A novel variant of osteogenesis imperfecta type IV and low serum phosphorus level caused by a Val94Asp mutation in *COL1A1*

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Abstract. Osteogenesis imperfecta (OI) is a rare congenital disorder characterized by bone fragility and fractures, and associated with bone deformity, short stature, dentin, ligament and blue-gray eye sclera. OI is caused by a heterozygous mutation in collagen α-1(I) chain *(COL1A1)* or collagen α-2(I) chain *(COL1A2)* genes that encode α chains of type I collagen. Collagen α chain peptide contains an N-propeptide, which has a role in assembly and processing of collagen. Point mutations in the N-propeptide domain appear to trigger OI. In the present study, a novel heterozygous missense mutation, c.281T>A (p.Val94Asp), was identified in the von Willebrand C domain of N-terminal of type I collagen in an individual with type IV OI. The majority of N-terminal mutations are associated with OI/Ehlers-Danlos syndrome (EDS); however, in the present study, the affected individual did not suffer from EDS and the level of serum phosphorus of the patient was low (0.67 mmol/l). A number of clinical phenotypes were observed at the same variation site or in the same region on the polypeptide chain of *COL1A*, which suggests that additional genetic and environmental factors may influence the severity of OI. The present study may provide insight into the phenotype-genotype association in collagen-associated diseases and improve clinical diagnosis of OI.

Introduction

Osteogenesis imperfecta (OI) (types MIM 166200, 166210, 259420 and 166220), or brittle bone disease, is a rare connective tissue disorder characterized by increased bone fragility, low bone mass, short stature and other connective tissue manifestations. Extra-skeletal features manifest to a variable degree, including muscle weakness, brittle teeth, bluish-gray sclera and hearing defects. The traditional classification is based on clinical manifestations and radiographic criteria. The hereditary patterns of OI were classified into four primary types (I-IV) with either autosomal-dominant or autosomal-recessive patterns of inheritance, exhibiting variable levels of clinical severity (1). Their phenotypic presentation varies from mild to lethal and the prevalence of OI is ~1/15,000 globally and 0.04% in China (2,3), among which half of the affected individuals are OI type I or IV while type IV has a broad phenotypic range overlapping types I and III (4).

Several factors have been reported to have a role in the severity of OI, including the location of the mutation on the polypeptide chain and the particular amino acid substitution (5). Over 90% of patients with OI exhibit heterozygous mutations in collagen α-1(I) chain *(COL1A1)* or collagen α-2(I) chain *(COL1A2)*, genes encoding two type I pro-collagen α chains, pro-α1(I) and pro-α2(I). Translated collagen α chains include N- and C-terminals and a core triple helical domain of the Gly-X-Y triplet repeat unit. The C-terminus is involved in the reorganization and assembly of type I collagen. N-terminal cleavage results in the formation of the α-1 (I) chain which is terminated by a N-telopeptide (6,7). All N-termini adhere to the surface of the fibrils preventing further accretion and limiting fibril diameter (8). Previous studies demonstrated that environmental factors also contribute to the severity of OI. Maternal smoking during pregnancy is associated with childhood bone fractures in offspring and diet is associated with the severity of OI (9,10). *COL1A1* and *COL1A2* are ~18 kb and 38 kb long, respectively and are located in 17q21.3-17q22, 7q21.3-7q22.3, respectively, both with >50 exons. To date, >1,000 different *COL1A1/COL1A2* mutations have been identified in patients with OI (11). The present study reports a Chinese patient with OI type IV disease, who carried a novel missense mutation c.281T>A in the N-propeptide of collagen type I α chain in the *COL1A1* gene. The present study may improve the disease-causing mutation database of collagen genes to improve the diagnosis of OI type IV.

Materials and methods

Patients. A 6-year-old boy was admitted to the Guangxi Zhuang Autonomous Region Women and Children Care Hospital in

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August 2014 for genetic counseling of constitutional bone disease. According to the description of proband's mother, this patient previously suffered three fractures in each limb. Consequently, he was diagnosed with OI type IV following careful physical examination, capturing of radiologic images and genetic diagnosis. The patient's pedigree chart is presented in Fig. 1. From January 2015-December 2016, 300 normal Han individuals (1:1 sex ratio) aged 1-60 years were recruited. All participants agreed to carry out a genetic analysis and singed written informed consent for the study approved by the Genetic and Metabolic Central Laboratory of Guangxi Zhuang Autonomous Region Women and Children Care Hospital (Nanning, China).

Genetic analysis. A peripheral blood sample (5 ml) was obtained from the patient, patient's parents and brother, and 300 normal controls. Genomic DNA was extracted from peripheral blood using Lab-Aid DNA kit (Zeesan Biotech Co., Ltd., Xiamen, China). DNA concentration was determined using NanoDrop ND-2000 spectrophotometer and software (NanoDrop 2000; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Polymerase chain reaction (PCR) primers were designed using an online tool Primer version 3 (frodo.wi.mit.edu) to include all exons and exon-intron boundaries of *COL1A1* and *COL1A2* genes (Tables II and III). Polymerase chain reaction was conducted in a 50 μl reaction volume consisting of: 10X buffer, 1.5 mmol/l magnesium chloride, 200 μmol/l dNTPs, 400 nmol/l of each primer, 200 ng/μl DNA and 2 U Taq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China). Initial denaturation occurred for 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 60 sec at 72°C. Final extension occurred for 7 min at 72°C. PCR products were stained with ethidium bromide and visualized on a UV transilluminator following 1.5% agarose gel electrophoresis and were sequenced directly in an ABI 3130 genetic analyzer (Thermo Fisher Scientific, Inc.). When a potential novel mutation was considered following alignment of the patient's genome sequence against the ClinVar (www.ncbi.nlm.nih.gov), HGMD (www.hgmd.cf.ac.uk/ac/), HPSD (liweilab.genetics.ac.cn/HPSD/), the SNP (www.ncbi.nlm.nih.gov/SNP) databases and osteogenesis imperfecta and Ehlers-Danlos syndrome variant databases (11), direct sequencing of the amplified PCR products from the same region of the 300 unaffected patients was performed to verify the possibility of the difference being caused by a polymorphism. Sorting Intolerant From Intolerant (12), PolyPhen 2.0 (13) and Mutation Taster (14) tools were used to evaluate the pathology of the novel mutations. Three-dimensional structures of wild type (WT) and mutant *COL1A1* were predicted using I-TASSER (15) by importing WT and mutant *COL1A1* amino acid sequences. Subsequently, DNAMAN version 8.0 was used to perform multiple sequence alignment of novel mutations of OCA2 protein from different species.

Results

Patient description. The patient diagnosed with OI was a 6-year-old boy of Han ethnicity from the Guangxi province in China. The proband began walking at the age of 16 months and suffered three bone fractures. The first fracture occurred
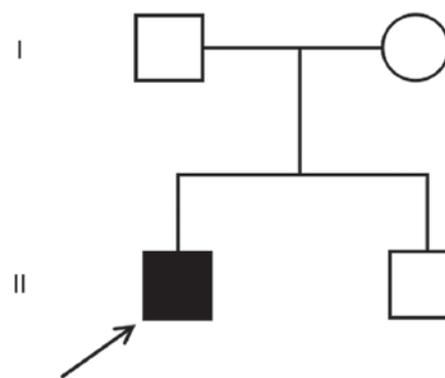

Figure 1. Pedigree chart of the family of the OI type IV patient. The black symbol indicates the patient with OI and white symbols represent unaffected individuals in the family. OI, osteogenesis imperfecta; L, left; R, right.

in the right tibia/fibula following a minor injury at 2 years of age. Subsequently, there have been two fractures, each caused by either a fall or collision. Physical examination revealed short stature. The boy was 98 cm tall and weighed 16 kg, with distinct signs of dentinogenesis imperfecta, genu varum, deformities of mild scoliokyphosis and joint hypermobility. X-ray analysis demonstrated hip joint femoral head fractures, osteoporosis and multiple fractures of four limbs (Fig. 2). Biochemical examination demonstrated that his calcium level (2.5 mmol/l) was within normal range (2.25-2.75 mmol/l), whereas the level of serum phosphorus (0.67 mmol/l) was lower than normal serum phosphorus range (1.29-1.94 mmol/l).

Mutation analysis. Sequence analysis revealed no functional mutations in *COL1A2* from the patient. However, a heterozygous mutation, c.281T>A (p.Val94Asp), was identified during sequence analysis of the *COL1A1* gene. This mutation was not identified in any other family members (brother, father and mother of the proband) or ethnically matched controls (Fig. 3). Notably, comparison with the osteogenesis imperfecta and Ehlers-Danlos syndrome variant databases, the Human Gene Mutation Database, HPSD and the SNP databases revealed that the mutation had not been previously identified. Functional predictions revealed that the novel missense variant may demonstrate deleterious effects by markedly altering the structure of the *COL1A1* protein. Multiple sequence alignments revealed that sequence conservation at residue 94 of pro-α1(I) was poor in different organisms (Fig. 4).

Further protein structure prediction and analysis using I-TASSER revealed that the proportion of regions folding into an α-helix and a β-sheet would increase and the proportion of random coils would decrease in mutant protein compared with the wild-type protein. Domains 1227-1230, 1266-1271, 1277-1279, 1390-1392, 1395-1398 and 1389-1400 were predicted to be altered from random coils into β-sheets, domains 3-5, 9 and 20-22 were predicted to be altered from random coils into α-helices, domains 97-98 would form random coils instead of β-sheets, and domains 67-68 and 1259-1263 would fold into random coils instead of α-helices. The overall fibril shape was predicted to be altered (Fig. 5).

Table I. Comparison of OI types with *COL1A1* mutations.

Location	Mutation	Location	Outcome	OI type	(Refs.)
2	c.111_117del	Deletion	Frameshift	OI IA	(28)
2	c.141C>A	Substitution	Nonsense	OI IA	(29)
2	c.174C>A	Substitution	Nonsense	unknown	(11)
2	c.182G>T	Substitution	Missense	OI I, OI III	(29,30)
2	c.189C>A	Substitution	Nonsense	unknown	(11)
2	c.198_204dup	Duplication	Frameshift	OI I	(31)
2	c.199A>T	Substitution	Nonsense	OI I	(11)
2	c.246delC	Deletion	Frameshift	OI I	(32)
2	c.266G>T	Substitution	Missense	OI III, OI IV	(11)
2	c.266_266delGinsCGCCGTCCCGGGGA	Insertion/Deletion	Frameshift	OI I	(11)
2	c.268_299-60del	Deletion	Other Site	OI I	(30)
2	c.285C>A	Substitution	Nonsense	OI I	(11)
02i	c.299-5_313del	Deletion	Splice site	OI I	(11)
02i	c.299-1G>C	Substitution	Splice site	OI I	(11)

Adapted from the osteogenesis imperfecta and Ehlers-Danlos syndrome variant databases (11). OI, osteogenesis imperfecta; *COL1A1*, collagen α-1(I) chain. 2, 2nd exon of *COL1A1*; 02i, 2nd intron of *COL1A1*.

Table II. PCR primers and conditions used for mutation analysis of the *COL1A1* gene.

Exon	Forward (5'-3')	Reverse (5'-3')	Product size (bp)	Annealing temp (°C)
1	GCTCTGATTGGCTGGGGCAC	CCCTTCCATTCCCGAGTCTCC	445	60
2	CTGAGGTTGGAGTTGGAAGCC	GTTTCTTGGTCGGTGGGTG	647	60
3-5	GGGAGCAGCATTAGCAAACCT	GCAAAAGAGCCTGATGTTAGCA	656	58
6-7	GTCAGTTTCTTCCATCCTCAGT	AGTCCCTGTCAACCTTCTCC	547	62
8-9	TGGTAAGATTGGAGAAGGTTG	TTCCTCTGAGTATCGTTCCC	437	58
10-12	TAGGCGGTGGTGGGGAGGCA	TGGTGGGACTCTGGGGATGTG	719	63
13-16	CCACATCCCCAGAGTCCCAC	ATCAGAGACGGAGAACCCAGG	808	64
17-20	GAGAGGCAAGGTTGGGTTTC	GTTCCTGGGGGTGTGGCA	802	60
21-23	TGCCACACCCCAGGAAC	AAGAGGAAGAAGATGCCCAGG	428	60
24-25	GGCATCTTCTTCCTCTTTTGGC	AAGTCTCAGGTGTGTTTGTCCC	640	60
26-29	TCTCACTTCAGCCCCCTCAAC	GCGTCTAACCTCAATCCCTCT	727	60
30-32	CAGACCCCAGGAGGAAGGACC	GAGATTCAAAGCAGGCAGAGATG	796	60
33-35	GGAAACCCAGACACAAGCAGAAC	CCAGTCGGTGATGAAAAATGATG	718	60
36-38	CTGCCTCCATTACTGCTCCTCC	ACCTTTGCCGCCTTCTTTGCC	709	62
39-40	TGACAGCCCCTCCTATCCTC	CAAGTCCTGTGATGGTTTTTCTC	450	60
41-43	GGAGCCAAGGAGAACAGATTT	TCCGACACCCATCCCCAG	661	60
44-45	CTGGGAGTTGGGAGAGATGG	GAGGGGGAAACTGAGGCGAAG	487	60
46-47	CAGAGAGGGATTATGGGAGAGG	GAGGGAAGAGAGTGGGGATTAC	774	60
48-49	CCCCTCATCCCCTCTGCTCAT	CCAGCTCTGTCCATCACCCTT	800	60
50-51	TAGCGGCTCACTCTTCCCTC	GTTTGGGTTGCTTGTCTGTTTC	634	60

PCR, polymerase chain reaction; bp, base pairs.

Discussion

Severity of OI mutation is determined by a number of genetic factors, including location, mutation type and mutated residues. Over 1,000 different *COL1A1/COL1A2* mutations have been identified in patients with OI (11), accounting for ~90% of OI cases in patients with OI type I-IV. Additionally, there are a number of mutations identified in genes encoding proteins that interact with type I collagen, affecting the development of bones and leading to different symptoms of OI. These

Figure 2. X-ray images from the patient with osteogenesis imperfecta type IV. X-ray images demonstrate the multiple fractures present in the (A) left upper limb, (B) right upper limb and (C) lower limbs. (D) Hip joint femoral head fractures and osteoporosis.
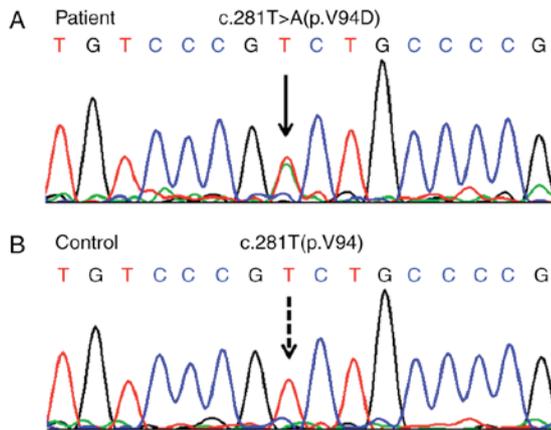

Figure 3. Sequence analysis of *COL1A1* exon 2. The nucleotide sequence of (A) the patient and (B) a representative normal subject. The patient exhibits a novel heterozygous T>A mutation at nucleotide 281 in exon 2 (c.281T>A), resulting in the substitution of valine with aspartic acid at codon 94 (p.V94D).
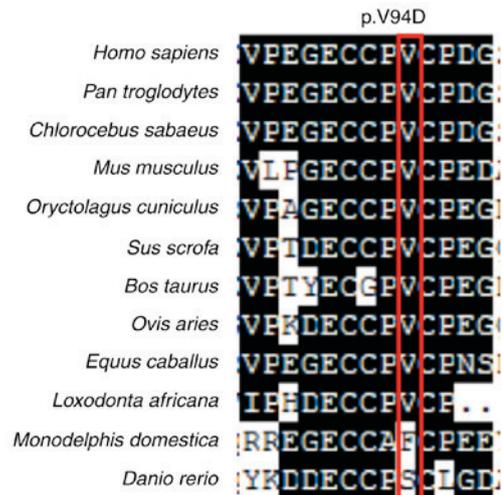

Figure 4. Multispecies alignment of *COL1A1* sequence. The novel mutation site (p.V94D) is indicated with a red box. The black background indicates complete sequence conservation; the white background indicates sequence variation.

genes include, cartilage-associated protein (MIM no. 605497), P3H1 (MIM no. 610339), serpin peptidase inhibitor clade F member 1 (MIM no. 613329), peptidyl-prolyl cis-trans isomerase B (MIM no. 123841); and the non-collagen associated genes, peptidyl-prolyl cis-trans isomerase FKBP10 (MIM no. 607063), pigment epithelium-derived factor (MIM 172860) and proto-oncogene Wnt-1 (MIM no. 615220) (3,17-21).

Type I collagen, encoded by *COL1A1* and *COL1A2*, consists of two pro-α1 chains and one pro-α2 chain, which interweave and form a dense structure. Each α chain contains three domains, one collagen triple helix repeat domain, one fibrillar collagen C-terminal propeptide and N-terminal propeptide. Collagens are generally extracellular structural proteins involved in formation of connective tissue structure. The sequence predominantly consists of repeats of the G-X-Y and polypeptide chains from triple helix motifs. The fibrillar C-terminal of collagen propeptide is the most conserved part of fibrillar collagens from invertebrates to vertebrates (22). The C-terminal propeptide, also termed the COLFI domain, has a role in tissue growth and repair by controlling intracellular assembly of procollagen molecules and extracellular assembly of collagen fibrils (23). The N-terminal propeptide is encoded by the first ~200 amino acids of collagen protein sequence and contains the von Willebrand C domain (vWFC) (24). The N-terminal amino acids of the α1(I) chain

has a role of a stabilizing anchor for the amino end of a type I collagen triple helix (25). Furthermore, the von Willebrand C (vWFC) domain has a role in binding to and regulating other components, including bone morphogenetic proteins of the extracellular matrix during development (26).

In the present study, the patient with OI type IV exhibited a thymine to adenine substitution mutation at nucleotide c.281 in the *COL1A1* gene, resulting in p.Val94Asp missense substitution. This variant was not identified in patient's parents, his brother, or 300 controls. The identified mutation was located in the second exon of the *COL1A1* gene which is a part of the N-terminal propeptide domain of the pro-α-1(I) collagen chain (16). A previous study revealed that most N-terminal mutations are associated with OI/EDS (27) and appear to alter the structure of N-anchor and conformation of the N-propeptide cleavage site, resulting in the incorporation of partially processed procollagen with uncleaved N-propeptides into fibrils (25). During proteosynthesis, the N-terminal propeptide is removed from the pro-α-1(I) chain by a specific proteinase to form the α-1(I) chain terminated by N-telopeptide. In the present study, the patient did not have EDS, as the substitution identified in his genome was not localized in close proximity to the N-proteinase cleavage site; thus, its occurrence was not

Table III. PCR primers and conditions used for mutation analysis of the COL1A2 gene.

| Exon	Forward (5'-3')	Reverse (5'-3')	Product size (bp)	Annealing temp
1	GGTTTCGGCTAAGTTGGAGG	GGTGCCCTCCCATCTAACC	248	60
2	TCCCTGCCATACTTTTGACCT	ATGTAACTTCTTCCCTTCCAAGA	282	60
3	TTCCAAAATAGGCGGGGCTA	TGCCTTCCATCTCCAGAATAAA	738	62
4	GCTTCCAATCCTCCAGCTGA	ATTCGCTTCTTCTGCAGTGC	399	60
5	TCTTAGGTTTCTACAGGGCCT	GCACACAAAGACCAGTCCTG	356	58
6	AGTTAAGGCAGAGGAAGGGC	TGGCGTGGTAAAATGTGACA	399	58
7-10	CACAACAATGGCACTGCTAAG	GTTCTGTCAGGCATATTCAGCT	548	60
11-12	ACTTTGGAGGGAAGAAGTCAC	TGGAGGTCATGGGGAATTTCA	768	60
13-16	TCTGTGTGTCTGGCATAATTGA	ACCTTCCCTCTCCTTCTGTC	1,436	62
17-19	CCAAGATGGCAGAATCAAGCA	AGGAAGGGCATGTCTGTGTG	872	60
20-23	AGCTTCTCTTTACCTTGACCCA	ACCTCATAGCCATTGTATCAAGT	1,236	60
24	ACTCTTTTCACACTTCCCAGC	TCTCTTGCATCCCCTTGTCA	462	62
25-26	TCATCCGTGGCAGCATCATA	CCTGGGGATGCCATCTTGAA	749	62
27-29	ATTTGGGCTTTCGTGGGAAC	GGCTCATTCTCTCCATCAGC	895	60
30	TGCACTCATGTAGATACTGCCA	AGAGACTTGTTGCAGGGTCA	267	60
31	TAAATGCAAACCAGGGCTCG	TCCACTGGAATCGGATTGCT	364	60
32	TAGCCCAGCCTTCTTTGTGT	GCATGGTTGACAGCTGTTCA	561	60
33	CTGCAGCCCATTGTGATGTT	TAGAGGTCCCGGCTTCCTAA	742	60
34	ATCCAACCAGAGTGCAGTGA	GCGCAACATGGAGAGACTTT	429	60
35-38	AGCCCCGTGTCCATCTAAAA	CAGGCAGAAGAAGGGGCATA	1,404	63
39	GCCTACCTCCTACTCCTTGG	GGGGACTGGTGTTCACATGA	403	60
40	ACCAGTGGCATGACATTGTT	TACTTAAACTGGGGAGGCGG	1,010	60
41	TGCCAAGATGTAAACTCACCG	TGCAGAGGAGTAAACGCAAT	565	60
42	TGAATGACACGAGGCTCACT	ATACTTGGGCCCAGTTTTGC	562	60
43-46	AGCTACAACATAGGGGCTGG	CAAGAGTGAGATGGAGTTAGCC	1,136	60
47-49	TGCACTGCTGAAATAGGTTGT	GAAATGAGGTTGGGTGCTGG	1,247	60
50	TTTTCATGGAGGAGGGGAGG	CCCAGGAAAGGAACAGGTCT	497	60
51	AGATGACCTTGCCTCAGTCT	AGGCCACTGAAGTCTCAAGA	812	60
52	CCCTCCCACTAAAGACACCC	TGTGCAGAAGAAATGGAAGGA	434	60

PCR, polymerase chain reaction; bp, base pairs.
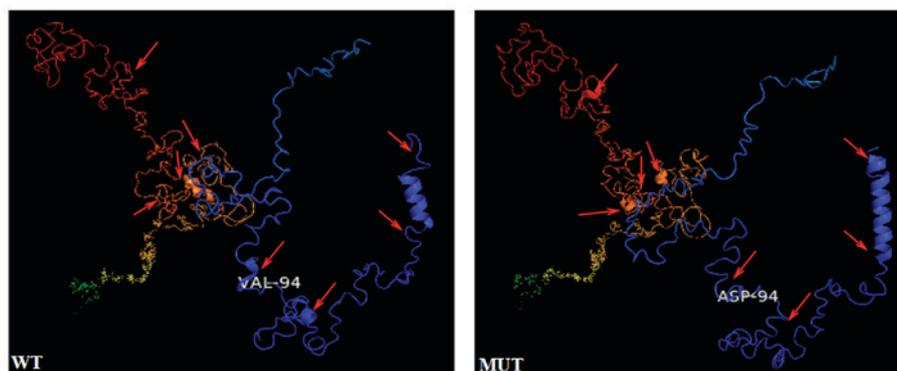
Figure 5. Three-dimensional structure estimation, indicating that in the variant protein there would be an increase in the proportion of α-helix and β-sheet regions, and that the random coil regions would be decreased in the mutant protein compared to the wild-type protein. The dimer alterations are indicated by an arrow. WT, wild-type; MUT, mutant.

likely to prevent N-propeptide removal. The mutation identified in the present study was predicted to lead to an increased number of α-helices and β-sheets, disruption of integrity of the N-terminal and delayed N-propeptide removal, decreased fibril

size and the phenotypic characteristics of OI. The substitution identified in the present study was also located in the vWF-c binding region (amino acid residues 38-96). The mutation was predicted to lead to delayed vWF-c binding and prevent normal regulation of components of the extracellular matrix during development. In addition to the symptoms of OI type IV, the patient also had low serum phosphorus levels (0.67 mmol/l), which may suggest an association between the vWF-c binding complex and regulation of serum phosphorus level. Further studies would be necessary to verify this hypothesis.

Similar mutations have been observed in the same region of the VWFC domain of N-terminal in the collagen type I α chain (Table I) in the majority of OI patients with mild symptoms. There are two splicing mutations, 5 non-sense mutations, 4 frameshift mutations, 1 site variation (c.268_299-60del) and 2 missense mutations. The majority of patients with these mutations presented with the same mild symptoms of OI type I (28-32). The phenotypes resulting from null variants are caused by haploinsufficiency (33). The other mutations causing OI changed the distinct activities of the N-propeptide of collagen I and its regulation of collagen fiber assembly and fibroblast function (34). Similar symptoms to those exhibited in the patient of the present study are also caused by the p.Val94Asp substitution, but to a milder degree. Patient with OI with missense mutations c.182G>T and c.266G>T, were diagnosed with OI type I or OI type III and OI type III or OI type IV, respectively (26,28). Smoking during pregnancy may affect the growth of fetus and lead to an increased risk of serious fractures in childhood (9). Furthermore, a study by Mertz *et al* (10) demonstrated that in heterozygous Col1a2$^{tm1Mcbr/J}$ (G610C) mice, a low protein diet had beneficial effects on osteoblast differentiation and bone matrix mineralization and it also affected bone modeling and suppressed overall animal growth. To a certain extent, the severity of OI is affected by environmental factors (10). Additionally, if the mutation occurs in a poorly conserved site, then the phenotype impact is relatively mild. The aforementioned findings of previous studies, combined with the description of the novel mutation c.281T>A described in the present study, suggest that the splicing, frameshift, nonsense and missense mutation pattern, the affected *COL1A1* region, additional genes and possibly other environmental factors including smoking and diet may influence the severity of OI.

In conclusion, a novel missense mutation c.281T>A, p.Val94Asp in exon 2 of *COL1A1* gene resulting in OI type IV and low serum phosphorus level was identified in a patient with sporadic OI. The novel missense mutation disrupted vWF-c binding and the integrity of the N-terminal of the collagen type I α chain. It remains to be elucidated how this substitution leads to the abnormal function of collagen I and low serum phosphorus levels. Detailed clinical features and molecular diagnostics have a role in expanding evidence of genetic and phenotypic heterogeneity and determining the phenotype-genotype associations in OI. The present study contributed to the understanding of *COL1A1* mutations in the Han Chinese population with OI type IV.

Acknowledgements

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