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Case Report

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Very Low Vitamin D in a Patient With a Novel Pathogenic Variant in the *GC* Gene That Encodes Vitamin D-Binding Protein

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Abbreviations: 25(OH)D, 25-OH vitamin D; DBP, vitamin D-binding protein; ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; PTH, parathyroid hormone; SNV, single-nucleotide variation.

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Abstract

Circulating plasma vitamin D metabolites are highly bound to vitamin D-binding protein (DBP), also known as *g*roup-specific *c*omponent or Gc-globulin. DBP, encoded by the *GC* gene, is a member of the albumin family of globular serum transport proteins. We previously described a homozygous *GC* gene deletion in a patient with apparent severe vitamin D deficiency, fragility fractures, and ankylosing spondylitis. Here, we report an unrelated patient free of fractures or rheumatologic disease, but with very low 25-hydroxyvitamin D and 1,25-hydroxyvitamin D, as well as undetectable DBP measured by liquid chromatography–tandem mass spectrometry. A whole gene deletion was excluded by microarray, and Sanger sequencing of *GC* revealed a homozygous pathogenic variant affecting a canonical splice site (c0.702-1G > A). These findings indicate that loss of function variants in *GC* that eliminate DBP, and severely reduced total circulating vitamin D levels, do not necessarily result in significant metabolic bone disease. Together with our previous report, these cases support the free-hormone hypothesis, and suggest free vitamin D metabolites may serve as preferable indicators of bone and mineral metabolism, particularly when clinical suspicion of DBP deficiency is high.

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Diagnosis of vitamin D deficiency based on low total 25-OH vitamin D (25[OH]D) levels is clinically common. Vitamin D deficiency has an estimated worldwide prevalence of approximately one billion, and is linked to a diverse array of illnesses including autoimmune and cardiovascular diseases, depression, and cancer [1]. Fatigue, myalgia, joint pain, weakness, and depression are common, nonspecific symptoms described by patients in general medicine and endocrinology clinics, and 25(OH)D levels are now routinely measured as part of their evaluation. As lipophilic steroid prohormones, hormone, and catabolites, vitamin D metabolites circulate primarily bound to serum proteins, notably vitamin D-binding protein (DBP) and albumin [2-4]. In large part due to historical challenges in measuring free vitamin D, total 25(OH)D levels are widely used as the biomarker of vitamin D deficiency or sufficiency [1]. However, caveats to using 25(OH)D as a biomarker of vitamin D status in different clinical populations, along with the utility of measuring DBP or free 25(OH)D levels, and the standardization of both assays, are increasingly appreciated as important issues in the field [5, 6]. Thus, there is a need to determine the optimal tests for clinical use.

Prior to our identification of a patient with homozygous deletion of the *GC* gene [7], no genetic variants were reported to abolish DBP function, despite it being a highly polymorphic gene. In fact, loss-of-function mutations were predicted to be lethal until knockout mice lacking DBP were found to be viable, fertile, and free of bone metabolism defects unless stressed with a diet low in vitamin D [8].

In this report, we describe a patient with undetectable DBP, and undetectable to very low 25(OH)D and 1,25(OH)D levels, who was found to harbor a novel homozygous mutation in the *GC* gene. Comparing our present patient with the previous *GC* deletion case suggests that apparent severe vitamin D deficiency can occur without skeletal abnormalities.

Materials and Methods

Vitamin D and Vitamin D-Binding Protein Assays

DBP levels were initially assessed by PAN Laboratories using a Human Vitamin D BP Quantikine ELISA Kit (Bio-Techne) [9]. DBP levels were also subsequently assessed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) Hoofnagle Lab, University of Washington) [10]. Routine total 25(OH)D measurements were performed by Beckman Coulter Dxl immunoassay (UAB Laboratories), unless sent for referral testing by LC-MS/MS as indicated (Quest Diagnostic Labs or Mayo Clinic Laboratories). Dihydroxyvitamin D concentrations (1,25-dihydroxyvitamin D) were initially determined by LC-MS/MS (Quest Diagnostics). Plasma calcium, phosphate, and alkaline phosphatase, were measured using standard clinical chemistry analyzers and parathyroid hormone (PTH) (intact) by immunoassay (Beckman Coulter Dxl); all were performed at UAB Laboratories. Tissue transglutaminase immunoglobulin A (IgA) was quantified using an INOVA enzyme-linked immunosorbent assay (ELISA) kit, IgA was quantified using a Binding Site assay (UAB Laboratories), and intact-fibroblast growth factor-23 was quantified using an ELISA from Immutopics/Quidel (Mayo Clinic Laboratories). Subsequent analyses of 25(OH)D and 1,25(OH)2D were performed at PAN Laboratories using immunoassay (DiaSorin). Free 25-hydroxyvitamin D levels were determined using an ELISA kit (DiaSource/Future Diagnostics) [11]. Additional confirmatory testing for vitamin D metabolites (25-hydroxyvitamin D; 24,25-dihydroxyvitamin D; and 1,25-dihydroxyvitamin D) was performed on one specimen by University of Washington Reference Laboratory [12].

Chromosomal Microarray

Chromosomal microarray analysis was performed on DNA extracted from peripheral blood using the Infinium CytoSNP-850K v1.2 array platform (Illumina), which contains 846 819 single-nucleotide variation (SNV; formerly single-nucleotide polymorphism [SNV]) markers spanning the entire genome. Analysis was conducted using the BlueFuse Multi Interpret Software v4 (Illumina) on the genome build NCBI Build 37/UCSC Human Genome, Feb. 2009 (GRCh37/hg19).

Sanger Sequencing

Sanger sequencing was performed to sequence the coding regions of the *GC* gene canonical complementary DNA transcript (GRCh37/hg19: NM_000583.4). Sequencing primers were manually designed using Alamut Visual software (SOPHiA Genetics) to cover all coding exonic regions and 20 nucleotides of the flanking intronic regions and were M13 tagged. Primers were evaluated using the UCSC Genome Browser Human BLAT Search to confirm absence of sequence homology to other genomic regions and examined using Alamut and Gene Tools SNPCheck to avoid regions containing highly polymorphic SNVs. Nucleotide

sequence of primers designed for this study are listed in Supplementary Table 1 [13]. Variants were described according to standard HGVS nomenclature and interpreted in accordance with American College of Medical Genetics and Genomics standards and guidelines for interpretation of sequence variants [14].

Results

Case

The patient is a 60-year-old Pakistani man with chronic undetectable to very low 25(OH) and 1,25(OH)D levels. He was first referred to Endocrinology at age 55, with a more than 10-year history of vitamin D deficiency that was refractory to multiple trials of high-dose vitamin D supplementation. He suffered from chronic fatigue and lower back pain following a motor vehicle accident, but lumbar spine and pelvic plain films revealed no fractures or vertebral height loss.

His past medical history was otherwise notable for hypertension, and obstructive sleep apnea treated with uvulopalatopharyngoplasty (age 58), for which he still used continuous positive airway pressure at night. He had gastroesophageal reflux disease, status post Nissen fundoplication at age 47, and a revision 2 years later. He had undergone lumbar spine surgery at age 50, and sinus surgery at age 53.

Review of outside records indicated he had taken several courses of 50 000 units of vitamin D weekly, but his 25(OH)D levels remained undetectable to very low. Reportedly, a trial of Rocaltrol (calcitriol) was stopped after a month because he developed hypercalcemia. Calcium, phosphorus, and alkaline phosphate levels were within normal limits (Fig. 1), as was serum albumin. His family history was remarkable for osteoarthritis in both parents, but negative for rheumatologic disease, bone disease, or familial calcium disorders.

Initial testing at our clinic revealed normal calcium, phosphate, albumin, and alkaline phosphatase levels, very low 25(OH)D by immunoassay, and undetectable 25(OH)D by LC-MS/MS, consistent with previous results (Fig. 1). Additionally, his 1,25(OH)D levels were undetectable, and PTH was slightly elevated, raising concern for primary hyperparathyroidism (see Fig. 1). A 24-hour urine study revealed normal urinary calcium, magnesium, and phosphate (Table 1; age 55), excluding renal phosphate wasting. A fibroblast growth factor-23 level was slightly elevated at 252 (normal ≤ 180 RU/mL). Malabsorption did not appear to be contributing to the low vitamin D levels because a negative tissue transglutaminase antibody test (< 5) and normal IgA (278) excluded celiac disease. Because of the elevated PTH, vitamin D supplementation was increased from 50 000 units vitamin D 3 times weekly to daily.

Over the next 5 years, the patient was maintained on high-dose vitamin D, ranging from 50 000 units weekly to 50 000 units daily. With more than 3-times-weekly dosing, 25(OH)D levels by immunoassay increased to within detectable levels, but remained below the low end of the normal range (UAB Laboratories), whereas PTH levels remained slightly elevated or at the upper end of the normal range (Fig. 1). Calcium, phosphate, PTH, and alkaline phosphatase remained within normal limits throughout (see Fig. 1), and $1,25(OH)_2D$ levels by LC-MS/MS were undetectable.

Additional Laboratory Evaluation of Vitamin D Status

Based on the undetectable $1,25(OH)_2D$, we decided to measure DBP levels using the Quantikine ELISA, which were extremely low (11; normal range, 104-477 mcg/mL) (Supplementary Table 2) [13]. Additionally, measurements of 25(OH)D and 1,25(OH)2D by DiaSorin immunoassay were very low (see Supplementary Table 2) [13]. Together with a serum albumin of 4.5 g/dL, this DBP level and metabolite concentrations were used to calculate a bioavailable 25(OH)D level of 5.2 ng/dL (normal range, 1.92-8.82 ng/dL) and bioavailable 1,25(OH)D level of 10.1 pg/mL (normal range, 3.44-14.16 pg/mL) using the method described by Powe and colleagues [15]. Because of the discordance between immunoassay and LC-MS/MS measurements of vitamin D metabolites, we next determined the concentration of DBP and vitamin D metabolites by LC-MS/MS. The concentration of DBP was undetectable. Indeed, none of the peptides monitored for DBP in the LC-MS/MS assay were observable, conclusively demonstrating that no DBP was present (Supplementary Fig. 1) [13]. In addition, vitamin D metabolite levels by a different LC-MS/MS assay were very low to undetectable (see Supplementary Fig. 1 and Supplementary Table 3), and a free 25(OH)D level by ELISA was 3.45 pg/mL, above the reference range lower limit of 2.4 pg/mL (see Supplementary Tables 2 and 3) [13].

The patient has never reported fractures or kidney stones. Symptoms of fatigue and joint pain were not improved with vitamin D supplementation or physical therapy. Other potential causes of long-standing fatigue, such as anemia, adrenal insufficiency, and hypothyroidism were excluded with normal hemoglobin levels, adrenocorticotropin stimulation test, and thyroid hormone levels, respectively. The patient was supplemented with 50 000 units vitamin D_3 5 days a week for some time following his initial evaluation in the endocrine clinic, but the dosage was reduced to 3 times a week

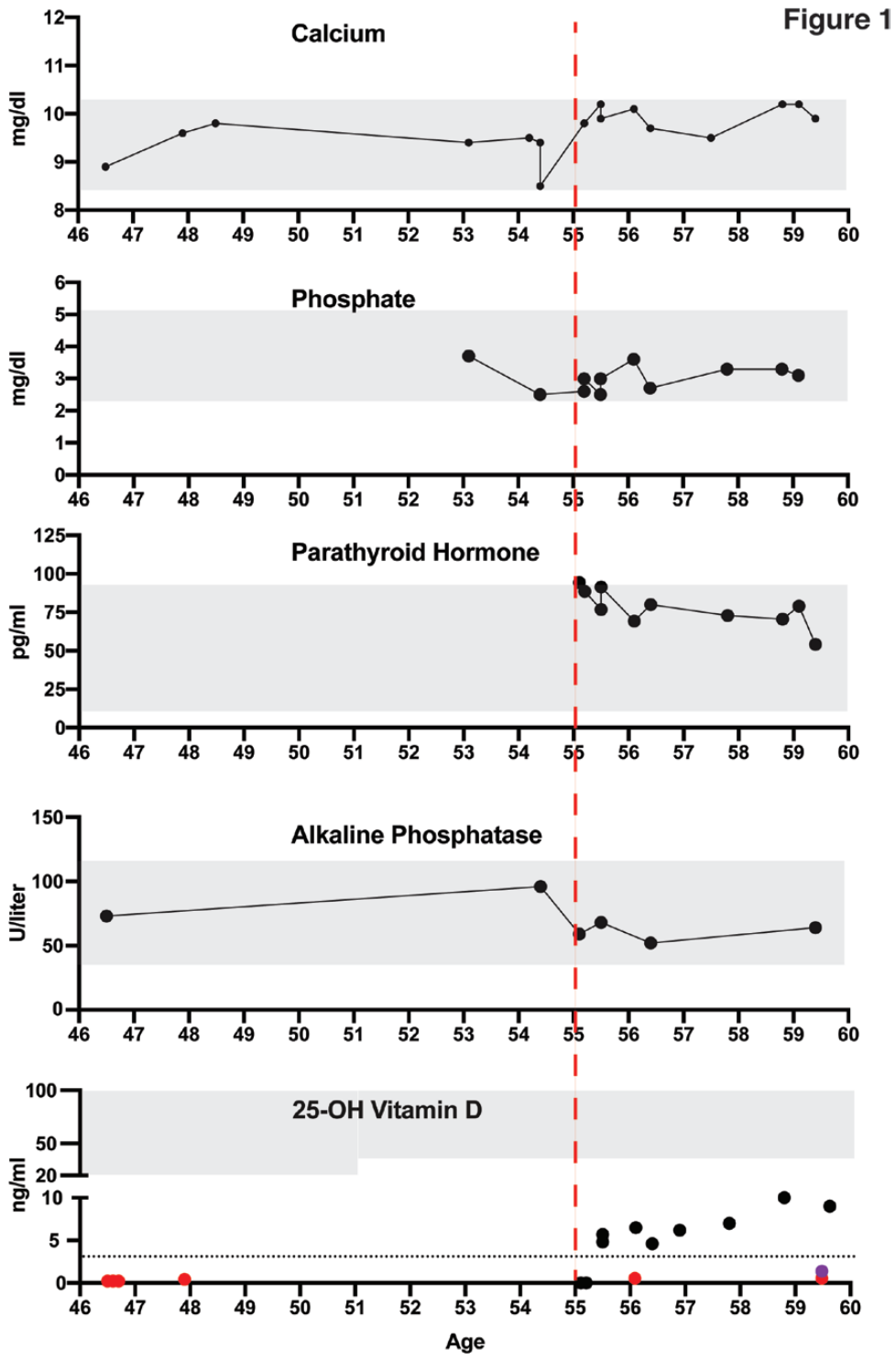

Figure 1. Laboratory data pertaining to calcium homeostasis. Clinical laboratory results for calcium, phosphate, parathyroid hormone, alkaline phosphatase, and total 25-OH vitamin D (25[OH]D). Total 25(OH)D measurements are indicated by method: immunoassay (black circles), or using liquid chromatography–tandem mass spectrometry by QUEST or Mayo Clinic Labs (red circles), and University of Washington Labs (purple). Gray-shaded areas indicate reference ranges as follows: calcium 8.4 to 10.2 mg/dL, phosphate 2.4 to 5.0 mg/dL, alkaline phosphatase (37-117 U/L), parathyroid hormone (PTH) 12 to 90 pg/mL, and 25(OH)D (20-100 or 30-100 ng/mL). Shifts in 25(OH)D references ranges indicate changes in instrumentation and assays in the clinical laboratory across the times the measurements were obtained; the lower limit of detection for the immunoassay is indicated by a dotted line. The dashed red line indicates age at initial visit to an endocrine clinic.

Table 1. 24-Hour urine studies

	Patient age 55 y	Patient age 59 y	Reference range
Urine chemistry			
Calculated U calcium	177.0	**671.0** (High)	100.0-240.0 mg/24 h
Ur calcium	17.7		
Total volume U Ca	1.000	2.200	L
H Col U calcium	24	24	24 h
Calculated U magnesium	91		72-103 mg/24 h
Ur magnesium	9		
Tot volume U magnesium	1.000		
H Col U magnesium	24		24 h
Calculated U phosphorus	1,110		700-1500 mg/24 h
Ur phosphorus	111		
Tot volume U phosphorus	1.000		
H Col U phosphorus	24		24 h
Calculated U creatinine	2000	**2024** (High)	1000-2000 mg/24 h
Ur creatinine	200		
Total volume U Creat	1.000	2.200	
H Col U creatinine	24	24	24 h

when urine calcium levels were elevated on 24-hour urine studies (see Table 1).

Skeletal Evaluation

The patient's complaints of joint and back pain led to further evaluation. Radiologic studies, including magnetic resonance imaging of the lumbar spine, and x-ray imaging of the lumbar spine and pelvis, revealed no fractures, osteomalacia, or deformities other than some stable loss of disc space and a grade 1 retrolisthesis at L4 to L5 (imaged at ages 53 and 57). A bone density scan at age 57 showed osteopenia with an L1 to L4 *T* score of –1.0 and left total hip of –0.3. Two years later, an interval scan revealed a lumbar spine increase of 5.2%, but no change at the hip or femoral neck. A urine N-terminal telopeptide (43; reference range, 9-60) and bone-specific alkaline phosphatase (54%; reference range, 28%-66%) both were normal, indicating normal bone turnover. Because of ongoing fatigue and chronic joint pain, the patient was evaluated in the rheumatology clinic. X-ray films both of the lumbar spine (a 5-year interval from initial studies) and of the sacroiliac joints were normal (Fig. 2). Rheumatologic exam revealed no signs of inflammation, swelling, or joint tenderness, concluding there was no evidence of inflammatory arthritis or connective tissue disease. Together, these results indicated no skeletal or bony abnormalities beyond a radiologic diagnosis of osteopenia.

Identification of *GC* Pathogenic Variant in a Region of Absence of Heterozygosity

The absence of DBP by LC-MS/MS led us to hypothesize that the patient likely carried a genetic alteration that would result in the complete absence of DBP protein. An initial SNV microarray revealed normal dosage across the genome, specifically with no deletion of the *GC* gene (Fig. 3), in contrast to our prior patient [7]. However, the microarray did identify multiple regions with absence of heterozygosity across the genome (see Fig. 3), encompassing 6.5% (180.1 Mb) of the haploid length (Supplementary Table 4) [13] and consistent with parental consanguinity (presumed first cousins).

Notably, the *GC* gene lies within a 43.3-Mb region of absence of heterozygosity on chromosome 4. Subsequent Sanger sequencing of the *GC* gene [16] revealed a homozygous c0.702-1G > A variant (see "Materials and Methods" and Fig. 3), classified as pathogenic according to American College of Medical Genetics and Genomics criteria [14], and expected to disrupt the canonical splice acceptor site of exon 7 based on the output of predictive algorithms (MaxEntScan, NNSPLICE, SpliceSiteFinder-like; Alamut Visual). The variant is rare in the general population, seen as one heterozygous allele in an individual of South Asian descent out of 249 470 alleles in the Genome Aggregation Database (gnomAD). The variant is not reported in ClinVar or the literature (PubMed search as of January 2021).

Discussion

Our patient exhibited extreme apparent vitamin D deficiency, manifesting as an absence of detectable 25(OH)D and 1,25(OH)D by certain LC-MS/MS assays. By immunoassay, there were detectable concentrations of 25(OH)D, which were determined to be falsely elevated results based on the LC-MS/MS assays. Similarly, extremely low DBP detected by ELISA appears to be a false positive based on its complete

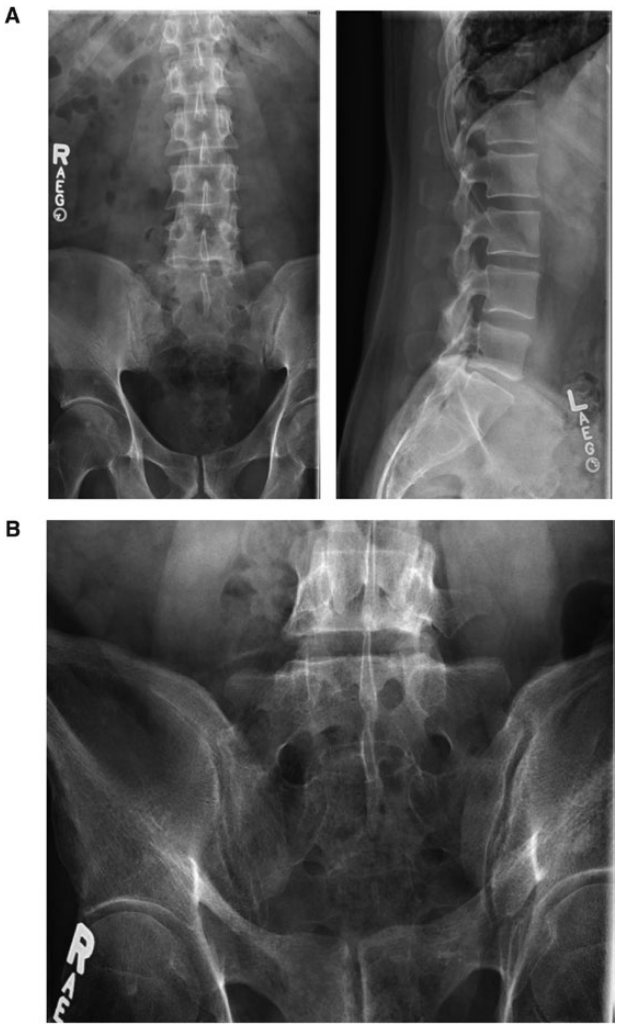

Figure 2. Normal skeletal imaging. A, Lumbar spine plain radiographs are normal. Left, Anteroposterior, and right, lateral views. B, Sacroiliac joints on plain radiographs in anteroposterior view are normal without narrowing, widening, erosion, or ankylosis.

absence in the LC-MS/MS. Together these results suggest that in patients without DBP, some immunoassays may yield misleading results. Subsequent testing by a very sensitive LC-MS/MS method demonstrated a very low concentration of 25(OH)D and a low concentration of $1,25(OH)_2D$. The concentration of $1,25(OH)_2D$ was similar to the concentration measured in our previous DBP-deficient patient, but the amount of 25(OH)D was significantly higher, which is surprising and supports the concept that other proteins besides DBP bind a small fraction of 25(OH)D in plasma. Further laboratory and radiologic studies demonstrated no clinically apparent bone metabolism or skeletal abnormalities that might be expected with profound vitamin D deficiency, such as rickets, osteomalacia, or osteoporosis.

This case differs clinically from the prior biallelic *GC* deletion case in that our present patient lacks both fragility fractures and rheumatologic disease such as ankylosing spondylitis. This distinction is important, as with the *GC* deletion case it was suggested by other groups that the ankylosing spondylitis may, in fact, be a manifestation of metabolic bone disease, or that the patient suffered from diffuse idiopathic skeletal hyperostosis [17, 18], claims refuted in our response on the basis of radiologic and laboratory findings [19]. Furthermore, this case leads us to suggest that the fragility fractures seen in the *GC*-deletion patient are perhaps more likely related to her severe ankylosing spondylitis rather than to apparent vitamin D deficiency. Our patient essentially phenocopies knockout mice lacking DBP [8], who similarly exhibit extremely low levels of vitamin D metabolites along with a decreased susceptibility to vitamin D toxicity and hypercalcemia. Our patient has not developed hypercalcemia despite a high-normal PTH level and 25(OH)D supplements of up to 50 000 units daily. Direct supplementation with calcitriol did lead to hypercalcemia, however, proving further that the patient has sufficient endogenous 1,25(OH)D activity. Alternatively, since the patient's albumin concentration was normal, vitamin D metabolites weakly bound to albumin could also be available for biological activity. This may be applicable both to 25(OH)D and 1,25(OH)D. While thought to represent a minor fraction of vitamin D (10%-15%) in normal circumstances, it may become physiologically critical in the absence of DBP; this is supported by the detectable levels of free 25(OH)D using ELISA. Both the previous [7] and present patient are expected to have a complete lack of DBP, the former owing to homozygous *GC* deletion, and the latter to homozygous splice-site mutations predicted to result in nonsense-mediated messenger RNA decay. In both cases the absence of DBP has been confirmed through LC-MS/MS. Observed clinical differences between these patients may also be due to variations in genetic modifiers that are beyond the scope of this report. The original case also included the deletion of nearby regulatory domains and the adjacent *NPFFR2* gene, the functional significance of which remains uncertain.

Interestingly, an analogous homozygous mutation in the 3′ splice acceptor site of intron 6 in the albumin gene (*ALB*) [20, 21] was previously shown to cause analbuminemia [22], thus demonstrating that loss of function variants in a gene with significant sequence homology can result in the absence of protein product. The intronic and exonic structure around exons 6 and 7 of *GC* is highly similar to that of the albumin and α-fetoprotein genes [3, 4]. Thus, the ability of an exon-splicing defect in a related gene within the albumin family, and at the same location with the gene's structure to cause complete absence of circulating protein, demonstrates proof of principle that our patient's defect should result in the absence of circulating DBP. Confirmation that the *GC* c0.702-1G > A variant results in exon 7 skipping, which would result in an out-of-frame shift in

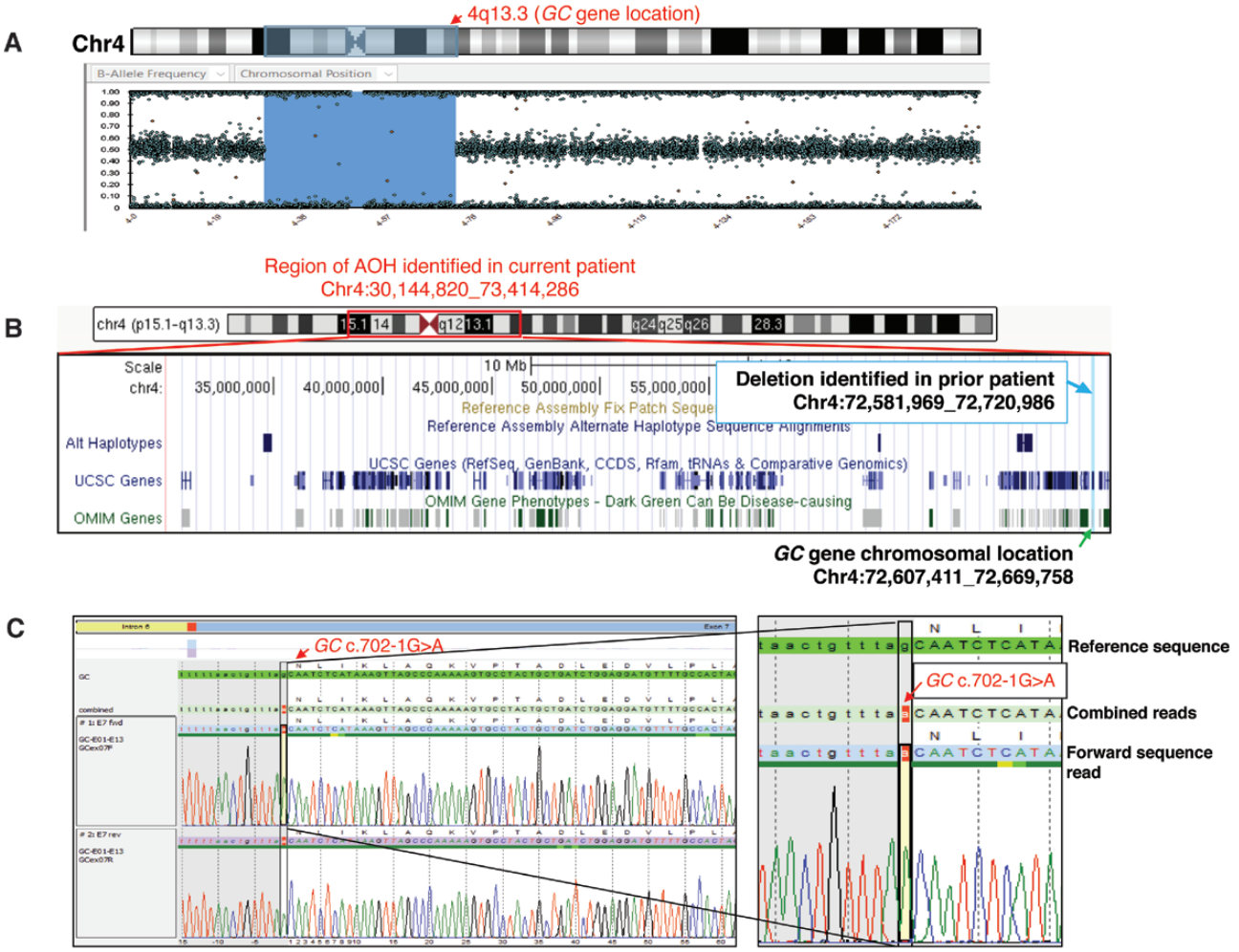

Figure 3. Chromosomal microarray and sanger sequencing of the *GC* gene. A, Single-nucleotide variation chromosomal microarray identified a 43.3-Mb region of absence of heterozygosity (AOH) on chromosome 4 (blue shaded region) overlapping the *GC* gene at 4q13.3. B, UCSC Genome Browser overlay of the region of AOH identified in the present case (red box) and homozygous deletion identified in the prior patient (light blue shaded box), both encompassing the *GC* gene (green arrow). C, Sanger sequencing of the *GC* gene, indicating the presence of a homozygous variant of the canonical splice acceptor site in exon 7.

the coding sequence, or other alterations in DBP structure or stability causing absence of DBP, will require additional studies in the future. Thus, loss of the exon 7 splice acceptor site is expected to lead to a loss of protein expression due to nonsense-mediated decay resulting in the absence of DBP that can bind meaningful amounts of vitamin D metabolites within the circulation. This, to our knowledge, is the first report of a homozygous loss-of-function sequence variant resulting in abolished DBP activity.

Our findings support the "free hormone hypothesis" (reviewed in [2, 5, 6, 23]). In the absence of circulating DBP, 25(OH)D and 1,25(OH)D both were undetectable or extremely low using gold-standard LC-MS/MS, yet the patient maintains normal calcium homeostasis. We concur with those who suggest that free vitamin D is a more appropriate biomarker of vitamin D sufficiency [5, 6, 23] and suggest that in patients in whom congenital DBP deficiency is suspected, or in scenarios like acute illness where acquired DBP deficiency is more common, measurement of free vitamin D metabolites and DBP levels be performed so as to avoid unnecessary or potentially harmful vitamin D supplementation.

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Additional Information

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Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in "References."

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