Original Article

Occurrence of P392L Mutation in Ethnic Thai with Chinese Descent Paget's Disease

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Background: The etiology of Paget's disease is still unclear. However, the available evidence shows that genetics plays an important role in its pathogenesis. Although the disease is genetically heterogeneous, mutations of *SQSTM1* gene, especially missense mutation P392L, are reportedly the most common genetic polymorphism associated with it. To the best of our knowledge, data regarding mutations of this gene have mainly been obtained from patients of European descent. The authors hypothesized that the P392L mutation might also play a role in Paget's disease among Asian ethnic groups. Unfortunately, due to the rarity of the cases, data regarding mutations among Asian ethnic groups, especially those from Southeast Asian countries are sparse in the literature.

Objective: To find the occurrence of P392L mutation in Paget's disease among ethnic Thai group.

Materials and Methods: A genetic analysis was carried out using archived material from four ethnic Thai patients with Paget's disease. DNA was extracted from formalin-fixed, paraffin embedded tissue for specific PCR targeting of 326 bp of the *SQSTM1* gene (exon 8). Amplicons were sequenced bidirectionally using an ABI PRISM 3730XL (Applied Biosystems, USA) sequencer using T7 promoter and SP6 primers.

Results: No P393L mutations in the SQSTM1 protein (exon 8) were found in any of these four cases.

Conclusion: These results were similar to those obtained from previous studies on patients of Chinese descent. A combined analysis of the present data and that from 21 previously-reported, sporadic cases of Paget's disease in Asian patients revealed absence of the P392L mutation but presence of one different mutation (1/25 = 4%).

Keywords: Sequestosome 1, Paget's disease, SQSTM1, Ethnic Thai, P392L, Chinese

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Paget's disease of bone [PDB] is common in some European countries, especially in patients of British descent, and affects approximately 3% of whites over 55 years of age(1). It is extremely rare in Asia, especially in Southeast Asian countries, where most of the population is of Chinese descent(2,3). The etiology of the disease is still unclear. However, it appears that genetic factors play a role in pathogenesis of the disease(4). Among the identified genetic loci reported to influence susceptibility to PDB is exon 8 (PDB3) of the gene sequestosome 1 [*SQSTM1*] that is located on chromosome 5q35-qter and codes for the protein SQSTM1 (also called P62). It is the most common and convincing locus associated with PDB, and P392L appears to be the most common associated mutation in this gene(5-7). Since PDB is fairly common in western countries, most data regarding molecular studies have been obtained from patients of European ethnic descent. Although PDB is quite rare in other ethnic groups, the authors hypothesized that it might also be associated with the P392L mutation in exon 8 (PDB3) of the gene *SQSTM1*. Here, the authors described the results of testing this hypothesis using archived samples from Thai PDB patients.

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Materials and Methods

Tissue samples

The present study protocol was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (Protocol number: ID 03-54-48). Demographic data of the cases was shown in Table 1. All of our cases were of Chinese descent and had no familial history of Paget's disease. The clinical and radiological diagnosis of Paget's disease of all the cases were confirmed by histopathological examination. Bone specimens were

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fixed in 10% buffered formalin and decalcified in 10% EDTA. Formalin fixed paraffin-embedded tissue [FFPE] of all cases were cut as 5 micron-thick sections and stained with haematoxylin and eosin [H&E]. The sections were reviewed and the blocks that contained characteristic lesions were selected by a specialized musculoskeletal pathologist. Five sections of 5-micron-thick scrolls from the selected paraffin blocks were then used for DNA extraction using QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA).

Sequence analysis of SQSTM 1

A 326 bp fragment of exon 8 of the *SQSTM1* gene was amplified by polymerase chain reaction [PCR] using Top Tag Master Mix kit (Qiagen,Valencia, CA, USA) and the following specific, forward and reverse PCR primers: forward primer 5'-CAC TCC TCA TGG CTT CCT TAC TG-3', reverse primer 5'-ATG GCT TCT TGC ACC CTA ACC-3'. Thermal cycling conditions involved an initial denaturation step for five minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 60 seconds at 72°C, with a final extension step at 72°C for 10 minutes. PCR products were electrophoresed on an agarose gel to ensure that the correct sizes of amplicons were obtained. Amplicons were then cut from the gel and purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The purified amplicon was subcloned into pPrime cloning vector (5 PRIME, Gaithersburg, USA) according to the manufacture's protocol. Colony PCR was used for positive colony screening and the same specific primers and thermo cycling conditions as above were used. The positive colonies were then cultured in 5 ml LB with ampicillin for 16 to 18 hours at 37°C. Plasmids were prepared by QIAGEN Plasmid Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturers protocol. The sequences were analyzed by ABI PRISM 3730XL (Applied Biosystem, USA) bidirectionally using T7 promoter and SP6 primer. The sequence of the wild type *SQSTM1* gene was obtained from GenBank (NM003900).

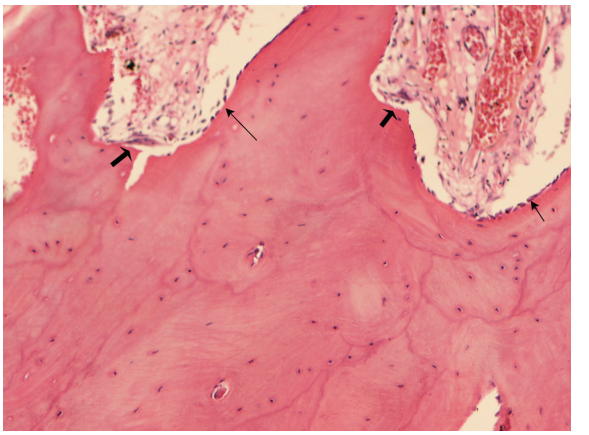

Figure 1. Photomicrograph showing the mosaic pattern of the bone matrix with abnormal osteoblastic (thin arrows) and osteoclastic (thick arrows) activities. H&E stained section at original magnification x200.

Results

Clinical information of the cases were summarized in Table 1. There were four cases, three males and one female. The range of ages was 35 to 64 years with a mean age of 44 years. All of the cases were asymptomatic and presented elevated alkaline phosphatase levels whereas serum electrolytes, especially calcium and phosphate, were within normal ranges. The X-rays findings of each case revealed multiple foci of either osteolytic or osteoblastic lesions or mixed types. The common lesion sites were pelvic bones and spines. The histologic findings of all cases exhibited typical features of PDB, showing mosaic patterns of the bone matrix and pronounced osteoclastic and osteoblastic activities (Figure 1). The SQSTM1 sequencing carried out showed that two out of four cases (cases 1 & 4) exhibited sequences similar to the wild type (Figure 2). The other two cases (cases 2 and 3) showed mutations in nucleotides but no changes in the corresponding,

Table 1. Clinical data of cases

Case No.	Age/gender	Ethnic/descent	Type of lesion	Presentation	Distribution of lesions
1	37 years/male	Thai/Chinese	Multiple foci	- Asymptomatic - Elevation of alkaline phosphatase	Skull, right scapula, whole spine, and bilateral pelvic bones
2	40 years/male	Thai/Chinese	Multiple foci	- Asymptomatic - Elevation of alkaline phosphatase	Right supraorbital ridge, left and right scapula, first rib, spine L4, left pubic rami, and right acetabulum
3	35 years/female	Thai/Chinese	Multiple foci	- Asymptomatic - Elevation of alkaline phosphatase	Pelvis, thoraco-sacral spine, and lumbosacral spine
4	64 years/ male	Thai/Chinese	Multiple foci	- Asymptomatic - Elevation of alkaline phosphatase	Right pelvic bone and spine

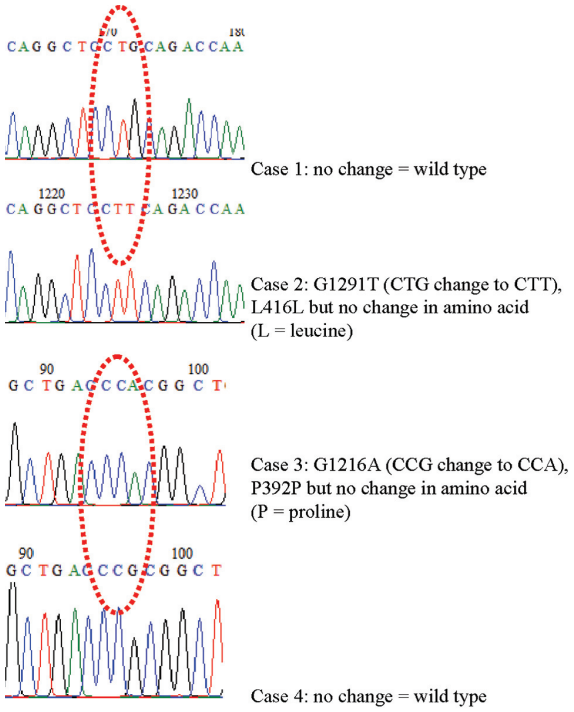

Figure 2. Sequencing analysis revealed mutations in nucleotides of 2/4 cases.

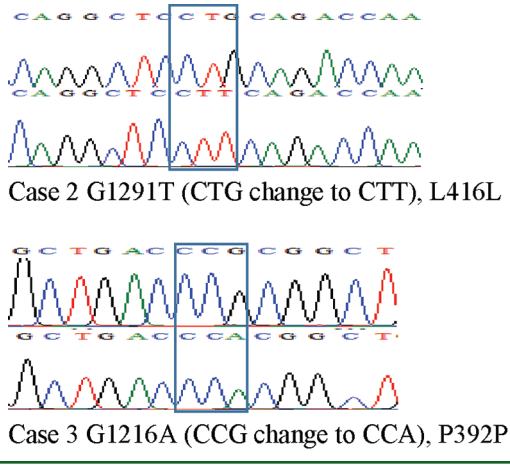

Figure 3. Silent mutation of case 2 and 3.

encoded amino acids. For example, case 1 showed substitution of a nucleotide at position 1291 from G to T (G1291T) but no change in the encoded amino acid Leucine. In case 4, there was change at position 1216 from G to A (G1216A) but no change in the encoded amino acid Proline (Figure 3).

Discussion

Paget's disease is not common in Thailand, and all of the reported cases in the literature were of ethnic Thai with Chinese descent. None had a known familial history of PDB, implying sporadic mutation[2,3]. It is known that the genetic etiology of this disease is considerably heterogeneous and may vary amongst different ethnic groups. However, the rare occurrence of cases in our region is a constraint for identifying the diverse susceptible chromosomal loci. Therefore, the authors focused on P392L in *SQSTM1* gene (exon 8) that is the most commonly cited focus for mutations associated with this disease[5-7]. Our study may be different from others in that we performed tests using biopsied specimens that we believed would provide more advantage than blood samples employed in previous studies, since genuinely affected tissue could be selected for genetic analysis. It is generally accepted that Paget's lesions are focal and it is still unclear as to whether PDB is related to somatic or germ-line mutations[8,9]. The notion of somatic mutations is supported by other studies showing that PBD gene mutations are found only in lesion tissue and not in unaffected tissue[9]. The *SQSTM1* gene encodes P62 protein and possesses 8 coding exons and 6 domains namely 1) a Phox and Bem1 [PB1], ZZ type zinc finger, 2) an SOD1 mutant interaction region [SMIR], 3) a TRAF6 binding [TB] motif, 4) a microtubule-associated protein 1 light chain 3 B [LC3], 5) an interaction region [LIR], and 6) a ubiquitin association [UBA] region[5]. P62 protein plays roles in many biological functions including 1) proteosomal degradation of proteins, 2) function as a scaffold protein in the RANKL, IL1, nerve growth factor, TNF-induced NF-B signaling pathways, 3) autophagy, and 4) apoptosis[5]. Mutations of SQSTM1 occur in 40% to 50% of Caucasian patients with familial PDB and in 8% to 20% of patients who do not have a family history of PDB. Approximately 89% of these were miss-sense mutations in P393L or a truncating mutation E396X[5]. Using genome wide searches, several human chromosomal loci were reported to increase susceptibility to the disease, including the PDB2 locus on chromosome 18q21, the PDB3 locus on chromosome 5q35, the PDB4 locus on chromosome 5q31, the PDB5 locus on chromosome 2p36, the PDB6 locus on chromosome 10p13, and the PDB7 locus on chromosome 18q23[4]. Among various mutations, almost 90% were found in domain UBA (amino acid position 394 to 440) in exon 8 of the *SQSTM1* gene[4,6,10]. As seen in our results, two of our four cases showed silent mutations in this region, signal 1216 G to A (resulting in P392P) and G1291T (CTG change to CTT) resulting in L416L. However, those

mutations did not change the encoded amino acids in the deduced protein. In other words, the authors did not find any missense mutations in exon 8, as has been found in most previous studies[10]. Similar to our study, Sankaran et al[11] found no mutation of Exon 8 of the *SQSTM1* gene from 8 Asian ethnic immigrants tested in New Zealand. These patients were predominately of south Asian origin and two of them were Chinese. Gu et al[12] found only one mis-sense mutation in this gene among 13 Paget's disease cases in mainland China. This was an M404T T-to-C transversion at position 1250 in exon 8 (1250 T>C) resulting in a methionine (ATG) to threonine (ACG) substitution at codon 404. The diversity of mutations in SQSTM1 varies from country to country, as has been previously reported, and haplotype analysis also indicates that the ubiquitous P392L mutation is probably an ancestral mutation widely distributed through populations of western European origin[13,14]. It was interesting that our four cases and all previously reported 21 cases of Asian ethnic groups with demographic and clinical data shown in Table 2 showed absence of P392L mutations[11,12]. In addition, there were reports showing other mutations, namely mutations (78dup27) in exon 1 of the *TNFRSF11A* gene encoding the receptor activator of nuclear factor kappa B [RANK] among Chinese and Japanese patients[15,16]. The authors realized that the sample of four cases, inevitably limited by the rarity of cases in our region, was too small to deduce the role of P392L in the pathogenesis of Paget's disease in ethnic Asian groups and still requires accumulation of data from other sources to establish any role for this mutation. The authors believe that the dissemination of the present study may stimulate interest in other centers and lead to accumulation of more data for assessing the possible role P392L mutations in PDB in ethnic Asians.

What is already known on this topic?

This P392L most likely plays a role in ethnic

Table 2. Demographic and clinical data of Asian descent cases tested for SQSTM1 gene mutations

Case No.	Age/gender	Ethnic	Descent	Clinical presentations	Level of ALK	Distribution of lesions	Reference
1	69 years/female	Chinese	Chinese	Enlarged skull, blurred vision	Not test	Skull	12
2	76 years/male	Chinese	Chinese	Bone pain, elevation of ALK	Not test	Pelvis	12
3	54 years/male	Chinese	Chinese	Bone pain, elevation of ALK	Elevate	Left tibia	12
4	67 years/male	Chinese	Chinese	Bone pain, elevation of ALK	Elevate	Pelvis	12
5	53 years/male	Chinese	Chinese	Bone pain, elevation of ALK	Elevate	Pelvis	12
6	51 years/male	Chinese	Chinese	Bone pain, elevation of ALK	Elevate	Spine, pelvis, left femur, left humerus	12
7	53 years/female	Chinese	Chinese	Bone pain	Elevate	Pelvis	12
8	36 years/male	Chinese	Chinese	Bone pain	Elevate	Right femur	12
9	61 years/female	Chinese	Chinese	Bone pain	Elevate	Right tibia	12
10	81 years/male	Chinese	Chinese	Bone pain	Elevate	Left tibia	12
11	78 years/female	Chinese	Chinese	Bone pain	Elevate	Bilateral tibia	12
12	85 years/female	Chinese	Chinese	Bone pain, deafness	Elevate	Bilateral femur, bilateral radius, scapula, spine	12
13	58 years/female	Chinese	Chinese	Bone pain	Elevate	Skull, pelvis	12
14	55 years/female	Sri Lanka	Sri Lanka	Asymptomatic	Elevate	3 bones (b)	11
15	61 years/male	Fijians	Indian	Asymptomatic	Elevate	2 bones (b)	11
16	48 years/male	Fijians	Indian	Yes (a)	Normal	1 bone (b)	11
17	67 years/male	Fijians	Indian	Yes (a)	Elevate	7 bones (b)	11
18	65 years/male	Chinese	Chinese	Yes (a)	Elevate	2 bones (b)	11
19	70 years/male	Indian	Indian	Asymptomatic	Normal	2 bones (b)	11
20	73 years/male	Fijians	Indian	Yes (a)	Elevate	1 bone (b)	11
21	(c)	(c)	(c)	(c)	(c)	(c)	11

ALK = alkaline phosphatase

(a) No type of symptoms described

(b) No specific locations mentioned

(c) One case was missed to specify in the reference No. 11

European patients. However, to our best knowledge, there is no report regarding this mutation found in Asian patients especially in Southeast Asian patients.

What this study adds?

The authors did not find this P392L in any of the study cases, which is different from that found in European groups. However, the authors may not be able to conclude that this mutation does not play role in the disease for Asian patients since the number of studied cases was small, which is inevitable due to the rare incidence of cases in our region. However, our data may stimulate interest in other centers and lead to accumulation of more data for assessing the possible role P392L mutations in PDB in ethnic Asians.

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Potential conflicts of interest

The authors declare no conflict of interest.

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