CASE REPORT

A novel missense mutation in *GALNT3* causing hyperostosis–hyperphosphataemia syndrome

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Abstract

Objective: Hyperostosis–hyperphosphataemia syndrome (HHS) is a rare hereditary disorder characterized by hyperphosphataemia, inappropriately normal or elevated 1,25-dihydroxyvitamin D₃ and localized painful cortical hyperostosis. HHS was shown to be caused by inactivating mutations in *GALNT3*, encoding UDP-*N*-acetyl- α -*D*-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNAc-transferase; *GALNT3*). Herein, we sought to identify the genetic cause of hyperphosphataemia and tibial hyperostosis in a 19-year-old girl of Colombian origin.

Methods: Genomic DNA was extracted and sequencing analysis of the *GALNT3* and fibroblast growth factor 23 (*FGF23*) genes performed. Serum levels of intact and C-terminal FGF23 were measured using two different ELISA methods.

Results: Mutational analysis identified a novel homozygous missense mutation in exon 6 of *GALNT3* (1584 G>A), leading to an amino acid shift from Arg to His at residue 438 (R438H). The mutation was not found in over 200 control alleles or in any single nucleotide polymorphism databases. The R438 residue is highly conserved throughout species and in all known GalNAc-transferase family members. Modelling predicted the substitution deleterious for protein structure. Importantly, the phosphaturic factor FGF23 was differentially processed, as reflected by low intact (15 pg/ml) but high C-terminal (839 RU/ml) serum FGF23 levels.

Conclusions: We report on the first missense mutation in *GALNT3* giving rise to HHS, since previous *GALNT3* mutations in HHS caused aberrant splicing or premature truncation of the protein. The R438H substitution likely abrogates *GALNT3* activity, in turn causing enhanced FGF23 degradation and subsequent hyperostosis/hyperphosphataemia.

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Introduction

Hyperostosis–hyperphosphataemia syndrome (HHS; OMIM #610233) is a rare autosomal recessive metabolic disorder first described in 1970 (1). It is characterized by persistent elevated serum inorganic phosphate (Pi), high or inappropriately normal 1,25-dihydroxyvitamin D₃ levels (with regard to hyperphosphataemia) and recurrent episodes of painful swellings of the long bones (2, 3). Radiological examinations reveal periosteal reactions with cortical hyperostosis and periosteal apposition of bone. The condition is usually painful and could also be associated with an inflammatory response in the skin at the sites of hyperostosis. In addition, some HHS patients present with dental engagement (4, 5).

HHS shares many biochemical similarities with hyperphosphataemic familial tumoral calcinosis (HFTC; OMIM #21900) (6, 7) including persistent hyperphosphataemia as a result of increased tubular reabsorption rate of phosphorus (TRP%), and normal or elevated levels of 1,25-dihydroxyvitamin D₃. By contrast, the main

clinical features of HFTC are ectopic calcified tumoral masses and vascular calcifications (6–10), which are not observed in HHS.

HHS and HFTC are ultimately caused by enhanced processing and inactivation of fibroblast growth factor 23 (FGF23), a circulating factor that negatively regulates serum levels of Pi and 1,25-dihydroxyvitamin D₃ (8, 9, 11, 12). The augmented processing of FGF23 is either due to inactivating mutations in *GALNT3* (10, 11), preventing proper O-linked glycosylation of FGF23, or directly by destabilizing mutations in *FGF23* (8, 9, 12, 13). Therefore, HHS and HFTC are thought to be two different manifestations of the same disorder (14).

Notably, since HHS is a very rare syndrome, only a few cases in the world have been described. All reported mutations in *GALNT3* causing HHS give rise to premature truncation of the protein or aberrant splicing with skipping of one or more exons (10, 14–16). In the present study, we sought to determine the genetic aetiology of severe tibial hyperostosis/sclerosis and hyperphosphataemia in a 19-year-old girl of Colombian origin.

Patients and methods

Study subject

Written informed consent was obtained from the patient before participation in the study. Serum biochemistries were assessed at Malmö University Hospital.

GALNT3 and FGF23 mutational analyses

Genomic DNA was extracted from blood samples using the Wizard Genomic DNA Purification Kit (STS Promega). All exons in the *GALNT3* and *FGF23* genes, including the intron–exon boundaries, were PCR amplified with AmpliTaq Gold DNA (Applied Biosystems, Foster City, CA, USA) polymerase using 200 ng genomic DNA. PCR conditions for all experiments were as follows: 10 min at 95 °C, followed by 40 cycles of 45 s at 95 °C, 1 min at 60 °C, 1 min at 72 °C and a final extension of 7 min at 72 °C. PCR products were electrophoresed on 1% agarose gels and visualized under u.v. light before gel purification with QIAquick Gel Extraction Kit (Qiagen). Purified products were directly sequenced from forward and reverse primers, using BigDye v3.1 and the 3130×1 Genetic Analyser (Applied Biosystems). Sequencing primers for *GALNT3* and *FGF23* are available upon request.

FGF23 serum assays

Intact FGF23 was analysed using an ELISA according to the manufacturer's protocol (Kainos Laboratories International, Tokyo, Japan) (17). FGF23 was also evaluated using a sandwich ELISA detecting both intact FGF23 and C-terminal FGF23 fragments (Immutopics Inc., San Clemente, CA, USA) according to the manufacturer's protocol (18).

Results

Clinical findings

The patient is a 19-year-old adopted Swedish girl of Colombian origin. She presented with painful cortical

lesions in the left tibia at 5 years of age. Biochemical analysis revealed marked hyperphosphataemia secondary to increased TRP% and normal 1,25-dihydroxyvitamin D₃. Calcium and alkaline phosphatase were also elevated, whereas parathyroid hormone was below the reference range. A summary of biochemical variables is presented in Table 1.

Radiological examinations indicated diaphyseal hyperostosis, periosteal reactions and oedema in the surrounding tissue of the affected area (Fig. 1). Nine months later she started suffering from pain in the right foot and lower leg. In the following years until the present time, she suffered from reoccurring episodes of skeletal pain in the lower extremities and continuously displayed a manifest hyperphosphataemia. In addition to analgesics, various treatments including phosphate binders and bisphosphonates were tested without convincing results.

To determine the role of FGF23 in this patient, we analysed C-terminal as well as intact FGF23 using two different ELISA techniques. Intact FGF23 was subnormal (15 pg/ml; normal range (mean ± s.d.) 28.9 ± 1.1 pg/ml) (17) while C-terminal FGF23 severely elevated (839 RU/ml; normal range (mean ± s.d.) 55 ± 50 RU/ml), supporting increased processing and inactivation of FGF23.

Mutation analysis

Due to the differential processing of FGF23 and biochemical features similar to HHS in our patient, we examined the *GALNT3* and the *FGF23* genes for mutations. No mutations were found in *FGF23* (data not shown). By contrast, we discovered a novel homozygous 1584 G>A substitution in exon 6 of *GALNT3*, causing an amino acid change from Arg to His at residue 438 (R438H; Fig. 2a). This mutation was not found in the NCBI single nucleotide polymorphism (SNP) database (www.ncbi.nlm.nih.gov/SNP/) or in the publicly available Japanese SNP database (<http://snp.ims.u-tokyo.ac.jp/>), neither was it found in 240 Chinese nor in 232 Nigerian control alleles (<http://www.hapmap.org/>). In concert,

Table 1 Biochemical characteristics of the hyperostosis–hyperphosphataemia syndrome (HHS) patient.

Variable	Reference range	Value at presentation
Intact FGF23 (pg/ml)	28.9 ± 1.1 (mean ± s.d.)	15^a
C-terminal FGF23 (RU/ml)	55 ± 50 (mean ± s.d.)	839^a
Phosphate (mg/dl)	3.1–5.6	8.0
Calcium (mg/dl)	8.8–10.4	10.92
1,25(OH) ₂ D ₃ (pmol/l)	24–154	83
25(OH)D ₃ (nmol/l)	38–200	57
Creatinine (mg/dl)	0.25–0.64	0.55
PTH (pg/ml)	10–65	<6.6
TRP (%)	78–98	98.5
ALP (μkat/l)	1.8–7.2	8.3

Note the low intact but markedly elevated C-terminal level of FGF23, indicating differential processing. Abnormal values are in bold. The given reference ranges for phosphate, creatinine and ALP are obtained from children aged 4–6 at Uppsala University Hospital.

^aMeasured in 2007.

Figure 1 Radiological examinations of the HHS patient. (a) Skeletal radiograph of the right tibia at 13 years of age shows hyperostosis with periosteal apposition and cortical thickening (left arrow) as well as severely increased diaphyseal sclerosis (right arrow). (b) MRI of tibiae at 11 years of age demonstrates hyperostosis with periosteal apposition and oedema (indicated by arrow) in the surrounding tissue.

the substitution was not present in 200 Caucasian control alleles, suggesting it is a novel mutation. Real-time PCR analysis suggested equal copy numbers of *GALNT3* alleles, excluding the possibility of a genomic deletion (data not shown).

Structural analyses

To determine the effects of the R438H substitution on *GALNT3* protein structure, we used SIFT software prediction (<http://blocks.fhcrc.org/sift/SIFT.html>). In this model, the R438H substitution was predicted deleterious for protein structure ($P < 0.01$; data not shown). The substitution is predicted to disrupt a C-terminal β -strand of *GALNT3* protein as determined by protein secondary structure prediction (<http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html>) and is located within a region highly conserved throughout species (Fig. 2b). Finally, residues homologous to the R438 in *GALNT3* of all known GalNAc-transferase protein family members were completely conserved (Fig. 2c).

We also analysed whether the R438H substitution affected any functional motifs employing the PROSITE prediction database (<http://au.expasy.org/prosite/>). The R438 residue did not comprise any known functional motifs (data not shown).

Discussion

In the present report, we confirmed that the *GALNT3* gene is responsible for HHS in a Swedish patient of Colombian origin. This is the first report describing a *GALNT3* mutation causing HHS in a patient from South America, since previously DNA sequenced HHS patients were from the Middle East and Europe respectively (14–16). Unfortunately, we were unable to establish a family pedigree since the patient was adopted and no information was available with regard to the relatives and family history.

The genetic cause of HHS has previously been determined only in very few patients. These had mutations affecting splice sites or resulted in a premature determination of translation (14–16). By contrast, our patient harboured a homozygous missense mutation in exon 6 leading to a single amino acid substitution. Importantly, this is the first reported missense mutation in *GALNT3* causing HHS. The substitution is not located within the enzymatic region of *GALNT3*, supporting that *GALNT3* activity is compromised through an altered protein structure, in turn leading to the enhanced FGF23 degradation.

Although we cannot entirely rule out the possibility that the 1584 G>A substitution is a rare ethnicity-specific polymorphism, we provide compelling evidence

Figure 2 (a) Sequencing analyses revealed a homozygous point mutation in exon 6 of *GALNT3* (indicated by the arrow), leading to an amino acid shift from an Arg to a His at residue 438 (R438H). The wild-type sequence of control alleles is shown above to the right. (b) Sequence alignment indicates that the R438 residue is highly conserved throughout species. Modelling suggests that the R438H substitution is detrimental to *GALNT3* protein structure, leading to the enhanced FGF23 degradation and subsequent hyperostosis/hyperphosphataemia. (c) Sequence alignment of all known GalNAc-transferase family members showing that the residues homologous to the R438 in *GALNT3* also are highly conserved.

that the 1584 G>A substitution is disease causing; it is not present in over 200 Caucasian control alleles; it is not found in any available SNP databases; it is predicted highly deleterious for protein structure; homologous residues of the affected R438 in *GALNT3* are completely conserved throughout species and known GalNAc-transferase protein family members; all patients with *GALNT3*/*FGF23* mutations display the same FGF23 serum profile as our HHS patient (19, 20); and the

known associative physiology of *GALNT3*, FGF23 and its relation to HHS.

Since we did not find any heterozygous changes in the coding regions of *GALNT3*, we cannot theoretically rule out that the mutation could be a combination of a missense mutation in one allele and a genomic deletion of the other. This is unlikely since real-time PCR analysis suggested equal copy numbers of *GALNT3* alleles in the HHS patient and a healthy control. Regardless, the allele

carrying the 1584 G>A substitution in our patient is presumably non-functional since HHS is a recessive disorder and previous studies support that a single *GALNT3* allele is sufficient to maintain a normal phosphate balance (10, 15, 16).

The biochemical phenotypic similarity of HHS and HFTC is striking since both disorders are characterized by hyperphosphataemia, increased TRP% and inappropriately normal or high levels of 1,25-dihydroxyvitamin D₃. By contrast, most HFTC patients display ectopic and/or vascular calcifications, whereas HHS patients predominantly suffer from cortical hyperostosis but no other apparent calcifications. A common pathogenesis of these disorders has been proposed based on the fact that some HFTC patients also harbour skeletal hyperostosis (21, 22), but also with regard to the augmented FGF23 processing causing a low serum intact/C-terminal FGF23 ratio (11, 14).

The hypothesis that HHS and HFTC are different manifestations of the same disorder has been further validated through molecular diagnostics. HFTC is caused by mutations in the *GALNT3*, *FGF23* or *KLOTHO* gene (8–10, 12, 23), whereas the small number of reported HHS patients only harbour *GALNT3* mutations (14–16). *GALNT3* has been shown to directly O-glycosylate FGF23 (11), preventing intracellular FGF23 cleavage by furine-like proprotein convertases. *KLOTHO* is a permissive FGF-receptor cofactor for FGF23 (24) and thus a determinant of FGF23 activity. Due to the similarities in molecular mechanisms underlying these disorders, the variations in phenotypic expression is likely dependent on other factors, e.g. genetic background, differences in local environment and counter-regulatory mechanisms.

The level of C-terminal FGF23 fragments in our patient was significantly lower when compared with previously reported patients with HHS and HFTC (8, 9, 11, 12, 15). This indicates that our mutation does not confer a complete loss of function, but that *GALNT3* retains some of its enzymatic activity. Therefore, it would be of interest to determine any possible genotype–phenotype correlations amongst HHS and HFTC patients and also to identify factors that determine the development towards a possible HHS (hyperostosis) or HFTC (ectopic/vascular calcification) phenotype.

In conclusion, we identified a novel missense mutation in *GALNT3* as the cause of HHS and increased FGF23 processing. Our data support that functional *GALNT3* is required for maintaining FGF23 stability. In addition, the aberrant processing of FGF23 in our patient further highlights the physiological role of FGF23 in bone and mineral homeostasis.

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