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ORIGINAL ARTICLE

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Hereditary hypophosphatemic rickets with hypercalciuria: a study for the phosphate transporter gene type IIc and osteoblastic function

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Abstract Two cases of hereditary hypophosphatemic rickets with hypercalciuria (HHRH) were reported in Japanese female siblings. Both of them manifested short stature and bowed legs, and biochemical examination revealed hypophosphatemia, phosphaturia, and hypercalciuria. The serum concentrations of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) were elevated. In the oral phosphate loading test, serum phosphate levels were markedly increased in the HHRH patients, and the elevation was much higher than that in patients affected with X-linked hypophosphatemic rickets (XLH), suggesting the increased gastrointestinal absorption of phosphate in HHRH. Bone histology studies showed increased osteoid surface and width in HHRH, which was compatible with osteomalacia. In the HHRH patients, there were no hypomineralized periosteocytic lesions, which was a hallmark of XLH in bone histology. In one of the HHRH patients, phosphate administration alone almost completely cured the osteomalacia within a year, although pharmacological doses of $1,25(OH)_2D_3$ had little effect. In osteoblasts isolated from a HHRH patient, basal alkaline phosphatase (ALP) activities and osteocalcin syntheses by a physiological concentration of $1,25(OH)_2D_3$ were not stimulated by the increased medium phosphate concentrations from 0.5 to 4 mM. In contrast, these two parameters were stimulated by the increased medium phosphate concentrations both in normal and XLH osteoblasts, although the regulatory patterns of increased osteocalcin syntheses were different from normal to XLH osteoblasts; 2 and 4 mM of phosphate concentrations at least were necessary for normal and XLH osteoblasts, respectively. The gene analysis of phosphate transporter revealed a novel heterozygous mutation (R564C) in the exon of phosphate transporter NPT type IIc. These lines of evidence suggested that the pathogenesis of osteomalacia in HHRH was different from XLH in terms of the utility of phosphate in osteoblasts. These abnormalities were speculated to be associated with the abnormal functions of phosphate transporter gene type IIc, although the exact roles of this phosphate transporter in the human osteoblast are still unknown.

Key words phosphate transporter · bone histomorphometry · osteoblast · phosphate · $1,25(OH)_2D$ · osteocalcin · HHRH · XLH

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Introduction

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is a rare disorder inherited in an autosomal recessive fashion, characterized by hypophosphatemia, short stature, rickets, and/or osteomalacia and secondary absorptive hypercalciuria. It was initially described as a new syndrome in a large group of a closely related tribe of Bedouins [1], and only a few cases were reported thereafter [2,3]. The primary defect of HHRH was believed to be associated with the phosphate transporter gene because of an animal model of phosphate transporter gene knockout [4]; however, this hypothesis was wrong because there were no abnormalities in the sodium-dependent phosphate transporter gene of NPT type IIa in HHRH patients [5]. We also confirmed this observation including the three isoforms of NPT type IIa genes (unpublished data). In fact, a sodium phosphate

transporter of NPT type IIa gene abnormality was reported to be a cause of hypophosphatemic rickets with nephrolithiasis [6]. Recently, a gene abnormality was shown in the intron of a phosphate transporter NPT type IIc [7,8]. However, the correlation between gene abnormalities and osteoblastic functions in human HHRH osteoblasts was still unclear.

In HHRH, hypophosphatemia resulted in the elevation of serum 1,25-dihydroxyvitamin D concentrations, which suggested no abnormal regulation of vitamin D metabolism. Thus, HHRH was fundamentally different from X-linked hypophosphatemia (XLH) or tumor-induced hypophosphatemia (TIO), because serum $1,25(OH)_2D$ levels in these diseases were low or low normal in the presence of hypophosphatemia [9]. Recently, a phosphaturic factor of fibroblast growth factor 23 (FGF-23) was found to be a causal facor for hypophosphatemia of XLH and TIO [10]; however, the plasma levels of FGF-23 were normal in HHRH, as we recently reported [11]. In HHRH, hypophosphatemia alone causes the impairment of bone mineralization in spite of the high level of serum $1,25(OH)_2D$. Moreover, it was reported that phosphate administration improved clinical and radiographic manifestations and also normalized the serum $1,25(OH)_2D$ and renal calcium excretion [3,12], except for renal phosphate loss. Such pathogenesis and the response to phosphate administration in HHRH patients suggested that phosphate played an important role for the bone-forming cells of osteoblasts.

In this article, we investigated two Japanese female siblings affected with HHRH for bone morphology and osteoblastic functions in terms of basal alkaline phosphatase (ALP) activities and osteocalcin synthesis by a physiological concentration of $1,25(OH)_2D_3$ in the presence of various medium phosphate concentrations in culture. We also investigated the presence of gene abnormalities in a phosphate transporter gene NPT type IIc.

Subjects and methods

Subjects

A 51-year-old woman (case 1) was admitted to the orthopedic unit of Osaka University Hospital for an operation on her knees. She manifested bowed legs and short stature; her height was 145 cm without any sign of malnutrition. She had three children who revealed no abnormal laboratory data. Her parents seemed to be normal by historical examination. She had been administered a large amount of calcitriol (9 μg/day) for years, but radiographic examination at admission revealed osteomalacia by rentogenogram. After discontinuance of calcitriol for 2 weeks, biochemical examination revealed normal serum calcium, 8.8 mg/dl; depressed serum phosphate, 2.0 mg/dl; elevated serum ALP, 311 IU/l, with normal renal function. Urinary calcium excretion was increased; calcium/creatinine ratio was 0.320 and the daily urinary calcium excretion was 5 mg/kg body weight. Tubular reabsorption of phosphate was obviously depressed to 65%. Serum concentration of parathyroid hormone (PTH, midportion assay: normal range, 180–560 pg/ml) was within the normal range at 469 pg/ml. Serum $1,25(OH)_2D$ level was increased to 130 pg/ml. Serum level of 25(OH)D was decreased to 5 ng/ml, which suggested the possible coexistence of vitamin D deficiency. However, both the presence of hypercalciuria and the ineffectiveness of several years of calcitriol therapy on osteomalacia and serum ALP activity suggested to us that vitamin D deficiency was not the main problem in this patient. Therefore, we abandoned calcitriol therapy and began phosphate therapy with three tablets K-phos (K-Phos No. 2; Beach Pharmaceuticals, Tampa, FL, USA), which contained 0.75 mg elemental phosphorus.

Case 2 was the sister of case 1, and she was 50 years old when she came to our hospital. She was 140 cm in height and had bowed legs. She was suffering from severe back and knee pain, but she had been never treated. Radiographic examination revealed osteomalacia. The laboratory data were normal serum calcium, 9.2 mg/dl; low serum phosphate, 2.4 mg/dl; depressed tubular reabsorption of phosphate, 55%; depressed serum PTH, 151 pg/ml. Urinary calcium excretion was 6 mg/kg body weight, and urinary calcium/creatinine ratio was 0.349. Serum ALP activity was 102 IU/l, and serum $1,25(OH)_2D$ was 57 pg/ml. Serum level of 25-OHD was 11 ng/ml. We diagnosed her also as HHRH, and began phosphate therapy with three tablets K-phos.

Oral phosphate loading test

To investigate gastrointestinal phosphate absorption in our HHRH patients, an oral phosphate loading test was performed. For a comparison, the test was also carried out in 8 normal male adults and 13 patients (5 males, 8 females) who were clinically diagnosed as X-linked hypophosphatemic rickets (XLH), the prototypical inherited hypophosphatemic osteomalacia. The ages of normal controls and XLH were 38 ± 3.3 ($n = 8$) and 16 ± 13 ($n = 13$), respectively ($P < 0.001$). These tests were performed after the discontinuance of all therapies for at least 2 weeks. The study protocol of the test was described previously [13]. Briefly, patients on therapy were taken off all medication for 2 weeks before the study. Blood samples were obtained in the morning after overnight fasting, then the subjects were administered K-phos tablets at 50 mg/kg body weight (maximum, 2000 mg) elemental phosphorus. Blood samples were collected over a period of 24 h after phosphate loading and serum phosphate concentrations were measured. Serum $1,25(OH)_2D$ levels of HHRH and XLH patients were also measured before the challenge test. Informed consents were obtained from the patients. This protocol was approved by the ethical committee of Osaka University.

Bone histomorphometry

Transiliac bone biopsies were performed before the phosphate therapy in both cases. In case 1, rebiopsy of bone was performed in a year. All the biopsies were performed at the

time of orthopedic surgery for the knee under general anesthesia. The bone specimens were fixed in neutral formaldehyde, dehydrated in ethanol, then embedded in methylmetacrerate, and an undecalcified section was prepared. The sections were stained in Villanueva bone stain, and static histomorphometric measurements were performed by computerized digitizing systems. The following parameters were calculated: bone volume/total volume (BV/TV), osteoid volume/total volume (OV/BV), osteoid volume/bone volume (OV/BV), osteoid surface/bone surface (OS/BS), erosion surface/bone surface (ES/BS), osteoid volume/osteoid surface (OV/OS), and mean osteoid thickness (O.Th).

Osteoblastic functions dependent on different medium phosphate concentrations

Osteoblasts were isolated using human cancellous bone fragment as previously reported [14,15]. Bone samples were obtained from a clinically diagnosed 8-year-old XLH patient and HHRH patient 1. In the XLH patient and a normal 12-year-old patient without metabolic bone disease, bone samples were obtained when orthopedic surgery was performed for correcting femur bone deformity. In HHRH, a part of the first iliac bone biopsy sample was used. Osteoblasts between the 4th and 8th passages were used. Cells were plated at a density of 2×10^4/well and cultured for 7–10 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). The media were then changed to 2% FCS-DMEM containing $1,25(OH)_2D_3$ or 0.1% ethanol, and cells were cultured for 72 h. We prepared five different phosphate concentrations (0.5, 1, 2, 3, and 4 mM) for the experiments. Informed consents were obtained from all the parents or the patients. This protocol was approved by the ethical committee of Osaka University. In the XLH patient, gene analysis for PHEX was not performed because the parents did not agree to the analysis. The method for the preparation culture media containing various phosphate concentrations was reported previously [16]. Osteoblastic functions were evaluated by using osteocalcin synthesis [15] and ALP activity [14] as previously reported. DNA content was also measured as previously reported [16].

Gene analysis of a phosphate transporter NPT type IIc

Genomic DNA was extracted from EB-transformant human lymphocyte from HHRH patients. Then the gene was amplified by polymerase chain reaction (PCR) in all exons using pairs of sense and antisense primers (Table 1). The PCR products were electrophoresed in an agarose gel and purified using a DNA Gel Extraction Kit (Qiagen). Approximately 100 ng each PCR amplicon was directly sequenced using capillary sequence with either sense or antisense primer, using a DYEnamic ET Terminator Kit (GE Healthcare) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Informed consents were obtained from the patients. This protocol was approved by the ethical committee of Osaka University.

Table 1. Primers used for polymerase chain reaction (PCR)

	Sequence (5′–3′)	
Exon 1	ACACAGGTAGGAGCTCCTAG	Sense
Exon 2	ATCTAGACCTGGGCCTGGG	Sense
	AAATCCATGCCGAGTT	Sense
Exon 3	TCCTCCAAGACTGGAGCAGA	Antisense
Exon 4	AGCCTGTACTTCTTCATCTG	Sense
Exon 5	AAGGACAACGTGGTGCTGTC	Sense
	GGTCACAGCCCTGGTGCAGA	Sense
	CATGCTGACCACGATGGAGGA	Antisense
	GGACAGTCAGCAGCTTAGCA	Antisense
Exon 6	GGACAGTCAGCAGCTTAGCA	Antisense
	TGCTGACTGTCCGGGTGTCT	Sense
	CTCTGAAATTCATCCCGGTC	Antisense
Exon 7	ATCTTCAACTGGCTCACAGTGCT	Sense
	AGCACTGTGAGCCAGTTGAAGAT	Antisense
	GCTAGCCCTGGGTGCCG	Sense
Exon 8	GTGCAGTTGGACTCCGACAT	Sense
	ACATGATCATGAGCAGTGCC	Sense
	GCCGCACCAGTGCTTAATGA	Antisense
Exon 9	CAGAGAAGAACAGCACAGCC	Sense
	GGCTGTGCTGTTCTTCTCTG	Antisense
	GGCTGTGCTGTTCCTCTCC	Antisense
Exon 10	CCTCATAGTCAAGCTGCTCA	Sense
	ATTGATGACTGTCCTCACGA	Antisense
Exon 11	CCTGACCTTCGCACTGCAGA	Sense
Exon 12	AACATCGGCACCACTACCAC	Sense
	GTGGTAGTGGTGCCGATGTT	Antisense
Exon 13	TACCTGCTGCTCGATTCCT	Sense
	CTCCAGAGAATGGAGCCAGA	Antisense
	CCAGATAACAGCCTGGTTTA	Antisense

List shows the sequence of the primer and the place of human slc34a3

Statistical analysis

Data were shown as mean ± standard deviation (SD), and significant differences were assessed using one- or two-way analysis of variance (ANOVA). Analyses were performed using Dr. SPSS II. The method of Tukey–Kramer or Dunnet was used as a post hoc test for the one-way ANOVA; the Games–Howell method was used as a post hoc test for the two-way ANOVA because of the nonhomogeneity of the data.

Results

Oral phosphate loading test

In oral phosphate loading test, the serum phosphate levels were increased in all subjects at 2 or 4 h after administration of phosphate. The maximal increase in serum phosphate and serum levels of $1,25(OH)_2D$ before the challenge were significantly higher in HHRH patients than in XLH patients: 3.4 ± 0.28 ($n = 2$) vs. 2.0 ± 0.74 mg/dl ($n = 13$); $P = 0.028$ for the maximal increase of phosphate and 94 ± 52 ($n = 2$) vs. 20 ± 9.1 pg/ml ($n = 13$); $P < 0.001$ for the basal $1,25(OH)_2D$ concentrations respectively (Table 2).

Bone histomorphometry

Microscopic bone findings are shown in Fig. 1; morphometric results are summarized in Table 3. The increased osteoid volume and osteoid seam thickness were compatible with osteomalacia. Hypomineralized periosteocytic lesions (HPL), a hallmark of bone histology of XLH [17], were not found in HHRH patients. In case 1, the only phosphate administration remarkably decreased OV/TV, OV/BV, OV/OS, and O.Th, suggesting improvement of osteomalacia (Table 3).

Osteoblastic functions dependent on medium phosphate concentrations

In osteoblasts isolated from HHRH patients, the responsiveness to a physiological concentration of 10^{-10} M $1,25(OH)_2D_3$ to produce osteocalcin showed no change (Fig. 2, Exp. 1) or a significant decrease at 3 mM medium phosphate concentration (Fig. 2, Exp. 2). In contrast, in normal (Fig. 3) and XLH (Fig. 4) osteoblasts, the osteocalcin synthesis by 10^{-10} M $1,25(OH)_2D_3$ was increased dependent on medium phosphate concentration; 2 mM ($P = 0.009$) and 4 mM ($P = 0.024$) phosphate concentration was necessary for normal and XLH osteoblasts, respectively.

ALP activities of osteoblasts isolated from a normal control at 0.5 mM phosphate concentration were 8.9 nmol paranitrophenol/μgDNA/30minutes (PNP/DNA), which were determined from the two independent experiments; these were 63.6 PNP/DNA and 95.7 PNP/DNA in XLH and HHRH osteoblasts from the three and two independent experiments, respectively. In the experiments, mean values of ALP activity at different medium phosphate concentrations were drawn from the data of at least triplicate deter-

Table 2. Results of oral phosphate loading test in HHRH, XLH, and normal controls

	ΔPi max (mg/dl)	Pi max (mg/dl)	Basal $1,25(OH)_2D$ (pg/ml)
HHRH	3.4 ± 0.28(2)	6.1 ± 1.3(2)	94 ± 52(2)
Vs. normal	$P = 0.473$	$P = 0.994$	$P = 0.002$
Vs. XLH	$P = 0.028$	$P = 0.011$	$P = 0.000$
XLH	2.0 ± 0.74 (13)	4.2 ± 0.77 (13)	20 ± 9.1 (13)
Vs. normal	$P = 0.039$	$P = 0.000$	$P = 0.111$
Normal	2.8 ± 0.45 (8)	6.0 ± 0.74 (8)	38 ± 14 (5)

HHRH, hereditary hypophospatemic rickets with hypercalcuria; XLH, X-linked hyphophosphatemic rickets
ΔPi max, maximal increase of serum phosphate concentrations
Pi max, maximal serum phosphate concentrations

Table 3. Histomorphometric parameters before and after phosphate therapy (case 1)

	Before	After	Normal range
Bone volume/total volume (%)	19.5	17.4	12 ± 4
Osteoid volume/total volume (%)	5.9	1.4	0.46 ± 0.12
Osteoid volume/bone volume (%)	30.3	8.3	2.37 ± 0.47

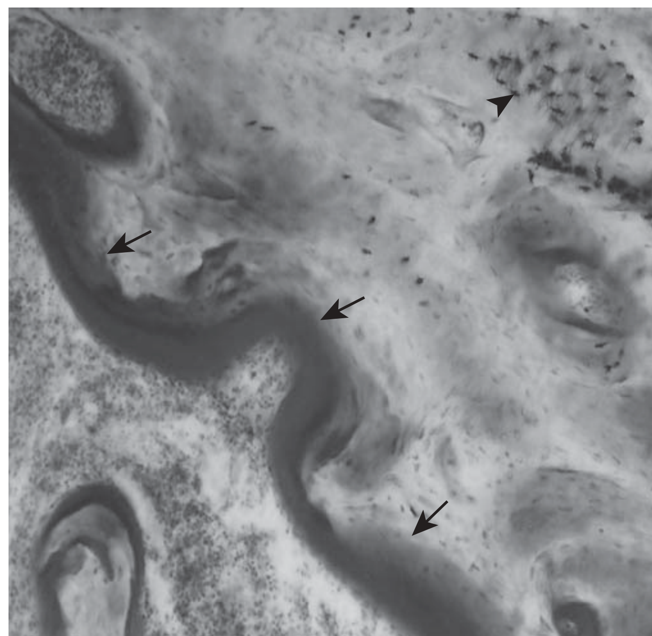

Fig. 1. Hislogical findings of the patient with hereditary hypophosphatemic rickets with hypercalcuria (HHRH) (case 1). Bone specimens were obtained from iliac crest biopsy. The sections were stained by villanueva bone stain. Note the marked increased osteoid tissues (*arrows*) and no hypomineralized periosteocytic lesions (HPL) in the specimen (*arrowhead*). HPL was defined as the presence of osteoid tissues in the lacnae regions of the osteocyte. ×25

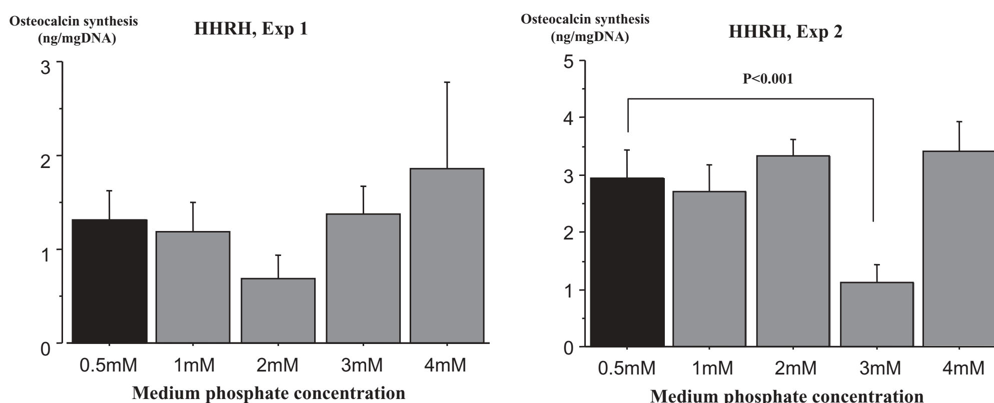

Fig. 2. Effect of phosphate concentrations on osteocalcin synthesis by osteoblasts isolated from a HHRH patient in the presence of 10^{-10} M $1,25(OH)_2D_3$. After 72 h culture at different medium phosphate concentrations in the presence of 10^{-10} M $1,25(OH)_2D_3$, we measured osteocalcin concentrations and DNA content. Data of two independent studies are shown (**2-1**: Exp 1; **2-2**: Exp 2)

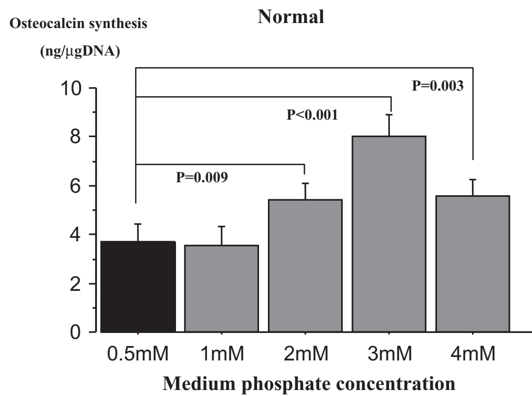

Fig. 3. Effect of phosphate concentrations on osteocalcin synthesis by osteoblasts isolated from normal controls in the presence of 10^{-10} M $1,25(OH)_2D_3$. Experimental protocol was same as Fig. 2 except that osteoblasts were isolated from normal human bone fragments

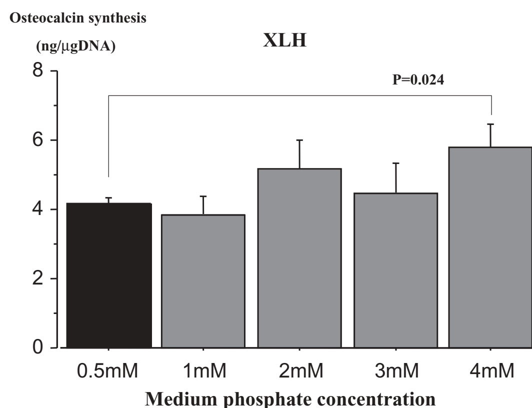

Fig. 4. Effect of phosphate concentrations on osteocalcin synthesis by osteoblasts isolated from an X-linked hypophosphatemic rickets (XLH) patient in the presence of 10^{-10} M $1,25(OH)_2D_3$. Experimental protocol was the same as Fig. 2 except that osteoblasts were isolated from bone fragments of an XLH patient

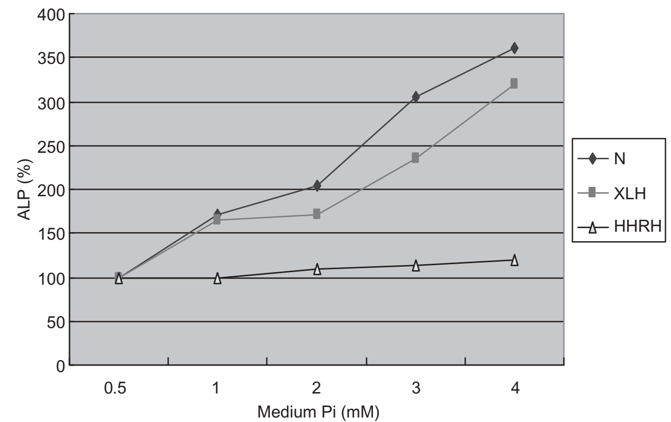

Fig. 5. Effect of phosphate concentrations on alkaline phosphatase (ALP) activity of osteoblasts isolated from normal (N), XLH and HHRH patients. After 72 h culture at different medium phosphate concentrations, ALP activities and DNA contents of osteoblasts were measured, respectively. *Vertical line* denotes percent changes of ALP activities. ALP activities of normal, XLH, and HHRH osteoblasts at 0.5 mM medium phosphate concentration were calculated as 100%. Mean values of percent changes in ALP activities are shown

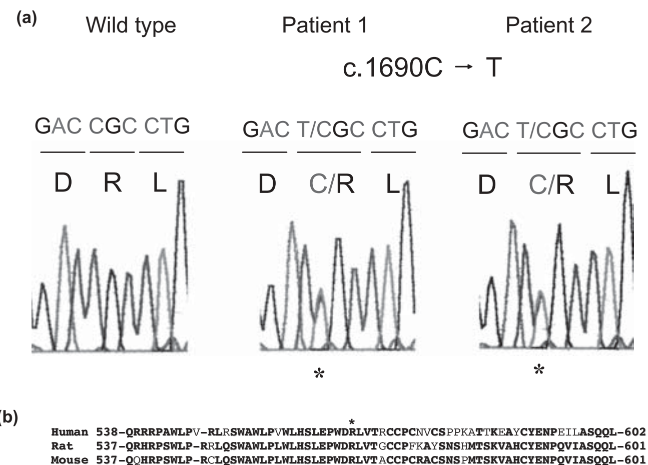

Fig. 6. Mutation analysis of NPT type IIc (SLC34A3) in HHRH patients. **a** The mutation is indicated above the electropherograms. *Asterisk* indicates the nucleotide of the mutation. **b** Amino acid sequence comparison of NPT type IIc (human, rat, and mouse) and location of the mutation in intracellular C-terminal loop. *Asterisk* indicates the amino acid of the mutation. Amino acid residues that are identical in two or more sequences are *bold letters*. Residue numbers are indicated beside the aligned sequences

minations. Both the medium phosphate concentration (P = 0.004) and the origin of osteoblasts (P = 0.003) significantly increased ALP activities (Fig. 5). There were no interactions between the effect of medium phosphate concentration and the origin of osteoblasts for ALP activities. A post hoc test revealed that ALP activities were significantly increased in normal (P = 0.013) and XLH (P = 0.020) compared to those in HHRH.

Gene analysis of a phosphate transporter NPT type IIc

Gene analysis of NPT type IIc revealed that both HHRH patients were affected with two single-nucleotide mutations including one amino acid mutation. One of the mutations, which has a C to T change at the 1689 nucleotide, is also a cysteine (C) to arginine (R) change at amino acid 564 (Fig. 6a). Both patients were heterozygous for the C1689T mutation. Another mutation, with a T to C change at 757, has no change in the amino acid. We were not able to find an abnormality of other allele including the area from exon 9 to 11, where intronic deletions in NPT type IIc gene were recently reported in HHRH patients by Ichikawa et al. [18]. As shown in Fig. 6b, the amino acid of the mutation that was located in the intracellular C-terminal loop was conserved in human, mouse, and rat.

Discussion

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) was initially described as a new syndrome in a large Bedouin tribe kindred [1]. It is a rare inheritable

hypophosphatemic rickets/osteomalacia characterized by increased renal phosphate loss, and elevated circulating $1,25(OH)_2D$ response to hypophosphatemia. Secondary hypercalcuria is also observed because of increased gastrointestinal absorption of phosphate and calcium. The existence of elevated $1,25(OH)_2D$ and secondary hypercalciuria distinguished HHRH from XLH; the latter manifested circulating $1,25(OH)_2D$ in the normal range, an inappropriate response to hypophosphatemia caused by increased renal phosphate clearance [9]. Moreover, abnormal function of osteoblastic cells was suggested in XLH patients by the existence of hypomineralized periosteocytic lesion (HPL) [17].

Both our patients described here manifested decreased tubular reabsorption of phosphate, hypophosphatemia, and osteomalacia. The family history suggested that they were affected with inheritable bone disorders such as XLH and HHRH. Both the observations such as elevated circulating $1,25(OH)_2D$ levels and increased amount of urinary calcium excretion in case 1 confirmed that her hypophosphatemic osteomalacia was compatible with HHRH complicated by vitamin D deficiency because of a low serum level of 25-OHD, although we did not clarify the reason for the vitamin D deficiency. The possible contribution of pharmacological doses of calcitriol therapy for the increased serum $1,25(OH)_2D$ level on admission in case 1 was implausible, because serum $1,25(OH)_2D$ levels were between 60 and 100 pg/ml during the long term of phosphate therapy for 10 years (data not shown). In case 2, the elevated circulating $1,25(OH)_2D$ levels and an obvious hypercalciuria on admission were compatible with HHRH. In Dent's disease, which is caused by the gene mutations of a chloride channel CLCN5 [19], the coexistence of hypophosphatemia and hypercalciuria was reported. However, this was implausible in our patients, because the urinary excretions of β2-microglobulin revealed normal (data not shown).

To prove increased gastrointestinal absorption of phosphate, we performed an oral phosphate loading test to investigate the maximal increase of serum phosphate levels in normal, XLH, and HHRH patients. Oral phosphate administration increased serum phosphate levels in all subjects; however, the maximal increases in serum phosphate concentrations after the tests were significantly higher in HHRH compared to XLH patients. These data suggested that the gastrointestinal absorption of phosphate in HHRH was increased compared to XLH. These speculations were supported by the data that basal serum $1,25(OH)_2D$ levels of HHRH patients were significantly increased compared to those in XLH patients ($P < 0.001$), although we could not adjust the ages of the patients (see Table 2).

Histological findings in bone biopsy specimens before phosphate therapy revealed increased osteoid volume in both case 1 and case 2, which were comparable with osteomalacia as reported by Gazit et al. [12]. However, we did not find hypomineralized periosteocytic lesions (HPL) in these patients, which was a characteristic of XLH [17]. HPL remained even after the combination therapy of calcitriol and phosphate therapy in XLH [20], which suggested osteoblast dysfunction in XLH. This hypothesis was proved by the experiments using osteoblasts from the HYP mice [21], which was an animal model for XLH [22]. Thus, the absence of HPL in HHRH was supposed to be useful to distinguish these two hereditary rickets.

In case 1, both osteoid volume and mean osteoid thickness were remarkably increased, although she had been treated with a large amount of calcitriol for a long time. However, phosphate therapy alone decreased both osteoid volume and osteoid thickness within a year. This observation was compatible with the report that phosphate therapy improved clinical and radiographic manifestations of HHRH patients [1]. In contrast, a combination therapy of phosphate and supraphysiological doses of calcitriol was necessary in XLH for the improvement of bone disorders [20], because both renal phosphate leak and impairment of vitamin D metabolism coexisted. Our results in bone morphometrical evaluations also proved that $1,25(OH)_2D_3$ was less effective for bone, but phosphate therapy alone could completely cure the osteomalacia in our HHRH patient. These findings in HHRH patients strongly suggested that hypophosphatemia in the absence of the impaired vitamin D metabolism played an important role for the pathogenesis of rickets/osteomalacia of HHRH.

In normal human osteoblasts, we showed previously that phosphate concentrations were an important regulating factor for the action of a physiological concentration of $1,25(OH)_2D_3$ in terms of osteocalcin syntheses [15]. In detail, they were significantly increased in the condition of medium phosphate concentrations higher than 2 mM. In this study, we showed that the threshold of phosphate concentration for the increased action of a physiological concentration of $1,25(OH)_2D_3$ was shifted to 4 mM in XLH osteoblasts as in HYP mice osteoblasts [16]. However, the osteocalcin syntheses in HHRH osteoblasts induced by a physiological concentration of $1,25(OH)_2D_3$ did not increase in response to the medium phosphate concentration. In addition, ALP activities of a normal osteoblast were markedly stimulated by the increased medium phosphate concentrations, which was previously reported by Farley [23] in a human osteoblastic cell line of SaOS-2. We also found the same phenomenon in XLH osteoblasts (see Fig. 5). However, the increased levels of ALP activities of HHRH osteoblasts in response to the medium phosphate concentrations were markedly smaller than those in normal ($P = 0.013$) and XLH osteoblasts ($P = 0.020$) (Fig. 5). These lines of evidence suggested the possibility of abnormal phosphate metabolism in HHRH osteoblasts. In Hyp mice osteoblasts, phosphate transport was reported to be normal [24], whereas no evidence was shown for osteoblastic phosphate transport in HHRH. Thus, abnormal phosphate metabolism of HHRH osteoblasts is a possible key to elucidate the mechanism of rickets/osteomalacia of HHRH patients. Recently, two groups reported several types of mutations for the phosphate transporter of NPT type IIc genes in a large family of HHRH patients [7,8]. In this article, we also showed a novel heterozygous mutation (R564C) in the exon of phosphate transporter NPT type IIc. Because HHRH was transmitted as autosomal recessive fashion, the present role of the novel heterozygous muta-

tion (R564C) is unknown. However, the mice model for HHRH, which was made by NPT type IIa gene knockout, failed to show the rickets/osteomalacia [25]. Also, we could not find bone abnormalities in a mice model made by the NPT type II c gene knock out (submitted), probably because the NPT type IIc gene was not expressed in murine bone, whereas the expression of the same gene in human bone has not been investigated. It was possible that a novel heterozygous mutation (R564C) in the NPT type IIc gene has an important role in cooperating with the unknown gene for the rickets/osteomalacia in humans. It was also plausible that the heterozygous R564C mutation has the dominant negative effect. Thus, the functional analyses of the NPT type IIc gene in human osteoblasts in the presence or absence of an unknown cooperating factor might be a future target to clarify the pathogenesis of the rickets/osteomalacia in HHRH patients.

In conclusion, the abnormal osteoblastic functions and a gene abnormality of a phosphate transporter gene NPT type IIc were reported in sporadic Japanese cases of HHRH. These data possibly suggested that the defective functions in a sodium-dependent phosphate transporter NPT type IIc played an important role for human osteoblasts, which could explain the pathophysiology of rickets/osteomalacia in HHRH patients.

References

1. Tieder M, Modai D, Shaked U, Samuel R, Arie R, Halabe A, Bab I, Gabizon D, Lieberman UA (1985) Hereditary hypophosphatemic rickets with hypercalciuria. N Engl J Med 312:611–617
2. Nishiyama S, Inoue F, Matsuda I (1986) Single case of hypophosphatemic rickets with hypercalciuria. J Pediatr Gastroenterol Nutr 5:826–829
3. Tieder M, Arie R, Bab I, Maor J, Liberman UA (1992) A new kindred with hereditary hypophosphatemic rickets with hypercalciuria: implications for correct diagnosis and treatment. Nephron 62:176–181
4. Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS (1998) Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities. Proc Natl Acad Sci USA 95:5372–5377
5. Jones A, Tzenova J, Frappier D, Crumley M, Roslin N, Kos C, Tieder M, Langman C, Proesmans W, Carpenter T, Rice A, Anderson D, Morgan K, Fujiwara T, Tenenhouse H (2001) Hereditary hypophosphatemic rickets with hypercalciuria is not caused by mutations in the Na/Pi cotransporter NPT2 gene. J Am Soc Nephrol 12:507–514
6. Prie D, Huart V, Bakouh N, Planelles G, Dellis O, Gerard B, Hulin P, Benque-Blanchet F, Silve C, Grandchamp B, Friedlander G (2002) Nephrolithiasis and osteoporosis associated with hypophosphatemia caused by mutations in the type 2a sodium-phosphate cotransporter. N Engl J Med 347:983–991
7. Lorenz-Depiereux B, Benet-Pages A, Eckstein G, Tenenbaum-Rakover Y, Wagenstaller J, Tiosano D, Gershoni-Baruch R, Albers N, Lichtner P, Schnabel D, Hochberg Z, Strom TM (2006) Hereditary hypophosphatemic rickets with hypercalciuria is caused by mutations in the sodium-phosphate cotransporter gene SLC34A3. Am J Hum Genet 78:193–201
8. Bergwitz C, Roslin NM, Tieder M, Loredo-Osti JC, Bastepe M, Abu-Zahra H, Frappier D, Burkett K, Carpenter TO, Anderson D, Garabedian M, Sermet I, Fujiwara TM, Morgan K, Tenenhouse HS, Juppner H (2006) SLC34A3 mutations in patients woth hereditary hypophosphatemic rickets with hypercalciuria predict a key role for the sodium-phosphate cotransporter NaPi-IIc in maintaining phosphate homeostasis. Am J Hum Genet 78:179–192
9. Econs MJ, Drezner MK (1994) Tumor-induced osteomalacia—unveiling a new hormone. N Engl J Med 330:1679–1681
10. Jonsson KB, Zahradnik R, Larsson T, White KE, Sugimoto T, Imanishi Y, Yamamoto T, Hampson G, Koshiyama H, Ljunggren O, Oba K, Yang IM, Miyauchi A, Econs MJ, Lavigne J, Juppner H (2003) Fibroblast growth factor 23 in oncogenic osteomalacia and X-linked hypophosphatemia. N Engl J Med 348:1656–1663
11. Yamamoto T, Imanishi Y, Kinoshita E, Nakagomi Y, Shimizu N, Miyauchi A, Satomura K, Koshiyama H, Inaba M, Nishizawa Y, Juppner H, Ozono K (2005) The role of fibroblast growth factor 23 for hypophosphatemia and abnormal regulation of vitamin D metabolism in patients with McCune–Albright syndrome. J Bone Miner Metab 23:231–237
12. Gazit D, Tieder M, Liberman UA, Passi-Even L, Bab IA (1991) Osteomalacia in hereditary hypophosphatemic rickets with hypercalciuria: a correlative clinical-histomorphometric study. J Clin Endocrinol Metab 72:229–735
13. Yamamoto T, Ozono K, Yamaoka K, Shima M, Yamamoto K, Michigami T, Matsumoto S, Hiroshima K, Okada S (1996) The role of IGF-I in the phosphate therapy to improve the short stature of patients with hypophosphatemic vitamin D resistant rickets. J Bone Miner Metab 14:222–228
14. Yamamoto T, Ecarot B, Glorieux FH (1991) In vivo osteogenic activity of isolated human bone cells. J Bone Miner Res 6:45–51
15. Yamamoto T, Ecarot-Charrier B, Glorieux FH (1990) Familial hypophosphatemia: altered regulation of osteocalcin synthesis in bone cells. In: Cohn DV, Glorieux FH, Martin TJ (eds) Calcium Regulation and Bone Metabolism, vol 10. Excerpta Medica, Amsterdam, pp 239–242
16. Yamamoto T, Ecarot B, Glorieux FH (1992) Abnormal response of osteoblasts from Hyp mice to 1,25-dihydroxyvitamin D_3. Bone (NY) 13:209–205
17. Frost HM (1958) Some observations on bone mineral in a case of vitamin D resistant rickets. Henry Ford Hosp Med Bull 6:300–310
18. Ichikawa S, Sorenson AH, Imel EA, Friedman NE, Gertner JM, Econs MJ (2006) Intronic deletions in the SLC34A3 gene cause hereditary hypophophatemic rickets with hypercalciuria. J Clin Endocrinol Metab 91:4022–4027
19. Morimoto T, Uchida S, Sakamoto H, Kondo Y, Hanamizu H, Fukui M, Tomino Y, Nagano N, Sasaki S, Marumo F (1998) Mutations in CLCN5 chloride channel in Japanese patients with low molecular weight proteinuria. J Am Soc Nephrol 9:811–818
20. Glorieux FH, Marie PJ, Pettifor JM, Delvin EE (1980) Bone response to phosphate salts, ergocalciferol, and calcitriol in hypophosphatemic vitamin D-resistant rickets. N Engl J Med 303:1023–1031
21. Ecarot-Charrier B, Glorieux FH, Travers R, Desbarats M, Bouchard F, Hinek A (1988) Defective bone formation by transplanted Hyp mouse bone cells into normal mice. Endocrinology 123:768–773
22. Eicher EM, Southard JL, Scriver CR, Glorieux FH (1976) Hypophosphatemia: mouse model for human familial hypophosphatemic (vitamin D resistant) rickets. Proc Natl Acad Sci USA 73:4667–4671
23. Farley JR (1995) Phosphate regulates the stability of skeletal alkaline phosphatase activity in human osteosarcoma (SaOS-2) cells without equivalent effects on the level of skeletal alkaline phosphatase immunoreactive protein. Calcif Tissue Int 57:371–378
24. Ecarot B, Caverzasio J, Desbarats M, Bonjour JP, Gloriuex FH (1994) Phosphate transport by osteoblast from X-linked hypophosphatemic mice. Am J Physiol 266(1 Pt 1):E33–E38
25. Beck L, Karaplis AC, Amizuka N, Hewson AS, Ozawa H, Tenenhouse HS (1998) Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciura, and skeletal abnormalities. Proc Natl Acad Sci USA 95:5372–5377