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WNT16 rs2908004 missense variant acts as eQTL of FAM3C in human primary osteoblasts

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Summary

Introduction: WNT16 is an important gene in bone homeostasis, found in a very complex locus that also includes neighboring genes: ING3, FAM3C, and CPED1. In addition to the clear role of WNT16 in determining bone mineral density (BMD), evidence has also been found for the importance of these three neighboring genes in bone metabolism. Therefore, it remains to be clarified whether the variants in WNT16 associated with BMD carry out their effect on WNT16 or if they do so by modifying the expression of these neighboring genes.
Material and methods: We have determined the expression levels of CPED1 and FAM3C in primary osteoblasts and we have verified whether WNT16 variants behave as loci of quantitative expression traits (expression quantitative trait loci; eQTL) of these genes.
Results: The amino acid change variant rs2908004 in WNT16 acts as the eQTL of FAM3C in primary osteoblasts under the dominant model hypothesis.
Discussion: It is possible that the effect of this variant on BMD is due to the modification of the expression levels of FAM3C in addition to or instead of a direct effect of the mutant WNT16 protein resulting from the amino acid change.

Key words: Bone mineral density, *WNT16*, osteoporosis, transcription.

Introduction

WNT16 is a ligand of the Wnt pathway that has been extensively studied for its importance in regulating bone homeostasis. This has been confirmed with the phenotype of knock-out (KO) mice and conditional KO mice in osteoblasts (cKO), which show spontaneous fractures due to low cortical bone mineral density (BMD), low bone strength and high cortical porosity, keeping the volume of trabecular bone unchanged[1-4]. On the contrary, Wnt16 overexpression in osteoblasts and osteocytes produces an increase in BMD and bone strength in both trabecular and cortical bone[5-7]. Despite this, the precise mechanism by which WNT16 acts is not known and different studies indicate that the effect on canonical and non-canonical Wnt pathways could be tissue specific[1,8-11]. In bone, WNT16 is expressed mainly by osteoblasts and carries out its function both by stimulating bone formation and by inhibiting its resorption indirectly through osteoprotegerin (OPG) or directly affecting the osteoclasts differentiation[1,12].

Several genome-wide association studies (GWAS) have shown an association between the locus containing *WNT16* and various skeletal phenotypes, including BMD and risk of fractures[2,3,13-26]. *WNT16* is found in a very complex locus, where several genes in the region show an important role in bone metabolism. The genes *ING3* and *CPED1* at 5 'and *FAM3C* at 3' of *WNT16* belong to this locus (figure 1).

ING3 (Inhibitor Of Growth Family Member 3) is responsible for the regulation of chromatin, since it is part of the NuA4 histone acetyltransferase (HAT) complex that recognizes the trimethylated form of lysine 4 of histone H3[27]. Other functions unrelated to chromatin regulation include apoptosis promotion, DNA repair, and modulation of cell mobility[27]. ING3 is expressed in a multitude of tissues, especially in those with a higher proportion of cell growth, bone being one of those with the highest expression of ING3[27]. The in vitro cellular model of ING3 KO mesenchymal cells shows involvement of osteoblastogenesis and stimulation of adipogenic differentiation[28].

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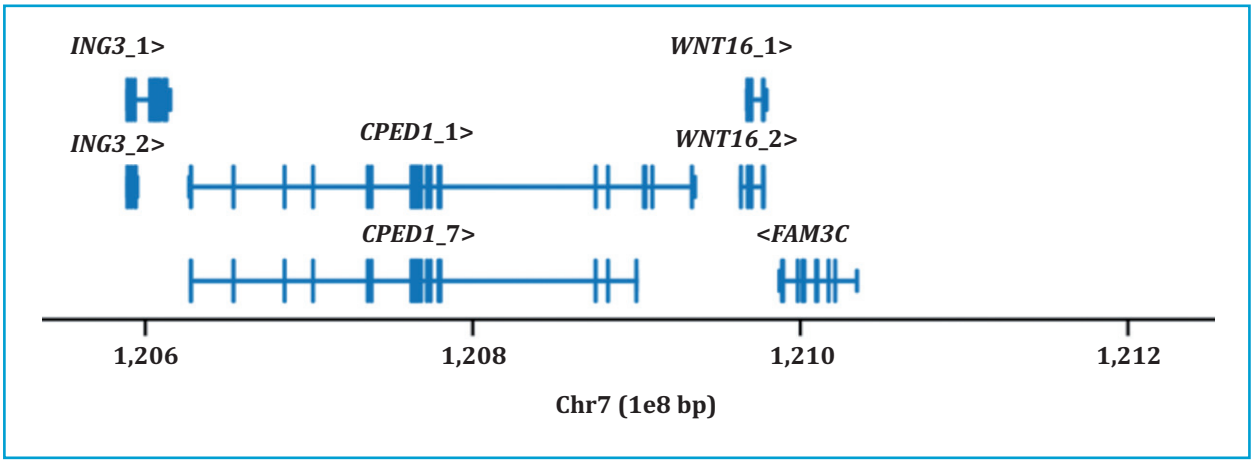
Figure 1. Genes at the locus: ING3_1 (ENST00000315870.5), ING3_2 (ENST00000339121.5), CPED1_1 (ENST00000 310396.5), CPED1_7 (ENST00000450913.2), WNT16_1 (ENST00000222462.2), WNT16_2 (ENST00000361301.2), and FAM3C (ENST00000359943.3) by GRCh37/hg19



No specific function is known for CPED1 (Cadherin Like And PC-Esterase Domain Containing 1) in humans or mice. In the latter, it is uniformly expressed in a variety of solid tissues, including bone, although it is not detected in the RAW264.7 cell line or in circulating leukocytes[29]. Furthermore, CPED1 presents different isoforms due to alternative splicing and three active promoter regions during osteogenic differentiation, indicating a complex regulation during differentiation[29].

FAM3C (Family of sequence similarity 3c) is a cytokine-like growth factor expressed in a multitude of tissues[30], which plays a very important role in the epithelial-mesenchymal transition (EMT) and subsequent metastasis during cancer progression[31]. Its relationship with bone metabolism has been confirmed with the KO mouse model, which presents alterations in the cortical and trabecular structure, with an increase in cortical BMD, which generates a decrease in bone resistance[30]. In in vitro studies, it was found that the mesenchymal cells extracted from these KO mice showed accelerated osteoblastogenesis[31].

The relationship of the genes present at the *'ING3-CPED1-WNT16-FAM3C'* locus with bone metabolism and their repeated association with BMD raises the question of whether there is a single causative gene and, if so, which is it, or if instead, all genes are contributing to the phenotype. To this end, in the present work we have determined whether those *WNT16* variants associated with BMD in a previous work by our group[32] are found to modify the expression of neighboring genes *CPED1* and *FAM3C.*

Material and methods

Cell culture

Human primary osteoblasts (hOB) were used for the loci assays that determine quantitative differences in gene expression (expression quantitative trait loci; eQTL). The hOBs were obtained from trabecular bone fragments discarded from knee replacement operations performed on women with osteoarthritis and who did not have any other disorder that could affect bone quality. The study was approved by the Clinical Research Ethics Committee of the Parc de Salut MAR (registration numbers: 2010/3882/I and 2013/5266/I) and was carried out in accordance with the Declaration of Helsinki, obtaining informed consent by written by all participants. The primary osteoblast culture protocol is described in Roca-Ayats et al.[33]. Briefly, bone samples were cut into small pieces and washed with phosphate buffered saline (PBS; Gibco, Life Technologies). These pieces were cultured in 140 mm plates with DMEM supplemented with 10% FBS, 1% w/s, 0.4% fungizone (Gibco, Life Technologies) and 100 µg/ml of ascorbic acid (Sigma-Aldrich). When the cells reached confluence, they were divided into three 75 cm^2 flasks, one for DNA extraction, one for RNA extraction, and the third for freezing and storage. Cells at passage 2 or lower were used for all extractions.

EQTL assay

DNA was extracted from cultured hOBs using the Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The concentration of the purified DNA and its quality was analyzed in a spectrophotometer (Nanodrop). The genotypes of the rs2908004, rs2707466, rs55710688 and rs142005327 variants were evaluated by Sanger sequencing using BigDye® Terminator v3.1 (Applied Biosystems) in the Genomics facilities of the CCiT of the University of Barcelona. The primers (Invitrogen, Thermo Fisher) were designed using Primer3 Input 0.4.0 (table 1). The total RNA of the cultured hOBs was extracted using the High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions and the quantification and quality of the RNA were checked using a Nanodrop spectrophotometer. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher), according to the manufacturer's specifications. RT-qPCR was carried out using UPL probes (Roche) on a LightCycler 480 Instrument II (Roche). *HMBS* gene expression was used as a normalization control and the relative quantification (fold change) was calculated using the second derivative method. The number and sequence of the probe used, as well as the primers used for the amplification of the *CPED1, FAM3C* and *HMBS* genes are shown in table 1.

Statistic analysis

For the statistical analysis of the eQTL, the WGassociation[34] function was used in RStudio. This function performs an association analysis between a given SNP and

a dependent variable (in this case the expression levels of *CPED1* and *FAM3C*) in five different genetic inheritance models: codominant [homozygous for major allele vs. heterozygous vs. homozygous for minor allele], dominant [homozygous for major allele versus (heterozygous + homozygous for minor allele)], recessive [homozygous for minor allele versus (heterozygous + homozygous for major allele)], over-dominant [heterozygous versus (homozygous for allele major + homozygous for minor allele)] and log-additive [each allele modifies the risk by an additive amount] .

Results

Cis-eQTL analysis

The variants rs2908004, rs2707466, rs55710688 and rs142005327 of *WNT16* have been described as cis-eQTLs, according to the GTEx database in different human tissues (table 2). Unfortunately, this database does not have information on any bone tissue. This is why, using our own database of human primary osteoblasts (n=45), we have tested whether these variants act as cis-eQTL of the neighboring genes of *WNT16: CPED1* and *FAM3C*. Only the rs2908004 variant has shown a significant association with *FAM3C* expression levels under the dominant hypothesis (p=0.03, table 3, figure 2). In addition, the rs2908004 and rs2707466 variants show a trend towards significance with *FAM3C* expression levels under the codominance hypothesis (p=0.05491) and under the dominant hypothesis (p=0.06954), respectively (table 3, figure 2). The presence of the G allele (rs2908004) and the C allele (rs2707466) are associated with an increase in the expression of *FAM3C* (table 3, figure 2). On the contrary, we have not found a significant association or trend between the *WNT16* variants analyzed and *CPED1* expression levels (table 3, figure 2).

Discussion

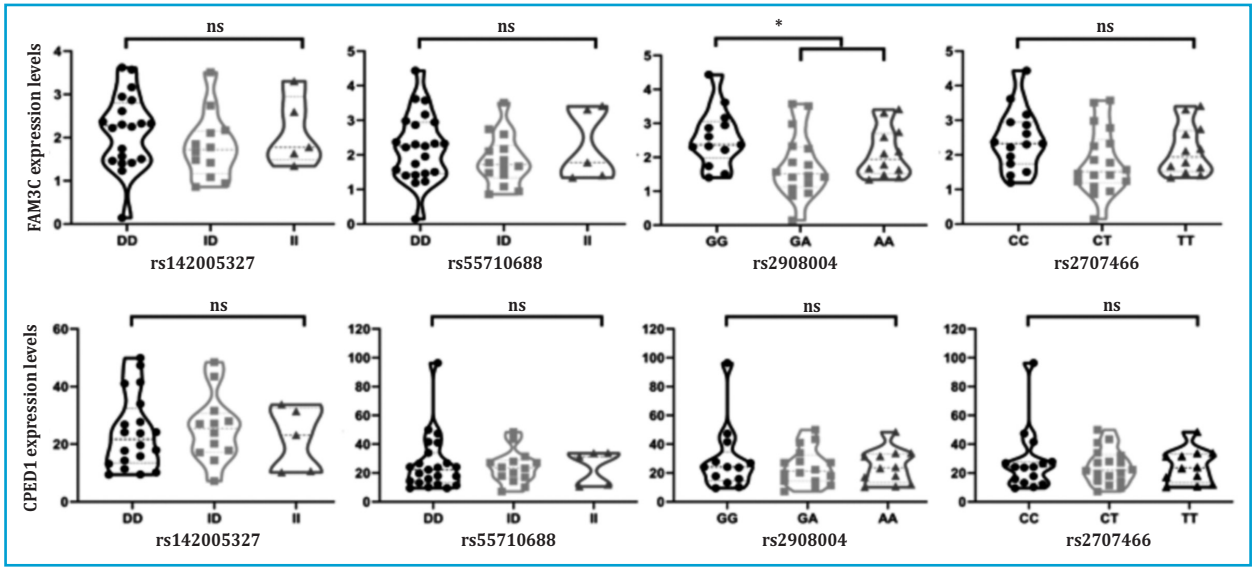
Over the past 20 years, many works have highlighted the importance of WNT16 in bone homeostasis. *WNT16* is found at position 7q31.31 along with 3 other genes also related to bone metabolism. In a previous study by our group, we found that the variants rs142005327, rs55710588, rs2908004, and rs2707466 were associated with BMD in a cohort of postmenopausal women from the Barcelona area (BARCOS)[32]. To determine whether these variants are related to an effect on *WNT16* or

Table 1. Primers used in the sequencing of four *WNT16* SNPs and for the RT-qPCR of *FAM3C, CPED1* and *HMBS*

Primer name	F (5'--> 3')	R (5'-->3')	Probe
WNT16-rs55710688	GGTAGCTCCAGTAAGAGATTC	CAGATTACCGTGTCTTTGGGT	
WNT16-rs2908004-rs142005327	ACTTTCAACTGAGGCTGGGG	CTGGAACTGGGGAGTCAGG	
WNT16-rs2707466	TGGGACAAAAACCAAAGGACG	TGACCACATGGGTGTTGTAAC	
FAM3C_qPCR	GGCAAATGGAAAAACAGGAG	TGTATGGCCTTCAGAAACTCAA	52
CPED1_qPCR	CCCAAGTCTGCCCTTGTTT	GAAGAAATAGGCTGTAACCCACA	6
HMBS_qPCR	TGCCCTGGAGAAGAATGAAG	CAGCATCATGAGGGTTTTCC	79

F: primer forward; R: primer reverse.

Figure 2. Violin plots of the expression levels of FAM3C (top) and CPED1 (bottom) according to the three genotypes (homozygous for the majority allele, heterozygous, homozygous for the minority allele) of the 4 variants studied



I: insertion; D: deletion.

to an effect on the expression of neighboring genes, we have verified whether they are acting as eQTL of *FAM3C* and *CPED1*. This work has allowed us to determine that the missense variant rs2908004 is acting as eQTL of the *FAM3C* gene under the hypothesis of a dominant model in human primary osteoblasts.

The missense variant rs2908004 (p.Gly72Arg/p.Gly82Arg) has been associated with different bone parameters by us and others[2,32,35-38]. This amino acid change from glycine to arginine is considered tolerated and benign by the pathogenicity predictors SIFT and PolyPhen-2, so its effect on BMD could be due to its role as eQTL and not to a change in the resulting WNT16 protein.

It should be taken into account that to obtain a more robust statistical significance having a bank with a greater number of primary osteoblasts is required. Unfortunately obtaining these samples is difficult and we have only managed to enter 45 samples into our bank.

In addition, it would be interesting to determine the expression levels of other neighboring genes that may be influencing BMD such as *ING3* or directly on the expression levels of *WNT16*, which have not been able to be quantified due to lack of RNA sample from primary osteoblasts.

Conclusion

Through this work we have determined that the variant rs2908004 of *WNT16* regulates the expression levels of the neighboring gene *FAM3C* under the hypothesis of a dominant model. If this association is confirmed in a larger primary osteoblast bank, it would indicate that the association of this variant with BMD could be due, at least in part, to the variation of *FAM3C* expression.

Table 2. Genes whose expression is modified by the variants rs2908004, rs2707466, rs55710688, rs142005327 in various human tissues (data extracted from the Genotype-Tissue Expression (GTEx) Portal)

SNP	Gen	Tissue
rs2908004	CPED1	Artery - Tibial
	CYCSP19	Testicle
	FAM3C	Skin - NE and E; Brain - Frontal Cortex (BA9); Esophagus - Muscularis
rs142005327	CPED1	Artery - Tibial
	FAM3C	Skin - NE and E; Muscle - skeletal; Heart - Left Ventricle; Chest - breast tissue; Heart - Atrial Appendix; Nerve - Tibial
	WNT16	Adipose - Subcutaneous
rs2707466	CPED1	Artery - Tibial
	CYCSP19	Testicle
	FAM3C	Skin - NE and E; Brain - Frontal Cortex (BA9)
rs55710688	CPED1	Artery - Tibial
	FAM3C	Skin - NE and E; Muscle - skeletal; Heart - Left Ventricle; Chest - breast tissue
	WNT16	Adipose - Subcutaneous

NE: not exposed to the sun (suprapubic); E: exposed to the sun (lower leg).

Table 3. Results of association of the 4 SNPs of *WNT16* and the expression of *CPED1* and *FAM3C*

				CPED1		*FAM3C*	
SNP	**A may**	**A min**	**HWE**	**Codominant**	**Dominant**	**Codominant**	**Dominant**
rs55710688	D	I	0.2597	0.90837	0.65906	0.44952	0.34621
rs2908004	G	A	0.2095	0.68815	0.38455	*0.05491*	**0.03061**
rs142005327	D	I	0.2325	0.84422	0.91336	0.49356	0.35142
rs2707466	C	T	0.2305	0.80454	0.50710	0.11531	*0.06954*

In bold, the significant associations (p<0.05) and in italics, those that show a trend.
A may: majority allele; A min: minor allele; HWE: Hardy-Weinberg equilibrium.

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

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