Human Molecular Genetics, 2022, Vol. 31, No. 7 1082–1095

https://doi.org/10.1093/hmg/ddab306
Advance Access Publication Date: 1 December 2021
Bioinformatics Article

BIOINFORMATICS ARTICLE

Novel susceptibility loci for steroid-associated osteonecrosis of the femoral head in systemic lupus erythematosus

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Received: July 17, 2021. **Revised:** October 14, 2021. **Accepted:** October 15, 2021

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Abstract

Osteonecrosis of the femoral head (ONFH) involves necrosis of bone and bone marrow of the femoral head caused by ischemia with unknown etiology. Previous genetic studies on ONFH failed to produce consistent results, presumably because ONFH has various causes with different genetic backgrounds and the underlying diseases confounded the associations. Steroid-associated ONFH (S-ONFH) accounts for one-half of all ONFH, and systemic lupus erythematosus (SLE) is a representative disease underlying S-ONFH. We performed a genome-wide association study (GWAS) to identify genetic risk factors for S-ONFH in patients with SLE. We conducted a two-staged GWAS on 636 SLE patients with S-ONFH and 95 588 non-SLE controls. Among the novel loci identified, we determined S-ONFH-specific loci by comparing allele frequencies between SLE patients without S-ONFH and non-SLE controls. We also used Korean datasets comprising 148 S-ONFH cases and 37 015 controls to assess overall significance. We evaluated the functional annotations of significant variants by *in silico* analyses. The Japanese GWAS identified 4 significant loci together with 12 known SLE susceptibility loci. The four significant variants showed comparable effect sizes on S-ONFH compared with SLE controls and non-SLE controls. Three of the four loci, *MIR4293/MIR1265* [odds ratio (OR) = 1.99, *P*-value = 1.1×10^{-9})], *TRIM49/NAALAD2* (OR = 1.65, *P*-value = 4.8×10^{-8}) and *MYO16* (OR = 3.91, *P*-value = 4.9×10^{-10}), showed significant associations in the meta-analysis with Korean datasets. Bioinformatics analyses identified *MIR4293, NAALAD2* and *MYO16* as candidate causal genes. *MIR4293* regulates a PPARG-related adipogenesis pathway relevant to S-ONFH. We identified three novel susceptibility loci for S-ONFH in SLE.

Introduction

Osteonecrosis of the femoral head (ONFH) involves necrosis of bone and bone marrow of the femoral head caused by ischemia (1). A Japanese nationwide study estimated that 11 400 patients developed ONFH in 2004, with an incidence of 2.51 per 100 000 person-years (2). The annual number of patients seeking medical care increased by 1.5 times in 2004 compared with 1994 (3). Hip pain from femoral head collapse in the progressive stage of ONFH is often the initial subjective symptom of the disease. Femoral head collapse usually produces gait disturbance and patients often require surgery, including total hip arthroplasty or joint-preserving procedures (4). ONFH often manifests in people aged from their 30s to 50s. Because these young-to-middle-aged patients frequently require surgical interventions, the disease has socioeconomic impacts. Consequently, there is a huge demand for determination of the elucidating disease mechanism and development of non-surgical treatments and predictive tools for its onset.

Steroid intake is the most prevalent risk factor for ONFH (one-half of patients have history of steroid administration), followed by excessive alcohol consumption (2). Systemic lupus erythematosus (SLE) is the major underlying disease (33.1%) for steroid-associated ONFH (S-ONFH) (2). Nevertheless, two-thirds of SLE patients with steroid administration did not develop ONFH (5,6), strongly suggesting that factors other than steroid administration also contribute to the disease onset.

Genetic factors are involved in ONFH, but only a few associated loci have been established. A Chinese nationwide epidemiological survey revealed that family history increased the risk of ONFH [odds ratio (OR) = 5.33, $P < 0.0001$)] (7). Many genetic analyses have focused on particular candidate genes or families (8–13). A genome-wide association study (GWAS) is a useful approach to identify genetic risk factors for complex diseases by comprehensively searching for association signals across genomes in a hypothesis-free manner. Previous GWASs for ONFH identified significant loci around *GRIN3A* (14), *BMP7*, *LINC00251*, *PROX1-AS1* (15) and *CACNA1E* (16). However, the studies involved very small cohorts (~400 cases) and did not produce consistent associations. We previously performed a GWAS for ONFH and identified two significant loci: 12q24 and 20q12 (17). Rs3858704 on 12q24 was significantly associated with alcohol consumption and alcohol-associated ONFH (OR = 2.73, *P*-value = 1.23×10^{-21}), while rs6038718 on 20q12 was significantly associated with ONFH regardless of risk factors and was suggestively associated with S-ONFH with a distinct effect size (OR = 1.12, *P*-value = 6.84×10^{-7}). These findings indicate that ONFH has multiple phenotypes with distinct genetic backgrounds.

Here, we conducted a two-staged GWAS on 636 SLE patients with S-ONFH [SLE_ON(+)] and 95 588 controls in Japan and conducted a subsequent meta-analysis using 148 SLE_ON(+) and 37 015 controls in Korea (Supplementary Material, Table S1, Fig S1). We also analyzed SLE patients without S-ONFH [SLE_ON(−)] to confirm S-ONFH-specific associations rather than associations with SLE susceptibility.

Results

GWAS for SLE_ON(+) in Japan

We conducted first GWAS on 436 SLE_ON(+) and 63 726 BioBank Japan (BBJ) controls which is composed on 47 common diseases (18,19) and conducted the second GWAS on 200 SLE_ON(+) and 31 862 BBJ that passed quality control (QC) in Japanese population. Then, we conducted meta-analysis. For these studies, we used very stringent criteria for cases, namely, patients who were diagnosed as ONFH by X-rays or MRI and as SLE by the 1982 or 1997 American College of Rheumatology (ACR) criteria (20,21), to ensure that the cases had common pathology and thus genetic backgrounds. We took the approach of comparing cases with BBJ controls rather than with SLE_ON(−) because the limited number of definitive controls (MRI was essential to exclude ONFH) restricted the statistical power of the study. Thus, we took advantage of data for definitive controls to confirm the associations, as shown later. As a result, we identified 16 significant loci (Fig. 1, Table 1). The genomic control inflation factor showed very slight inflation of chi-square statistics (lambda: 1.047) and the linkage disequilibrium score regression (LDSC) analysis indicated minimal bias in the results (estimated mean χ^2: 1.0575 and intercept: 1.012) (22). Therefore, we did not apply genomic control correction.

The association results apparently contained statistics for susceptibility to SLE and susceptibility to SLE_ON(+). We hypothesized that susceptibility to SLE_ON(+) is distinct from susceptibility to SLE and excluded 12 significant loci which were previously reported as SLE-susceptibility loci. As a result, we identified four remaining significant loci: *RREB1* on 6p24.3 (OR = 3.11, *P*-value = 1×10^{-8}), *MIR4293/MIR1265* on 10p13 (OR = 2.1, *P*-value = 2×10^{-9}), *TRIM49/NAALAD2* on 11q14.3 (OR = 1.78, *P*-value = 1.2×10^{-8}) and *MYO16* on 13q33.3 (OR = 4, *P*-value = 2.8×10^{-10}) (Table 1). Their ORs were quite comparable between first and second GWAS. While we did not find systemic inflation of statistics (extreme case–control imbalance would result in false-positives, especially for rare variants by violating assumptions of logistic regression model), we confirmed the association signals by firth logistic regression and down-sampling (case–control ratio of 1:19) analyses (Supplementary Material, Tables S2 and S3). Their effect sizes and *P*-values are consistent with logistic regression model.

We evaluated the associations between these four variants and SLE by referring to our recent meta-analysis in Asian populations (23). As a result, regardless of the very large sample size of the latest meta-analysis, we did not

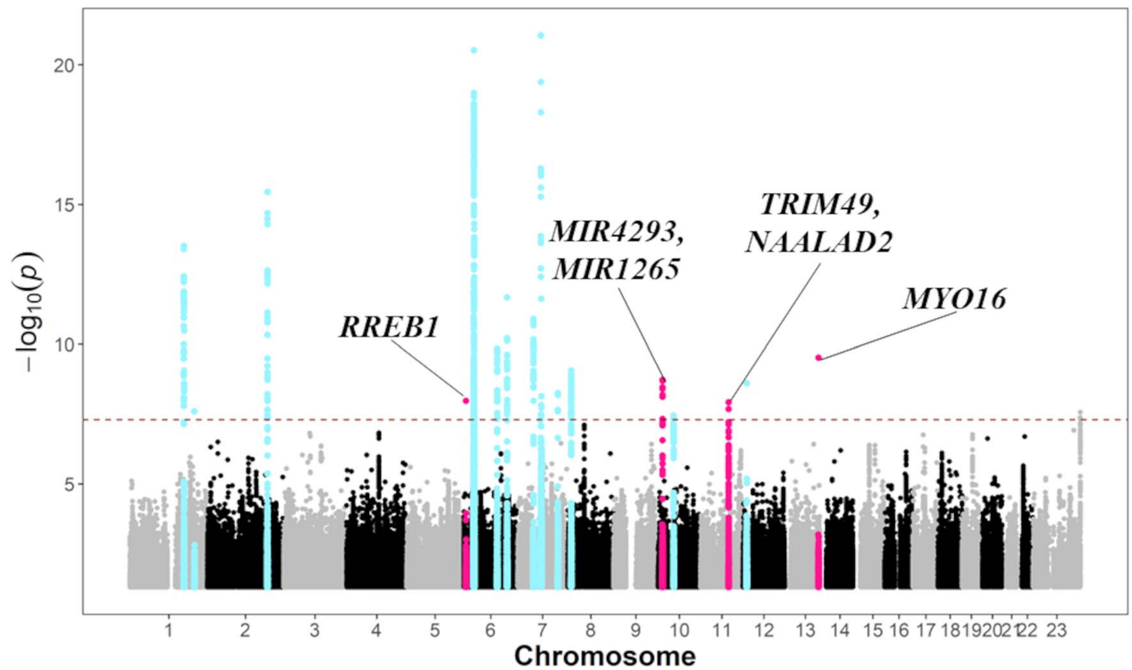

Figure 1. Manhattan plot of the GWAS comprising 636 SLE patients with S-ONFH and 95 588 BBJ controls. X- and Y-axes indicate genomic positions and minus $\log_{10}$-transformed association P-values, respectively. Dashed line shows the genome-wide significant threshold ($P < 5 \times 10^{-8}$). Red and blue plots indicate SLE_ON(+)-associated loci and known SLE susceptibility loci, respectively. The nearest genes are shown in SLE_ON(+)-associated loci.

find even trends for associations between the variants and susceptibility to SLE [ORs for nearby 1 being <1 in contrast to ORs ≥ 1.78 in SLE_ON(+)], indicating that these associations were not explained by SLE susceptibility (Supplementary Material, Table S4, Supplementary Material, Fig S2). To further confirm that these loci were associated with SLE_ON(+), we evaluated the associations of the four variants by comparing 636 SLE_ON(+) and 683 SLE_ON(−) or 95 588 BBJ controls, assuming similar allele frequencies between the BBJ controls and SLE_ON(−) patients. We conducted GWAS on the first study composing of 436 SLE_ON(+) and 377 SLE_ON(−) and conducted GWAS on the second study of composing 200 SLE_ON(+) and 306 SLE_ON(−) and conducted meta-analysis for these two GWASs (Supplementary Material, Table S5). All four variants had comparable effect size with association study between SLE_ON(+) and BBJ (or rather enhanced associations in terms of ORs) and P-values satisfying the nominal threshold (P-value < 0.0018) (Supplementary Material, Table S5, Fig. 2A–D). We conducted GWAS on the first study composing of 377 SLE_ON(−) and 64 103 BBJ controls and conducted the second study composing of 306 SLE_ON(−) and 31 862 BBJ controls and also meta-analysis. As a result, we confirmed the lack of associations between the four variants and SLE_ON(−) by comparing 683 SLE_ON(−) and 95 588 BBJ controls (Supplementary Material, Table S5). Conditional analyses showed no additional signals (data not shown).

Statistical power analysis for Japanese GWAS

We evaluated the statistical power for this study. The association study between SLE_ON(+) and BBJ controls had >99% power to identify variants with minor allele frequency (MAF) of 0.01 and OR of 4.02 and with MAF of 0.1 and OR of 1.87, with significance at P-value $= 5 \times 10^{-8}$ (Fig. 2E). This statistical power was not obtained when SLE_ON(−) patients were used as controls (Fig. 2E). Because there were no significant variants with MAF between 0.1 and 0.5 (after excluding SLE-associated variants), common variants (MAF > 0.1) with large effects (OR > 1.87) on SLE_ON(+) were unlikely to be present.

Association study in Korean subjects and meta-analysis with Japanese GWAS

We took advantage of Korean data for 148 SLE_ON(+) and 37 015 population controls to assess consistent associations of the four variants (24). Although rs7911501 on *MIR4293/MIR1265* (OR = 1.52) and rs372605131 on *TRIM49/NAALAD2* (OR = 1.14) are not significant (P > 0.05), they showed the same direction for their effects as the Japanese study (Supplementary Material, Table S6). Especially, rs7911501 had almost the same effect size (OR = 1.61) in association between SLE_ON(+) and SLE_ON(−), and opposite direction of effect size (OR = 0.9) in association between SLE_ON(−) and population control, which was the same trend as the Japanese study (Supplementary Material, Table S6). We noted that rs145720245 was too rare in Koreans (MAF = 0.005) to find even a single heterozygote in the limited number of case subjects assuming a similar effect size. Rs2714333 on *RREB1* did not show even a trend of the same direction of association as the Japanese study (Supplementary Material, Table S6).

When we evaluated overall significance in a meta-analysis of the two datasets composing 784 SLE_ON(+) and 132 603 controls, rs7911501 on *MIR4293/MIR1265* (OR = 1.99, P-value $= 1.1 \times 10^{-9}$), rs372605131 on *TRIM49/NAALAD2* (OR = 1.65, P-value $= 4.8 \times 10^{-8}$) and rs145720245 on *MYO16* (OR = 3.91, P-value $= 4.9 \times 10^{-10}$) showed genome-wide significance (Table 2). We did not observe heterogeneity among the three studies (P-heterogeneity > 0.05). However, rs2714333 on *RREB1* did not satisfy genome-wide significance. Thus, we regarded *MIR4293/MIR1265*, *TRIM49/NAALAD2* and *MYO16* loci as statistically significant. To further evaluate potential shared genetic components among ONFH, in general,

Table 1. Significant loci in two-staged GWAS in 636 SLE_ON(+) and 95 588 BBJ controls

CHR POS	EA NEA	Gene	Function	Meta-analysis		First GWAS			Second GWAS			R2	Novel or known
				OR [95%CI]	P-value	EAF case control	OR [95%CI]	P-value	EAF case control	OR [95%CI]	P-value		
SLE_ON(+)-associated loci													
6	T	*RREB1*	Intronic	3.11	1×10^{-8}	0.032	3.34	2×10^{-7}	0.027	2.57	0.014	0.46	Novel
7 236 620	C			[2.11–4.59]		0.016	[2.12–5.26]		0.015	[1.22–5.45]			
10	A	*MIR4293*	Intergenic	2.1	2×10^{-9}	0.056	2.12	4.6×10^{-7}	0.055	2.05	0.0011	1	Novel
14 436 836	G	*MIR1265*		[1.65–2.67]		0.028	[1.58–2.83]		0.027	[1.33–3.16]			
11	C	*TRIM49*	Intronic	1.78	1.2×10^{-8}	0.128	1.77	3.1×10^{-6}	0.13	1.8	0.0011	0.66	Novel
89 538 685	T	*NAALAD2*		[1.46–2.16]		0.091	[1.39–2.24]		0.092	[1.27–2.56]			
13	A	*MYO16*	Intronic	4	2.8×10^{-10}	0.019	3.86	3.5×10^{-7}	0.019	4.32	1.9×10^{-4}	0.73	Novel
109 491 871	G			[2.6–6.15]		0.006	[2.29–6.49]		0.006	[2.01–9.31]			
SLE susceptibility loci													
1	T	*TNFSF4*	Intergenic	1.62	3.7×10^{-14}	0.264	1.59	2.1×10^{-9}	0.277	1.69	3.2×10^{-6}	1	Known
173 191 475	G	*LOC100506023*		[1.43–1.84]		0.183	[1.37–1.85]		0.182	[1.35–2.11]			
1	A	*RASSF5*	Intronic	3.66	9.8×10^{-9}	0.022	4.37	8.8×10^{-9}	0.013	1.96	0.16	0.76	Known
206 735 148	C			[2.35–5.71]		0.007	[2.64–7.22]		0.007	[0.76–5.06]			
2	T	*STAT4*	Intronic	1.6	3×10^{-16}	0.411	1.62	4.3×10^{-12}	0.405	1.57	1.4×10^{-5}	1	Known
191 943 742	C			[1.43–1.79]		0.302	[1.41–1.86]		0.306	[1.28–1.92]			
6	A	*HLA-DQA1*	Intergenic	2.19	4.3×10^{-21}	0.209	2.1	4.2×10^{-13}	0.226	2.4	1.3×10^{-9}	0.58	Known
32 614 963	G	*HLA-DQB1*		[1.86–2.58]		0.144	[1.72–2.56]		0.143	[1.81–3.18]			
6	A	*PRDM1*	Intergenic	1.47	1.7×10^{-10}	0.372	1.48	7×10^{-8}	0.373	1.44	6×10^{-4}	0.94	Known
106 564 236	G	*ATG5*		[1.3–1.65]		0.291	[1.28–1.71]		0.293	[1.17–1.78]			
6	T	*TNFAIP3*	Intronic	1.93	1.3×10^{-12}	0.113	1.9	1.1×10^{-8}	0.113	2.01	2.6×10^{-5}	0.91	Known
138 190 533	C			[1.61–2.32]		0.065	[1.52–2.37]		0.064	[1.45–2.77]			
7	A	*C7orf72*	Intergenic	1.49	1.1×10^{-11}	0.612	1.48	2.6×10^{-8}	0.625	1.5	9.3×10^{-5}	0.99	Known
50 307 334	G	*IKZF1*		[1.33–1.67]		0.517	[1.29–1.7]		0.518	[1.22–1.84]			
7	A	*NCF1*	Intronic	3.42	1.2×10^{-22}	0.099	2.7	7.3×10^{-10}	0.13	4.91	1.6×10^{-15}	0.36	Known
74 196 185	C			[2.67–4.37]		0.065	[1.97–3.7]		0.065	[3.32–7.26]			
7	T	*KCP*	Intergenic	1.59	3.9×10^{-9}	0.158	1.6	6.4×10^{-7}	0.154	1.56	0.0016	0.99	Known
128 575 797	TCTTAG[illegible]TTGCTC	*IRF5*		[1.36–1.85]		0.105	[1.33–1.92]		0.107	[1.18–2.05]			
8	T	*BLK*	Intronic	1.55	7.8×10^{-10}	0.777	1.56	2.2×10^{-7}	0.767	1.51	9.3×10^{-4}	0.95	Known
11 354 097	C			[1.35–1.78]		0.69	[1.32–1.85]		0.691	[1.18–1.94]			
10	T	*WDFY4*	Intronic	1.36	3.8×10^{-8}	0.484	1.4	8.6×10^{-7}	0.467	1.29	0.011	1	Known
50 076 325	G			[1.22–1.52]		0.406	[1.22–1.6]		0.406	[1.06–1.57]			
12	G	*CDKN1B*	3'UTR	2.64	2.4×10^{-9}	0.96	2.57	1.4×10^{-6}	0.961	2.81	4.3×10^{-4}	0.84	Known
12 874 462	A			[1.92–3.63]		0.918	[1.75–3.76]		0.917	[1.58–4.99]			

SLE_ON(+), SLE with S-ONFH; CHR, chromosome; POS, position (hg19); EA, effect allele; NEF, non-effect allele; EAF, effect allele frequency.
R^2 shows imputation quality.

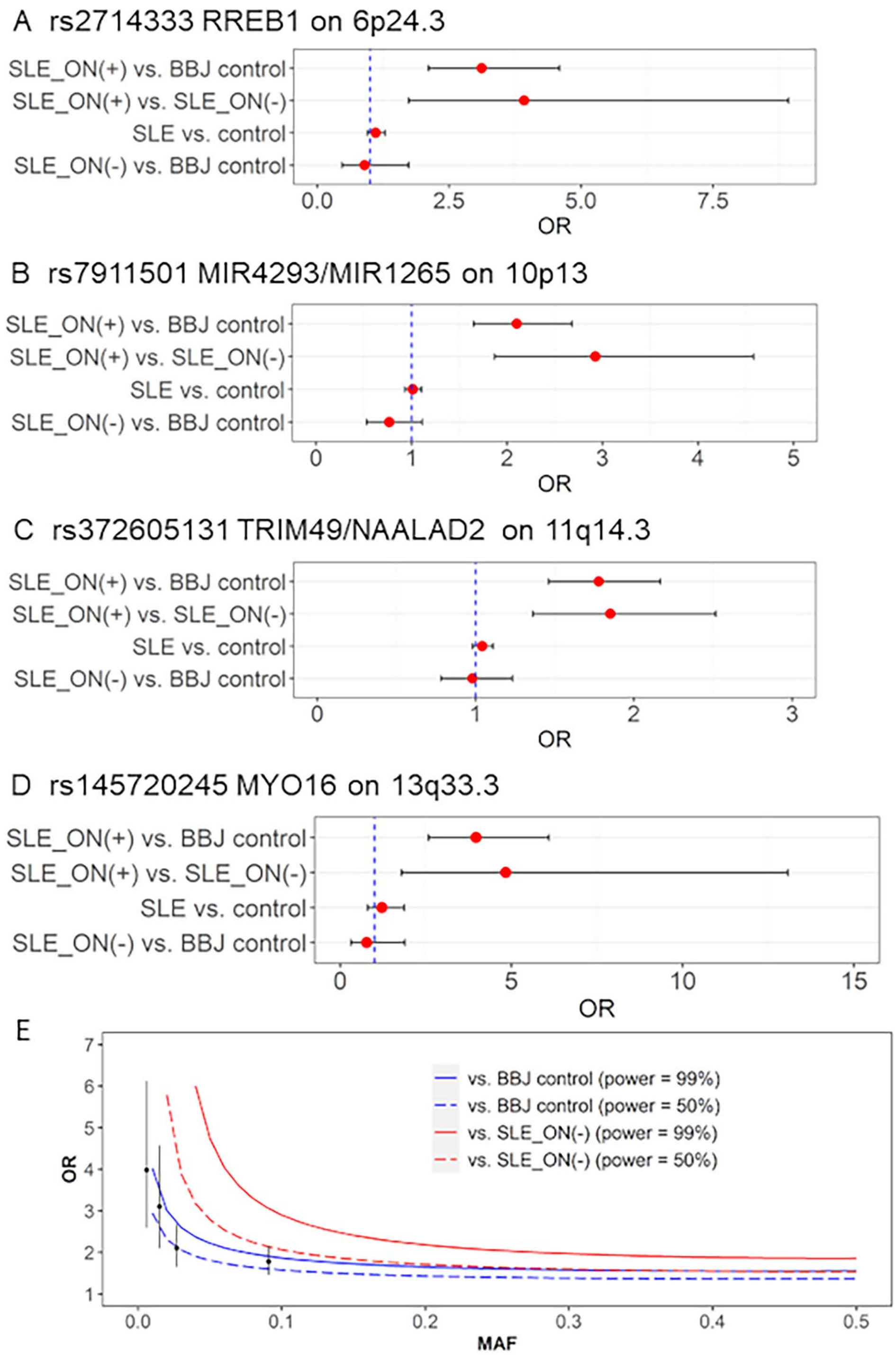

Figure 2. Associations of four S-ONFH associated loci in SLE. (**A–D**) The four association results in Japan, namely, SLE_ON(+) versus BBJ controls, SLE_ON(+) versus SLE_ON(−), SLE versus controls (23) and SLE_ON(−) versus BBJ controls, are shown for four SLE_ON(+)-associated variants. The numeric data are shown in Supplementary Material, Table S2. The statistical analysis for GWAS using SLE_ON(+) and SLE_ON(−) or BBJ controls is shown in (**E**). X- and Y-axes indicate MAFs and ORs, respectively. Alpha-error rate and statistical power are set to 5×10^{-8} and 0.99 or 0.5, respectively. Red and blue lines show statistical power of association studies of 636 SLE_ON(+) compared with 683 SLE_ON(−) and 95 588 BBJ controls, respectively. Solid and dashed lines show statistical power of 0.99 and 0.5, respectively. Plots and bars indicate ORs and 95% confidence intervals (CIs) of SLE_ON(+)-associated variants in current Japanese study, respectively.

we evaluated associations of the three significant variants with idiopathic ONFH (I-ONFH) which have neither excessive alcohol consumption nor steroid intake (see Materials Methods). As a result, all three variants showed comparable directions of effects with SLE_ON(+) (Supplementary Material, Table S7).

Identification of potentially causal genes

We identified sets of 95% credible variants by fine-mapping in *MIR4293/MIR1265* and *TRIM49/NAALAD2* locus (Supplementary Material, Table S8) by using the FINEMAP software (25). As a

Table 2. Meta-analysis of two GWASs comprising 784 SLE_ON(+) and 132 603 controls in Japan and Korea

CHR	POS	rsID	EA/NEA	Gene	Function	EAF	OR [95%CI]	P-value	Phet
Significant in the meta-analysis									
10	14 436 836	rs7911501	A/G	*MIR4293/MIR1265*	Intergenic	0.028	1.99 [1.59–2.48]	1.1×10^{-9}	0.56
11	89 538 685	rs372605131	C/T	*TRIM49/NAALAD2*	Intronic	0.089	1.65 [1.38–1.98]	4.8×10^{-8}	0.21
13	109 491 871	rs145720245	A/G	*MYO16*	Intronic	0.006	3.91 [2.54–6]	4.9×10^{-10}	0.34
Not significant in the meta-analysis									
6	7 236 620	rs2714333	T/C	*RREB1*	Intronic	0.015	2.76 [1.9–4]	8.4×10^{-8}	0.1

SLE_ON(+), SLE with S-ONFH; rsID, reference single-nucleotide polymorphism ID; Phet, P heterogeneity among Japanese 2 studies and Korean study.
Rs372605131 locates in intron of *TRIM49*; however, it is an eQTL of *NAALAD2*.

result, *TRIM49/NAALAD2* locus had a candidate causal variant [rs372605131; posterior probability (PP) > 0.5], on the other hand, *MIR4293/MIR1265* did not (PP < 0.154), because of the LD structure. (*MYO16* locus did not have significant variants <1 Mb from the lead variant (rs145720245).) We checked the functional annotations of rs145720245 on *MYO16* locus, rs372605131 on *TRIM49/NAALAD2* locus and a set of credible variants on *MIR4293/MIR1265* and tried to investigate potentially causal genes in three significant loci (Fig. 3A–C). As a result, these variants did not include missense or loss-of-function mutations. In *TRIM49/NAALAD2* locus, eQTL analysis using GTEx v8 (26) revealed that rs372605131 was significantly associated with increased *NAALAD2* expression in the tibial artery (Fig. 4A and B, Supplementary Material, Fig S3, Supplementary Material, Table S9). Though most of GTEx v8 consist of European descent (26), Spearman's rank correlation coefficient analyses showed correlated association signals (Spearman's rank correlation = 0.438) and Locus compareR showed suggestive colocalization signal (PP.H4 = 38.3%) between GWAS and eQTL regardless of different LD structures between populations, suggesting that eQTL of *NAALAD2* in the tibial artery might be colocalized with the SLE_ON(+) association signal (Fig. 4C). Because other novel loci had no variants with evidences of missense, loss-of-function or eQTL, we regarded *MYO16* and *MIR4293*, the nearest genes from the lead or a set of 95% credible variants, as potentially causal genes (Fig. 3A and C).

Pathway enrichment analysis for target genes of MIR4293

MIR4293 is a type of microRNA and is considered to regulate other genes (27). We identified 111 target genes with target score ≥ 80, which are likely regulated by *MIR4293* by using miRDB (Supplementary Material, Table S10) (28). *MIR4293* regulates *SREBF2* in the sterol regulatory element-binding protein (SREBP) signaling pathway, which includes *SREBP2* as a previously reported S-ONFH susceptibility gene by candidate gene analysis (9). Moreover, we conducted pathway enrichment analysis for 111 target genes by using FUMA (29). As a result, we found that a PPARG-related adipogenesis pathway, a relevant pathway for S-ONFH (30,31), was significantly enriched in the target genes regulated by *MIR4293* (Supplementary Material, Table S11). Meanwhile, 47 genes regulated by *MIR1265*, another gene near the variant, did not show enrichment of relevant pathways.

LDSC-heritability enrichment analysis

We found some types of helper T cells and hematopoietic cell groups with strong enrichment of heritability in SLE_ON(+) [SLE_ON(+) vs. BBJ controls, Supplementary Material, Tables S12 and S13] by LDSC software with baseline model version2.2 (32). However, strong enrichment of SLE heritability in these cell types and cell groups could apparently explain the findings (33). Similarly, we did not find SLE_ON(+)-specific significant genetic correlations between SLE_ON(+) [SLE_ON(+) vs. BBJ controls] and 99 other traits (59 complex traits) (34) (40 diseases) (35)) (Supplementary Material, Table S14).

Discussion

The majority of ONFH patients have no evident cause of ischemia, such as decompression sickness (36), sickle cell disease (37) or trauma. How steroid administration leads to ischemia remains unknown. Many genetic studies have been performed on S-ONFH, but no definite susceptibility genes have been identified. One possible reason for the lack of established genetic loci for S-ONFH is that S-ONFH has various underlying diseases, including SLE, polymyositis/dermatomyositis, nephrotic syndrome and mixed connective tissue disease, and that the genetic backgrounds of these diseases can influence GWAS results. Another reason could be the low power of previous studies. Difficulty in recruiting S-ONFH cases and controls with harmonized backgrounds, especially from the viewpoint of underlying diseases, has made it difficult to identify consistent associations with S-ONFH.

In this study, we addressed two problems in GWAS for ONFH. First, we focused on S-ONFH cases stringently restricted to SLE patients. Second, we took the approach to use controls irrespective of their backgrounds (disease-mixed controls; a common strategy for GWAS (18,19)) in the initial stage to maximize statistical power and showed comparable effect sizes of significant variants using definitive controls with the same backgrounds as cases. As a result, three loci showed significant GWAS signals in the meta-analysis of the Japanese and Korean studies. Our study could serve as a model to identify susceptibility loci for intra-disease phenotypes. In the current study, the information of steroid dosage is not available. Since the steroid dosage tends to be related to SLE_ON(+) occurrence, whether the genetic risk of SLE_ON(+) is modulated by steroid dosage should be evaluated in detail in future studies.

Though the statistical power analysis showed that our association study should identify variants with MAF > 0.1 and OR > 1.87, our study did not identify such significant variants. This result indicates that common variants (MAF > 0.1) with large effect for SLE_ON(+), which may serve as biomarkers and predictive markers, are unlikely to be present as far as SLE_ON(+) associations are distinct from those for SLE susceptibility. It is therefore necessary to collect further samples and to identify common variants with a weak effect size or rare variants with modest to strong effect size.

We conducted association study for three significant variants of SLE_ON(+) on I-ONFH and BBJ controls to evaluate whether

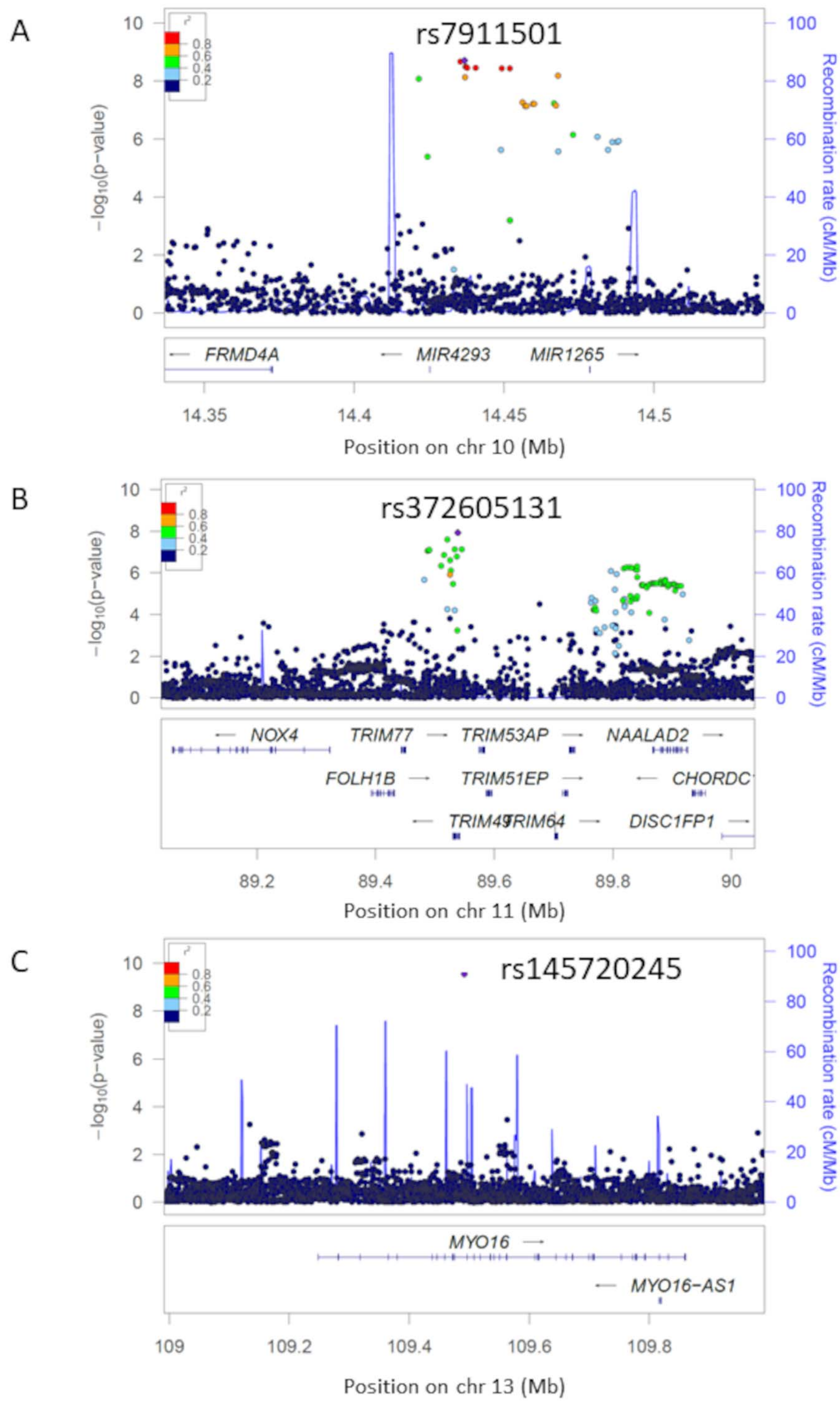

Figure 3. Regional plots of SLE_ON(+) susceptibility loci. Regional plots of SLE_ON(+) susceptibility loci. *X*- and *Y*-axes indicate genomic positions and minus $\log_{10}$-transformed association *P*-values, respectively. Colors of dots show strength of linkage disequilibrium (*R*-square) with the lead variant in each locus. (**A**) *MIR4293*/*MIR1265* region on 10p13. (**B**) *TRIM49*/*NAALAD2* region on 11q14.3. (**C**) *MYO16* region on 13q33.3.

these variants have effect to other type of ONFH without SLE. As a result, all of variants shared effects between I-ONFH and SLE_ON(+). This result suggests that SLE_ON(+) and I-ONFH have partially common genetic background and pathology and may be compatible with the previous study reporting ONFH in some SLE patients without steroid administration and excessive alcohol consumption (38). We should increase sample sizes of SLE_ON(+) and I-ONFH to replicate the current findings in future studies. Moreover, because current data are composed on East Asian population, we attempted to replicate significant variants on UK BioBank (UKBB) data. However, UKBB did not have the data of ONFH. In the future, we should conduct a trans-ethnic meta-analysis among multiple populations.

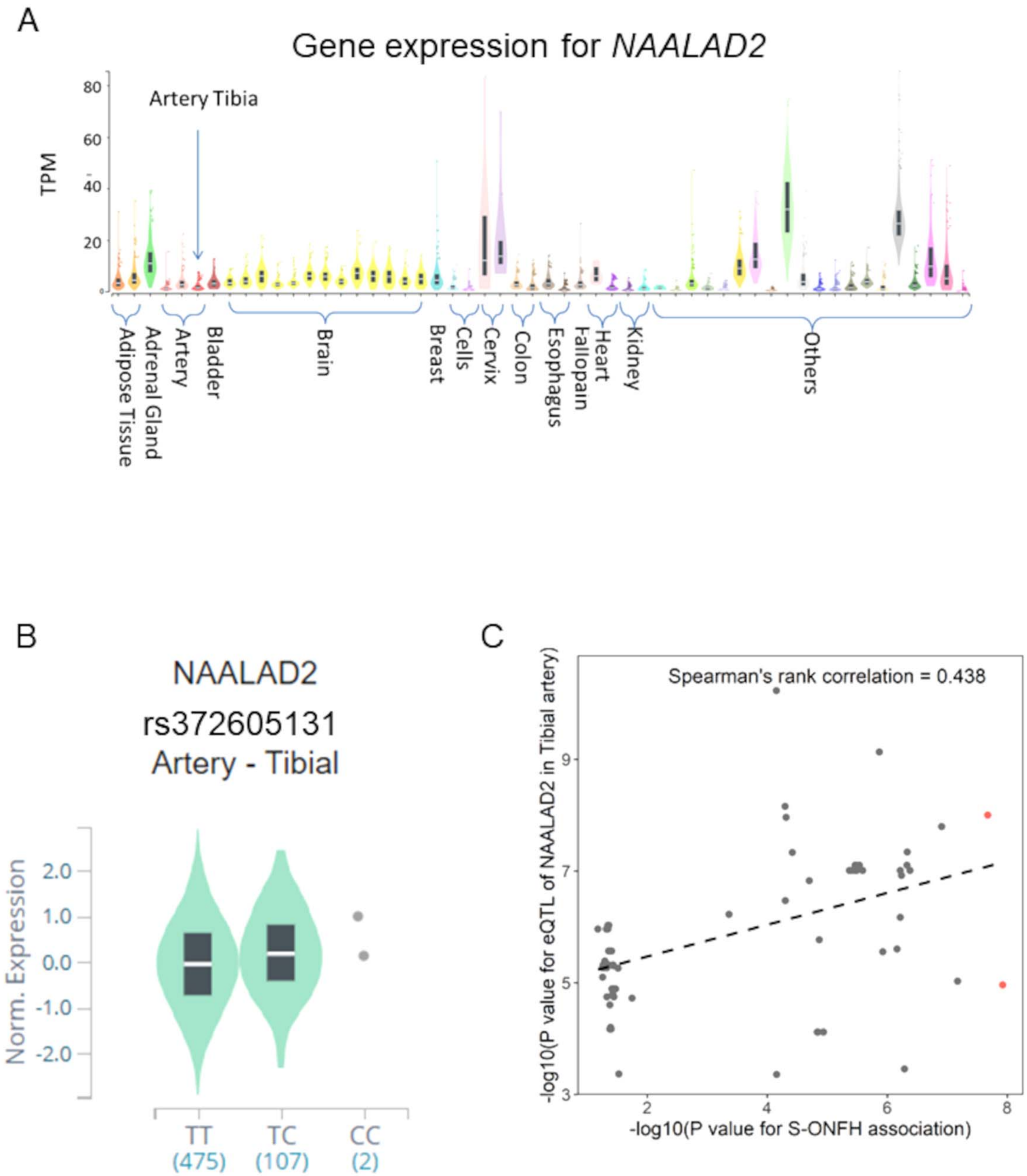

Figure 4. Functional annotation for SLE_ON(+) susceptibility locus on 11q14.3. (**A**) Expression levels of *NAALAD2* in 54 tissues within GTEx v8. X- and Y-axes show groups of tissues and transcripts per million, respectively. Box plots show median and 25th and 75th percentiles. (**B**) Violin plot of *NAALAD2* expression levels in the tibial artery for each genotype of rs372605131. X- and Y-axes show haplotype and normalized gene expression levels, respectively. Box plots show median and 25th and 75th percentiles. (**C**) Scatter plot of variants in the susceptibility locus. X-axis is minus $\log_{10}$-transformed association *P*-values for the association study between SLE_ON(+) and BBJ controls in Japan, and Y-axis is minus $\log_{10}$-transformed eQTL *P*-values for *NAALAD2* in GTEx v8 in the tibial artery. Red dots indicate significant variants in the association study ($P < 5 \times 10^{-8}$). Dashed line indicates the regression line.

We identified *MIR4293*, *NAALAD2* and *MYO16* as candidate causal genes for SLE_ON(+). *MIR4293* regulates *SREBF2* in the lipid metabolism pathway, including *SREBP2*, a previously reported S-ONFH susceptibility gene (9). Moreover, pathway enrichment analysis indicated that the 111 genes regulated by *MIR4293* were significantly enriched in a PPARG-related adipogenesis pathway. *PPARG* was reported as a susceptibility gene for ONFH (16,30). A previous study indicated that glucocorticoid administration induced promotive differentiation of adipocytes and increased intraosseous pressure in steroid-treated rabbits (31). As a result, the femoral head of the rabbits exhibited osteonecrosis. From these results, we concluded that *MIR4293* is a plausible candidate causal gene for SLE_ON(+). There were no available data for *MIR4293* in GTEx v8, and we were unable to evaluate the eQTL effect of the lead variant on *MIR4293*. To clearly verify that *MIR4293* is associated with SLE_ON(+), it is necessary to investigate gene expression and eQTL in ONFH-relevant tissues by *in vitro* or *in vivo* assays. Although most previous candidate gene analyses focused on protein-coding genes, recent transcriptome studies demonstrated that S-ONFH was correlated with non-coding RNAs, such as microRNAs (39,40) and long-non-coding RNAs (41). These results appear compatible with the present and previous findings. Actually, our previous GWAS (17) for ONFH identified, as a susceptibility gene, long non-coding RNA 01370 whose regulatory function to lipid-metabolism pathway was suggested in *in silico* analyses. *NAALAD2* is a member of the N-acetylated alpha-linked acidic dipeptidase gene family and is ubiquitously expressed in tissues. A previous genetic study demonstrated that *NAALAD2* was associated with basophil count (42). However, its detailed function was not revealed. We found an eQTL association of the lead variant with *NAALAD2* in the tibial artery. The eQTL of *NAALAD2* may share an effective variant with GWAS signals (Spearman's correlation = 0.438, PP.H4 = 38.3%). High expression of *NAALAD2* in arteries may increase the risk of SLE_ON(+). However, the colocalization signal is not very convincing. Since most of eQTL data are derived from European subjects, further colocalization analysis by using East Asian eQTL data and functional analyses in arterial tissues should be conducted to confirm the association. *MYO16* is a member of the myosin superfamily and it may have an important role in neural development, although the detailed function has not yet been revealed (43). The allele frequency of rs145720245 is quite low, and the accuracy of the association of this locus should be evaluated in further samples. In conclusion, we identified three susceptibility loci for SLE_ON(+) and three candidate causal genes. Our approach will be useful for the detection of loci associated with intra-disease phenotypes. The present findings suggest that common variants with large effects on SLE_ON(+) are unlikely. We must collect further samples to identify susceptibility genes for SLE_ON(+) and elucidate the biological mechanisms underlying SLE_ON(+) and other types of ONFH.

Materials and Methods

Japanese subjects in the GWAS

We collected a total of 648 SLE_ON(+) and 697 SLE_ON(−) patients with SLE diagnosed by the 1982 (20) or 1997 ACR criteria (21). SLE_ON(+) was defined as ONFH findings on X-rays or MRI. SLE_ON(−) was defined as no ONFH findings on MRI at >6 months after starting steroid administration because almost all ONFH occurs within 3 months of steroid administration (5,6). Clinical information and peripheral blood for SLE_ON(+) and SLE_ON(−) patients were collected in 32 hospitals across Japan from 2012 to 2019. We used genome-wide screening data in BBJ controls for subjects with 47 common diseases as controls (18,19). After excluding SLE- and S-ONFH-related diseases, like autoimmune diseases and osteoporosis, data for 95 588 subjects were used as BBJ controls. Written informed consent was obtained from all patients and/or their guardians. The study was approved by the Institutional Review Board of Kyushu University, RIKEN and from all participating institutes for sample collection. All methods were carried out in accordance with relevant guidelines and regulations.

QC in Japanese GWAS

Genomic DNA was extracted from peripheral blood using a standard protocol. Genotyping was performed by Illumina HumanOmniExpressExome, HumanOmniExpress or HumanExome BeadChips (Supplementary Material, Table S1).

For QC of subjects, subjects with call rate < 0.98, high-degree relatedness with other subjects and outliers of East Asian ethnicity were excluded. For QC of variants, variants with call rate < 0.99, *P*-value for Hardy–Weinberg equilibrium $< 1.0 \times 10^{-6}$ and minor allele count < 10 were excluded (Supplementary Material, Table S1).

Imputation in Japanese GWAS

The reference panel for imputation was composed of 1000 Genomes Project phase 3 (version 5) (44) and Japanese whole-genome sequence data (Flaganan *et al*., manuscript in preparation) with 3256 high-depth subjects (≥30 read counts) and 4216 low-depth subjects (≤15 read counts). We performed pre-phasing using EAGLE2.4.1 (https://alkesgroup.broadinstitute.org/Eagle/) to determine the haplotypes. Genotypes were imputed using Minimac4 (version1.0.0) (45). After imputation, variants with MAF < 0.005 and low imputation quality ($R^2 < 0.3$) were excluded (Supplementary Material, Table S1).

GWAS in Japanese subjects

We used SLE_ON(+) samples collected from 2012 to 2016 as first GWAS as we previously published (17) and from 2017 to 2019 as second GWAS. The BBJ control samples were used for the first and second studies roughly proportionally to the case subjects. GWAS was conducted by logistic regression with the top 10 principal components and sex (only for variants in autosome) as covariates using PLINK2.0 software (46) for first and second GWAS, separately. For meta-analysis comprising first and second GWAS, an inverse-variance-based method was performed by METAL (version2011-03-25) (47). Variants satisfying *P*-value $< 5 \times 10^{-8}$ were regarded as significant for SLE_ON(+). We regarded multiple significant loci as independent when they were apart from each other at least 1Mbp. We conducted conditional analyses for all significant variants conditioned for a lead variant in the same locus using GCTA-COJO (version1.93.1) (48). Variants with *P*-value $< 5 \times 10^{-8}$ in the conditional analyses were regarded as independent from the lead variant. To distinguish confounding bias from polygenic effects, we performed LDSC with LD scores from East Asian population of 1000 Genomes Project descendants using LDSC software (version1.0.0) (22). Variants were restricted to Hapmap3 (49) as reliable variants for the test. An intercept nearby 1 indicated that inflation of the genomic control inflation factor was not derived from bias, but from polygenicity. We performed firth logistic regression analysis for significant variants with current sample size and down-sampled controls (case–control ratio of 1:19) by using PLINK2.0 software (50,51).

Comparison of effect size in with association studies using SLE_ON(−)

We excluded regions within 1Mbp from the significant variants (*P*-value $< 5 \times 10^{-8}$) in known SLE susceptibility loci using GWAS catalog (https://www.ebi.ac.uk/gwas/), GWASkb (http://gwaskb.stanford.edu) and significant loci in our recent SLE international meta-analysis (23) from significant loci. GWAS catalog was obtained on 13 November, 2020. We regarded remaining variants as candidates for SLE_ON(+) susceptibility. We compared the allele frequencies of candidate variants between SLE_ON(+) and SLE_ON(−) or BBJ controls. We conducted the association study for SLE_ON(+)-associated variants between SLE_ON(−) and SLE_ON(+) or BBJ using the same approach of the SLE_ON(+) GWAS (we took the two-staged GWAS depending on collection dates for SLE subjects, Supplementary Material, Fig S1). Meta-analysis of the first and second studies was conducted by METAL.

Statistical power analysis

We evaluated the statistical power of our datasets [SLE_ON(+) vs. BBJ controls and SLE_ON(+) vs. SLE_ON(−)] using the Genpwr package (version1.0.2) in R software as an additive model (https://cran.r-project.org/web/packages/genpwr/index.html). We set the type 1 error rate and statistical power to 5×10^{-8} and 0.99 or 0.5, respectively.

Korean association study and meta-analysis with Japanese GWAS

We conducted association study using Korean data to assess consistent associations of the SLE_ON(+) associated variants in East Asian populations. We collected 148 SLE_ON(+) and 1158 SLE_ON(−) in Hanyang University Hospital for Rheumatic Diseases (Seoul, Korea). Subjects were diagnosed as SLE by the 1997 ACR criteria (21). SLE_ON(+) was defined as ONFH findings on X-rays or MRI. SLE_ON(−) was defined as no hip pain. We used 37 015 samples in KoGES and the Hanyang University Hospital for Rheumatic Diseases database as population controls (24). Written informed consent was obtained from all patients and/or their guardians. The study was approved by the Institutional Review Board of Hanyang University Hospital. The procedures for genotyping, QC and imputation were described previously (24).

We focused on significant variants in the Japanese study and evaluated their associations in the Korean dataset using (1) 148 SLE_ON(+) and 37 015 population controls, (2) 148 SLE_ON(+) and 1158 SLE_ON(−) and (3) 1158 SLE_ON(−) and 37 015 population controls. We conducted logistic regression analysis with the top 10 principal components and sex (for only variants in autosome) as covariates using EPACTS software (v3.2.6 or 3.3; http://genome.sph.umich.edu/wiki/EPACTS) (52). Because Korean SLE_ON(+) and SLE_ON(−) genotyping data were composed of two datasets, the association studies were further adjusted for the datasets. For the meta-analysis comprising SLE_ON(+) and controls in Japan and Korea, an inverse-variance-based method was performed by METAL (version2011-03-25) (47). *P*-heterogeneity among Japanese 2 studies and Korean study was evaluated using Cochran Q test and PLINK1.90 software (46). Finally, variants satisfying *P*-value $< 5 \times 10^{-8}$ were regarded as significant for SLE_ON(+).

Evaluation for effect of significant variants to I-ONFH

We evaluated the associations of significant variants with 127 I-ONFH (17) cases using the same BBJ controls and statistical framework we used in SLE_ON(+) analysis.

Evaluation of significant associations and potentially causal genes

For each significant locus, we conducted bioinformatics analysis to identify potentially causal genes. At first, to identify candidates of causal markers, we performed fine-mapping analyses calculating PP of being causal variants using the FINEMAP software (25). LD structure of reference panel restricting to Japanese whole-genome sequence data and 1000 Genomes Project phase 3 (version 5) (44) was used for the analyses. We analyzed the two regions (the *TRIM49/NAALAD2* and *MIR4293/1265* regions) containing multiple GWAS significant variants in the current study. We regarded a variant as causal if it showed PP > 0.5. If none of variants showed PP > 0.5, a 95% credible set of variants was generated. We functionally annotated the causal or a 95% credible set of variants by using Annovar (version: 2017-07-17) (53). If the locus has no another significant variants, the lead variants is functionally annotated. We analyzed whether these variants were missense or loss-of-function variants. Then, we checked whether these variants have been reported as significant eQTL in 49 tissues in GTEx v8 (where >80% of its subjects are of European descent) (26). We checked the colocalization signal between eQTL and GWAS by Spearman's rank correlation and Locus CompareR (54). If annotated variants were missense, loss-of-function or eQTL variants for surrounding genes, we regarded the genes as potentially causal genes. If not, we regarded the nearest genes from the lead variants as potentially causal genes.

Pathway enrichment analysis for target genes of microRNA

In general, a microRNA downregulates several genes by binding to their 3′UTRs (27). If microRNA was identified as potentially causal gene, we investigated genes regulated by microRNA using miRDB (version 6.0) (28), a computational prediction tool that searches for microRNA target genes. We defined genes satisfying target score ≥80 as target genes, representing reliable microRNA-regulated genes. To evaluate the pathway in which target genes are accumulated, we performed pathway enrichment analysis for target genes using FUMA (version 1.3.6) (29) and regarded pathways satisfying false discovery rate (FDR) < 0.05 as significant.

LDSC-heritability enrichment analysis

Partition heritability enrichment analysis for SLE_ON(+) was conducted in 10 specific cell groups and 220 combinations of cell types and histone marks using LDSC software with baseline model version 2.2 (32). Genetic correlation analyses between SLE_ON(+) and 99 other traits (59 complex traits (34) and 40 diseases (35)) were conducted using LDSC software (22). For both partition heritability enrichment and genetic correlation analysis, Japanese GWAS results for SLE_ON(+) and BBJ controls were used as association data for SLE_ON(+), and the significance threshold was set at FDR < 0.05. We defined significant results as SLE_ON(+)-specific heritability enrichment or genetic correlations only if observed in the SLE_ON(+) study and not observed in SLE susceptibility studies (23,33).

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

We acknowledge the participants in this study. We thank Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript. We appreciate members of Japanese Research Committee on Idiopathic Osteonecrosis of the Femoral Head: Yasuharu Nakashima, Kazuyuki Karasuyama, Kazuhiko Sonoda, Yusuke Kubo, Takeshi Utsunomiya, Hiroyuki Hatanaka, Shuji Baba, Koichiro Kawano, Noriko Yamamoto, Yukihide Iwamoto, Satoshi Ikemura and Ryosuke Yamaguchi (Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University), Hiroshi Kataoka and Makoto

Kondo (Department of Rheumatology & Clinical Immunology, Sapporo City General Hospital), Tsuyoshi Asano, Tohru Irie and Norimasa Iwasaki (Department of Orthopaedic Surgery, Faculty of Medicine and Graduate School of Medicine, Hokkaido University), Tatsuya Atsumi (Department of Rheumatology, Endocrinology and Nephrology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University), Shunji Kishida (Department of Orthopaedic Surgery, Seirei Sakura Citizen Hospital), Shigeo Hagiwara (Department of Orthopaedic Surgery, Graduate School of Medicine, Chiba University), Ichiei Narita (Division of Clinical Nephrology and Rheumatology, Kidney Research Center, Niigata University Graduate School of Medical and Dental Sciences), Koichi Akashi (Faculty of Medical Sciences, Kyushu University), Hiroshi Tsukamoto (Shin-Kokura Hospital), Yojiro Arinobu and Hiroki Mitoma (Kyushu University Hospital), Mitsuteru Akahoshi (Faculty of Medicine, Saga University), Masahiro Ayano (Graduate School of Medical Sciences, Kyushu University), Atsuhiro, Yoshiaki Toyama and Atsushi Funayama (Department of Orthopaedic Surgery, Keio University School of Medicine), Kunihiro Yamaoka (Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine), Hironari Hanaoka (Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine), Yasushi , Hisashi Yamanaka, Tetsuji Hosozawa and Shigeki Momohara (Institute of Rheumatology, Tokyo Women's Medical University), Fumio Sekiya and Masakazu Matsushita (Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine), Megumi Matsuhashi and Kazuhide Tanimura (Hokkaido Medical Center for Rheumatic Diseases), Takashi Sakai (Department of Orthopaedic Surgery, Yamaguchi University Graduate School of Medicine), Wataru Ando, Takashi Nishii and Masaki Takao (Department of Orthopaedic Medical Engineering, Osaka University Graduate School of Medicine), Ryosuke Nakanishi (Department of Orthopaedic Surgery, Fujigaoka Hospital, Showa University School of Medicine), Yukiharu Hasegawa (Department of Rehabilitation, Kansai University of Welfare Science), Kazuyoshi Saito (Tobata General Hospital), Kazuhisa Nakano (The First Department of Internal Medicine, University of Occupational and Environmental Health, Japan), Tetsuya Sakamoto (Department of Orthopaedic Surgery, Faculty of Medicine, Fukuoka University), Keiichiro Ueshima (Kyoto Interdisciplinary Institute Hospital of Community Medicine), Yoshitomo Kajino (Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University), Yoshiharu Amasaki (Department of Rheumatology, Kuriyama Red Cross Hospital), Hiroaki Nakamura and Shigekazu Mizokawa (Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine), Shinichiro Kume (Orthopedis and Joint Surgery Center, Kurume Univ. Medical Center), Akihiro Sudo and Masahiro Hasegawa (Department of Orthopaedic Surgery, Mie University Graduate School), Toru Ichiseki (Department of Orthopaedic Surgery, Kanazawa Medical University), Takuma Yamasaki (Department of Orthopaedic Surgery, National Hospital Organization Kure Medical Center and Chugoku Cancer Center).

Conflict of Interest statement. The authors declare no conflicts of interest associated with this manuscript.

Funding

This study was supported in part by the Practical Research Project for Rare/Intractable Diseases from Japan Agency for Medical Research and development, AMED, by RIKEN Junior Research Associate Program (for H.S.), by Grant of Japan Orthopaedics and Traumatology Research Foundation, No. 350 (for Y.S.), by Center for Genome Science, Korea National Institute of Health, Republic of Korea (4845-301, 3000-3031) (for B-J Kim), by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A6A1A03038899) (for S-C.B) and Hanyang University Institute for Rheumatology Research (for S-C.B). The study sponsors had no involvement in this study.

Patient consent for publication

Not required.

Ethics approval

The study was approved by the Institutional Review Boards at RIKEN Center for Medical Sciences and by the Hanyang University Hospital of Rheumatic Diseases.

Data sharing statement

The meta-analysis summary association statistics in the present study are available from the corresponding author on reasonable request.

Authors' contributions

H.S. and K.K. contributed equally to this work. C.T. conceived the study design. S.-C.B. and C.T. acquainted the financial support. H.S., K.K., S.-C.B. and C.T. wrote the manuscript. H.S. and K.K. conducted all of the analyses with the help of M.K., S.I., Y.K. and C.T. T.T., Y.S., N.S., M.M., K.O., D.T., K.K., T.M., J.N., G.M., T.K., H.N., T.M. K.K., S.-Y.B., J.-M.S., J.S.K., Y.-K.L., D.J.P., G.-Y.A., T.T., Y.-C.K., J.K., K.I., K.A., Y.T., K.Y., M.S., T.A., T.S., Y.T., T.K., R.H., T.Y., M.Y., T.K., T.K., K.O., T.O., Y.N., Y.O., A.K., Y.Y., K.O., B.-J.K., H.-S.L., S.I., S.-C.B., and the Japanese Research Committee on Idiopathic Osteonecrosis of the Femoral Head collected case samples and clinical information, or generated genetic data. K.M. provided genetic data of BioBank Japan. T.K. made Japanese reference panel for imputation. S.-C.B. and C.T. managed the cohort data. S.-C.B. and A.S. conducted procedure of Institutional Review Board. All authors reviewed and approved the manuscript.

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