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Effect of genetic variants of OPTN in the pathophysiology of Paget's disease of bone

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ABSTRACT

Paget's disease of bone (PDB) is the second most frequent metabolic bone disease after osteoporosis. Genetic factors play an important role in PDB, but to date PDB causing mutations were identified only in the *Sequestosome 1* gene at the *PDB3* locus. OPTN has been recently associated with PDB, however little is known about the effect of genetic variants in this gene in PDB pathophysiology. By sequencing *OPTN* in *SQSTM1* non-carriers PDB patients we found 16 SNPs in regulatory, coding and non-coding regions. One of those was found to be associated with PDB in our cohort - rs2234968. Our results show that rs2238968 effect may be explained by a change in *OPTN* splicing that give rise to a predicted truncated protein. We also performed functional studies on the variants located in *OPTN* promoter – rs3829923 and the rare variant −9906 – to investigate putative regulators of *OPTN*. Our results show that *OPTN* expression seems to be regulated by SP1, RXR, E47, and the E2F family. In conclusion, our work suggests a potential pathophysiological role of SNPs in *OPTN*, giving a new perspective about the regulatory mechanisms of this gene. Ultimately we discovered a new variant associated with PDB in *OPTN*, reinforcing the relevance of this gene for the development of this bone disease.

1. Introduction

Optineurin was first identified by Li's group [1] using a yeast two-hybrid system and named FIP-2 - interacting protein of Adenovirus E3 14.7-kDa protein. Later, in 2002, mutations in this gene were found to be associated with primary open-angle glaucoma, a disease that causes irreversible bilateral blindness, and the gene was renamed "optineurin" (*OPTN*) [2]. The human *OPTN* gene is located in the short arm of chromosome 10 (13099449–13138308) and spans about 39 Mbp of genomic DNA. It contains three noncoding exons in the 5′ untranslated region (UTR) and 13 exons that code for a 577 amino acid protein. Alternative splicing in the 5′ UTR generates at least four different transcripts (NM_001008211.1, NM_001008212.1, NM_001008213.1, and NM_021980), but all have the same open reading frame. Alternative splicing in the coding region give rise to three different protein isoforms with 571 (ENST00000378764), 126 (ENST00000424614) and 107 (ENST00000486862) amino acids. Also, two partial transcripts were also described, but there is no indication of being protein coding. In addition, in 2012 a 'new first exon' was described, upstream from the previously known exon 1, and was labelled as exon 1a [3]. The OPTN protein consists of a NEMO-like domain, a leucine zipper motif, multiple coiled-coil motifs, an ubiquitin binding domain (UBD), a microtubule associated protein 1 light chain 3 (LC3)-interacting motif, and a C-terminal zinc finger [4]. OPTN is known to undergo posttranslational modifications and after being ubiquitinated it is processed through the ubiquitin-proteasome pathway [5]. It is also phosphorylated at Ser177, which is adjacent to the LC3 interacting region (LIR) site [6]. OPTN is a multifunctional protein involved in several biological functions such as NF-κB regulation, autophagy, membrane trafficking, exocytosis, vesicle transport, transcriptional activation, and reorganizing of actin and

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microtubules, since it interacts with several proteins, including Rab8, huntingtin, transcription factor IIIA, myosin VI, and TANK binding protein 1 (TBK1). OPTN biological role is not yet fully understood and appears to be complex and involve different mechanisms and pathways (reviewed in [7]). Besides glaucoma, a number of diseases such as neurodegenerative diseases (like Alzheimer's disease, Parkinson's disease [8] and amyotrophic lateral sclerosis [9]) and Paget's disease of bone (PDB) [3,10] have been associated with mutations in *OPTN*. PDB is the second most frequent metabolic bone disorder, after osteoporosis [11], affecting between 1% and 3% of individuals over the age of 55 years [12,13]. This disease is characterized by focal abnormal bone remodelling, with increased bone resorption and accelerated, excessive, and disorganized new bone formation. The pathophysiology of PDB is currently an area of intensive investigation, and this disease seems to have both genetic and non-genetic causes. Fifteen to 40% of affected patients have a first-degree relative with PDB, and numerous studies have described extended families with PDB exhibiting an autosomal dominant mode of inheritance [14–16]. Linkage studies in these families have identified a number of susceptibility loci on chromosomes *6p21* (*PDB1*) [17], *18q21.1–22* (*PDB2*) [18], *5q35* (*PDB3*), *5q31* (*PDB4*) [15], *2q36* (*PDB5*) [19], *10p13* (*PDB6*) [20] and *18q23* (*PDB7*) [19]. The regions identified are typically large and contain several genes that could be possible candidates based on their known functions. Moreover, a genome-wide scan in British families with PDB has shown a linkage to the *10p13* (*PDB6*) locus [20,21]. Recently, reanalysis of data from this genome-wide scan confirmed a genetic association to the *10p13* locus [10], namely to the rs1561570 SNP located in *OPTN* gene, but no PDB causal mutation has been reported to date in this locus. Our group replicated the strong and statistically significant genetic association of rs1561570 (p-value = 5.65×10^{-7}) with PDB in the French-Canadian population [3], and also identified a functional SNP in *UCMA/GRP*, but with a marginal association with PDB, and a rare variant in *OPTN* promoter predicted to alter the putative binding of bone transcription factors [3]. In this work, we have assessed the possible contribution of each significant variant identified in the *OPTN* gene and have selected rs3829923 and rs2234968 to perform the association study and functional analysis in order to determine the involvement of these variants in the PDB pathophysiology. We also analysed the effect of a rare variant (RV −9906) found in *OPTN* promoter and described in our previous work [3].

2. Material and methods

2.1. Study participants and candidate gene sequencing

This study was approved by the CHU de Québec-Université Laval Ethics Committee and all participants have signed a consent form before inclusion 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. Clinical characteristics of these cohorts were previously published [22–24]. For each individual, peripheral blood was obtained as described in [3]. All patients and healthy donors studied here were non-carrier of the *P392L* mutation within the *SQSTM1* gene (*PDB3* locus). RNA from total blood was collected as described in [3].

2.2. OPTN genetic variation screening

The strategies used throughout this study are briefly summarized in Fig. S1. To search for variants within the *OPTN* gene, the exons, their exon–intron boundaries, 5′ and 3′-UTRs and the basal promoter were sequenced as previously described [3]. Thirty samples from patients with PDB and five healthy controls from the French-Canadian population underwent Sanger sequencing, which we refer as the discovery group. For the association study, the SNPs rs3829923 and rs2234968 were selected to be genotyped in a group of 225 controls and 298 patients, based on their frequency in the discovery group and location in the gene. The allele frequencies were calculated as previously described [3].

2.3. Bioinformatic analysis

To identify the potential effect of the variants found in the coding regions we used the online translate tools Expasy (http://web.expasy.org/translate/), SIFT (http://siftdna.org/www/SIFT_dbSNP.html), Condel (http://bg.upf.edu/fannsdb/), Polyphen (http://genetics.bwh.harvard.edu/pph2/) and Mutation taster software (http://www.mutationtaster.org/). Potential changes in the splice sites were predicted using the Human Splicing Finder tool (http://www.umd.be/HSF/). To identify transcription factor binding sites (TFBSs) for the variants located in promoter regions we used TFsearch (http://diyhpl.us/~bryan/irc/protocol-online/protocol-cache/TFSEARCH.html) and Consite (http://consite.genereg.net/). To analyse the effect in the mRNA secondary structure we used RNA fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). To search for SNPs in *linkage disequilibrium* (LD) we used the SNAP tool (https://www.broadinstitute.org/mpg/snap/).

2.4. Preparation of human in vitro-differentiated mature osteoclasts

Human mature osteoclasts were differentiated *in vitro* using mononuclear cells from blood of healthy controls and patients with PDB. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Ficoll-Hypaque. The cells were re-suspended (3×10^6 cells/mL) in OPTI-MEM containing 10% FBS (Life technologies). The cell suspension was added to 6-well plates (9×10^6 cells/well) and to Lab-Tek 8 well-slides ($3–6 \times 10^5$ cells/well). After 24 h, the cells were washed thoroughly and lymphocyte-free adherent cells were incubated for three weeks with M-CSF (25 ng/ml, Life technologies) and RANKL (30 ng/ml, Fitzgerald).

2.5. Quantitative real-time PCR

In order to test if rs2234968 had an impact on *OPTN* gene expression, we performed qPCR as previously described [3]. We used RNA from PBMCs of six patients and three healthy donors. A quantity of cDNA corresponding to 10 ng of total RNA was used in these analyses. The primers used are displayed in Table S1. For every assay, a negative control was performed in the absence of cDNA template. Fluorescence was measured at the end of each extension cycle and melting profiles of each reaction were performed to check for unspecific product amplification. Relative mRNA expression was calculated using the ΔΔCt method [25]. Normalization was performed using the reference gene peptidylprolyl isomerase B (*PPIB*), since it was defined as a suitable reference gene for mRNA quantification in peripheral whole blood [26].

2.6. Western blot analyses

Osteoclasts derived from PBMCs from patients and controls were washed once in PBS and lysed using Trizol. The protein concentrations were determined using the Bradford reagent (Bio-Rad). Proteins were separated by an 8% SDS-polyacrylamide gels and transferred onto PVDF membranes (Life technologies). After transfer, the membranes were blocked in 0.1% TBS/Tween 20 containing 5% nonfat dried milk at room temperature for 1 h. The membranes were then incubated overnight (ON) at 4 °C with anti-OPTN (1:500, #100000 Cayman), or anti-αTubulin (1:2000, #2144 Cell Signaling) in 0.1% TBS/Tween 20, followed by extensive washing using 0.1% TBS/Tween 20 and an incubation with HRP-conjugated secondary antibody (1:2000, Cell