



Whole exome sequencing implicates *PTCH1* and *COL17A1* genes in ossification of the posterior longitudinal ligament of the cervical spine in Chinese patients

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ABSTRACT. Ossification of the posterior longitudinal ligament (OPLL) of the cervical spine is a complex multifactorial disease. Patients with OPLL commonly present with symptoms in their 40s or 50s. The genetic basis of OPLL remains poorly understood. Exome capture combined with massively parallel DNA sequencing has been proposed as an efficient strategy to search for disease-causing genes of both monogenic and multigenic disorders. To identify candidate pathogenic genes associated with OPLL, we performed whole exome sequencing (WES) on two unrelated southern Chinese OPLL patients.

The entire DNA coding region of the candidate genes was amplified by PCR and Sanger sequenced. The common single nucleotide polymorphisms were analyzed by association studies. WES revealed p.T265S/*PTCHI*, p.P1232L/*PTCHI*, and p.T902S/*COL17A1* mutants in the two female cases with mixed OPLL. These were confirmed by Sanger sequencing. p.P1232L/*PTCHI*, p.N1374D/*COL17A1* and p.T902S/*COL17A1* were subsequently identified in three males with continuous OPLL and one female with mixed OPLL. The association studies indicated that the SNPs rs805698 and rs4918079 in *COL17A1* were significantly associated with OPLL. This study suggests that WES may be a practical approach to revealing significant genetic involvement in OPLL. Variants of the *PTCHI* and *COL17A1* genes may contribute to the development of OPLL.

Key words: Ossification of the posterior longitudinal ligament; Whole exome sequencing; Susceptible gene; *PTCHI*; *COL17A1*

INTRODUCTION

Ossification of the posterior longitudinal ligament (OPLL; OMIM #602475) is characterized by pathological ectopic ossification of the cervical and thoracic spine ligament, leading to myeloradiculopathy as a result of chronic pressure on the spinal cord and nerve roots (Schmidt et al., 2002). OPLL was first described in detail by Tsukimoto and colleagues in 1960, and has a reported prevalence of 1.9-4.3% in the Japanese population (Firooznia et al., 1982; Stapleton et al., 2011). OPLL in Taiwan Chinese has been reported to occur at 2.8%, based on a review of hospital cervical spine radiographs (Tsai et al., 1978). Despite a long-standing predominance in Japan, this disease has also been recognized in other geographic regions and ethnicities, although its prevalence in the US and Europe is only 0.01-1.7% (Stapleton et al., 2011). The incidence increases with age, with most cases occurring after age 40. In both males and females, the average age at onset is approximately 50 years, and the reported male:female ratio is roughly 2:1 (Stapleton et al., 2011). OPLL of the cervical spine can be subdivided radiographically into 4 types, based on the location of the spinal lesion in lateral plane radiographs: continuous, mixed, segmental, and unclassified or localized (Mizuno and Nakagawa, 2006). A diagnosis noting calcification of the posterior longitudinal ligament is typically made in the appropriate clinical setting using either planar radiographs or computed tomography (CT). Patients with serious symptoms require surgical treatment. However, the prognosis is often unsatisfactory (Horikoshi et al., 2006).

Early clinical and epidemiological studies conducted in Japan suggested that the underlying cause of OPLL is multifactorial in nature, reflecting the interplay of numerous genetic and environmental factors (Terayama, 1989; Sakou et al., 1991). Several non-genetic factors, including mechanical stress, nutrition, glucose intolerance, and high body mass index probably contribute to the etiology of OPLL (Iwasaki et al., 2004; Kobashi et al., 2004; Okamoto et al., 2004). Genetic studies of OPLL have revealed several gene loci that may be involved in the pathogenesis of this disease (Yan et al., 2010; Karasugi et al., 2013). In 2006, a large-scale genetic association study of OPLL in the Japanese population indicated that three SNPs in the

TGF- β 3, *AHSG*, and *ESR1* genes showed significant association with OPLL (Horikoshi et al., 2006). Although a host of gene products has been implicated as the root cause, several genes and proteins have emerged over the years as promising targets for future investigation and intervention, including *NPPS*, *COL11A2*, *COL6A1*, *BMP2*, *BMP4*, *TGF- β 1*, *TGF- β 3*, and so on (Wang et al., 2008; Stapleton et al., 2011; Karasugi et al., 2013). Recently, novel mutations in the *BMP4* gene and a specific haplotype, TGGGCTT, were demonstrated to contribute to the risk of developing OPLL in the Chinese population (Meng et al., 2010; Ren et al., 2012). OPLL is believed to arise because of endochondral bone formation, and each of the aforementioned genetic targets has been shown to critically regulate a crucial step in chondrogenesis, osteogenesis, or bone mineralization (Koga et al., 1998; Inamasu et al., 2006). Identification of pathogenic genes for OPLL will allow new approaches to the management of OPLL.

Recent advances in genetic laboratory technology as well as the sequencing of the human genome, have enabled more specific studies to determine gene mutations predisposing to or causing OPLL (Karasugi et al., 2013). Whole exome capture and next-generation sequencing (WES) represent a cost-effective, reproducible, and robust strategy for the sensitive and specific identification of variants causing protein-coding changes in individual human genomes (Ng et al., 2009). Exome sequencing of a small number of unrelated affected individuals is a powerful, efficient strategy for identifying the genes underlying rare Mendelian disorders, and will likely transform the genetic analysis of monogenic traits (Ng et al., 2010). Here, we performed WES on two unrelated OPLL patients, coupling the Agilent whole-exome capture system to the Illumina HiSeq 2000 DNA sequencing platform, to attempt the identification of pathogenic genes for OPLL. We used Sanger DNA sequencing to validate the variants disclosed by WES. Common single nucleotide polymorphisms were analyzed by association study. Our data indicate that WES has potential as a practical method for the identification of genes determining susceptibility to OPLL.

MATERIAL AND METHODS

Subjects

This study involved 28 unrelated Han Chinese patients and 100 unrelated healthy controls from Zhejiang Province, southeast China. Among the 28 cases (18 male, 10 female; mean age, 57 years; range, 40-75), 10 were affected with continuous OPLL, 9 were diagnosed with mixed OPLL, 5 presented segmental OPLL, and 4 fell into the unclassified category. All affected individuals were inpatients at the Department of Orthopedics, Hangzhou Red Cross Hospital, Hangzhou, and the Department of Orthopedic Surgery, Changzheng Hospital, Second Military Medical University of PLA, Shanghai, China. OPLL diagnosis was based on radiological findings, including radiographs, CT, and magnetic resonance imaging (MRI) of the cervical spine, using the criteria stipulated by Tsuyama (Tsuyama, 1984). One hundred biologically unrelated healthy individuals, 66 male and 34 female, aged 28-85 years (average age: 64 years), with a similar ethnic background, were enrolled as the control group. All the control subjects over 60 years of age had no signs of spinal ossification when examined by standard radiograph, thereby excluding most unmanifested diseases.

This study was conducted with the approval of the Medical Ethics Committee of Hangzhou Red Cross Hospital and Zhejiang University, in accordance with the tenets of the Declaration of Helsinki. Informed consent was given by all participating individuals.

Exome capture and massively parallel DNA sequencing

The peripheral blood genomic DNA samples from each of 2 unrelated female cases with mixed OPLL (O1 and O2) were randomly fragmented with a Covaris ultrasonicator (Covaris, Inc. Woburn, MA, USA) to provide DNA fragments with a size distribution peak at 150 to 200 bp. Adapters were ligated to both ends of the resulting fragments. The adapter-ligated templates were purified by Agencourt AMPure SPRI beads (Agencourt, Inc. Beverly, MA, USA), and fragments ~200 bp were excised. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the SureSelect Biotinylated RNA Library (Agilent Technologies, Inc. Santa Clara, CA, USA) for enrichment. Hybridized fragments were bound to streptavidin beads and non-hybridized fragments were washed out. Captured LM-PCR products were screened on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA) to estimate the magnitude of enrichment. Each captured library was then loaded onto the HiSeq 2000 platform (Illumina, Inc. San Diego, CA, USA). We performed GA sequencing for each captured library independently to ensure each sample had at least 50-fold coverage. Raw image files were processed by Illumina Pipeline v1.6 (Illumina, Inc. San Diego, CA, USA) for base-calling using the default parameters. The sequences of each individual were generated as 90 bp paired-end reads.

SOAPaligner (soap2.21) (BGI, Shenzhen, Guangdong, China) was used to align the clean reads to the human reference genome. Based on the SOAP alignment results, the SOAP-snp software was used to assemble the consensus sequence and call genotypes in target regions (Li et al., 2008). We filtered the SOAPsnp results as follows: base quality >20, depth 4-200, estimated copy number ≤2, and the distance between two SNPs ≥ 5. Detailed information on each parameter for the two algorithms is available at <http://soap.genomics.org.cn/>.

Data from the dbSNP129 and 1000 Genome Project (20100208 release) were downloaded from the NCBI FTP site (<http://www.ncbi.nlm.nih.gov/Ftp/>). Target regions were from the captured regions of the Agilent Exome Array (http://www.nimblegen.com/downloads/annotation/seqcap_exome/index.html). The exome variation set of eight HapMap samples was obtained from “Supplementary data 2” for 12 exomes in Ng et al., 2009.

Confirmation of variants

Sanger DNA sequencing was used to confirm the accuracy of variants identified by WES.

Association study

Analysis of sequence variation in the coding regions of the *PTCH1* and *COL17A1* genes in the 28 Chinese patients with OPLL revealed several common SNPs. Genotypic frequencies of each SNP were compared between the case patients and control subjects. Data was analyzed with the SPSS 13 software (SPSS, Chicago, IL, USA). Unless indicated otherwise, a P value < 0.05 was considered to be statistically significant.

Pathogenic mutation prediction

In an effort to assess the functional significance of the gene variations identified in this

study, we used public databases as well as web-based software. The pathogenic potential was evaluated computationally by examining interspecies sequence variations, using PolyPhen (<http://genetics.bwh.harvard.edu/pph>) and Genomic Evolutionary Rate Profiling (GERP) to evaluate the evolutionary conservation of the resulting amino-acid substitutions (Ramensky et al., 2002; Adzhubei et al., 2010).

RESULTS

Variant confirmation

We generated an average of approximately 3.8 billion bases of sequence per individual affected as paired-end, 90-bp reads, and the capture specificity was about 68.41%, with a 72-fold average sequencing depth on target. At this depth of coverage, more than 99% of the targeted bases were sufficiently covered to pass our thresholds for variant calling (Table 1). We focused on nonsynonymous (NS) variants, splice acceptor, and donor site mutations (SS), anticipating that synonymous variants would be far less likely to be pathogenic. Filtering against public SNP databases, the 1000 Genome Project, eight HapMap exomes, and the in-house exome database Beijing Genomics Institute (BGI) provided, we identified 13 genes harboring 13 different NS/SS variants that were shared by the 2 patients (O1 and O2). The 13 variants included *PALMD*, *GON4L*, *KIAA1462*, *COL17A1*, *CCDC38*, *HERC2*, *HELZ*, *MIB1*, *DSCI*, *PNMAL1*, *C20orf152*, *MAGEF1*, and *PTCHI*.

Table 1. Overview of whole exome sequencing data production.

Exome capture statistics	O1	O2
Target region (bp)	37,640,396	37,640,396
Raw reads	60,854,576	62,823,314
Raw data yield (Mb)	5,426.00	5,637.00
Reads mapped to genome	52,350,649	55,244,951
Reads mapped to target region	36,325,989	36,435,219
Data mapped to target region (Mb)	2,710.54	2,705.91
Mean depth of target region	70.53	71.89
Coverage of target region (%)	97.97%	99.36%
Average read length (bp)	89.72	89.62
Rate of nucleotide mismatch (%)	0.28	0.30
Fraction of target covered $\geq 10\times$	92.82	94.34
Fraction of target covered $\geq 50\times$	56.65	56.72
Capture specificity (%)	71.36	68.41
GC rate	46.23	48.01

After functional significance analysis of these 13 genes, we focused on the *PTCHI* and *COL17A1* genes. WES disclosed c.C794G (p.T265S)/*PTCHI* and c.A2704T (p.T902S)/*COL17A1* variants in O1 and c.C3695T (p.P1232L)/*PTCHI* and c.A2704T (p.T902S)/*COL17A1* variants in O2 (Table 2). c.C3695T (p.P1232L)/*PTCHI*, c.A4120G (p.N1374D)/*COL17A1* and c.A2704T (p.T902S)/*COL17A1* were separately identified in three males with continuous OPLL, and in one female with mixed OPLL (Figure 1, Table 2). Examination of 100 unrelated control individuals failed to detect the mutated allele. Several SNPs of *PTCHI* and *COL17A1* were also found in our patients (Table 3). No genetic information or SNPs were noted in other OPLL susceptibility genes, in contrast to a previous report (Stetler et al., 2011).

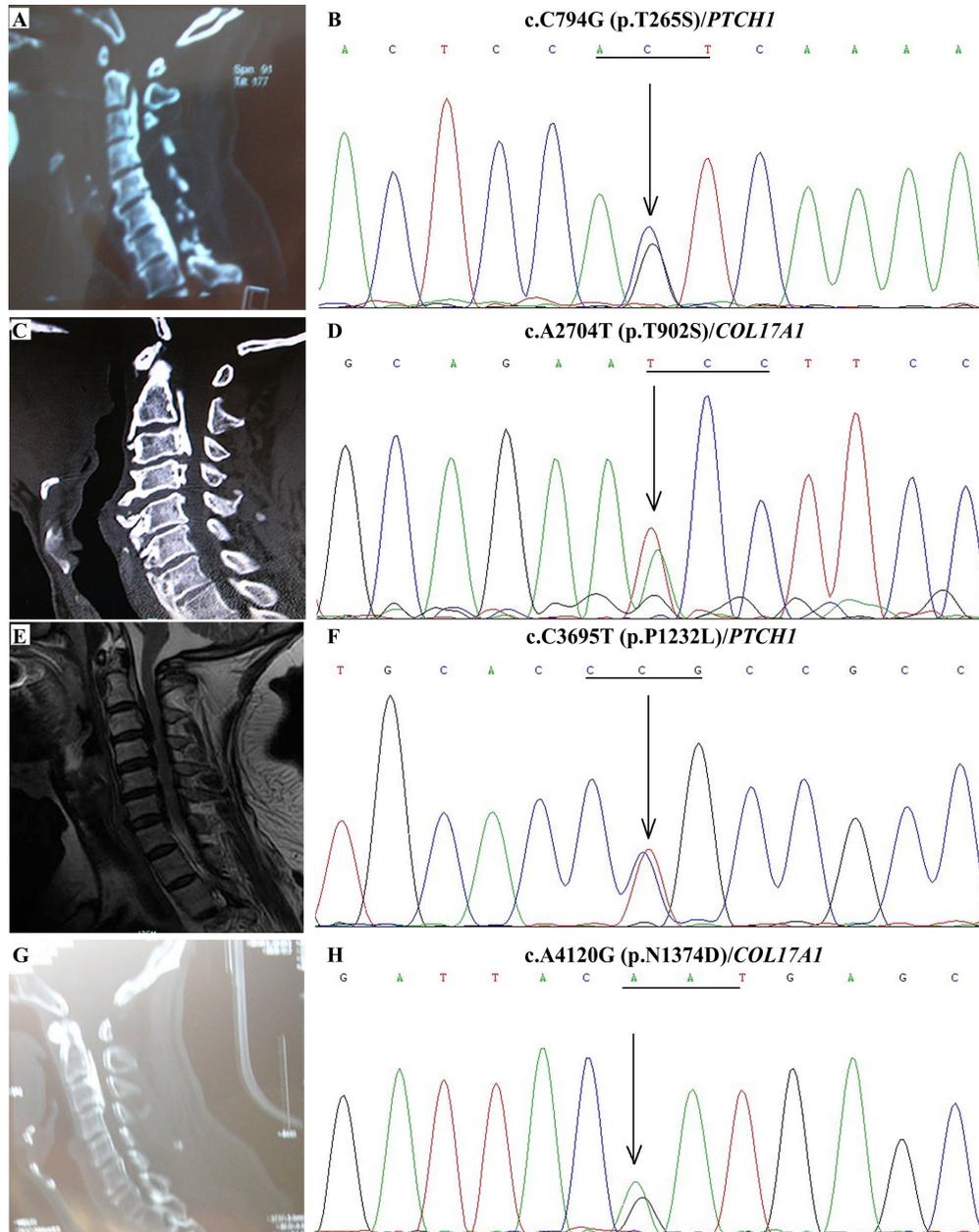


Figure 1. Two dimensional computerized tomography (2D-CT) and the *PTCH1* and *COL17A1* gene sequencing results of OPLL patients. **A.** Patient 01 presented mixed OPLL at C2-C4, C5, C6-C7. **B.** Patient 01 exhibits c.C794G (p.T265S)/*PTCH1* variant. **C.** Patient 02 presented mixed OPLL at C2-C3, C4. **D.** Patient 02 exhibits c.A2704T (p.T902S)/*COL17A1* variant. **E.** Patient 03 presented continuous OPLL at C2-C5. **F.** Patient 03 exhibits c.C3695T (p.P1232L)/*PTCH1* variant. **G.** Patient 04 presented continuous OPLL at C2-C4. **H.** Patient 04 exhibits c.A4120G (p.N1374D)/*COL17A1* variant.

Table 2. Clinical symptoms and confirmed variants.

Patients	Age/Gender	OPLL subtype	Ossified vertebrae	Variants
O1	58/F	Mixed	C2-C4, C5, C6-C7	p.T265S/ <i>PTCHI</i> + p.T902S/ <i>COL17A1</i>
O2	62/F	Mixed	C2-C3, C4	p.P1232L/ <i>PTCHI</i> + p.T902S/ <i>COL17A1</i>
O3	60/M	Continuous	C2-C5	p.P1232L/ <i>PTCHI</i>
O4	63/M	Continuous	C2-C4	p.N1374D/ <i>COL17A1</i>
O5	65/M	Continuous	C2-C4	p.T265S/ <i>PTCHI</i>
O6	64/F	Mixed	C2-C3, C4	p.N1374D/ <i>COL17A1</i>

Association between SNPs and OPLL

Two of the 11 common SNPs in *COL17A1*, rs805698 and rs4918079, demonstrated significant associations with OPLL (Table 3). Minor allele carriers (heterozygous and homozygous) of rs805698 and rs4918079 had increased risks for OPLL, with odds ratios (ORs) of 7.722 (95% confidence interval [CI] = 3.004-19.848; P = 0.00023) and 4.000 (95%CI = 1.646-9.723; P = 0.003), respectively.

Table 3. SNPs of *PTCHI* and *COL17A1* in Chinese patients with OPLL.*

Gene	SNP	Amino acid variation	Genotype	Case (N = 28)	Control (N = 100)	OR (95%CI)	P value
<i>PTCHI</i>	rs1805155	c.T1212C (p.N404N)	TT	23	83	1.061 (0.354-3.185)	1.000
			TC+CC	5	17		
	rs2066836	c.C1233T (p.A411A)	CC	23	85	1.232 (0.405-3.746)	0.770
			CT	5	15		
	rs2229062	c.T2460C (p.Y820Y)	TT	27	98	1.815 (0.159-20.779)	0.526
			TC	1	2		
	rs76234253	c.T2688G (p.L896L)	TT	24	88	1.222 (0.361-4.133)	0.750
			TG	4	12		
	rs357564	c.C3491T (p.P1164L)	CC	5	30	1.971 (0.685-5.676)	0.238
			CT+TT	23	70		
<i>COL17A1</i>	rs805708	c.C628T (p.T210M)	CC	15	51	0.902 (0.389-2.089)	0.834
			CT+TT	13	49		
	rs805701	c.C1062T (p.A354A)	CC	16	55	0.917 (0.393-2.136)	1.000
			CT+TT	12	45		
	rs805698	c.G1282A (p.G428S)	GG	13	87	7.722 (3.004-19.848)	0.000
			GA+AA	15	13		
	rs805722	c.A2107G (p.M703V)	AA	15	66	1.682 (0.719-3.937)	0.270
			AG+GG	13	34		
	rs4918079	c.C2595T (p.R865R)	CC	14	80	4.000 (1.646-9.723)	0.003
			CT+TT	14	20		
	rs2274100	c.A2902G (p.P968P)	AA	16	65	1.393 (0.593-3.271)	0.508
			AG+GG	12	35		

*Genotypic frequencies were compared between the case patients and control subjects by use of a χ^2 test for 2-by-2 contingency tables with the SPSS software (version 13). 95%CI = 95% confidence interval; OR = odds ratio.

Functional significance of the gene variations

The SNP c.C3491T (p.P1164L) (rs357564) and the variant c.C3695T (p.P1232L) of *PTCHI* can be considered as possibly pathogenic mutations based on the PolyPhen and GERP predictions. GERP indicated c.C794G (p.T265S)/*PTCHI* is located in the highly conserved region of the PTCH molecule (Table 4).

Table 4. Functional significance of the missense variants.

Genes	SNP	Variants	PolyPhen	GERP*
<i>PTCHI</i>	Novel	c.C794G (p.T265S)	Benign	5.280
<i>PTCHI</i>	rs357564	c.C3491T (p.P1164L)	Possibly damaging	4.370
<i>PTCHI</i>	Novel	c.C3695T (p.P1232L)	Possibly damaging	4.320
<i>COL17A1</i>	rs805708	c.C629T (p.T210M)	NA	0.327
<i>COL17A1</i>	rs805698	c.G1282A (p.G428S)	Benign	1.140
<i>COL17A1</i>	rs805722	c.A2107G (p.M703V)	Benign	-1.550
<i>COL17A1</i>	rs117837187	c.A2704T (p.T902S)	Benign	2.940
<i>COL17A1</i>	rs151151613	c.A4120G (p.N1374D)	NA	4.520

*Number between -11.6 and 5.82 that describes the degree of sequence conservation among 34 mammalian species.

DISCUSSION

Many common human diseases have a strong heritable component. The accumulation of mildly deleterious missense mutations in individual human genomes has been proposed to be a genetic basis for complex diseases. The plausibility of this hypothesis depends on quantitative estimates of the prevalence of mildly deleterious *de novo* mutations and polymorphic variants in humans, and on the intensity of selective pressure against them (Kryukov et al., 2007). Several recent studies have reported a significant excess of rare missense variants in candidate genes or pathways in individuals with extreme quantitative phenotypic values. These results would be unlikely if most rare variants were neutral, or if rare variants were not a significant contributor to the genetic component of phenotypic inheritance (Kryukov et al., 2007). A mutation that damages protein structure does not necessarily lead to a detectable human-disease phenotype, and a mutation that predisposes an individual toward a disease is not necessarily evolutionarily deleterious (Kryukov et al., 2007). Here, we used WES as an instructive method to identify candidate pathogenic genes for OPLL, and the association study to confirm specific susceptible loci in the expanded samples. The data indicate that *PTCHI* and *COL17A1* variants possibly contribute to the pathogenesis of OPLL.

The *PTCHI* gene (homolog 1 of *Drosophila* patched) on chromosome 9q22.32 spans ~74 kb of genomic DNA, with 23 exons. It encodes PTCH, a 1447-amino-acid integral membrane protein with 12 transmembrane regions, five forming a sterol-sensing domain, and two extracellular loops. PTCH is a transmembrane protein that represses the transcription of genes encoding members of the TGF- β and Wnt families of signaling proteins in specific cells. The human *PTCHI* gene is a typical tumor suppressor gene for all basal cell carcinomas and medulloblastomas, the loss of which causes increased signaling through the human sonic hedgehog protein (SHH) pathway (Johnson et al., 1996; Nagao et al., 2005). Thibert et al. demonstrated that the proapoptotic activity of unbound PTCH and the positive effect of SHH-bound PTCH on cell differentiation probably cooperate to achieve appropriate spinal cord development (Thibert et al., 2003). Ohba et al. found that adult *Ptch1*-deficient (*Ptch1*^{-/-}) mice and nevoid basal cell carcinoma syndrome patients show high bone mass. In culture, *Ptch1*^{+/-} cells show accelerated osteoblast differentiation and enhanced responsiveness to runt-related transcription factor 2, which is closely associated with OPLL (Kishiya et al., 2008; Ohba et al., 2008). OPLL results in increased bone formation in ligament tissue, and there is some evidence showing a correlation between OPLL and increased systemic bone mineral density, especially in the women over 60 years of age in our study (Yamauchi et al., 1999). p.T265S

and p.P1232L are located in the highly conserved superfamily and C-terminal domains of PTCH, respectively. p.T265S is a serine substitution for a threonine. These two amino acids are both polar and hydrophilic, differing from one another by a methyl group. The amino acid substitution might cause a conformational change, resulting in a protein with altered signaling ability, and contributing to the pathogenesis of this high-bone-mass disease.

We selected *COL17A1* as a gene potentially causing OPLL based on previous studies in which genetic linkage and linkage disequilibrium analyses have associated *COL11A2* and *COL6A1* with susceptibility to OPLL (Furushima et al., 2002; Tanaka et al., 2003). The *COL17A1* gene (collagen XVII- α 1) is located on chromosome 10q24.3-q25.1, spanning ~54 kb of genomic DNA, with 56 exons. Collagen XVII (COL17), a type II transmembrane protein, is a structural trimer of hemi-desmosomes. It consists of an intracellular N-terminal domain of 466 amino acids, a transmembrane domain of 23 amino acids, and an extracellular C-terminus of 1008 amino acids (Nonaka et al., 2000). COL17 acts as a cell surface receptor and a matrix component to mediate the adhesion of keratinocytes and other epithelial cells to the underlying basement membrane (Franzke et al., 2002). Mutations in *COL17A1* are associated with the autoimmune skin disease bullous pemphigoid, for which the phenotypes exhibit a wide range of severity that, in addition to skin blistering, include different degrees of mucosal involvement, enamel defects, nail dystrophy, and alopecia (Fine et al., 2008). Seppanen et al. (2006) demonstrated the presence of collagen XVII in neurons of the human brain, and proposed that it may be involved in the pathogenesis of various disorders affecting neuronal migration or synaptic plasticity. Murrell et al. reported that *COL17A1* heterozygotes have enamel defects, implying that reduced amounts of or mutated collagen XVII might be sufficient for dermal-epidermal stability, but not for proper tooth development (Murrell et al., 2007). Our data suggest that *COL17A1* possibly contributes to the genesis of OPLL. However, the pathogenesis mechanism needs further investigation, owing to the limited sample size and pathogenicity exclusion of c.A2704T (p.T902S) and c.A4120G (p.N1374D) by PolyPhen and GERP.

OPLL patients are rare individuals, and the pathways in which the genes involved act are very complex. Unrelated individuals from similar environments with similar OPLL manifestations could and probably do have different causal variants, since the metabolic pathways are quite complicated. A WES study alone cannot provide sufficient analytical power to pinpoint the susceptibility genes. Further research is therefore necessary to assess the significance of the above findings.

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Conflicts of interest

The authors declare no conflict of interest.

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