

## A familial case of Paget's disease of bone with mutation at exon 8 of the sequestosome 1 (*SQSTM1/p62*) gene

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### Introduction

Paget's disease of bone (PDB) is a disorder of bone metabolism reported to affect up to 3% of Caucasian over 55 years of age (1). PDB is a genetically heterogeneous disorder characterized by abnormal osteoclastic activity leading to bone destruction and macroscopic deformities, which cause bone pain and pathological fractures. There is evidence of genetic abnormalities in its pathogenesis, and at least 8 different human chromosomal loci have been correlated to PDB (2). The PDB 3 locus in chromosome 5q35-qter hosts the *SQSTM1/p62* gene, whose mutations account for most of the sporadic and familial forms of PDB reported in the literature (2). *SQSTM1/p62* gene encodes the *SQSTM1/p62* protein, that is component of the NF- $\kappa$ B signaling pathway crucial for osteoclastic differentiation and activation (3). The exon 8 DNA sequence accounts for the ubiquitin protein-binding domain (UBA) and represents a mutational hot spot area. An abnormal UBA region is reported to account the inability to bind to ubiquitin with consequential accumulation of sequestosome protein (4). Different mutations of *SQSTM1/p62* have been reported in French Canadian PDB families (5), and a recent observation seems to confirm the causal relationship between this gene and Paget's disease of bone also in sporadic Italian patients (2).

We report a case of a family whose members are affected by polyostotic PDB with mutation at exon 8 of *SQSTM1/p62* gene.

### Materials and methods

#### Patients

We monitored for a long period 3 members of a family affected by polyostotic PDB at the Bone Metabolic Unit of the IRCCS Ospedale Maggiore di Milano. The members of this family were a woman (proband), her brother, their father and the brother's daughter (Fig. 1).

Brother, sister and their father were visited since 1987. Diagnosis of PDB was confirmed by X-rays, bone scintigraphy and bone markers measurements. Total alkaline phosphatase (AP) and its bone isoenzyme (BAP) were used to assess disease activity. The woman (VB), born in 1928, is affected by polyostotic PDB, diagnosed in 1987, involving dorsal spine (D9, D11, D12) and left hemipelvis. She also presented a colloid goitre since 1989, developed in hypothyroidism in 2003 and treated with L-thyroxin (100  $\mu$ g/day). A benign M-component hypergammaglobulinemia was diagnosed in 2002. VB underwent several courses of salmon calcitonin (rCT) till 1992 and thereafter nine courses of bisphosphonate treatment (clodronate till 2001 and neridronate in 2002).

The brother (VG), born in 1942, is affected by polyostotic PDB diagnosed in 1980 involving skull, right occipital and parietal bone, lumbar spine (L3), left ulna and left hemipelvis. He reported a traumatic lumbar spine fracture (L1) in 1972 and a traumatic left olecranon fracture in 1980. Moreover, he under-

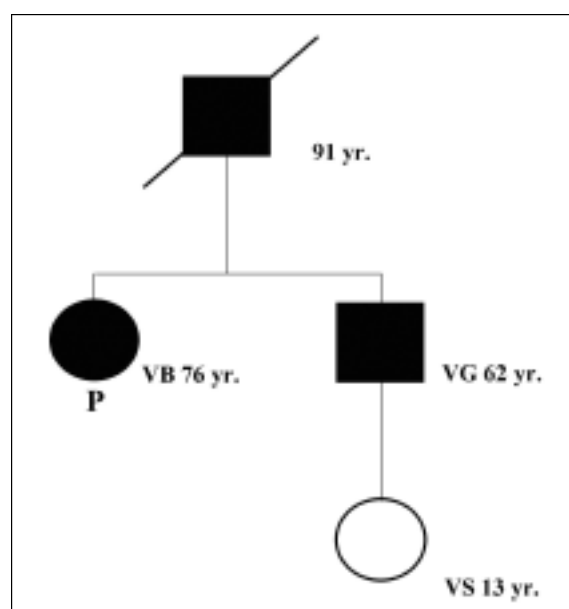


Figure 1 - Pedigree of the family. Black symbols indicate the affected PDB subjects (squares = males; circles = females). P indicates the proband.

went a left hemicolectomy for a dysplastic adenoma in 2001. He was treated with several courses of sCT till 1989, and with 9 courses of clodronate thereafter.

Their father, born in 1901, had also polyostotic PDB, involving right proximal humerus, pelvis and proximal distal femoral epiphysis. He suffered *ictus cerebri* in 1986 with right hemiparesis. For PDB he underwent a single course of i.m. sCT for 4 months in 1987. He died in 1992 consequently to PDB's cardiovascular complication.

VG's daughter (VS), born in 1981, is not affected by PDB.

### Treatment

In all patients pharmacological treatment was prescribed when serum AP and BAP levels firstly increased at least 25% above the upper limit of the normal range. During the whole observation period we used in these PDB patients synthetic sCT at the dosage of 100 UI i.m./day for 6 months and bisphosphonates (clodronate, 300 mg i.v./day for 5 days, and neridronate, 100 mg/day i.v. for 2 days). As regards sCT, another course of treatment was prescribed after the sixth month of follow-up when total serum AP activity newly increased above the upper limit of the normal range by at least 25%.

Overall, regarding bisphosphonates therapy, we have treated about 50 PDB outpatients. The nadir of our patients' serum AP and BAP levels were observed between the third and the fifth month after therapy and the zenith usually after the ninth month (data not published). Disease activity was evaluated by the means of serum total AP and BAP determined at three, five, and nine months after therapy. Another course of drugs was prescribed when, after the ninth month, total serum AP and BAP had an increase of at least 25% above the upper limit of the normal range. After 5 months of treatment with clodronate, the patients showing serum AP and BAP above the upper limit of the normal range, although exhibiting a reduction of the values to 50% or more of the pre-treatment value, were classified as non-responders to the therapy and switched to neridronate.

### Mutational analysis

Genetic tests were performed in the affected patients VB and VG, and in VS, not affected. Genetic tests of their father were not possible, because the study started ten years after his death and no biological samples were available.

After administration of an informed consent form approved by the Local Ethical Committee, peripheral blood was obtained and genomic DNA was extracted using a microvolume extraction method, QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

PCR protocol was performed as follows. Exons 7 and 8 of the *SQSTM1/p62* gene were amplified by PCR (I-Cycler, Bio-Rad Laboratories, Milan, Italy) using two couples of primers located in the flanking introns: respectively 5'-GACTGTCT-GCCAGGAGCC-3'/5'-CCCTGCAGTGGAGAATCT-3' for exon 7 and 5'-CAGTGTGGCTGTGAGGAC-3'/5'-CAGT-GAGCCTTGGGTCTCG-3' for exon 8. For each patient we used 0.1 mg of DNA, in a final buffer volume of 50 ml [67 mM Tris-HCl, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween-20, 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxyribonucleotides, 0.2 mM of each primer and one unit of Polytaq (Polymed, Florence, Italy)]. Thirty-five PCR cycles were performed at 94 °C for 30 seconds, 58 °C (exon 7) or 55 °C (exon 8) for 30 seconds and 72 °C for 1 minute, after a first denaturing cycle at 94 °C for 3 minutes. A final extension cycle of 5 minutes at 72 °C was performed.

PCR products were tested by 2% ethidium bromide-stained agarose gel electrophoresis, purified using the High Pure PCR

Product Purification Kit (Roche, Indianapolis, IN, USA) and finally sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing reaction consisted of twenty-five repeated cycles of denaturation for 10 seconds at 96 °C, annealing for 5 seconds at 55 °C and extension for 2 minutes at 60 °C. The sequencing products were purified with DyeEx 2.0 Spin Kit (Qiagen, GmbH, Hilden, Germany) to remove the excess of dye terminator. Five microliters of each purified sequence were then resuspended in 15 ml of formamide and denaturated for 2 minutes at 95 °C. Analysis of the forward and reverse sequences was performed on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared to reference sequence NM\_003900.

### Results

Mutational screening of the exons 7 and 8 of the *SQSTM1/p62* gene revealed the presence of a C/T transversion at position 1215 in exon 8 (Fig. 2) in the affected individuals (VB and VG). This mutation causes the substitution proline/leucine at codon 392 (P392L), and it has been described by other reports in different populations (2, 5-10). No mutation was found in the not affected subject VS.

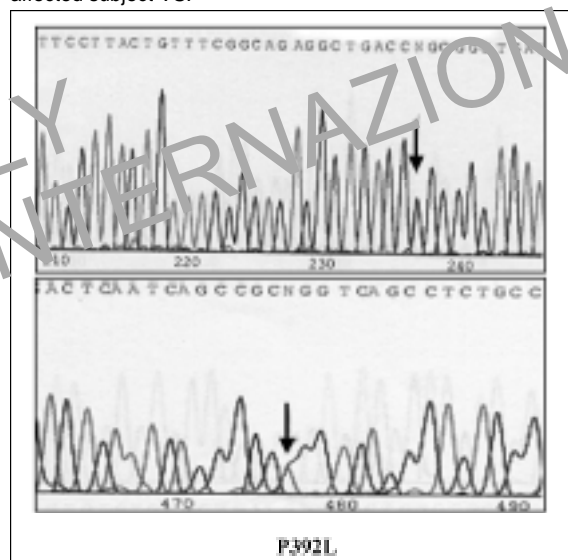


Figure 2 - *SQSTM1* gene mutation (bottom) detected in two Italian PDB patients from the same family. Specific forward and reverse sequences have been reported. Arrows indicate the presence of mutation, a C>T transition at exon 8.

### Discussion

The aminoacidic residue 392 was conserved in the mouse and rat homologues. The P392 residue is located in the C-terminal end of the p62 protein flanking the ubiquitin associated domain (UBA) and could thus be important for the conformation and/or function of this region. The UBA domain of *SQSTM1/p62* consists of three anti-parallel  $\alpha$ -helices; P392 is the first residue of helix 1. P392L mutation modifies the secondary structure of the UBA domain by extending the N-terminus of helix 1, and this could result in an altered conformation and/or function of p62 protein (11).

However, the molecular mechanisms by which SQSTM1/p62 UBA domain mutations cause PDB remain undetermined. The ubiquitination pathway plays a crucial role in the regulation of signal transduction by targeting key components for degradation by proteasome. It is possible that mutations in the UBA domain of SQSTM1/p62 result in an accumulation of signaling intermediates in cytosol that are involved in the regulation of osteoclastogenesis and/or osteoclast activation such as NF- $\kappa$ B (8). Further studies will be required to elucidate the exact mechanism involved.

The report of two novel mutations, other than P392L, at exon 8 of the SQSTM1/p62 gene in sporadic Italian PDB patients by Falchetti et al. (2) confirms the evidence of a clustered mutational area at this level also in PDB patients of Italian origin, indicating the role of the UBA domain in the biological properties of SQSTM1/p62 protein. Mutational analysis of these subjects provided the opportunity to demonstrate the co-segregation of P392L mutation and PDB trait.

Data about this PDB family strongly confirm the importance of genetic analysis in order to early identify asymptomatic carriers, making possible to prevent or limit the occurrence of PDB. The creation of a large SQSTM1/p62 Italian mutational database, together with functional studies, will provide the opportunity to assess a possible correlation between the type of mutation and the response to therapy, as well as to set-up new therapeutic strategies.

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