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ORIGINAL ARTICLE

Mutational analysis of *PHEX*, *FGF23*, *DMP1*, *SLC34A3* and *CLCN5* in patients with hypophosphatemic rickets

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This study aimed to identify the underlying genetic mutation in patients with hypophosphatemic rickets (HR). Genomic DNA was analysed for mutations in *PHEX*, *FGF23* and *CLCN5* by polymerase chain reaction (PCR) followed by denaturing high-performance liquid chromatography (dHPLC). Bi-directional sequencing was performed in samples with deviating chromatographic profiles. *DMP1* and *SLC34A3* were sequenced, only. In addition, a multiplex ligation-dependent probe amplification (MLPA) analysis was performed to detect larger deletions/duplications in *PHEX* or *FGF23*. Familial cases accounted for 12 probands while 12 cases were sporadic. In 20 probands, mutations were detected in *PHEX* of which 12 were novel, and one novel frameshift mutation was found in *DMP1*. Three *PHEX* mutations were identified by the MLPA analysis only; that is, two large deletions and one duplication. No mutations were identified in *FGF23*, *SLC34A3* or *CLCN5*. By the methods used, a disease causing mutation was identified in 83% of the familial and 92% of the sporadic cases, thereby in 88% of the tested probands. Genetic analysis performed in HR patients by PCR, dHPLC, sequencing and in addition by MLPA analysis revealed a high identification rate of gene mutations causing HR, including 12 novel *PHEX* and one novel *DMP1* mutation.

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INTRODUCTION

Hypophosphatemic rickets (HR) comprises a group of rare inherited diseases with an incidence of 3.9 per 100 000 live births and a prevalence of 1:21 000.[1] The first description of the disease was by Albright in 1937.[2] Characteristically, children present during the first 1–2 years of age with bowing of the weight-bearing extremities and growth failure. With increasing age, many patients experience painful joints, arthrosis, enthesopaties (calcification of ligaments and their attachment to bone) and recurrent spontaneous abscesses of the teeth.[3] The most common form is X-linked HR (XLHR; MIM 307800), exhibiting a dominant trait of inheritance. The causative gene coding for the phosphate-regulating endopeptidase homologue, X-linked, (*PHEX*; MIM 300550) was identified in 1995.[4] Today, 300 different mutations in *PHEX* are listed in the *PHEX*db (hhtp://www.phexdb.mcgill.ca/, accessed April 2012). Subsequently, mutations in several other genes responsible for rare forms of HR have been identified. In the year 2000, the principal regulator of the phosphate homoeostasis, fibroblast growth factor 23 (FGF23) was isolated and a mutation in *FGF23* (MIM 605380) was associated with autosomal dominant HR (ADHR; MIM 193100).[5] In 2006, a mutation in the gene encoding for the dentin matrix protein (*DMP1*; MIM 600980) was identified in patients with autosomal recessive HR (ARHR1; MIM 241520),[6] and a mutation in ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*; MIM 173335) was in 2010 shown to cause autosomal recessive HR (ARHR2; MIM 613312).[7,8] XLHR, ADHR, ARHR1 and ARHR2 share identical biochemical characteristics of excessive renal phosphate wasting and low-serum phosphate associated with elevated levels of serum FGF23 and accompanied by inappropriately low serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$).[8,9] Two types of HR differ biochemically from the four described types, as they are characterised by hypercalciuria: Hereditary HR with hypercalciuria (HHRH; MIM 241530), where the hypercalciuria is due to increased serum $1,25(OH)_2D$. The inheritance is autosomal recessive and the disease is caused by a mutation in the sodium-cotransporter gene (*SLC34A3*; MIM 609826), identified in 2006.[10,11] The second type is X-linked recessive HR (MIM 300554), characterised by proximal renal tubulopathy and Fanconi syndrome caused by a mutation in the gene coding for the chloride channel 5 (*CLCN5*; MIM 300008).[12,13]

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Current recommendations on medical treatment of the HR types without hypercalciuria are intermittent oral phosphate supplementation in combination with alfacalcidol, carefully adjusted to avoid the development of secondary hyperparathyroidism or nephrocalcinosis.[14] Medical treatment improves the bowing of extremities and the stunted growth in children, and prevents the recurring dental abscesses and the dentin malformation, but early treatment onset is crucial for obtaining sufficient efficacy.[15–17] In the HR types characterised by hypercalciuria, treatment is oral phosphate supplementation alone.[9]

The aim of this study was to identify the underlying genetic mutation in patients with HR by use of the traditional methods of genetic analysis and in addition the recently introduced MLPA method. The finding of a genetic diagnosis enables genetic counselling and early diagnosis ensures early treatment of affected offspring.

MATERIALS AND METHODS

Patients

The HR patients were recruited from a cross-sectional study in Denmark.[3] As the study has been described in detail elsewhere, only a brief account of the method of patient inclusion is given. Originally, the patients were identified in the Danish National Patient Registry by a search based on the diagnosis codes of vitamin D-resistant rickets. The inclusion area was Jutland and Funen, covering approximately 3.0 million inhabitants and thereby 55% of the total Danish population. The diagnosis was confirmed by review of the patients' medical files. By contact to the treating doctors, patients with HR who did not appear in the register were identified. Finally, family screening added additional cases. The inclusion criteria were biochemically verified HR, and in addition a history of childhood rickets or spontaneous dental abscesses was required to exclude acquired HR, for example, Tumour-induced osteomalacia (TIO). The biochemical criterion of HR was at least one of the following parameters: serum phosphate below normal range, low renal threshold value for reabsorption of phosphate in the urine (TPO_4/GFR) or elevated serum FGF23. Patients with secondary rickets due to malabsorption, TIO, or hereditary vitamin D-dependent rickets type 1 (VDDR type 1) were excluded.

Genetic Analysis

Genomic DNA was extracted from full blood using a DNA purification robot (Maxwell Promega, Ramcon, Denmark). All DNA samples from the probands were initially analysed for mutations in *PHEX*. If no mutations were identified, we proceeded with consecutive analyses of *FGF23*, then *CLCN5* followed by *DMP1* and finally *SLC34A3* was analysed if no mutations were found in the previously analysed genes. For the analysis of *PHEX*, *FGF23* and *CLCN5*, polymerase chain reaction (PCR) was used covering all introns and intron/exon-boundaries. This was followed by denaturing high-performance liquid chromatography (dHPLC) (WAVE 3500HT High Sensitivity System; Transgenomic Inc, Elancourt, France) testing for small deletions, insertions or point mutations in all exons and exon–intron boundaries of all genes. Samples with deviating chromatographic profiles were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analysed on a 3730XL DNA Analyser (Applied Biosystems). Sequence analysis was performed using SeqMan Software (DNA STAR, Madison, WI, USA). *DMP1* and *SLC34A3* were analysed by direct bidirectional sequencing only. Mutational analysis of *PHEX* and *FGF23* was performed by use of the primers published by Goji *et al.*,[18] and mutational analysis of *CLCN5* was performed by use of the primers published by Lloyd *et al.*[19] We designed primers using Primer Select Software (DNA STAR) for mutational analysis of *DMP1*, *FGF23* and *SLC34A3*. To detect larger deletions of *PHEX* and *FGF23*, a Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed in patients with no mutations detected by dHPLC. The MLPA procedure was performed according to the manufacturer's recommendations (Salsa MLPA, P223 PHEX, MRC-Holland, Amsterdam, the Netherlands) and run on the 3730XL DNA analyser using GeneMarker software (Softgenetics, State College, PN, USA) for the analysis.

The genetic analysis was first performed in the probands. When a *PHEX* mutation was identified, DNA from all first-degree family members and symptomatic second-degree family members were then screened for the detected mutation. Due to the X-linked mode of inheritance, DNA from the fathers of male probands was not analysed for the identified *PHEX* mutation. If the *PHEX* mutation was not detected in the DNA from the parents of the proband, and both parents had no clinical or biochemical signs of HR, this proband was classified as sporadic.

We determined a mutation to be disease causing: (1) when the mutation was previously described in the *PHEX*db (accessed April, 2012) or characterised in publications, but not yet appearing in the *PHEX*db;[20–23] (2) when the mutation identified was present in all family members with clinically and biochemically verified HR, but not in any of the asymptomatic family members; and/or (3) when the mutation type was predicted to cause a non-functional protein as frameshift, deletion, duplication, nonsense or abnormal splicing. Missense mutations were initially tested by the prediction software PolyPhen ('polymorphism phenotyping', http://genetics.bwh.harvard.edu/pph/) and SIFT ('sorting intolerant from tolerant', http://sift.jcvi.org/) to predict the impact of missense mutations on protein structure and function based on sequence alignments. *PHEX* belongs to the M13 subfamily of mammalian metallopeptidases further including *ECE1, ECE2, KELL* and *NEP*. The overall sequence homology of these metallopeptidases is sufficiently high to indicate a common origin and a similar folding pattern.[24] Clustal W (MegAlign, DNA STAR) was used to test the evolutionary conservation of the substituted amino acid in the missense mutations by alignment to the mammalian M13 subfamily of membrane metallopeptidases.[25] Additionally, the molecular visualisation software PyMol 0.99rc6 (www.pymol.org) was used to predict the functional consequence of the missense mutation on the tertiary structure at the site of the mutation. The 3D structure of NEP was described by Oefner *et al.*[26]

Mutations fulfilling criteria two and/or three only were considered novel.

Biochemical Analyses

Serum FGF23 (reported as mean ± s.d.) was analysed by enzyme-linked immunosorbent assay (Kainos Laboratories, Tokyo, Japan). Further details of the biochemical analyses performed have been described previously.[3]

RESULTS

A total of 59 patients with biochemically and clinically confirmed HR were included. They originated from 12 families comprising 47 patients, and 12 sporadic patients. Thus, a total of 24 probands were included in this report. Patients of Danish decent accounted for 23 probands, while one proband originated from Lebanon.

We identified 20 different *PHEX* mutations, of which 12 were novel (Figure 1). In addition, one novel frameshift mutation in exon 5 *DMP1* (c.247_248delGG) was identified in the Lebanese family exhibiting a recessive trait (Figure 2). Table 1 lists a complete description of the mutations identified. Overall, gene mutations were identified in 10 of 12 familial probands (83%), and in 11 of 12 sporadic probands (92%); that is, in 21 of the 24 total probands (88%). The *PHEX* mutations were six abnormal splicing (30%), five frameshifts (25%), four nonsense (20%), two missense (10%), two deletions (10%), and one duplication (5%). Three *PHEX* mutations were identified by the MLPA analysis only; that is, two large deletions and one duplication. No mutations were identified in *FGF23*, *SLC34A3* or *CLCN5*.

The mean S-FGF23 level in patients with a *PHEX* mutation was $198 \pm 401\ pg\,ml^{-1}$, range 46 to $2430\ pg\,ml^{-1}$. Two patients from two different families with proven *PHEX* mutation had normal S-FGF23 levels of 46 and $49\ pg\,ml^{-1}$, respectively. The S-FGF23 value for the sporadic probands and the mean (s.d.) for families are provided in Table 1.

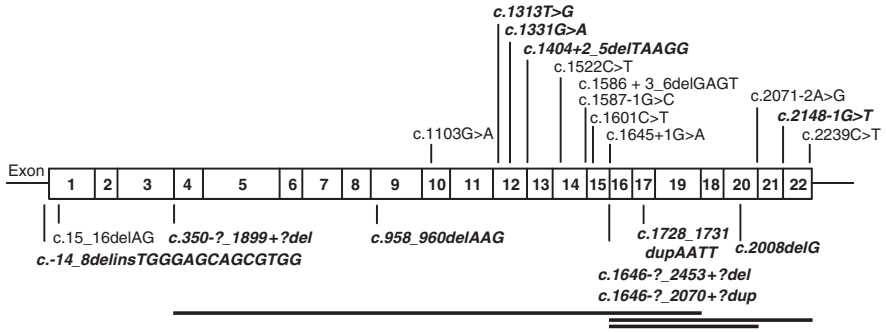

Figure 1 Distribution of mutations identified in *PHEX* in this study. The numbers in the boxes indicate the 22 exons in *PHEX*. The novel mutations are in bold/italics. Abnormal splicing, missense and nonsense mutations are represented above the gene, frameshift, deletions and duplications below. Thick, black lines indicate the large deletions and duplication identified by MLPA.

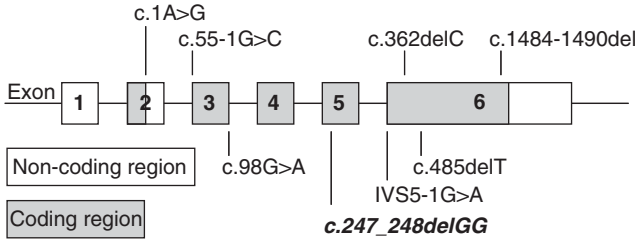

Figure 2 The figure depicts the six exons of *DMP1* and the approximate location of the mutations previously identified (c.1A>G,[6,33] c.55-1G>C,[6] c.98G>A,[34] IVS5-1G>A,[35] c.362delC,[6] c.485delT,[36] c.1484-1490del[33]). The novel *DMP1* mutation identified in our study, c.247_248delGG is in bold/italics.

The proband homozygous for the *DMP1* mutation was the child of clinically and biochemically healthy parents, both heterozygous for the *DMP1* mutation identified. The parents were immigrants from Lebanon and reported being non-consanguineous. At the age of 1.5 years the patient complained of painful legs and had genu varus. Due to complete renal calcium retention he was primarily suspected of nutritional rickets and was treated as such from the age of 2.3 until the diagnosis of HR was established at the age of 3.5 years, where after treatment with phosphate and Alphacalcidol was commenced. The patient was 6.2 years of age when examined for this study. He was short statured with a height s.d. of -1.4, showed a disproportioned sitting height ratio of 3.8 s.d., and had genu varus of 5 cm (3.3 s.d.). He had no history of dental problems. Biochemically, his S-phosphate was 0.9 mmol l^{-1} (normal range: 1.16–1.81 mmol l^{-1}), S-FGF23 was 71 pg ml^{-1} (10–50 pg ml^{-1}), the tubular reabsorption of phosphate per glomerular filtration rate was 0.67 nmol ml^{-1} (1.16–1.81), and S-parathyriod hormone was 1.9 pmol l^{-1} (1.1-6.9 pmol l^{-1}).

DISCUSSION

We identified 20 *PHEX* mutations of which 12 were novel, and in addition one novel frameshift mutation in *DMP1* in HR probands. The addition of the MLPA analysis ensured a high detection rate of gene mutations, and revealed three novel *PHEX* mutations.

The frequency of the different types of mutations identified in this study differs slightly from the frequencies reported by PHEXdb (in brackets): abnormal splicing 30% (23%), frameshifts 25% (25%), nonsense 20% (18%), missense 10% (22%), deletions 10% (8%), and duplications 5% (0%). We identified fewer missense mutations, but the overall frequency of mutation types identified by the MLPA analysis (that is, deletions and duplications) was higher in our study.

We report two missense mutations, the Pro534Leu also reported by several other groups,[27–32] and a novel Leu438Trp mutation. Pro534 is conserved between the related mammalian M13 subfamily of membrane metallopeptidases *KELL*, *ECE1*, *ECE2* and *PHEX*. In *NEP* this position is substituted by an Alanine. Pro534 is in immediate juxtaposition to the active site of the enzyme (Figure 3A). The alignment using Clustal W predicts a conformational change caused by the substitution p.Pro534Leu due to its close proximity to Y478 in the linker between β-strand II1 and α-helix D3. This conformational change involves the highly conserved VNA motif of the ligand-binding domain situated immediate juxtaposed to β-strand III1 (Figure 3B). Leu438 is conserved between *KELL*, *ECE1*, *NEP* and *PHEX*. In *ECE2* this position is substituted by a Methionine (Figure 3C). The alignment depicts the position of Leu438 relative to the consensus C1 α-helical structure. Substituting Leu438 with an aromatic Tryptophane is not compatible with the α-helical structure and possible detrimental to protein function (Figure 3D). As evident from this illustration, the substitution p.Leu438Trp will result in a conformational change relocating the HEITH motif caring α-helix F1. This is predicted to influence Zn binding and probably abolish the protein function. This novel missense mutation was present in three members of a family where the proband (male), his mother and his daughter, all had biochemically and clinically confirmed HR. A clinically and biochemically unaffected sister of the proband did not carry the missense mutation.

We report a novel *DMP1* mutation identified in one proband from a Lebanese immigrant family living in Denmark and exhibiting a recessive trait of inheritance. Including this novel frameshift mutation, a total of seven mutations to date has been reported in *DMP1* (Figure 2).[6,33–36]

As *ENPP1* was not yet detected as a gene causative of autosomal recessive HR when this study was performed, we did not analyse this gene for mutations. Of the two familial probands without a genetic diagnosis, one originated from a large family comprising 12 patients exhibiting an X-linked dominant trait and a genome-wide linkage scan has revealed strong evidence of linkage to the *PHEX* locus.[3] The second family, which also showed a dominant trait, consists of a mother and her son, both of which have disease. The analysis of *ENPP1* was therefore only relevant for the one sporadic proband in whom no mutations were found in the five genes studied.

Table 1 The *PHEX* mutations and one *DMP1* mutation identified in HR patients in this study

Gene	Method	Kindred	Gender, proband	S-FGF23[a]	Location	DNA-level	Nucleotide change	Protein level	Type	Mutation	Amino acid change
PHEX	dHPLC/ sequencing	F-1	Male	69 (±22)	Ex 01	c-14_8delinsTGG GAGCAGCGTGG	Substitution	p.?	Frameshift	Novel	No transcript
PHEX	dHPLC/ sequencing	S-4	Female	128	Ex 01	c.15_16delAG	Deletion	p.5ThrfsX44	Frameshift	Not novel	Truncated protein
PHEX	MLPA	F-5	Male	112 (±42)	Ex 04–18	c.350-?_1899+?del	Deletion	p.Lys118_655del	Deletion	Novel	Truncated protein
PHEX	dHPLC/ sequencing	F-8	Female	95 (±27)	Ex 09	c.958_960delAAG	Deletion	p.Lys320del	Frameshift	Novel	Truncated protein
PHEX	dHPLC/ sequencing	S-15	Female	103	Ex 10	c.1103G>A	Substitution	p.Trp368X	Nonsense	Not novel	Trp to Ter
PHEX	dHPLC/ sequencing	F-3	Male	51 (±6)	Ex 12	c.1313T>G	Substitution	p.Leu438Trp	Missense	Novel	Leu to Trp
PHEX	dHPLC/ sequencing	S-23	Male	180	Ex 12	c.1331G>A	Substitution	p.Trp444X	Nonsense	Novel	Trp to Ter
PHEX	dHPLC/ sequencing	S-28	Female	67	Inv 12 and Ex 12[b]	c.1404+2_5delTAAGG	Deletion	p.?	Abnormal splicing	Novel	Splice donor
PHEX	dHPLC/ sequencing	S-19	Female	98	Ex 14	c.1522C>T	Substitution	p.Gln508Ter	Nonsense	Not novel	Gln to Ter
PHEX	dHPLC/ sequencing	S-22	Male	73	Inv 14	c.1586 + 3_6delGAGT	Deletion	p.?	Abnormal splicing	Not novel	Splice donor
PHEX	dHPLC/ sequencing	F-14	Female	331 (±368)	Inv 14	c.1587-1G>C	Substitution	p.?	Abnormal splicing	Novel	Intron retention/exon skipping
PHEX	dHPLC/ sequencing	F-9	Female	310 (±331)	Ex 15	c.1601C>T	Substitution	p.Pro534Leu	Missense	Not novel	Pro to Leu
PHEX	dHPLC/ sequencing	F-6	Female	91 (±59)	Inv 15	c.1645+1G>A	Substitution	p.?	Abnormal splicing,	Not novel	Splice donor
PHEX	MLPA	S-20	Female	56	Ex 16–22	c.1646-?_2453+?del	Deletion	p.Gly590_Trp749del	Deletion	Novel	Truncated protein
PHEX	MLPA	F-10	Female	68 (±20)	Ex 16–20	c.1646-?_2070+?dup	Duplication	p.Val634_H690dup	Duplication	Novel	Truncated protein
PHEX	dHPLC/ sequencing	S-18	Male	321	Ex 17	c.1728_1731dupAATT	Duplication	p.Ile577fsX5	Frameshift	Novel	Truncated protein
PHEX	dHPLC/ sequencing	S-34	Female	246	Ex 20	c.2008delG		p.Glu679FsX17	Frameshift	Novel	Truncated protein
PHEX	dHPLC/ sequencing	S-16	Female	72	Inv 20	c.2071-2A>G	Substitution	p.?	Abnormal splicing	Not novel	Splice acceptor
PHEX	dHPLC/ sequencing	S-13	Male	2430	Inv 21	c.2148-1G>T	Substitution	p.?	Abnormal splicing	Novel	Splice acceptor
PHEX	dHPLC/ sequencing	F-12	Female	70 (±13)	Ex 22	c.2239C>T	Substitution	p.Arg747X	Nonsense	Not novel	Arg to Ter
DMP1	dHPLC/ sequencing	F-21	Male	71	Ex 5a	c.247_248delGG	Deletion	p.G82FsX2	Frameshift	Novel	Truncated protein

Abbreviations: Ex, exon; F, familial; dHPLC, denaturing high-performance liquid chromatography; HR, hypophosphatemic rickets; Inv, intervening sequence; MLPA, multiplex ligation-dependent probe amplification; S, sporadic.
[a]S-FGF23 (pg ml^{-1}), mean (±s.d.), normal values (10–50 pg ml^{-1}).
[b]Inv 12 and Ex 12: overlap between intron 12 and exon 12.

Our detection rate of 88% of gene mutations causing HR was high and only exceeded by the recent study by Morey *et al.*,[37] where the MLPA analysis was also performed. The detection rate in familial probands of 83%, and especially our detection rate in probands with sporadic HR of 92% were prominent. The overall median detection rate of gene mutations reported in previous studies comprising 15 or more HR probands was 66% (range 43–100%),[20,27–32,37–41] in familial probands 66% (44–100%), and in sporadic probands the detection rate was 50% (29–100%).[20,27–30,32,37,38] In our study, the MLPA analysis added three *PHEX* mutations not identified by PCR, dHPLC or sequencing, thus increasing our overall detection rate from 78 to 88%, in familial probands from 67 to 83% and in sporadic probands from 83 to 92%. Our high detection rate of gene mutations, especially among the sporadic patients, may also be due to the robust inclusion/exclusion criteria of this study.

S-FGF23 may be used as a biochemical marker distinguishing the FGF23-mediated types of HR (XLHR, ADHR, ARHR1 and ARHR2) from the non-FGF23-mediated types (HHRH, and X-linked recessive HR).[42] In our study population only FGF23-mediated types of HR were identified, but two patients with proven XLHR displayed levels slightly below the upper limit of the normal range of 10–50 pg ml^{-1}.[43] Normal S-FGF23 levels in genetically verified XLHR has been published in a few cases[43,44] but in the vast majority of HR patients, S-FGF23 levels are elevated, and this significantly more in patients receiving medical treatment.[45,46] Patients with TIO display even higher values of S-FGF23 than the XLHR patients in this study,

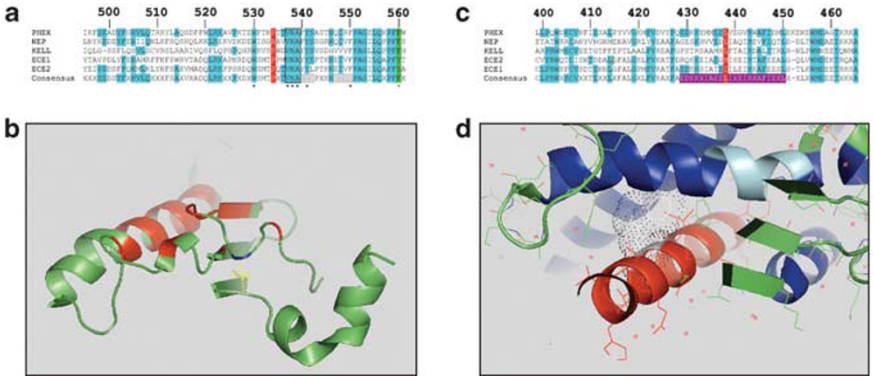

Figure 3 Multiple sequence alignment of *PHEX* against the mammalian M13 subfamily of membrane metallopeptidases. (**a**) The position of p.Pro534 is indicated in red, the active site by asterisks, ligand-binding residues are boxed, beta sheets in grey and residues highly conserved relatively to *PHEX* are indicated in turquoise. (**b**) The three-dimensional (3D) structure of a part of *PHEX* based on the published 3D structure of *NEP1* (PBD 1DMT).[26] Highlighted in red are residues of the active site. Pro534 is indicated in blue. The distance to Y478 is indicated. Rendering was performed using Pymol 0.99rc6. (**c**) The position of p.Leu438 is indicated in red, α-helix C1 in purple. Conserved residues relative to *PHEX* in turquoise. (**d**) The 3D structure of part of *PHEX* based on the published 3D structure of *NEP1* (PBD 1DMT). Highlighted in red are α-helix C1. p.Leu438 is indicated in cyan. The spacing of p.Trp438 is indicated by dots and sticks. A-helix F1 is indicated in blue and the HEITH motif in aquamarine. Rendering was performed using Pymol 0.99rc6.

mean 934 ± 1115 pg ml^{-1} vs 198 ± 401 pg ml^{-1}.[47] Thus, if patients suspected to have HR display S-FGF23 levels exceeding approximately 4–500 pg ml^{-1}, one should bear the differential diagnosis of TIO in mind. One patient in this study had a markedly elevated S-FGF23 of 2430 pg ml^{-1}. He was treated with very-large doses of phosphate ranging from 100–200 mg kg^{-1} per day as the age of 1.8 years and nephrocalcinosis and hyperparathyroidism were demonstrated at the age of 10. He developed tertiary hyperparathyroidism demanding surgical removal of the glandulae parathyroidea at the age of 17 years. He had a decreased creatinine clearance glomerular filtration rate most likely contributing to the markedly elevated S-FGF23 as demonstrated in patients with chronic kidney disease.[48] TIO was not suspected due to the clinical history and the finding of a *PHEX* mutation.

Identification of the causative gene mutation in HR patients is not mandatory for the diagnosis of HR, but it reveals the underlying disease mechanism and confirms the clinical and biochemical diagnosis of HR. If no mutation in the known HR genes is identified, differential diagnoses implying renal phosphate wasting should be considered. Furthermore, a genetic diagnosis allows early detection of affected offspring. This enables early intervention with medical treatment, which is important for optimal therapy.

In conclusion, we extend the spectrum of mutations in *PHEX* and *DMP1* causing HR. Our high detection rate of gene mutations was achieved by addition of the MLPA analysis and also by robust inclusion/exclusion criteria. We encourage testing HR patients for gene mutations as this allows early detection of affected offspring and ensures early treatment intervention.

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