

# Domain-specific mutations in sequestosome 1 (SQSTM1) cause familial and sporadic Paget's disease

Lynne J. Hocking<sup>1</sup>, Gavin J.A. Lucas<sup>1</sup>, Anna Daroszewska<sup>1</sup>, Jon Mangion<sup>2</sup>, Mark Olavesen<sup>2</sup>, Tim Cundy<sup>3</sup>, Geoff C. Nicholson<sup>4</sup>, Lynley Ward<sup>5</sup>, Simon T. Bennett<sup>2</sup>, Wim Wuyts<sup>6</sup>, Wim Van Hul<sup>6</sup> and Stuart H. Ralston<sup>1,\*</sup>

<sup>1</sup>Department of Medicine and Therapeutics, University of Aberdeen, UK, <sup>2</sup>Oxagen Ltd, Abingdon, UK, <sup>3</sup>Department of Medicine, University of Auckland, New Zealand, <sup>4</sup>Department of Clinical and Biomedical Sciences, Barwon Health, Geelong Hospital, University of Melbourne, Australia, <sup>5</sup>Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Western Australia and <sup>6</sup>Department of Medical Genetics, University of Antwerp, Belgium

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**Paget's disease of bone (PDB) is a common disorder characterized by focal abnormalities of increased and disorganized bone turnover. Genetic factors are important in the pathogenesis of PDB, and in previous studies, we and others identified a locus for familial PDB by genome-wide search on 5q35-qter (*PDB3*). The gene encoding sequestosome 1 (*SQSTM1/p62*) maps to within the *PDB3* critical region, and recent studies have identified a proline-leucine amino acid change at codon 392 of *SQSTM1* (P392L) in French-Canadian patients with PDB. We conducted mutation screening of positional candidate genes in the *PDB3* locus in patients with PDB, and also identified mutations in the gene encoding *SQSTM1* as a common cause of familial and sporadic PDB. Three different mutations were found, all affecting the highly conserved ubiquitin-binding domain. The most common mutation was the P392L change in exon 8, which was found in 13 of 68 families (19.1%). Another mutation—a T insertion that introduces a stop codon at position 396 in exon 8—was found in four (5.8%) families. A third mutation affecting the splice donor site in intron 7 was found in one (1.5%) family. The P392L mutation was also found in 15 of 168 (8.9%) of patients with sporadic PDB and 0 of 160 of age- and sex-matched controls ( $P < 0.0001$ ). These studies confirm that mutations affecting the ubiquitin-binding domain of *SQSTM1* are a common cause of familial and sporadic Paget's disease of bone.**

## INTRODUCTION

Paget's disease of bone (MIM 167250, 602080; see <http://www.ncbi.nlm.nih.gov/OMIM>) is a common disorder, affecting up to 3% of individuals above the age of 55 years in the UK and other Caucasian populations (1,2). The disease is characterized by focal areas of increased osteoclastic bone resorption, coupled to increased and disorganized new bone formation. Whilst many patients are asymptomatic, up to 30% have symptoms related to the disease, such as bone pain, bone deformity, pathological fracture and deafness (3,4). Accumulating evidence suggests that PDB is largely determined by genetic influences. Familial clustering is common in PDB, and between 15% and 40% of individuals have an affected first-degree relative (5–7). Many families have been described where PDB is inherited in an autosomal dominant manner (5,8,9), as is the rare bone dysplasia familial expansile

osteolysis (FEO) (MIM 174810), which shares many clinical features with PDB (10). The gene responsible for FEO was mapped to a locus on chromosome 18q21–22 by Hughes and colleagues (11) in 1994, and some PDB kindreds were subsequently found to be linked to the same region (8,9). It is now clear that FEO, expansile skeletal hyperphosphatasia and some cases of familial Paget's disease that present below the age of 20 years are allelic disorders caused by different activating mutations affecting the signal peptide region of the receptor activator of NFκB (RANK) (12,13). Whilst this demonstrates that abnormalities in the NFκB signaling pathway can cause a PDB-like phenotype, mutations of the osteoprotegerin and RANK genes have been excluded as a common cause of familial and sporadic PDB (14,15).

Several other susceptibility loci for familial PDB have been identified by genome-wide search on chromosomes 5q35 (*PDB3*) (16,17), 5q31 (*PDB4*) (16), 2q36 (*PDB5*) (17),

\*To whom correspondence should be addressed at: Department of Medicine and Therapeutics, University of Aberdeen Medical School, Foresterhill, Aberdeen AB25 2ZD, UK. Tel: +44 1224553025; Fax: +44 1224554761; Email: s.ralston@abdn.ac.uk

**Table 1.** Details of families with *SQSTM1* mutations

Mutation	Country/region	Family ID*
P392L	Australia/Geelong	34
	Australia/Perth	58, 65
	Hungary/Budapest	53
	New Zealand/Auckland	38, 42, 44
	UK/N. England	18, 19
	UK/S. England	20
	UK/Scotland	14, 23
E396X	Australia/Geelong	68
	Australia/Perth	54
	New Zealand/Auckland	48
	UK/N. England	5
IVS7+1 G-A	Australia/Perth	55

\*Numbers correspond to those on the bone research group's website (see Materials and Methods).

10p13 (*PDB6*) (17) and 18q23 (*PDB7*) (18). The sequestosome 1 gene (*SQSTM1*), which encodes a component of the NF $\kappa$ B signaling pathway, maps to the *PDB3* locus on chromosome 5q35, and recently Laurin *et al.* (19) identified a recurrent mutation of *SQSTM1* in a population of French-Canadian patients with PDB. In this study, we also report the identification of mutations in the ubiquitin-binding domain of *SQSTM1* as a common cause of familial and sporadic PDB.

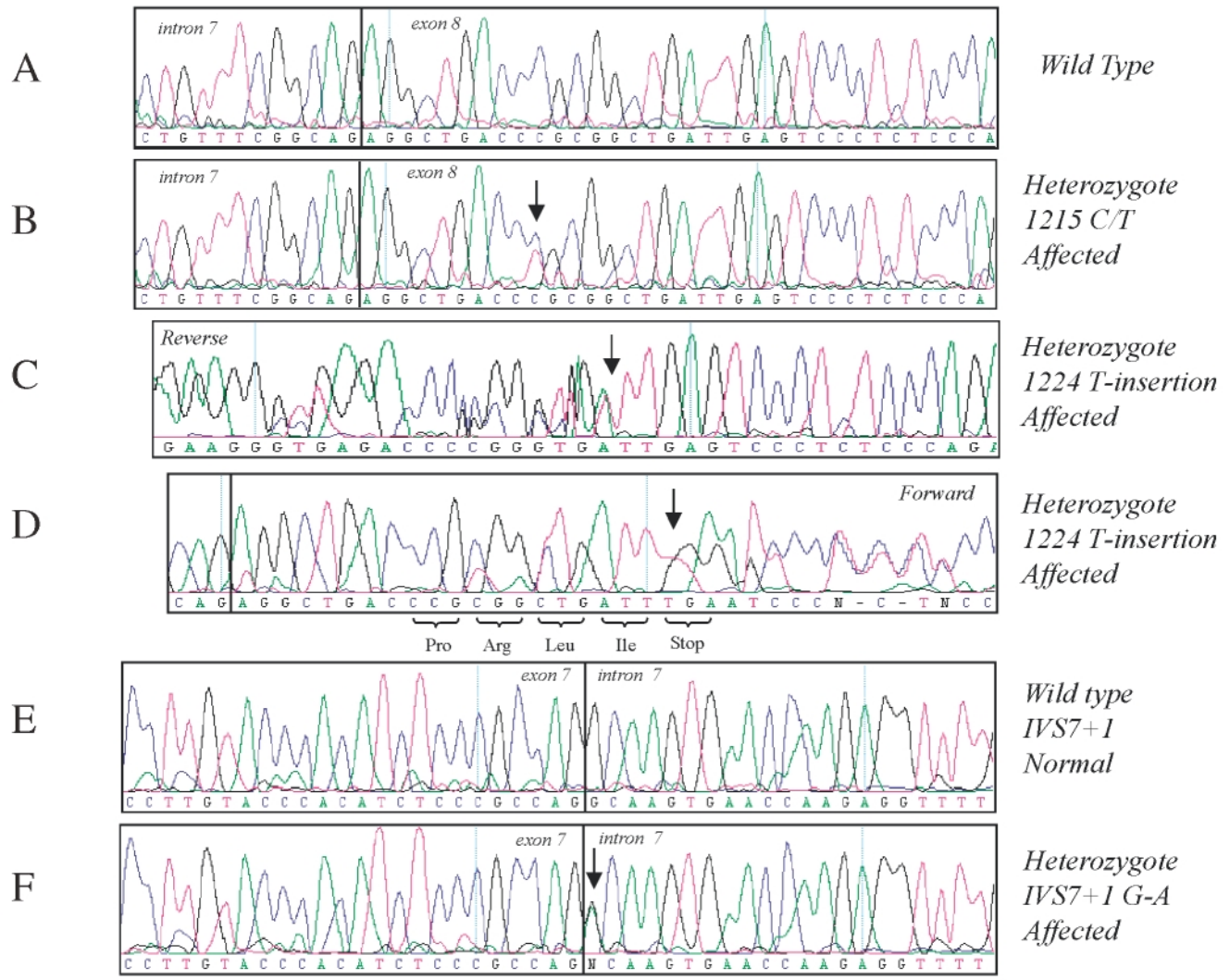
## RESULTS

Mutation analysis of the positional candidate genes *FLT4*, *ADAMTS12*, retinoid X activating protein, *MSX2* and calnexin in affected individuals from 10 families with a high probability (>70%) of linkage to the *PDB3* locus showed no disease-specific mutations (data not shown). Mutation screening of the sequestosome 1 gene showed three different mutations, which segregated with the disease in affected family members. One was a C/T transversion at position +1215 on the cDNA sequence (NM\_003900), resulting in a proline-leucine amino acid substitution at codon 392 (P392L; Fig. 1A and B); the second was a T insertion at position +1224, which introduces a stop codon in place of glutamic acid 396 (E396X; Fig. 1C and D); the third was a G/A mutation affecting the splice donor site (IVS 7+1) at the start of intron 7 (Fig. 1E and F). This mutation would be predicted to disrupt splicing, potentially resulting in the production of a truncated protein of 390 amino acids, terminating at an inframe stop codon at position +6–9 within intron 7 (Fig. 1F). All three of these mutations affect the ubiquitin-associated (UBA) domain of the protein, which is involved in ubiquitin binding (20). No disease-specific mutations were detected in other regions of the *SQSTM1* gene, and no mutations were found in spouses of the affected individuals. In order to assess the frequency of sequestosome 1 mutations in familial PDB, we carried out DNA sequencing of the coding region of exon 8 in the remaining kindreds with familial Paget's disease that were available for analysis. The P392L mutation was found in affected individuals from 13 families (19.1%), the E396X mutation was found in affected individuals from 4 families (5.8%), and the intron 7 mutation was found in 1 family (1.5%), giving a total of 18 families (26.5%) in which PDB was

associated with sequestosome 1 mutations (Table 1). Mutation screening of the same region of exon 8 in 168 unrelated patients with sporadic Paget's disease identified the P392L mutation in 15 individuals (8.9%). This mutation was not found in 160 age- and sex-matched controls ( $\chi^2 = 15.0$ ,  $df = 1$ ,  $P = 0.0001$ ). None of the patients with sporadic PDB or the controls had the E396X mutation or the intron 7 splice site mutation. All three mutations segregated with the disease in affected individuals from each of the families studied. There was one exception, which was in family 51, where only two out of the four affected family members carried the P392L mutation. There were no clear phenotypic differences in terms of disease distribution and age at onset between subjects in family 51 who carried the P392L mutation (individuals 381 and 384; for pedigree diagram, see [http://www.abdn.ac.uk/medicine\\_therapeutics/bone/paget%20pedigrees.hti](http://www.abdn.ac.uk/medicine_therapeutics/bone/paget%20pedigrees.hti)) and those that did not (individuals 383 and 382). The only notable feature in this family was that the father of individual 381 (deceased) had a history of osteosarcoma. Clinical evaluation of other families revealed no obvious differences in disease distribution or age at diagnosis in PDB families with *SQSTM1* mutations as compared with those who did not have *SQSTM1* mutations. We were also unable to discern any significant differences in phenotype between affected individuals with the P392L *SQSTM1* mutation as compared with those who carried other mutations in the *SQSTM1* gene.

## DISCUSSION

PDB is a common bone disease of ageing that is characterized by foci of increased and disorganized bone remodeling predominantly affecting the axial skeleton (2). Whilst many patients are asymptomatic, others present with bone pain, deformity, pathological fracture, deafness, nerve compression syndromes and osteoarthritis (4). Osteosarcoma is a rare complication of PDB (4), but almost all osteosarcomas occurring in adults do so in patients with PDB (21). Genetic factors are important in the pathogenesis of PDB, and many families have been described where the disease segregates as an autosomal dominant trait with incomplete penetrance (5,8,9,22,23). It has been estimated that between 15% and 40% of pagetic individuals have an affected first-degree relative (5–7), but the true prevalence of familial PDB is difficult to ascertain, because the condition is often asymptomatic or presents with non-specific symptoms that are attributed to other causes. Several potential susceptibility loci for familial PDB have been identified on chromosomes 6p21, (*PDB1*) (24), 18q21–22 (*PDB2*) (8,9,11), 5q35 (*PDB3*) (16,17), 5q31 (*PDB4*) (16), 2q36 (*PDB5*) (17), 10p13 (*PDB6*) (17) and 18q23 (*PDB7*) (18). Involvement of the *PDB2* locus seems to be restricted to early-onset PDB-like syndromes, and recent work has indicated that FEO, expansile skeletal hyperphosphatasia and some cases of early-onset familial PDB are allelic disorders, caused by different activating mutations in the signal peptide region of the RANK molecule (12,13). Involvement of the *PDB2* locus and RANK is rare in late-onset familial Paget's disease (16,23,25). In contrast, the *PDB3* locus on 5q35 has been found to be linked to late-onset PDB in families of both French-Canadian (16) and British descent (17), consistent with the presence of a major susceptibility gene in this chromosomal



**Figure 1.** Sequestosome 1 mutations in Paget's disease of bone. (A) Wild-type sequence at the start of exon 8. (B) Heterozygous C/T mutation giving rise to the P392L amino acid substitution. (C) and (D) Heterozygous T insertion mutation at position 1224 sequenced in forward and reverse orientations in the same individual. Note the double tracking of the sequence immediately after the mutation. (E) Wild-type splice donor sequence (GC) at the start of intron 7. (F) Heterozygous G/A mutation at position +1 of intron 7 that disrupts the splice donor site, potentially resulting in the production of a truncated protein, terminated by the stop codon at IVS7+6-8 (underlined). The positions of the mutations are indicated by arrows in each panel.

region. Recently Laurin *et al.* (19) were able to narrow the *PDB3* critical region to a 300 kb interval between D5S2073 and D5S408 in French-Canadian families. This resulted in the identification of a recurrent P392L mutation of *SQSTM1* as the cause of PDB in ~46% of French-Canadian families with PDB, as well as 16% of individuals who apparently had sporadic PDB (19). The findings reported here confirm the importance of *SQSTM1* as an important susceptibility gene for familial and sporadic PDB. We found the same P392L mutation as reported by Laurin *et al.* in 13 of 68 (19.1%) of the families studied and 8.9% of British patients with sporadic Paget's disease. A novel mutation that introduces a premature stop codon at position 396 was also found in a further 4 families (5.8%). A mutation affecting the splice donor site at the start of intron 7 was found in 1 (1.5%) family. Whilst further studies will be required to determine whether this mutation affects splicing, the site of the mutation and its predicted consequences, coupled with the fact

that it segregated with the disease in family members, and was absent from non-pagetic controls, suggests that this was a disease-causing mutation, rather than a benign polymorphism. Thus, *SQSTM1* mutations occurred in 26.5% of cases of familial PDB in this series of Caucasian patients from different geographical regions. We found no evidence to suggest that affected individuals shared an ancestral common haplotype, which is consistent with the view put forward by Laurin *et al.* (19) that this region of the *SQSTM1* gene may be a mutational hotspot.

In all families, the mutations co-segregated with the disease, and were not found in spouses or a large series of non-pagetic controls, which lends support to the hypothesis that the mutations were causal. In one family, however, mutations were found in only 2 of 4 of the affected individuals. This is in keeping with the known genetic heterogeneity of PDB, and presumably reflects the fact that the other affected family

members may have had mutations in a different susceptibility gene. In families carrying *SQSTM1* mutations, disease-associated mutations were also present in younger individuals (age <50 years) who were not thought to have PDB on the basis of biochemical screening. We did not routinely carry out bone scan examinations in these subjects unless they had elevated alkaline phosphatase values, and therefore we cannot exclude the possibility that they may have had localized Paget's disease which was not detected by biochemical screening. Another possibility is that they may not yet have developed the disease, since PDB rarely presents clinically before the age of 55 years.

The mechanisms by which these domain-specific mutations in *SQSTM1* cause PDB remain to be elucidated, but the fact that all the mutations described so far affect the ubiquitin-binding region is likely to be significant. Ubiquitination plays a crucial role in the regulation of signal transduction, by targeting key cellular proteins for degradation through the proteasome (26). Deletion of residues 361–440 completely abolishes the ability of sequestosome 1 to bind to ubiquitin, whereas deletion of residues 392–440 significantly inhibits ubiquitin binding (20). The PDB-causing mutations described in this paper and by Laurin *et al.* (19) all affect this region of the molecule. The functional consequences of the P392L mutation remain to be established, but the intron 7 splice site mutation and the E396X mutation are likely to result in a truncated protein, lacking much of the ubiquitin-binding domain. Since removal of this region inhibits the ability of sequestosome 1 to bind to ubiquitin (20), a possible consequence would be impaired degradation of sequestosome 1 and its accumulation within the cytoplasm. Previous studies have shown that sequestosome 1 facilitates tumour necrosis factor (TNF)-induced NF $\kappa$ B activation by linking the adaptor protein RIP to members of the atypical protein kinase C family (aPKC) (27), and also facilitates interleukin-1 (IL-1)-induced signalling by linking TRAF6 to aPKC (28). Moreover, inhibition of sequestosome 1 expression by antisense RNA has been shown to inhibit NF $\kappa$ B signalling *in vitro* (27,28). It is tempting to speculate that the mutations that have been observed in PDB cause activation of NF $\kappa$ B signalling by increasing levels of sequestosome 1, although further studies will be required to investigate this hypothesis.

Irrespective of the underlying mechanisms, the data presented here show that sequestosome mutations are a common cause of PDB, and provide evidence in support of the hypothesis that PDB (19) and related syndromes such as FEO and expansile skeletal hyperphosphatasia (12,13) may occur as the result of mutational events in different components of the RANK–NF $\kappa$ B signalling pathway.

## MATERIALS AND METHODS

### Family recruitment and disease ascertainment

The families described in this paper comprised 62 of those included in the genome-wide search reported previously (17), plus an additional 6 families with autosomal dominant PDB. Pedigree diagrams are available at the Bone Research Group's website at the University of Aberdeen ([http://www.abdn.ac.uk/medicine\\_therapeutics/bone/paget%20pedigrees.hti](http://www.abdn.ac.uk/medicine_therapeutics/bone/paget%20pedigrees.hti)). We

**Table 2.** Primers for amplification of sequestosome 1

Exon	Primers	Annealing temp (°C)
1F	GGTAGCGGGGAAGGGGAGAGTAG	60
1R	AGAAGGGAGGGGCGCCAC	
2F	CCTCAGCCATTCCAGCAGC	60
2R	CCTCGGCTCCCAAAGTGC	
3F	CCTGCCCTCTGTGGCTCAAG	60
3R	TTAGTAGCGACAAGGTTTCACCATG	
4F	ACAGCCCCACAGTGACGACAGA	60
4R	GGCAGGCAGTGAGCGGGTC	
5F	GGCCTTGCTGGCAGTGACAG	60
5R	GCCCTGGGGGAGGAATTAGC	
6F	TGTAGGGTCTGCGAGGGAAAG	58
6R	CTTGCAGGGTCTGTGTGATTG	
7F	GGAACACAGGGACCTTGGCAAGA	62
7R	TTATCCAACCAATGCCAGCTGA	
8F	TTAGCTGCTTGTGGGACTGAACG	60
8R	CGGGTTGTAAAGTGGGGCTGCA	

screened for the presence of PDB in family members by measurement of serum total alkaline phosphatase (AP). Individuals above the age of 55 years in whom AP values were <65 u/l (reference range 40–105 u/l) were considered as unaffected, whereas those with AP levels above this were considered as diagnosis unclear and were investigated further where possible. These individuals were scored as affected if evidence of PDB was found on radionuclide bone scan examinations and/or skeletal radiographs. Unless PDB had been confirmed radiologically or scintigraphically, individuals under the age of 55 years were considered as 'diagnosis unclear' even in the presence of normal AP levels to take account of fact that PDB does not usually present clinically until the fifth or sixth decade. The patients with sporadic PDB were recruited from UK clinic referrals and had been diagnosed on the basis of typical radiographic and scintigraphic findings. As controls, we used a combination of clinic referrals undergoing investigation of suspected osteoporosis and normal healthy individuals taking part in population-based surveys of osteoporosis risk. All subjects gave informed consent to being included in the studies, which were approved by the Grampian Research Ethics Committee and the Research Ethics Committees of the other contributing centres.

### Mutation analysis of positional candidate genes

We conducted mutation screening of the proximal promoter, coding exons and intron–exon boundaries of candidate genes within the PDB3 locus between D5S2034 and the telomere using automated DNA sequencing, in affected individuals from 10 families where analysis with the HOMOG program (29) had shown a high probability (>70%) of linkage to chromosome 5q35. Primers used in mutation analysis of the sequestosome 1 gene are shown in Table 2. We used 35 cycles of amplification with annealing temperatures as shown in Table 2 using Qiagen *Taq* polymerase according to the manufacturer's instructions. The PCR products were treated with ExoSAP-IT (Amersham)

and sequenced in forward and reverse directions using DYEnamic ET terminator chemistry on a MegaBACE 1000 automated DNA sequencer. Primers used in the mutation screening of FLT4, ADAMTS12, retinoid X activating protein, MSX2 and calnexin are available from the authors on request.

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