CIHR IRSC Canadian Institutes of Health Research Instituts de recherche en santé du Canada

Submitted by CIHR
Déposé par les IRSC

Bone. Author manuscript; available in PMC 2012 December 08.

Published in final edited form as:
Bone. 2012 October ; 51(4): 720–728. doi:10.1016/j.bone.2012.06.028.

Genetic association study of *UCMA/GRP* and *OPTN* genes (*PDB6* locus) with Paget's disease of bone

Laëtitia Michou[1], **Natércia Conceição**[2], **Jean Morissette**[3], **Edith Gagnon**[3], **Gabriel Miltenberger-Miltenyi**[4], **Ethel S. Siris**[5], **Jacques P. Brown**[1], and **M. Leonor Cancela**[2,6]

[1]Department of Medicine, Laval University, CHUQ (CHUL) Research centre and Division of Rheumatology, CHUQ (CHUL), Quebec City, QC, Canada

[2]Centre of Marine Sciences (CCMAR), University of Algarve, Faro, Portugal

[3]CHUQ (CHUL) Research centre, Quebec City, QC, Canada

[4]Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon, Lisbon, Portugal

[5]Columbia University Medical Centre, New York City, NY, USA

[6]Dept of Biomedical Sciences and Medicine, University of Algarve, Faro, Portugal

Abstract

We performed a genetic association study of rare variants and single nucleotide polymorphisms (SNPs) of *UCMA/GRP* and *OPTN* genes, in French-Canadian patients with Paget's disease of bone (PDB) and in healthy controls from the same population. We reproduced the variant found in the *UCMA/GRP* basal promoter and tested its functionality using *in vitro* transient transfection assays. Interestingly, this SNP rs17152980 appears to affect the transcription level of *UCMA/GRP*. In addition, we have identified five rare genetic variants in *UCMA/GRP* gene, four of them being population-specific, although none were found to be associated with PDB. Six Tag SNPs of *UCMA/GRP* gene were associated with PDB, particularly the SNP rs17152980 (uncorrected $P=3.8 \times 10^{-3}$), although not significant after Bonferroni's correction. More importantly, we replicated the strong and statistically significant genetic association of two SNPs of the *OPTN* gene, the rs1561570 (uncorrected $P=5.7 \times 10^{-7}$) and the rs2095388 (uncorrected $P=4.9 \times 10^{-3}$), with PDB. In addition, we identified a very rare variant found to be located close to the basal promoter of the *OPTN* gene, at −232 bp from its distal transcription start site. Furthermore, depending on the type of allele present (*G* or *A*), the binding of several important nuclear factors such as the vitamin D or the retinoic acid receptors is predicted to be altered at this position, suggesting a significant effect in the regulation of transcription of the *OPTN* gene. In conclusion, we identified a functional SNP located in the basal promoter of the *UCMA/GRP* gene which provided a weak genetic association with PDB. In addition, we replicated the strong genetic association of two already known SNPs of the *OPTN* gene, with PDB in a founder effect population. We also identified a very rare variant in the promoter of *OPTN*, and through

Joint corresponding authors: Laëtitia Michou, Rhumatologie-S763, CHUQ-CHUL, 2705 boulevard Laurier, Québec, Québec, G1V 4G2, Canada. Phone: 1-418-654-2178. Fax: 1-418-654-2142, laetitia.michou@crchul.ulaval.ca. Leonor Cancela, Dept of Biomedical Sciences and Medicine, University of Algarve, Faro 8005-139, Portugal. Phone: +351-289800971 Fax: +351-289800076, lcancela@ualg.pt.

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

bioinformatic analysis, identified putative transcription factor binding sites likely to affect *OPTN* gene transcription.

Keywords

Paget's disease of bone; *UCMA/GRP* gene; *OPTN* gene; Sox2 binding site; transcription regulation

Introduction

Paget's disease of bone (PDB) is the second most frequent metabolic bone disorder after osteoporosis. The prevalence of PDB increases with age, affecting up to three percent of adults over 55 years of age [1]. PDB is characterized by focal increases in bone turnover, resulting in abnormal bone architecture and weakened bone strength. Approximately 30% of PDB patients experience disabilities due to bone pain, osteoarthritis secondary to bone deformities, fractures, or nerve root compression [2]. Genetic factors play a key role in PDB, and one-third of patients with PDB have a familial form transmitted in an autosomal dominant pattern of inheritance with incomplete penetrance [3]. Genetic heterogeneity has been demonstrated in familial forms of PDB, which have been linked to several chromosomal regions. In the *5q35-qter* (*PDB3*) locus [4], the first and still most common mutation, *P392L*, within the *Sequestosome 1* (*SQSTM1*) gene was reported in French-Canadian PDB patients [5]. The *10p13 (PDB6)* locus was suggested in a genome-wide scan linkage analysis in British PDB families [6, 7], but no PDB-causing mutation has been reported in this locus until now. Furthermore, this locus was not suggested either in the genome-wide scan of three French-Canadian families, who were linked to *PDB3* in one family and to the *PDB4* locus in the two others [4]. However, linkage to *PDB6* was not investigated in the remaining PDB families in the French-Canadian population. Albagha *et al*. reported recently that the stronger association, within the *10p13 (PDB6)* locus, was with three single nucleotide polymorphisms (SNPs), particularly the rs1561570 which is located in the *Optineurin* (*OPTN)* gene [8]. This genetic association with the gene, coding for a NEMO-related protein, was already known to be mutated in two other aging-related disorders: adult-onset primary open angle glaucoma and amyotrophic lateral sclerosis [9, 10]; it was further confirmed with PDB in replication studies [11, 12].

The *Upper zone of growth plate and Cartilage Matrix Associated/Gla-Rich Protein (UCMA/GRP)* gene, located within the linkage interval of the *10p13* (*PDB6)* locus, only 83kb distant from the *OPTN* gene, encodes a recently described vitamin K-dependent protein, which was suggested to be a modulator of calcium in the extracellular environment [13]. This highly conserved protein may be involved in the negative control of osteogenic differentiation of osteochondrogenic precursor cells in peripheral zones of fetal cartilage and at the cartilage-bone interface as well as in the early phase of chondrocyte differentiation [14, 15]. More recently, the UCMA/GRP protein was suggested to directly influence mineral formation and to play a role in processes involving soft tissue mineralization and abnormal calcification in the vascular system [16]. Investigation for alternatively spliced transcripts of *UCMA/GRP* in mice led to the identification of 4 isoforms, two of them probably secreted and two others

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

reported to form aggregates in a structure similar to aggresome, an organelle where aggregated proteins are stored or degraded by autophagy [17]. Interestingly, the catabolic process of macroautophagy may be involved in PDB pathophysiology since SQSTM1/p62 protein plays a central role in autophagy, acting as an adaptor allowing specific molecules to undergo selective degradation by autophagy [18, 19]. The *UCMA/GRP* gene was then considered as a candidate gene of PDB because of its location within the *PDB6* linkage interval and its proposed functions in the negative control of osteogenic differentiation, in modulation of mineral formation and maybe in autophagy processes (reviewed in [20]). In the present study, we performed a genetic association study of the *UCMA/GRP* gene and of the *OPTN* gene, in French-Canadian patients affected by PDB and in healthy controls from the same population. Taking advantage of the influence of genetic drift and the strong founder effect of the French-Canadian population, we first performed bidirectional sequencing to search for rare variants in coding sequences, exon-intron boundaries and in the basal promoter of *UCMA/GRP* and *OPTN* genes. Second, we genotyped Tag single nucleotide polymorphisms (Tag SNPs) and rare variants identified to test for genetic association with each variant separately, followed by haplotype analysis. We also analyzed the structure of all known isoforms for *OPTN* and identified, through bioinformatic analysis, several isoforms derived from alternative promoter usage or alternative splicing. These allowed us to position the rare variant identified for this gene within its distal promoter and predict its involvement in the transcription regulation of this gene. Finally, we performed functional *in vitro* studies to determine the effect of the variant rs17152980 found in the *UCMA/GRP* basal promoter on transcription transactivation of this gene.

Materials and methods

Patients

The present study was approved by the Centre Hospitalier de l'Université Laval (CHUL) Ethics Committee and by the Columbia University Medical Center Institutional Review Board. All individuals signed an informed-consent document before entering in the study. Phenotype assessment comprised a complete bone evaluation, including total serum alkaline phosphatase, a total body bone scan and skull and pelvis x-rays. We investigated patients with familial form of PDB (one patient per family), unrelated PDB patients and healthy controls, all from the French-Canadian population. Unrelated PDB patients, living in the New York City area, with a more heterogeneous genetic background and no founder effect population, were used for comparison to the French-Canadian population. Clinical characteristics of these cohorts were previously published [3, 5, 21]. For each individual, peripheral blood was obtained by venipuncture and DNA was extracted from blood samples, using standard procedures. All patients and healthy donors studied here were non-carrier of the *P392L* mutation within the *SQTM1* gene (*PDB3* locus).

UCMA/GRP and OPTN sequencing

To search for rare variants, the exons of *UCMA/GRP* and *OPTN* genes, their exon-intron boundaries and the basal promoters were PCR amplified. Amplification products were purified before bidirectional sequencing on a 3730 ABI sequencer using the Big Dye Deoxy Terminator Cycle Sequencing kit (ABI). Both strands were analyzed using STADEN

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

package 1.1 [22]. We considered here the DNA coding strand (mRNA like strand) as the reference sequence, corresponding to the minus strand for *UCMA/GRP* and the plus strand for *OPTN*. Rare variants were suggested in the presence of a nucleotide variation not reported in the SNP database of the NCBI web site (http://www.ncbi.nlm.nih.gov/snp). We sequenced a first sample consisting of 31 PDB patients, each one belonging to a different PDB multiplex family (five of them were previously linked to the *PDB4* locus), and four healthy controls from the French-Canadian population. Seventy unrelated PDB patients from the New York population were further sequenced for the promoter and exons 2-3 of *UCMA/GRP* gene PCR products in which rare variants were suggested in the first sample to estimate whether suggested rare variants were population-specific. In patients with a familial form of PDB in which rare variants were suggested, PCR products from several available relatives were further sequenced in order to investigate if those rare variants segregated with the disease within the family. For the association study, all rare variants identified in at least one PDB patient were further determined by bidirectional sequencing in 240 unrelated PDB patients and 297 unrelated healthy controls from the French-Canadian population.

Tag SNPs selection, genotyping and in silico prediction of function

Tag SNPs selection was based on the data provided by the HAPMAP database (http://www.hapmap.org/). We selected a region of 28.01 kb surrounding *UCMA/GRP* gene (HapMap Data Phase III/Rel#2, Feb09, on NCBI B36 assembly, dbSNP b126; chr10:13301779..13329790). We selected Tag SNPs with minor allele frequency 0.05 and r^2 at 0.8, picked out for the population CEU, with the aggressive Tagger program. Genotyping of the Tag SNPs was performed by Sequenom MassARRAY SNP Multiplex Technology in unrelated PDB patients and healthy controls from the French-Canadian population. Purified DNA solution containing multiplexed primer-based extension reaction (iPLEX reaction) products was dispensed from the 384-well microplate onto a 384-pad silicon microchip using the MassARRAY nanodispenser. The mass of each SNP allele was detected on the MassARRAY Compact MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time of Flight) mass spectrometer, and the results were analyzed with MassARRAY Typer software. Duplicated samples were included to verify the allele calls. All Tag SNPs and rare variants were in Hardy-Weinberg equilibrium, except rs17152975 which was removed from the analyses. The three SNPs of the *OPTN* gene previously reported to be associated with PDB [8], *i.e.* rs1561570, rs825411 and rs2095388, were also genotyped by the use of the same method and technology. *In silico* prediction of function was searched by Human Splicing Finder (http://www.umd.be/HSF/) [23] for each intronic rare variant or PDB-associated SNP and by TFsearch for variants located in promoter or flanking regions (http://www.cbrc.jp/research/db/TFSEARCH.html).

Haplotype analyses

Haplotypes composed of the Tag SNPs with an uncorrected P 0.05 in the genetic association study by individual Tag SNP of the *UCMA/GRP* gene, of the *OPTN* gene and of both *UCMA/GRP* and *OPTN* genes, were inferred in nineteen nuclei of French-Canadian PDB families by the use of SIMWALK 2.89. Then we inferred haplotypes by Bayesian inference with PHASE software in the sample of unrelated PDB patients and unrelated healthy controls from the French-Canadian population. Although the *UCMA/GRP* gene was

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

only 83kb distant from the *OPTN* gene, the HAPMAP linkage disequilibrium plots in this region (Figure 1) indicated that both genes were located in different blocks and possible recombination hot spots>20cM/Mb were recently reported in the literature between *OPTN,* and *MCM10* and *UCMA/GRP* genes [8].

Power calculation

The power of our sample of 240 PDB patients and 297 healthy controls to provide an association with an OR 1.5 is of 85% considering the hypothesis of one gene following an additive model of inheritance with a risk allele frequency of 0.25, as determined by the use of the computer program QUANTO 1.2.4 (http://hydra.usc.edu/gxe).

Statistical analysis

We tested for genetic association for each Tag SNP and each rare variant separately in the *UCMA/GRP* and the *OPTN* genes, between PDB patients and healthy controls. Allelic ORs, 95% Confidence Interval and P values (df=1) were calculated. Further genotype relative risks (RR) were calculated in case of uncorrected P-value of < 0.01 in the comparison of minor allele frequencies. Search for genotype-phenotype correlations, relying on Chi-squared or Fisher exact tests when appropriate for nominal values and t test for continuous variables, were performed for the most significantly PDB-associated SNP in each gene. Conservative Bonferroni correction was applied for multiple testing. Haplotype analyses relied first on a WHAP omnibus analysis, in which all haplotypes with a frequency 1% were handled simultaneously. In case of P-value of the omnibus analysis <0.05, a haplotype-specific analysis (df=1) was performed for the *UCMA/GRP* gene, for the *OPTN* gene and for both *UCMA/GRP* and *OPTN* genes [8].

Real-time quantitative PCR of the UCMA/GRP gene

In order to test if the most strongly associated Tag SNP of the *UCMA/GRP* promoter, rs17152980, had an impact on gene expression, total intracellular RNA from whole blood was extracted by the use of the PAXgene Blood RNA kit from PAXgene Blood RNA tubes (Qiagen) in 45 PDB patients and 43 healthy donors. Total RNA was measured in duplicate by Nano Drop and RNA quality was validated by Bioanalyzer (Agilent). Primers for the *UCMA/GRP* gene were designed by the use of the GeneTools software (Biotools Inc.), sense GCGAGTGAAGATGCAAAACAGAAGATT and antisense CCTCGTAATATTCTCTCCGCAGCT, and were synthesized by Integrated DNA Technologies. cDNA was synthesized by reverse transcriptase using 0.5–3 μg of total RNA in a reaction containing 200 U of Superscript III Rnase H-RT (Invitrogen Life Technologies). A quantity of cDNA corresponding to 20 ng of total RNA was used for the quantification of mRNA, by the use of a LightCycler 480 (Roche Diagnostics). *G6PD*, *PPIB* and *18S* genes were used as internal controls. The LightCycler 480 v1.5 software was used to determine the Crossing point by the second derivative calculation, as previously reported [24]. Real-time quantitative PCR analyses were performed by the Q_RTPCR platform of the CHUQ research centre (Quebec City, QC, Canada).

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Cloning of the human UCMA/GRP promoter variants in the reporter vector

A 1.8 kb fragment of the *UCMA/GRP* promoter was amplified from human genomic DNA using the primer set 5′-TAAATAGACATGGGGGTCTCGCTA-3′ and 5′-TTGCAGAGGTAGGGGCTCCG-3′. The amplified PCR product was cloned into pCR^{II}TOPO (Invitrogen) and the fidelity of the sequence was confirmed by DNA sequencing. This 1.8 kb insert, ranging from −1705 to +71 of the *UCMA/GRP* gene and corresponding to the C allele, was then cloned between the *Xho*I and *Hind*III sites of the pGL3-Basic luciferase reporter gene vector (Promega), resulting in the pHsGRP(−493C) construct. A point mutation in this sequence was generated by PCR using the QuickChange Lightning site-directed mutagenesis kit (Stratagene), and primers: 5′-TCCAGTCATTATGAGCCCTT**G**TTGACTGACATTTAGATCAA-3′ (forward) and 5′-TTGATCTAAATGTCAGTCAA**C**AAGGGCTCATAATGACTGGA-3′ (reverse) according to the manufacturer's protocol. Mutated bases are indicated in bold. Mutations in the resulting construct were confirmed by DNA sequencing. The resulting plasmid construct was named pHsGRP(−493G) and contains a 1-bp mutation corresponding to the polymorphism identified in the *UCMA/GRP* promoter at position −493 (*G* allele). The Sox2 expression plasmid pCMV-Taq2-Sox2 was the generous gift of Dr. Alka Mansukhani (New York University School of Medicine).

Cell transfections

Human embryonic kidney HEK 293 cells cultured on 12 well plates were transiently transfected using the standard calcium phosphate coprecipitation technique, with 20, 100, 200 or 500ng of either *UCMA/GRP* reporter gene pHsGRP(−493G or −493C) or empty reporter vector (pGL3-basic, 500ng), at a total concentration of 2μg DNA per well. A renilla luciferase reporter (Promega), 20 ng/well, was used to control for transfection efficiency. Co-transfection of an additional expression construct (50ng) was performed using a similar approach. Cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were lysed and luciferase activity was assayed using a Dual-luciferase Reporter Assay kit (Promega) in accordance with the manufacturer's instructions. All luciferase activities were normalized to the *Renilla* luciferase reporter pRL-TK Luc plasmid (Promega).

Comparative promoter transcription factor-binding sites (TFBSs) analysis

A set of thirteen mammalian *UCMA/GRP* genes, derived from human (*Homo sapiens*), Ord's kangaroo rat (*Dipodomys ordii*), American pikas (*Ochotona princeps*), African elephant (*Loxodonta africana*), common bottlenose dolphin (*Tursiops truncatus*), dog (*Canis familiaris*), bat (*Pteropus vampyrus*), gorilla (*Gorilla gorilla*), chimpanzee (*Pan troglodytes*), common marmoset (*Callithrix jacchus*), rhesus monkey (*Macaca mulatta*), gray mouse lemur (*Microcebus murinus*), and mouse (*Mus musculus*) were selected for this analysis. For each promoter pair (human plus other species), the DNA Block Aligner (DBA) software (http://www.ebi.ac.uk/Tools/Wise2) was used to extract conserved blocks of nucleotide sequences using the default parameter settings. The promoter sequences were then assessed for the TFBSs by running the web-based prediction program MatInspector (http://

www.genomatix.de/), with thresholds for core and matrix similarity set to 0.85 and 0.90, respectively.

Collection of OPTN sequences and promoter analysis

For the analysis of the *OPTN* gene and the different transcripts the Ensembl genome and NCBI databases were used. The promoter sequences were assessed for the TFBSs by running MatInspector, with thresholds for core and matrix similarity set to 0.85 and 0.90, respectively.

Results

Rare variant identification

Bidirectional sequencing in the discovery sample allowed us to identify in the *UCMA/GRP* gene, fourteen SNPs already reported in the SNP database (NCBI), and five variants previously unknown: three were detected in the promoter, in intron 2 and in intron 3, respectively. A deletion GT/– at position −618 was identified in three healthy individuals, but absent from PDB patients. This variant was unlikely to be associated with PDB and was not further investigated in the genetic association study. A second variant in the promoter, −448C/T, was identified in a PDB patient with a familial form of the disease but was absent from healthy controls. Further sequencing of available relatives of this patient showed that the −448C/T variant did not segregate with the affected phenotype in family and was unlikely to be a PDB causal mutation. However, this promoter rare variant may be functionally relevant through dominant effects on *UCMA/GRP* gene expression. The third variant in the promoter, −222C/T, was identified in a French-Canadian patient, and was absent from controls. This −222C/T variant was considered as a possible rare variant with functional relevance on gene expression. The variant in intron 2, IVS2+20A/C, was identified in a French-Canadian patient, and was absent from healthy controls. The change of the *A* into the *C* allele of this variant was *in silico* predicted to create a potential new acceptor site for splicing (new splice motif cccccggggcagGG) and to break a potential branch point (loss of branch point motif cacccAc), therefore this variant was further investigated in the genetic association study with PDB. The last identified variant was located in the intron 3, IVS3+56C/T, and was *in silico* predicted to break a potential branch point (loss of branch point motif cccccCg). This variant is the only rare variant which was not French-Canadian population-specific, since it was identified in one French-Canadian patient and in one PDB patient from the New York population, but was absent from healthy controls.

Bidirectional sequencing of the *OPTN* gene in the discovery sample allowed us to identify eleven SNPs already reported in the SNP database (NCBI) (Table 1), and one unknown variant located in the *OPTN* 5′ flanking region (−9906G/A) identified in one French-Canadian patient and absent from controls. Analysis of the structure of the various *OPTN* transcripts present in public databases allowed us to locate this variant within −232bp of the most distal transcription start site of this gene and therefore within a basal promoter region (Supplementary Fig 1 and Fig 2). We were unable to study the segregation of this variant within the family since no other DNA from affected family member was available. The

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

change of the *G* into the *A* allele of this variant was *in silico* predicted to affect binding of several transcription factors relevant for *OPTN* function (Table 2), therefore this variant was further investigated in the genetic association study with PDB.

Individual Tag SNP and rare variant genetic association analysis

The genetic association study of the nineteen Tag SNPs selected from the HAPMAP database and rare variants identified within the *UCMA/GRP* gene, demonstrated that six Tag SNPs were associated with PDB (uncorrected P 0.05 for rs7917620, rs727518, rs4750328, rs17152980, rs2476981 and rs533672) (Table 3). The most strongly associated Tag SNP being the SNP rs17152980 (10% of PDB patients carried the *G* allele of this Tag SNP versus 16% of healthy controls, uncorrected $P=3.8 \times 10^{-3}$, OR=0.58 (0.39–0.85)), but none of these allelic associations remained statistically significant following conservative Bonferroni's correction. A difference in the RR of the *GC* and *GG* genotypes when compared to the *CC* genotype of rs17152980, was also observed (20% in PDB patients versus 30% in controls, RR=0.57; 95% CI: 0.38–0.85, uncorrected $P=5.5 \times 10^{-3}$).

More importantly, the statistically significant association, even after conservative Bonferroni's correction, was replicated in two SNPs of the *OPTN* gene, particularly for the rs1561570 (Table 4). 36% of PDB patients carried the *C* allele of this SNP versus 52% of healthy controls, uncorrected $P=5.7 \times 10^{-7}$, OR=0.53 (0.42–0.69). The *C* allele instead of a *T* in the SNP rs1561570 was *in silico* predicted to create a potential new acceptor site for splicing (new splice motif tctgtagtatagAC), and to break a potential branch point (loss of branch point motif tggtcAc). A statistically significant difference in the RR of the *TC* and *CC* genotypes when compared to the *TT* genotype of rs1561570, was observed (58% in PDB patients versus 77% in controls, RR=0.41; 95% CI: 0.28–0.60, uncorrected $P=1.9 \times 10^{-6}$). An association of the 3′ flanking region SNP rs2095388 (uncorrected $P=4.9 \times 10^{-3}$) with PDB was also replicated, and a statistically significant difference in the RR of the *AG* and *GG* genotypes when compared to the *AA* genotype of rs2095388, was observed (43% in PDB patients versus 57% in controls, RR=0.57; 95% CI: 0.41–0.81, uncorrected $P=1.4 \times 10^{-3}$). The rare variant of the *OPTN* 5′ flanking region (–9906G/A), which was found in one patient with a familial form of PDB and absent from the other French-Canadian families with PDB, was not found in 246 unrelated pagetic patients and was identified in one healthy control out of 293 healthy individuals, from the same population.

Searches for genotype-phenotype correlations in the most significantly PDB-associated SNP in each gene, *ie* rs17152980 for *UCMA/GRP* gene and rs1561570 for *OPTN* gene, suggested that the mean age at diagnosis was younger in PDB patients carrying at least one *G* allele of the rs17152980 (59.7 ± 13.5 years in patients carrying a *G* allele versus 63.6 ± 10.5 in patients carrying the ancestral genotype *CC*, uncorrected P=0.04). There was a trend for a higher mean number of affected bones in patients carrying at least one G allele of this SNP (3.1 ± 3.5 in patients carrying a G allele versus 2.4 ± 1.8 in patients carrying the ancestral genotype *CC*, uncorrected P=0.06) (Table 5), but those results were not significant after Bonferroni's correction.

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

Haplotype analyses

The six Tag SNPs of the *UCMA/GRP* gene resulted in eleven different haplotypes with a frequency 1%. The WHAP omnibus analysis (df=10), in which all haplotypes with a frequency 1% were handled simultaneously, provided an uncorrected P=1.8×10^{-2}, suggesting a difference in the distribution of haplotypes between PDB patients and healthy donors. The haplotype-specific analysis provided that one haplotype, *GTCCAT*, which also contains the ancestral *C* allele of SNP rs17152980, was associated with PDB (27% in patients versus 19% in controls, uncorrected P=2.5×10^{-3}, OR=1.57 (1.14–2.16)) (Table 6), and remained statistically significant after conservative Bonferroni's correction.

Haplotypes of the *OPTN* gene were formed by rs1561570 and rs2095388, which resulted in four haplotypes with a frequency 1%. The WHAP omnibus analysis (df =3) provided a significant difference in the distribution of haplotypes between PDB patients and healthy donors (uncorrected P=9.5×10^{-5}). The haplotype-specific analysis indicated that three haplotypes were associated with PDB, and remained statistically significant after conservative Bonferroni's correction, particularly the *TA* haplotype (61% in patients versus 47% in controls, uncorrected P=1.2×10^{-5}, OR=1.73 (1.35–2.22)) (Table 7).

Haplotypes constituted by eight SNPs from both *UCMA/GRP* and *OPTN* genes resulted in 27 haplotypes with a frequency 1%. The WHAP omnibus analysis (df =26) provided a significant difference in the distribution of haplotypes between PDB patients and healthy donors (uncorrected P=6.5×10^{-3}). The haplotype-specific analysis provided that five haplotypes were associated with PDB, but none of them remained statistically significant after conservative Bonferroni's correction (Table 8).

Real-time quantitative PCR of the UCMA/GRP gene

The real-time quantitative PCR of the first 24 samples out of the 88 samples collected from PDB patients and healthy donors showed no detectable level of expression for the *UCMA/GRP* gene in total RNA from whole blood, suggesting that this gene was unlikely to be expressed in the blood tissue of humans. Therefore real-time quantitative PCR was not carried out in the remaining samples.

Functional analysis of the SNP rs17152980 (UCMA/GRP gene)

To directly determine the allele-specific effect of *UCMA/GRP −493C/G* polymorphism (rs17152980) on native promoter activity, two luciferase reporter gene constructs were generated, spanning 1.8 Kb of the *UCMA/GRP* promoter region and containing either a *G* or a *C* at the −493 polymorphic site. As shown in Figure 2, and following transient transfections of HEK 293 cells, the C allele of the *UCMA/GRP* promoter had a significantly higher activity than the *G* allele at all used DNA concentrations. These results suggest that the presence of the mutation corresponding to the *−493 G* allele decreases the transcriptional activity of the *UCMA/GRP* gene.

As noted, the SNP rs17152980 is located in the basal promoter of the *UCMA/GRP* gene. Bioinformatic analysis using the web-based prediction program MatInspector (http://www.genomatix.de/) identified a putative binding site for Sox2 transcription factor

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

overlapping the SNP containing the *G*, whereas it was absent in the sequence containing the *C*. We hypothesized then that Sox2 might bind to the less widely spread *G* allele of this promoter and affect the expression of *UCMA/GRP*. To investigate this hypothesis, we tested the ability of Sox2 to transactivate the GRP promoter containing either the *C* or the *G* allele using cotransfection experiments. We observed a significant induction of LuC expression only when using the latter (Figure 3). No significant difference in transcriptional activity was obtained when transfecting the pHsGRP(−493C) construct in the presence or absence of Sox2 expression vector (Figure 3), indicating that the results obtained are specific of the G allele.

Next, we performed a comparative analysis of genomic sequences using DNA Block Aligner (DBA, see Materials and methods), that aligns two sequences under the assumption that they share a number of colinear blocks of conservation separated by potentially large and varied lengths of DNA in each of the two sequences. Using the default setting of DBA, we compared the human *UCMA/GRP* promoter with those of its orthologs from other mammalian species to identify conserved blocks. A highly conserved block was found to be located in mammals, within the region of the SNP rs17152980. Among these, the *C* allele was found to be the most widely distributed among mammalian species analyzed (Supplementary Fig 3).

Discussion

In the present study, we identified five rare genetic variants located in putative functionally important regions of the *UCMA/GRP* gene, and four of them were French-Canadian population-specific, but none of them were found to be significantly associated with PDB. Among the nineteen Tag SNPs which were genotyped for the *UCMA/GRP* gene, the *G* allele of the SNP rs17152980 was found to be associated with PDB (10% in PDB patients versus 16% in healthy controls, uncorrected $P=3.8 \times 10^{-3}$, OR=0.58 (0.39–0.85)) and the mean age at diagnosis was suggested to be younger in PDB patients carrying at least *G* allele of the rs17152980 (uncorrected P=0.04), although not statistically significant after conservative Bonferroni's correction. Considering the potential protective effect of this allele, results of the genotype phenotype correlation analysis may reflect an underpowered analysis. More importantly, we replicated the strong and statistically significant genetic association of two SNPs of the *OPTN* gene, the rs1561570 (36% of PDB patients carried the *C* allele of this SNP versus 52% of healthy controls, uncorrected $P=5.7 \times 10^{-7}$, OR=0.53 (0.42–0.69)) and the 3′ flanking region SNP rs2095388 (uncorrected $P=4.9 \times 10^{-3}$), with PDB; these SNPs were recently reported to be associated with PDB in several West-European countries [8, 11, 12]. We also identified one very rare variant in the 5′ flanking region of the *OPTN*, not reported in NCBI database. This rare variant is located at −232bp and at −865bp from the first and second transcription start sites of the *OPTN* gene and, by *in silico* analysis, we have detected the presence of putative transcription factor-binding sites (TFBSs) overlapping this SNP region. We found that the presence of a *G* was consistent with TFBSs for VDR/RXR, Sp1 and PAX4 and the presence of an *A* was consistent with TFBSs for PPARG and HNF4 (Table 2). Since PAX4 has been reported to be strongly expressed in the retina of the rat [25] and SP1 has been involved in the regulation of genes in the lens [26], this putative regulation of *OPTN* by these TFs might be relevant. It is worth noting that

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

OPTN is highly expressed in the brain, retina and skeletal muscle. In addition VDR and PPARG are known regulators of bone and cartilage metabolism [27, 28].

Then, considering the six more associated Tag SNPs of the *UCMA/GRP* gene, we performed a haplotype analysis which provided that the *GTCCAT* haplotype, was more frequent in PDB patients when compared to healthy controls (uncorrected $P=2.5 \times 10^{-3}$). The two PDB-associated SNPs of the *OPTN* gene determined four haplotypes with a frequency >1%, which provided a statistically significant difference in their distribution between PDB patients and healthy controls (uncorrected $P=9.5 \times 10^{-5}$). Three of the four haplotypes, *TA*, *CA* and *CG*, provided a statistically significant association with PDB (uncorrected $P=1.2 \times 10^{-5}$, uncorrected $P=3.5 \times 10^{-3}$ and uncorrected $P=4.3 \times 10^{-3}$, respectively). However, after conservative Bonferroni's correction, haplotypes constituted by both *UCMA/GRP* and *OPTN* gene SNPs failed to provide statistically significant associations with PDB.

Since the *OPTN* gene is only 83kb distant from the *UCMA/GRP* gene and although both genes are not on the same linkage disequilibrium blocks in HAPMAP database, we cannot exclude that Tag SNPs of the *UCMA/GRP* gene studied here may be in linkage disequilibrium with polymorphisms located in the *OPTN* gene and strongly associated with PDB. The other hypothesis may be that *UCMA/GRP* gene is a genetic factor, although minor, weakly associated with PDB, and the lack of significant genetic association after conservative's Bonferroni's correction may be explained by the large number of polymorphisms tested in this study. Because only a few studies have reported the tissue distribution of UCMA/GRP in adult humans, it is not possible yet to correlate changes in tissue expression of this gene with bone pathologies. Until now different levels of UCMA/GRP expression and/or accumulation were essentially detected at sites of abnormal calcifications (reviewed in [20]).

To examine whether the SNP rs17152980, located in the basal promoter of the *UCMA/GRP* gene has any effect on the transcriptional regulation of the gene, a luciferase reporter assay was performed. The *in vitro* result offered strong evidence that the *G* allele containing construct displayed markedly lower promoter activity compared with the *C* allele, suggesting that it may reduce the *UCMA/GRP* levels of expression. We have used an *in silico* approach to detect the TFBSs overlapping this SNP region. We found that the presence of a *G* (instead of a *C*) was consistent with a putative binding site for the transcription factor (TF) Sox2. This possibility was further analyzed using a luciferase *in vitro* assay, and our data confirmed that the promoter construct corresponding to the *G* allele allows binding of Sox2 and transactivation of the *UCMA/GRP* promoter. Moreover, this effect was not observed when using the promoter construct overlapping the *C* allele, thus indicating that Sox2 binding was specific of the *G* allele. The transcription factor Sox2, a member of the SY-related, HMG box family, plays a critical role in embryonic development and maintenance of pluripotency and self-renewal of embryonic stem cells [29, 30]. Furthermore, Sox2 is also involved in the maintenance of self-renewal of the osteoblastic lineage [31]. Recently, it was shown that mesenchymal stem cells derived from human umbilical cord constitutively express SOX2 and are capable of differentiating into osteoblast as well as adipocytes, indicating the involvement of SOX2 in osteoblast differentiation [32].

Since one of the cellular abnormalities in PDB involves an increase in bone-forming osteoblast activity [33], and expression of *UCMA/GRP* gene has been detected in osteoblasts at levels comparable to those of osteocalcin [13], the transactivation by Sox2 of only one of the two *UCMA/GRP* promoter alleles analyzed could be of relevance to PDB. It also suggests that this functional SNP of *UCMA/GRP* could be further explored as a possible candidate biomarker of PDB susceptibility and/or severity. In contrast, osteocalcin, another gla protein associated with mineralized matrix of bone and a known marker of bone formation, has proven disappointing in PDB. Its levels are less consistently raised in active disease than other formation markers due either to altered synthesis by pagetic osteoblasts or increased incorporation by the high mineral content of woven bone with decreased release into the circulation [34].

In conclusion, we identified one functional SNP located in the basal promoter of the *UCMA/GRP* gene which provides a weak genetic association with PDB, and replicated the strong genetic association of two already known SNPs of the *OPTN* gene, with PDB in a founder effect population. Furthermore, we identified a very rare variant not previously described for *OPTN* gene, located in one of its basal promoters and within the putative binding sites of several nuclear factors likely to be relevant for OPTN function. Further replication studies and functional analyses are required to confirm those genetic associations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Dr. Michou is supported by a career award from the Fonds de la Recherche du Québec –Santé (FRQS), Canada. Dr. Natércia Conceição is the recipient of a post-doctoral fellowship from Portuguese Science and Technology Foundation (SFRH/BPD/48206/2008), Portugal. This study was funded by a Catalyst Grant (Bone Health) from the Canadian Institutes of Health Research (Canada), and in part by the CHUQ Foundation (Canada), the Groupe de Recherche en Maladies Osseuses (Canada), the Canadian Foundation for Innovation (Canada), the FRSQ (Canada), the Laval University (Canada), the CHUQ (CHUL) Research Centre (Canada) and by the Centre of Marine Sciences (CCMAR) funding (Portugal).

References

1. van Staa TP, Selby P, Leufkens HG, Lyles K, Sprafka JM, Cooper C. Incidence and natural history of Paget's disease of bone in England and Wales. J Bone Miner Res. 2002; 17:465–71. [PubMed: 11878305]
2. Rousiere M, Michou L, Cornelis F, Orcel P. Paget's disease of bone. Best Pract Res Clin Rheumatol. 2003; 17:1019–41. [PubMed: 15123049]
3. Morissette J, Laurin N, Brown JP. Sequestosome 1: mutation frequencies, haplotypes, and phenotypes in familial Paget's disease of bone. J Bone Miner Res. 2006; 21 (Suppl 2):38–44.
4. Laurin N, Brown JP, Lemainque A, Duchesne A, Huot D, Lacourciere Y, Drapeau G, Verreault J, Raymond V, Morissette J. Paget disease of bone: mapping of two loci at 5q35-qter and 5q31. Am J Hum Genet. 2001; 69:528–43. [PubMed: 11473345]
5. Laurin N, Brown JP, Morissette J, Raymond V. Recurrent mutation of the gene encoding sequestosome 1 (SQSTM1/p62) in Paget disease of bone. Am J Hum Genet. 2002; 70:1582–8. [PubMed: 11992264]

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

6. Hocking LJ, Herbert CA, Nicholls RK, Williams F, Bennett ST, Cundy T, Nicholson GC, Wuyts W, Van Hul W, Ralston SH. Genomewide search in familial Paget disease of bone shows evidence of genetic heterogeneity with candidate loci on chromosomes 2q36, 10p13, and 5q35. Am J Hum Genet. 2001; 69:1055–61. [PubMed: 11555792]
7. Lucas GJ, Riches PL, Hocking LJ, Cundy T, Nicholson GC, Walsh JP, Ralston SH. Identification of a major locus for Paget's disease on chromosome 10p13 in families of British descent. J Bone Miner Res. 2008; 23:58–63. [PubMed: 17907922]
8. Albagha OM, Visconti MR, Alonso N, Langston AL, Cundy T, Dargie R, Dunlop MG, Fraser WD, Hooper MJ, Isaia G, Nicholson GC, del Pino Montes J, Gonzalez-Sarmiento R, di Stefano M, Tenesa A, Walsh JP, Ralston SH. Genome-wide association study identifies variants at CSF1, OPTN and TNFRSF11A as genetic risk factors for Paget's disease of bone. Nat Genet. 2010; 42:520–4. [PubMed: 20436471]
9. Rezaie T, Child A, Hitchings R, Brice G, Miller L, Coca-Prados M, Heon E, Krupin T, Ritch R, Kreutzer D, Crick RP, Sarfarazi M. Adult-onset primary open-angle glaucoma caused by mutations in optineurin. Science. 2002; 295:1077–9. [PubMed: 11834836]
10. Maruyama H, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuura S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R, Kawakami H. Mutations of optineurin in amyotrophic lateral sclerosis. Nature. 2010; 465:223–6. [PubMed: 20428114]
11. Albagha OM, Wani SE, Visconti MR, Alonso N, Goodman K, Brandi ML, Cundy T, Chung PY, Dargie R, Devogelaer JP, Falchetti A, Fraser WD, Gennari L, Gianfrancesco F, Hooper MJ, Van Hul W, Isaia G, Nicholson GC, Nuti R, Papapoulos S, Montes Jdel P, Ratajczak T, Rea SL, Rendina D, Gonzalez-Sarmiento R, Di Stefano M, Ward LC, Walsh JP, Ralston SH. Genome-wide association identifies three new susceptibility loci for Paget's disease of bone. Nat Genet. 2011; 43:685–9. [PubMed: 21623375]
12. Chung PY, Beyens G, Boonen S, Papapoulos S, Geusens P, Karperien M, Vanhoenacker F, Verbruggen L, Fransen E, Van Offel J, Goemaere S, Zmierczak HG, Westhovens R, Devogelaer JP, Van Hul W. The majority of the genetic risk for Paget's disease of bone is explained by genetic variants close to the CSF1, OPTN, TM7SF4, and TNFRSF11A genes. Hum Genet. 2010; 128:615–26. [PubMed: 20839008]
13. Viegas CS, Simes DC, Laize V, Williamson MK, Price PA, Cancela ML. Gla-rich protein (GRP), a new vitamin K-dependent protein identified from sturgeon cartilage and highly conserved in vertebrates. J Biol Chem. 2008; 283:36655–64. [PubMed: 18836183]
14. Surmann-Schmitt C, Dietz U, Kireva T, Adam N, Park J, Tagariello A, Onnerfjord P, Heinegard D, Schlotzer-Schrehardt U, Deutzmann R, von der Mark K, Stock M. Ucma, a novel secreted cartilage-specific protein with implications in osteogenesis. J Biol Chem. 2008; 283:7082–93. [PubMed: 18156182]
15. Tagariello A, Luther J, Streiter M, Didt-Koziel L, Wuelling M, Surmann-Schmitt C, Stock M, Adam N, Vortkamp A, Winterpacht A. Ucma--A novel secreted factor represents a highly specific marker for distal chondrocytes. Matrix Biol. 2008; 27:3–11. [PubMed: 17707622]
16. Viegas CS, Cavaco S, Neves PL, Ferreira A, Joao A, Williamson MK, Price PA, Cancela ML, Simes DC. Gla-rich protein is a novel vitamin K-dependent protein present in serum that accumulates at sites of pathological calcifications. Am J Pathol. 2009; 175:2288–98. [PubMed: 19893032]
17. Le Jeune M, Tomavo N, Tian TV, Flourens A, Marchand N, Camuzeaux B, Mallein-Gerin F, Duterque-Coquillaud M. Identification of four alternatively spliced transcripts of the Ucma/GRP gene, encoding a new Gla-containing protein. Exp Cell Res. 2010; 316:203–15. [PubMed: 19819238]
18. Pankiv S, Lamark T, Bruun JA, Overvatn A, Bjorkoy G, Johansen T. Nucleocytoplasmic shuttling of p62/SQSTM1 and its role in recruitment of nuclear polyubiquitinated proteins to promyelocytic leukemia bodies. J Biol Chem. 2010; 285:5941–53. [PubMed: 20018885]
19. Goode A, Layfield R. Recent advances in understanding the molecular basis of Paget disease of bone. J Clin Pathol. 2009; 63:199–203. [PubMed: 19858527]

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

20. Cancela ML, Conceiçao N, Laizé V. Gla rich protein, a new player in tissue calcification? Adv. Nutr. 2012; 3:174–181.
21. Michou L, Morissette J, Gagnon ER, Marquis A, Dellabadia M, Brown JP, Siris ES. Novel SQSTM1 mutations in patients with Paget's disease of bone in an unrelated multiethnic American population. Bone. 2011; 48:456–60. [PubMed: 21073987]
22. Staden R. The Staden sequence analysis package. Mol Biotechnol. 1996; 5:233–41. [PubMed: 8837029]
23. Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res. 2009; 37:e67. [PubMed: 19339519]
24. Luu-The V, Paquet N, Calvo E, Cumps J. Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction. Biotechniques. 2005; 38:287–93. [PubMed: 15727135]
25. Rath MF, Bailey MJ, Kim JS, Coon SL, Klein DC, Moller M. Developmental and daily expression of the Pax4 and Pax6 homeobox genes in the rat retina: localization of Pax4 in photoreceptor cells. J Neurochem. 2009; 108:285–94. [PubMed: 19012751]
26. Singh DP, Bhargavan B, Chhunchha B, Kubo E, Kumar A, Fatma N. Transcriptional Protein Sp1 Regulates LEDGF Transcription by Directly Interacting with Its Cis-Elements in GC-Rich Region of TATA-Less Gene Promoter. PLoS One. 2012; 7:e37012. [PubMed: 22615874]
27. Fahmi H, Martel-Pelletier J, Pelletier JP, Kapoor M. Peroxisome proliferator-activated receptor gamma in osteoarthritis. Mod Rheumatol. 2011; 21:1–9. [PubMed: 20820843]
28. Chen K, Aenlle KK, Curtis KM, Roos BA, Howard GA. Hepatocyte growth factor (HGF) and 1,25-dihydroxyvitamin D together stimulate human bone marrow-derived stem cells toward the osteogenic phenotype by HGF-induced up-regulation of VDR. Bone. 2012; 51:69–77. [PubMed: 22521434]
29. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 2003; 17:126–40. [PubMed: 12514105]
30. Lefebvre V, Dumitriu B, Penzo-Mendez A, Han Y, Pallavi B. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. Int J Biochem Cell Biol. 2007; 39:2195–214. [PubMed: 17625949]
31. Basu-Roy U, Ambrosetti D, Favaro R, Nicolis SK, Mansukhani A, Basilico C. The transcription factor Sox2 is required for osteoblast self-renewal. Cell Death Differ. 2010; 17:1345–53. [PubMed: 20489730]
32. Tong CK, Vellasamy S, Tan BC, Abdullah M, Vidyadaran S, Seow HF, Ramasamy R. Generation of mesenchymal stem cell from human umbilical cord tissue using a combination enzymatic and mechanical disassociation method. Cell Biol Int. 2011; 35:221–6. [PubMed: 20946106]
33. Layfield R, Shaw B. Ubiquitin-mediated signalling and Paget's disease of bone. BMC Biochem. 2007; 8 (Suppl 1):S5. [PubMed: 18047742]
34. Shankar S, Hosking DJ. Biochemical assessment of Paget's disease of bone. J Bone Miner Res. 2006; 21 (Suppl 2):22–7.

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

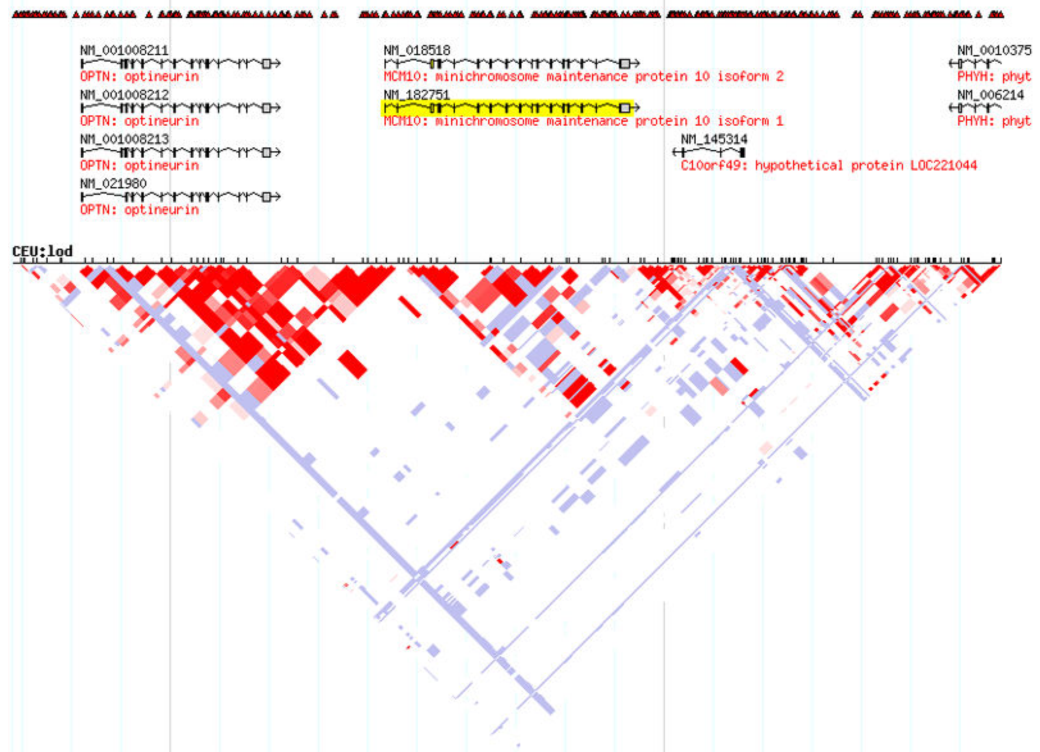

Figure 1. Linkage disequilibrium plots within the PDB6 locus

(from HAPMAP database HapMap Data Rel 27 Phase II+III, Feb 09, on NCBI B38 assembly, dbSNP b126; chr10:13168347.13368346; accessed on the 3rd of February 2011). *C10orf49* is an alias of the *UCMA/GRP* gene.

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

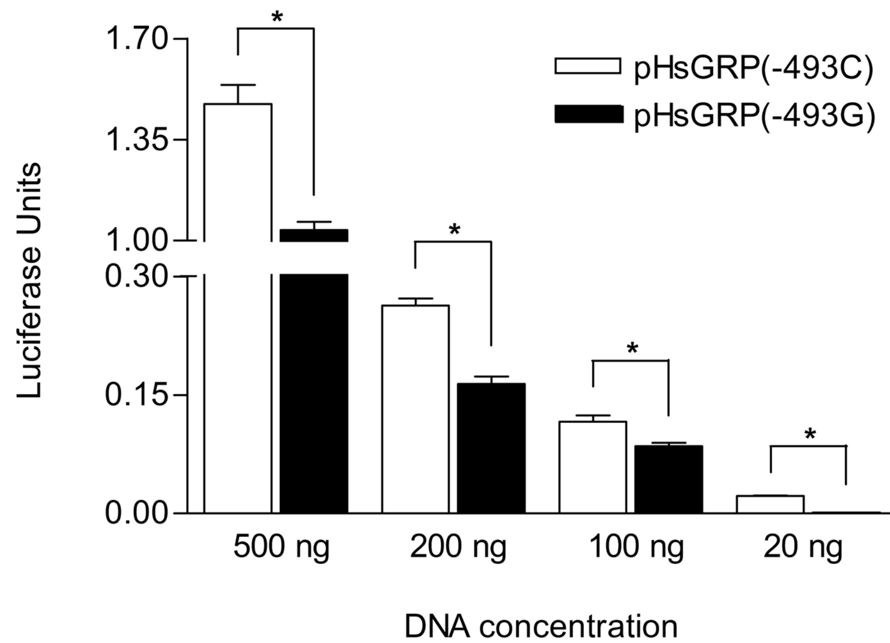

Figure 2. Allele dependent Ucma/GRP promoter activity in vitro

HEK293 cells were transfected with a luciferase reporter gene with the 1.8 kb GRP promoter containing either the −493C or the −493G allele. Luciferase expression in each case was normalized for an internal transfection control (renilla). Data are mean ± SD of at least five experiments. Ucma/GRP −493C allele, *solid bars*; Ucma/GRP −493G allele, *gray bars*. Significance was determined by One-way Anova. Asterisk * indicates that values are statistically different ($p<0.05$).

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

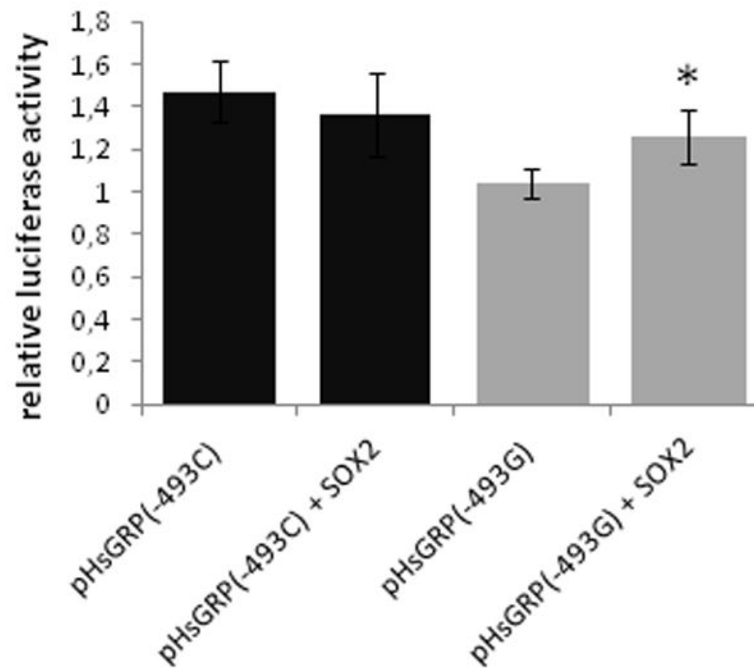

Figure 3. Allele dependent binding of Sox2 to Ucma/GRP promoter in vitro

HEK 293 cells were cotransfected with human *Ucma/GRP* promoter constructs including either the −493C or the −493G allele and Sox2 expressing plasmid. Data are mean ± SD of at least five experiments. Luciferase expression levels controlled by Ucma/GRP promoter alleles −493C or −493G in the presence of Sox2 expression vector are relative to luciferase expression mediated by each promoter allele alone. Significance was determined by One-way Anova. Asterisk * indicates that the value is statistically different ($p<0.05$).

CIHR Author Manuscript

CIHR Author Manuscript

CIHR Author Manuscript

Table 1

Results of the detection of genetic variants in the *OPTN* gene in the discovery sample consisting in 33 PDB patients non-carrier of a *SQSTM1/P392L* mutation and five healthy non-mutated donors.

SNP*	**Minor allele frequency**	
	PDB patients	**Healthy controls**
rs3829924 G/A	0.018	0.000
rs2234968 G/A	0.306	0.417
rs11258194 T/A	0.031	0.083
rs72043574 delT	0.422	0.333
rs2244380 C/T	0.156	0.083
rs11258211 G/A	0.016	0.000
rs765884 T/C	0.375	0.583
rs489040 A/G	0.422	0.250
rs523747 A/G	1.000	1.000
rs676302 G/T	0.179	0.100
rs10906310 C/A	0.219	0.083

*The rare variant identified in the 5′ flanking region of the *OPTN* gene is reported in the Results section.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Table 2

Results of the *in silico* analysis of transcription factor-binding sites (TFBSs) for the three SNPs and the rare variant located close to the promoter of the *OPTN* gene.

Variant name	Region (position)	Flanking region	TFBSs[#] only in Major allele	TFBSs[#] only in Minor allele
rs1561570	Intron 7	tataga[c/t]ggt	LyL1-e12.01	NUDR.01
rs825411	Intron 13	tgatac[c/t]gtt	-	MIF1.01 FHXB.01
rs2095388	3′ Flanking region	aattaa[a/g]tta	MSX.01 CART1.01 LMX1A.01 EN1.01 HOXC4.01 LBX2.01 EN1.01 SHOX2.01 TST1.01 BRIGHT.01	S8.01 BRN5.02 SL2.01 BARX2.01 PSE.02 HNF1.04
Rare variant[*] −9906G/A	5′ flanking region	gggcag[g/a]gtt	VDR_RXR.06 SP1.01 PAX4PD.01[**]	PPARG.02 HNF4.03

[#]TFBSs: Transcription Factor-Binding Sites

[*]This position corresponds to −9906bp from the ATG (located in the exon 5 of *OPTN* gene), and is located at −232bp from Transcription Start Site 1 and at −865bp from Transcription Start Site 2.

[**]Transcription Factor associated to ocular development.

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Table 3

Results of the genetic association analysis of the *UCMA/GRP* gene (*PDB6* locus) by individual Tag SNP and rare variant in 240 PDB patients non-carrier of a *SQSTM1/P392L* mutation versus 297 healthy non-mutated donors.

Tag SNP or rare variant (RV)	Minor allele	Minor allele frequency		Uncorrected P#	OR	95% CI
		PDB patients	Healthy controls			
rs7917620 G/A	*A*	0.25	0.31	0.02	0.73	0.55 – 0.96
rs727518 T/C	*C*	0.29	0.37	0.01	0.72	0.55 – 0.93
rs3740210 G/C	*C*	0.39	0.45	0.05	0.78	0.61 – 1.01
rs4589172 T/C	*C*	0.29	0.32	0.29	0.86	0.66 – 1.13
rs3886695 A/T	*T*	0.08	0.10	0.34	0.80	0.51 – 1.24
rs942413 A/G	*G*	0.41	0.46	0.08	0.80	0.63 – 1.03
rs11258275 G/A	*A*	0.36	0.40	0.16	0.84	0.65 – 1.08
rs4750320 C/T	*T*	0.40	0.42	0.76	0.96	0.74 – 1.23
RV IVS3+56C/T	*T*	0.011	0.014	0.77	0.79	0.17 – 2.96
rs4750328 C/T	*T*	0.21	0.26	0.04	0.74	0.55 – 1.00
RV IVS2+20A/C	*C*	0.003	0.000	0.39	∞	0.04 – ∞
RV −222C/T*	*T*	0.005	0.004	1.00	1.47	0.11–20.30
rs2093847 G/A	*A*	0.14	0.14	0.86	1.03	0.72 – 1.49
RV −448C/T*	*T*	0.003	0.000	0.41	∞	0.04 – ∞
rs17152980 C/G	*G*	0.10	0.16	3.82×10^{-3}	0.58	0.39 – 0.85
rs11258281 A/G	*G*	0.62	0.59	0.38	1.12	0.87–1.45
rs10906326 T/C	*C*	0.49	0.44	0.12	1.21	0.95 – 1.56
rs2181841 A/C	*C*	0.31	0.28	0.34	1.14	0.86 – 1.49
rs2476981 G/A	*A*	0.39	0.33	0.04	1.30	1.01 – 1.69
rs10906331 A/G	*G*	0.04	0.06	0.18	0.68	0.37 – 1.21
rs2025450 A/G	*G*	0.46	0.45	0.76	1.04	0.81 – 1.34
rs528320 A/G	*G*	0.11	0.14	0.23	0.80	0.54 – 1.17
rs533672 T/A	*A*	0.04	0.07	0.04	0.55	0.29 – 1.00

* Promoter position was determined from the ATG position within the first exon of the *UCMA/GRP* gene.

\# None of these uncorrected P-values remained statistically significant after conservative Bonferroni's correction (threshold of P-value after correction= 2.17×10^{-3}).

CIHR Author Manuscript
CIHR Author Manuscript
CIHR Author Manuscript

Table 4

Replication study of three single nucleotide polymorphisms (SNPs) of the *OPTN* gene (*PDB6* locus) previously reported to be associated with PDB in the literature, in 240 PDB patients non-carrier of a *SQSTM1/P392L* mutation versus 297 healthy non-mutated donors.

SNPs	Minor allele	Minor allele frequency		Uncorrected P	OR	95%CI
		PDB patients	Healthy controls			
Rs1561570 T/C	*C*	0.36	0.52	5.65×10^{-7}*	0.53	0.42 – 0.69
Rs825411A/G	*G*	0.59	0.53	0.056	1.27	0.99–1.63
Rs2095388 A/G	*G*	0.25	0.33	4.87×10^{-3}*	0.68	0.52 – 0.90

*These uncorrected P-values remained statistically significant after conservative Bonferroni's correction (threshold of P-value after correction=1.67×10^{-2}).

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Table 5

Genotype-phenotype correlations in patients with Paget's disease of bone (PDB) for the SNP rs17152980 of *UCMA/GRP* gene and for the SNP rs1561570 of *OPTN* gene.

	SNP rs17152980 (*UCMA/GRP* gene)			**SNP rs1561570 (*OPTN* gene)**		
	Patients carrier of *CG* or *GG* genotypes	**Patients carrier of the ancestral genotype (*CC*)**	**Uncorrected P**	**Patients carrier of *CT* or *CC* genotypes**	**Patients carrier of the ancestral genotype (*TT*)**	**Uncorrected P**
Male sex, n (%)	24 (54.5%)	115 (59.9%)	0.61	80 (59.7%)	59 (57.8%)	0.79
Positive family history of PDB, n (%)	5 (11.4%)	18 (9.4%)	0.78	14 (10.4%)	9 (8.8%)	0.82
Age at diagnosis, mean (± SD)	59.7 ± 13.5	63.6 ± 10.5	0.04	62.8 ± 11.2	63.2 ± 11.5	0.79
Number of affected bones, mean (± SD)	3.1 ± 3.5	2.4 ± 1.8	0.06	2.4 ± 2.2	2.6 ± 2.3	0.53
Renier's index, mean (± SD)	10.0 ± 10.0	9.8 ± 7.8	0.89	9.4 ± 7.8	10.5 ± 8.7	0.33

SD = standard deviation

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Table 6

Results of the haplotype-specific analysis with haplotypes =1% of frequency, formed by the six Tag SNPs* of the *UCMA/GRP* gene with an uncorrected P= 0.05 in the individual genetic association study in 240 PDB patients non-carrier of a SQSTM1/*P392L* mutation versus 297 healthy non-mutated donors.

Haplotype*	Haplotype frequency		Uncorrected P	OR	95% CI
	PDB patients	Healthy controls			
GTCCGT	0.26	0.25	0.76	1.06	0.78 – 1.43
GTCCAT	0.27	0.19	2.5×10^{-3}#	1.57	1.14 – 2.16
GTTCGT	0.12	0.13	0.52	0.87	0.59 – 1.30
ACCCGT	0.10	0.90	0.72	1.15	0.73 – 1.82
ACCGGT	0.05	0.08	0.04	0.56	0.32 – 0.98
ACTCAT	0.04	0.06	0.06	0.62	0.33 – 1.16
GTCGGT	0.05	0.06	0.50	0.84	0.45 – 1.56
GCCCAT	0.04	0.06	0.38	0.72	0.37 – 1.37
ACTCGA	0.02	0.04	0.13	0.51	0.21 – 1.16
ACCCAT	0.03	0.03	0.79	1.13	0.49 – 2.64
GTTCAT	0.02	0.01	0.14	2.81	0.67 – 16.54

*Haplotypes were formed by rs7917620, rs727518, rs4750328, rs17152980, rs2476981 and rs533672 of the *UCMA/GRP* gene.

#This uncorrected P-value remained statistically significant after conservative Bonferroni's correction (threshold of P-value after correction=4.55×10^{-3}).

Table 7

Results of the haplotype-specific analysis with haplotypes 1% of frequency, formed by the two Tag SNPs[*] of the *OPTN* gene, with an uncorrected P 0.05 in the individual genetic association study in 240 PDB patients non-carrier of a *SQSTM1/P392L* mutation versus 297 healthy non-mutated donors.

Haplotype[*]	Haplotype frequency		Uncorrected P	OR	95% CI
	PDB patients	Healthy controls			
TA	0.61	0.47	1.22×10^{-5}#	1.73	1.35 – 2.22
TG	0.03	0.02	0.54	1.31	0.53 – 3.23
CA	0.13	0.20	3.52×10^{-3}#	0.62	0.44 – 0.88
CG	0.23	0.31	4.25×10^{-3}#	0.68	0.51 – 0.90

[*] Haplotypes were formed by rs1561570 and rs2095388 within the *OPTN* gene.

[#] These uncorrected P-values remained statistically significant after conservative Bonferroni's correction (threshold of P-value after correction=1.25×10^{-2}).

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript

Table 8

Results of the haplotype-specific analysis with haplotypes 1% of frequency, formed by the eight Tag SNPs* of *UCMA/GRP* and *OPTN* genes, with an uncorrected P 0.05 in the individual genetic association study in 240 PDB patients non-carrier of a *SQSTM1/P392L* mutation versus 297 healthy non-mutated donors.

Haplotype*	Haplotype frequency		Uncorrected P#	OR	95% CI
	PDB patients	Healthy controls			
TAGTCCGT	0.20	0.15	0.033	1.40	1.00 – 1.96
TAGTCCAT	0.17	0.10	1.9×10^{-3}	1.86	1.27 – 2.74
TAGTTCGT	0.10	0.09	0.55	1.09	0.70 – 1.69
CGGTCCAT	0.07	0.07	0.92	0.97	0.58 – 1.60
CGGTCCGT	0.04	0.06	0.12	0.70	0.38 – 1.25
CAGTCCGT	0.03	0.05	0.15	0.58	0.27 – 0.17
CGACCCGT	0.04	0.04	0.81	1.11	0.56 – 2.17
CGGTCGGT	0.02	0.04	0.20	0.63	0.27 – 1.37
TAACCCGT	0.04	0.02	0.26	1.67	0.77 – 3.66
TAGCCCAT	0.03	0.03	0.82	1.05	0.47 – 2.30
CGACCGGT	0.02	0.04	0.029	0.40	0.14 – 0.97
TATCCGGT	0.02	0.03	0.65	0.87	0.36 – 2.03
CAGTTCGT	0.02	0.03	0.34	0.67	0.26 – 1.61
CAACTCAT	0.02	0.03	0.15	0.56	0.21 – 1.37
TAACTCAT	0.02	0.03	0.16	0.55	0.19 – 1.43
CAACCGGT	0.02	0.03	0.22	0.55	0.19 – 1.43
TAGTCGGT	0.02	0.02	0.69	0.85	0.30 – 2.28
CAACCCAT	0.02	0.02	0.62	1.28	0.47 – 3.47
CAACTCGA	0.00	0.03	5.1×10^{-3}	0.16	0.02 – 0.67
CGGCCCAT	0.01	0.02	0.28	0.53	0.14 – 1.62
CAACCCGT	0.01	0.01	0.66	0.96	0.27 – 3.17
CAGTCCAT	0.01	0.01	0.49	1.09	0.30 – 3.83
CGGTTCGT	0.01	0.01	0.43	0.56	0.13 – 2.04
TAGTTCAT	0.02	0.01	0.042	3.88	0.96 – 22.41
TGGTCCAT	0.01	0.01	0.78	1.28	0.34 – 4.82

Haplotype*	Haplotype frequency		Uncorrected P#	OR	95% CI
	PDB patients	Healthy controls			
CGACTCGA	0.01	0.01	0.59	0.64	0.14 – 2.39
TAACCCAT	0.01	0.01	0.50	1.54	0.39 – 6.41

*Haplotypes were formed by rs1561570 and rs2095388 (*OPTN* gene), and rs7917620, rs727518, rs4750328, rs17152980, rs2476981 and rs533672 (*UCMA/GRP* gene).

#None of these uncorrected P-values remained statistically significant after conservative Bonferroni's correction (threshold of P-value after correction=1.85×10^{-3}).

CIHR Author Manuscript CIHR Author Manuscript CIHR Author Manuscript