serum FT4 levels were significantly correlated to the outcome of the weight reduction therapy. The trend test analysis for groups according to serum FT4 levels with 0.1 pg/mL increments (Supplemental Table 2) revealed that baseline serum FT4 levels within reference range were significantly correlated to the changes in BW and BMI.

Baseline serum leptin levels in female obese patients are higher than those in the male patients. Furthermore, serum leptin levels are significantly decreased only in female obese patients after the 6-month weight reduction therapy and serum adiponectin levels, which reflects the mass of visceral fat were not significantly different after the therapy both in the male and the female patients (Supplemental Table 1). Since serum leptin levels are correlated with the percentage of body fat, especially subcutaneous fat [20], these data suggest that more adiposity in the female patients compared to the male patients and that the body weight reduction after the therapy could be due to the reduction of subcutaneous fat in the female patients. On the other hand, since leptin leads to an increase in type 1 deiodinase (D1) activity, which results in the conversion of T4 to T3 [21], we speculate that in the female patients, D1 would be more activated to convert T4 to T3, thereby promoting the energy expenditure. In fact, since serum FT3 levels were significantly decreased only in the female patients, we speculated that during the 6-month weight reduction therapy, the reduction of adipose tissue leads a decrease in serum leptin levels followed by a decrease in D1 activity, which might result in a decrease of serum FT3 levels.

As it is known that in humans, subtle changes in deiodinase enzyme activity might affect energy metabolism and as such BMI or risk for obesity, D1 polymorphisms between the male and the female patients should be taken into the consideration. However, so far, no effect of D1 polymorphisms on BMI was found in a cohort of healthy elderly men [22, 23].

In addition, since baseline serum FT4 levels were significantly correlated to the outcome of the weight reduction therapy only in the premenopausal female patients, a female sex hormone could be another factor, which affects the difference between the male and female patients.

In our study, baseline serum FT4 and TSH levels in subjects prior to the weight reduction therapy were not correlated to baseline BW and BMI, which is somewhat inconsistent with previous studies [8, 24]. However, the reported findings on the influences of the biological variability of thyroid hormones on weight are still contradictory [6, 8, 11, 24, 25].

Additionally, in the present study, the changes in IRI and HOMA-R during the therapy showed significant negative correlations to baseline serum FT4 levels in obese female patients, suggesting that elevated serum FT4 levels prior to the therapy would predict the improvement of insulin resistance upon the successful weight reduction therapy.

Some limitations of this study merit consideration. Firstly, the follow-up used in the weight reduction program was relatively short, so it is necessary to conduct a long-term prospective cohort study with a larger sample size in order to clarify the long-term effectiveness of the baseline serum FT4 levels to predict the efficacy of the weight reduction therapy. Secondly, in the current study, the fat composition such as visceral and subcutaneous fat was not evaluated before and after the weight reduction therapy. To elucidate the sex difference in the correlation of the efficacy of the weight reduction therapy with baseline serum FT4 levels, the analysis of the fat composition should be performed in further study.

In conclusion, the present study suggested that baseline serum FT4 levels, even though they are within the reference range, would predict the outcome of the weight reduction therapy in obese female patients.

Acknowledgements

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Disclosure

The authors declare that there is no conflict of interest associated with this manuscript.

Supplemental Table1 Effect of body weight reduction on metabolic parameters in obese patients after the 6-month weight reduction therapy

	Male		Female	
	Baseline	After 6 months	Baseline	After 6 months
n	40		57	
Age (years)	50.3 ± 2.2		50.0 ± 1.7	
BW (kg)	101 ± 3.7	95 ± 3.4**	81 ± 2.3	76 ± 2.1**
BMI (kg/m^2)	35 ± 1.2	32 ± 1.1**	33 ± 0.9	31 ± 0.9**
Waist (cm)	110 ± 2.4	106 ± 2.3**	104 ± 2.1	98 ± 2.0**
SBP (mmHg)	147 ± 3.0	133 ± 2.3**	145 ± 2.8	136 ± 2.4**
DBP (mmHg)	90 ± 1.9	82 ± 1.7**	86 ± 1.7	80 ± 1.5**
FBS (mg/dL)	120 ± 6.3	103 ± 3.7**	113 ± 4.4	102 ± 2.8**
HbA1c (NGSP) (%)	6.3 ± 0.2	5.8 ± 0.1**	6.5 ± 0.2	5.9 ± 0.1**
IRI (μU/mL)	36.5 ± 8.5	16.2 ± 2.4*	21.7 ± 3.2	14.2 ± 1.8*
HOMA-R	12.0 ± 3.0	4.2 ± 0.7*	6.6 ± 1.1	3.8 ± 0.6**
AST (IU/L)	38.1 ± 3.6	30.2 ± 2.5**	30.1 ± 2.7	21.6 ± 1.0**
ALT (IU/L)	60.3 ± 7.6	41.8 ± 5.4**	36.2 ± 3.6	21.3 ± 1.2**
γ-GTP (IU/L)	75.7 ± 8.2	59.6 ± 6.8*	45.0 ± 8.8	30.7 ± 4.3
TC (mg/dL)	191 ± 5.7	178 ± 4.7*	202 ± 4.9	196 ± 3.7
TG (mg/dL)	173 ± 12.7	145 ± 17.2	152 ± 10.0	120 ± 8.0**
HDL-C (mg/dL)	49 ± 1.5	50 ± 1.5	57 ± 1.7	60 ± 1.6*
LDL-C (mg/dL)	119 ± 5.8	108 ± 3.9*	124 ± 3.5	119 ± 3.1
BUN (mg/dL)	14.2 ± 0.5	16.0 ± 0.9*	13.1 ± 0.5	13.2 ± 0.5
Cre (mg/dL)	0.83 ± 0.03	1.13 ± 0.24	0.59 ± 0.02	0.61 ± 0.01*
eGFR	83 ± 3.7	75 ± 3.5**	88 ± 3.1	83 ± 2.6*
Leptin (ng/mL)	11.7 ± 1.7	11.3 ± 2.2	22.7 ± 2.2	17.5 ± 1.7*
Adiponectin (μg/mL)	7.4 ± 1.0	6.0 ± 0.4	7.7 ± 0.7	7.7 ± 0.5
hs-CRP (μg/mL)	2.06 ± 0.41	1.91 ± 0.48*	2.36 ± 0.42	2.11 ± 0.55

** p<0.010, * p<0.05 by t-testing. eGFR, mL /min/1.73 m^2; Data are expressed as mean ± SE.

Supplemental Table 2 Changes in BW and BMI according to baseline serum FT4 levels with 0.1pg/mL increments in the female patients

FT4 (pg/mL)	n	ΔBW	ΔBMI
≤ 1.0	12	-3.2 ± 1.2	-1.2 ± 0.5
1.1	14	-5.5 ± 1.0	-2.1 ± 0.4
1.2	10	-5.7 ± 2.0	-2.2 ± 0.7
1.3	8	-5.0 ± 1.4	-2.0 ± 0.6
1.4	10	-5.9 ± 2.4	-2.3 ± 0.9
1.5 ≤	3	-13.2 ± 3.8	-5.3 ± 1.6
Total	57	p=0.043	p=0.030

Data are expressed as mean ± SE. (trend test by ANOVA)

Supplemental Table 3 Correlations of changes of clinical and metabolic parameters with baseline serum FT3 after the 6-month weight reduction therapy

	Male (n=14)		Female (n=29)	
	FT3		FT3	
	r	p	r	p
ΔBW	-0.067	0.819	0.107	0.579
ΔBMI	-0.075	0.799	0.082	0.673
ΔWaist	0.080	0.785	0.069	0.721
ΔFBS	0.187	0.523	0.480	0.008
ΔHbA1c	0.011	0.971	0.242	0.206
ΔIRI	-0.195	0.523	0.174	0.396
ΔHOMA-R	-0.187	0.542	0.363	0.068

Supplemental Table 4 Correlations of changes of clinical and metabolic parameters with baseline serum FT4 and TSH levels in pre- and post-menopausal females those who reduced their BW by more than 5% from their baseline BW after the 6-month weight reduction therapy

	Premenopausal (n=17)				Postmenopausal (n=19)			
	FT4		TSH		FT4		TSH	
	r	p	r	p	r	p	r	p
ΔBW	-0.537	0.026	0.105	0.688	-0.195	0.425	0.208	0.392
ΔBMI	-0.587	0.013	0.099	0.705	-0.243	0.317	0.225	0.355
ΔWt	-0.564	0.018	-0.079	0.762	-0.234	0.335	0.438	0.061
ΔFBS	0.517	0.034	0.117	0.656	-0.350	0.142	-0.189	0.440
ΔHbA1c	0.134	0.608	0.168	0.518	-0.280	0.245	-0.028	0.910
ΔIRI	-0.362	0.204	-0.429	0.126	-0.377	0.112	0.157	0.520
ΔHOMA-R	-0.272	0.347	-0.276	0.339	-0.438	0.061	0.061	0.805

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RESEARCH ARTICLE

Detection of Hereditary 1,25-Hydroxyvitamin D-Resistant Rickets Caused by Uniparental Disomy of Chromosome 12 Using Genome-Wide Single Nucleotide Polymorphism Array

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Data Availability Statement: All relevant data except for SNP array data are within the paper and its Supporting Information files. Although we could not obtain informed consent for depositing SNP array data, the de-identified participant-level dataset is available upon request to corresponding authors, and with an appropriate approval of human genomic DNA research ethics committee of institutions to which researchers involved in the data analyses belong. A minimized, anonymous dataset can be provided on request to any researcher.

Abstract

Context

Hereditary 1,25-dihydroxyvitamin D-resistant rickets (HVDRR) is an autosomal recessive disease caused by biallelic mutations in the vitamin D receptor (VDR) gene. No patients have been reported with uniparental disomy (UPD).

Objective

Using genome-wide single nucleotide polymorphism (SNP) array to confirm whether HVDRR was caused by UPD of chromosome 12.

Materials and Methods

A 2-year-old girl with alopecia and short stature and without any family history of consanguinity was diagnosed with HVDRR by typical laboratory data findings and clinical features of rickets. Sequence analysis of *VDR* was performed, and the origin of the homozygous mutation was investigated by target SNP sequencing, short tandem repeat analysis, and genome-wide SNP array.

Results

The patient had a homozygous p.Arg73Ter nonsense mutation. Her mother was heterozygous for the mutation, but her father was negative. We excluded gross deletion of the father's allele or paternal discordance. Genome-wide SNP array of the family (the patient and her parents) showed complete maternal isodisomy of chromosome 12. She was successfully treated with high-dose oral calcium.

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Competing Interests: The authors have declared that no competing interests exist.

Conclusions

This is the first report of HVDRR caused by UPD, and the third case of complete UPD of chromosome 12, in the published literature. Genome-wide SNP array was useful for detecting isodisomy and the parental origin of the allele. Comprehensive examination of the homozygous state is essential for accurate genetic counseling of recurrence risk and appropriate monitoring for other chromosome 12 related disorders. Furthermore, oral calcium therapy was effective as an initial treatment for rickets in this instance.

Introduction

Hereditary 1,25-dihydroxyvitamin D (1,25[OH]$_2$D)-resistant rickets (HVDRR) (OMIM #277440), also known as vitamin D-dependent rickets type 2A (VDDR 2A), is a rare disorder characterized by early onset rickets, hypocalcemia, and secondary hyperparathyroidism, and alopecia when severe [1]. Patients with HVDRR have high circulating levels of 1,25(OH)$_2$D, and are resistant to 1,25(OH)$_2$D$_3$ and 1α(OH)D$_3$ treatment. Elevated 1,25(OH)$_2$D levels differentiate HVDRR from 1α-hydroxylase deficiency, which is known as vitamin D-dependent rickets type 1A [2].

HVDRR is caused by mutations in the vitamin D receptor (VDR) gene on chromosome 12q13.11 [3]. HVDRR shows autosomal-recessive inheritance and the patients usually have biallelic mutations in the *VDR* inherited from each parent. Because of the rarity of the disease, most cases arise in consanguineous families and have homozygous mutations [4]. VDR is a member of the steroid/nuclear receptor superfamily of ligand-activated transcription factors, and it is composed of an N-terminal DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [5]. Patients with a mutation in the DBD usually show severe vitamin D resistance associated with alopecia [6], whereas those with a mutation in the LBD show various degrees of vitamin D unresponsiveness and can occasionally respond to high-dose vitamin D. Patients with alopecia showing resistance to high-dose vitamin D therapy usually require intravenous calcium infusions to treat clinically overt rickets at their first presentation [6].

Some recessive disorders have been reported to be caused by uniparental disomy (UPD) of a single parent allele with a mutation [7]. UPD refers to a condition in which both homologues of a chromosomal region or segment are inherited from only one parent. To date, most cases of UPD have been found in imprinting diseases such as Prader-Willi syndrome, and the incidence of UPD of any chromosome is estimated to be as frequent as 1 per 3,500 live births [8,9]. Conversely, UPD causing recessive diseases have only been reported in a limited number of cases. Moreover, no cases have been reported in HVDRR and complete isodisomy of chromosome 12 is extremely rare [7,10,11].

In this report, we used genome-wide single nucleotide polymorphism (SNP) array analysis to determine whether HVDRR was caused by UPD of chromosome 12. Furthermore, we observed the effectiveness of high oral calcium therapy for the treatment of rickets in this severe HVDRR patient.

Materials and Methods

Clinical case

A 2-year 1-month-old girl presented to hospital with fever, at which point she was noted to have short stature, alopecia (Fig 1), and gait instability. Her parents were non-consanguineous

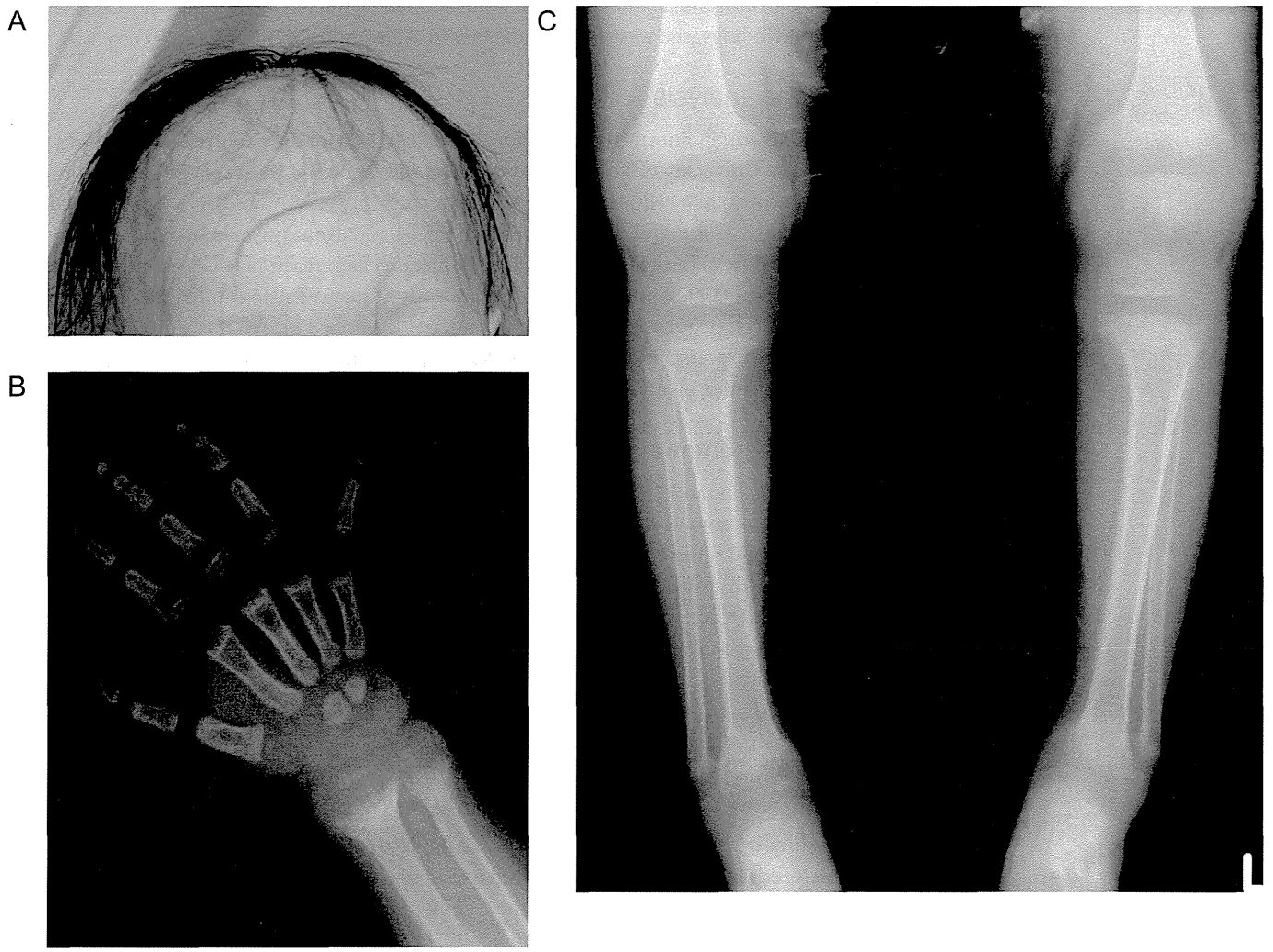

Fig 1. Alopecia and Rickets in the Proband at Presentation. (A) Alopecia. (B,C) A bone roentgenogram of the arm (B) and legs (C) showing cupping, fraying, and flaring at the end of the long bones.

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and approximately 30 years old when she was born. She had no family history of rickets or unresolved pain. Her mother got a natural conception, and the pregnancy and delivery was uneventful. Her birth weight was 2,868 g (-0.8 standard deviations [SD]), birth length 51 cm (+0.6 SD), gestational age 41 weeks. She had no episode of convulsion and her psychomotor development was normal until she started walking alone at 1 year 3 months of age, but she could not run by age 2. Her body height at presentation was 74.8 cm (−3.5 SD), and her body weight, 9.7 kg (−1.2 SD). She had symptoms of rickets such as bow-legs and enlargement of the limb joints, but had no other external malformation, dysmorphic features, or ataxia. Her verbal developmental quotient (DQ) was 81 and cognitive DQ 94.

Laboratory data revealed hypocalcemia (7.7; reference: 8.5–10.5 mg/dL), hypophosphatemia (3.0; reference: 4.5–6.5 mg/dL), markedly elevated levels of serum alkaline phosphatase (8,891; reference: 300–1,239 IU/L), and intact parathyroid hormone (PTH) levels (576; reference: 10 –65 pg/mL). Her serum $1,25(OH)_2D$ level was high (137; reference: 20–70 pg/mL) with a normal 25(OH)D level (20.1 ng/mL). Serum levels of fibroblast growth factor 23 (FGF23) were

low (<10 pg/mL), and a bone roentgenogram showed characteristic findings of rickets (Fig 1). From these findings, she was clinically diagnosed as having HVDRR.

VDR gene analysis

We obtained written informed consent for DNA analysis from the parents, and the Ethics Committee of The University of Tokyo approved the study. Genomic DNA was extracted from peripheral white blood cells of the patient and parents using a QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). The entire coding region and exon-intron boundaries of the *VDR* were amplified from the genomic DNA by polymerase chain reaction (PCR) using the specific primers (S1 Table). PCR products were subsequently sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and the forward and reverse primers from the PCR amplification. Direct sequencing in both directions was performed on an autosequencer (PE Applied Biosystems 3130x1, Genetic Analyzer).

Analysis of common gene polymorphisms

Common SNPs in the *VDR* (rs10735810, rs7975232, rs2853562 rs731236, rs12717991), other genes on chromosome 12 (rs2259820, rs2464196, rs1169289, rs1169288, rs1169301, rs1169304, rs10877012), and other chromosomes (rs4588, rs7041, rs116930, rs1155563, rs2060793, rs3829251, rs6013897, rs6599638, rs10741657, rs12785878, rs17217199) were analyzed by sequencing the PCR products as previously reported [12,13]. Short tandem repeat (STR) analysis was performed using AmpFLSTR Identifiler kit (Identifiler, Applied Biosytems, Foster City, CA, USA), which included 16 STR markers (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, and FGA), according to the manufacturer's protocol.

Genome-wide SNP array

Using of the Affymetrix Axiom ASI 1 array (Affymetrix, CA, USA) in accordance with the manufacturer's instructions, we genotyped a total of 600,307 SNPs for the three individuals. Genotype calls were determined using the Genotyping Console 4.1.4 software with the Birdseed v2 algorithm provided by the manufacture. In addition to data from the patient and her parents, we also used 474 Japanese individuals to ensure reliable genotype calling. Signal intensities for alleles A and B were observed by using Affymetrix Power Tools [14]. B allele frequency was calculated by using of the intensities of both alleles: BAF = B / (A + B).

Results

Identification of the *VDR* mutation

Sequencing the *VDR* in the patient revealed a single homozygous base pair substitution, c.217C>A (Fig 2A). This substitution was predicted to result in a nonsense mutation p.Arg73-Ter, which is a premature stop codon in the DBD (Fig 2B). This mutation has been reported in 5 other patients with HVDRR and is functionally inactive [15–18]. From these findings, we considered that HVDRR in this patient was caused by a homozygous nonsense mutation in the *VDR*.

Analysis of the genesis of homozygosity

Most cases of homozygosity among rare mutations are caused by consanguinity, but the parents in this case denied consanguinity; therefore, we performed a genetic analysis of the parents. The mutation was heterozygous in the mother only, and was not present in the father (Fig 2A).

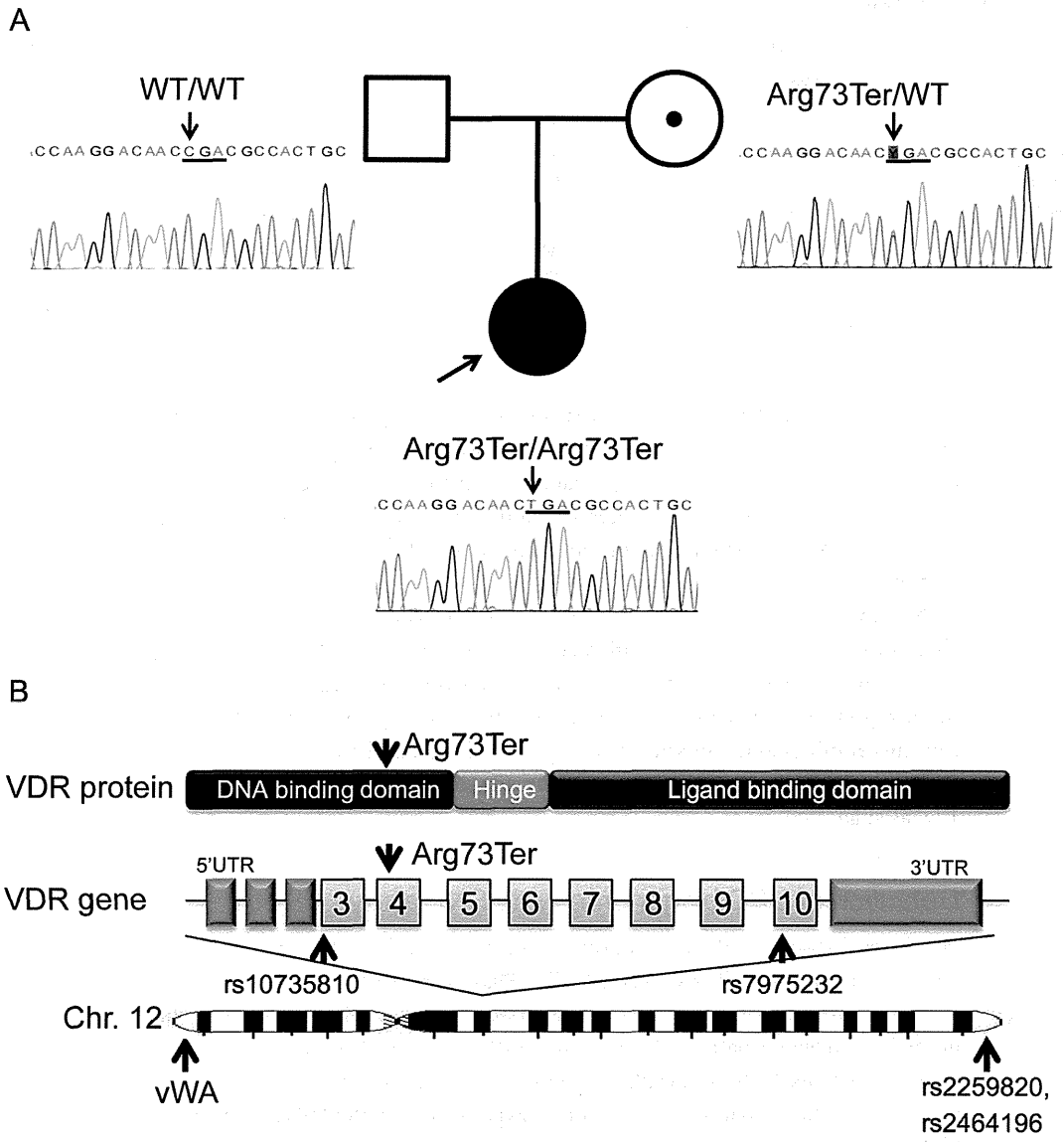

Fig 2. *VDR* Analysis of the Proband and her Parents, with the Position of the Target SNPs on Chromosome 12. (A) Proband pedigree with chromatograms of the *VDR* mutation. The *VDR* analysis showed a homozygous Arg73Ter mutation in the proband, a heterozygous mutation in the mother, and no mutation in the father. Mutations were checked by bidirectional sequencing. A black symbol indicates the proband, a dot symbol indicates a carrier, a square indicates a male, and a circle indicates a female. (B) Diagram of the VDR protein and gene, and chromosome 12 with positions of the mutation and the sequence-analyzed SNPs. The nonsense mutation, Arg73Ter, is located in the DNA binding domain of the VDR protein in exon 4. The indicated common SNPs were analyzed in the family; vWA indicates the position of a marker included in the STR analysis.

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To assess the possibility of a gross deletion around the mutation in the father's allele, we sequence-analyzed several common target SNPs in the *VDR*, in other genes on chromosome 12, and in other chromosomes studied in our laboratory. Table 1 shows the results of the SNPs which identify the parental origin of the proband's allele. Two SNPs in the *VDR*, one near the mutation (rs10735810) and another within approximately 20 kb (rs7975232) (Fig 2B), demonstrated homozygous alleles in the proband that were derived only from the mother (Table 1). Moreover, another 2 SNPs located at the opposite end of the long arm of chromosome 12

Table 1. Sequence Analysis of the Common Target SNPs that Could Identify Parental Origin.

Gene	SNP	Location	Proband	Father	Mother
VDR	rs10735810	12q13.11	CC	TT	CT
VDR	rs7975232	12q13.11	GG	TT	GG
HNF1A	rs2259820	12q24.31	CC	TT	CC
HNF1A	rs2464196	12q24.31	GG	AA	GG
NADSYN1	rs3829251	11q13.4	GA	GG	AA
GC	rs7041	4q13.3	TG	TG	GG
GC	rs1155563	4q13.3	CT	CC	TT

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(rs2259820, rs2464196) (Fig 2B) were also homozygous and derived only from the mother. However, SNPs on chromosomes 4 and 11 showed a normal pattern of allele inheritance from the parents. These results indicated that at least the long arm of chromosome 12 consists of alleles derived only from the mother. G-banding karyotype analysis of the proband revealed a normal 46,XX karyotype without monosomy of 12q. These findings suggested that gross deletion of the father's allele was unlikely.

Next, we assessed the biological paternity by conventional STR analysis. STRs located on chromosomes other than 12 confirmed that the father was the biological father. Interestingly, the STR of a gene located on the short arm of chromosome 12 (vWA; 12p12-pter) showed a homozygous maternal allele (proband 19; father 14, 16; mother 16, 19). Taken together, these findings eliminated the possibility of a gross deletion and paternal discordance, and suggested that *de novo* mutation was unlikely. Finally, maternal UPD of the entire chromosome 12 was suggested.

Detection of UPD by genome-wide SNP array

For the evaluation of UPD, we conducted a genome-wide SNP array of the proband and the parents. The overall call rates were 99.47%, 99.64%, and 99.57% for the proband, the father, and the mother, respectively. All chromosomes other than chromosome 12 showed a normal homo/heterozygous pattern. There were 29,197 SNPs on chromosome 12 on the array, of which 13,940 SNPs showed multiple genotypes among the trio of samples (proband, mother, and father). The proband was called homozygous for 13,848 SNPs and heterozygous for 92 SNPs; however, we found that these heterozygous SNPs were miss-calls caused by the genotype calling algorithms, and the proband was considered homozygous for all SNPs on chromosome 12. After linkage disequilibrium pruning (LD pruning) with 474 samples, a total of 8,933 SNPs remained [19,20], except for bad clusters. Fig 3 shows the B allele frequencies for chromosome 12, which represents the distribution of each proband, maternal, and paternal allele. On chromosome 12, the allele segregation revealed to be composed of only homozygous AA and BB combinations, and no AB combinations (loss of heterozygosity). Allele segregation of chromosome 12 showed a heterozygous pattern in her parents. In the proband's diagram, the pink spots represent the maternal SNPs (1,514 SNPs) and the blue spots, paternal (none) (Fig 3). It was obvious that all of the homozygous SNPs on chromosome 12 derived from the mother. Moreover, the 2 alleles in the proband were 100% identical to those in the mother by identical-by-descent analysis, whereas none were identical to those in the father [21]. The signal intensity of chromosome 12 was sufficient to conclude that the chromosome was diploid, compared with other chromosomes. Thus, we concluded that the proband had complete maternal uniparental isodisomy of chromosome 12 with a nonsense mutation.

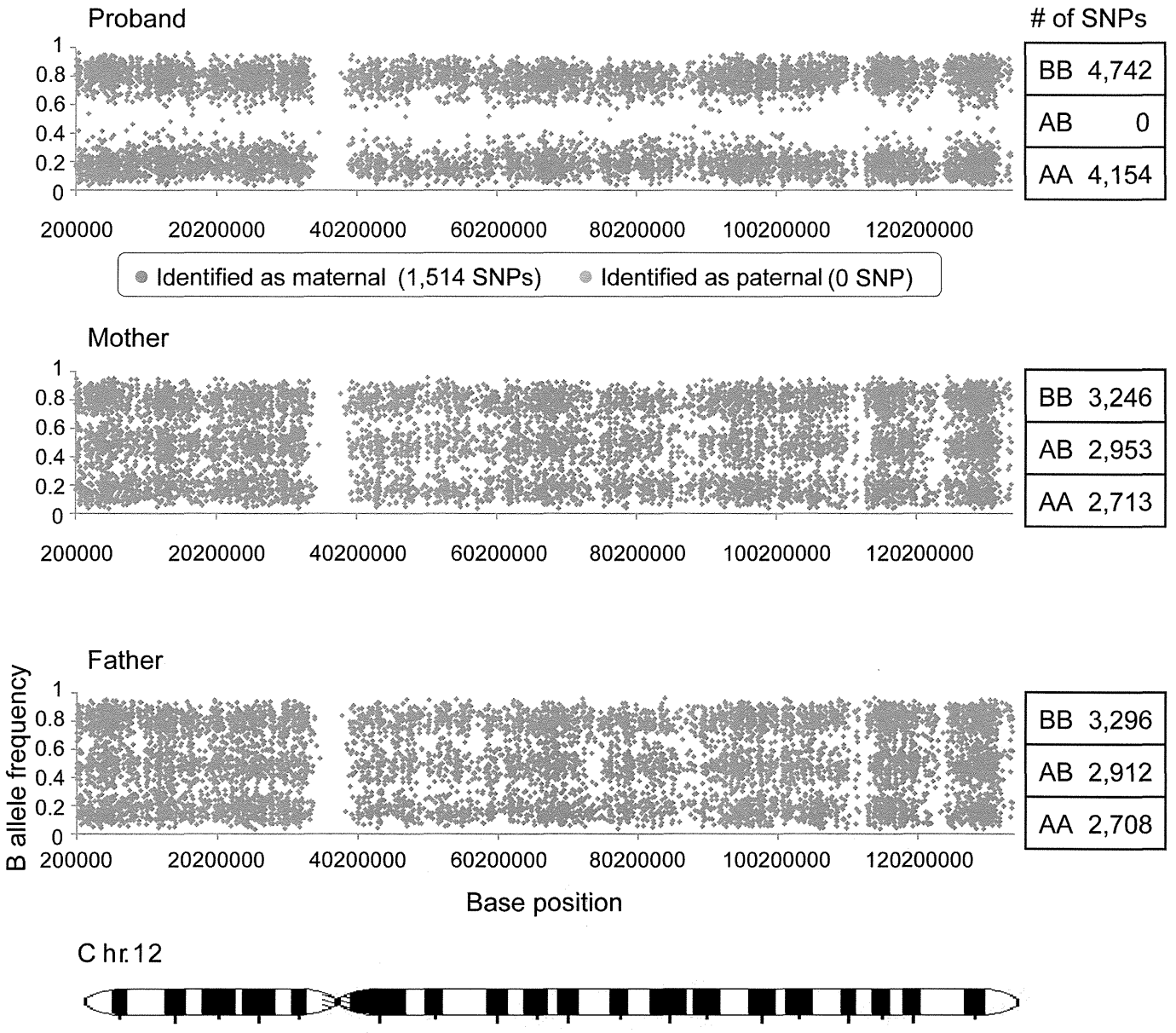

Fig 3. Genome-wide SNP Array Indicating Complete Maternal Isodisomy of Chromosome 12. B allele frequencies of chromosome 12 in the proband, the mother, and the father are shown. Allele segregation in the proband comprised AA (4,154 SNPs) and BB (4,742 SNPs) homozygotes only, and loss of heterozygosity (AB; 0 SNP). The parental allele segregation of chromosome 12 showed a heterozygous pattern. All other chromosomes had normal homo/heterozygous patterns. In the diagram for the proband, the pink spots (1,514 SNPs) represent SNPs identified as maternal, with no evidence of paternal SNPs (which would be blue).

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Clinical course

Initial treatment with increasing amounts of alfacalcidol up to 5 μgkg/day with oral calcium 60 mg/kg/day failed to improve her symptoms. After detecting the *VDR* mutation, she was admitted to another hospital for further treatment. After increasing her oral elemental calcium (calcium lactate) dose to 300 mg/kg/day (divided 3 times), her serum calcium and PTH levels improved (Fig 4A). The oral calcium was subsequently reduced to 240 mg/kg/day when high

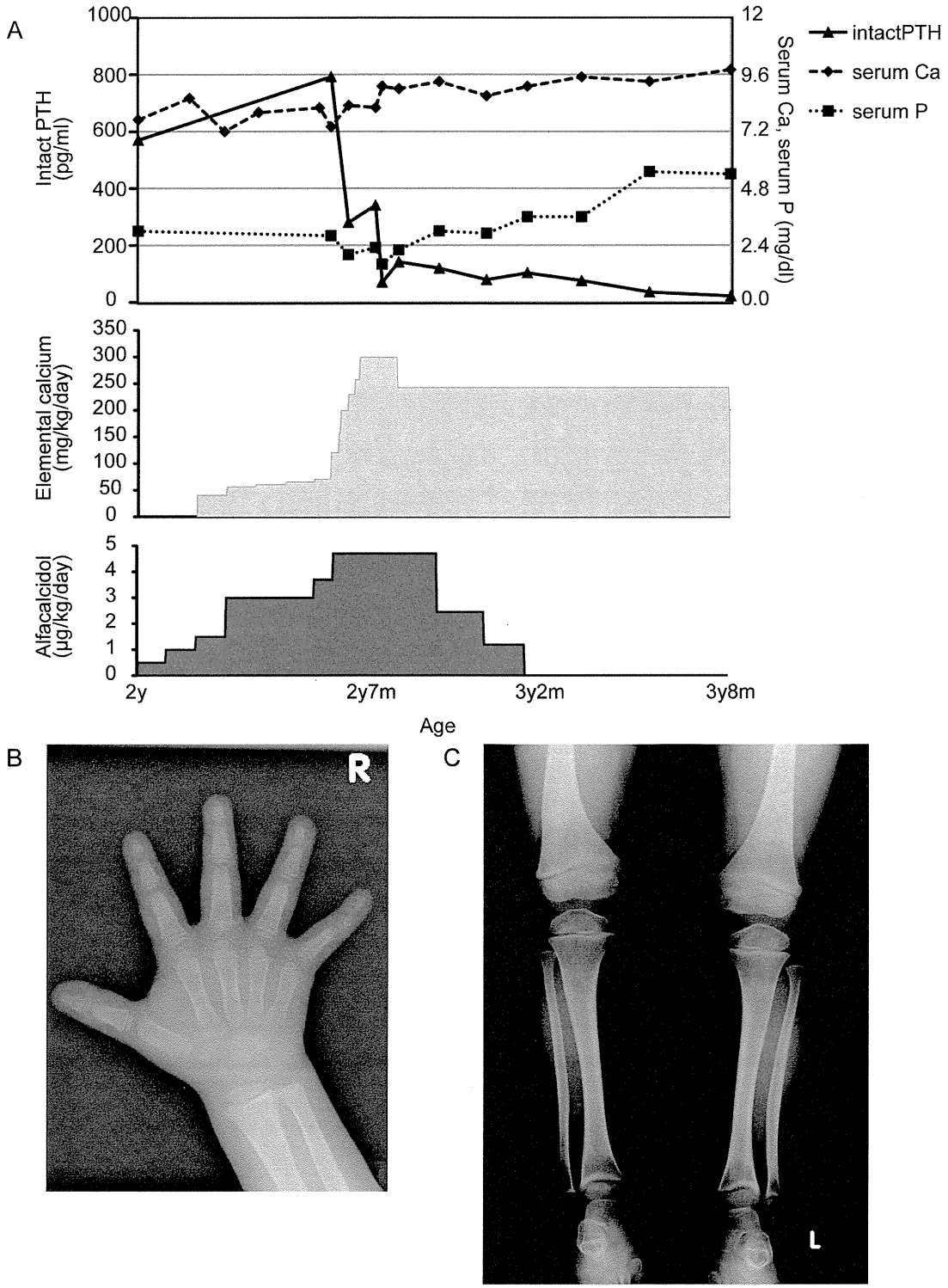

Fig 4. After Treatment with High-dose Oral Calcium Supplementation. (A) Treatment course and laboratory data are shown. (B) Bone roentgenogram at 3 years and 8 months of age, showing markedly improved signs of rickets.

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urinary calcium excretion started; the alfacalcidol dose was stopped because it was considered ineffective based on the genetic analysis. After 12 months of therapy, her laboratory data, including alkaline phosphatase levels, had normalized, her height gain improved, she started to run, and a repeat bone roentgenogram showed an improvement in the features of rickets (Fig 4B and 4C). Her most recent $1,25(OH)_2D$ level was 20 pg/mL, whereas her FGF23 was 12 pg/mL, and her urine calcium/creatinine ratio was 0.5. Although her rickets improved, alopecia has remained. Her amblyopia was noticed at age three.

Discussion

This is the first report of an HVDRR caused by UPD of the mutant allele. We also found that genome-wide SNP array was useful for the detection of the complete isodisomy. We first noted an unusual homozygous state in this non-consanguineous family, and found that only the mother was heterozygous for the mutation. Such non-Mendelian inheritance implied the possibility of gross deletion of the father's allele, non-paternity, UPD, or some *de novo* mutation. However, common SNP sequencing and STR analysis suggested a homozygous chromosome 12 and confirmed paternity, whereas subsequent G-band karyotyping excluded gross deletion of chromosome 12. Finally, the genome-wide SNP array confirmed that complete maternal isodisomy of chromosome 12 was present. This molecular diagnosis is fundamental to ensure accurate genetic counseling of the risk of recurrence in the next child, which would be far less than 25%, as would typically be the case for a recessive disorder.

Uniparental isodisomy refers the inheritance of two identical copies of the same chromosome, and different from uniparental heterodisomy, which is the inheritance of the two different homologous chromosomes from one parent. Both of isodisomy and heterodisomy can cause genome imprinting disorders, but only uniparental isodisomy can cause recessive disorders if the chromosome harbors a disease-causing mutation. As a result, the mutated allele of her mother became homozygous in the proband to present non-Mendelian inheritance of the autosomal recessive disease, HVDRR. Although high maternal age is suggested as a risk factor for maternal UPD in imprinting disorders, it is mostly meiotic nondisjunction causing heterodisomy and differs for this case of isodisomy [22].

For the molecular detection of UPD, microsatellite marker analysis has been used conventionally, which needs parents' samples to demonstrate UPD [23]. Recently, the new technology of genome-wide SNP array had facilitated the detection of UPD [9,24–26]. SNP array can detect isodisomy by loss of heterozygosity of the segment or whole chromosome without parents' samples. It is sufficient to demonstrate UPD if there are no chromosomal aberration by cytogenetic analysis. More recently, using a signal intensity analysis of the SNP array, it is possible to evaluate the copy number changes, so that even chromosome banding is unnecessary. Furthermore, we have shown it possible to detect the origin of the homozygous chromosome by analyzing together with the parents' allele. For example, if the proband's allele is AA, mother's AA or AB, and father's allele BB, the proband's allele reveals to be derived only from the mother. In this point, our study is distinguished from others.

There are only two reported cases of chromosome 12 UPD leading to recessive disorders, one maternal and one paternal [27,28], making this the third case. The occurrence of UPD in each chromosome does not appear to be equivalent, and is rare in chromosome 12. UPD of chromosomes 6, 7, 11, 14, 15, and 20 has been reported to cause imprinting disorders; however, none is known for chromosome 12. Although we show the limited period of one case, the fact that our patient shows no other than typical symptoms of HVDRR by the age of 3, suggests that there are no genomic imprinting diseases caused by UPD of maternal chromosome 12. Moreover, it is also suggested that the UPD allele in the proband contained no other disease-

causing mutations that would present by the age of 3. In theory, there may be at least 3 other recessive disease-causing mutations in this homozygous chromosome (average mutations > 200 per genome, maternal chromosome 12 constitution approximately 2%) [26,29]. The discovery of UPD will require careful observation for late manifestations of other chromosome 12 related genetic disorders. Furthermore, we consider from this study that, although there are relatively few reports of recessive diseases caused by UPD, this phenomenon may be more frequent and only have not analyzed.

Our patient with deleterious *VDR* mutation was successfully treated with high-dose oral calcium. She had no trouble taking large doses of calcium lactate orally after every meal. Most of the reported cases with severe HVDRR have required intravenous calcium infusions for initial treatment, which often lead to prolonged hospitalizations and increased risks of catheter-related complications [6,30]. Although oral calcium with vitamin D therapy is reportedly effective in some cases, it is mainly reserved for use as a maintenance therapy [18,31]. Although this is one unusual case with UPD, the clinical course of our case suggested that oral calcium therapy is effective in the initial treatment in some cases of severe HVDRR.

Among the intestinal calcium absorption, active transport of calcium through calcium transporters is induced by VDR and $1,25(OH)_2D$. On the other hand, high dietary calcium with lactose can induce passive transport, which is considered to be VDR-independent [32,33]. It has been shown in the VDR-null mice that bone abnormalities can be rescued by high calcium diet [34,35]. We consider that although the mutant VDR in this case is inactive, high calcium diet induced passive calcium transport at the intestine and improved her rickets. However, alopecia is considered as VDR-mediated but not calcium-mediated phenotype, and was unresponsive to high calcium diet, which was also similar to the observations on VDR-null mice [36].

Conclusions

HVDRR in this case was caused by a rare and complete UPD of maternal chromosome 12 with a *VDR* mutation. Genome-wide SNP array helped to detect the isodisomy and parental origin of the allele. Such comprehensive examination of the homozygous state is essential for accurate genetic counseling of recurrence risk and appropriate monitoring for other chromosome 12 related disorders. The treatment course suggested that oral calcium therapy is effective as an initial treatment for rickets in some cases with severe HVDRR.

Supporting Information

S1 Table. Primers and PCR conditions used to amplify the coding region of *VDR*.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SK. Performed the experiments: TI MK KT SK. Analyzed the data: MT TI MK KT SK. Contributed reagents/materials/analysis tools: MT TI MK HY KY TK NN KO KT SK. Wrote the paper: MT TI MK KT SK. Study supervision: AO KO KT SK.

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Global Consensus Recommendations on Prevention and Management of Nutritional Rickets

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Background: Vitamin D and calcium deficiencies are common worldwide, causing nutritional rickets and osteomalacia, which have a major impact on health, growth, and development of infants, children, and adolescents; the consequences can be lethal or can last into adulthood. The goals of this evidence-based consensus document are to provide health care professionals with guidance for prevention, diagnosis, and management of nutritional rickets and to provide policy makers with a framework to work toward its eradication.

Evidence: A systematic literature search examining the definition, diagnosis, treatment, and prevention of nutritional rickets in children was conducted. Evidence-based recommendations were developed using the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) system that describe the strength of the recommendation and the quality of supporting evidence.

Process: Thirty-three nominated experts in pediatric endocrinology, pediatrics, nutrition, epidemiology, public health, and health economics evaluated the evidence on specific questions within five working groups. The consensus group, representing 11 international scientific organizations, participated in a multiday conference in May 2014 to reach a global evidence-based consensus.

Results: This consensus document defines nutritional rickets and its diagnostic criteria and describes the clinical management of rickets and osteomalacia. Risk factors, particularly in mothers and infants, are ranked, and specific prevention recommendations including food fortification and supplementation are offered for both the clinical and public health contexts.

Conclusion: Rickets, osteomalacia, and vitamin D and calcium deficiencies are preventable global public health problems in infants, children, and adolescents. Implementation of international rickets prevention programs, including supplementation and food fortification, is urgently required. (***J Clin Endocrinol Metab*** **101: 394–415, 2016)**

Summary of Consensus Recommendations[1]

Section 1: Defining nutritional rickets and the interplay between vitamin D status and calcium intake

1.1. Definition and diagnosis of nutritional rickets

- Nutritional rickets, a disorder of defective chondrocyte differentiation and mineralization of the growth plate and defective osteoid mineralization, is caused by vitamin D deficiency and/or low calcium intake in children. (1⊕⊕⊕)
- The diagnosis of nutritional rickets is made on the basis of history, physical examination, and biochemical testing, and is confirmed by radiographs. (1⊕⊕⊕)

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Grading of evidence: 1 = strong recommendation; and 2 = weak recommendation. Quality of evidence: ⊕⊕⊕, high; ⊕⊕◯, moderate; and ⊕◯◯, low quality.

Abbreviations: ALP, alkaline phosphatase; DXA, dual-energy x-ray absorptiometry; NR, nutritional rickets; 25OHD, 25-hydroxyvitamin D_2 and/or D_3 or 25-hydroxycholecalciferol D_2 and/or D_3; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D_2 and/or D_3 or 1,25-dihydroxycholecalciferol D_2 and/or D_3 (calcitriol); PTH, parathyroid hormone; RCT, randomized controlled trial; UVB, ultraviolet B; Vitamin D_2, ergocalciferol; Vitamin D_3, cholecalciferol.

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1.2. Vitamin D status

- The panel recommends the following classification of vitamin D status, based on serum 25-hydroxyvitamin D (25OHD) levels. (1⊕⊕⊕)
  - Sufficiency, >50 nmol/L
  - Insufficiency, 30–50 nmol/L
  - Deficiency, <30 nmol/L

1.3. Vitamin D toxicity

- Toxicity is defined as hypercalcemia and serum 25OHD > 250 nmol/L, with hypercalciuria and suppressed PTH. (1⊕⊕⊕)

1.4. Dietary calcium intake to prevent rickets

- For infants 0–6 and 6–12 months of age, the adequate calcium intake is 200 and 260 mg/d, respectively. (1⊕⊕⊕)
- For children over 12 months of age, dietary calcium intake of <300 mg/d increases the risk of rickets independent of serum 25OHD levels. (1⊕⊕○)
- For children over 12 months of age, the panel recommends the following classification of dietary calcium intake. (1⊕⊕○)
  - Sufficiency, >500 mg/d
  - Insufficiency, 300–500 mg/d
  - Deficiency, <300 mg/d

1.5. Vitamin D deficiency and fractures

- Children with radiographically confirmed rickets have an increased risk of fracture. (1⊕⊕○)
- Children with simple vitamin D deficiency are not at increased risk of fracture. (1⊕⊕○)

Section 2: Prevention and treatment of nutritional rickets and osteomalacia

2.1. Vitamin D supplementation for the prevention of rickets and osteomalacia

- 400 IU/d (10 μg) is adequate to prevent rickets and is recommended for all infants from birth to 12 months of age, independent of their mode of feeding. (1⊕⊕⊕)
- Beyond 12 months of age, all children and adults need to meet their nutritional requirement for vitamin D through diet and/or supplementation, which is at least 600 IU/d (15 μg), as recommended by the Institute of Medicine (IOM). (1⊕⊕⊕)

2.2. Target for vitamin D supplementation

- In healthy children, routine 25OHD screening is not recommended, and consequently, no specific 25OHD threshold for vitamin D supplementation is targeted in this population. (1⊕⊕⊕)

2.3. Candidates for preventative vitamin D supplementation beyond 12 months of age

In the absence of food fortification, vitamin D supplementation should be given to:

- Children with a history of symptomatic vitamin D deficiency requiring treatment. (1⊕⊕⊕)
- Children and adults at high risk of vitamin D deficiency, with factors or conditions that reduce synthesis or intake of vitamin D. (1⊕⊕⊕)
- Pregnant women (see *Section 3.1*).

2.4. Dose of vitamin D and calcium for the treatment of nutritional rickets

- For treatment of nutritional rickets, the minimal recommended dose of vitamin D is 2000 IU/d (50 μg) for a minimum of 3 months. (1⊕⊕⊕)
- Oral calcium, 500 mg/d, either as dietary intake or supplement should be routinely used in conjunction with vitamin D in the treatment regardless of age or weight. (1⊕⊕⊕)

2.5. Appropriate route of administration and duration of therapy

- We recommend oral treatment, which more rapidly restores 25OHD levels than IM treatment. (1⊕⊕⊕)
- For daily treatment, both D_2 and D_3 are equally effective. (1⊕⊕⊕)
- When single large doses are used, D_3 appears to be preferable compared to D_2 because the former has a longer half-life. (1⊕⊕⊕)
- Vitamin D treatment is recommended for a minimum of 12 weeks, recognizing that some children may require longer treatment duration. (1⊕⊕⊕)

Section 3: Prevention of nutritional rickets/ osteomalacia: identification of risk factors

3.1. Dietary practices and nutrient intakes among mothers associated with nutritional rickets in infants

- Maternal vitamin D deficiency should be avoided by ensuring that women of childbearing age meet intakes of 600 IU/d recommended by the IOM. (1⊕⊕⊕)
- Pregnant women should receive 600 IU/d of vitamin D, preferably as a combined preparation with other recommended micronutrients such as iron and folic acid. (2⊕⊕○)

3.2. Early feeding, supplementation, complementary feeding, and nutrient intake associated with rickets in infants

- In addition to an intake of 400 IU/d of vitamin D, complementary foods introduced no later than 26 weeks should include sources rich in calcium. (1⊕⊕⊕)

- An intake of at least 500 mg/d of elemental calcium must be ensured during childhood and adolescence. (1⊕⊕⊕)

3.3. Association of sunlight exposure to nutritional rickets

- Because UVB rays trigger epidermal synthesis of previtamin D_3, restricted exposure to sun increases the risk of vitamin D deficiency and nutritional rickets. (1⊕⊕⊕)
- Environmental factors, such as latitude, season, time of day, cloud cover, and pollution affect availability of UVB, whereas personal factors, such as time spent outdoors, skin pigmentation, skin coverage, age, body composition, and genetics affect the dose-response of UVB exposure and circulating 25OHD. (2⊕⊕○)
- No safe threshold of UV exposure allows for sufficient vitamin D synthesis across the population without increasing skin cancer risk. (2⊕⊕○)

Section 4: Prevention of osteomalacia during pregnancy and lactation and congenital rickets

4.1. The relationship between vitamin D during pregnancy and infant growth and bone mass

- Pregnant women should receive 600 IU/d of supplemental vitamin D. This will ensure adequacy of maternal 25OHD, especially in women at risk of deficiency, to prevent elevated cord blood alkaline phosphatase (ALP), increased fontanelle size, neonatal hypocalcemia and congenital rickets, and to improve dental enamel formation. (2⊕⊕○)
- There is little evidence that maternal supplementation with vitamin D will protect or improve birth anthropometry (2⊕○○) and no evidence that supplementation with vitamin D will protect or improve short- or long-term growth or bone mass accretion. (2⊕⊕○)

4.2. The effect of calcium supplementation during pregnancy on infant bone mass

- Pregnant women do not need calcium intakes above recommended non-pregnant intakes to improve neonatal bone. (1⊕⊕⊕)

4.3. Influence of calcium or vitamin D supplementation in pregnancy or lactation on breast milk calcium or vitamin D

- Lactating women should ensure they meet the dietary recommendations for vitamin D (600 IU/d) for their own needs, but not for the needs of their infant. (1⊕⊕⊕)
- Lactating women should not take high amounts of vitamin D as a means of supplementing their infant. (2⊕⊕○)
- Pregnant and lactating women should meet the recommended intakes of calcium. Maternal calcium intake during pregnancy or lactation is not associated with breast milk calcium concentrations. (1⊕⊕⊕)

4.4. Causes and therapy of congenital rickets

- Supplementing mothers with 600 IU/d of vitamin D and ensuring they receive recommended calcium intakes, or appropriate therapy of maternal conditions predisposing to hypocalcemia or vitamin D deficiency, prevents congenital rickets. (2⊕○○)

Section 5: Assessing the burden of nutritional rickets and public health strategies for prevention

5.1. Assessment of disease burden

- The prevalence of rickets should be determined by population-based samples, by case reports from sentinel centers, or by mandatory reporting. (1⊕⊕⊕)
- Screening for nutritional rickets should be based on clinical features, followed by radiographic confirmation of suspected cases. (1⊕⊕⊕)
- Population-based screening with serum 25OHD, serum ALP, or radiographs is not indicated. (1⊕⊕⊕)

5.2. Public health strategies for rickets prevention

- Universally supplement all infants with vitamin D from birth to 12 months of age, independent of their mode of feeding. Beyond 12 months, supplement all groups at risk and pregnant women. Vitamin D supplements should be incorporated into childhood primary health care programs along with other essential micronutrients and immunizations (1⊕⊕⊕), and into antenatal care programs along with other recommended micronutrients. (2⊕⊕○)
- Recognize nutritional rickets, osteomalacia, and vitamin D and calcium deficiencies as preventable global public health problems in infants, children, and adolescents. (1⊕⊕⊕)
- Implement rickets prevention programs in populations with a high prevalence of vitamin D deficiency and limited vitamin D and/or calcium intakes, and in groups of infants and children at risk of rickets. (1⊕⊕⊕)
- Monitor adherence to recommended vitamin D and calcium intakes and implement surveillance for nutritional rickets. (1⊕⊕⊕)
- Fortify staple foods with vitamin D and calcium, as appropriate, based on dietary patterns. Food fortification can prevent rickets and improve vitamin D status of infants, children, and adolescents if appropriate foods are used and sufficient fortification is provided, if fortification is supported by relevant legislation, and if the process is adequately monitored. Indigenous food sources of calcium should be promoted or subsidized in children. (1⊕⊕⊕)

- Promote addressing the public health impact of vitamin D deficiency as both a clinical and a public health issue. (1⊕⊕⊕)

5.3. Economic cost/benefits of prevention programs

- The cost-effectiveness of supplementation and food fortification programs needs further study. (1⊕⊕◯)

Nutritional rickets (NR), secondary to vitamin D deficiency and/or dietary calcium deficiency, remains a significant global, public health problem despite the availability of supplementation and numerous published guidelines for its prevention (1–8). This is concerning because NR can have a major impact on the health of infants, children, and adolescents, with ramifications that persist into adulthood. The morbidity and mortality associated with NR can be devastating, with substantial but poorly recognized consequences for society and health economics. Features of NR and osteomalacia include: 1) hypocalcemic seizures and tetanic spasms; 2) life-threatening hypocalcemic cardiomyopathy; 3) bone pain and muscle weakness; 4) limb and pelvic deformities; 5) failure to thrive; 6) developmental delay; and 7) dental anomalies (9, 10). Alarmingly, NR can also lead to death from heart failure caused by hypocalcemic cardiomyopathy, even in developed countries (11). In addition, narrowing of the pelvic outlet after NR in childhood can result in obstructed labor and maternal and fetal death (12).

Despite intense focus around the role of vitamin D status in health and disease, there has been a worldwide failure to implement public health guidance and eradicate the most severe manifestations of vitamin D and calcium deficiency in our most vulnerable population—NR and osteomalacia of childhood. Therefore, the goal of this Consensus Statement is to provide clinicians with clarity and recommendations on the recognition, societal burden, and treatment of NR and osteomalacia, and to enable clinicians and health policy leaders to establish appropriate clinical and public health interventions to prevent this debilitating, costly, and under-recognized global health problem.

Methods

In recognition of the considerable variation in the definition, diagnosis, and management of NR worldwide, the European Society for Pediatric Endocrinology decided to examine current best practice in NR and to formulate evidence-based recommendations. Experts were assembled from the following societies: the Pediatric Endocrine Society (PES), the Asia Pacific Pediatric Endocrine Society (APPES), the Japanese Society for Pediatric Endocrinology (JSPE), the Sociedad Latino-Americana de Endocrinología Pediátrica (SLEP), the Australasian Pediatric Endocrine Group (APEG), the Indian Society for Pediatric and Adolescent Endocrinology (ISPAE), the African Society for Pediatric and Adolescent Endocrinology (ASPAE), the Chinese Society of Pediatric Endocrinology and Metabolism (CSPEM), the British Nutrition Society, and the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN). This consensus paper includes the cumulative evidence up to the end of 2014.

Participants included individuals from Europe, North America (United States and Canada), Latin America, Asia, Africa, and Australia, with a balanced spectrum of professional seniority and expertise. In addition, an expert on the development of evidence-based guidelines served in an advisory capacity. Panel members declared any potential conflict of interest at the initial meeting of the group. Thirty-three participants were assigned to one of five groups to which topics 1–5 were allocated, and a chairperson was designated for each group. Each participant prepared an evidence-based summary of the literature relating to a particular question distributed before the conference (which was held over 3 days in May 2014).

Each group presented the revised summaries for discussion to the full conference. This report is based on the questions addressed. A detailed description of the GRADE classification has been published elsewhere (13). Recommendations were based on published findings and on expert opinion when appropriate.

The best available research evidence was used to develop recommendations. Preference was given to articles written in English, identified by PubMed searches with MeSH terms. For each point, recommendations and evidence are described, with a modification in the grading evidence as follows: 1 = strong recommendation (applies to most patients in most circumstances, benefits clearly outweigh the risk); and 2 = weak recommendation (consensus opinion of working group or should be considered; the best action may depend on circumstances or patient values, benefits, and risks closely balanced or uncertain). Quality of evidence is indicated as follows: ⊕⊕⊕, high quality (prospective cohort studies or randomized controlled trials [RCTs] at low risk of bias); ⊕⊕◯, moderate quality (observational studies or trials with methodological flaws, inconsistent or indirect evidence); and ⊕◯◯, low quality (case series or nonsystematic clinical observations) (13).

The target audience for these guidelines includes general and specialist pediatricians, other professionals providing care for patients with NR, and health policy makers, particularly in countries with developing economies.

1.0. Defining Nutritional Rickets and the Interplay between Vitamin D Status and Calcium Intake

1.1. Definition and diagnosis of nutritional rickets

1.1.1. Recommendations

- Nutritional rickets, a disorder of defective chondrocyte differentiation and mineralization of the growth plate and defective osteoid mineralization, is caused by vitamin D deficiency and/or low calcium intake in children. (1⊕⊕⊕)
- The diagnosis of nutritional rickets is made on the basis of history, physical examination, and biochemical testing and is confirmed by radiographs. (1⊕⊕⊕)