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Serum levels of sclerostin, Dickkopf-1, and secreted frizzled-related protein-4 are not changed in individuals with high bone mass causing mutations in LRP5

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Abstract

Summary—We compared circulating levels of Wnt inhibitors among patients with high bone mass mutations in LRP5, unaffected kindred, and unrelated normal controls. Inhibitors were unchanged in affected and unaffected kindred. We saw no meaningful differences between

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controls and affected individuals. LRP5 signaling may not influence circulating levels of these inhibitors.

Introduction—It is thought that gain-of-function mutations in LRP5 result in high bone mass syndromes because these allelic variants confer resistance to the actions of endogenous inhibitors of Wnt signaling. We therefore attempted to determine if circulating levels of Wnt inhibitors are altered in patients with gain-of-function mutations in LRP5.

Methods—This is a cross-sectional study in a university research center. Serum was collected from consented volunteers known to have either the G171V or N198S gain-of-function mutations in LRP5, kindred members affected with either mutation, unrelated kindred, and unrelated normal age-matched controls. BMD was provided or measured on site.

Results—There were no significant differences found in the serum levels of sclerostin (SOST), Dickkopf-1 (Dkk-1), or secreted frizzled-related protein-4 (SFRP-4) in affected vs. unaffected individuals from different kindreds or when compared to age-matched unrelated normal individuals. Mean serum SOST values in affected and unaffected kindred members and unrelated normal controls were 52.7 ± 6.1 , 36.5 ± 9.6 , and 54.8 ± 5.4 , respectively. For Dkk-1, the values were 25.9 ± 3.4 , 25.7 ± 3.0 , and 17.3 ± 2.3 and for SFRP-4, 38.1 ± 2.3 , 39.8 ± 3.6 , and 28.5 ± 1.7 . Serum levels of RANKL and osteoprotegerin (OPG) were not different in the three groups.

Conclusions—Circulating levels of endogenous Wnt inhibitors do not change in patients with gain-of-function mutations in LRP5 including Dkk1, which is suppressed by Wnt signaling. It may be that circulating levels of Wnt inhibitors do not reflect changes in target tissues. It is also possible that other mechanisms besides or in addition to resistance in Wnt inhibitors explains the skeletal effects of these mutations.

Keywords

HBM mutations; High bone mass; LRP5; Wnt inhibitors

Introduction

It is now established that Wnt signaling plays an important role in regulating skeletal metabolism. In humans, mutations in the Wnt co-receptors LRP5 and LRP6 are associated with dramatic changes in bone mass. In particular, mutations in the first beta-propeller loop of the extracellular domain of LRP5 cause extremely high bone mass [1–4]. Bone formation rates in individuals with these mutations have not been reported, but mice engineered to express high bone mass (HBM)-causing mutations in LRP5 have an increased rate of bone formation [5]. In contrast to these findings, individuals with loss-of-function mutations in LRP5 have extremely low bone mass and suffer fragility fractures [6]. Mice with loss-of-function mutations in LRP5 have low bone formation rates [7]. Consistent with an important role for LRP5 in skeletal metabolism, allelic variants in this gene have been identified as contributors to the heritability of bone mass in GWAS studies [8]. The precise cellular and molecular mechanisms that result in high bone mass in humans with the above-noted LRP5 mutations remains largely unstudied and controversial, although extensive work in cellular and animal models has resulted in several putative signaling pathways by which this may occur [9, 10].

The canonical Wnt signaling cascade involves one of several Wnt ligands binding to a receptor complex consisting of the seven transmembrane-spanning Wnt receptors, Frizzled, and a co-receptor LRP5 or LRP6. Ligand binding results in the cellular accumulation and nuclear translocation of beta-catenin that binds to specific members of the Tcf/Lef and FoxO families of transcription factors, resulting in transcriptional activation of target genes [11, 12]. Several endogenous inhibitors of this pathway have been identified, and changes in their activities also profoundly affect bone mass. Loss-of-function mutations in the endogenous Wnt signaling inhibitor sclerostin have sclerosteosis, a disorder characterized by extremely high bone mass [6, 13]. Mice engineered with haploinsufficiency of another endogenous Wnt inhibitor, Dickkopf-1 (Dkk-1), have high bone mass [14]. Inhibition of the circulating Wnt inhibitor, secreted frizzled-related protein 1, results in increased bone mass in mice [15, 16], while transgenic overexpression of Wnt inhibitors including Dkk-1, secreted frizzled-related protein-4 (SFRP-4), and Kremin causes osteopenia [17–19]. SFRP-4 knockout mice have increased bone density [20].

It is thought that mutations in the first beta-propeller loop of the extracellular domain of LRP5 result in high bone mass syndromes because these allelic variants confer resistance to the actions of endogenous inhibitors, in particular Dkk-1. Dkk-1 acts to inhibit Wnt signaling by binding to LRP5 at the first beta-propeller [21] as well as several other sites [22]. This prevents the formation of the LRP5/Wnt/Fzl trimolecular signaling complex. Sclerostin (SOST), a product of osteocytes, is also thought to directly bind to LRPs and prevent ligand binding [23, 24]. SFRP-1 and SFRP-4 are thought to competitively inhibit binding of Wnts to the LRP/Frizzled complex by acting as decoy receptors [25].

Of these inhibitors, only one, Dkk-1, is a known direct cellular target of canonical Wnt signaling [26]. Wnt signaling suppresses Dkk-1 expression, and therefore, one might predict that HBM mutations in LRP5 would suppress Dkk-1 expression. Alternatively, it could be argued that resistance to the actions of endogenous inhibitors might lead to a compensatory increase in the levels of those inhibitors. Consequently, we wondered if circulating levels of known Wnt inhibitors might be altered in patients with HBM mutations in LRP5.

Materials and methods

Study subjects and sample collection

Blood samples were collected after an overnight fast from 16 individuals recruited from two kindreds with known high bone mass-causing and heterozygous mutations in LRP5 [1, 27], 13 unaffected individuals from the same kindreds, and 24 unrelated, normal, age-matched controls. Serum was stored frozen at -70°C until analyzed. This study was approved by the Yale Human Resource Protection Program at Yale University, and every subject gave informed written consent.

Assays

Serum levels of sclerostin, Dkk-1, soluble RANKL (RANKL), and osteoprotegerin (OPG) were determined by ELISA using commercially available kits (manufactured by Biomedica, Vienna, Austria and distributed by ALPCO Diagnostics, Salem, NH, USA). Secreted

frizzled-related protein-4 was measured by ELISA (USCN Life Science Inc. Wuhan, China distributed by Cedarlane Laboratories USA, Burlington, NC). Serum C-terminal telopeptide of type 1 collagen (CTX) was determined by ELISA and procollagen type 1 aminoterminal propeptide (P1NP) by radioimmunoassay (UniQ, Orion Diagnostica). Both kits were obtained from Immunodiagnostic Systems Ltd, Scottsdale, AZ. Vitamin D metabolites were measured by radioimmunoassay (DiaSorin Inc. Stillwater, MN). Parathyroid hormone was measured as previously reported using an in-house mid-molecule RIA [28]. The normal range for PTH in this assay is 10–25 nLeq/mL.

Bone mineral density

BMD in affected individuals and unaffected members of their kindreds was measured by DXA on either a Hologic 4500W densitometer at the Yale Bone Center or using either Hologic or Lunar densitometers in the study subjects' local communities. BMD in unrelated normal subjects was measured in the Yale Bone Center.

Statistical analysis

Data were stored and analyzed in Prism V6.0 (GraphPad Software, San Diego, CA) except for the analyses of covariance (ANCOVA) which were performed using SPSS (PASW Statistics 18, 2009 SPSS Inc. Quarry Bay, Hong Kong). Data was assessed for normality by the Shapiro-Wilk test. A *p* value for the Shapiro-Wilk test <0.05 was considered evidence that data were not normally distributed. The primary outcome for this study was the difference in circulating levels of sclerostin, Dkk-1, and SFRP-4 in three groups, the individuals with HBM mutations (affected individuals), the unaffected individuals from the same kindreds, and age-matched normal controls. Secondary analyses were conducted based on genotype of the affected individuals.

One-way analysis of variance (ANOVA) was used for the primary analysis of the data. Tukey's test for multiple comparisons was used for post hoc testing. Where appropriate, ANCOVA analyses with pair-wise comparisons and a Bonferroni correction were conducted to control for the difference in age between the unrelated members of the two kindreds and the normal controls.

Results

Demographics and serum biochemistries

The table summarizes the demographics and mineral metabolism findings in the study subjects. The 16 affected kindred members and the 24 unrelated normal controls were very similar in age, while the unaffected kindred members were somewhat younger. There was a borderline significant group effect for age by one-way ANOVA, although post hoc testing revealed no significant between-group differences. As expected, the affected members of the kindreds had markedly increased bone density with average Z-score of 6.4, while the two other study groups had Z-scores that were in the normal range. Serum PTH, 25-hydroxy vitamin D, and 1,25-dihydroxyvitamin D were not different in the three groups. There were no differences in CTX or P1NP values among the three groups.

Serum levels of sclerostin, DKK-1, and SFRP-4

There was no group effect for sclerostin when analyzed by one-way ANOVA (Table 1, Fig. 1). There was a barely significant group effect for Dkk-1 ($p=0.05$) but no between-group differences (Table 1, Fig. 1). In particular, the mean serum values for Dkk-1 were nearly identical in the affected individuals and their unaffected family members (25.9 ± 3.4 and 25.7 ± 3.0 pmol/L, respectively) while both values tended to be higher than the mean value in the normal subjects (17.3 ± 2.3 pmol/L). Similar changes were observed for serum SFRP-4. Thus, there was a significant group effect for SFRP-4 ($p= 0.002$, Table 1, Fig. 1). By post hoc testing, serum SFRP-4 values were higher in subjects with HBM LRP5 mutations than in unrelated normal controls (38.1 ± 2.3 vs. 28.5 ± 1.7 ng/ mL, $p = 0.05$). In unaffected kindreds members, the mean serum SFRP-4 was 39.8 ± 3.6 ng/mL, which was also significantly higher than the value in unrelated controls by post hoc testing ($p = 0.001$). Since the mean age of the unaffected members of the two kindreds was higher than that of the normal controls, an ANCOVA analysis was conducted to determine if controlling for age affected these latter results. When age was controlled for, the serum SFRP-4 values in both the affected and unaffected kindreds members remained significantly higher than the mean value in controls ($p=0.01$ for both comparisons). As noted, although there were no between-group differences for circulating levels of Dkk-1, there was an overall group effect by one-way ANOVA. When an ANCOVA analysis was conducted for Dkk-1, controlling for age did not affect the results in any pair-wise comparisons among the three groups, all of which remained nonsignificant.

Analysis by genotype

Figure 2 summarizes the circulating levels sclerostin, Dkk-1, and SFRP-4 in affected individuals with either the G171V or N198S mutation in LRP5. There were relatively few individuals in the N198S group. That notwithstanding, there were no differences noted in circulating levels of any of the inhibitors based on genotype. Similarly, there were no significant differences in serum levels of PINP or CTX.

Serum levels of RANKL and OPG

To investigate the impact of HBM mutations in LRP5 on the RANKL/OPG system, serum RANKL and OPG were measured in the groups. As summarized in Table 1, there were no significant differences in circulating levels of RANKL and OPG in the three study groups. Similarly, the ratio of circulating RANKL to OPG was not different based on group, although there was a trend in the OPG/RANKL ratio data ($p= 0.09$) and the mean value appeared lower in the normal individuals than in the other two groups.

Influence of sex on outcome measures

Since the sex distribution of the unrelated normal controls is different from that of the affected and unaffected individuals, we determined whether there was an influence of sex on the key outcome measures in this study. Serum levels for sclerostin, Dkk-1, SFRP-4, RANKL, and OPG were measured in five women and five age-matched men from the unrelated normal control group. The mean ages of these two groups were 62 ± 8.7 and 63 ± 13.2 ; $p=0.97$; female vs. male, respectively. Mean levels of sclerostin were 57.5 ± 5.2 and

77.0±19.3 pmol/L, $p=0.4$; for Dkk-1 15.7±0.7 and 15.3±2.5 pmol/L, $p=0.9$; for SFRP-4 24.2±3.3 and 25.1±3.9 ng/mL, $p=0.9$; for RANKL 0.50±0.20 and 0.94±0.41 pmol/L, $p=0.4$; and for OPG 6.8±0.9 and 9.1±2.5 pmol/L, $p=0.4$; female vs. male, respectively). Our sclerostin data are consistent with data from a recent large clinical trial involving 170 men and women. This study found no difference in mean circulating levels of sclerostin in men and women [29].

Discussion

This study demonstrates that circulating levels of three important inhibitors of the canonical Wnt signaling cascade are not influenced by the presence or absence of HBM mutations in LRP5. Although there was a group effect observed for SFRP-4, unaffected family members had mean serum values that were nearly identical to values in affected members of these kindreds. Controlling for age in the analyses did not influence this result. Thus, it is unlikely that the presence of a HBM mutation in LRP5 influenced circulating level of these molecules since members of the same kindred without the mutation had similar levels. In the general population, some data suggest that there is a positive relationship between circulating levels of sclerostin and bone mass, with higher bone mass associated with higher serum sclerostin levels [30, 31]. Sclerostin levels were not higher in our study subjects with extraordinarily high bone mass compared to controls. Mean values for sclerostin in both the affected individuals and the normal controls were higher than the values in unaffected individuals, but these differences were not significant. We studied two kindreds with different mutations in LRP5, but segregating our data based on genotype did not change our results.

Serum CTX values were not different among the three groups. The mean serum P1NP tended to be higher in the affected individuals compared to the other two groups, although the differences were not significant. Nonetheless, this is interesting given the data, albeit limited, that there may be an increase in bone formation rates in vivo with HBM mutations in LRP5 [32]. It is also of interest that the RANKL/OPG ratio was higher in the affected individuals than in the other two groups, although this too was not significant. One might interpret this as a compensatory effort to increase the rate of skeletal resorption in the face of an ongoing increase in bone formation. Such a compensatory change would be advantageous and consistent with the clinical observation that individuals with HBM mutations in LRP5 do not generally show progressive increases in bone mass as adults. Similarly, cranial or spinal and nerve entrapment, although reported, are uncommon in these individuals and clinically significant marrow compromise has not been described.

Our results differ from those of Frost et al. who found significantly higher serum sclerostin levels in subjects with a T2531 HBM mutation in LRP5 compared to controls [33]. They also found that serum CTX and P1NP were significantly lower in the affected individuals in their study population. Although both studies used the same assays for sclerostin and Dkk-1, in 2011, both assays were reformulated by the manufacturer using the same primary antibodies (personal communication from Biomedica Medizinprodukte, Vienna, Austria). Since it is likely that Frost et al. performed their analyses before this reformulation, the absolute values for these two cytokines may not be strictly comparable in the two studies.

Our differing conclusions are less likely explained on this basis. It is possible that the different genotypes of the study subjects in the two reports contributed to the divergent findings.

Our results indicate that circulating levels of endogenous Wnt inhibitors do not change in patients with HBM mutations in LRP5. These data suggest that if the mechanism of action of the HBM mutations in LRP5 is conferred by resistance to the actions of endogenous inhibitors, it is not reflected in circulating levels of the three molecules measured in this study.

Our study has some limitations. The number of individual with the N198S mutations recruited was small. Further, we did not study any patients with the T2531 mutation in which different results were reported.

It may be that circulating levels of Wnt inhibitors do not reflect changes in target tissues or that changes in LRP5 signaling do not influence circulating levels of these molecules.

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Fig. 1.

Circulating levels of the three Wnt signaling inhibitors in the three study groups. There was no group effect for sclerostin by one-way ANOVA. While there was an overall group effect for Dkk-1 ($p=0.05$), there were no significant differences found by Tukey's multiple comparisons post hoc test. There was a significant group effect for SFRP-4 ($p=0.002$). By post hoc testing, mean values for SFRP-4 in affected and unaffected kindred members were significantly higher than the mean value in unrelated normal subjects. There was no difference in the mean values for affected and unaffected kindred members.

Fig. 2.

Circulating levels of the three Wnt signaling inhibitors as well as of serum CTX and P1NP in the affected individuals based on genotype. There was no genotype effect for any of the three inhibitors or for CTX or P1NP. G171V $N=12$ for all Wnt signaling inhibitors and markers of bone turnover except for Dkk-1 $N=9$. N198S $N=4$ for all Wnt signaling inhibitors and markers of bone turnover

Table 1

Demographics and serum biochemistries in the three study groups

	Affected kindred members (N)	Unaffected kindred members (N)	Unrelated controls (N)	Reference range	ANOVA <i>p</i> value
Age (years)	60±5 (16)	42±5.5 (13)	58±4.2 (24)	N/A	0.05
Sex (M/F)	(11/5)	(5/8)	(5/19)	N/A	
Z-scores	6.4±0.3 (15)	0.8±0.5 (10)	1.4±0.4 (23)	-1.0–+1.0	<0.0001 ^{a,b}
Sclerostin (pmol/L)	52.7±6.1 (16)	36.5±9.6 (13)	54.8±5.4 (24)	11.9–47.9	NS
Dkk-1 (pmol/L)	25.9±3.4 (13)	25.7±3.0 (11)	17.3±2.3 (23)	25.7–65.7	0.05
SFRP-4 (ng/mL)	38.1±2.3 (16)	39.8±3.6 (13)	28.5±1.7 (24)	5.5–79.8	0.002 ^c
PLNP (ug/L)	65.6±22.3 (16)	53.0±5.5 (13)	40.1±3.1 (24)	16–96	NS
CTX (ng/mL)	0.453±0.116 (16)	0.499±0.068 (13)	0.520±0.109 (18)	0.122–1.351	NS
PTH (nLeq/mL)	23.6±3.0 (16)	20.1±2.2 (13)	17.9±1.8 (23)	10–25	NS
25(OH) D (ng/mL)	34.2±2.8 (16)	32.0±2.8 (13)	28.7±2.3 (22)	30–50	NS
1,25(OH) ₂ D (pg/mL)	58.7±10.0 (9)	51.0±6.7 (9)	44.1±4.4 (23)	25–66	NS
RANKL (pmol/L)	0.98±0.21 (16)	0.82±0.18 (13)	0.73±0.14 (23)	Not established	NS
OPG (pmol/L)	5.82±1.03 (16)	5.14±0.71 (13)	6.98±0.82 (23)	Not established	NS
RANKL/OPG ratio (%)	29.9±9.1 (16)	19.2±4.7 (13)	12.7±2.6 (23)	Not established	0.09

Data are presented as mean±SE

^a The *p* values presented in the table are those for the one-way ANOVA. The *p* values for the Tukey's multiple comparisons are presented in the "Results" section^b Affected significantly different than the other two groups by Tukey's multiple comparisons post hoc test. Unaffected kindred members are not different from controls^c Affected and unaffected kindred members significantly different from controls by Tukey's multiple comparisons post hoc test. Affected and unaffected kindred members are not different from each other

NS= not significant