Experimental research

Rare variations in WNT3A and DKK1 may predispose carriers to primary osteoporosis

Johanna Korvala a,*, Marika Löijä a, Outi Mäkitie b, Etienne Sochette c, Harald Jüppner d,e, Dirk Schnabel f, Stefano Mora g, William G. Cole h, Leena Ala-Kokko a,i, Minna Männikkö a

a Oulu Center for Cell-Matrix Research, Biocenter and Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, P.O. Box 5000, 90014 University of Oulu, Finland
b Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
c Laboratory of Pediatric Endocrinology, BioNetwork, Division of Metabolic and Cardiovascular Sciences, San Raffaele Scientific Institute, Milan, Italy
d Department for Pediatric Endocrinology and Diabetes, Otto-Heubeiner-Centrum für Kinder- und Jugendmedizin, Charité, University Medicine Berlin, Berlin, Germany
e Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
f Department for Pediatric Endocrinology and Diabetes, Otto-Heubeiner-Centrum für Kinder- und Jugendmedizin, Charité, University Medicine Berlin, Berlin, Germany
g Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA
h Hospital for Sick Children, University of Toronto, Toronto, ON, Canada
i Oulu Center for Cell-Matrix Research, Biocenter and Department of Medical Biochemistry and Molecular Biology, Institute of Biomedicine, P.O. Box 5000, 90014 University of Oulu, Finland

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Childhood-onset primary osteoporosis is manifested as reduced bone mineral density, peripheral fractures and/or vertebral compression fractures. Until now, only mutations in LRPS have been shown to cause the disorder. Candidate gene analyses were performed on 15 patients with primary osteoporosis and 80 healthy controls using CSGE and sequencing. The genes studied included DKK1, DKK2, WNT3A, WNT10B, AXIN1, SOST, TPH1 and 5-HTR1B. Two rare variants in WNT3A (c.152A > G, p.K51R) and DKK1 (c.359G > T, p.R120L) were identified in two patients and their affected family members, but not in control subjects, suggesting a significance for the skeletal phenotype. The in vitro studies of variants showed reduced signaling activity in p.K51R-Wnt3a, while no differences were observed between the WT and variant forms of DKK1. This study addresses the role of other components of the canonical Wnt signaling pathway besides LRPS in primary osteoporosis, and putatively associates WNT3A and DKK1 variants with the disorder. Future functional studies are needed to elucidate the functional effects of the variants.

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1. Introduction

Primary osteoporosis without features of osteogenesis imperfecta (OI) (i.e. non-OI primary osteoporosis) is a rare bone disorder affecting young children. Its exact prevalence is not known, but it has been estimated to be rarer than OI (with prevalence of 6–7:100 000 [1]). It is manifested in low bone mineral density (BMD), vertebral compression fractures and/or long bone fractures resulting from low-energy trauma [2,3]. The etiology and genetic factors behind the disorder have remained largely unknown, and so far only the gene encoding low-density lipoprotein receptor-related protein 5 (LRPS), a co-receptor in the canonical Wnt signaling pathway, has been shown to cause non-OI primary osteoporosis in children [4,5].

Several components of the canonical Wnt signaling pathway are associated with BMD [6] and various other bone disorders ranging from osteoporosis [7,8] to the high bone mass (HBM) phenotype [9,10]. Canonical Wnts, e.g. WNT3A and WNT10B, activate the pathway and have been shown to affect osteoblast differentiation and to stimulate the growth of osteoblast precursors, while Axin-1 belongs to the complex responsible for β-catenin degradation [11]. Mutations in two inhibitors of the Wnt pathway result in opposite bone phenotypes: mutations in sclerostin (SOST) have been found to cause van Buchem disease and sclerosteosis [10], whereas DICKKOPF-1 (DKK1) is associated with the development of bone lesions in multiple myeloma (MM) [12]. In addition another inhibitor of the pathway, DKK2, participates in orchestrating osteoblast proliferation and maturation [13].

The most intensively studied component, LRPS, has another bone-related function along with its crucial role in the canonical
Wnt signaling pathway, in that it inhibits the expression of tryptophan hydroxylase 1 (TPH1) [14], which catalyzes the conversion of L-tryptophan to 5-hydroxy-L-tryptophan, a serotonin precursor. Moreover, it is the rate-limiting enzyme for serotonin synthesis in the enterochromaffin cells of the duodenum. Serotonin inhibits the proliferation of osteoblasts acting through the 5-hydroxytryptamine (serotonin) receptor 1B (HTR1B) [14].

Our aim here was to search for putative disease-causing mutations in genes involved in the canonical Wnt signaling pathway (WNT3A, WNT10B, AXIN1, DKK1, DKK2, SOST) and in TPH1 and 5-HTR1B in 15 children with non-OL primary osteoporosis, and to perform in vitro studies to examine the effects of the observed sequence variants.

2. Material and methods

2.1. Subjects

The series includes 15 patients who had been referred to a pediatric bone health clinic because of recurrent long bone fractures, bone pain, findings of osteopenia on imaging and/or low BMD. The clinical features of these patients (designated OP1–OP4, OP5, OP9–OP15 and OP17–OP19) are presented in Hartikka et al. [4]. The other patients in the original series, those with previously identified LRPS mutations (OP5, OP8, OP16) [4], were excluded from the current study. Furthermore, samples from two patients (OP7, OP20) [4] were not available for the current study. The diagnosis of non-OL primary osteoporosis was based on the following criteria: I) clinical and genetic exclusion of OI, II) exclusion of secondary causes of osteoporosis, and III) low BMD, defined as a Z-score below −2.0, a history of recurrent peripheral fractures (>3 fractures) caused by low impact trauma, and/or findings of vertebral compression fractures on X-ray films [2,3].

The control group comprised of 80 patients diagnosed with skeletal dysplasia (Schmid type of metaphyseal dysplasia, multiple epiphyseal dysplasia, or Ehlers–Danlos syndrome) from the same geographical area as the patients. The diagnosis of skeletal dysplasia had been clinically and/or radiographically confirmed, and none of the controls presented osteoporosis. The variants identified were also screened in affected and unaffected members of the probands’ families. The study was approved by the local ethics committee, and signed informed consent was obtained from each subject.

2.2. Genetic analyses

Genomic DNA was extracted from EDTA blood samples by standard procedures. Eight candidate genes were amplified using a polymerase chain reaction (PCR) with AmpliTaq Gold DNA polymerase (Applied Biosystems) and specific primers (available on request). The genes examined included DKK1, DKK2, WNT3A, WNT10B, AXIN1, SOST, TPH1, 5-HTR1B and the DKK1 promoter [15]. Samples were screened for mutations using conformation-sensitive gel electrophoresis (CSGE) [16]. Heteroduplexes found in CSGE and amplicons exceeding the optimal range of CSGE (200–450 bp) were confirmed by sequencing. The samples were sequenced using the ABI PRISM 3100 Genetic Analyzer and BigDye terminator cycle sequencing chemistry (Applied Biosystems). Mutation nomenclature is in accordance with the guidelines by den Dunnen et al. [17]. WNT3A and DKK1 protein sequences were aligned using the NCBI HomoloGene system.

2.3. DKK1 and Wnt3a constructs

The wild-type DKK1 construct containing a Flag tag (WT-DKK1-flag) originally made in Dr. Xi He’s laboratory was received from Dr. Warman’s laboratory and the wild-type mouse Wnt3a (WT-Wnt3a) in pRES2-EGFP vector was received from Prof. Vainio’s laboratory. The DKK1 c.359G>T; p.R120L and WNT3A c.152A>G; p.K51R variants were introduced into the WT-DKK1-flag and WT-Wnt3a constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions with specifically designed primers (available on request). Constructs were confirmed by direct sequencing.

The wild-type construct for full-length LRP5 with a mycHis-tag (WT-LRP5-mycHis) was received from Dr. Warman’s laboratory [18]. The reporter constructs, SuperTOPflash (STF) and β-galactosidase (β-Gal–CMV), were provided by Prof. Vainio, and used to study the effect of DKK1-conditioned media and Wnt3a constructs on the activity of the canonical Wnt signaling pathway.

2.4. Producing DKK1 (DKK1–CM) and Wnt3a-conditioned media (WNT3A–CM)

Conditioned media were produced for WT-DKK1 (DKK1–CM) and the DKK1 variant (R120L-DKK1–CM), by culturing Chinese hamster ovarian (CHO) cells in Dulbecco’s modified Eagle’s medium (DMEM) (BIOCHROM AG) with 10% fetal bovine serum (FBS) (HyClone) and plating the cells on a10 cm plates at 2 × 10^5 cells/plate followed by transfection 24 h later with 3 µg of WT- or R120L-DKK1 construct using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The DKK1-conditioned media were collected 48 h later and stored in −70 °C for later use. A control medium was produced by transfecting CHO cells with 3 µg of pcDNA3.1- vector. Expression of DKK1 was confirmed by Western blotting and the DKK1 flag was detected using the anti-FLAG M2 antibody (Stratagene).

Wnt3a-conditioned medium (WNT3A–CM) was produced and collected from mouse L1 cells expressing Wnt3a (L Wnt3a; ATCC CRL-2647) following the manufacturer’s instructions. The control-conditioned medium (L1–CM) was prepared from a normal L1 cell line (ATCC CRL-2648) using the same protocol as for the L Wnt3a cells.

2.5. Transfections and luciferase assay

CHO cells were plated onto a 24-well plate at 2 × 10^4 cells/well 24 h prior to transfection. To study the effect of WT-DKK1 and R120L-DKK1 on the activity of the canonical Wnt signaling pathway, the WT-LRP5 construct (20 ng), STF (100 ng) and β-Gal–CMV (5 ng) were co-transfected into the cells using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The pcDNA3.1- vector was used to raise the total amount of transfected DNA to 250 ng/well. Five hours later 150 µl of either WT-DKK1–CM or R120L-DKK1–CM and 150 µl of Wnt3a-CM or L1–CM and 200 µl of 10% FBS-DMEM were added to each well. The cells were harvested 48 h after the transfections. Each transfection was performed in triplicate and repeated separately at least three times.

To study the effect of WT-Wnt3a and K51R-Wnt3a on signaling activity, the CHO cells were plated for transfections as described above and co-transfected with 10 ng of WT-Wnt3a, K51R-Wnt3a or pRES2-EGFP, 20 ng of WT-LRP5, 100 ng of STF construct and 5 ng of β-Gal–CMV using Lipofectamine (Invitrogen). In addition, pcDNA3.1- vector was used to complete the total amount of transfected DNA to 200 ng. The cells were harvested 48 h after transfection.

2.6. Measurement of luciferase activity

The cells were lysed using Cell culture lysis reagent (CCLR) according to the manufacturer’s protocol (Promega) and luciferase (STF/pGL3 Basic luc) activity was measured using a luciferase assay.
A heterozygous missense variant c.152A > G, p.K51R was detected in WNT3A in patient OP4 (Fig. 1A III:1), in his affected sister (Fig. 1A II:2) and in his mother (Fig. 1A II:1), but not in his healthy siblings (Fig. 1A III:3 & III:4) or his father (Fig. 1A II:2). The father had fractures, and there was also a history of osteoporosis on the paternal side of the family. The patient’s paternal grandmother had decreased BMD after the menopause (Fig. 1A II:1) and his paternal aunt has had two fractures following moderate traumas (Fig. 1A II:3).

3.2. Patient OP17

Patient OP17 (Fig. 1B II:1) had his first fracture (of the collar bone) at the age of 5 years after falling on concrete. In 2002, at 10 years, he slipped on ice and sustained fractures of the T5 and T7 vertebrae. BMD was first measured in 2002, showing a mild reduction, 0.564 g/cm², corresponding to an age-matched Z-score of −1.9 in the lumbar spine (L1−L4). His alkaline phosphatase levels were slightly elevated (192–434 IU/l) during the years 2002–2005. Bone biopsies from two sites in the right iliac crest performed in 2003 confirmed the diagnosis of osteoporosis, and treatment with pamidronate was started.

Patient OP17 (Fig. 1B II:1) had a heterozygous c.359G > T, p.R120L missense variant in DKK1, as did his affected sister (Fig. 1B II:2). Both parents were also affected, but the father (Fig. 1B I:2) did not carry the variant, while the mother (Fig. 1B I:1) was not willing to participate in the genetic analysis. The patient’s sister (Fig. 1B II:2) has osteoporosis (Z-score −2.1) and has suffered vertebral compression fractures. His mother (Fig. 1B I:2) is osteopenic (Z-score −1) and his father (Fig. 1B I:1) has been diagnosed with osteoporosis (Z-score −2.7).

3.3. Functional significance

The functional significances of the WNT3A and DKK1 variants were examined in vitro in terms of the effect of K51R-Wnt3a and R120L-DKK1 on the activity of the Wnt signaling pathway. The activity of the pathway was significantly reduced in CHO cells transfected with K51R-Wnt3a as compared to those transfected with WT-Wnt3a (P = 0.01) (Fig. 2A). Wild-type DKK1 (WT-DKK1-
CM) and variant (R120L-DKK1-CM)-conditioned media inhibited the pathway equally (Fig. 2B), and the activity of the pathway did not differ between CHO cells treated with WT-DKK1-CM and R120L-DKK1-CM in any of the culture media used (Fig. 2B).

4. Discussion

We present here rare variations in the components of the canonical Wnt signaling pathway, WNT3A and DKK1, associated with a similar bone phenotype (i.e. non-OI primary osteoporosis) as caused by LRP5 mutations [4,5], supporting a role for canonical Wnt signaling in the development of the disorder.

A c.152A > G, p.K51R variant in the WNT3A gene was found concurrently with the bone phenotypes in patient OP4 and his affected mother and sister, but not in his healthy siblings. The father did not have the variant, but had suffered fractures in connection with sports and had a family history of osteoporosis. In the case of DKK1, a c.359G > T, p.R120L variant was observed in OP17 and his affected sister. Both parents had osteoporosis and the father did not have the variant, while the mother’s DNA was not available for the study. Hence the origin of the variant remained uncertain. In both families (Fig. 1A and B) the affected children’s phenotypes were more severe than any of those in the parents, having arisen in early childhood. The same observation was made by Hartikka et al. [4] and Korvala et al. [5]. The differences in the severity of the bone phenotypes and in the onset of osteoporosis could be explained by variations in mutation penetrance, for example, or by presence of some other underlying genetic factors that predispose carriers to bone deficiencies [19,20], but it may equally well be that the disorder is multigenic [21].

The significance of these findings is supported by the evolutionary conservation of the variant sites between species (Fig. 2A and B), and previous research has shown the importance of WNT3A and DKK1 for bone health. Both have been associated with MM [12,22,23] and the development, differentiation and/or maturation of osteoblasts [13,22]. Our in vitro studies of Wnt3a showed that the variant p.K51R-Wnt3a reduced signaling activity as compared to the WT-Wnt3a, which may contribute to the bone formation and hence affected phenotype. No statistically significant differences between the variant and wild-type DKK1 constructs were revealed in terms of signaling activity, but it is possible that the variant exercises its influence through mechanisms that were not examined here. In fact the DKK1 variant locates on N-terminal cysteine-rich domain (Dkk_N) that does not affect Wnt signaling (consistent with our results), but has shown independent signaling functions and may employ unknown receptors on other pathways [24]. Hence further research is required to elucidate the effect of the variants.

The study suggests that variants in other components of the canonical Wnt signaling pathway in addition to LRP5 may contribute to the development of primary osteoporosis. The results support the significance of WNT3A and DKK1 in bone biology and indicate a possible role for the WNT3A and DKK1 variants in the etiology of primary osteoporosis. Finally, as the disorder is rare and findings are rare, the results cannot be readily applied in larger populations, but a better understanding of the effects and significance of the WNT3A and DKK1 variants will be achieved through the necessary additional in vitro studies.

Accession numbers

The GenBank accession numbers for the reference sequences of each candidate gene were NM_012242.2 (DKK1), NM_014421.2 (DKK2), NM_033131.3 (WNT3A), NM_003394.3 (WNT10B), NM_181050.2 (AXIN1), NM_025237.2 (SOST), NM_004179.2 (TPH1) and NM_000863.1 (5-HTR1B). Accession number for WNT3A p.K51R in the NCBI database is rs145797401.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ejmg.2012.06.011.

References


Web resources