Rev Osteoporos Metab Miner. 2021;13(1):17-20

Search for variants of the *LRP4* gene in women with high bone mass and in patients with Chiari type I malformation

DOI: http://dx.doi.org/10.4321/S1889-836X2021000100004

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Date of receipt: 22/12/2020 - Date of acceptance: 01/03/2021

This study was awarded a scholarship to attend the 2019 ASBMR meeting (Orlando, USA)

Summary
Objetive: LRP4 is an essential facilitator in sclerostin-specific inhibition of the canonical Wnt pathway. Mutations in LRP4 have been associated with various conditions, including bone growth disease, sclerosteosis, and Chiari type I malformation (CMI).
Material and methods: The *LRP4* has been re-sequenced in two patient cohorts with high bone mass phenotype (HBM) and with CMI aimed at finding causal variants.
Results: Among the mutations found, we would highlight: 1) a missense mutation in a patient with CMI, which does not co-segregate with the phenotype in the family; 2) a previously undescribed intronic mutation (c.3364+16A>C) in a woman with HBM; and 3) an intronic mutation in a woman with HBM with a very low frequency in the European control population.
Conclusions: Although we have not found variants in LRP4 to explain the HBM or CMI phenotype in the patients studied, we encourage other researchers to analyze the *LRP4* gene in their patients as it is a good functional candidate for both phenotypes.

Key words: LRP4, HBM, Chiari malformation type I, bone mineral density, sclerostin.

Introduction

The Wnt signalling pathway is involved in a wide range of processes, including bone development and homeostasis[1]. In accordance with this, mutations have been identified in various components of the Wnt pathway that cause different musculoskeletal diseases[2]. The canonical Wnt pathway begins with the formation of a heterotrimeric complex between a co-receptor, LRP5/6, a ligand, WNT, and a receptor, FZD, which produces an accumulation of β-catenin that, once in the nucleus will activate the transcription of numerous important target genes for bone[1]. This activation is finely regulated by a series of extracellular inhibitors such as DKK1 and sclerostin that bind to LRP5/6, preventing the formation of the heterotrimeric complex. For DKK1 and sclerostin to exert their inhibitory activity, they must form another heterotrimeric complex with LRP5 and KREMEN1/2 or LRP4, respectively. Although in the case of DKK1 the presence of KREMEN does not seem to be necessary to carry out a correct inhibition, the presence of LRP4 is essential for the inhibitory function of sclerostin[3,4].

LRP4 mutations have been described in humans which cause different diseases that affect not only bone mass, but also the regulation of the extremities and kidneys among other, depending on the position of where the mutation occurs[2]. Specifically, mutations in the central cavity of the third β-propeller cause sclerosteosis, characterized by variable syndactyly and progressive

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bone overgrowth, particularly severe in the facial skeleton and skull. These patients present an increase in the Wnt pathway in osteoblasts, generating an increase in bone formation[4-6]. In addition to these mutations, in 2017, Merello et al.[7] described a mutation in the second β-propeller domain (p.Thr851Arg) that cosegregated with the phenotype in a family with type I Chiari malformation (CMI). This is a malformation of the central nervous system characterized by a caudal displacement of the cerebellar tonsils, which generates varied symptoms both in onset and in severity (Orphanet, https://www.orpha.net/consor/cgi-bin/ index.php). Although some patients with CMI may be asymptomatic, others can present with a suboccipital headache and neck pain, among others. It is interesting to note that this malformation was described in a patient who presented a phenotype of high bone mass (HBM) due to gain-of-function mutations in LRP5, thus suggesting a possible relationship between CMI, the HBM phenotype and Wnt pathway[8]. Taking into account the role of LRP4 on the Wnt pathway and the important role that this pathway has in determining bone mineral density (BMD) and in the development of the skull, we hypothesized that mutations in LRP4 could be the cause of the HBM in women with this phenotype or of causing disease in patients with CMI. In this study, we have carried out a re-sequencing of the *LRP4* gene in a cohort of 10 women with the HBM phenotype and in a cohort of 12 patients with CMI.

Material and methods

Cohorts studied

Sarrión et al. describe the HBM cohort, in a study of 10 women with HBM phenotype[9], in which this phenotype is defined as the sum of the Z-score values of the lumbar spine and femoral neck are equal or greater than 4.

The CMI cohort studied here consists of 12 patients with unrelated CMI, diagnosed and treated at the Hospital del Mar in Barcelona. The diagnosis of CMI is based on the position of the cerebellar tonsils by brain magnetic resonance imaging (Achieva 3.0 T, Philips, Amsterdam, The Netherlands) with a herniation equal to or greater than 5 mm on a mid-sagittal T1-weighted image in the presence of signs or symptoms indicating neural compression at the cranio-vertebral junction, syringohydromyelia, cerebellar dysfunction or intracranial hypertension. In addition, information is available on 8 relatives of 4 of the patients with CMI.

LRP4 re-sequencing

Genomic DNA from CMI cases and their relatives and from HBM women was isolated. women was isolated from peripheral blood leukocytes using the Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA), according to with the manufacturer's instructions. Regarding the re-sequencing, specifically, we have amplified the exons that code for the mutations that cause Cenani-Lenz syndrome (p.D137N, p.C160Y, p. D449N, p. T461P, p.L473F, p. D529N, p.L953P, p.C1017R, p.R1277H, p.E1233K) and the third β-propeller domain where the mutations causing myasthenic syndrome and sclerosteosis are located. Amplification of each of these fragments was done by polymerase chain reaction (PCR) using GoTaq Flexi DNA polymerase (Promega). The PCR fragments were analyzed by agarose gel electrophoresis method and their purification was done on MultiScreen™ Vacuum Manifold 96-well plates (Merck Millipore). The purified PCR products were sequenced using the Sanger method in the genomics service of the CCiTUB (Genómica, Parc Científic, Barcelona, Spain). The labeling kit used was BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher), detection and electrophoresis were carried out on the 3730 Genetic Analyzer and 3730xl Genetic Analyzer (ThermoFisher) automatic capillary sequencers models. The design of the primers was based on the consensus sequence ENSG00000134569 (LRP4; GRCh37.p13). Their sequence is presented in table 1.

Bioinformatic analysis and *in silico* predictions of the effect of the variants

To identify and characterize all the variants, we have used information culled from the Ensembl database GRCh37.p13 and ENCODE. The minor allele frequency (MAF) of each of the variants is extracted from the non-Finnish European population of gnomAD v2.1.1. We have used SIFT, (http://sift.bii.a-star.edu.sg/) and PolyPhen (http://genetics.bwh.harvard.edu/pph2/) to test the effect of the change variants of amino acid (missense). The GTEx database (www.gtexportal.org/home/) has been used to identify variants that act as eQTLs.

Results

Re-sequencing of *LRP4* in women with HBM phenotype and in patients with CMI

In the *LRP4* re-sequencing have identified 12 variants, of which one was not previously described (c.3364+16A>C; table 2). This variant has been found in heterozygosity in the HBM2 woman who presents a Z-score sum (lumbar spine and femoral neck) of 4.6. Six of the 12 variants identified are found both in the cohort of women with the HBM phenotype and in the cohort of patients with CMI, with a similar frequency in both cohorts to that of the European population. Furthermore, we have found 4 variants only present in the HBM cohort and 2 variants only present in the CMI cohort. All the variants described, except rs558515201 and the variant c.3364+16A>C, appear as eQTL of different genes and tissues in the GTEx database. Specifically, the rs17790156, rs61898529, rs2306028, rs964551 and rs2306032 variants are *LRP4* eQTL.

Discussion

Different studies have highlighted the role of the *LRP4* gene in determining BMD, as mutations in it generate a phenotype of bone overgrowth. Furthermore, it has also been associated with CMI in a report on whole exome sequencing[7]. In the study presented here, we have re-sequenced the regions of the gene that contain mutations associated with different conditions. In it, we have only found a missense mutation in *LRP4* in a patient with CMI that does not co-aggregate with the phenotype in the family, thus ruling out its ability to cause the disease (Figure 1). It is interesting to note that we have found a variant not previously described (c.3364+16A>C) and the variant rs558515201, which has a very low frequency in the European population (MAF=0.00006480), in heterozygosity in two women with HBM. Furthermore, the rs3751097 and rs540384558 variants, present in both HBM and CMI patients, are found in a cis regulatory element categorized as a distal enhancer-like signature by ENCODE. On the other hand, contrary to expectations, we found in both cohorts a slightly lesser frequency for the minor allele of the rs2306032 variant

Table 1. Primers used in *LRP4* re-sequencing

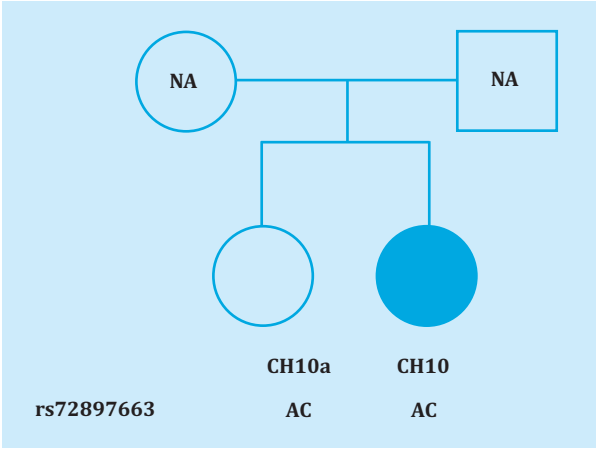
Primer	Forward	Reverse
LRP4_Frag1	GGGCTTTAAGTCAGGCTTCC	CAACCCAACAGCCTGAGGT
LRP4_Frag2	GAGTGGGAGGACGACAGAAG	TTGCAAACCACTGGCCTATT
LRP4_Frag3	ATAGTGCCTGGCCCAAAAA	GCCCAGCTACACCACACTTT
LRP4_Frag4	TCGCCTTAAATTATGGTTGC	ACCACTGGGTTAGGGTCTCC
LRP4_Frag5	AGTGGGAGAGCTGCTTTCTG	CCATCTGCAAGGAAGGAAGA
LRP4_Frag6	TGCGTTTTCCTTGATTTCCT	GATGCAAGCTTCCTCTCCAC
LRP4_Frag7	AAGGTTGAGATAATGCACATGAA	ACAGGTCACCGTCTTTCTGG

Table 2. Variants found in *LRP4* re-sequencing in women with HBM phenotype and in patients with CMI. The genotypes in the first column are indicated by the nucleotides of the coding strand of the *LRP4* gene, which is the reverse of that which is used in a standard way to indicate genomic SNVs. For this reason, for example, the A allele of c.431-12G> A is equivalent to the T allele of rs139371503, and the same for the other variants

LRP4	Number rs	Type	MAF			Effect
			EUR	HBM	CMI	
c.431-12G>A	rs139371503	I	0.011 (T)	0.05 (T)	-	eQTL
c.1309+24G>A	rs3751097	I	0.101 (T)	0.2 (T)	0.125 (T)	eQTL
c.1309+87_1309+91dup	rs540384558	I	0.038 (dup)	0.05 (dup)	0.083 (dup)	eQTL
p.Asn501His	rs72897663	M	0.044 (G)	-	0.042 (G)	eQTL T;B
p.Lys546=	rs10838631	S	0.011 (T)	0.05 (T)	-	eQTL
c.2507-73C>T	rs558515201	I	<0.01 (A)	0.05 (A)	-	
c.2507-204C>T	rs61898529	I	0.093 (A)	-	0.042 (A)	eQTL
c.2612+104T>A	rs17790156	I	0.09436 (T)	0.2 (T)	0.125 (T)	eQTL
c.3004+18C>A	rs2306028	I	0.127 (T)	0.1 (T)	0.042 (T)	eQTL
c.3364+16A>C	-	I	-	0.05 (C)	-	
c.3536+22C>A	rs964551	I	0.227 (G)	0.15 (G)	0.083 (G)	eQTL
c.3700-21C>G	rs2306032	I	0.329 (G)	0.25 (G)	0.21 (G)	eQTL

MAF: minority allele frequency; EUR: non-Finish European population of gnomAD v2.1.1.; HBM: high bone mass phenotype; CMI: Chiari type I malformation; dup: dup: duplication; M: missense variant; I: intronic variant; S: synonymous variant; eQTL: described as eQTL in Gtex; T: tolerated by SIFT; B: benign by Polyphen-2.

Figure 1. Pedigree of the family of the CMI CH10 patient. NA: no data on the parents. Genotypes are indicated with the nucleotides of the coding strand of the *LRP4* gene, which is the reverse of that used in a standard way to indicate genomic SNVs. Therefore, allele C is equivalent to allele G of rs72897663



which has been defined as a protector in whole genome association study with total BMD[10].

The low number of patients must be taken into account as a limitation of our study. This could explain both the absence of causal variants in the *LRP4* gene in these two cohorts and the results contrary to those expected in allele frequency in the SNP rs2306032. Thus, increasing the size of the cohorts would provide us with a deeper understanding of the role of LRP4 on these phenotypes.

In conclusion, although *LRP4* is undoubtedly an important gene for bone biology, we have not found any variant that could explain the HBM or CMI phenotype. Despite these negative results, it is interesting to consider *LRP4* in the selection of candidate genes that may explain HBM or CMI-causing phenotypes.

Conflict of interests: The authors declare no conflict of interest.

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