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VARIATION IN THE PTH2R GENE IS ASSOCIATED WITH AGE RELATED DEGENERATIVE CHANGES IN THE LUMBAR SPINE

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ABSTRACT

In the elderly, degenerative changes in the lumbar spine are common, contributing to falsely elevated bone mineral density (BMD) values. The parathyroid hormone (PTH) system plays an important role in the regulation of bone turnover and we explore the hypothesis that polymorphisms (SNPs) within genes in this pathway (*PTH, PTHLH, PTH1R* and *PTH2R*) contribute to degenerative manifestations of the spine in elderly women. The study participants were 1004, 75 year old Swedish women from the population based OPRA cohort who attended follow-up at 5 and 10 years. Lumbar spine BMD was assessed by DXA and each individual vertebra was evaluated visually on the DXA image for apparent degenerative manifestations. Six SNPs in *PTH*, 3 SNPs each in *PTH1R, PTH2R* and *PTHLH* were analysed. Among women with degenerative manifestations at the lumbar spine, there was an over-representation at baseline of those carrying the *PTH2R* SNP rs897083 A-allele (p=0.0021; Odds Ratio 1.5 95%CI (1.2-2.0)) and across the duration of follow-up (p=0.0008). No association with degenerative manifestations and variation in the other genes was observed. None of the PTH hormone system genes were associated with vertebral fracture. Variation in the *PTH2R* gene (Chr2q34, rs897083) may contribute to the age associated degenerative manifestations that develop at the lumbar spine.

Key Words: Genetic, polymorphism, PTH2R, degenerative changes, spine

INTRODUCTION

Osteoporosis is characterised by low bone mineral density (BMD) and fragility fractures. Diagnosis is based on the World Health Organisation definition of "BMD 2.5 standard deviations or more below the average value for young healthy adults" as measured using dual energy X-ray absorptiometry (DXA) [1]. The preferred measurement site for the diagnosis of spinal osteoporosis and prediction of vertebral fracture risk is the lumbar spine, however, it has long been observed that spinal DXA assessment is less reliable in the elderly as a result of degenerative manifestations including osteophytes, disc degeneration and subchondral sclerosis, vertebral compression fractures, degenerative scoliosis and aortic calcification [2, 3]. The consequence is an artificially elevated spine BMD [4, 5]. To assess the influence of apparent degenerative spine changes on clinical DXA assessment over time, in a previous study, we evaluated degenerative manifestations in each individual lumbar vertebra over a 10 year period. These changes accelerated over time, were most pronounced in the distal segments and had significant impact on diagnosing osteoporosis in elderly women [6].

The characteristics of degenerative manifestations at the lumbar spine are indicative of increased bone growth or increased bone density and are frequently referred to as spondylarthritis, although the deterioration arises from a variety of causes with osteoarthritis at one end of the spectrum and vertebral deformities related to osteoporosis at the other.

On this basis, we hypothesised that genetic variation in genes belonging to the parathyroid hormone (PTH) pathway could be associated with these age related lumbar spine degenerative manifestations. The rationale for studying genes in the PTH pathway is based on their particular roles in bone metabolism. PTH, the key regulator of calcium homeostasis, induces bone loss and osteoporosis at continuously elevated levels [7], but conversely has bone anabolic properties when administered intermittently [8, 9]. Parathyroid hormone-like hormone (PTHLH) contributes to skeletal growth and development [10] in children. The PTH receptors were also included in this analysis because *PTH1R* binds with PTH/PTHLH promoting osteoclastogenesis [11], while *PTH2R* has been implicated in endochondral bone formation [12] and is specific for PTH rather than PTHLH. Since degeneration in the lower spine may be linked to both osteoporosis and osteoarthritis resulting from the anatomical structure and loading pattern on the spine, there may be pleiotropic gene-effects associated with the manifestations.

In the current investigation, our aim was to explore the hypothesis of a possible relationship between age related lumbar spine degenerative manifestations and variation in the *PTH*, *PTHLH*, *PTH1R* and *PTH2R genes* using the OPRA population-based cohort of postmenopausal Swedish women aged 75 at inclusion.

MATERIALS AND METHODS

Subjects

The study cohort comprised the Malmö Osteoporosis Prospective Risk Assessment study (OPRA) of 75 year old women, which has been previously described in detail [13]. Briefly, these women were randomly selected from the city files in Malmö, included between December 1995 and May 1999 and followed at 5 and 10 years. 1044 (65%) subjects attended baseline investigation and no exclusion criteria were applied. The vast majority were self-ambulatory, and predominantly of Swedish origin (99%) [13, 14]. As part of this study, baseline bone mineral density (BMD) was assessed and blood samples were collected for DNA and biochemical analysis. At all visits the participants also answered a detailed questionnaire covering lifestyle, health and fracture risk factors.

Participants gave written, informed consent and the Regional Ethical Review Board in Lund approved the study, which was performed according to the principles of the Helsinki declaration.

DXA measurement, visual assessment of spine degenerative manifestations and vertebral fracture

At baseline and all follow-up visits, BMD at the lumbar spine was assessed using a Lunar® DPX-L DXA scanner (Madison, WI, USA). DXA scans were analysed with software versions 1.33 and 1.35 at baseline, version 4.7b at 5 years and version 4.7e at 10 years. Calibration using the manufacturer's phantom was performed 3 times per week with precision coefficients of 1.45% at the lumbar spine and 4.01% at the femoral neck [15].

All DXA scans of the lumbar spine were evaluated visually, by a single operator, an experienced spine surgeon (MT). Each individual vertebra (L1, L2, L3 and L4) was evaluated for the presence of degenerative manifestations defined as visible vertebral osteophytes, disc space narrowing, asymmetric subchondral sclerosis or facet joint sclerosis using a semi-quantitative score as follows: Degeneration: grade 0, none; grade 1, mild; grade 2, severe (e.g. deformation of the vertebra in addition to the listed criteria). In addition, scoliosis was graded:

grade 0,- none; grade 1, yes (i.e. Cobb angel L1-L4 >10 degree) and fracture: grade 0 - none; grade 1 - suspected; grade 2 – yes [6]. The sensitivity of detecting X-ray verified degenerative changes on the DXA image was moderately high in L2-L4 (70-82%) but lower in L1 (42%) as previously reported [6].

For the current analysis women were classified as having degenerative spine changes if evidence of degenerative manifestations were detected at any vertebrae level (L1-L4) at DXA baseline or at respective visits. Due to technical reasons or surgery, the number of assessable DXA scans varied slightly at each time point (baseline n=973-976; 5y n=691-698; 10y n=377-380) [6].

At inclusion in the study, self-reported adult fractures (age 20-75) were recorded and were verified from radiological files as previously reported [16]. Incident fractures during the follow-up period (until November 2006) were recorded and verified by review of the related medical records [17]. In this report, clinical symptomatic vertebral fractures from age 50 upwards were analysed.

Genotyping

The polymorphisms were selected based on information from Ensembl (www.ensembl.org), the details of which have previously been reported in full [18]. In brief, SNPs were selected based on information available at the time (prior to completion of HapMap), according to the criteria: relative position to the gene, minor allele frequency and distance between the SNPs, in order to obtain maximum genetic information content for the most likely functional parts of the genes. For PTH, polymorphisms were additionally selected to encompass the gene and its surroundings, representing the most commonly occurring haplotypes according to Haploview.

Genotyping of the PTH, PTHLH, PTH1R and PTH2R genes was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) and genotypes were determined using an ABI Prism 7900HT sequence detector (Applied Biosystems). The samples were genotyped blind with 4.5% of the samples routinely repeated by the same method, with 100% concordance. The SNPs studied were: *PTH* ((11p15.2) rs307253; rs307247; rs6254; rs1459015; rs10500783 and rs10500784); *PTHLH* ((12p11.22) rs805512, rs10492364, rs1268693); *PTH1R* ((3p21.31) rs6442037, rs724449, 7652849); and *PTH2R* ((2q34) rs9288393, rs10497900, rs897083) [18]. Haplotypes, defined by the six PTH SNPs were determined by indirect haplotyping using the program PHASE v2.02 [19]. As previously described, haplotypes occurring with frequency >0.10 and probability >0.8 were included in the analyses [18]. In this

report we present *PTHLH*, *PTH1R*, *PTH2R* genotype data for 1004 women. PTH SNPs were genotyped in 741-1001 women enabling a valid haplotype for 750.

Statistical Analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc.). The χ^2 test was used to analyze association between measures of vertebral degeneration and SNPs or haplotypes comparing major allele homozygotes vs. heterozygotes vs. minor allele homozygotes and additionally major allele homozygotes vs. carriers of the minor allele). Odds ratios and 95% confidence intervals for *PTH2R* rs897083 were calculated using regression analysis. For analysis of clinical vertebral fractures, binary logistic regression analysis was used.

The *a priori* power calculation for this study indicates >80% power to detect a difference in BMD of 0.056 BMD units based on the assumption of a SD of 0.13 g/cm2 and a rare genotype frequency of 4%. The study is therefore adequately powered to detect association with degenerative spine changes since an average of 63% of the women in the cohort were affected over the 10 year follow-up period.

Multiple statistical tests were performed, however the phenotypes and several of the markers studied are dependent (i.e. correlated or in linkage disequilibrium) making a Bonferroni correction too stringent, therefore we took into account and adjusted for the number of genes (n=4) and phenotypes (n=2). Associations were considered nominally significant if $p<0.008$.

RESULTS

The genotype distributions for *PTH*, *PTHLH, PTH1R* and *PTH2R* were all in Hardy-Weinberg equilibrium as reported previously [18]. Genotype frequencies are reported in **Table 1**. Minor allele frequencies (MAF) were similar to those reported in HapMap (www.hapmap.org). The basic clinical characteristics of the OPRA study participants including bone density have been reported in detail [13, 18], as has the prevalence of apparent degenerative changes, scoliosis and vertebral fractures from visual assessment of the DXA scans [6]. Characteristics of the women attending at baseline and at 5 year and 10 year follow-up visits are shown in **Table 2.**

The proportion of women classified with visible degenerative manifestations was 43%, 67%, and 80% at baseline, 5 and 10 years, respectively. Of these women, the proportion who also displayed evidence of scoliosis was 23%, 22%, and 32% at baseline, 5 and 10 years respectively. Scoliosis was predominantly a degenerative manifestation since only 8 women displayed

scoliosis with no other signs of degeneration in the lumbar spine, barring their analysis as a separate group. Circulating levels of PTH were not associated with visible degenerative manifestations (data not shown).

Genetic variation close to all four genes was analyzed for association with vertebral degenerative manifestations. There were no differences in *PTH* genotype or haplotype distribution between women with and without visual evidence of vertebral degenerative manifestations. Similarly, no differences were observed for *PTHLP*, or *PTH1R*. In the *PTH2R* gene an association between rs897083 and vertebral degenerative manifestations was observed. The minor A-allele was over-represented among women with evidence of degenerative manifestations, which increased per copy of the A-allele (**Table 3).** Although the number of homozygotes for the A-allele was low, under both analytical models there was an association at baseline (χ^2 p= 0.0021-0.0006) and overall, i.e. anytime during the study, between baseline and 10-year follow-up (χ^2 p= 0.0021-0.0008).

Among women who had degenerative manifestations of the spine, the proportion who had a clinical vertebral fracture was smaller compared to those free from degenerative manifestations (13% v 39%; χ^2 p=0.032). There was no evidence of association between clinical vertebral fracture and any of the studied polymorphisms, however the number of women with clinical vertebral fractures was low (n=152) and therefore underpowered to detect association (data not shown).

DISCUSSION

In this study we examined the genetic association between SNP-markers for four PTH pathway genes and apparent degenerative manifestations of the spine in the large population-based OPRA cohort of elderly women.

From a clinical viewpoint this phenotype is interesting since vertebral deformity and clinical vertebral fractures are the most debilitating complications of osteoporosis. Vertebral deformity, defined by reduced vertebral height, as assessed by vertebral morphometry on lateral spinal radiographs, has demonstrated vertebral deformation in over one third of women between the ages of 70-79 and over 60% of those over 80 [20]. Furthermore, degenerative manifestations in the lumbar spine becomes increasingly common with advancing age and we have recently reported a gradient of vertebra being affected by these changes (L1 least and L4 most affected), with up to 36% of all L4 vertebrae having degenerative manifestations in elderly women [6]. Vertebral degenerative manifestations are

a phenotype not commonly investigated in genetic studies of osteoporosis, yet in addition to the diagnostic implications of falsely elevated BMD readings [3, 21], there is also the possibility that in genetic studies using elderly cohorts, potential associations with BMD may be masked.

The *PTHLH* and *PTH1R* were not associated with spine degenerative manifestations and despite the potentially anabolic role of PTH, variation within this gene did not contribute to the incidence of degenerative manifestations at the lumbar spine. We have previously shown that PTH SNPs were not associated with spine BMD [18]. The *PTH2R* gene was associated with degenerative vertebral changes in this cohort of elderly women. Although there is no equivalent study against which to draw direct comparisons, there are similarities in bone anabolic activity between degenerative changes of the spine and other skeletal sites as observed in osteoarthritis. These changes occur most commonly at the bone-cartilage transition line and interestingly, the *PTH2R* gene is known to be involved in endochondral bone formation [12]. Evidence for linkage of the 2q33 locus, in which the *PTH2R* gene resides, for familial early-onset generalised osteoarthritis (FOA) has been reported [22]. Although a rare missense mutation in the *PTH2R* gene (c.786G>T; p.A225S) co-segregating with FOA was discounted as contributing significantly to the FOA phenotype, there was nevertheless evidence of a possible association with generalised radiographic osteoarthritis in the general population. While this does not provide definitive evidence for a role of *PTH2R in* OA, it does support our finding of a potential role for *PTH2R* in age-related spine degenerative manifestations, in lieu of true replication. These findings are of general interest since degenerative changes in the lumbar spine may be associated with increased hip BMD [23], supporting the inverse relationship between osteoporosis and osteoarthritis [24]. However, in our previous studies we found no evidence of association between *PTH2R* variation and osteoporosis related phenotypes including BMD, osteoporosis, or any type of fracture [18].

Our results support the idea of pleiotropic genes for BMD and degenerative manifestations which may or may not be due to osteoarthritis [25, 26], while biological and mechanistic evidence implicates PTH2R as a candidate for degenerative changes related to osteophyte development and subchondral sclerosis. Furthermore, PTH2R is expressed in endocrine cells, indicating its role in the secretion of growth hormone and pituitary hormones in general [12].Despite our interesting finding, we acknowledge that using a candidate gene approach, the results must be interpreted with caution in the absence of replication in a similarly aged cohort since the contribution from *PTH2R* is modest in the absence of stringent adjustment for multiple testing. Furthermore, we can only speculate on whether *PTH2R* genotype is

associated with newly developed degenerative manifestations during follow-up since the numbers are too low to facilitate meaningful statistical comparison.

In the future, analyses of gene-gene interactions are also likely to prove highly interesting. While genes in the immediate vicinity of *PTH* have not been implicated in bone metabolism, interactions between SNPs in other calcium regulatory pathway genes, e.g. calcium-sensing receptor (*CaSR*), *Klotho* and fibroblast growth factor-23 (*FGF23*), could be considered and further explored. Other candidates include Wnt signalling pathway genes, e.g. the frizzled-related protein (*FRZB*) which localises nearby the 2q33 locus and could contribute to degenerative changes associated with bone formation. In addition, in view of our findings it will be interesting to determine if the *PTH2R* gene is associated with osteoarthritis at other skeletal sites in our cohort and it is hoped that further studies will explore the anabolic contribution of PTH to osteoarthritis.

The design of the cohort is a particular strength of the study, containing as it does approximately 1000 women of homogeneous origin, at a single age (75) and in whom there is already a high prevalence of spine degenerative manifestations at baseline. The investigation relies on DXA images and hence a semi-quantitative method of scoring degenerative manifestations, however we have previously shown that this visual estimation reliably identifies degenerative manifestations compared to X-ray [6]. While inclusion of spinal radiographs or MRI scans in the study protocol might have afforded better resolution to detect degenerative manifestations, even these methods have their limitations [27, 28]. Furthermore, to our knowledge, such a comprehensive exploration of the relationship between variation in the PTH system genes and the major bone phenotypes; BMD, fracture, bone dimensions and now spine degenerative manifestations [28, 36]; has not previously been undertaken.

On the grounds that degenerative manifestations in the lower spine are associated with bone growth and increased density, the PTH pathway could play a role, hence this study is a first test of this hypothesis. We conclude that variation in the region of the *PTH2R* gene may contribute to the age associated degenerative changes that commonly develop at the spine, encouraging further exploration into the mechanisms contributing to age related bone changes.

CONFLICT OF INTEREST

The authors declare that they have no competing interests

AUTHORS CONTRIBUTIONS

Study design and co-ordination: KÅ, HL, PG, MT, FMG; Data collection: KÅ, PG, MT; Data analysis: FMG; Data interpretation: FM and KÅ; Drafting manuscript: FMG, KÅ; Revising manuscript content and approving final version: FM, KÅ, HL, PG, MT

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Table 1 Gene and SNP information for PTH pathway genes

	PTH						PTHLH			PTH1R			PTH2R		
SNP ID	**rs_6254**	**rs307253**	**rs_307247**	**rs_1459015**	**rs_10500783**	**rs_10500784**	**rs_10492364**	**rs_1268693**	**rs_805512**	**rs_6442037**	**rs_724449**	**rs_7652849**	**rs_10497900**	**rs_897083**	**rs_9288393**
Major allele homozygous	305 (41%)	331 (44%)	374 (44%)	561 (66%)	721 (72%)	408 (41%)	532 (53%)	294 (30%)	380 (38%)	436 (44%)	373 (37%)	797 (79%)	310 (31%)	660 (66%)	554 (56%)
Heterozygous	325 (44%)	325 (43%)	377 (44%)	250 (30%)	259 (26%)	446 (44%)	395 (40%)	520 (52%)	477 (48%)	445 (44%)	478 (48%)	187 (19%)	478 (48%)	313 (31%)	389 (39%)
Minor allele homozygous	111 (15%)	93 (12%)	108 (13%)	37 (4%)	21 (2%)	145 (15%)	73 (7%)	182 (18%)	136 (14%)	120 (12%)	150 (15%)	17 (2%)	213 (21%)	27 (3%)	54 (5%)
MAF (OPRA)	0.37	0.34	0.35	0.19	0.15	0.37	0.27	0.44	0.38	0.34	0.39	0.11	0.45	0.18	0.25
MAF (HapMap)	0.34	0.34	0.34	0.21	0.15	0.34	0.29	0.45	0.33	0.37	0.38	0.11	0.49	0.21	0.27
Total genotypes	**741**	**749**	**859**	**848**	**1001**	**999**	**1000**	**996**	**993**	**1001**	**1001**	**1001**	**1001**	**1000**	**997**

Table 2. General clinical characteristics of the OPRA cohort at baseline

Variable	Baseline* (n=1004)	5 yr Follow-up* (n=715)	10 yr Follow-up * (n=382)
Age (years)	75.2 (0.2)	80.2 (0.2)	85.2 (0.1)
Weight (kg)	67.8 (11.5)	66.1 (11.4)	63.7 (10.6)
Height (m)	161 (6)	159 (6)	158 (6)
BMI (kg/m^2)	26.3 (4.2)	26.1 (4.2)	25.5 (3.9)
Current use of bisphosphonates	31 (3%)	58 (8%)	55 (12%)
Current smokers	138 (14%)	85 (10%)	33 (7%)
Serum vitamin D (ng/mL)	95.1 (30.0)	77.9 (30.0)	78.9 (26.0)
Serum PTH (pmol/L)	4.6 (2.1)	4.4 (3.2)	5.2 (4.2)
Serum calcium (mmol/L)	2.4 (0.1)	2.4 (0.1)	2.34 (0.1)

*Values are mean (SD) except for bisphosphonate use, and smoking which are number (%)
Vitamin D, PTH and calcium measurements were available for 954, 967 and 975 individuals, respectively

Table 3. PTH2R polymorphism rs897083 and spine degenerative changes at baseline, 5 years, 10 years and overall i.e. any time between BL-10 years

	BASELINE			5 YEAR FOLLOWUP			10 YEAR FOLLOWUP			ANY TIME (Baseline-10y)		
PTH2R **rs897083 (G/A)**	**Spine Degenerative Manifestations**			**Spine Degenerative Manifestations**			**Spine Degenerative Manifestations**			**Spine Degenerative Manifestations**		
Genotype	**YES**	**NO**	**Total**	**YES**	**NO**	**Total**	**YES**	**NO**	**Total**	**YES**	**NO**	**Total**
GG	244 (39%)	379 (61%)	623	299 (64%)	166 (36%)	465	178 (76%)	57 (24%)	235	420 (66%)	220 (34%)	640
GA	139 (47%)	152 (52%)	291	151 (72%)	60 (28%)	211	109 (85%)	19 (15%)	128	225 (76%)	73 (24%)	298
AA	18 (72 %)	7 (28%)	25	17 (89%)	2 (11%)	19	9 (90%)	1 (10%)	10	22 (85%)	4 (15%)	26
Total genotypes	401	538	939	467	228	695	296	77	373	667	297	964
P-value* (GG vs. GA vs. AA)	0.0006			0.0195			0.075			0.0021		
P-value* (GG vs. A-allele)	0.0021			0.0209			0.0245			0.0008		
OR 95%CI‡	1.5 (1.2-2.0)			1.5 (1.1-2.1)			1.9 (1.1-3.3)			1.7 (1.2-2.3)		

* Pearson $\chi 2$ p-values

‡ Odds Ratio calculated based on GG v carriers of the minor A-allele