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Functional studies of DKK1 variants present in the general population

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Summary

Objective: In recent decades, genes associated with bone mass and osteoporotic fracture risk have been identified, several of which belong to the Wnt pathway. In this project, the functionality of 7 missense mutations of the gene *DKK1* –an inhibitor of the Wnt pathway– present in the general population was studied.
Material and methods: In vitro studies of the luciferase reporter gene were carried out to measure Wnt pathway activity in the presence or absence of wild-type or mutated DKK1, and western blot studies, to evaluate if the different mutations affect its synthesis and/or stability.
Results: The DKK1 protein with the p.Ala41Thr variant shows lower pathway inhibitory activity compared to the wild-type protein. Significant differences were also observed between the experiments performed in the absence of DKK1 and those that include DKK1 with the p.Ala41Thr mutation. Western blots showed that the amount of protein was similar for all variants, both mutated and "wild-type, so the loss of p.Ala41Thr activity did not seem to be due to a lack of protein. The rest of the mutations did not show different behavior from that of the wild DKK1 protein.
Conclusions: The missense variant p.Ala41Thr of the DKK1 protein, with a population frequency of 0.013%, shows a partial loss of its inhibitory function, which is not due to the lack of expression. This gene variant could lead to an increase in bone mineral density in those people in the general population who carry this mutation.

Key words: *DKK1, functional studies, missense variants, luciferase, Wnt pathway, High Bone Mass (HBM), osteoporosis.*

Introduction

The Wnt pathway's role in regulating bone remodeling has been demonstrated in multiple studies. On the one hand, polymorphisms have been described in several genes of the Wnt pathway that show an association with bone mineral density (BMD) and the risk of fracture[1-6]. Rare or infrequent mutations have also been described in various genes of the Wnt pathway, which cause more rare bone phenotypes, such as osteoporosis-pseudoglioma (OPPG, OMIM 259770)[7], autosomal recessive osteogenesis imperfecta of type XV (OMIM 615220)[8], and osteosclerosis (OMIM 144750)[9]. The Wnt pathway begins with the formation of a heterotrimeric complex between the Frizzled receptor, the LRP5 co-receptor and the Wnt ligand. Once this complex is formed, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it can activate the transcription of numerous target genes. In osteoblasts, the Wnt pathway has been shown to activate the transcription of genes that clearly contribute to bone formation[10]. In addition, this pathway is finely regulated by a series of extracellular inhibitors, including the protein sclerostin, encoded by the *SOST* gene, and the DKK1 protein, encoded by a gene with the same name. These two proteins perform their function, preventing the formation of the heterotrimeric complex. The proteins sclerostin and DKK1 thus form other heterotrimeric complexes, together with LRP5 and LRP4 (in the case of sclerostin)[11,12] and together with LRP5 and Kremen (in the case of DKK1)[13].

The transgenic over-expression of the *DKK1* gene in osteoblasts produces a relative decrease in the number of osteoblasts compared to that of osteoclasts, thus producing a decrease in bone formation. Similarly, in mice, the homozygous deletion of the *DKK1* gene is lethal, but deletion in heterozygosis presents a phenotype of bone overgrowth (high bone mass)[14,15].

In the past decade, thanks to the direct effect on osteoblastogenesis inhibition and the indirect activation of osteoclastogenesis[16], sclerostin and DKK1 have become interesting targets in osteoporosis treatment. Regarding DKK1, antibodies have been developed (BHQ880, DKN-01 and PF-04840082), the first of which in the clinical trial phase in postmenopausal women with low bone mineral density (BMD)[17-19].

In our group's previous study[20], *DKK1* was sequenced to identify variants that could explain the high bone mass (HBM) phenotype , defined by a femoral + lumbar Z-score > 4, present in 15 women. In one of them, a missense mutation was found (p.Tyr74Phe) that co-segregated with the HBM phenotype in the family. In another study of *DKK1* gene sequencing in postmenopausal women of the BARCOS cohort, we found another missense mutation (p.Arg120Leu) in another woman with HBM[21]. In addition to these mutations, in the general population there are other variants of change of direction in DKK1 (http://exac.broadinstitute.org/), whose effect in terms of bone mass has not been reported.

In the present work, we have conducted *in vitro* studies of the mutations p.Arg120Leu and p.Tyr74Phe, together with other missense mutations of DKK1 frequent in the general population (p.Met16Leu, p.Ala41Thr, p.Pro84Leu, p. Ala106Thr, p.Ser157Ile), to assess their possible involvement in bone phenotypes.

Material and method

Expression and mutagenesis vectors

The Wnt1-V5 mouse expression vectors, mesdc2, human wild-type LRP5, pRL-TK, PGL3-OT and DKK1-FLAG22, were courtesy of Dr. Wim van Hul (Antwerp, Belgium). Mutations p.Met16Leu, p.Ala41Tyr, p.Tyr74Phe, p.Pro84Leu, p.Ala106Thr, p.Arg120Leu, p.Ser157Ile were introduced into the DKK1-FLAG expression vector using the Quick Change Site-Directed Mutagenesis kit (Stratagene). The presence of mutations and the absence of errors were verified by Sanger sequencing.

Cell culture, production of conditioned medium and western blot

HEK293 cells, cultured with DMEM medium supplemented with FBS (10% V/V, Gibco, LifeTechnologies) and 1% streptomycin-penicillin (Gibco, LifeTechnologies) and maintained in incubators at 37°C at 5% in CO_2. Were used. 3×10^5 cells were seeded per well in 6-well plates, 24 h before transfection. 2,000 ng/well of the mutated or wild-type DKK1-FLAG plasmids were transfected. The transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 24 h, the medium was changed, reducing from 2 to 1 ml of DMEM, without FBS (Fetal Bovine Serum) or antibiotics. 48 hours after transfection, the supernatant of each condition was collected. The proteins of the conditioned medium were concentrated using Amicon Ultra filters (Millipore) and quantified by the BCA assay (Pierce). The proteins from the conditioned media (4.5 µg/lane) were separated by electrophoresis in a polyacrylamide gel with SDS (SDS-PAGE) and transferred to a nitrocellulose membrane. For the western blot analyses, Abcam ab109416 antibodies against DKK1 and ab2413 were used against the extracellular protein fibronectin, as a load control. The images were developed using a secondary antibody conjugated with peroxidase (Sigma-Aldrich). For each mutant conditioned medium was obtained in 2 different days and the analysis was carried out by western blot 2 times with these conditioned media.

Gene reporter assays

HEK293 cells were used, cultured as indicated in the previous section. 10^4 cells were seeded per well in 96-well plates, 24 h before transfection. Up to 5 plasmids were cotransfected in HEK293 cells: Mouse Wnt1-V5 (3.2 ng), mesdc2 (6.4 ng), human wild-type LRP5 (6.4 ng), pRL-TK (8 ng), and pGL3-OT (160 ng). In addition, depending on the experiment, the wild or mutated plasmid DKK1-FLAG (0.6 ng) was also co-transfected. If necessary, the

empty vector pcDNA3 was used to equal the total amount of DNA from each experiment. The transfection was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 48 h after transfection, the cells were lysed and the luciferase activity of Photinus pyrali and Renilla reniformis was measured using a Glomax Multi+luminometer (Promega) following the instructions of the Dual-luciferase reporter assay (Promega). Each experiment included 5 replicates and was repeated independently in 3 separate experiments.

Statistical analysis

A one-way blocked ANOVA model was carried out for each mutant taking into account the test factor, the day as a blocking factor and the response variable the relationship between the activities of the luciferases (Photinus pyrali vs. Renilla reniformis). Blocking is a technique to deal with the nuisance factor and this can influence response. For each mutant protein, the test factor has the following levels: control (refers to the activity of the luciferase resulting from the endogenous Wnt pathway), the activator (luciferase activity produced by the Wnt pathway in the presence of Wnt and exogenous LRP5), the inhibitor (activity in the presence of the wild-type DKK1 inhibitor) and mutant (each of the mutant DKK1 proteins). The TukeyHSD test was used to carried out the post hoc test for multiple group comparisons. The ANOVA tests were done using the program R studio v.3.4.0, and values of $p < 0.05$ were considered significant. All data were evaluated for normality, homogeneity of variance and detection of outliers.

Results

Expression, secretion and stability of mutated proteins

A western blot assay was performed to check if the DKK1 mutant proteins are correctly located in the extracellular space, using a culture of HEK293 cells, which express high amounts of wild-type DKK1 or mutated DKK1. The results show that, in all cases, the different mutated DKK1 proteins were detected in the extracellular space (Figure 1) and at levels equal to or higher than those of the wild-type protein.

Activity of mutated DKK1 proteins

To test the inhibitory activity of mutant DKK1 proteins on the Wnt pathway, we performed a reporter gene assay (luciferase), specific for this pathway (Figure 2).

The results of the endogenous condition in which the plasmids pRLTK and pGL3-OT have been co-transfected are shown in Figures 2A and 3 which represent the Wnt pathway activity in HEK293 cells.

In the active condition, in addition to pRLTK and pGL3-OT, the vectors expressing Wnt1 and LRP5 have been co-transfected. Wnt1 acts as a ligand activator of the pathway and LRP5 as a co-receptor, two essential elements for the pathway activation. In this condition (Figure 2B) the activity of luciferase has been increased 3 times, on average, compared to the endogenous pathway (Figure 3, activator).

The inhibited pathway contained the same vectors as the active condition but in addition the vector expressing the protein DKK1-WT (wild-type protein) was co-transfected. In this condition, the Wnt pathway has been inhibited, by sequestering the LRP5 co-receptor (Figure 2C). In these experiments, luciferase activity has been increased 2.2-fold over the endogenous condition and has been significantly lower than that of the activated pathway (Figure 3, inhibitor).

When the functionality of the mutants of DKK1 has been verified, the different vectors of the inhibited pathway have been co-transfected, but substituting that of DKK1-WT for those expressing the different mutated DKK1. For the mutant proteins DKK1-p.Met16Leu, DKK1-p.Tyr74Phe, DKK1-p.Pro84Leu, DKK1-p.Ala106Thr, DKK1-p.Arg120Leu and DKK1-p.Ser157Ile no significant differences were found in the inhibitory activity compared with the DKK1 WT protein (data not shown).

In contrast, in the presence of the mutant protein DKK1-p.Ala41Thr, luciferase activity has been observed which is significantly greater than that of the pathway inhibited by DKK1-WT (Figure 3), and in turn significantly lower than that of the active route.

Discussion

The *DKK1* gene encodes a protein of the same name, which acts in the extracellular space as an inhibitor of the Wnt signaling pathway. Numerous studies have associated the Wnt pathway with bone formation, while blocking it with sclerostin or DKK1 has been associated with greater bone loss and risk of fracture. The search for gene variants that may explain Wnt pathway regulation in the general population may open a very relevant field of research in osteoporosis study. In this work, we have studied the inhibitory function of 7 mutant DKK1 proteins on the Wnt pathway and we have observed that the mutant protein DKK1-p.Ala41Thr shows a partial loss of its inhibitory function, which is not due to the loss of its expression. The activity of the DKK1-p.A41T protein is reduced by approximately 50% compared to the protein DKK1-WT. The mutation, at amino acid 41, is not found in the LRP5 binding domain (amino acids 189-263), but it does affect the NAIKN motif (amino acids 40-44), which is crucial for binding to LRP5 and LRP6 proteins and it is conserved in all inhibitors of the Wnt pathway[23].

According to the ExAC database, the population frequency of the variant p.Ala41Thr is 15 heterozygotes in 60,000 adult individuals free of serious diseases. Given our result of loss of inhibitory activity of this variant of DKK1, we could infer that the associated phenotype would be of a higher non-pathogenic bone density. From this frequency, we estimate that in Spain there are about 6,000 carriers of this variant in heterozygo-

sis. On the other hand, there is a single reference to the mutation p.Ala41Thr that associates it with pathology, specifically the Chiari type I malformation (CMI)[24]. This disease is characterized by a defect in the development of the occipital bone and the posterior fossa (PF) and the consequent hernia of the cerebellar amygdala. It will be interesting to study the possible relationship between mutations in *DKK1* and this disease, which, in many cases, is asymptomatic and undiagnosed.

No differences were found in the activity of the remaining mutant proteins and the DKK1 WT protein. These results coincide with the results found by Korvala et al.[25] for the mutation p.Arg120Leu. These authors found this mutation in a patient with primary osteoporosis, a phenotype diametrically opposed to the phenotype of the HBM woman where we found the mutation. This same mutation is found in patients with Paget's disease (PDB)[26] and its frequency in patients is twice that of controls, although the difference is not significant.

None of the seven mutations tested is in the domain that affects the Wnt signaling pathway (LRP5 binding domain: amino acids 189-263), and only p.Ala41Thr affects the NAIKN motif. This

Figure 1. Expression levels of the wild-type or mutated DKK1 protein analyzed by western blot. HEK293 cells were transfected with expression vectors of the different DKK1 variants indicated in each lane. The resulting conditioned media, properly concentrated, was used for this analysis. In each lane 4.5 ug of total protein was loaded. The extracellular protein fibronectin has been used as load control
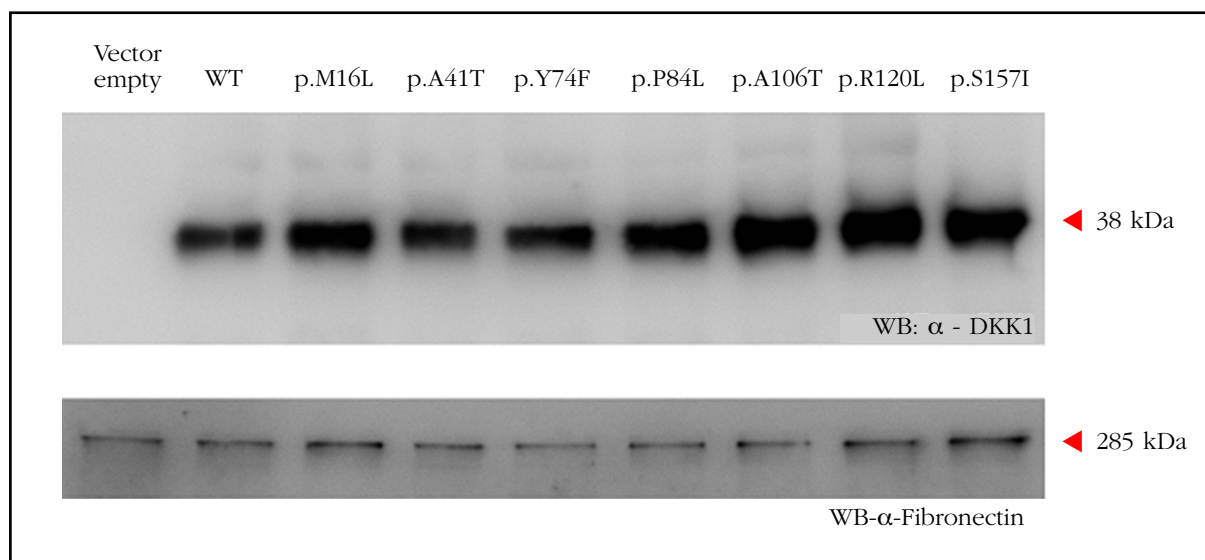

Figure 2. Reporter gene assay design. A) Endogenous condition: we transfected the pRLTK and pGL3OT plasmids. B) Active condition: we transfected plasmids pRLTK, pGL3OT, Wnt1, LRP5 and mesdc2. C) Inhibited condition: we transfected plasmids pRLTK, pGL3OT, Wnt1, LRP5, mesdc2 and DKK1-WT. In gray the endogenous elements of the HEK293 cells of the Wnt pathway, in color the transfected elements in each condition
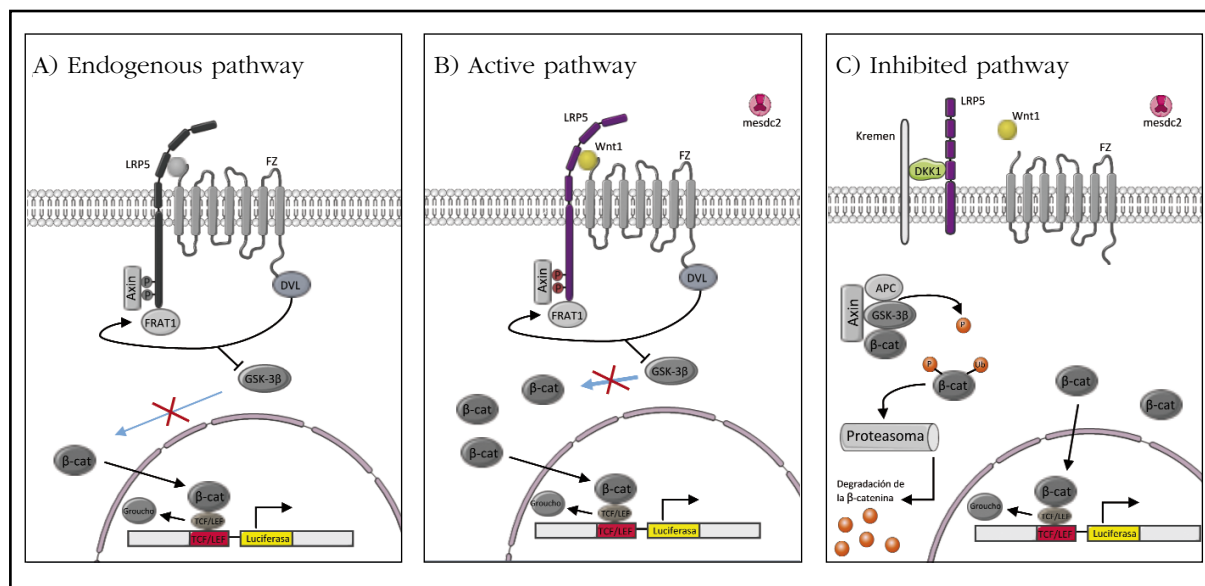
could be a reason why no differences in inhibitory activity have been observed in 6 of the 7 mutated DKK1. Alternatively, these DKK1 mutants would show differences in inhibitory activity lower than those that can be detected with the sensitivity of the reporter gene assay that has been used. A limitation of the study would be that the assay carried out involves the co-transfection of several vectors to have high values of luciferase activity, which gives it a high variability. Another limitation would be that the expected effect of these mutations is small, since they are variants present in the general population. This question can only be solved when there is a trial with a higher sensitivity.

In conclusion, in our study, DKK1 protein (p.Ala41Thr) shows a partial loss of its inhibitory function, which is not due to its lack of expression. This could lead to an increase in bone mineral density in people of the general population who carry this mutation.
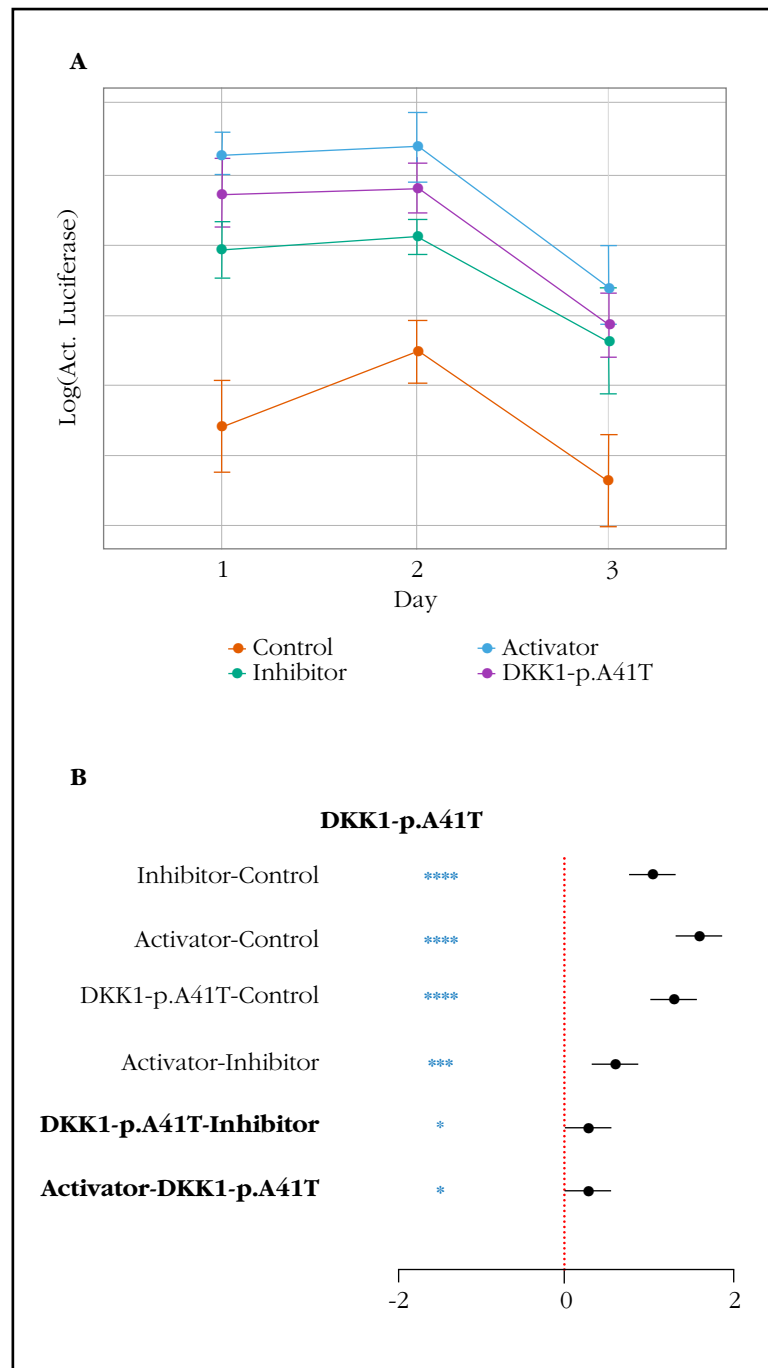
Figure 3. Activity assay of the DKK1-p.Ala41Thr protein. A) Graph of interactions following the model, $Y_{ij} = \mu + day + test + day{:}test + \epsilon_{ijk}$. The logarithm of the mean ratio of luciferase activities (Photinus pyrali/Renilla reniformis) with its confidence interval is shown on the Y axis. in the X axis the three days in which the experiment has been tested are shown. B) Tukey test of multiple comparisons between the different conditions tested. Significance levels are shown by means of the code: (****)<0.000001, (***)<0.0001, (**)<0.01, (*)<0.05

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