

A patient with combined pituitary hormone deficiency and osteogenesis imperfecta associated with mutations in *LHX4* and *COL1A2*



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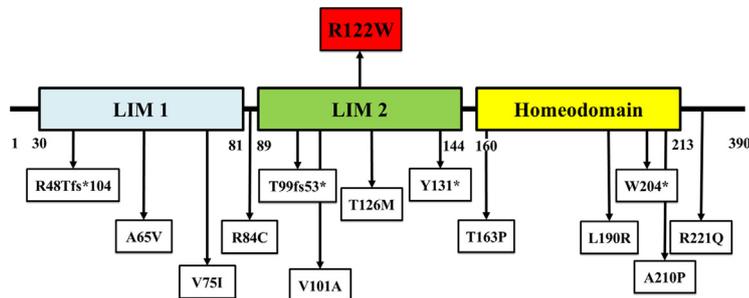
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HIGHLIGHTS

- The mutations in two different genes should be sought in the patients with complex phenotypes.
- The c.1531G > T in *COL1A2* leading to OI and c.364C > T (p.R122W) in *LHX4* to CPHD were found in a Thai boy.
- The incomplete penetrance and loss-of-function are the features of p.R122W mutation in *LHX4*.
- The mutation spectra of *COL1A2* and *LHX4* and pathomechanism of *LHX4* are expanded.

GRAPHICAL ABSTRACT



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ABSTRACT

Genetic disorders have been shown to co-occur in individual patient. A Thai boy with features of osteogenesis imperfecta (OI) and combined pituitary hormone deficiency (CPHD) was identified. The causative mutations were investigated by whole exome and Sanger sequencing. Pathogenicity and pathomechanism of the variants were studied by luciferase assay. The proband was found to harbor a novel *de novo* heterozygous missense mutation, c.1531G > T (p.G511C), in *COL1A2* leading to OI and a heterozygous missense variant, c.364C > T (p.R122W), in *LHX4*. The *LHX4* p.R122W has never been reported to cause CPHD. The variant was predicted to be deleterious and found in the highly conserved LIM2 domain of *LHX4*. The luciferase assays revealed that the p.R122W was unable to activate *POU1F1*, *GH1*, and *TSHB* promoters, validating its pathogenic effect in CPHD. Moreover, the variant did not alter the function of wild-type *LHX4*, indicating its hypomorphic pathomechanism. In conclusion, the novel *de novo* heterozygous p.G511C mutation in *COL1A2* and the heterozygous pathogenic p.R122W mutation in *LHX4* were demonstrated in a patient with OI and CPHD. This study proposes that the mutations in two different genes should be sought in the patients with clinical features unable to be explained by a mutation in one gene.

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Introduction

Osteogenesis imperfecta (OI) is a rare disease characterized by bone fragility. The prevalence of OI is 6-7/100,000 [1]. The disease is inherited in an autosomal dominant, autosomal recessive, or X-linked recessive manner. Alterations in at least 18 genes have been associated with OI [1,2]. Mutations in *COL1A1* or *COL1A2*, which encode the pro- α 1 or pro- α 2 chain of type I collagen, account for more than 85% of disease-causing variants. Glycine substitutions within the Gly-X-Y repeats of collagen chains are the most common type of mutations leading to abnormal collagen structure [1].

Combined pituitary hormone deficiency (CPHD) is a condition in which the pituitary gland produces insufficient amounts of several hormones, including growth hormone (GH), prolactin production (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), adrenocorticotropic hormone (ACTH), and/or thyroid-stimulating hormone (TSH). Its prevalence is 1/8000 [3]. Up to 2500 variants in 30 genes including *PROPI*, *POU1F1*, *HESX1*, *OTX2*, *GLI2*, *LHX3*, and *LHX4* have been associated with CPHD [3]. The *LHX4*, which is the LIM homeodomain transcription factor, plays an important role in the development of anterior pituitary gland and nervous system. Its expression is found in the Rathke's pouch. It can regulate *POU1F1*, *GH1*, *PRL*, α *GSU*, *FSHB*, and *TSHB* genes [4]. To date, only thirteen of *LHX4* variants have been investigated for their effects in CPHD [3–6].

A Thai boy manifesting the combined features of OI and CPHD was identified. The study aimed to identify the causative mutations leading to two different Mendelian diseases and to investigate the pathogenicity and pathomechanism of the identified variant causing CPHD.

Materials and methods

Patient characterization and mutation analysis

A Thai boy diagnosed with both OI and CPHD and his parents were recruited. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB500/61), Faculty of Medicine, Chulalongkorn University. Written informed consents for publication of their clinical details and images were obtained from the participants. Genomic DNA isolated from the peripheral blood was subjected for mutation analyses using whole exome sequencing (WES) according to previous publication [7]. The identified variants were validated using Sanger sequencing.

Pathogenic effect of *LHX4* variants

The pTracer-LHX4_WT-HA was a gift from Marie Legendre, France. The *LHX4* mutant vectors of p.R122W (the mutation identified in this study) and of two previously reported p.T163P [4] and p.L190R [5] were generated using Q5[®] Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). Three luciferase reporter vectors were selected. The proximal promoter regions of human *GH1*, *POU1F1*, or *TSHB* were amplified and cloned into the pGL4.10[luc2] vector (Supplementary Table 1) [4,5]. Commercial Chinese hamster ovary (CHO)-K1 cells (ATCC[®]CCL-61TM) were used for transient transfection using X-tremeGENE 9 DNA Transfection Reagent (Roche, Mannheim, Germany).

Pathomechanism of *LHX4* variants

The CHO-K1 cells were cotransfected with an equal amount of wild-type and each mutant *LHX4*. The empty pTracer-CMV expres-

sion vector was used as the control. *POU1F1* promoter was selected for this experiment. After 48 h, the transcription activity was measured using the Luciferase Assay System (Promega, Madison, WI) and SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA). Total amount of protein was measured by the Pierce[™] BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The results of three independent experiments were reported as mean \pm SD. The *P*-value was <0.01.

Western blot analyses

Protein extracted from the cellular lysates of CHO-K1 cells transfected with *LHX4* expression vectors were prepared. 20 μ g of protein per sample was separated on 12% sodium dodecyl sulfate polyacrylamide gels and then transferred by iBlot[™] Transfer Stack, PVDF, regular size (Invitrogen, Carlsbad, CA). Monoclonal anti-HA antibody at 1:250 dilution (cat no. H3663, Sigma-Aldrich, St Louis, MO) was used as the primary antibody to detect *LHX4*-HA proteins, followed by anti-mouse IgG antibody at 1:2000 dilution (cat no. 7076, Cell Signaling Technology, Danvers, MA) as the secondary antibody. Anti-actin antibody (cat no. Mab1501, Sigma-Aldrich, St Louis, MO) was used as a positive control to determine gel loading equivalency. Results were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) and chemiluminescence camera (ImageQuant LAS 4000, Amersham).

Results

Patient characteristics

A 6-year-old Thai boy was born at 37 weeks by caesarian section due to breech presentation, intrauterine growth retardation, and oligohydramnios. His birth weight was 1,840 g (<3rd centile) and length was 41 cm (<3rd centile). His head circumference was 32 cm (25–50th centile). Apgar scores at 1 and 5 min were 5 and 10, respectively. At birth, the proband exhibited deformed limbs, bowed legs, wide anterior fontanelle (4x4 cm), frontal bossing, and blue sclerae. He also developed neonatal sepsis, seizure, and respiratory distress caused by subcostal retraction and chest wall instability. Fractures of his left humerus and angulation of long bones was radiologically observed (Fig. 1A, B). The result of hearing test was normal. Intravenous pamidronate (7.2 mg/kg/year) was started at the age of 2 months and then administered every 2 months. He started to walk unaided at 2 years of age but showed an abnormal gait. Pectus carinatum and flat feet were observed at the age of 3 years (Fig. 1C, D). His primary teeth showed dentinogenesis imperfecta and were severely deteriorated (Fig. 1E, F). He broke his right femur at the age of 5 years 11 months and his left femur at 6 years 2 months caused by falls (Fig. 1G, H). Bone density scans showed that his lumbar spine BMD at the age of 19 months was 0.343 g/cm² (<-3SD) and at the age of 4 years was 0.491 g/cm² (-2SD, +42.9%).

Apart from short stature and bone fragility which are the main features of OI, slow growth velocity was noticed (Fig. 1I). Physical examination of the proband at the age of 3 years 5 months showed that his upper lower body ratio was 1.19:1 and arm span was 72 cm. Further endocrine evaluation revealed that the proband had low level of free thyroxine (FT4) (0.51 ng/dl; normal 1.0–1.8). The levels of insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-binding protein 3 (IGFBP3) were in the low end of normal range. Growth hormone (GH) stimulation test showed the peak value of GH at 1.01 ng/ml (normal > 10). The levels of cortisol and adrenocorticotropic hormone (ACTH) were within normal limit (Table 1). Renal function test and blood

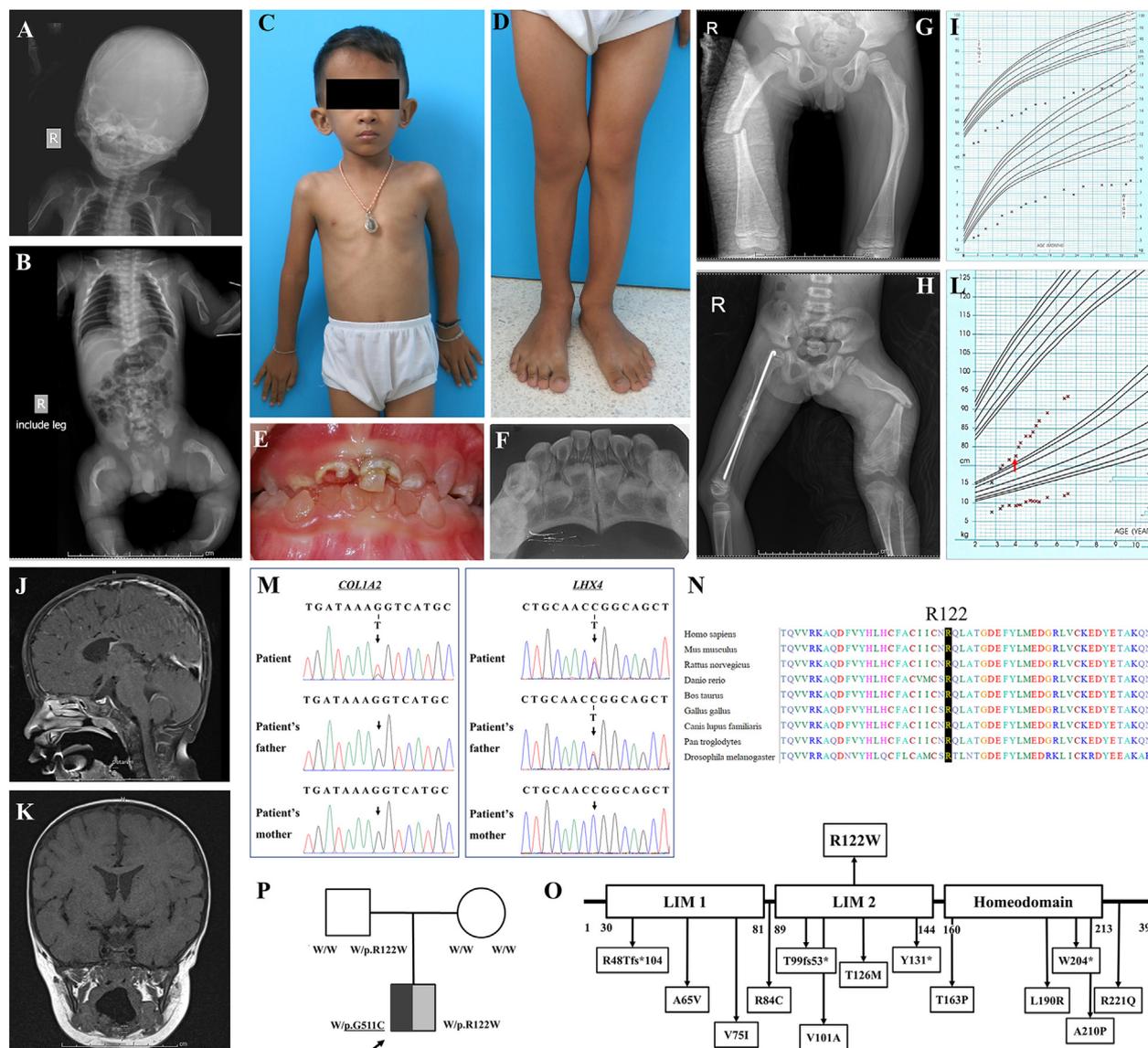


Fig. 1. Phenotype and genotype of the proband. (A, B) Radiographs at birth showed the fractures of the left humerus and deformities of lower extremities (C, D) The proband at 6 years of age exhibited pectus carinatum and flat feet. (E, F) Oral photographs and radiographs at the age of 4 years showed dentinogenesis imperfecta. (G, H) Radiographs showed right femur fracture at the age of 5 years 11 months and left femur fracture at the age of 6 years 2 months. (I) Growth curve from birth to 3 years of age. (J, K) Brain MRI showed hypoplasia of the pituitary gland. (L) Growth curve from 2.5 to 6.5 years of age. Red arrow indicated the initiation of growth hormone and thyroid hormone therapy. (M) Sanger sequencing showed a novel *de novo* heterozygous missense p.G511C (c.1531G > T) mutation in *COL1A2* and a heterozygous missense p.R122W (c.364C > T) variant in *LHX4* which was inherited from his healthy father. (N) Alignment of the amino acid sequence of *LHX4* among several species. (O) Schematic diagram of *LHX4*. The top panel showed the p.R122W variant identified in this study. The bottom panel showed the variants previously reported with functional studies. (P) Family pedigree of the proband. Symbol filled with black represents a subject with osteogenesis imperfecta; symbol filled with gray represents a subject with combined pituitary hormone deficiency; and empty symbols represent healthy subject. An arrow indicates the proband. Underlined letters are genotypes of *COL1A2* while those which are not underlined are genotypes of *LHX4*. W, wild-type allele.

biochemistry were normal. Brain MRI identified the hypoplastic pituitary gland and stalk and mildly diffused parenchymal volume loss (Fig. 1J, K). Based on the clinical and laboratory investigations, the proband was diagnosed with the deficiencies of growth hormone and thyroid hormone, indicating CPHD. The administration of growth hormone (Norditropin® 0.3 mg/day subcutaneously) and thyroid hormone (Eltroxin, 25 ug/day) was started when he was 3 years 9 months. Since hormonal replacement was initiated, his growth had been improved (Fig. 1L).

The proband was the only son of non-consanguineous and healthy Thai parents. They were 31 years of age when the proband was born. Paternal height was 164.8 cm and maternal height was 159 cm. Physical and dental manifestations of the parents were unremarkable. The proband's father had normal growth and no infertility problems. His sexual maturity was at Tanner stage V.

Endocrine evaluation of the father at age 37 years showed normal levels of IGF1, FT4, thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), and testosterone.

Mutation analyses

Exome sequencing revealed that the proband possessed a novel *de novo* heterozygous missense mutation, c.1531G > T (p.G511C), in *COL1A2*. The variant was not found in ExAC and our in-house database of 1,876 Thai exomes. Several lines of evidences have supported the pathogenicity of the *COL1A2* p.G511C. The variant was 1) *de novo* which is a strong evidence of its etiologic role, 2) absent from controls in multiple variant databases and in-house database, 3) highly conserved among several species, 4) predicted to be deleterious based on multiple lines of computational evi-

Table 1
Hormonal tests of the proband.

Treatment	Age	TSH mIU/l	FT4 (ng/dl)	FT3 (pg/dl)	IGF1 (ng/ml)	IGFBP3 (ug/ml)	GH (ng/ml)	Cortisol (ug/dl)	ACTH (pg/ml)
No treatment	3 days	7.52 (0.7–15.2)	1.02 (0.9–2.5)	–	–	–	–	–	–
	3 years 5 months	4.3 (0.7–6.0)	0.51* (1.0–1.8)	–	<25 (22–229)	0.9 (0.9–4.3)	–	4.2* (5–25)	–
	3 years 6 months	5.09 (0.7–6.0)	0.62* (1.0–1.8)	–	–	–	0.50* [1] (>10)	6.2 [1] (5–25)	29.9 (7.2–63)
	–	–	–	–	–	–	0.66* [2] (>10)	16.6 [2] (5–25)	–
	–	–	–	–	–	–	0.88* [3] (>10)	–	–
Norditropin® and Eltroxin	3 years 9 months	<0.005 (0.7–6.0)	1.31 (1.0–1.8)	4.68 (2.8–4.4)	–	–	–	–	–
	3 years 11 months	–	1.11 (1.0–1.8)	–	<25 (22–229)	1.2 (0.9–4.3)	–	–	–
	5 years 10 months	–	–	–	45 (39–250)	2.4 (1.1–5.2)	–	–	–
	6 years 6 months	<0.0025 (0.6–4.8)	1.38 (1.0–1.7)	–	107 (47–275)	–	–	–	–
	6 years 9 months	–	1.41 (1.0–1.7)	–	–	–	–	–	–
	6 years 10 months	–	1.77 (1.0–1.7)	–	79.6 (47–275)	–	–	–	–
	–	–	–	–	–	–	0.85* [4] (>10)	–	–
–	–	–	–	–	–	1.01* [5] (>10)	–	–	

*not in normal range

Numbers in parentheses are normal ranges.

dences, 5) corresponding to the patient's phenotype for OI, and 6) located in the triple helical domain of alpha 2 chains of type I collagen. According to American College of Medical Genetics and Genomics (ACMG) guidelines, the p.G511C variant is considered pathogenic [8]. Changes of glycine, the smallest amino acid, in Gly-X-Y triplets of collagen chain have been shown to disturb triple helical assembly and collagen chain stability [9,10]. These therefore provide compelling evidence that p.G511C in *COL1A2* is the causative variant of OI in the proband. In addition, a missense variant, c.364C > T (p.R122W), in *LHX4* was detected in the proband and his healthy father (Fig. 1M). The p.R122W variant was found in 1 out of 121,208 alleles in ExAC. However, it was not observed in our in-house exome database and had never been reported to cause any diseases. The p.R122W was highly conserved among several species and located in the LIM2 domain of *LHX4* (Fig. 1N, O). Both *COL1A2* and *LHX4* variants were predicted to be deleteri-

ous (Sorting Intolerant From Tolerant/SIFT), probably damaging (Polymorphism Phenotyping/PolyPhen), and possibly pathogenic (Mendelian Clinically Applicable Pathogenicity/M-CAP). The phenotypic and genotypic findings of the proband lead to the diagnosis of combined OI and CPHD (Fig. 1P). Mutation analyses were demonstrated in Tables 2, 3.

Pathogenicity of the *LHX4* missense variant, p.R122W

Western blotting showed that the wild-type, p.L190R, p.T163P, and p.R122W *LHX4* proteins were detected at similar sizes (50 kDa) (Supplementary Fig. 1). The luciferase assays showed that the wild-type *LHX4* was able to activate the *POU1F1*, *GH1*, and *TSHB* promoters. In contrast, the luciferase activities of the p.R122W variant and two previously reported mutations, p.T163P and p.L190R, were significantly lower than the wild-type (Fig. 2A–C).

Table 2
Filtering criteria for the exome sequencing of the proband.

Total number of variants after exclusion of variants with quality score <20		87,959			
After exclusion of variants with read depth <10		72,310			
After exclusion of variants not in or close to the coding regions		12,478			
After exclusion of variants with allele frequency >1% in the database (Exome Variant Server, Exome Aggregation Consortium, 1000 Genomes Project Consortium, dbSNPs)		1,969			
After exclusion of variants found in an in-house database of 1,876 Thai exomes		726			
After selection of variants associated with phenotypes (Supplementary Table 4)	osteogenesis imperfecta	combined pituitary hormone deficiency			
	1	4			
Gene	<i>COL1A2</i>	<i>LHX4</i>	<i>WNT4</i>	<i>ALK</i>	<i>IGF1R</i>
Variant	G > G/T	C > C/T	G > G/A	T > T/C	G > G/A
Coordinate	7:94042422	1:180235642	1:22447789	2:29449866	15:99473472
Genotype	heterozygous	heterozygous	heterozygous	heterozygous	heterozygous
Transcript	NM_000089.3	NM_033343.3	NM_030761.4	NM_004304.4	NM_000875.3
Consequence	missense variant	missense variant	missense variant	missense variant	missense variant
cDNA	c.1531G > T	c.364C > T	c.503C > T	c.2989A > G	c.2894G > A
Protein	p.G511C	p.R122W	p.S168L	p.M997V	p.S965N
Sift	deleterious (0)	deleterious (0)	deleterious (0.042)	tolerated (0.184)	tolerated (0.438)
PolyPhen-2	probably damaging (1)	probably damaging (1)	possibly damaging (0.867)	benign (0.001)	benign (0)
M-CAP	possibly pathogenic (0.956)	possibly pathogenic (0.196)	possibly pathogenic (0.096)	likely benign (0.021)	likely benign (0.011)
Associated diseases	OI	CPHD	Mullerian aplasia and hyperandrogenism SERKAL syndrome	Neuroblastoma, susceptibility to, 3	Insulin-like growth factor I, resistance to

Table 3
Gene lists associated with osteogenesis imperfecta and combined pituitary hormone deficiency.

Phenotype	Genes
Osteogenesis imperfecta	<i>FKBP10, LEPRE1, PPIB, BMP1, COL1A1, COL1A2, CREB3L1, CRTAP, IFITM5, MBTPS2, PLOD2, SERPINF1, SERPINH1, SP7, TMEM38B, WNT1, SEC24D, SPARC</i>
Combined pituitary hormone deficiency	<i>AAAS, AANAT, AES, ALK, ANXA1, AR, ARNT2, BGLAP, BLVRB, CDA, CRH, CSF2, CSHL1, CUL4B, CYP19A1, DRD2, ELANE, EPO, ERBB2, ESR1, F2, FOXA2, GH1, GH2, GHR, GHRH, GHRHR, GHSR, GLI2, GLIS3, HESX1, IGF1, IGF1R, IGFBP3, IGHD, IGSF1, INSM1, INSR, KLK3, LHX3, LHX4, MC2R, MCHR1, MRAP, NBEA, NF1, NKX2-1, NKX2-5, NOG, OTP, OTX2, PAX8, PLAT, POMC, POU1F1, POU3F2, PRL, PROP1, PTTG1IP, REN, SERPINA1, SHOX, SHOXY, SIM1, SIX6, SLC6A3, SOX2, SOX3, SST, STAT5B, SYTL4, TBG, THRA, TNFSF11, TRH, TSHB, TSHR, VWF, WNT4, XRCC4, ZIC2 (HP:0000871, HP:0000824, HP:0000851)</i>

Pathomechanism of the LHX4 missense variant, p.R122W

Cotransfection of wild-type and each mutant LHX4 (p.R122W, p.T163P, and p.L190R) showed comparable luciferase activities to the cotransfection of wild-type LHX4 and empty pTracer-CMV expression vector. These suggest that these LHX4 mutations do not have a dominant negative effect on the wild-type function and they are likely to cause the disease by loss-of-function mechanism (Fig. 2D).

Discussion

A Thai boy with the complex phenotypes was identified. His main features including short stature, bone deformity, skeletal fracture, low BMD, blue sclerae, and dentinogenesis imperfecta led to the primary diagnosis of OI. After intravenous pamidronate was administered every 2 months, his BMD had increased. However, low height velocity was observed. Further laboratory investi-

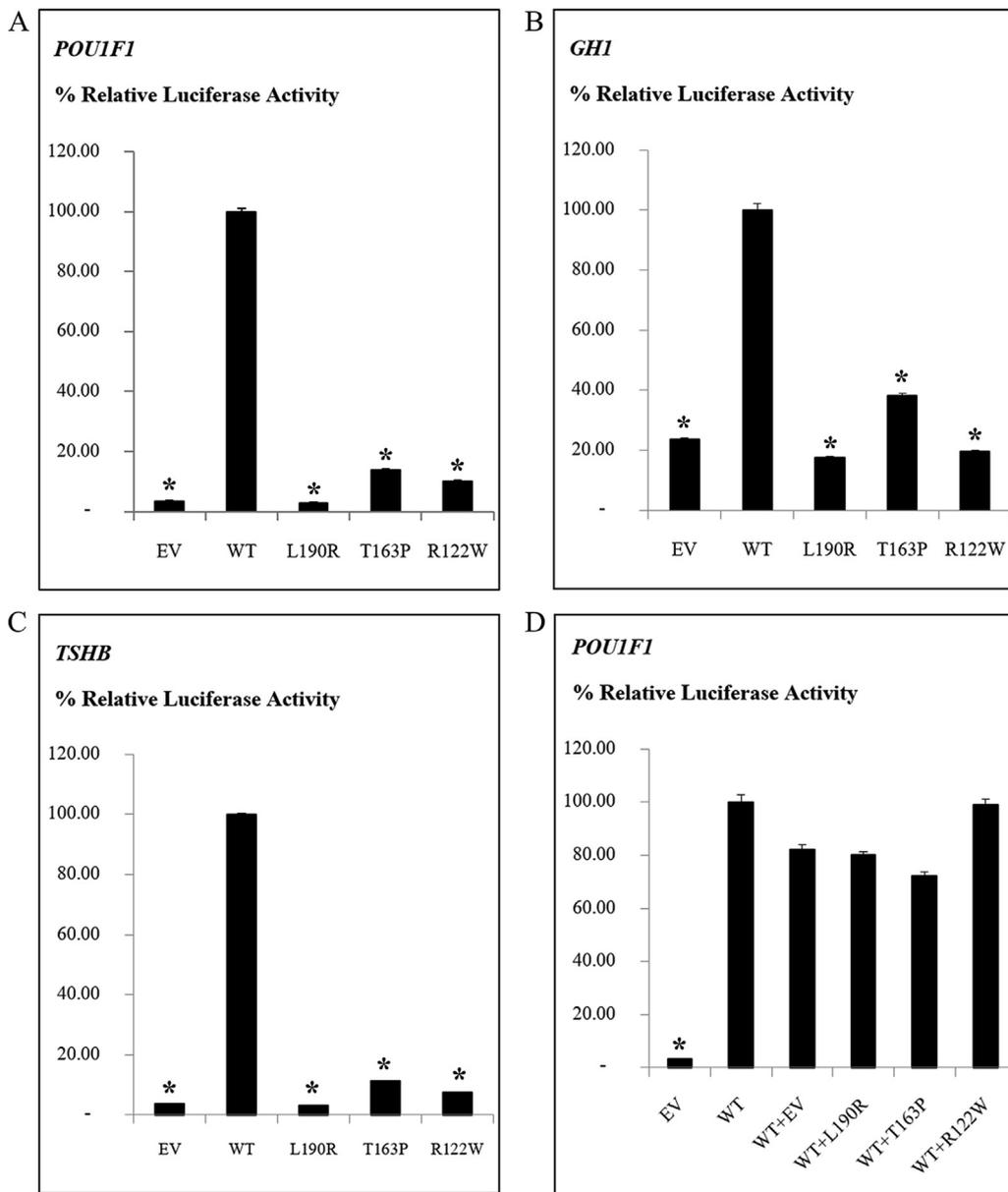


Fig. 2. Luciferase reporter assays demonstrating pathogenicity and pathomechanism of the p. R122W variant in LHX4. (A–C) The LHX4 mutations reduced the transcriptional activities of *POU1F1*, *GH1*, and *TSHB* promoters, compared to the wild-type LHX4. (D) The mutations did not show a dominant negative effect on the wild-type LHX4. EV, empty vector; WT, wild-type LHX4; p.L190R and p.T163P, the variants previously reported; p.R122W, the variant identified in this study; (A–C) $P < 0.01$ compared with WT (*); (D) $P < 0.01$ compared with WT + EV (*).

gations showing pituitary hypoplasia and deficiencies of GH and TSH indicated the secondary diagnosis of CPHD. These suggest that attentive monitoring a child's growth benefits an early diagnosis of combined rare diseases. In addition, the replacement therapy with GH apart from IV bisphosphonate results in an increased height velocity for this patient.

More than forty OI patients have been identified in our Genetics Clinic of King Chulalongkorn Memorial Hospital. Those were associated with the mutations in various genes including *COL1A1*, *BMP1*, *P4HB*, *WNT1*, and *MBTPS2* [2,11–13]. In the proband, WES analysis revealed the novel *de novo* heterozygous missense mutation, c.1531G > T (p.G511C), in *COL1A2*, corresponding to OI. This *COL1A2* variant was not found in his healthy parents. Glycine is considered to be the smallest amino acid allowing it to be incorporated in the triple helix structure of collagen. Therefore, the change of glycine to cysteine is expected to impede the folding and formation of collagen resulting in abnormal collagen structure.

The complex phenotype of the proband was unexplainable by the single mutation in *COL1A2*. He was the only one out of our 40 Thai OI patients that exhibited CPHD features. Further genetic investigation revealed that the proband also possessed the heterozygous missense mutation, c.364C > T (p.R122W), in *LHX4*, which was inherited from his healthy father. Interestingly, the mutations in *LHX4* causing CPHD have been reported with a high rate of incomplete penetrance [4,14]. The pathogenicity of *LHX4* variant, p.R122W, was then investigated. The p.R122W was predicted to be deleterious and located in the highly conserved LIM2 domain playing important role in protein-protein interactions [15]. Using the luciferase reporter assays, the *LHX4* p.R122W was shown to lose its ability to activate *POU1F1*, *GH1*, and *TSHB* promoters, validating its pathogenicity. These explain the deficiencies of GH and TSH found in the proband. Western blot analyses showed that the wild-type and mutant *LHX4* proteins of expected size (50 kDa) were detected. The level of expression of p.R122W was lower than that of the wild-type. However, this might not be the cause for its low level of luciferase activity as the control mutants that were expressed at higher levels also showed low activities. Next, the pathomechanism study of p.R122W showed that it did not interfere with the function of wild-type *LHX4*, similar to the other two *LHX4* mutations, L190R and T163P. These indicate that it possesses loss-of-function or haploinsufficiency mechanism rather than dominant negative effect in CPHD. Notably, *LHX4* has been proposed to have random monoallelic expression [16]. The single expression of normal *LHX4* allele could therefore explain the absence of disease in the proband's father who also carries the heterozygous p.R122W variant.

Patients with mutations in two genes leading to clinical manifestations of two or more Mendelian disorders are not uncommon. Recently, it was reported that these patients accounted for five percent of individuals with informative exome [17].

In conclusion, this study demonstrated the first patient with combined OI and CPHD. While OI was caused by the *de novo* heterozygous *COL1A2* mutation, CPHD was by the heterozygous *LHX4* mutation inherited from the healthy father. The incomplete penetrance and loss-of-function are the characteristics of p.R122W mutation in *LHX4*. This study expands the mutation spectra of *COL1A2* and *LHX4* and demonstrates the pathogenicity of the *LHX4* p.R122W mutation. We propose here that exome sequencing could be a promising tool to discover pathogenic variants for complex phenotypes leading to precise diagnosis of combined disorders.

Compliance with ethics requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimen-

tion (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

Author's contributions

Hemwong N, Shotelersuk V, Pornraveetus T contributed to study design, data analysis, and drafting the manuscript; Phokaew C, Srichomthong C, Tongkobpetch S, Suphapeetiporn K contributed to analysis and interpretation of data; Srilanchakon K, Supornsilchai V contributed to patient's examination and data analysis. All authors revised the manuscript critically, gave final approval, and agreed to be accountable for all aspects of the work.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2019.10.006>.

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