RUNX2 Polymorphisms Associated with OPLL and OLF in the Han Population

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Abstract

Background Runt-related transcription factor 2 (RUNX2), BMP-2, COL6A1, and VDR are four genes that may be related to ossification of the spinal ligament. However, their pathogenetic relevance remains unclear. Most cases of ossification of the posterior longitudinal ligament (OPLL) and ossification of the ligamentum flavum (OLF) have been reported in Asian populations, but the polymorphic loci in these genes may vary among people of different races.

Purposes We identified (1) polymorphic loci in four genes (RUNX2, BMP-2, COL6A1, and VDR) in OPLL and OLF in Chinese Han patients and (2) identified loci related to these diseases.

Methods We analyzed 19 single nucleotide polymorphisms (SNPs) in four candidate genes in 200 Han individuals (82 patients and 118 control subjects) by the Sequenom system. The genotype distribution and allele frequency of each SNP in the control and patient groups were compared. We then determined the relationships between the loci and the occurrence of OPLL and OLF.

Results Genotyping showed RS1321075 and RS12333172 in RUNX2 differed between the patients and the control subjects. Both loci were located on chromosome 6 and exhibited linkage disequilibrium. One of the two blocks was a haplotype, thus suggesting a link between this block and increased incidence of OPLL and OLF.

Conclusion Although the detailed mechanism of the SNP is unclear, our data suggest RUNX2 could be responsible for ectopic bone formation in the spinal ligament in the Chinese Han population. However, we found no obvious connection between polymorphic loci of COLA1, BMP-2, and VDR and the diseases.

Clinical Relevance Molecular genetic studies have identified several candidate genes that may be responsible for increased susceptibility to the diseases. Information regarding SNPs among the certain candidate genes may improve understanding of the disease and assist in developing new diagnostic gene tools during early episodes of the disease.

Introduction

OPLL and OLF are common diseases in East Asia [5, 9], with an incidence rate between 2% to 4% [11]. OLF has many epidemiologic, pathologic, and etiologic features in common with OPLL, and the two often coexist [1–3, 14]. The pathologic feature of OPLL and OLF is endochondral ossification. Owing to the formation of a progressively enlarging mass of ligament and bone in the spinal canal floor, endochondral ossification causes spinal cord compression. The majority of OPLL is located in the cervical spine, whereas the thoracic spine is a common site of OLF. Reported risk factors implicated in OPLL include gender, trauma, hormonal imbalance, and dietary habits [5, 9, 13, 15].

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Genetic background is considered to be the predominant factor in the etiology of OPLL. Of 1030 family members of 347 patients with OPLL, 27% of the parents and 29% of the siblings also had OPLL [18]. The incidence of OPLL among family members with two-strand HLA haplotypes was 53%, whereas for those with single-strand haplotypes only 24% had OPLL [12]. This indicated that the mutations responsible for OPLL may be located near the HLA gene on chromosome 6.

With the advent of genetic research tools, the cause of OPLL and of OLF has been attributed to variations in polymorphic SNP loci in several candidate genes. Tanaka et al. performed multipoint linkage analysis on the genomes of Japanese patients with OPLL [17]. Seven genes on chromosome 21q22.3 were reportedly associated with the disease; additional linkage disequilibrium analysis showed SNPs in the collagen 6A1 gene (COL6A1) were closely related to OPLL. Tsukahara et al. [19] subsequently found one SNP at intron 32 (−29) correlated to Japanese patients with diffuse idiopathic skeletal hyperostosis (DISH), indicating that COL6A1 was related to OPLL and DISH among the Japanese population. A SNP analysis of COL6A1 among Han Chinese patients with OPLL and OLF by Kong et al. [10] showed a greater allele frequency of a SNP in the promoter (−572T) in the OPLL and OLF groups than in the control group, suggesting that the COL6A1 gene might influence the occurrence of OPLL and OLF in Han populations.

Wang et al. [20] reported that two SNPs (Ser37Ala and Ser87Ser) in BMP-2 were correlated with the predisposition to and severity of OPLL in the Chinese population. Two studies [6, 7] showed that the expression of the gene RUNX is more robust in patients with ossification of the ligaments. RUNX plays a critical role in regulating the differentiation of osteoclasts, and RNA interference targeting RUNX reduces the expression of angiopoietin-1 in cultured OPLL cells, suggesting angiopoietin-1 is important in heterotopic ossification. One study by Japanese researchers [8] found a variant of the VDR genotype, family history of myocardial infarction, and high body mass index at age 40 in conjunction with other lifestyle factors such as long working hours to be independent risk factors for OPLL.

We (1) identified polymorphic loci in four genes (RUNX2, BMP-2, COL6A1, and VDR) related to Chinese Han patients with OPLL and OLF, and (2) identified the haplotype in four genes including RUNX2, BMP-2, COL6A1, and VDR related to these diseases.

Patients and Materials

We identified patients diagnosed with OPLL and/or OLF and control subjects without these disorders from January

2007 to September 2008. The diagnosis of OPLL and OLF was made by surgeons on the basis of clinical symptoms and radiographic examinations of the cervical, thoracic, and lumbar spines. All patients had CT or MRI before surgery to ascertain the diagnosis. The same group of doctors diagnosed OPLL and OLF based on clinical symptoms, physical examinations, and AP and lateral plain films of the spine. All patients had CT and/or MRI to identify compression of the spinal cord, shape of the vertebral canal, and the size and scope of the ossification. The control group consisted of patients with spinal disease other than OPLL or OLF as confirmed by radiographs of the cervical, thoracic, and/or lumbar vertebrae admitted to the hospital during the corresponding period. We excluded patients older than 60 years with ankylosis, DISH, spinal infections, or tumors, and/or who were taking medications that influence long-term bone metabolism. There were 82 patients (65 men, mean age 57 years; 17 women, mean age 55 years) and 118 control subjects (78 men, mean age 53 years; 40 women, mean age 53 years) recruited for this study. Patients in the disease group were divided into three subsets: 48 patients with OPLL but not OLF, 12 with OLF but not OPLL, and 22 with OPLL and OLF. Except for two in the thoracic spine, most of the OPLL was located in the cervical spine and involved levels 1 to 7, with 4.1 level being average. With the exception of two located in the cervical spine, most of the OLF was located in the thoracic spine and involved levels 3 to 8, with an average at the 4.8 level. OPLL and OLF were located in the thoracic and cervical spine and involved levels 3 to 11, with an average at the 5.6 level. The study protocol was approved by the ethical committees for human subjects in the hospital affiliated with the Second Military Medical University (Shanghai Changzheng Hospital). All patients gave written informed consent for participation in the study.

We obtained EDTA anticoagulated peripheral blood samples for DNA extraction from each participant with informed consent. Genomic DNA samples were extracted from peripheral leukocytes with the standard procedure using a TIANamp Blood DNA kit (TIANGEN, DP318-03 Tiangen Biotech, Beijing, China).

SNPs were genotyped by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy using the MassARRAY system (Sequenom, San Diego, CA, USA) according to the manufacturer's instructions. Two preparatory steps were required before MALDI-TOF analysis: amplification of the regions of interest, and primer extension using the homogeneous MassEXTEND (hME) assay (Sequenom Inc). The size of the reaction products were determined with a Bruker Biflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA), to yield genotype information.

Table 1. PCR primers used in the multiplex PCR system

Gene	SNP ID	Forward primer sequence	Reverse primer sequence
COL6A1	RS9978314	ACGTTGGATGCAGACAGGAGGCATCTAGTG	ACGTTGGATGTGCAGGAGCTTCACAGCCAG
	RS2276255	ACGTTGGATGTGCTTCCCTCCAAGGTCAC	ACGTTGGATGACCCTGAGGAGGAAGAGGAG
BMP-2	RS1005464	ACGTTGGATGTAGTGGCCTTAAGAAAGGAG	ACGTTGGATGAGTGGGAAGGTCAGAGAAAG
RUNX2	RS3749863	ACGTTGGATGACCCCGCTTCTCAGCACAG	ACGTTGGATGGTGGAGTTGCCTCTCGACT
	RS6908650	ACGTTGGATGAAACAGGTTTCCAGGTTGGG	ACGTTGGATGAACCCGAACCTCCACTCTAAG
	RS7771889	ACGTTGGATGCTAATCTTACCTGGACCTCC	ACGTTGGATGTGAAAACCACTTCTTTGGGC
	RS16873379	ACGTTGGATGGCCTCCACAATGTGTATGTC	ACGTTGGATGCCTAGCTCTTTACTGCTTCC
	RS16873437	ACGTTGGATGGGAAATCATGAAAGGGAACG	ACGTTGGATGAAACTGCCTTTGCAGACACC
	RS2677108	ACGTTGGATGATTGGTTAGCAAAGAGAGGC	ACGTTGGATGCTAGTTTAGACTCCTGGCTG
	RS1321075	ACGTTGGATGTTAGTTTGAGGCGGGTGG	ACGTTGGATGTACTGTGAGAGGCTCCAGAT
	RS967588	ACGTTGGATGGGGTTCAGCTATACTATTTTCG	ACGTTGGATGGTTTAGCATAATCTCATG
	RS1406846	ACGTTGGATGCTGATGCAGAGATAACTCCC	ACGTTGGATGCTGTCACTGACATGTTGTTTG
	RS9296459	ACGTTGGATGGAACGACTTGACAGAAATGC	ACGTTGGATGCAGCTTTGCTATTTATTGG
VDR	RS12333172	ACGTTGGATGGGGCCCCACATAAAGAGTTT	ACGTTGGATGCTGTACTTAAAATACACTGC
	RS2189480	ACGTTGGATGGATCTGAGTTCCAGAGAGCC	ACGTTGGATGATTACGCTCTGGAGCGTGG
	RS11168287	ACGTTGGATGTACGTGGAGGAAAAGTCCAG	ACGTTGGATGGGGTGAGGAGAAAATGAGTTG
	RS3847987	ACGTTGGATGCTAGTGGGGTCTCCCTCTC	ACGTTGGATGTGGGCACTGAAGGGGGTGG
	RS12721370	ACGTTGGATGTGACCCAACAGTCAAGGAAG	ACGTTGGATGCTTGGGTGATGAGGGTTGTC
	RS11574079	ACGTTGGATGTGAGGAAGAGCCTATGCTGG	ACGTTGGATGGAGGGAAGCACCATCTCTTG

SNPs distributed among the four genes were selected based on findings from previous association studies. Genomic regions containing the SNPs of interest were amplified by multiplex PCR. Primers for multiplex PCR were designed with Assay Design v3.1 (Sequenom Inc) (Table 1). Genomic DNA (10 ng/ μ L) was mixed with 12.5 nm of each primer, 25 mmol/L $MgCl_2$, 25 mmol/L dNTPs, and 0.15 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) in a total volume of 10 μ L. Thermocycling conditions were 94°C for 15 minutes, followed by 45 cycles of denaturation at 94°C for 20 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final extension at 72°C for 3 minutes. Amplified products were mixed with Arctic shrimp alkaline phosphatase (SAP) to dephosphorylate residual nucleotides, and incubated at 37°C for 40 minutes followed by incubation at 85°C for 5 minutes. For the hME assay, PCR products were mixed with 0.625 μ mol/L–1.25 μ mol/L of the hME primer mix (Table 2) and a reaction cocktail containing an iPLEX terminator, iPLEX enzyme, and iPLEX buffer plus (Table 3).

The final extension products were diluted with 16 μ L of H_2O and treated with 6 mg of Clean Resin (Sequenom Inc) to remove buffer salts. Samples then were dripped on the sample target and underwent spontaneous nucleation. SNPs were genotyped by MALDI-TOF mass spectroscopy using the MassARRAY system and the data were analyzed with MassARRAY Typer Version 3.4 (Sequenom Inc).

The Hardy-Weinberg equilibrium [16] was used to describe the equilibrium status of a locus distribution in a

population. The frequency distribution of an allele and genotyping of a single locus were calculated with the direct counting method. Pearson's chi square test was used to determine the correlation between the locus and the disease based on the relative occurrence ratio (OR) of alleles and its 95% confidence interval. Haploview software (Massachusetts Institute of Technology, Cambridge, MA, USA) was used to calculate the linkage disequilibrium between loci; D' and R^2 were used to describe this relationship. The confidence interval method was adopted to set the haplotype block. This algorithm was taken from Gabriel et al. [4], in which 95% confidence bounds on D' were generated and each comparison was labeled "strong LD" (linkage disequilibrium), "inconclusive," or "strong recombination". We presumed a pair of SNPs would be in strong LD if the one-sided upper 95% confidence bound on D' was greater than 0.98 and the lower bound was greater than 0.7. A pair of SNPs was defined as strong evidence for historical recombination if the upper confidence bound on D' was less than 0.9. If 95% of informative (ie, noninconclusive) comparisons were strong LD, a block was created. In each block, the frequency of the haplotype was estimated with the EM method. In cases where haplotypes had a frequency greater than 1% in the two blocks, risk indicators and the chi square test were used to determine the relationship between the haplotype and the disease. In cases in which the p value was determined with a two-tailed test, a difference was defined as $p < 0.05$. All statistical analyses were performed using Haploview, SHEsis

Table 2. Extension primer sequences and masses for unextended and extended products

SNP ID	Extension primer sequence	Mass of unextended primer (Da)	Variant alleles	Mass of extended primer (Da)
RS9978314	CTGGGTGCTTGCACAGT	5217.4	C/T	5464.6/5544.5
RS2276255	cttCGTGAGGGGAGCAGGCT	6214	G/A	6461.2/6541.1
RS1005464	gaatGTCAGAGAAAGATCTTCGTATC	8018.2	A/G	8289.4/8305.4
RS3749863	CCTCTCGACTCCTCCCT	4993.2	C/A	5280.5/5320.3
RS6908650	TTCAGGCCGTCTTCCTT	5103.3	A/G	5374.5/5390.5
RS7771889	CTTCTTTGGGCCTCAGCAG	5770.7	G/C	6017.9/6058
RS16873379	TGCTTCCATTGTGGTTGGC	6121	T/C	6392.2/6408.2
RS16873437	GGCTTCCCTTCTCATGATTCT	6314.1	G/T	6601.3/6641.2
RS2677108	ATTCTTCTGGTTAGTCTTAACG	6706.4	C/T	6953.6/7033.5
RS1321075	ggtggGAGGCTCCAGATCCTAAA	7113.6	C/A	7360.8/7384.8
RS967588	TTAGCATAATCTCATGCATACTTG	7301.8	T/C	7573/7589
RS1406846	gaTGACATGTTGTTTGAATAAAAA	7438.9	T/A	7710.1/7766
RS9296459	gTTTGCTATTTATTGGAAATTTTC	7658	A/G	7929.2/7945.2
RS12333172	ttccCTGCAGTAATAATTACAATTCC	7864.1	T/C	8135.3/8151.4
RS2189480	GTGGGGTCAGTGTTTCAGG	5626.6	C/A	5913.9/5953.7
RS11168287	TTGTTCTAATCTCTCTCCTAACA	6899.5	A/G	7170.7/7186.7
RS3847987	ccggtTGGGGTGGGAGCTGTGGG	7248.7	C/A	7495.9/7519.9
RS12721370	ctgtGTGATGAGGGTTGTCACAGCAG	8082.2	G/T	8369.5/8409.3
RS11574079	tcttGAAGCACCATCTCTGCAGTAGC	8210.3	A/G	8481.5/8497.6

Table 3. Extension primer sequences and masses for unextended and extended products

Temperature (°C)	Time (seconds)	Cycles
94	30	1
94	5	1
52	5	40
80	5	5
72	180	1
4	∞	1

(Bio-X Life Science Research Center, Shanghai, China), and Microsoft Excel 2003 (Microsoft Corp, Redmond, WA, USA).

Results

We found no disease-related loci in the allelic analysis. However, genotyping revealed that RS1321075 and RS12333172 were related to the disease ($p = 0.0339$, $p = 0.0428$). Both loci are in the gene RUNX2 on

Fig. 1 A graph shows the statistical distribution of loci of 19 SNPs. No disease-related loci were found in the allelic analysis, however, genotyping revealed that RS1321075 and RS12333172 were related to the disease ($p = 0.0339$, $p = 0.0428$). Both loci are in the gene RUNX2 on chromosome 6. The X-axis represents the locus of each

SNP and the Y-axis represents the corresponding $-\log_{10} p$ value for each SNP locus. Two SNP loci (RS1321075 and RS12333172) are disease-related according to the genotyping results but not according to the allele results.

Table 4. Statistical association between loci and OPLL status

Gene	SNP ID	Genotypes and alleles	Patient (n = 82)	Control (n = 118)	Chi square	p Value	OR (95%CI)
BMP2	RS1005464	AA	8	17	1.66	0.4354	
		AG	46	57			
		GG	27	44			
RUNX2	RS12333172	A:G	62:100	91:145	0.003	0.9538	0.99 (0.65,1.49)
		CC	53	85	6.77	0.0339	
		CT	28	25			
		TT	1	8			
	RS1321075	C:T	134:30:00	195:41:00	0.06	0.8128	0.94 (0.55,1.58)
		AA	5	2	6.31	0.0428	
		AC	19	43			
	RS1406846	CC	50	60			
		A:C	29:119	47:163	0.4	0.5255	0.84 (0.5,1.42)
		AA	14	17	1.85	0.396	
	RS16873379	AT	40	69			
		TT	28	32			
		A:T	68:96	103:133	0.188	0.6646	0.91 (0.61,1.37)
		CC	5	8	2.92	0.2328	
	RS16873437	CT	33	61			
		TT	44	49			
		C:T	43:121	77:159	1.89	0.169	0.73 (0.47,1.14)
		GG	6	7	0.23	0.8906	
	RS2677108	GT	31	43			
		TT	45	68			
		G:T	43:121	57:179	0.22	0.6387	1.12 (0.71,1.76)
		CC	10	12	1.33	0.5155	
	RS3749863	CT	37	63			
		TT	35	43			
		C:T	57:107	87:149	0.19	0.6657	0.91 (0.60,1.38)
		AA	28	33	2.36	0.3068	
	RS6908650	AC	42	73			
		CC	12	12			
		A:C	98:66	139:97	0.03	0.8637	1.04 (0.69,1.55)
		AA	13	22	2.1	0.3506	
	RS7771889	AG	46	54			
		GG	23	42			
		A:G	72:92	98:138	0.22	0.6362	1.10 (0.73,1.64)
		CC	39	59	0.12	0.944	
	RS9296459	CG	35	48			
		GG	8	11			
		C:G	113:51:00	166:70	0.09	0.7584	0.93 (0.61,1.44)
		AA	1	1	1.34	0.513	
	RS967588	AG	15	15			
		GG	64	100			
		A:G	0.807639	0.857639	1.3	0.2542	1.5 (0.74,3.04)
		CC	43	49	2.64	0.2678	
		CG	33	61			
		GG	5	8			
		C:G	119:43:00	159:77	1.69	0.1939	1.34 (0.86,2.09)

Table 4. continued

Gene	SNP ID	Genotypes and alleles	Patient (n = 82)	Control (n = 118)	Chi square	p Value	OR (95%CI)
VDR	RS11168287	AA	13	19	0.5	0.7796	
		AG	30	48			
		GG	39	50			
	RS11574079	A:G	56:108	86:148	0.29	0.5933	0.89 (0.59,1.36)
		AA	0	0	3.37	0.0663	
		AG	9	5			
	RS2189480	GG	73	113			
		A:G	0.482639	0.36875	3.25	0.0714	
		AA	39	64	0.89	0.6417	
	RS3847987	AC	34	42			
		CC	9	12			
		A:C	112:52:00	170:66	0.65	0.4197	
	RS12721370	AA	10	17	0.36	0.8345	
		AC	44	58			
		CC	20	26			
	RS12721370	A:C	64:84	92:110	0.18	0.6687	0.91 (0.59,1.4)
		GG	45	73	0.94	0.6238	
		GT	33	40			
COL6A1	RS9978314	TT	3	4			
		G:T	123:39:00	186:48:00	0.71	0.4	
		CC	58	81	3.46	0.177	
		CT	19	35			
	RS2276255	TT	5	2			
		C:T	135:29:00	197:39:00	0.09	0.7618	
		AA	66	90	1.8	0.4058	
		AG	13	26			
		GG	3	2			
		A:G	145:19:00	206:30:00	0.11	0.7354	1.11 (0.6,2.05)

chromosome 6. Two SNP loci (RS1321075 and RS12333172) appeared to be disease-related by genotyping results, but not according to the allele results (Fig. 1).

We identified no polymorphic haplotypes with obvious connection to the diseases in COL6A1, BMP-2, or VDR. However, a haplotype (CTCG) made up of RS967588, RS16873379, RS3749863, and RS6908650 had a patient and control subject frequency rate of 1.75 and a risk rate of 0.81, indicating the possibility that this block is associated with increased incidence of OPLL (Table 4). All 19 of the SNP genotypes in the four candidate genes were found in the Hardy-Weinberg equilibrium and minor allele frequency (MAF). The LD coefficients (D') of the five SNP loci on chromosome 12 ranged from 0.043 to 1, and the R^2 values ranged from 0 to 0.185. Five loci on chromosome 12 did not form a haplotype block. For the two loci on chromosome 21, $D' = 0.92$ and $R^2 = 0.57$. No haplotype

block was found on chromosome 21. The two blocks were formed by 11 SNP loci on chromosome 6 (Fig. 2). Block 1 included RS967588, RS16873379, RS3749863, and RS6908650 and had a range of 51 kb. The LD coefficient D' between these four loci ranged from 0.964 to 1 and the R^2 ranged from 0.299 to 1 (Table 5). These four loci combined to give four haplotypes with a frequency greater than 0.01 (Table 6). The haplotype CTCG was present in 14% of patients and 8% of control subjects, respectively, with a patient/control ratio of 1.75 (risk factor 0.81, $p = 0.259$). Block 2 contained RS1321075, RS1406846, and RS2677108 and spanned 11 kb. The linkage disequilibrium coefficient D' for the three loci ranged from 0.951 to 1, and the R^2 value ranged from 0.348 to 0.753 (Table 7). The three loci formed four haplotypes with a frequency greater than 0.01 (Table 8); the risk ratios of each haplotype suggested no correlation with the disease.

Discussion

OPLL and OLF are common disorders among the elderly population and are the major cause of spinal myelopathy in Asian populations in contrast to populations of Western countries. Because genetic factors appear to play a crucial role in OPLL and OLF, molecular genetic studies were considered an important approach to investigating the etiologies of the diseases. However, as multiple genetic and

environmental components seem to be correlated to the development of OPLL and OLF, it has been difficult to understand all susceptible genes.

The purpose of this study is to identify the polymorphic loci and the haplotype in four candidate genes reportedly correlated with OPLL and OLF: RUNX2, BMP-2, COL6A1, and VDR.

Our study has numerous limitations. First, most patients came from the southeast of China, which represents a geographically limited section of the whole Han population. Further collaborative study with centers in additional regions of China would be required to confirm whether our findings were generalizable to the entire population. Further, we cannot ensure our findings reflect those of other races. Second, although variations of two SNPs in the RUNX2 gene were associated with an elevated incidence of OPLL and OLF, the detailed mechanism by which this occurs remains obscure. Further investigation is needed to pinpoint the specific function of the RUNX2 gene in the development of OPLL and OLF.

Four SNPs in the COL6A1 gene [10] were recently reported, with the allele frequency of the promoter (−572T) SNP in the OPLL/OLF group outnumbering the allele frequency in the control group and the total haplotype frequency of the promoter (−572), intron 32 (−29) and intron 33 (+20) SNPs markedly different between the OPLL/OLF group and the control group. This suggests COL6A1 might be involved in the occurrence of ossification of spinal ligament (OSL) in the Chinese Han population. However, our data suggests no correlation between the genotypes of two SNP loci in COL6A1 in the patient and the control groups. This result may be attributed to differences in selection of loci, differences in

Fig. 2 The graph shows the linkage disequilibrium of 11 SNP loci and haplotype blocks on chromosome 6. The upper part of this figure shows the names of the five SNP loci. The linkage disequilibrium between loci is estimated with D' . The dark squares represent the linkage disequilibrium between the two loci; the darker the color, the greater the linkage disequilibrium. The white squares indicate no linkage disequilibrium between two loci. The values shown in the squares represent the D' of loci on the upper left and upper right; the larger the value, the greater the linkage disequilibrium and vice versa. Loci that are not covered in the squares have $D' = 1$. Loci having $MAF < 0.001$ and $HW < 0.001$ are excluded from the linkage disequilibrium analysis. Two haplotype blocks are observed on chromosome 6 and were analyzed separately. Haplotypes are indicated by thick lines. No polymorphic haplotypes with obvious connection to the diseases were identified in COL6A1, BMP-2, or VDR, however, a haplotype (CTCG) made up of RS967588, RS16873379, RS3749863, and RS6908650 has a patient/control frequency rate of 1.75 and a risk rate of 0.81, indicating the possibility that this block is associated with increased incidence of OPLL.

Table 6. Linkage disequilibrium statistics for each locus in Block 1 (D'/R^2)

Loci	RS967588	RS16873379	RS3749863	RS6908650
RS967588	—	1	0.629	0.304
RS16873379	1	—	0.623	0.299
RS3749863	1	1	—	0.472
RS6908650	0.971	0.971	0.964	—

Table 5. Linkage disequilibrium statistics for loci on chromosome 12 (D'/R^2)

Loci	RS3847987	RS11574079	RS12721370	RS2189480	RS11168287
RS3847987	—	0.024	0.185	0.034	0.005
RS11574079	1	—	0.01	0.087	0.004
RS12721370	0.725	1	—	0.056	0.003
RS2189480	0.316	1	0.69	—	0
RS11168287	0.104	0.424	0.078	0.043	—

Table 7. Analysis of linkage disequilibrium, risk, and significance of each locus in Block 1 (Chromosome 6).

RS967588	RS16873379	RS3749863	RS6908650	Patient	Control	Risk	Chi square value	p Value
C	T	A	A	0.432	0.41	0.075407403	0.027	0.8694
T	C	C	G	0.255	0.326	-0.35437472	0.853	0.3557
C	T	A	G	0.166	0.179	-0.10877635	0.0024	0.961
C	T	C	G	0.14	0.08	0.807354922	1.2742	0.259

Table 8. Linkage disequilibrium statistics for each locus in Block 2 (D'/R^2)

Loci	RS1321075	RS1406846	RS2677108
RS1321075	—	0.348	0.444
RS1406846	0.97	—	0.753
RS2677108	0.951	1	—

research methods, or inherent differences found in populations from different regions that may have affected the obtained results.

In another study, Kishiya et al. [7] observed elevated levels of RUNX2 expression in patients with OPLL and OLF. They also showed RNAi targeting RUNX2 caused an obvious decrease in the expression of angiopoietin-1 when administered to OPLL cells in vitro, indicating that RUNX2 plays an important role in heterotopic ossification of the spinal ligament. In our study, genotyping revealed RS1321075 and RS12333172 in the gene RUNX2 on chromosome 6 were related to the disease. A haplotype (CTCG) made up of RS967588, RS16873379, RS3749863, and RS6908650 had a patient/control frequency rate of 1.75 and a risk rate of 0.81. Our experimental results are supported by those of Kishiya et al. [7] mentioned above, with both studies revealing that RUNX2 and OPLL are closely related. The differing CTCG percentages between patient and control groups suggest that in conjunction with environmental factors, people with CTCG of the two SNPs may be predisposed to the disease.

The functional impact of RS1321075 and RS12333172 in the RUNX2 gene on chromosome 6 has not been fully investigated. RUNX2 is a key regulator of osteocalcin and alkaline phosphatase gene promoters and cooperates with BMP-specific Smads [21], which are important signal transducers regulated in osteoblast differentiation. Certain variants of the SNPs in RUNX2 may affect the process of bone formation, but additional study is needed to identify the exact mechanism by which this occurs.

The RUNX2 gene could be associated with the occurrence and development of OSL. Although the detailed mechanism of the SNP is unclear, our data suggest that RUNX2 may be responsible for ectopic bone formation in the spinal ligament.

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