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Development of a molecular test of Paget's disease of bone

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ABSTRACT

Depending on populations, 15 to 40% of patients have a familial form of Paget's disease of bone (PDB), which is transmitted in an autosomal-dominant mode of inheritance with incomplete penetrance. To date, only *SQSTM1* gene mutations have been linked to the disease. Several single nucleotide polymorphisms (SNPs) have been associated with PDB in patient non-carriers of *SQSTM1* mutations, but they have minor size effects. The current clinical practice guidelines still recommend to measure total serum alkaline phosphatase (sALP) for PDB screening. However, genetic or bone biomarkers alone may lack sensitivity to detect PDB. Thus, the objective of this study was to develop a molecular test of PDB, combining genetic and bone biomarkers, in order to detect PDB, which is frequently asymptomatic. We genotyped 35 SNPs previously associated with PDB in 305 patients, and 292 healthy controls. In addition, serum levels of 14 bone biomarkers were assayed in 51 patients and 151 healthy controls. Bivariate and multivariate logistic regression models with adjustment for age and sex were fitted to search for a combination of SNPs and/or bone biomarkers that could best detect PDB in patient non-carriers of *SQSTM1* mutations. First, a combination of five genetic markers gave rise to the highest area under the ROC curve (AUC) with 95% confidence interval [95% CI] of 0.731 [0.688; 0.773], which allowed us to detect 81.5% of patients with PDB. Second, a combination of two bone biomarkers had an AUC of 0.822 [0.726; 0.918], and was present in 81.5% of patients with PDB. Then, the combination of the five genetic markers and the two bone biomarkers increased the AUC up to 0.892 [0.833; 0.951], and detected 88.5% of patients with PDB. These results suggested that an algorithm integrating first a screen for *SQSTM1* gene mutations, followed by either a genetic markers combination or a combined genetic and biochemical markers test in patients non-carrier of any *SQSTM1* mutation, may detect the PDB phenotype better than biomarkers already available in the clinical practice.

1. Introduction

Paget's disease of bone (PDB) is characterized by focal abnormal bone remodeling, with increased bone resorption coupled with an increased and disorganized new bone formation, resulting in abnormal bone architecture and weakened bone strength. This disease affects up to 3% of Caucasians over 55 years of age, which makes it the second most frequent metabolic bone disorder after osteoporosis ([20]). In most cases, patients with PDB are asymptomatic. However, 10 to 30% of patients will develop symptoms and complications, such as bone pain, bone deformities, fractures, deafness, or nerve root compression. The development of an osteosarcoma is the most severe complication, and occurs in less than 1% of patients with PDB ([28]).

PDB has a strong genetic component. Depending on populations, 15 to 40% of patients have a familial form of the disease, which is transmitted in an autosomal dominant mode of inheritance with incomplete penetrance ([28]). Although genetic heterogeneity has been demonstrated in familial forms of PDB, only the *Sequestosome 1* (*SQSTM1*) gene at the *5q35* locus has been linked to PDB, with nearly 30 disease-causing mutations identified so far [19,26]. Overall, these mutations in *SQSTM1* gene are present in about 35% of familial forms of PDB, and 7% of unrelated patients [14]. Two genome wide association studies (GWAS) performed in PDB-affected patients without *SQSTM1* mutations identified seven loci associated with PDB [2,4]. These associations were then replicated in other

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populations, including the French–Canadian population [5,8,10,21,22]. In this population, some rare variants were also associated with PDB in patient non-carriers of *SQSTM1* mutations [5,6]. Environmental factors were reported to play an important role in PDB pathogenesis. Although controversial in the literature, viral infection would contribute to the development of the disease, as osteoclasts expressing the gene encoding for the measles virus nucleocapsid protein (MVNP) develop a complete pagetic phenotype, both in cellular and animal models [17].

Currently, a decline in the prevalence and severity of PDB is observed in many countries previously known to have a high prevalence of PDB [11,12]. This may increase the proportion of affected individuals who remain asymptomatic, especially in familial forms. Given the high risk of developing an osteosarcoma on pagetic bone, this disease is a formal contraindication to the prescription of bone anabolic agents, such as teriparatide. Indeed, the excessive stimulation of osteoblasts in individuals affected but asymptomatic or predisposed to PDB may increase the risk of developing an osteosarcoma or more likely give rise to the occurrence of a symptomatic PDB, representing serious adverse events of these drugs. With the incoming introduction of anabolic agents targeting natural inhibitors of osteoblasts indicated for osteoporosis treatment, it will be crucial to exclude accurately the presence of PDB, including in asymptomatic individuals. The current clinical practice guidelines still recommend to measure total serum alkaline phosphatase (sALP) for PDB screening [29]. However, total sALP levels may be within the normal range, especially in patients with monostotic or metabolically inactive disease, and this should not exclude a diagnosis of PDB ([25]). Since genetic or bone biomarkers alone may lack sensitivity to predict the clinical diagnosis of PDB, we hypothesized that the combination of both kind of markers would be better. Thus, the objective of this study was to develop a molecular test of PDB, including a combination of genetic and bone biomarkers, in order to detect PDB.

2. Materials and methods

2.1. Individuals

The present study was approved by the CHU de Québec Ethics Committee and all participants, affected or not, signed a consent form before entering the study. A complete bone evaluation, including total sALP measurement, skull and pelvis X-rays and a whole-body bone scan, was performed for each patient. The criteria used to diagnose PDB were: 1) an increase in total sALP level and/or 2) a typical aspect of PDB on the bone X-rays and/or 3) an abnormal whole-body bone scan, as previously reported [18]. Patients with PDB originated from two different countries: Canada (French–Canadian from a 120 km area around Quebec City) and France (Angers, Paris, Saint-Etienne areas). They had either a familial form of PDB, with only one affected per family being included in this study, or they were unrelated affected individuals. Clinical characteristics, including sex, family history of PDB, total sALP levels (expressed as the number of times the midpoint of normal range, in order to normalize results between patients), the age at PDB diagnosis, the number of bone sites affected by PDB and the skeletal extension calculated by the Reniers' index, were collected for each patient [27]. General characteristics of patient cohorts and controls are provided in Supplementary Table 1. Briefly, in the French–Canadian patients, the mean age at inclusion was 69.2 ± 9.6 years, 57.1% were male, and 42.7% had a monostotic disease [19,24]. In the French population, the mean age of PDB patients at inclusion was 62.7 ± 13.9 years, 50.0% were male, and 22.2% had a monostotic disease [21]. An affected-only cohort of validation included PDB patients from United States (New York city area). In this population, 44.3% were male, and 54.3% had a monostotic disease [23]. Controls were healthy individuals from the French–Canadian population living in an area of 120 km around Quebec City without any personal or familial history of PDB based on a questionnaire, and with normal total sALP levels at inclusion. Bone scans were not done in this population. The mean age of these healthy individuals at inclusion was 64.8 ± 10.9 years, and 28.8% were male. For each participant, DNA from peripheral blood mononuclear cells (PBMCs) was extracted according to standard procedures.

2.2. SNP selection and genotyping

All study participants were previously screened for germinal mutations in exons 7 and 8 of the *SQSTM1* gene, and 47 patients with PDB were carriers of a germinal *SQSTM1* mutation: all patients with a mutation were carriers of the *SQSTM1/P392L* germinal mutation, except for two French individuals; one was carrier of *SQSTM1/A390X* mutation, and the other of both *SQSTM1/P392L* and *SQSTM1/A390X* mutations. In addition, 15 participants were carriers of the *SQSTM1/P392L* post-zygotic but not germinal mutation [15]. Thirty-five SNPs previously shown to be associated with PDB in the literature were genotyped (Table 1) ([2,4–10,22]. Genotyping of SNPs relied on two different methods: Sanger sequencing or Sequenom MassARRAY SNP Multiplex Technology. The SNPs genotyped by the Sanger sequencing method were first amplified using a polymerase chain reaction (PCR). Amplification products were purified and sequenced with Big Dye Deoxy Terminator v 3.1 Cycle (Applied Biosystems) on an ABI 3730xl sequencer, and the DNA sequences obtained were analyzed with Staden package version 1.6 ([30]). For the Sequenom MassARRAY SNP Multiplex Technology, the purified DNA solution containing multiplexed primer-based extension reaction (iPLEX reaction) products was dispensed from the 384-wells

Table 1
Single nucleotide polymorphisms (SNPs) genotypes available for this study.

Loci	Physical position	SNP number	Gene
	110,201,580	Rs650985	*GSTM4*
1p13	110,352,477	Rs10494112[b]	*EPS8L3/CSF-1*
	110,361,682	Rs499345	*EPS8L3/CSF-1*
	110,366,083	Rs484959	*EPS8L3/CSF-1*
3q24	18,160,060	Rs4688903	*LOC339862*
5q31	141,019,830	Rs11742646[a]	*RELL2*
7q33	135,293,128	Rs4294134	*NUP205*
	104,388,446	Rs35500845	*CTHRC1*
8q22	105,359,432	Rs2458413	*TM7SF4*
	105,367,264	Rs62620995	*TM7F4*
	119,950,668	Rs1485286[b]	*TNFRSFIIB*
	119,952,765	Rs11573871	*TNFRSFIIB*
8q24	119,953,158	Rs11573869	*TNFRSFIIB*
	119,955,111	Rs6415470	*TNFRSFIIB*
	119,964,283	Rs2073617	*TNFRSFIIB*
9p13	35,054,586	Rs565070	*VCP*
	13,141,144	Rs3829923	*OPTN*
	13,151,224	Rs2234968	*OPTN*
10p13	13,155,726	Rs1561570	*OPTN*
	13,169,374	Rs825411	*OPTN*
	13,184,045	Rs2095388	*OPTN*
	13,276,751	Rs17152980	*UCMA*
	54,074,757	Rs2241529	*DKK1*
10q11	54,076,271	Rs1569198	*DKK1*
	54,086,453	Rs11001604	*DKK1*
10q23q24	100,322,658	Rs477950	*HPSE2*
14q32	93,103,309	Rs10498635[a]	*RIN3*
15q24	74,336,633	Rs5742915	*PML*
17q11	41,829,296	Rs851062	*SOST*
	59,751,331	Rs4941107	*PIGN*
18q21	59,979,135	Rs2980996	*KIAA1468*
	60,082,093	Rs3018362	*RPL17P14*
	60,021,761	Rs35211496[a]	*TNFRSFIIA*
18q22	60,027,241	Rs1805034	*TNFRSFIIA*
	60,060,735	Rs2957128	*TNFRSFIIA*

[a] SNPs departing from Hardy–Weinberg equilibrium in the control group.
[b] SNPs removed from further analyses because of complete linkage disequilibrium ($D' = 1$) with other SNPs studied.