Usategui-Martín R[1,2], Corral E[1], Alonso M[1], Calero-Paniagua I[2,3], Carranco-Medina TE[2,3], Quesada-Moreno A[2,3], Sánchez-González MD[2,3], Hidalgo-Calleja C[2,3], Pérez-Garrido L[2,3], Montilla Morales C[2,3,4], Mirón-Canelo JA[5], González-Sarmiento R[1,2,4], del Pino-Montes J[2,3,4]

1 Unidad de Medicina Molecular - Departamento de Medicina - Universidad de Salamanca

2 Instituto de Investigación Biomédica de Salamanca (IBSAL)

3 Servicio de Reumatología - Hospital Universitario de Salamanca

4 Red Temática de Investigación Cooperativa en Envejecimiento y Fragilidad (RETICEF)

5 Departamento de Medicina Preventiva, Salud Pública y Microbiología Médica - Universidad de Salamanca

Study of the deletions in the GSTM1 and GSTT1 genes and of the *Ile*105*Val* polymorphism of the GSTP1 gene in patients with Paget's disease of bone

Correspondence: Javier del Pino Montes - Servicio de Reumatología - Hospital Universitario de Salamanca - Pº San Vicente, 182 - 37007 Salamanca (Spain)
e-mail: jpino@usal.es

Date of receipt: 29/08/2014
Date of acceptance: 23/11/2014

Summary

Background: Paget's disease of bone (PDB) is a disorder focussed on the bone with an increase in the number, size and activity of the osteoclasts. Some epidemiological data support the theory of its relationship with toxic or infectious environmental agents, whose interaction with some predisposing genetic alterations may lead to PDB. The glutathione S-transferases (GST) are involved in the metabolism of toxins, by catalysing the nucleophilic attack of the physiological substrate, reduced glutathione or GSH (g-Glu-Cys-Gly) on the electrophilic centre of a great number of toxic structures. We studied whether the variability of the GSTM1, GSTP1 and GSTT1 genes is related to the risk of developing PDB.

Patients and methods: We analysed 148 patients diagnosed with PDB, and 207 control individuals matched in sex and age with no history of bone alterations. Using genomic DNA obtained from peripheral blood the presence-absence of the GSTM1 and GSTT1 genes was studied by means of multiplex PCR. The study of the *Ile105Val* GSTP1 gene was carried out using PCR and subsequent digestion with the restriction enzyme BsmAI. The distribution of genotypes was analysed by means of the Pearson chi-square test. When statistically significant differences were found we carried out a multivariate logistical regression to determine the risk which the presence of a particular genotype could generate. We used the CSPSS 21.0 program. Differences were considered to be statistically significant when the value of $p<0.05$.

Results: We found differences in the distribution of the presence-absence of the deletion in the GSTM1 gene; not being a carrier for the deletion or being a heterozygous carrier in the GSTM1 gene confers a lower risk of developing PDB (OR=0.56, 95% CI: 0.36-0.87; p=0.011). In the study of the GSTT1 and GSTP1 genes there were no significant differences.

Conclusion: The detoxifying activity diminishes when two copies of the GSTM1 gene with deletions are inherited by reducing in enzyme activity, which has been associated with a greater susceptibility to some cancers, alcoholic hepatopathy and other inflammatory problems. We are not aware of any description of its association with PDB. PDB is observed more frequently in carriers of the homozygous deletion in the GSTM1 gene. This fact could explain the epidemiological findings which link PDB to exposure to certain environmental agents.

Key words: *Paget's disease of bone, glutathione S-transferase, genetics, polymorphism.*

Introduction

Paget's disease of bone is the most common bone metabolic disease after osteoporosis[1]. It is a bone disorder characterised by an increase in bone turnover in a disorganised way: a large increase in bone resorption, followed by bone formation of the same proportions. The result is bone with a structure which is irregular and anarchic, which alters its morphology and mechanical properties. Some patients are asymptomatic, while others have pain, degenerative arthropathy, fractures, bone deformities, deafness or other syndromes of nerve compression. The main change resides in the osteoclasts which increase in number size and activity[2,3].

There are currently two etiopathogenic hypotheses which attempt to explain the origin of PDB: the influence of environmental factors and the existence of genetic determinants[1].

There is evidence that genetic changes play a significant role in the development of the disease. There is a strong tendency to family aggregation (15-40%), with a seven-fold increase in relative risk of suffering the disease in families of patients with PDB[3-6]. In most of these families there is an autosomal dominant pattern with high penetrance in the sixth decade[3]. Recently, alterations in the genes SQSTM1, CSF1, OPTN, TNFRSF and TM7SF4[7,8], have been associated with a higher risk of developing PDB. Some epidemiological data, such as its heterogeneous distribution, or more recent changes in its incidence and seriousness, support the idea of the influence of environmental factors in the development of the disease. Its association with diets poor in calcium and vitamin D during infancy[9,10], exposure to environmental toxins[11], contact with animals during infancy[12-14], consumption of non-controlled meat[15], consumption of untreated water[16] and infectious agents such as viruses (paramixoviridae)[14,17,18], have been reported.

Neither environmental nor genetic factors alone explain the etiopathogeny of this disease. The most accepted model considers PDB to be the result of the synergetic action of both environmental and genetic factors. The genetic determination would explain the individual susceptibility to developing the disease following the exposure to the participating environmental factor[2].

Involved in the metabolization of toxins are the glutathione S-transferases (GSTs). These are a family of enzymes which participate in cell detoxification. These enzymes catalyse the nucleophilic attack of the physiological substrate, reduced glutathione or GSH (g-Glu-Cys-Gly) on the electrophilic centres of a great number of toxic structures, allowing their degradation. They are classified in seven families (alpha, kappa, mu, pi, sigma, theta and zeta) which are differentiated both in their sequences, as well as in their immunological properties and physiological roles[19,20]. GSTM1, GSTP1 and GSTT are the most studied GSTs, and those which have been most commonly associated with human pathologies[21].

The role of environmental factors, some of which are toxic, appears to be significant in the development of the disease. Given that the individual response to the toxic factors is genetically determined, we have designed this study with the aim of trying to determine whether the variability of the GSTM1 GSTP1 and GSTT1 genes (involved in the metabolization of exogenous toxins) is related to the risk of developing PDB.

Materials and methods

Patients and controls

We studied 148 patients diagnosed with PDB. In the case of patients with family history of the disease, we only selected one patient per family to avoid familial genotype bias. The patients were diagnosed by the rheumatology service of the University Hospital of Salamanca. As a control group, we analysed 207 individuals, matched in sex and age with the group of patients, without history of bone alterations, and coming from the same geographic area. From each of the patients were gathered clinical characteristics such as sex, age at diagnosis, family history, number of bones affected, presence of fractures, affectation of skull and affectation of cranial nerve pairs. All the subjects studied, both in the group of patients and the control group, gave their informed consent to participate in the study, which was approved by the ethics committee of the hospital.

DNA extraction and analysis of polymorphisms

In both the patient and control groups the extraction of genomic DNA from peripheral blood was carried out using the standard phenol-chloroform procedure.

The study of the presence-absence of the deletions in the GSTM1 and GSST1 genes was carried out using multiplex PCR under conditions described in Table 1. The study of the *Ile*105*Val* polymorphism of the GSTP1 gene was conducted using PCR and subsequent digestion with the restriction enzyme BsmAl. The conditions used are set out in Table 1.

Statistical analysis

The distribution of genotypes among patients and controls was analysed using the Pearson chi-square test. In those polymorphisms in which statistically significant differences were found we carried out a multivariate logistical regression to determine the risk which the presence of a particular genotype could generate. The statistical analysis was carried out using the SPSS 21.0 program. Those differences whose p value was <0.05 were considered as statistically significant.

Results

We studied a total of 148 patients and 207 controls. The clinical characteristics of the patients are set out in Table 2. The distribution of the presence-absence of deletions in the GSTM1 and GSST1 genes and the distribution of the genotypes for the *Ile*105*Val* polymorphism in the GSTP1 gene, and

their relationship to the risk of developing PDB are shown in Table 3.

We found statistically significant differences in the distribution of the presence-absence of deletion in the GSTM1 gene: not being a carrier for the homozygous deletion in the GSTM1 gene confers a lower risk of developing PDB (OR=0.56, CI 95%: 0.36-0.87; p=0.011). In the study of the GSTTT1 and GSTP1 genes we found no statistically significant differences (Table 3).

No statistically significant differences were found in the analysis of the clinical characteristics of the patients in relation to the variability of the GSTM1, GSTT1 and GSTP1 genes.

Discussion

PDB lesions occur as the result of an increase in bone resorption followed by an increase in its formation. The main change is located in the osteoclasts which increase in number, size and activity. There is a range of evidence which indicates that the etiopathogeny of the disease is a synergy between a series of environmental factors and the existence of certain genetic determinants[2]. Through a study of the variability of the GSTM1, GSTT1 and GSTP1 genes (involved in the metabolism of endogenous toxins) we intended to evaluate the relationship between these variables and the risk of developing PDB. As far as we know, this is the first work which examines the influence of the changes in these genes on the development of this disease.

The GSTM gene is located in the 1p13 chromosome, and to date, five allelic variants are known: GSTM1, GSTM2, GSTM3, GSTM4 and GSTM5. A reduction in detoxification activity occurs when the deletion in gene GSTM1 is inherited, meaning that being a homozygous carrier of a deletion in the GSTM1 gene causes a reduction in enzyme activity. The theta class of GSTs comprises two genes which code for the two proteins GSTT1 and GSTT2. As with the GSTM1 gene, if a homozygous deletion in the GSTT1 gene is inherited, there is a reduction in detoxification activity. In terms of the sub-family of GSTP, it comprises a single gene GSTP1 in which have been described two allelic variants which differ in the base 313 of the cDNA, one adenine (A) being substitute by a guanine (G). This difference results in a change of a valine (Val) to an isoleucine (Ile) in the 105 codon of the amino-acid sequence, causing a defective bond between the enzyme and the substrate, and thus, a reduction detoxification activity[19,20,22,23].

Being a homozygous carrier for deletion in the GSTM1 and/or GSTT1 genes has been associated with a greater susceptibility to developing different types of cancer[21,22,24], alcohol-related liver disease[25] and other inflammatory diseases[25,26], because it causes poorer metabolization of toxic agents, with the synthesis of free radicals which damage DNA[20]. Our results show that not being a homozygous carrier for the deletion in the GSTM1 gene brings a lower risk of suffering PDB. In the study of the GSTT1 and GSTP1 genes we found no statistically significant difference between the patient and control groups. We found no statistically significant differences between the clinical expression, extent and activity of the disease in relation to the variability in the GSTM1, GSTT1 and GSTP1 genes in the group of patients with PDB.

One of the causes postulated as the origin of PDB is exposure to environmental toxins from the production of cotton, meat or drinking water without adequate control of sanitation, which may alter the maturation and activity of the osteoclasts, the increase in activity fostering the development of PDB[11,15,16]. Our hypothesis is that to have the homozygous deletion in the GSTM1 gene assumes poor metabolization of environmental toxins which, by a mechanism yet unknown, may increase the function of the osteoclasts and osteoclast precursors which, combined with other genetic changes not yet well described, could result in the development of PDB.

In conclusion, in those individuals who are carriers of the GSTM1 gene with homozygous deletions, PDB is more frequently observed. This fact could explain the epidemiological findings which associate PDB with exposure to certain environmental agents. Even so, functional studies of these polymorphisms are required in order to validate our hypothesis.

Bibliography

1. Ralston SH, Layfield R. Pathogenesis of Paget Disease of Bone. Calcif Tissue Int 2012;91:97-113.
2. Singer FR, Mills BG, Gruber HE, Windle JJ, Roodman GD. Ultrastructure of bone cells in Paget's disease of bone. J Bone Miner Res 2006;21(Suppl 2):P51-4.
3. Morales-Piga AA, Rey-Rey JS, Corres-González J, García-Sagredo JM, López-Abente G. Frequency and characteristics of familial aggregation of Paget's disease of bone. J Bone Miner Res 1995;10:663-70.
4. Morissette J, Laurin N, Brown JP. Sequestosome 1: mutation frequencies, haplotypes, and phenotypes in familial Paget's disease of bone. J Bone Miner Res 2006;21(Suppl 2):P38-44.
5. Siris ES, Ottman R, Flaster E, Kelsey JL. Familial aggregation of Paget's disease of bone. J Bone Miner Res 1991;6:495-500.
6. Hocking LJ, Herbert CA, Nicholls RK, Williams F, Bennett ST, Cundy T, et al. Genomewide search in familial Paget disease of bone shows evidence of genetic heterogeneity with candidate loci on chromosomes 2q36, 10p13, and 5q35. Am J Hum Genet 2001;69:1055-61.
7. Albagha OM, Visconti MR, Alonso N, Langston AL, Cundy T, Dargie R, et al. Genome wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget's disease of bone. Nat Genet 2010;42:520-4.
8. Albagha OME, Wani SE, Visconti MR, Alonso N, Goodman K, Brandi ML, et al. Genome-wide association identifies three new susceptibility loci for Paget's disease of bone. Nat Genet 2011;43:685-9.
9. Barker DJ, Gardner MJ. Distribution of Paget's disease in England, Wales and Scotland and a possible relationship with vitamin D deficiency in childhood. Br J Prev Soc Med 1974;28:226-32.
10. Siris ES. Epidemiological aspects of Paget's disease: family history and relationship to other medical conditions. Semin Arthritis Rheum 1994;23:222-5.
11. Lever JH. Paget's disease of bone in Lancashire and arsenic pesticide in cotton mill wastewater: a specula-

Table 1. Amplification conditions and digestion for GSTM1 GSTM1, GSTT1 and GSTP1 genes

Amplification conditions GSTM1 and GSTT1 genes
Primers
C(+):
Sense: 5′-CGCCATCTTGTGCTACATTGCCCG-3
GSTM1:
Sense: 5`-ATCTTCTCCTCTTCTGTCTC-3′
Anti sense: 5′-TCACCGGATCATGGCCAGCA-3′
GSTT1:
Sense: 5′-TTCCCTTACTGGTCCTACATCTC-3
Anti sense: 5′-TCACCGGATCATGGCCAGCA-3
PCR program
95ºC 5 minutes
30 cycles (94ºC 30 seconds/58ºC 30 seconds/72ºC 45 seconds)
72ºC 8 minutes
Resulting PCR fragments and correspondence with the genotype
231, 450 y 158 pb: GSTM1(+)/GSTT1(+)
231 y 158 pb: GSTM1(+)/GSTT1(-)
450 y 158 pb: GSTM1(-)/GSTT1(+)
158 pb: GSTM1(-)/GSTT1(-)
Amplification conditions and digestion for GSTP1 gen
Primers
Sense: 5′-ACCCCAGGGCTCTATGGGAA-3
Anti sense: 5′-TGAGGGCACAAGAAGCCCCT-3′
PCR program
95ºC 5 minutes
30 cycles (94ºC 30 seconds/55ºC 30 seconds/72ºC 30 seconds)
72ºC 5 minutes
Amplicon: 176pb
Enzyme: BsmaI **Digestion:** 37ºC / 4 hours
Fragments resulting from digestion and correspondence with genotype
176 pb: AA
176, 91 y 85 pb: AG
91 y 85 pb: GG

tive hypothesis. Bone 2002;31:434-6.
12. Merlotti D, Gennari L, Galli B, Martini G, Calabrò A, De Paola V, et al. Characteristics and familial aggregation of Paget's disease of bone in Italy. J Bone Miner Res 2005;20:1356-64.
13. López-Abente G, Morales-Piga A, Elena-Ibáñez A, Rey-Rey JS, Corres-González J. Cattle, pets, and Paget's disease of bone. Epidemiology 1997;8:247-51.
14. O'Driscoll JB, Anderson DC. Past pets and Paget's disease. Lancet 1985;2:919-21.
15. Mills BG, Singer FR. Nuclear inclusions in Paget's disease of bone. Science 1976;194:201-2.
16. Mirón-Canelo JA, Del Pino-Montes J, Vicente-Arroyo M, Sáenz-González MC. Epidemiological study of Paget's disease of bone in a zone of the Province of Salamanca (Spain). The Paget's disease of the bone study group of Salamanca. Eur J Epidemiol 1997;13:801-5.
17. Rebel A, Malkani K, Basle M, Bregeon C, Patezour A, Filmon R. Ultrastructural characteristics of osteoclasts in Paget's disease. Rev Rhum Mal Osteoartic 1974;41:767-71.
18. Mills BG, Singer FR, Weiner LP, Suffin SC, Stabile E, Holst P. Evidence for both respiratory syncytial virus and measles virus antigens in the osteoclasts of patients with Paget's disease of bone. Clin Orthop Relat Res 1984:303-11.
19. Strange RC, Spiteri MA, Ramachandran S, Fryer AA. Glutathione-S-transferase family of enzymes. Mutat Res 2001;482:21-6.
20. Strange RC, Jones PW, Fryer AA. Glutathione S-transferase: genetics and role in toxicology. Toxicol Lett 2000;112-113:357-63.
21. Parl FF. Glutathione S-transferase genotypes and cancer risk. Cancer Lett 2005;221:123-9.
22. Ye Z, Song H. Glutathione s-transferase polymorphisms (GSTM1, GSTP1 and GSTT1) and the risk of acute leukaemia: a systematic review and meta-analysis. Eur J Cancer 2005;41:980-9.
23. Frova C. Glutathione transferases in the genomics era: New insights and perspectives. Biomol Eng 2006;23:149-69.
24. White DL, Li D, Nurgalieva Z, El-Serag HB. Genetic variants of glutathione S-transferase as possible risk factors for hepatocellular carcinoma: a HuGE systematic review and meta-analysis. Am J Epidemiol 200815;167:377-89.
25. Brind AM, Hurlstone A, Edrisinghe D, Gilmore I, Fisher N, Pirmohamed M, et al. The role of polymorphisms of glutathione S-transferases GSTM1, M3, P1, T1 and A1 in susceptibility to alcoholic liver disease. Alcohol Alcohol 2004;39:478-83.
26. Miller EA, Pankow JS, Millikan RC, Bray MS, Ballantyne CM, Bell DA, et al. Glutathione-S-transferase genotypes, smoking, and their association with markers of inflammation, hemostasis, and endothelial function: the atherosclerosis risk in communities (ARIC) study. Atherosclerosis 2003;171:265-72.

Table 2. Clinical characteristics of patients with PDB

		Patients (N)
Sex	Man Woman	79 69
Age at diagnosis	More than 60 years Less than 60 years	115 33
Family history	Sporadic Family	129 19
Number of affected bones	Less than three More than three	103 45
Presence of fractures	Yes No	9 139
Involvement of cranium	Yes No	61 87
Involvement of cranial nerve	Yes No	25 123

Table 3. Distribution of genotypes of the studied polymorphisms in genes GSTM1, GSTT1 and GSTP1 and its association with the risk of developing PDB

SNP	Genotype	Patients PDB N (%)	Controls N (%)	Value of p	OR (IC 95%)
GSTM1	-/-	98 (66.2%)	109 (52.7%)	0.011	1.00
	+/+ ; +/-	50 (33.8%)	98 (47.3%)		0.56 (0.36-0.87)
GSTT1	-/-	28 (18.9%)	49 (23.7%)	0.299	----
	+/+ ; +/-	120 (81.1%)	158 (76.3%)		
GSTP1	AA	70 (47.3%)	76 (39.0%)	0.280	----
	AG	65 (43.9)	97 (49.7%)		
	GG	13 (8.8%)	22 (11.3%)		
	AA+AG	135 (91.2%)	173 (88.7%)	0.477	----
	GG	13 (8.8%)	22 (11.3%)		
	AA	70 (47.3%)	76 (39.0%)	0.125	----
	AG+GG	78 (52.7%)	119 (61.0%)		