TR α is expressed at high levels in bone and cartilage, whereas TR β predominates in the hypothalamus and pituitary, where it mediates feedback control of the hypothalamic–pituitary–thyroid (HPT) axis (4).

The syndrome of resistance to thyroid hormone (RTH) was described in 1967 (5) and the first *THRB* mutations affecting TR β were identified in 1989 (6). Heterozygous mutations of *THRA* were finally reported in three families in 2012 and 2013 (7, 8, 9, 10). Affected individuals all had grossly delayed skeletal development and constipation but variable motor and cognitive abnormalities. All had normal serum TSH with low/normal T₄ and high/normal T₃ concentrations, and a characteristically reduced free T₄ (fT₄):free T₃ (fT₃) ratio. A 6-year-old girl had skeletal dysplasia, growth retardation with delayed bone age and tooth eruption, patent skull sutures, macrocephaly, disproportionate short stature, epiphyseal dysgenesis and defective bone mineralisation (7). A 3-year-old girl displayed a similar phenotype and also had congenital hip dislocation. Her 47-year-old father was short with height 3.77 s.d. below normal, and he had acquired hearing loss due to otosclerosis (9, 10). A 45-year-old female was described more recently with disproportionate short stature and macrocephaly, together with skull vault and long-bone cortical thickening (8). These reports define a new genetic disorder characterised by profound developmental abnormalities of the skeleton due to mutations of *THRA*, although limited information is available regarding the phenotypic consequences in adults.

In patients with RTH, a spectrum of dominant-negative *THRB* mutations of varying severity have been defined, and they result in heterogeneous phenotypes. Nevertheless, because TR β is expressed in the hypothalamus and pituitary, even the least severe *THRB* mutations result in characteristic disruption of the HPT axis such that over 3000 affected individuals have been reported and many more have been recognised (11). By analogy, it is likely that individuals with a spectrum of *THRA* mutations will be identified in the future. Significantly, the *THRA* mutations described so far result in a severe phenotype due to the expression of truncated TR α proteins with no T₃-binding capability but potent dominant-negative activity (7, 8, 9, 10). Recognition of individuals with milder mutations and phenotypes will be much more difficult because disruption of *THRA* does not affect the HPT axis significantly and alteration of circulating thyroid status is likely to be subtle.

Importantly, the phenotypes of individuals with *THRA* mutations recapitulate those reported in mice with dominant-negative mutations affecting *Thra*. Juvenile *Thra*^{PV/+}

mice have a reduced T₄:T₃ ratio, delayed closure of the skull sutures with enlarged fontanelles, severe growth retardation and delayed bone age (12, 13). Mice with a less severe *Thra*^{R384C} mutation are euthyroid and have a much milder phenotype with only transiently delayed ossification and growth retardation, but they have reduced osteoclast numbers and display increased bone mass and cortical thickness in adults (14, 15). Similarly, TR α ^{0/0} mice that lack TR α are euthyroid but have impaired osteoclastic bone resorption and high bone mass (16), and D2KO mice that lack DIO2 have increased bone mineralisation and brittle bones but normal circulating T₃ (17). Together with the phenotypes reported in individuals with *THRA* mutations, these data indicate that impaired T₃ action in the skeleton arising from defective TR α signalling or reduced T₃ availability results in high bone mass in adults without significant effect on systemic thyroid status.

We hypothesised that adults with mutations affecting *THRA* or *DIO2* would have increased bone mass and mineralisation but normal euthyroid status, and that such individuals might be identified by screening healthy euthyroid individuals with high bone mineral density (BMD). We tested this hypothesis by sequencing the *THRA* and *DIO2* genes in 100 euthyroid subjects with high BMD from the Osteoporosis and Ultrasound Study (OPUS) (18).

Subjects and methods

Osteoporosis and Ultrasound Study

OPUS is a prospective population-based study from five European centres and detailed information relating to the population demography and participant recruitment has been published previously (19). The study was approved at each institution according to the Declaration of Helsinki and written consent was obtained from all participants. A total of 2374 post-menopausal women were recruited between April 1999 and April 2001.

Measurement of BMD

Dual-energy X-ray absorptiometry scans of the lumbar spine (L2–L4) and hip were performed on all participants in postero-anterior projection (Hologic QDR-4500, Hologic, Inc., Bedford, MA, USA) in Kiel, Paris and Sheffield, or in antero-posterior projection (Lunar Expert devices; GE Lunar Corp., Madison, WI, USA) in Aberdeen and Berlin. Results were corrected for longitudinal changes and for differences among centres, and were expressed as standardised BMD (sBMD) as described (18).

Study cohort

The current study is a cohort study of healthy euthyroid post-menopausal women selected from the OPUS population. Thyroid status was determined in all individuals and reference intervals for fT_3 , fT_4 and TSH calculated as described (18). The following subjects were excluded initially in order to define a healthy euthyroid cohort: individuals with overt thyroid disease (TSH > 10 mU/l and fT_4 < 9 pmol/l; or TSH > 10 mU/l and fT_3 < 2.5 pmol/l; or TSH < 0.1 mU/l and fT_3 > 6 pmol/l), renal disease or cancer; and those with sick thyroid syndrome defined as subjects with an isolated fT_3 concentration < 2.5 pmol/l associated with a malabsorption syndrome including coeliac or Crohn's disease, rheumatoid arthritis, bone disease other than osteoarthritis, psoriasis or asthma. Subjects taking T_4 , antiepileptics, glucocorticoids or other drugs affecting bone metabolism were also excluded, resulting in 1278 healthy euthyroid post-menopausal women not receiving drugs affecting bone metabolism (18). The sBMD values of all 1278 individuals were ranked, and the 100 subjects with the highest sBMD from whom stored DNA samples were available were selected (Fig. 1). Sequencing of *THRA* was performed in all 100 subjects (200 alleles), and sufficient DNA was available for further sequencing of *DIO2* in only 48 of these 100 subjects (96 alleles).

DNA samples

Genomic DNA was extracted from stored blood samples in Sheffield and stored in 50 μ l aliquots at -80°C before shipment and analysis at Imperial College. The DNA concentration in each sample was determined using a Picogreen DNA quantitation kit (Cambridge Biosciences, Cambridge, UK), according to the manufacturer's instructions. Fluorescence intensity was measured with a Wallac Victor² 1420 Multichannel counter (PerkinElmer, Waltham, MA, USA).

THRA and DIO2 sequencing

Primers (Table 1) were designed (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to amplify the coding exons of *THRA* (exons 2–10 within genomic reference sequence ENSG00000126351) and *DIO2* (exons 1,2,a,b) and the selenocysteine insertion sequence (SECIS) within the UTR of *DIO2* (genomic reference sequence ENSG00000211448). PCRs for primer sequencing were carried out in 25 μ l vol with 30 ng/ μ l DNA template and 1.0 unit Platinum Taq High Fidelity (Invitrogen, Life Technologies Ltd), 1 $\times$ High-Fidelity PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl_2 and 0.2 μ M primers. PCR amplifications were incubated at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, $52\text{--}60^\circ\text{C}$ for 30 s, 72°C for 30 s

Figure 1

Exclusion criteria to define the study cohort of 1278 healthy euthyroid post-menopausal women not receiving drugs affecting bone metabolism, and the 100 subjects with the highest standardised bone mineral density from whom stored DNA samples were available.

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Table 1 Sequencing primers.

Exon	Forward 5'–3'	Reverse 5'–3'	Product size
<i>THRA</i>			
2	CAGTCTCTTGGCGTGCTGGA	GCCAGACAAAGGTAGGCAGA	242
3	GCTACTCGAAGACTGCTCTG	ACTGTTCCACATCCAGGTCC	203
4	TGGGCAGTCAGAAGAACTGA	GCGGGCTTTAGGTGACATAG	380
5	ATCACAGCCTGCTCCATCTC	TGGCTCTATTCCTCCACCT	210
6	TGCCATGCGTTAGACCTTGT	TGCCACCTGGCTACTGCTCT	285
7	GAGCCTCAGTGAGAGGCTGA	CCTCCAGCACAGCATCACAT	252
8	GGCTCCCGTAGGACACTCTA	CTCCCGACTAATCAGCAGGT	364
9	TCACGCCCTCTTCCCTCAC	CCTTCTCTCCAGGCTCCTCC	329
10	CCAGAGGCTCATCTTGAATATT	AAGGTATGGCACTCCTTCTCCTT	485
<i>DIO2</i>			
1	GCAATCCTGGCCAAAGTAAA	TGGCGTACTCGTCCCTAATC	502
2	TCAGGTGAAATTGGGTGAGG	CACAATGCACACACGTTCAA	481
a	GACCAAATGGGGAGATGAGA	TCTGGTCCCCAGCATATGAG	554
b	CCCCATAAAGAGCATTACGAA	GCCTAGGCAAGAGAAAATGC	287
SECIS	GACCAGTGTGCGGATGATAA	GGGCAAGTTCTAGGGAGAAA	371

and incubation at 72 °C for 2 min. PCR products were purified using ExoSAP, with 20 U/μl exonuclease I (New England Biolabs, Hitchin, UK) and 1 U/μl shrimp alkaline phosphatase (Promega UK) in preparation for direct sequencing. Mixtures containing 10 ng/100 bp of purified PCR product and 3.2 pmol of primer were prepared in 10 μl reaction volumes for cycle sequencing with BigDye v3.1 (Applied Biosystems, Life Technologies Ltd) in the Genomics Core Laboratory at Hammersmith Campus, Imperial College. The samples were denatured at 94 °C for 1 min followed by 30 cycles of 94 °C for 10 s, 55 °C for 15 s and 60 °C for 4 min. The products were precipitated in EDTA-ethanol, suspended in Hi-Di formamide (Applied Biosystems) and sequenced using a 3730xl DNA Analyzer (Applied Biosystems). Sequences analysis was performed using Sequencher v4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analysis

Analyses were performed using SPSS v.21 (SPSS, Inc.) or GraphPad Prism v5 (GraphPad Software, Inc., San Diego, CA, USA). The Kolmogorov–Smirnov test was used to test data for normal distribution. All normally distributed data were presented as mean ± s.d., whereas non-parametric data (logarithmically transformed TSH values) were presented as median and range. Comparisons between groups were performed using Student's *t*-test or Mann–Whitney *U* test. Stepwise regression analysis was performed to investigate the associations between $fT_4:fT_3$ ratio and BMD or bone turnover markers.

Results

Study cohort and high BMD groups

Compared with the total cohort of 1278 healthy euthyroid post-menopausal women, the group of 100 subjects with increased hip and lumbar spine BMD, in which the *THRA* ($n=100$) or *DIO2* ($n=48$ of these 100 subjects) genes were sequenced, had reduced concentrations of bone resorption (serum C-terminal telopeptide (sβCTX), urinary N-terminal telopeptide of type I collagen (uNTX)) and formation (osteocalcin (OC), procollagen type I N-terminal propeptide (PINP)) markers. By contrast, the $fT_4:fT_3$ ratio and TSH, fT_3 , fT_4 , vitamin D and parathyroid hormone (PTH) concentrations did not differ between groups (Table 2). The $fT_4:fT_3$ ratio was not associated with changes in hip and lumbar spine BMD or bone turnover markers (Table 3).

Analysis of *THRA*

Sequencing of intron–exon boundaries and coding exons of *THRA* in 100 subjects with high BMD revealed no coding sequence or splice site mutations in *THRA* (NCBI mRNA reference sequences: NM_199334.3 and NM_003250.5). The rs2230701 single nucleotide polymorphism in exon 5 (GCC → GCT; Ala/Ala) was identified at allele and genotype frequencies (Table 4) consistent with previous reports (20, 21) and established databases, including HapMap (<http://hapmap.ncbi.nlm.nih.gov>) and dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Concentrations of OC and sβCTX were higher in individuals who were heterozygous or homozygous for the rs2230701 minor allele (GCT)

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Table 2 Study population characteristics. Comparisons between healthy euthyroid post-menopausal women ($n=1278$) and groups in which *THRA* or *DIO2* was sequenced (mean $\pm$ s.d. except TSH shown as median and range. Text in bold highlights differences that are statistically significant).

	Euthyroid cohort	<i>THRA</i> group	<i>DIO2</i> group
<i>n</i>	1278	100	48
LS BMD (mg/cm ²)	1028 $\pm$ 180	1335 $\pm$ 151[†] (Z score + 1.7)	1322 $\pm$ 162[†] (Z score + 1.6)
Hip BMD (mg/cm ²)	839 $\pm$ 131	1113 $\pm$ 109[†] (Z score + 2.1)	1119 $\pm$ 126[†] (Z score + 2.1)
Age (years)	68 $\pm$ 7	65 $\pm$ 6*	65.7 $\pm$ 6
TSH (mU/l)	0.88 (range, 0.15–3.64)	0.89 (range, 0.19–2.89)	0.95 (range, 0.14–4.40)
ft ₄ (pmol/l)	12.9 $\pm$ 2.0	12.5 $\pm$ 1.8	12.6 $\pm$ 1.9
ft ₃ (pmol/l)	3.7 $\pm$ 0.8	3.7 $\pm$ 0.8	3.7 $\pm$ 0.8
ft ₄ :ft ₃ ratio	3.58 $\pm$ 0.79	3.44 $\pm$ 0.56	3.48 $\pm$ 0.64
Vitamin D (ng/ml)	20.9 $\pm$ 10.0	22.5 $\pm$ 8.8	21.5 $\pm$ 8.3
PTH (pg/ml)	44.2 $\pm$ 23.0	40.5 $\pm$ 16.3	42.8 $\pm$ 16.8
OC (ng/ml)	26.0 $\pm$ 10.6	20.0 $\pm$ 6.7[†]	20.4 $\pm$ 6.7[†]
PINP (ng/ml)	55 $\pm$ 20.0	44.0 $\pm$ 16.0[†]	44.7 $\pm$ 16.1[†]
sβCTX (ng/ml)	0.271 $\pm$ 0.248	0.181 $\pm$ 0.119[†]	0.196 $\pm$ 0.125*
uNTX/Cr (nM/mM)	56.6 $\pm$ 31.0	44.9 $\pm$ 21.9[†]	47.25 $\pm$ 22.8*

Student's *t*-test, * $P<0.05$, [†] $P<0.01$, [‡] $P<0.001$. LS, lumbar spine; PTH, parathyroid hormone; OC, osteocalcin; PINP, procollagen type I N-terminal propeptide; sβCTX, serum C-terminal telopeptide; uNTX/Cr, urinary N-terminal telopeptide of type I collagen/creatinine.

compared with subjects homozygous for the major allele (GCC); however, there were no differences in PINP and uNTX/Cr levels and hip or lumbar spine BMD among subjects of differing rs2230701 genotype (Table 4).

Analysis of *DIO2*

Sequencing of *DIO2* in 48 of the 100 subjects with high BMD also revealed no coding sequence or splice site mutations in *DIO2* (NCBI mRNA reference sequences: NM_013989.4; NM_000793.5; NM_001007023.3; NM_001242502.1 and NM_001242503.1). The rs225014 polymorphism in exon 2 (ACA $\rightarrow$ GCA; Thr92Ala) was

identified at allele and genotype frequencies (Table 4) consistent with previous reports (22, 23, 24) and established databases. Bone turnover marker concentrations and hip or lumbar spine BMD was not different among subjects of differing rs225014 genotype (Table 4).

Discussion

The recent identification of severely delayed skeletal development in patients with potent dominant-negative mutations of *THRA* (7, 8, 9, 10) recapitulated the findings in juvenile mice with *Thra* mutations (12, 13, 14, 16). Studies in mice with deletion or mutation of *Thra* or *Dio2*

Table 3 Relationships between ft₄:ft₃ ratio and BMD and bone turnover markers.

	ft ₄ :ft ₃ ratio		
	<i>R</i> ²	β coefficient (95% CI) (lower, upper, per s.d. change)	<i>P</i>
<i>THRA</i> group, <i>n</i> = 100			
LS BMD	0.008	−0.087 (−82.820 to 35.790)	0.432
Hip BMD	0.045	0.213 (−0.656 to 83.826)	0.054
OC	0.003	−0.058 (−3.413 to 2.012)	0.609
PINP	0.007	−0.084 (−8.831 to 4.025)	0.459
sβCTX	0.000	−0.018 (−0.052 to 0.044)	0.875
uNTX/Cr	0.015	−0.122 (−13.478 to 3.937)	0.279
<i>DIO2</i> group, <i>n</i> = 48			
LS BMD	0.007	−0.085 (−99.505 to 56.318)	0.579
Hip BMD	0.022	0.147 (−31.224 to 89.432)	0.336
OC	0.000	0.008 (−3.256 to 3.423)	0.960
PINP	0.010	−0.098 (−10.410 to 5.453)	0.531
sβCTX	0.014	−0.019 (−0.085 to 0.038)	0.446
uNTX/Cr	0.046	−0.214 (−18.421 to 3.102)	0.158

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Table 4 Relationships between *THRA* and *DIO2* polymorphisms and BMD and bone turnover markers. Text in bold highlights differences that are statistically significant.

	LS BMD	Hip BMD	OC	P1NP	sβCTX	uNTX/Cr
<i>THRA</i> minor allele (rs2230701) ^a						
rs2230701 (n = 11)						
Mean	1332.00	1126.7	24.31	53.18	0.29	49.04
S.D.	168.89	190.18	5.28	13.77	0.12	28.98
WT (n = 89)						
Mean	1335.34	1111.34	19.38	42.74	0.17	44.33
S.D.	149.91	95.60	6.74	15.98	0.11	20.97
t-test						
P	0.948	0.680	0.030	0.053	0.002	0.528
<i>DIO2</i> minor allele (rs225014) ^b						
rs225014 (n = 25)						
Mean	1264.67	1108.07	19.12	47.49	0.21	53.04
S.D.	136.08	98.30	7.91	20.97	0.13	22.17
WT (n = 23)						
Mean	1342.57	1114.79	21.06	42.89	0.20	39.00
S.D.	143.21	99.34	5.49	15.44	0.15	16.41
t-test						
P	0.523	0.374	0.830	0.642	0.224	0.247

^an = 11 heterozygous subjects vs WT major allele (n = 89 homozygous subjects).

^bn = 25 (seven homozygous, 18 heterozygous subjects) vs WT major allele (n = 23 homozygous subjects).

demonstrated further that impaired T_3 action in bone results in high BMD in adults despite normal circulating thyroid status (14, 16, 17). Despite this, little information is available regarding the adult skeletal consequences of severe *THRA* mutations, whilst no individuals with less deleterious *THRA* mutations or patients with *DIO2* mutations have been described.

A candidate gene association study of 862 men over 65 years of age investigated the relationships between genetic variation in *THRA* and BMD at the femoral neck and lumbar spine (25, 26), whilst a much larger study investigated the *THRA* locus in relation to BMD, fracture risk and bone geometry in 27 326 individuals from the Genetic Factors for Osteoporosis (GEFOS) consortium and the Rotterdam Study 1 and 2 populations (21). These studies did not identify any relationships between variation in bone parameters and genetic variation across the *THRA* locus. Furthermore, osteoporosis genome-wide association (GWA) studies have not identified associations between *THRA* or *DIO2* and BMD or fracture (27, 28, 29, 30). This is not surprising because GWA studies investigate common variation among very large numbers of SNPs across the entire genome to identify genetic associations with quantitative traits (31), and they are not designed to detect rare sequence variants or causative mutations for single gene disorders (32). By contrast, studies of phenotype extremes in humans have been instrumental in identifying molecular mechanisms underlying rare single gene disorders as well as common diseases, including diabetes and obesity.

Such studies have resulted in novel treatments that revolutionise the lives of affected individuals (33, 34, 35).

We adopted such a strategy by investigating whether screening of euthyroid adults with high BMD could be a novel and practical approach to identify patients with *DIO2* mutations or individuals with mild mutations affecting *THRA*. These studies did not identify coding sequence or splice site mutations affecting *THRA* or *DIO2* in 100 individuals with high BMD from the cohort of healthy euthyroid post-menopausal women, indicating that such mutations are unlikely to be a common cause of increased BMD in healthy euthyroid post-menopausal women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A Gogakos, J G Logan and J A Waung performed sequence analyses. J H D Bassett and G R Williams designed the experiments. C C Glüer, D M Reid, D Felsenberg, C Roux and R Eastell initiated OPUS and collected and provided samples and data from the study. G R Williams measured thyroid function tests and wrote the manuscript. All authors read and provided comments on the final manuscript.

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