ORIGINAL ARTICLE

Observational Study

Gene testing for osteonecrosis of the femoral head in systemic lupus erythematosus using targeted next-generation sequencing: A pilot study

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Abstract

BACKGROUND

Previous publications indicated that genetic predisposition might play important roles in the onset of osteonecrosis of the femoral head (ONFH) in systemic lupus erythematosus (SLE). Some gene loci such as complement C3d receptor 2 (*CR2*), nitric oxide synthase 3 (*NOS3*), collagen type II alpha 1 chain (*COL2A1*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), and transient receptor potential cation channel subfamily V member 4 (*TRPV4*) were reported to be involved in this process.

AIM

To investigate whether the risk of ONFH in SLE is associated with single nucleotide variations (SNVs) in these five genes.

METHODS

SNVs in the *CR2*, *NOS3*, *COL2A1*, *PTPN22*, and *TRPV4* genes were examined by using FastTarget and Illumina Miseq sequencing technologies in 49 cases of SLE with ONFH. Burrows–wheeler aligner was used to align the sequencing reads to hg19, and GATK and Varscan programs were used to perform SNV calling. PolyPhen-2, SIFT, and MutationTaster were used to assess the functional effects of non-synonymous SNVs.

RESULTS

Six of the 49 patients were confirmed to have low frequency SNVs, including one patient with SNVs in *NOS3* (exon 6: c.814G>A: p.E272K and exon 7: c.814G>A: p.E272K.), four in *COL2A1* (rs41263847: exon 29: c.1913C>T: p.T638I, exon 28: c.1706C>T: p.T569I, and rs371445823: exon 8: c.580G>A: p.A194T, exon 7:

c.373G>A: p.A125T), and one in *CR2* (rs45573035: exon 2: c.200C>G: p.T67S).

CONCLUSION
The onset of ONFH in SLE might be associated with the identified SNVs in *NOS3*, *COL2A1*, and *CR2*.

Key words: Single nucleotide variations; Osteonecrosis of the femoral head; Systemic lupus erythematosus; Nitric oxide synthase 3; Collagen type II alpha 1 chain; Complement C3d receptor 2

Core tip: Genetic predisposition might play important roles in the onset of osteonecrosis of the femoral head (ONFH) in systemic lupus erythematosus (SLE). Some gene loci such as complement C3d receptor 2 (*CR2*), nitric oxide synthase 3 (*NOS3*), collagen type II alpha 1 chain (*COL2A1*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), and transient receptor potential cation channel subfamily V member 4 (*TRPV4*) were reported to be involved in this process. We investigated whether the risk of ONFH in SLE is associated with single nucleotide variations (SNVs) in these five genes by using FastTarget and Illumina Miseq sequencing technologies in 49 cases. Six patients were confirmed to have low frequency SNVs, including one in *NOS3*, four in *COL2A1*, and one in *CR2*. The onset of ONFH in SLE might be associated with the identified SNVs in *NOS3*, *COL2A1*, and *CR2*.

INTRODUCTION

Both osteonecrosis of the femoral head (ONFH) and systemic lupus erythematosus (SLE) are multifactorial and complex diseases caused by both genetic alterations and environmental exposures[1]. Risk factors for secondary ONFH are known to involve the intake of corticosteroids, autoimmune disease, alcohol abuse, and radiation. Epidemiological studies suggest that SLE is the most common autoimmune disease to be the primary cause of steroidinduced ONFH[2]. However, only some patients with SLE receiving steroid administration ultimately develop ONFH, and there are a number of patients with SLE with ONFH who have no experience of corticosteroid treatment[3]. These reports indicated that other reasons, such as genetic variations between individuals, might also be involved in the onset of ONFH in SLE. Indeed, gene polymorphisms that affect coagulation, metabolic factors, mechanical stresses, immunologic factors, and fibrinolytic systems have been identified[4,5] and some of these genes have been suggested to be involved in SLE with ONFH.

Complement receptor type 2 (CR2) is a transmembrane glycoprotein expressed in mature B and follicular dendritic cells. CR2 plays a role in complement activation, antigen targeting, and B cell activation. Endothelial nitric oxide synthase (NOS3) regulates bone formation and osteoblast function. It has been reported that in Korean patients with SLE, CR2 and NOS3 contribute to ONFH susceptibility[6,7]. Collagen type II alpha-1 gene (*COL2A1*) is a causal gene of skeletal dysplasia and epiphyseal dysplasia. *COL2A1* mutations cause familial idiopathic ONFH[8]; however, the relationship was not found in Japanese idiopathic ONFH patients[9]. *PTPN22* encodes protein tyrosine phosphatase nonreceptor 22, a lymphoid protein, mutations in which might promote T cell activation and thus cause autoimmune diseases, such as rheumatoid arthritis, SLE, and juvenile idiopathic arthritis[10,11]. Transient receptor potential vanilloid 4 (TRPV4) forms a calciumpermeable non-selective cation channel and plays a role in vasoregulation and osteoclast differentiation. A novel *TRPV4* mutation and altered calcium homeostasis have been observed in ONFH[12].

In this study, we investigated whether patients with SLE with ONFH have a genetic predisposition to ONFH in SLE, the identification of which might lead to more

efficient diagnosis, evaluation, and even prevention of the disease. Using FastTarget and Illumina Miseq sequencing technologies, we analyzed single nucleotide variations (SNVs) in *CR2, NOS3, COL2A1, PTPN22*, and *TRPV4* genes of patients with SLE with ONFH.

MATERIALS AND METHODS

Patients included in the study

We enrolled 49 patients with SLE with ONFH (4 males and 45 females; mean age: 33.57 ± 11.24 years) visiting the Department of Rheumatology and Immunology of Shandong Provincial Hospital Affiliated to Shandong University. All patients met the criteria of the American College of Rheumatology as revised in 1997[13]. Individuals provided samples of peripheral blood, 2 mL of which was used for genomic DNA isolation using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's standard procedure. The local Ethics Review Board approved the current study, and informed consent was provided by all participants.

Mutation analysis

Primers were designed using Primer 3. To cover all the coding sequences and most of the untranslated regions of the five genes that might be responsible for ONFH in SLE, 243 oligonucleotide pairs were produced. A first round of primer design using the most stringent conditions (*e.g.*, no single nucleotide polymorphisms in the primer annealing region, an amplicon comprising 200-270 bp, and a GC content of 30%-80%) allowed us to put the 243 oligonucleotide pairs into 15 multiplex PCR panels to amplify the target regions from the five genes. An ABI 2720 Thermal Cycler (Life Technologies Corporation, Carlsbad, CA, United States) was used to carry out the amplification reactions using following cycling program: 95°C for 2 min; 11 cycles of 94 °C for 20 s, 63.5 °C for 40 s, and 72 °C for 1 min; 24 cycles of 94 °C for 20 s, 65 °C for 30 s, and 72 °C for 1 min; and final incubation at 72 °C for 2 min. An 8 bp barcode was incorporated into each PCR product, and then all the libraries for each sample were pooled. Cluster generation and hybridization of the sequencing primers were followed by nucleotide sequencing carried out on a MiSeq Benchtop Sequencer (Illumina, Inc, San Diego, CA, United States) in one single lane, following the manufacturer's standard protocols. For each sequencing read, 300 cycles were carried out to produce paired-end reads including 300 bp at each end and the 8-bp index tag.

Bioinformatic analysis

The Burrows–wheeler aligner[14] method was used to align the sequencing reads to human genome version hg19. The GATK[15] and Varscan programs[16] were used to call the SNVs, the data for which were then combined. SNVs were annotated using the Annovar program[17]. PolyPhen-2, SIFT, and MutationTaster[18-20] were used to assess the functional effects of non-synonymous SNVs. Significantly non-benign non-synonymous SNVs were identified as those with a Polyphen-2 score of > 0.85, a SIFT score of < 0.05, or a MutationTaster score of > 0.85. Perl scripts then filtered the SNVs against those of dbSNP135 to segregate benign polymorphisms from potentially deleterious variants. Benign polymorphisms were considered as those SNVs present in dbSNP135 with a minor allele frequency of ≥ 1% in a Chinese population from the 1000 genome database, and were removed from subsequent analysis.

Statistical analysis

The two-tailed Student's *t*-test and Fisher's exact test or χ^2 test were used when appropriate. Differences were considered significant if the *P* value was less than 0.05. The odds ratio and 95% confidence interval (95%CI) were calculated.

RESULTS

SNV identification in patients with SLE with ONFH

To identify SNVs, VarScan (http://varscan.sourceforge.net/) and GATK HaplotypeCaller (https://software.broadinstitute.org/gatk/best-practices//) were used to analyze single nucleotide polymorphisms and insertion-deletions (InDels) in the samples. A total of 92 SNVs and 17 InDels were identified using both approaches, with 4 SNVs and 1 InDel being identified only by VarScan, and another 16 SNVs and 2 InDels only by GATK HaplotypeCaller.

The identified SNVs and InDels were then grouped by the corresponding regions of the genome, including: Intron (40.15%), exon (21.21%), intergenic (13.64%), ncRNA

(non-coding)_intron (7.58%), 3′ untranslated region (UTR; 5.3%), splicing site (10 bp around a splice junction) (4.55%), upstream (1 kb upstream of the transcription start site; 3.03%); downstream (between one gene's upstream and another gene's downstream; 1.52%), 5′ UTR (1.52%), exonic splicing (0.76%), and ncRNA (non-coding)_splicing (0.76%). In this study, 15 SNVs were nonsynonymous (51.72%), while the other 14 SNVs were synonymous (48.28%). Gene transition (Ts) and transvertion (Tv) (both as annotated by dbSNP) of the SNVs were also analyzed. There were 61 (54.46%) Ts events and 29 (25.89%) Tv events, giving a Ts:Tv ratio of 2.1034. There were 15 (13.39%) Novel_Ts events (not annotated by dbSNP) and 7 (6.25%) Novel_Tv events (not annotated by dbSNP), giving a Novel_Ts:Tv ratio of 2.1429.

For all the identified InDels, 2 insertions comprised 1-5 bp (10%), while 17 deletions comprised 1-5 bp (85%) and 1 deletion was 6-10 bp (5%). The genomic distributions of the SNVs for each sample are shown in Table 1.

Low frequency SNVs in patients with SLE with ONFH

Among the 49 patients, six (12.25%) were confirmed to have low frequency functional mutations, including one patient with a mutation in the *NOS3* gene, four patients with a mutation in the *COL2A1* gene, and one patient with a mutation in the *CR2* gene; and the first priority (Take the highest priority of SNVs if the mutation is dominant or homozygous; take the lower one from the top two highest priority SNVs if it is a heterozygous recessive pattern) of these gene mutations were third, first1, and second, respectively. However, we failed to detect significant differences in the frequency of these mutations in comparison with controls (Table 2).

The low frequency functional mutations in *NOS3* are shown as follows: NM_001160109: exon 6: c.814G>A: p.E272K, NOS3: NM_000603: exon 7: c.814G>A: p.E272K, NOS3: NM_001160110: exon 6: c.814G>A: p.E272K, and NOS3: NM_001160111: exon 6: c.814G>A: p.E272K (Figure 1), which had never been reported previously. Most of these mutations here comprised nonsynonymous SNVs and were predicted as tolerated (SIFT Score Pred), possibly damaging (POLYPHEN Score Pred), and disease_causing damaging (MutationTaster Score Pred) (Table 3).

We revealed two rare functional mutations of *COL2A1*, both of which are nonsynonymous SNVs. One is rs41263847: COL2A1: NM_001844: exon 29: c.1913C>T: p.T638I; COL2A1: NM_033150: exon 28: c.1706C>T: p.T569I, the first priority of which is second; and was predicted as tolerated (SIFT Score Pred), benign (POLYPHEN Score Pred), and disease_causing (MutationTaster Score Pred) damaging. The other is rs371445823 COL2A1: NM_001844: exon 8: c.580G>A: p.A194T, COL2A1: NM_033150: exon 7: c.373G>A: p.A125T (Figure 2), the first priority of which is first1; and was predicted as tolerated (SIFT Score Pred), possibly damaging (POLYPHEN Score Pred), and disease_causing damaging (MutationTaster Score Pred) (Table 3).

One SNV identified in the *CR2* gene is also a nonsynonymous SNV with the first priority being second: rs45573035: CR2: NM_001006658: exon 2: c.200C>G: p.T67S, CR2: NM_001877: exon 2: c.200C>G: p.T67S (Figure 3). The variations were predicted as tolerated (SIFT Score Pred), possibly damaging (POLYPHEN Score Pred), and polymorphism damaging (MutationTaster Score Pred) (Table 3).

The phenotypic features of patients with SLE with ONFH are listed in Table 4. The patient with mutations in *NOS3* was a 36-year-old woman who had suffered from SLE for 4 years and ONFH for 1 mo, who had skin rashes and arthritis. For the patient with mutations in *CR2*, she also had skin rashes and arthritis, and a renal disorder. The four patients with *COL2A1* mutations (G4, G6, G34, and G42) had a relatively low SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) and none of them had interstitial pneumonia, renal disorders, or neurological disorders; however, three of them had arthritis and anemia. The C3 and C4 levels, and 24-hour urinary protein levels were normal in all six patients.

DISCUSSION

The known secondary risk factors for ONFH comprise rheumatic diseases, alcohol abuse, and the use of corticosteroids. Among autoimmune diseases, SLE has a higher ONFH incidence, ranging from 5% to 30%, compared with that in the general population. Moreover, the treatment of SLE deteriorated with the onset of ONFH. Details of the pathogenesis of ONFH in SLE are unclear because patients with SLE who have not taken corticosteroids also develop ONFH. To investigate whether patients with SLE with ONFH have a genetic predisposition, we used next generation sequencing technology to analyze SNVs in reported risk genes, including *CR2*, *NOS3*, *COL2A1*, *PTPN22*, and *TRPV4*. Bioinformatic analyses identified 112 SNVs and 20

Table 1 Genotype distribution of single nucleotide variants						
Sample	Het_SNV	Hom_SNV	Novel_SNV	Het_InDel	Hom_InDel	Novel_InDel
G1	25	22	2	4	0	7
G2	29	11	0	7	0	7
G3	21	9	1	5	0	7
G4	9	14	1	4	0	7
G5	17	12	2	6	0	5
G6	26	15	1	3	0	7
G7	14	24	1	4	0	7
G8	14	11	2	4	0	7
G9	25	21	0	3	0	5
G10	21	9	1	4	0	6
G11	19	25	0	5	1	6
G12	30	12	0	4	0	4
G13	17	14	1	7	0	7
G14	26	12	0	3	0	5
G15	17	14	0	4	0	6
G16	31	11	4	4	0	8
G17	16	19	1	7	0	6
G18	18	24	1	7	0	7
G19	18	22	2	5	0	4
G20	22	13	1	5	0	6
G21	5	17	4	4	0	8
G22	12	11	2	5	0	6
G23	20	26	1	3	1	5
G24	31	14	2	5	0	6
G25	19	13	2	5	0	7
G26	16	20	1	6	0	4
G27	6	16	2	4	0	6
G28	14	24	1	3	0	4
G29	45	8	0	6	0	6
G30	22	13	1	7	0	6
G31	20	23	3	5	0	6
G32	31	15	0	6	0	5
G33	23	13	0	4	0	4
G34	31	7	2	5	0	6
G35	17	13	2	6	0	6
G36	19	10	0	4	0	5
G37	19	25	1	5	1	7
G38	26	20	1	6	0	6
G39	26	13	2	4	0	8
G40	21	10	1	5	0	6
G41	30	12	1	6	0	7
G42	26	17	1	4	0	7
G43	31	14	1	7	0	7
G44	18	13	0	4	0	6
G45	16	21	0	6	0	7
G46	5	17	4	4	0	6
G47	19	25	0	4	1	6
G48	21	9	2	5	0	4
G49	9	14	0	5	0	4

Het_SNV: Heterozygous genotype SNP (annotation by dbSNP); Hom_SNV: Homozygous genotype SNP (annotation by dbSNP); Novel_SNV: Novel SNV (no annotation by dbSNP); Het_InDel: Heterozygous genotype insertion-deletion (InDel) (annotation by dbSNP); Hom_InDel: Homozygous genotype InDel

(annotation by dbSNP); Novel_InDel: Novel InDel (no annotation by dbSNP).

InDels. Most of these genomic variations were localized in coding sequence and more than the half were nonsynonymous. Almost all insertions and deletions were 1-5 bp, except for 1 deletion that was 6-10 bp. Low frequency functional mutations of *NOS3*, *COL2A1*, and *CR2* were found, although the differences between the patients and controls were not significant.

NOS3 deficiency results in impaired osteoblast function and reduced bone formation. The mutations in the *NOS3* identified in the present study were all previously unreported nonsynonymous SNVs. The corresponding regions of the genome were exon 6: c.814G>A: p.E272K and exon 7: c.814G>A: p.E272K. Nitric oxide, catalyzed by endothelial nitric oxide synthase (eNOS), is involved in ONFH pathogenesis by regulating angiogenesis, thrombosis, smooth muscle proliferation, and bone turnover. Excessive nitric oxide production occurs during SLE and certain other autoimmune diseases[21]. A recent study in Korean patients with SLE suggested that exonic *NOS3* polymorphisms, such as rs1549758 (Asp258Asp; exon 6) and rs1799983 (Glu298Asp; exon 7) might increase the risk of ONFH[7]. SNP Glu298Asp in *NOS3* exon 7 is also associated with idiopathic ONFH in Korean patients[22,23]. The c.814G>A: p.E272K mutation in exons 6 and 7 of NOS3 was also predicted to alter protein function and predicted as tolerated, possibly damaging and disease_causing damaging using several online tools. Mutations affecting the N-terminal domain of NOS3 might alter its function, leading to alterations in the enzymatic activity or expression of eNOS, thus causing ONFH in SLE.

The *COL2A1* gene is 31.5 kb, comprising 54 exons that encode a protein of 1487 amino acids with a molecular mass of 134.4 kDa. Mutations in *COL2A1* result in skeletal dysplasias because of failure of cartilage development and growth, which further cause epiphyseal dysplasia of the femoral head and spinal deformity.

The cause of familial idiopathic ONFH has been reported to be four types of COL2A1 mutation in six families: c.3508G>A (p.Gly1170Ser, rs121912891); c.1888G>A (p.Gly630Ser); c.2149G>A (p.Gly717Ser, rs387906558); and c.4148C>T (p.Thr1383Met, rs138498898)[24-26]. We identified two rare functional mutations in the *COL2A1* gene: rs41263847: exon 29: c.1913C>T: p.T638I, exon 28: c.1706C>T: p.T569I, and rs371445823: exon 8: c.580G>A: p.A194T, exon 7: c.373G>A: p.A125T. Exons 6-48 of the *COL2A1* gene encode the core region in the 330-Gly-X-Y triple-helical domain. Previous studies demonstrated that genetic mutations in the triple-helical domain can cause damage to cartilage homeostasis and long bone development. The mutations identified in *COL2A1* in the present study also mapped to this domain and might impair the assembly, folding, intracellular transport, or secretion of the type II collagens in patients with SLE, ultimately resulting in ONFH.

The membrane glyocprotein CR2 binds degraded C3 fragments that are generated during complement activation. In normal immunity, CR2 has many important functions and is believed to play a role in autoimmune disease development. Previous data suggested that rs1876453 in *CR2* affects gene regulation and decreases susceptibility to lupus[27]. The nonsynonymous *CR2* SNP rs17615 in exon 10 (G/A, Ser639Asn) is a conserved SNV in sheep, rats, and mice, and might affect CR2 receptor function. This mutation is associated with an increased risk of ONFH in Korean patients with SLE, possibly through impairing the normal expression of CR2[6]. In the present study, we found a nonsynonymous SNV in *CR2*: rs45573035: exon 2: c.200C>G: p.T67S. This variation was predicted to be possibly damaging and polymorphism damaging. Thus, these mutations in exon 2 might change the function of CR2 and increase the susceptibility of patients with SLE to ONFH.

With the aid of predictive bioinformatics tools, we identified four possible pathogenic variants. Even though the size of the patient group was small, we are the first to use next generation sequencing data to identify SNVs of *CR2*, *NOS3*, *COL2A1*, *PTPN22*, and *TRPV4* genes in patients with SLE and ONFH. Based on bioinformatic studies, we identified mutations of *NOS3* (exon 6: c.814G>A: p.E272K and exon 7: c.814G>A: p.E272K), COL2A1 (rs41263847: exon 29: c.1913C>T: p.T638I, exon 28: c.1706C>T: p.T569I, and rs371445823: exon 8: c.580G>A: p.A194T, exon 7: c.373G>A: p.A125T) and *CR2* (rs45573035: exon 2: c.200C>G: p.T67S) that are likely to be associated with the development of ONFH in SLE. These findings may have important pharmacogenetic implications. However, the detailed mechanisms of the associations need to be determined in further studies.

Ethics approval and consent to participate
All the procedures that involved human participants were performed according to the ethical standards of the institutional and/or national research committee and with the

Table 2 Low frequency functional mutations

Gene	First priority	SNV count	Sample count	Mutation (0|1|2)	Mutation 1	GENESKY Control DB SNV Count	GENESKY Control DB Mutation (0|1|2)	P1	P2	P3	P4
NOS3	Third	1	1	44|1|0	G19	14	203|15|2	0.399	0.3257	1	0.2235
COL2A1	First1	2	4	45|4|0	G34, G42, G6, G4	10	193|26|1	0.6773	0.6202	1	0.4848
CR2	Second	1	1	48|1|0	G2	5	209|11|0	0.6625	0.7007	1	0.7038

CR2: Complement C3d receptor 2; NOS3: Nitric oxide synthase 3; COL2A1: Collagen type II alpha 1 chain; SNV: Single nucleotide variants. First priority: Take the highest priority of SNVs if the mutation is dominant or homozygous; take the lower one from the top two highest riority SNVs if it is a heterozygous recessive pattern. SNV COUNT: The number of loci contained in the gene. Sample count: The number of samples containing mutaed genes. Mutation (0|1|2): The number of mutations; 0 indicates no mutation, 1 indicates one mutation, and 2 represents at least two mutations; Control: GENESKY database containing 220 samples. $P1$: The P value for the 2 × 3 χ^2 test of the sample numbers from the case and control groups with 0, 1, and 2 or more mutations, respectively. $P2$: The P value for the 2 × 3 χ^2 test of the sample numbers from the case and control groups with 0, 1, and 1 times or more mutations, respectively. $P3$: The P value for the 2 × 2 χ^2 test of the sample numbers from the case and control groups with 0 or 1, and 2 times or more mutations, respectively. $P4$: The P value for the 2 × 2 χ^2 test of the sample numbers from the case and control groups with 0 or 1, and 1 times or more mutations, respectively. Mutation 1: Only 1 mutation in the gene.

1964 Helsinki declaration and its later amendments or comparable ethical standards. All individual participants included in the study provided informed consent.

Table 3 Single nucleotide variant information for low frequency functional mutations

Gene	NOS3	COL2A1	COL2A1	CR2
First priority	Third	Second	First1	Second
SNP ID		rs41263847	rs371445823	rs45573035
Ref allele	G	G	C	C
Alt allele	A	A	T	G
Chrs	7	12	12	1
Position	1.51E+08	48377898	48390360	2.08E+08
Strand orientation	+	−	−	+
Gene region	Exonic	Exonic	Exonic	Exonic
Function	Nonsynonymous SNV	Nonsynonymous SNV	Nonsynonymous SNV	Nonsynonymous SNV
SIFT score	0.128	0.24	0.061	0.958
SIFT Score Pred	T	T	T	T
POLYPhen V2 Score	0.845	0.356	0.938	0
POLYPhen V2 Score pred	P	B	P	B
MutationTaster	1	1	1	1
MutationTaster Pred	D	D	D	N
Cadd	3.677832	2.954573	3.523652	−1.25999
Dann	0.999	0.995	0.996	0.129
Eigen	0.1917	−0.0992	0.1764	−1.5893
Kaviar_20150923		0.002186	1.29E-05	0.00066
1000g_chbs		0.0203		0.0254
esp6500		0.000077	0.000077	
ExAC03		0.0026	1.65E-05	0.0007
ExAC03_EAS		0.0307	0	0.0097

CR2: Complement C3d receptor 2; NOS3: Nitric oxide synthase 3; COL2A1: Collagen type II alpha 1 chain.

Table 4 Demographics of patients with systemic lupus erythematosus with osteonecrosis of the femoral head

	Patients (*n* = 49)	G19	G4	G6	G34	G42	G2
Age (yr)	34.1 ± 11.2	36	34	45	36	33	44
Sex (female/male)	45/4	Female	Female	Female	Female	Female	Female
Disease duration of SLE (mo)	61.8 ± 49.8	48	48	204	168	84	72
Disease duration of ONFH (mo)	14.2 ± 18.8	1	2	2	24	1	48
Fever, *n* (%)	4 (8.2)	N	N	Y	Y	N	N
Skin rashes, *n* (%)	28 (57.1)	Y	Y	N	N	N	Y
Photosensitivity, *n* (%)	8 (16.3)	N	Y	N	N	N	N
Raynaud phenomenon, *n* (%)	11 (22.4)	N	N	Y	N	Y	N
Oral ulcer, *n* (%)	5 (10.2)	N	N	Y	N	N	N
Arthritis, *n* (%)	37 (75.5)	Y	N	Y	Y	Y	Y
Polyserositis, *n* (%)	12 (24.5)	N	Y	N	N	N	N
Interstitial Pneumonia, *n* (%)	8 (16.3)	N	N	N	N	N	N
Renal disorder, *n* (%)	21 (42.9)	N	N	N	N	N	Y
Neurological disorder, *n* (%)	6 (12.2)	N	N	N	N	N	N
Anemia, *n* (%)	27 (55.1)	N	Y	Y	N	Y	N
Thrombocytopenia, *n* (%)	6 (12.2)	N	N	N	N	Y	N
Leukopenia, *n* (%)	8 (16.3)	N	N	Y	N	N	N
dsDNA, *n* (%)	28 (57.1)	N	N	Y	N	N	N
AnuA, *n* (%)	25 (51.0)	N	N	Y	N	N	N
Smith, *n* (%)	19 (38.8)	N	N	N	N	N	N
AHA, *n* (%)	16 (32.7)	N	N	Y	N	N	N
rRNP, *n* (%)	8 (16.3)	N	N	Y	N	N	N
ESR (mm/h), *n* (%)	46 (83.7)	21	16	34	76	24	59

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Low C3, *n* (%)	11 (22.4)	N	N	N	N	N	N
Low C4, *n* (%)	10 (20.4)	N	N	N	N	N	N
24-hour urine protein *n* (%)	32 (65.3)	N	N	N	N	N	N
SLEDAI	1-21 (8.9 ± 4.1)	6	4	8	5	5	10

AHA: Antihistone antibody; AnuA: Antinucleosome antibody; C3: Complement 3; C4: Complement 4; dsDNA: Anti-double-stranded DNA antibody; ESR: Erythrocyte sedimentation rate; rRNP: Antiribosome ribonucleoprotein antibody; SLE: Systemic lupus erythematosus; SLEDAI: Systemic lupus erythematosus disease activity index; Smith: Anti-Smith antibody; Y: Yes or positive; N: No or negative. Except where otherwise indicated, values are expressed as the mean ± standard deviation.
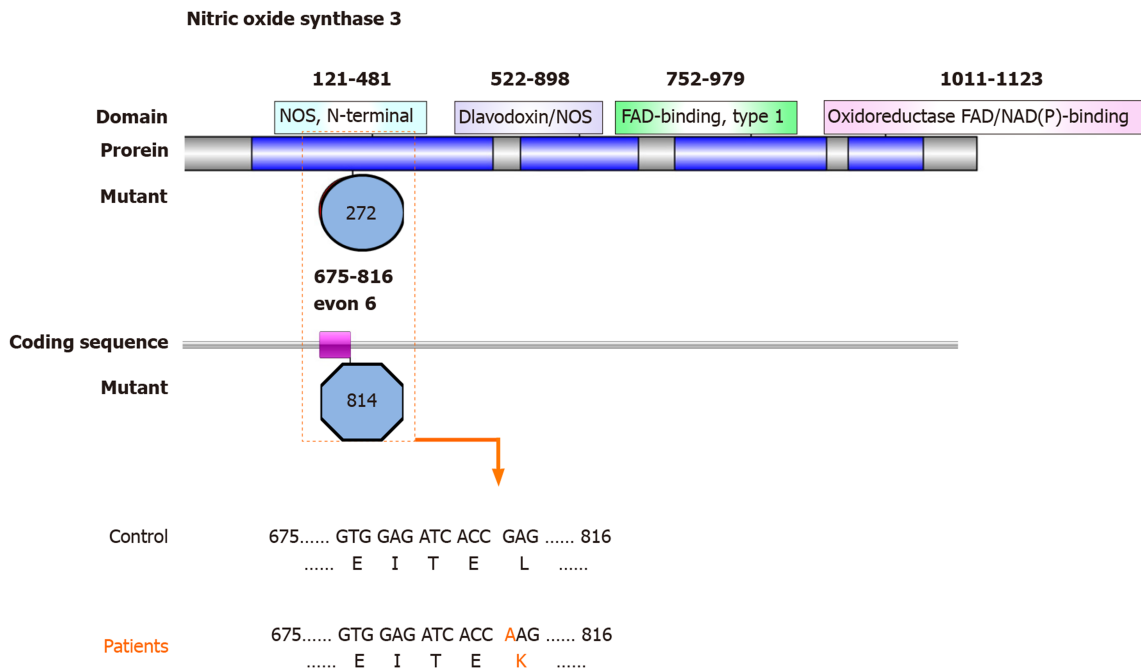
Figure 1 Map showing the nitric oxide synthase N-terminal domain with nitric oxide synthase 3 mutations identified in patients with osteonecrosis of the femoral head in systemic lupus erythematosus. Top: Diagram of the nitric oxide synthase 3 (NOS3) protein structure. Nitric oxide synthase (NOS) comprises a NOS N-terminal domain (amino acids 121-481), a flavodoxin/NOS domain (amino acids 522-698), a flavin adenine dinucleotide-binding, type 1 domain (amino acids 752-979), and an oxidoreductase flavin adenine dinucleotide/nicotinamide adenine dinucleotide (P)-binding domain (amino acids 1011-1123). Middle: The novel mutation of the *NOS3* gene coding sequence identified in this study, located in exon 6 (encoding amino acids 675-816). Bottom: Mutated nucleotides in exon 6 of *NOS3* are shown in orange. Mutated amino acids in the NOS N-terminal domain of NOS3 are shown in orange. FAD-binding: Flavin adenine dinucleotide-binding; NAD: Nicotinamide adenine dinucleotide.

Collagen type II alpha 1 chain
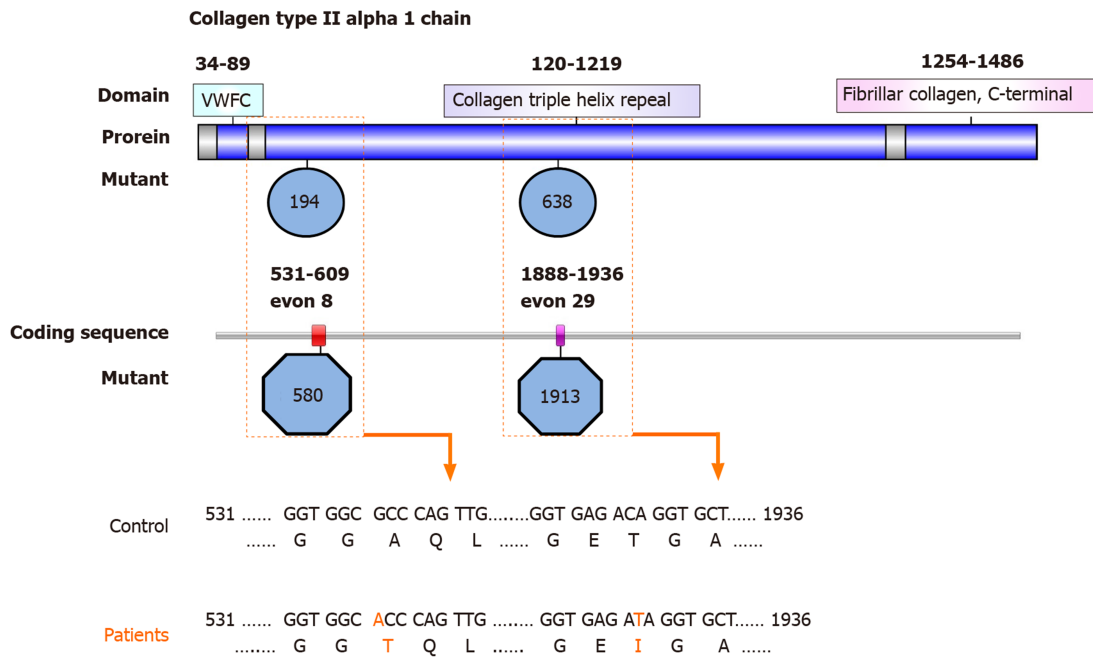
Figure 2 Map showing the collagen triple helix repeat domain with collagen type II alpha 1 chain mutations identified in patients with osteonecrosis of the femoral head in systemic lupus erythematosus. Top: Diagram of the collagen type II alpha 1 chain (COL2A1) protein structure. The COL2A1 comprises a von Willebrand Factor C (VWFC) domain (amino acids 34-89), a collagen triple helix repeat domain (amino acids 120-1219), and a fibrillar collagen C-terminal domain (amino acids 1254-1486). Middle: The novel mutations identified in the *COL2A1* gene coding sequence in this study are located in exon 8 (encoding amino acids 531-609) and exon 29 (encoding amino acids 1888-1936). Bottom: Mutated nucleotides in exons 8 and 29 of the *COL2A1* gene are shown in orange. Mutated amino acids in the VWFC and the collagen triple helix repeat domains of COL2A1 are shown in orange.
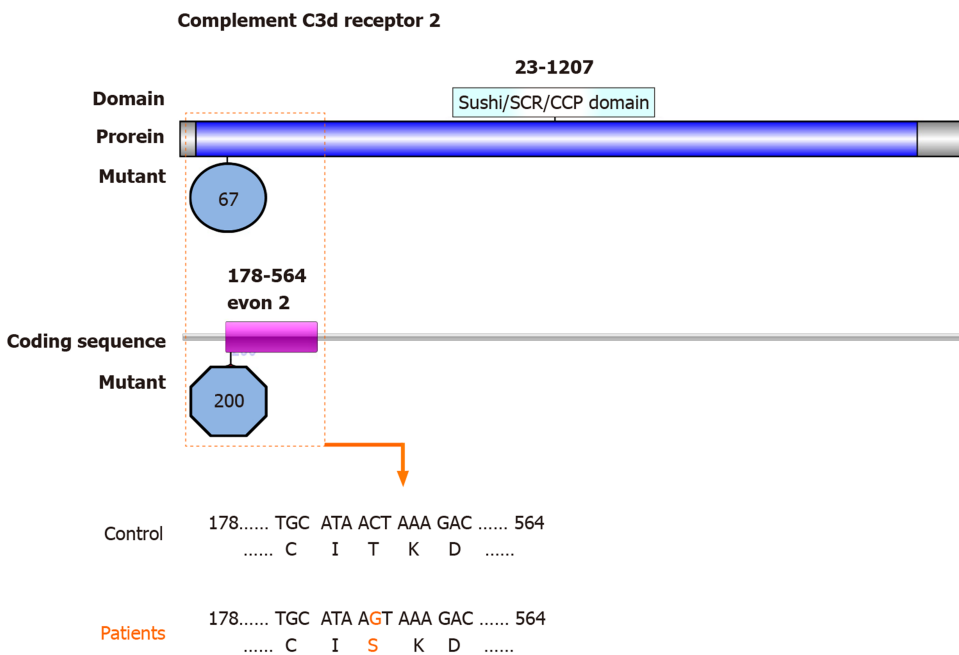
Figure 3 Map of the Sushi/short consensus repeat/complement control protein domain of complement C3d receptor 2 with mutations identified in patients with osteonecrosis of the femoral head in systemic lupus erythematosus. Top: Diagram of the complement C3d receptor 2 (CR2) protein structure. CR2 comprises a Sushi/short consensus repeat/complement control protein domain (amino acids 23-1027). Middle: The novel mutation identified in the *CR2* gene coding sequence in this study is located in exon 2 (encoding amino acids 178-564) Bottom: Mutated nucleotides in exon 2 of *CR2* are shown in orange. Mutated amino acids in the Sushi/short consensus repeat/complement control protein domain of CR2 are shown in orange. CCP: Complement control protein; SCR: Short consensus repeat.

ARTICLE HIGHLIGHTS

Research background

Previous publications indicated that genetic predisposition might play important roles in the onset of osteonecrosis of the femoral head (ONFH) in systemic lupus erythematosus (SLE).

Research motivation

Complement C3d receptor 2 (*CR2*), nitric oxide synthase 3 (*NOS3*), collagen type II alpha 1 chain (*COL2A1*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), and transient receptor potential cation channel subfamily V member 4 (*TRPV4*) were reported to be involved in the onset of ONFH in SLE.

Research objectives

To investigate whether the risk of ONFH in SLE is associated with single nucleotide variations (SNVs) in *CR2*, *NOS3*, *COL2A1*, *PTPN22*, and *TRPV4*.

Research methods

SNVs in the *CR2*, *NOS3*, *COL2A1*, *PTPN22*, and *TRPV4* genes were examined by using FastTarget and Illumina Miseq sequencing technologies in 49 cases of SLE with ONFH. Burrows–wheeler aligner was used to align the sequencing reads to hg19, and GATK and Varscan programs were used to perform SNV calling. PolyPhen-2, SIFT, and MutationTaster were used to assess the functional effects of non-synonymous SNVs.

Research results

Six patients were confirmed to have low frequency SNVs, including one patient with SNVs in *NOS3* (exon 6: c.814G>A: p.E272K and exon 7: c.814G>A: p.E272K.), four in *COL2A1* (rs41263847: exon 29: c.1913C>T: p.T638I, exon 28: c.1706C>T: p.T569I, and rs371445823: exon 8: c.580G>A: p.A194T, exon 7: c.373G>A: p.A125T), and one in *CR2* (rs45573035: exon 2: c.200C>G: p.T67S).

Research conclusions

The onset of ONFH in SLE might be associated with the identified SNVs in *NOS3, COL2A1,* and *CR2*. And the low frequency functional mutations in *NOS3* had never been reported previously.

Research perspectives

These findings may have important pharmacogenetic implications. But the detailed mechanisms of the associations need to be determined in further studies.

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