

ASSOCIATION OF SEROTONIN TRANSPORTER GENE POLYMORPHISM 5HTTVNTR WITH OSTEOPOROSIS

Joana T. Ferreira*, Pilar Q. Levy*, Claudia R. Marinho*, Manuel P. Bicho****, Mário Rui G. Mascarenhas****

Abstract

Aim: To study the association of serotonin transporter gene polymorphisms and osteoporosis.

Material and Methods: Blood samples were collected from 186 individuals with normal bone mineral density and 89 with osteoporosis. Serotonin transporter gene polymorphisms 5HTTVNTR and 5HTTLPR were studied by PCR and statistical analyses used to test the association between groups.

Results: The frequency of 12/10 and 12/12 genotypes of 5HTTVNTR was significantly higher among the osteoporotic patients (OR=2,620 CI 95% [1,112-6,172], P=0,037). For 5HTTLPR we did not find significant differences between the two studied groups.

Conclusions: As far as we know, this is one of the few studies that report an association between 5HTTVNTR and osteoporosis opening the hypothesis that the determination of this specific serotonin transporter gene polymorphism may contribute to the identification of individuals at high risk for the development of osteoporosis.

Keywords: Osteoporosis; Bone Remodeling; Serotonin Transporter Polymorphisms.

Introduction

Osteoporosis is a severe metabolic bone disease characterized by microarchitectural deterioration and a decrease in bone strength leading to an increased risk of fractures. It occurs as a consequence of poor bone mass acquisition during growth or more frequently as a consequence of bone loss due to increased bone resorption. In post-menopausal

osteoporosis there is an imbalance between osteoblastic and osteoclastic activity favoring bone resorption¹ mainly due to variations in hormonal factors with an impact in bone, with a especially relevant role for estrogens. Many other factors intervene in the regulation of bone metabolism, including the components of neuronal system like leptin, neuropeptide Y receptors and neurotransmitters and its transporters like glutamate and glutamate/aspartate transporter²⁻⁴.

Serotonin (5HT) is a neurotransmitter with relevant functions in the central nervous and cardiovascular systems (CNS; CVS) and in the gastrointestinal tract (GIT)^{1,5,6}. It was postulated that the serotonergic neurotransmitter system may have, somehow, a role in bone metabolism⁷⁻⁹ and more recently a relationship with leptin was also highlighted¹⁰.

Although some studies support the hypothesis that 5HT is also produced by bone cells, this is still unclear¹.

5HT synaptic and extracellular concentrations are strictly regulated by a specific serotonin transporter (5HTT, SLC6A4) responsible for its reuptake after release¹¹.

5HTT gene is located in 17p11.2 region and is organized in 14 exons spanning 31Kb¹². Two polymorphic regions have been reported in this gene. One located in the promoter (5HTTLPR) is an insertion/deletion polymorphism of 44 bp leading to long (L) and short (S) alleles which differ in their transcriptional activity (allele L has the highest)¹³. The other polymorphism sited in the intron 2 (5HTTVNTR) consists of a variable number of tandem repeats (VNTR) containing 9, 10 or 12 copies of a 17 bp element. This gives rise to 3 different alleles (9, 10 and 12) and 6 different genotypes (12/12, 12/10, 12/9, 10/10, 10/9, 9/9). Allele 9 is the less common and may not even appear in some populations. Although located in an intronic region, recent studies have shown that VNTR operates as an enhancer of gene transcription leading to quantitative and qualitative differences in transcriptional rate. Allele 12 is re-

*Genetics Laboratory, Centre of Metabolism and Endocrinology, Lisbon Medical School, University of Lisbon, Lisbon, Portugal

**Clinic of Endocrinology, Diabetes and Metabolism of Lisbon (CEDML, Lda), Portugal

***Endocrinology Department, CHLN, Lisbon, Portugal

****Instituto Bento da Rocha Cabral, Lisbon, Portugal

portedly associated with higher gene transcription¹⁴. Regarding the organization of the human 5HTT gene, the role of VNTR in the regulation of 5HTT expression is probably related to the fact that this polymorphism is followed by an activating protein (AP-1) motif, a putative binding site for a transcription factor comprising the heterodimer c-fos / c-jun, which may play a role in the regulation of 5-HTT expression. Alternatively, a small number of VNTR repeats may influence the stability of messenger RNA transcription¹².

Studies on the role of 5HT in the regulation of bone metabolism have been advanced by the identification of functional 5HT receptors in osteoblasts, osteocytes and in a population containing osteoblast precursor cells. Serotonin transporter (5HTT) was described in osteoblasts, osteoclasts and osteocytes^{7,8}. 5HTT is responsible for 5HT uptake in osteoblasts and osteocytes. In osteoclasts, it seems to regulate cell differentiation but not its activation^{7,8}.

Recent studies demonstrated a direct relation between selective 5HT reuptake inhibitors (SSRIs) and a decrease in bone mass¹⁵. On the other hand, other studies revealed that a 5HTT blockade reduces osteoclast differentiation but not activation⁷. Both studies support the hypothesis that 5HT has a relevant role in the regulation of bone metabolism. The 5HT transporter seems to play a role, but the exact biological mechanisms involved remain unclear.

The aim of this study was to evaluate the association of 5HTT gene polymorphisms 5HTTVNTR and 5HTTLPR with osteoporosis.

Materials and Methods

Subjects

Blood samples were obtained from 275 Caucasian individuals, 218 Females and 57 Males (Clínica de Endocrinologia, Diabetes e Metabolismo de Lisboa) after they were assessed for bone mineral density (g/cm²) by dual X-ray absorptiometry (DXA) at the lumbar spine (L1-L4), proximal femur and distal forearm, using the QDR Discovery W densitometer (Hologic Inc.). T-scores at lumbar spine, femoral neck and total hip were used to classify the bone mineral density, according to the World Health Organization operational definition of osteoporosis. Patients having at least one T Score $\leq -2,5$ at one or more measurement sites were classified as osteoporotic (regardless whether they had

primary or secondary osteoporosis). Those with a T-Score ≥ -1 were included in the normal bone mass group. Osteopenic individuals were excluded. All of them signed an informed consent. Some clinical characteristics of controls (normal bone mass) and patients (osteoporotic) are summarized in Table I.

DNA Extraction

Peripheral Blood was collected into 5 ml tubes containing EDTA and stored at -20°C until analysis. DNA was isolated from leukocytes by an adapted non-enzymatic DNA extraction procedure¹⁶.

Genetic polymorphisms identification. 5HTT 2nd intron VNTR and 5HTT promoter variant analysis

Genomic DNA was amplified by polymerase chain reaction (PCR) to identify two different polymorphisms in 5HTT gene, one located within intron 2 (5HTTVNTR) and the other in the promoter (5HTTLPR). Primers used as well as PCR conditions were designed by the authors. For 5HTTVNTR we used the primers 5'GTCAGTATCACAGGCTGC-GAG3' and 5'TGTTCTAGTCTTACGCCAGTG3' and the amplification reaction was made in a final volume of 50 μl containing 200 ng DNA, 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 2U Taq Polymerase. The amplification program comprised an hot start of $94^{\circ}\text{C}/2$ min, 35 cycles of denaturation $94^{\circ}\text{C}/30\text{s}$, annealing $57^{\circ}\text{C}/30\text{s}$ and extension $72^{\circ}\text{C}/30\text{s}$ and a final extension of $72^{\circ}\text{C}/5\text{min}$. For 5HTTLPR we used the primers 5'GGCGTTGCCGCTCTGAATGC3' and 5'GGGACT-GAGCTGGACAACCAC3' and the amplification reaction followed the protocol above using 1U Taq Polymerase and an annealing temperature of 61°C .

Both polymorphisms resulted in different amplicons as determined by 4 or 3% agarose gel (Figure 1).

Statistical analysis

Statistical analysis was performed with the program SPSS 16.0. Continuous variables showed a normal distribution (One-Sample Kolmogorov-Smirnov Test) and so we used T-test and ANOVA to compare controls and patients. To compare allele and genotype frequencies we used Chi-square test and Odds Ratio.

For 5HTTVNTR, as alleles 10 and 9 are the ones associated with a decrease in gene transcriptional rate when we compared allele frequencies between groups we did it in two different ways. We started by analyzing the three alleles separately and then we assessed combined allele 9 and 10 in just one

Table I. Characterization of normal bone mass and osteoporosis groups

	Normal bone mass			Osteoporosis			P (χ^2 test ^a and ANOVA)
	Female (F)	Male (M)	F+M	Female (F)	Male (M)	F+M	
N	150 (80,6%)	36 (19,4%)	186	68 (76,4%)	21 (23,6%)	89	0,540 ^a
Age (years)	47,01±12,40	54,40±12,67	48,44±12,79	64,59±10,34	61,45±7,11	63,85±9,73	<0.001
Body Mass Index (BMI) (kg/m ²)	29,99±5,61	30,96±4,12	30,18±5,38	26,66±4,63	27,62±4,07	26,89±4,50	<0.001
BMD Lumbar spine (L1-L4) (g/cm ²)	1,07±0,10	1,19±0,14	1,10±0,12	0,74±0,11	0,84±0,15	0,76±0,13	<0.001
BMD Trochanter (g/cm ²)	0,77±0,09	0,90±0,12	0,79±0,11	0,58±0,08	0,67±0,14	0,60±0,10	<0.001
BMD Neck of the femur (g/cm ²)	0,90±0,11	1,00±0,14	0,92±0,12	0,67±0,10	0,77±0,14	0,70±0,12	<0.001
BMD Total Femur (g/cm ²)	1,01±0,11	1,16±0,13	1,04±0,13	0,77±0,09	0,92±0,15	0,81±0,12	<0.001
BMD Distal forearm (g/cm ²)	0,70±0,05	0,80±0,08	0,71±0,07	0,57±0,08	0,67±0,06	0,59±0,09	<0.001

group. In respect to genotype frequencies we studied the 4 genotypes separately and then we did different genotype combinations.

Differences between groups were considered significant for *P*-values < 0.05.

Results

Table I shows the characterization of the two studied groups in terms of age, Body Mass Index (BMI) and Body Mineral Density (BMD) in different parts of the skeleton. It shows that the two studied groups (osteoporosis and normal bone mass) have

a similar gender distribution and that they differ significantly in terms of age and BMI.

To account for this differences, we compare age and BMI of each 5HTTVNTR genotype (12/9, 10/10, 12/10, 12/12) in each studied population separately (OST and NBM) and in all individuals (OST + NBM). We did not find association between age or BMI and 5HTTVNTR (Tables II and III).

Table IV shows allele and genotype frequencies for the intronic polymorphism (5HTTVNTR) in patients and controls. These two groups were in Hardy-Weinberg equilibrium for genotype distribution (data not shown).

Regarding allele frequencies, although we did not find significant differences, we could be able to observe higher frequency of allele 12 among osteoporotic patients.

In genotype frequencies, as we expected by knowing how this polymorphism affects gene transcription, we just found significant differences when we included individuals 10/10 and 12/9 (homozygotes for one of the shorter alleles, allele 10 and carriers of allele 9) in one group and individuals 12/10 and 12/12 (homozygotes for the long allele and heterozygotes) in another group. Significant higher frequencies of 12/10 and 12/12 genotypes were detected in the osteoporosis group (92.1% vs 81.7%). They show a risk factor of 2,620

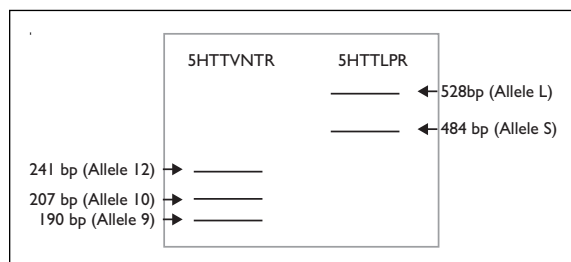


Figure 1. Diagram representing an agarose gel showing the number of base pairs of each allele for the two studied polymorphisms

Table II. 5HTTVNTR genotype frequencies according to age

	12/9	10/10	12/10	12/12	P (ANOVA)
OST	–	57,80±15,94 (n=7)	66,10±8,57 (n=42)	62,54±9,14 (n=40)	0,057
NBM	45,08±8,21 (n=4)	50,79±15,40 (n=30)	48,37±11,90 (n=82)	47,71±12,89 (n=70)	0,680
OST+NBM	45,08±8,21 (n=4)	52,12±15,53 (n=37)	54,37±13,74 (n=124)	53,10±13,66 (n=110)	0,494

OST – Osteoporosis group; NBM – Normal Bone Mass group

Table III. 5HTTVNTR genotype frequencies according to Body Mass Index (BMI)

	12/9	10/10	12/10	12/12	P (ANOVA)
OST	–	28,84±6,84 (n=7)	26,82±3,65 (n=42)	26,62±4,86 (n=40)	0,484
NBM	30,95±4,09 (n=4)	30,91±5,83 (n=30)	29,75±5,42 (n=82)	30,32±5,24 (n=70)	0,754
OST+NBM	30,95±4,09 (n=4)	30,52±5,99 (n=37)	28,76±5,08 (n=124)	28,98±5,39 (n=110)	0,300

OST – Osteoporosis group; NBM – Normal Bone Mass group

(OR=2,620 CI 95% [1,112-6,172], P=0,037) for the development of this pathology comparing with individuals with genotypes 10/10 and 12/9.

No association was observed in the osteoporosis group for the polymorphism in the promoter – 5HTTLPR (Table V).

Discussion

As Serotonin has been implicated in the control of eating behavior and body weight¹⁷, changes in its metabolism, for example, as a result of 5HTTVNTR, may induce differences in BMI. In spite of this, in our study, this variable doesn't seem to be manipulated by 5HTTVNTR.

Previous studies from our research group pointed to a deregulation of 5HT metabolism in osteoporosis as we demonstrated that intraplatelet 5HT is increased in osteoporotic patients (data not published).

We hypothesize that individuals carrying genotypes 12/12 or 12/10 of 5HTTVNTR have higher amounts of 5HTT. If we consider what is known about the role of this transporter in osteoblasts, we

may assume that the reuptake process by these cells should be activated leading to an increase in bone mass as reported by other authors¹⁵. On the other hand, if we regarded the role of 5HTT in both formation and differentiation of osteoclasts we may assume that in these individuals these processes are activated leading to a deregulation of bone homeostasis through an increase of osteoclastic function.

Although our results are in accordance with some data already published⁷, they are contradictory with other studies, especially those regarding the role of SSRIs in bone mass¹⁶. In that way, our study may be assumed as a support for the improvement of studies regarding the role of SSRIs in the regulation of bone mass and as a contribution for the knowledge about the role of 5HT metabolism in bone remodeling process.

Osteoporotic patients, as we expected, are older than the ones with normal bone mass. This may represent an important limitation to the study and compromise our conclusion. We can not predict whether individuals with NBM will still in the same group if they were older or if they will have osteoporosis or not. It would be interesting if we could

Table IV. Allele and genotype 5HTTVNTR frequencies

	Allele 12	Allele 10	Allele 9	P (χ^2 test)	Allele 12	Allele 9+10	P (χ^2 test)
OST (n= 89)	122 (68,5%)	56 (31,5%)	0	0,102	122 (68,5%)	56 (31,5%)	0,093
NBM (n= 186)	226 (61,4%)	142 (38,6%)	4 (1,1%)		226 (60,7%)	146 (39,3%)	
	12/9	10/10	12/10	12/12	P (χ^2 test)		
OST (n= 89)	0	7 (7,9%)	42 (47,2%)	40 (44,9%)	0,308		
NBM (n= 186)	4 (2,2%)	30 (16,1%)	82 (44,1%)	70 (37,6%)			
	12/9+10/10	12/10	12/12	P (χ^2 test)			
OST (n= 89)	7 (7,9%)	42 (47,2%)	40 (44,9%)	0,070			
NBM (n= 186)	34 (18,3%)	82 (44,1%)	70 (37,6%)				
	12/9+10/10	12/10+12/12	P (χ^2 test)/Odds Ratio				
OST (n= 89)	7 (7,9%)	82 (92,1%)	0,037 OR=2,620 CI 95% [1,112-6,172]				
NBM (n= 186)	34 (18,3%)	152 (81,7%)					
	12/9+12/10+12/12	10/10	P (χ^2 test)				
OST (n= 89)	82 (92,1%)	7 (7,9%)	0,091				
NBM (n= 186)	156 (83,9%)	30 (16,1%)					
	12/9+10/10+12/10	12/12	P (χ^2 test)				
OST (n= 89)	49 (55,1%)	40 (44,9%)	0,305				
NBM (n= 186)	116 (62,4%)	70 (37,6%)					

OST – Osteoporosis group; NBM – Normal Bone Mass group

Note: Numbers above the percentages represent the number of individuals within the group

Table IV. Allele and genotype 5HTTLPR frequencies

	L/L	L/S	S/S	P (χ^2 test)	Allele L	Allele S	P (χ^2 test)
OST (n= 73)	25 (34,2%)	37 (50,7%)	11 (15,1%)	0.883	87 (59,6%)	59 (40,4%)	0.704
NBM (n= 179)	56 (31,3%)	93 (52,0%)	30 (16,7%)		205 (57,3%)	153 (42,7%)	

OST – Osteoporosis group; NBM – Normal Bone Mass group

Note: Numbers above the percentages represent the number of individuals within the group

perform a prospective study of these individuals or if we could pare the ages of the two studied groups (OST and NBM).

In spite of this limitation, as far as we know, this is one of the few studies that report a possible association between 5HTTVNTR and osteoporosis opening the hypothesis that the determination of this specific polymorphism of serotonin transporter gene may contribute to the identification of individuals at high risk for the development of osteoporosis.

Correspondence to

Joana T. Ferreira
Genetics Laboratory, Centre of Metabolism and Endocrinology, Lisbon Medical School, University of Lisbon, Lisbon, Portugal
Avenida Prof. Egas Moniz
Faculdade de Medicina de Lisboa
Edifício Egas Moniz, Piso 1C
1649-028 Lisboa, Portugal
Fax: +351217999451
E-mail: jogtff@iol.pt

References

1. Warden SJ, Bliziotis MM, Wiren KM, Eshleman AJ and Turner CH. Neural regulation of bone and the skeletal effects of serotonin (5-hydroxytryptamine). *Mol Cell Endocrinol* 2005; 242: 1-9.
2. Ducy P, Amling M, Takeda S, Prieme M et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* 2000; 100: 197-207.
3. Baldock PA, Sainsbury A, Couzens M et al. Hypothalamic Y2 receptors regulate bone formation. *J Clin Invest* 2002; 109: 915-921.
4. Gu Y and Publicover S.J. Expression of functional metabotropic glutamate receptors in primary cultured rat osteoblasts. Cross-talk with N-methyl-D-aspartate receptors. *J Biol Chem* 2000; 275: 34252-34259.
5. Kroeze WK, Kristiansen K, Roth BL. Molecular biology of serotonin receptors structure and function at the molecular level. *Curr Top Med Chem* 2002; 2: 507-528.
6. Talley NJ. Serotonergic neuroenteric modulators. *Lancet* 2001; 358: 2061-2068.
7. Battaglini R, Fu J, Spate U, Ersoy U, Joe M, Sedaghat L, Stashenko P. Serotonin regulates osteoclast differentiation through its transporter. *J Bone Miner Res* 2004; 19: 1420-1431.
8. Bliziotis MM, Eshleman AJ, Zhang XW, Wiren K.M. Neurotransmitter action in osteoblasts: expression of a functional system for serotonin receptor activation and reuptake. *Bone* 2001; 29: 477-486.
9. Warden SJ, Robling AG, Sanders MS, Bliziotis MM, Turner C.H. Inhibition of the serotonin (5-hydroxytryptamine) transporter reduces bone accrual during growth. *Endocrinology* 2005; 146: 685-693.
10. WBodarski K, Włodarski P. Leptin as a modulator of osteogenesis. *Ortop Traumatol Rehabil* 2009; 11:1-6.
11. Watts SW. 5-HT in systemic hypertension: foe, friend or fantasy? *Clin Sci (Lond)* 2005; 108: 399-412.
12. Lesch KP, Balling U, Gross J et al. Organization of the human serotonin transporter gene. *J Neural Transm Gen Sect* 1994; 95: 157-162.
13. Heils A, Teufel A, Petri S et al. Allelic variation of human serotonin transporter gene expression. *J Neurochem* 1996; 66: 2621-2624.
14. MacKenzie A, Quinn J. A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *Proc Natl Acad Sci USA* 1999; 96: 15251-15255.
15. Weintrob N, Cohen D, Klipper-Aurbach Y, Zadik Z, Dickerman Z. Decreased growth during therapy with selective serotonin reuptake inhibitors. *Arch Pediatr Adolesc Med* 2002; 156: 696-701.
16. Lahiri DK, Nurnberger JI Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 1991; 19:5444.
17. Leibowitz SF and Alexander JT. Hypothalamic serotonin in control of eating behavior, meal size, and body weight. *Biological Psychiatry* 1998; 44 : 851-864.

12º Congresso da SPMFR

Algarve, Portugal

10 a 12 Março 2011