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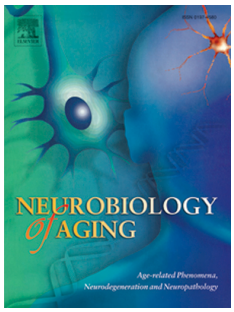

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ABSTRACT

Paget's disease of bone (PDB) is a focal bone disorder affecting the skeleton segmentally. A strong genetic component has been shown in PDB, and variants in several genes, as *SQSTM1, VCP,* and *OPTN,* have been associated with the disease. Mutations in the same genes have also been reported in patients with frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). Hexanucleotide repeat expansions in the *C9ORF72* gene have been shown to be responsible for both familial and sporadic FTD/ALS. Thence, we evaluated the frequency of the *C9ORF72* hexanucleotide repeat expansions in a cohort of 191 Italian PDB patients and in 106 controls. The pathogenic repeat expansion was detected in 2 PDB patients (1.0%). During the follow-up period, both PDB patients did not develop any sign of mental decline and/or motor neuron disease. Our study suggests that repeat expansions in the *C9ORF72* gene are rare in patients with Paget's disease of bone.

Keywords: Paget's disease of bone, C9ORF72, hexanucleotide repeat expansions, SQSTM1

Introduction

Paget's disease of bone (PDB) (OMIM 602080) is the second most common skeletal disorder after osteoporosis. PDB generally affects approximately 1% of adults in Western Europe, increasing significantly with age to affect about 5% of people aged 85 years. The disease is characterized by focal areas of abnormal bone metabolism, due to increased activity of both osteoclasts and osteoblasts, resulting in formation of disorganized bone (Galson et al., 2014). Additional abnormalities include bone marrow fibrosis and increased vascularity of affected bones. The most commonly affected bones are the pelvis, the spine, the femora and the skull but any bone of the skeleton may be affected. Thence, the disease may be diagnosed incidentally by skeletal radiographs or by biochemical finding of an unexplained elevation of serum alkaline phosphatase. About one third of PDB patients have only one affected bone and are asymptomatic. The commonest symptoms are pain, due to pagetic lesions in bone itself or caused by pathological fractures, bone enlargement, deformity, and deafness.

The etiology of PDB has remained largely unknown for several decades. Recent studies suggested that the disease is due to a complex interaction between several genetic and environmental risk factors. Epidemiologic studies have indicated a strong genetic component in PDB: 15 to 40% of patients have a familial form of the disease, which is transmitted in an autosomal-dominant model of inheritance with incomplete penetrance (Ralston et al., 2014). To date, causative PDB mutations have been identified mainly in the *SQSTM1* gene, coding for the p62 protein, with a frequency of 25–50% in familial and 5–10% in sporadic patients (Laurin et al., 2002). In addition, mutations in the *VCP* gene have been associated with a complex phenotype that comprises inclusion body myopathy, Paget's disease of bone and Frontotemporal dementia (IBMPFD) (Weihl et al., 2009). Finally, genetic

variants in several genes, including *CSF1*, *TNFRSF11A*, *TNFRSF11B*, and *OPTN*, have also been implicated in the pathogenesis of the disease.

In the last few years, mutations in some of the genes involved in PDB have also been described in patients with frontotemporal dementia (FTD) and/or amyotrophic lateral sclerosis (ALS), suggesting the presence of overlapping pathogenetic mechanisms. *SQSTM1* gene mutations have been found in approximately 2.5% of patients with ALS and 3% of patients with FTD (Rainero et al., 2017). An UK kindred segregating the P392L mutation in the *SQSTM1* gene showed the coexistence of both ALS and PDB phenotypes (Kwok et al., 2014). Finally, mutations in the *OPTN* gene were also recently reported in patients with ALS. Taken together, these data suggest that genes involved in the ALS/FTD spectrum might be investigated as candidate genes also in PDB patients (Rea et al., 2014).

Few years ago, a pathogenic expansion of hexanucleotide repeats in the *C9ORF72* gene was identified as a major cause of familial FTD and ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The mechanisms through which the *C9ORF72* expansion contributes to these disorders include both loss and gain-of-function mechanisms, driven by toxic RNA. These mechanisms have been linked to both defective nucleocytoplasmic trafficking and nuclear stress (Zhang et al., 2016). Interestingly, in a family with FTD and concomitant PDB, the co-occurrence of the P392L mutation in *SQSTM1* and the *C9ORF72* pathological expansions was recently reported (Almeida et al., 2016). Therefore, the aim of this study was to evaluate the frequency of *C9ORF72* expansion in a cohort of Italian patients with Paget's disease of bone and to evaluate the correlation with clinical phenotype.

Material and methods

One hundred ninety one unrelated patients with Paget disease of bone (103 men, 88 women; mean age± SD = 68.6± 12.8 years), attending the Unit of Geriatrics and Metabolic Bone Disorders,

Department of Internal Medicine, University of Torino, were recruited in the study. The diagnosis of PDB was made according to the established criteria, based both on alkaline phosphatase levels and bone radiologic/scintigraphic examinations. A positive family history, defined as at least one first-degree relative having PDB, was recorded in 22% of PDB patients. The age of onset was 64.2± 9.5 years in the female cohort, and of 59.8± 10.1 in the male subgroup. About 50% patients fulfilled the diagnostic criteria for polyostotic disease. Phenotypic data of PDB patients were summarized in Table 1. A group of 106 healthy subjects (56 men, 50 women; mean age± SD= 70.9± 9.2 years), served as controls. Patients and controls were of Caucasian origin and came from the same area of Northern Italy. Written informed consent was obtained from all participants, and the study was approved by Hospital Ethics Committee.

The genomic DNA was purified from whole blood leukocytes using a DNA extraction kit (QIAGEN, Germany). The presence of a pathological hexanucleotide expansion GGGGCC in the *C9ORF72* gene was evaluated using repeated primed PCR, as previously described (Xi et al., 2014), and a characteristic amplification pattern on the electropherogram was considered evidence of a pathogenic repeat expansion. Furthermore, PCR reagents were optimized for the amplification of the *C9ORF72* hexanucleotide repeats (AmplideX®PCR/CE C9ORF72 Kit, Asuragen, Inc.). The genomic DNA was amplified using a three-primer G4C2-Repeat Primed (RP)-PCR configuration, followed by fragment sizing on a 3100 Genetic Analyzer (Thermo Fisher). ROX 1000 was used for sizing by capillary electrophoresis and the size of the PCR products were converted to the number of G4C2 repeats using size and mobility conversion factor with Gene Mapper v 4.1 software (Thermo Fisher). In addition, the presence of mutations in *SQSTM1* or in other genes known to be associated with PDB-related syndromes was investigated. The statistical analyses were performed using SPSS software version 21.0.

Results

The complete analysis of the *C9ORF72* gene was conducted on a total of 297 subjects. The pathogenic *C9ORF72* expansion was detected in two out of 191 patients with Paget's disease of bone, with a frequency of 1.0%. Electropherograms of heterozygous samples with expanded alleles were shown in Fig. 1 in the Supplemental Material. No *C9ORF72* expansion was observed in the control group. The first subject carrying the *C9ORF72* expansion did not carry any other mutations in known PDB genes, while the second patient also presented the Y383X mutation (c.1149 C>A) in exon 7 of *SQSTM1* gene.

The two patients had a long-lasting diagnosis of sporadic PDB. The first patient is a male that, at age 70, developed a polyostotic disorder. Total body bone scintigraphy showed skull, vertebral and pelvic involvement. During the clinical follow-up, no clinical signs of motor neuron disease were observed, and the patient died at 83 years for myocardial infarction, without showing any sign of mental decline.

The second patient carrying both the *C9ORF72* expansion and the mutation in *SQSTM1*, is a 77-years female patient, with an age at onset of PDB at 62 years, who presented with polyostotic disorder (left scapula and pelvis). In 2008, the patient has been treated with 5-mg dose of intravenous zoledronate once a year with a clinical positive response. In 2018, serum bone alkaline phosphatase was 5.1 mcg/l, parathyroid hormone was 77 pg/ml, and urinary calcium was in normal range. A positive family history for Alzheimer's disease was reported, with a deceased brother with an age at onset of dementia at 64 years (no genetic investigations are available). At clinical examination, no signs of motor neuron disease were detected. Mini Mental State Examination was 28/30, and an extended neuropsychological examination did not show any sign of cognitive impairment.

Discussion

In this study we analyzed the presence of *C9ORF72* expansions in patients with Paget's disease of bone, and we found pathologic expansions with a frequency of approximately 1% of our dataset of PDB patients. Our study does not support a major role for *C9ORF72* gene in PDB, but suggests that this gene could be involved in the pathogenesis of Paget's disease of bone. In addition, our study confirmed the co-occurrence of *SQSTM1* and *C9ORF72* gene mutations in a patient with PDB.

This is the first study that investigated the prevalence of *C9ORF72* expansions in Paget's disease of bone, and we recognize a few limitations. The major limitation of our study is the low number of genotyped patients; therefore, the study could be not sufficiently powered. In addition, we did not have the possibility to carry out a Southern blot to quantify the length of the expansion, but we used a novel methodology that allowed us to quantify the *C9ORF72* expansion in the two PDB patients as >145 repeats.

Paget's disease of bone is characterized by abnormal bone remodeling, leading to osteolytic lesions, bone deformities, and pathologic fractures. An initial surge of osteoclastic activity leads to focalized resorption of bone, followed shortly thereafter by osteoblast hyperactivity. There are several evidences suggesting that the development of PDB may be related to a deregulation of autophagy (Shapiro et al., 2014). This process plays an essential role in homeostasis by providing energy and recycling cellular components, but also by facilitating lysosomal degradation, and aggregates of misfolded proteins. Osteoclasts from PDB patients show both nuclear and cytoplasmic inclusions that have been associated with protein aggregates, increasing the evidences of a possible deregulation of autophagy in the development of the disease. Both *SQSTM1* and *VCP* are involved in autophagy and in forming protein aggregates, indicating that a disturbance of these processes might be a risk factor for the pathogenesis of PDB. Recent experimental and clinical studies provided evidence that also the

C9ORF72 gene plays a critical role in autophagy (Todd et al., 2016). In addition, polymorphisms in autophagy genes have been found to be associated with Paget's disease of bone (Usategui-Martin et al., 2015). Intriguingly, in an experimental model, a decreased expression of *C9ORF72*, through an inhibition of autophagy, leads to accumulation of cytoplasmic aggregates of p62/SQSTM1 (Webster et al., 2016). At present, however, no data on the possible role of *C9ORF72* gene in bone metabolism are available.

In this study, we confirmed that mutations in *SQSTM1* gene and *C9ORF72* expansions may co-occur in patients with PDB, as previously described (Almeida et al., 2016). Therefore, it is possible that both genes may converge into a common pathogenetic mechanism. Mutations in the *SQSTM1* gene combined with a *C9ORF72* expansion could act in an additive manner to predict the extent and severity of PDB. However, owing to the limited number of double mutation carriers described, we cannot establish if the co-occurrence of mutations influences the clinical expression of PDB. Moreover, age at onset was not clearly different from patients carrying only one of the mutations. Finally, we did not find any *C9ORF72* expansion in our control group. In literature, in an extensive study investigating *C9ORF72* expansions in several neurodegenerative diseases, the authors detected an expansion of 0.15% in their control population (Beck et al., 2013). However, control samples involved in *C9ORF72* studies were mainly screened for neurological disorders. It could be of interest to review the clinical characteristics of positive *C9ORF72* control subjects, in order to exclude also a diagnosis of Paget's disease of bone.

In the present study, we identified a small number of *C9ORF72* expansions in a cohort of Italian PDB patients. Even if our findings do not support a major role for this gene in PDB pathogenesis, additional clinical and experimental studies are warranted to further elucidate the involvement of *C9ORF72* gene in Paget's disease of bone.

Disclosure statement

All authors state that they have no conflict regarding this study.

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Table 1. Clinical characteristics of patients with Paget's disease of bone.

	Males	**Females**
Number	103	88
Age (years ± SD)	66.9 ± 9.9	70.9 ± 9.2
Age at onset (years ± SD)	59.8 ± 10.1	64.2 ± 9.5
Familiarity for PDB (%)	18.8%	21.2%
Patients with polyostotic disease (%)	53.4%	49.4%
Patients with bone pain (%)	43.9%	63.3%
Number of bone localizations (mean ± SD)	2.2 ± 2.6	2.3 ± 2.7
Patients with arthrosis (%)	32.8%	38.6%
Patients with bone fractures (%)	31.2%	38.6%
Patients with bone deformations (%)	21.2%	25.6%
Patients with hearing loss (%)	25.6%	22.6%
Patients with skull involvement (%)	3.7%	4.2%
Patients with nephrolithiasis (%)	18.9%	26.3%

Highlights

 We evaluated for the first time the frequency of the *C9ORF72* hexanucleotide repeat expansions in a cohort of Italian PDB patients and in healthy controls.

 The pathogenic repeat expansion was detected in 2 out of 191 PDB patients (1.0%). One of the two patients showed the coexistence of both *C9ORF72* expansion and *SQSTM1* gene mutation.

 Our study does not support a major role for *C9ORF72* gene in PDB, but suggests that this gene could be involved in the pathogenesis of Paget's disease of bone.