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Genome-wide association study of systemic sclerosis identifies CD247 as a novel susceptibility locus

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#See supplementary notes

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Abstract

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis of the skin and internal organs that leads to profound disability and premature death. To identify novel SSc susceptibility loci we conducted the first genome wide association study (GWAS) in a population of Caucasian ancestry including a total of 2296 SSc patients and 5171 controls. Analysis of 279,621 autosomal single nucleotide polymorphisms (SNPs) followed by replication testing in an independent case-control set of European ancestry (2,753 SSc patients / 4,569 controls) identified a new susceptibility locus for systemic sclerosis at *CD247* (1q22-23; rs2056626, $P = 2.09 \times 10^{-7}$ in the discovery samples, $P = 3.39 \times 10^{-9}$ in the combined analysis). Additionally, we confirm and firmly establish the role of *MHC* (2.31×10^{-18}), *IRF5* ($P = 1.86 \times 10^{-13}$) and *STAT4* ($P = 3.37 \times 10^{-9}$) gene regions as SSc genetic risk factors.

Systemic sclerosis (SSc) is a profoundly disabling autoimmune disease characterized by vascular damage, altered immune responses and abnormal fibrosis of skin and internal organs leading to premature death in affected individuals ¹. SSc etiology is complex and poorly understood, but similar to most autoimmune conditions it is widely accepted that the involvement of environmental and a multiplicity of genetic factors leads to disease. Data from familial, twin and ethnicity studies support the relevance of the genetic component in SSc etiology ². Previous studies aimed at dissecting the genetic factors underlying SSc genetic susceptibility so far have used the candidate gene association study approach ³. In spite of the several years of research this strategy yielded a very limited characterization of SSc genetic risk factors. Except for the major histocompatibility complex (*MHC*) genes, that are relevant genetic markers for SSc across populations, few other loci outside the *HLA* region demonstrated strong and reproducible associations with SSc susceptibility ^{3,4}. Only very recently, large case-control association studies have identified *STAT4* and *IRF5* genes as novel genetic factors contributing to SSc susceptibility ⁵⁻⁸. Similar to other complex genetic disorders it is expected that several genetic markers contribute to SSc predisposition with modest effects, and large sample sizes are required to detect novel disease associated loci ⁹.

Therefore, we aimed more comprehensively to identify novel SSc susceptibility loci and thus conducted the first genome wide association study (GWAS) in SSc including a total of 2296 SSc patients and 5171 healthy controls from four case-control series of Caucasian ancestry (USA, Spain, Germany and The Netherlands) (Supplementary Table 1). Genotyping of SSc case sets and Spanish controls was performed using the Illumina Bead-Array platform with chips of different single nucleotide polymorphism (SNP) densities (Supplementary Table 1). The genotypes of North American controls were obtained from the Cancer Genetic Markers of Susceptibility (CGEMS) studies and Illumina iControlDB database

(www.illumina.com/iControlDB, Illumina, San Diego, CA), German and Dutch control groups were extracted from previous studies or public databases^{10–13}.

After rigorous genotyping quality control filters, a total of 279,621 SNPs shared between the four case-control series were extracted for analysis (Supplementary Table 1).

Genomic inflation factor (λ) was estimated for the complete data set showing evidence of a modest inflation of test statistics ($\lambda = 1.069$). When the *HLA* region was excluded, the inflation of test statistics somewhat decreased ($\lambda = 1.066$) (Supplementary Figure 1). To adjust for potential population stratification we applied a genomic control correction to the test statistics. The potential effect of population substructure was tested by deriving principal components on a population-specific basis. We observed that case and control individuals in each population were not significantly different by principal components and were therefore well genetically matched. We also performed an inverse variance based meta-analysis, adjusting the odds ratios for the first five country-specific principal components. This analysis showed little variation from genomic control corrected *P* values (Table 1).

The Mantel-Haenszel test under an allelic model revealed several SNPs reaching *P* values at genome-wide significance after genomic control correction ($P \leq 5 \times 10^{-7}$) (Figure 1). The strongest association signal was observed for a cluster of SNPs in an extended region at 6p21 locus within the MHC region, where the rs6457617 SNP located in the *HLA*DOB1* gene region gave the highest *P* value (*P* GC corrected = 2.31×10^{-18}) (Figure 1 & Supplementary Table 2). Outside the MHC region, five loci showed association at $P < 10^{-7}$ namely *TNPO3/IRF5* region in 7q32, *STAT4* in 2q32, *CD247* in 1q22-23, *CDH7* in 18q22 and *EXOC2/IRF4* near 6p25. The trend observed for all these loci were consistent across the different study populations (Supplementary table 3). Furthermore, the *TNPO3/IRF5* locus obtained genome wide significance in the single US cohort and was further corroborated in the European cohorts (Supplementary table 3). SNPs mapping to the region of *TNPO3/IRF5* and *STAT4* achieved the strongest association observed for non-HLA genes (rs10488631 $P = 1.86 \times 10^{-13}$ OR 1.50 95 % CI 1.35–1.67 and rs3821236 $P = 3.37 \times 10^{-9}$ OR 1.30 95 % CI 1.18–1.44, respectively) (Table 1 & Supplementary table 3). Therefore, these results confirm the previously reported role of *MHC*, *STAT4* and *IRF5* genes as genetic risk factors for systemic sclerosis and identified three new candidate loci^{3–8}.

We then aimed to confirm the association of the *CD247*, *CDH7* and *EXOC2/IRF4* loci with SSc susceptibility using a large independent replication case-control set comprising 2753 SSc patients and 4569 controls of Caucasian ancestry (Supplementary table 4). The SNPs showing the strongest GWAS association on each region (rs2056626 for *CD247*, rs10515998 for *CDH7* and rs4959270 for *EXOC2/IRF4*) were genotyped in the replication cohorts using TaqMan 5' allelic discrimination assay technology. The association analysis by Mantel-Haenszel test revealed a significant association of the rs2056626 genetic variant in the *CD247* region ($P = 3.07 \times 10^{-3}$ OR 0.89 95 % CI 0.83–0.96) (Table 2 & figure 2). The combined analysis of the GWAS and replication cohort for this SNP revealed highly significant association ($P = 3.39 \times 10^{-9}$ OR 0.86 95 % CI 0.81–0.90). The association of the SNPs in the *CDH7* and *EXOC2/IRF4* regions was not confirmed in this replication cohort (Table 2 and Supplementary figure 2). Considering that the frequency observed for the *CDH7* rs10515998 genetic variant, is quite low (around 5%) the population size of the replication cohort reached only 13% of statistical power to detect an association at a significance level similar to that observed in the replication analysis (OR 1.05). Therefore, the possible implication of the *CDH7* locus in SSc genetic predisposition should be still further investigated. In contrast, due to the high MAF frequency of the rs4959270 polymorphism in the *EXOC2/IRF4* region, great heterogeneity of the association was observed in the replication cohorts (supplementary figure 1). These findings are concordant with previous GWAS studies in which great population

allelic heterogeneity has been reported for *EXOC2/IRF4* genetic variants leading to not true disease associations, as possibly occurred in our screening phase¹⁴. Interestingly, the novel identified SSc susceptibility locus, *CD247*, participates in the regulation of the immune response and thus could have a role in SSc pathogenesis. The *CD247* gene encodes the T-cell receptor zeta (CD3 ζ) subunit, a component of the T cell receptor (TCR)/CD3 complex¹⁵. This chain plays an important role in the assembly and transport of the TCR/CD3 complex to the cell surface and is crucial to receptor signaling function. It has been observed that the expression of the CD3 ζ chain is altered in chronic autoimmune/inflammatory disorders and that its low expression results in impaired immune response^{16–18}. Notably, the *CD247* gene has been associated with susceptibility to systemic lupus erythematosus (SLE), another systemic autoimmune disease^{19,20}. Moreover, genetic variants in the 3' untranslated region of this gene have shown functional implication leading to a reduced expression of this molecule that could be manifested by systemic autoimmunity¹⁹. Therefore, further studies aiming to dissect the exact role of this molecule in SSc will be of interest.

This work represents the first large GWAS study conducted to date in SSc. Of note, the results obtained confirm and firmly establish the role of *HLA*, *STAT4* and *IRF5* in the genetic predisposition to SSc, genes that are also known to be risk factors for several other autoimmune conditions. In addition, a new susceptibility locus not previously considered as susceptibility factors for SSc has been identified. All these findings support the strong autoimmune component underlying SSc pathogenesis and highlight that the development of SSc seems to be determined by shared common genetic and pathogenic mechanisms with other autoimmune diseases and specific disease pathways that should be further characterized.

METHODS

Subjects

Because SSc is a relatively rare autoimmune disorder (estimated prevalence in Caucasian populations ~ 0.01 %) large sets of SSc patients can best be recruited through international collaboration. Consequently, to reach the total of 2296 SSc patients and 5171 healthy control individuals analyzed in the present study, four series with Caucasian European-ancestry were included: USA, Spain, Germany and The Netherlands. The North American cases (initial $n=1,678$; after applying quality control criteria, $n=1486$; 179 men, 1307 women; mean age=54.5 (median, 55.0); SD=12.9) were obtained from May, 2001 to December, 2008 from three U.S. sources: University of Texas (UT) Health Science Center-Houston, The Johns Hopkins University Medical Center and Fred Hutchinson Cancer Center, each enrolling patients from a US-wide catchment area. Whole genome genotyping data from USA control individuals (initial $n=5,520$) were obtained from the following 3 publicly available databases: (1) breast cancer controls from the Cancer Genetic Markers of Susceptibility (CGEMS) studies; (2) prostate cancer controls from CGEMS; and (3) controls from Illumina iControlDB. After sex-matching and applying quality control criteria, 419 men and 3058 women controls were analyzed.

The initial European SSc cases series came from previously established collections with nationally representative recruitment of in total 380 Spanish, 288 German and 190 Dutch SSc patients. Main demographical and clinical data of European SSc patients have been described previously^{5,21}. As control population, healthy unrelated individuals of Spanish (initial $n=414$), German (initial $n=678$) and Dutch (initial $n=643$) origin were included in the study. Whole genome genotyping data from German controls were from the Popgen Biobank and from a previous study in the case of Dutch^{12,13}.

To further confirm associations found on GWAS stage we collected a large independent replication cohort of individuals with Caucasian ancestry from Belgium, Spain, Holland,

Germany, Italy, Norway, Sweden, UK and USA. A total of 2753 SSc patients and 4569 healthy controls were recruited for this second stage (supplementary table 4).

All cases either met the American College of Rheumatology Preliminary criteria for the classification of SSc or had at least 3 of the 5 CREST (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactyly, Telangiectasias) features²². Main clinical features of SSc patients are included in supplementary table 5.

Collection of blood samples and clinical information from case and control subjects was undertaken with informed consent and relevant ethical review board approval from each contributing center in accordance with the tenets of the Declaration of Helsinki.

Genotyping

The GWAS genotyping of the Spanish SSc and controls together with Dutch and German SSc patients was performed at the Department of Medical Genetics of the University Medical Center Utrecht (The Netherlands) using the commercial release Illumina human CNV370K BeadChip that contains 300,000 standard SNPs with an additional 52,167 markers designed to specifically target nearly 14,000 copy number variant (CNV) regions of the genome, for a total of over 370,000 markers. This system delivers high genomic coverage of the SNPs from Phase I and II of the HapMap Project (www.hapmap.org), capturing 81% of the HapMap variation at $r^2 > 0.8$ in Caucasian populations. Genotype data for Dutch and German controls was obtained from the Illumina Human 550K BeadChip available from a previous study^{12, 13}. The North American SSc patient group was genotyped at Boas Center for Genomics and Human Genetics, Feinstein Institute for Medical Research, North Shore LIJ Health System, using the Illumina Human610-Quad BeadChip capturing 89% of the HapMap CEU variation at $r^2 > 0.8$. CGEMS and Illumina iControlDB controls were genotyped on Illumina Hap550K-BeadChip. For the replication phase, SNPs reaching GWAS significance located in novel potential SSc susceptibility loci (rs2056626 for *CD247*, rs10515998 for *CDH7* and rs4959270 for *EXOC2/IRF4*) were genotyped in the replication cohorts using Applied Biosystems' TaqMan SNP genotyping Assays on an Abiprism 7900 HT real-time thermocycler. Markers with call rates of 95% or less were excluded, as were markers whose allele distributions deviated strongly from Hardy-Weinberg equilibrium in controls ($P < 10^{-5}$). Only markers with minor allele frequencies of $\geq 1\%$ in both cases and controls were included in the analyses.

Statistical analysis

Statistical analyses were undertaken using R (v2.6), STATA (v8; State College, Texas, US) and PLINK (v1.06) software (<http://pngu.mgh.harvard.edu/purcell/plink/>)²³. All reported P values are two-sided. Using PLINK we identified and excluded pairs of genetically related subjects or duplicates and excluded pair members with lower call rate. To identify individuals who might have non-Western European ancestry, we merged our case and control data with the data from the HapMap Project (60 western European (CEU), 60 Nigerian (YRI), 90 Japanese (JPT) and 90 Han Chinese (CHB) samples). We used principal component analysis as implemented in HelixTree, plotting the first two principal components for each individual. All individuals who were not clustering with the main CEU cluster (deviating more than 4 SD from cluster centroids) were excluded from subsequent analyses. The principal components derived on the resulting sample look typical for populations of European origin (Supplementary Figure 3)²⁴. Additionally, we excluded individuals with low call rate (11 on US, 24 on Spanish, 1 on German and 1 on Dutch), relatedness (50 on US, 2 on Spanish, 1 on German and 1 on Dutch), non-Caucasian ancestry (42 on US, 5 on Spanish, 6 on German and 4 on Dutch) and inconsistent gender (83 on US, 2 on Spanish, 2 on German and 2 on Dutch). Then we filtered for SNP quality, removing SNPs with less than 98% genotyping success call rate and those showing minor allele frequency below 1%. Deviation of the genotype frequencies in the

controls from those expected under Hardy-Weinberg equilibrium (HWE) was assessed by a χ^2 test or Fisher's exact test when an expected cell count was <5 . SNPs strongly deviating from HW equilibrium ($P < 10^{-5}$) were eliminated from the study. For the combined analysis of the four datasets the same quality controls per individual and per SNP were re-applied except HW equilibrium. The genotyping success call rate on the merged data set after all these quality filters was 99.83% in the GWAS cohorts. In the replication cohorts, genotyping success call rate was 98.16% after quality filters. The association between each SNP and risk of scleroderma in each set of data was assessed by the Cochran-Armitage trend test. Odds ratios and associated 95% CIs were calculated by unconditional logistic regression.

To determine if genome wide significant associated SNPs belonged to extensive linkage disequilibrium (LD) blocks we investigated the LD pattern (using r^2 parameter) on a 1 Mb length region surrounding significant SNPs (Supplementary figures 4A–E). No strong LD ($r^2 > 0.8$) was observed among investigated SNPs and other variants on the region, except rs12537284 which was on LD with rs10488631 ($r^2 = 0.82$) in TNPO3/IRF5 region, both found to be genome wide significantly associated with SSc (table 1). The Meta-analysis of the four study series was conducted using standard methods based on Cochran-Mantel-Haenszel test. Breslow-Day test was performed for all SNPs to assess heterogeneity of the effect in different populations. We tested for the population structure and possibility of differential genotyping of cases and controls using quantile-quantile plots of test statistics and calculated inflation factor λ , by dividing the median of the test statistics by the expected median from a χ^2 distribution with one d.f. There was evidence of modest inflation of the test statistics ($\lambda = 1.069$, or 1.066 after excluding the *HLA* region), indicating a potential effect of the population substructure on the results. We therefore applied a genomic control correction to our results. Alternatively, we also derived principal components on a population-specific basis using HelixTree software, and applied an adjustment for the five first principal components as well as gender, separately for each country using logistic regression, after which the effects for each SNP were combined by meta-analysis, using inverse variance method (corresponding *P*-values are presented in Table 1 and Supplementary table 2). The results from this analysis were consistent with the results from the GC corrected Mantel-Haenszel meta-analysis. We then proceeded analyzing three new SNPs association found on the GWAS screen on the replication cohorts. Data was filtered according to same proceedings as the GWAS stage. Analysis was carried out by Mantel-Haenszel meta-analysis of all the independent replication cohorts to control for differences between groups. We then did meta-analysis of all the replication and GWAS cohorts for these SNPs with the same Mantel-Haenszel statistical procedure. Results are shown in table 2.

Supplementary Material

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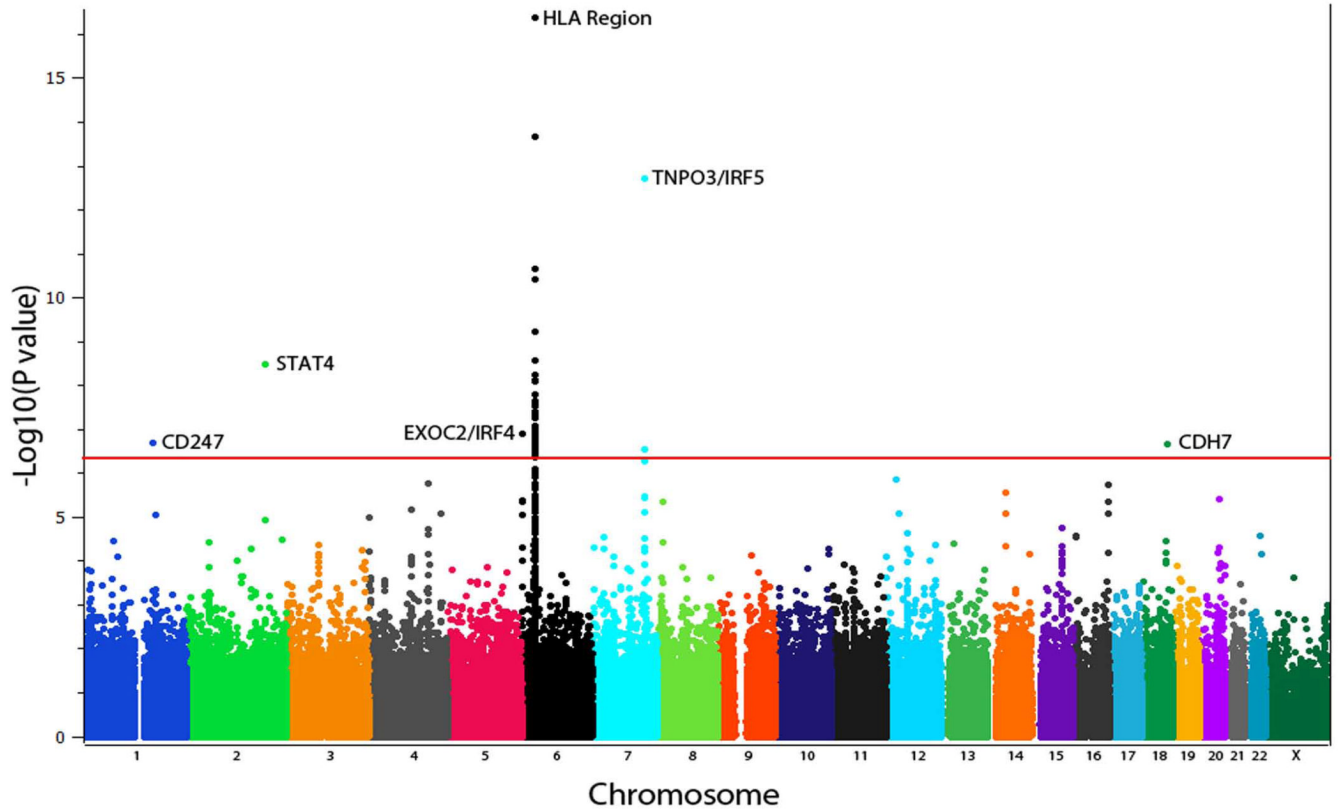


Figure 1.

Manhattan plot of the Genome wide association study of the discovery cohort comprising 2346 SSc patients and 5193 healthy controls. The $-\log_{10}$ of the Mantel-Haenszel test P value of 279.621 SNPs after correction by λ is plotted against its physical chromosomal position. Chromosomes are shown in alternate colors. SNPs above the red line represent those with a P-value of $5 < 10^{-7}$. Plot corresponds to the combined analysis of the study cohorts.

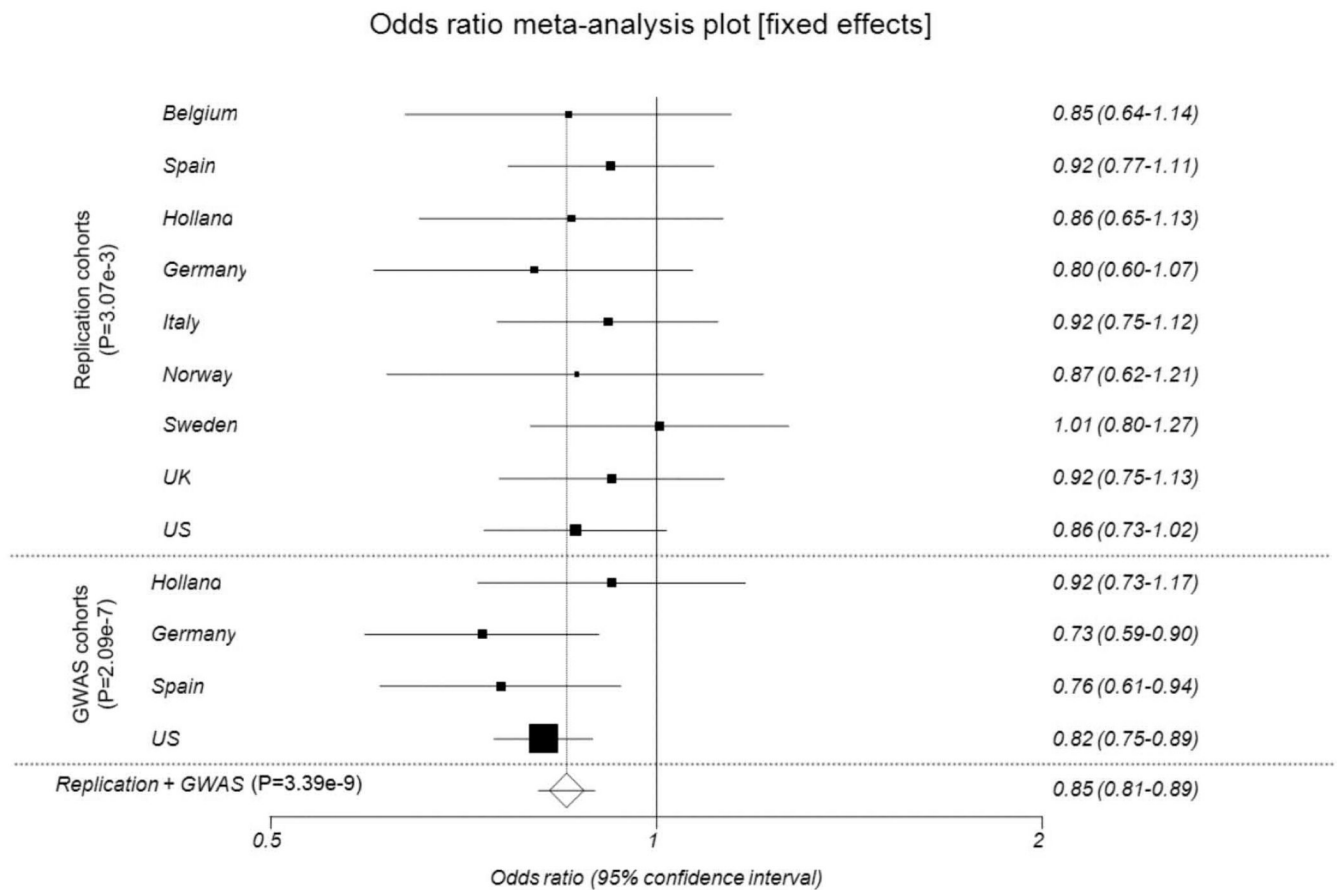


Figure 2. Forest plot showing the odds ratios and confidence intervals of the CD247 association in the various populations studied both in the discovery and replication cohorts.

Table 1

Loci showing the strongest association signal with SSc susceptibility outside the MHC region.

Chr.	Gene	SNP	Location	BP	Minor allele	MAF Case/Control	GC corrected P value	PC corrected P value	OR 95 % CI
7q32	TNPO3/IRF5	rs10488631 rs12537284	Downstream Intergenic	128,381,419 128,505,142	C A	0.145/1.102 0.162/0.129	1.86×10^{-13} 2.74×10^{-7}	3.84×10^{-14} 1.49×10^{-7}	1.50 (1.35–1.67) 1.30 (1.18–1.44)
2q32	STAT4	rs4728142 rs3821236	Upstream Intronic	128,361,203 191,611,003	A A	0.494/0.445 0.247/0.202	5.21×10^{-7} 3.37×10^{-9}	1.81×10^{-7} 3.93×10^{-9}	1.21 (1.12–1.29) 1.30 (1.19–1.41)
1q22-23	CD247	rs2056626	Intronic	165,687,049	G	0.370/0.421	2.09×10^{-7}	3.27×10^{-7}	0.82 (0.76–0.88)
18q22	CDH7	rs10515998	Intergenic	61,521,202	G	0.062/0.040	2.25×10^{-7}	1.01×10^{-7}	1.53 (1.31–1.76)
6p25	EXOC2/IRF4	rs4959270	Intronic	402,748	A	0.445/0.494	1.23×10^{-7}	9.06×10^{-8}	0.82 (0.77–0.88)

Table 2

Association results for three loci genotyped in the replication samples.

CHR	Gene	SNP	Position	Minor Allele	Stage	N (case/control)	MAF (case/control)	P value	OR 95 % CI
1q22-23	CD247	rs2056626	165,687,049	G	GWAS	2296/5014	0.370/0.421	2.09×10 ⁻⁷	0.82(1.76-0.88)
					Replication	2566/4387	0.366/0.394	3.07×10 ⁻³	0.89(0.83-0.96)
					Combined	4867/9401	0.368/0.409	3.39×10 ⁻⁹	0.86(0.81-0.90)
18q22	CDH7	rs10515998	61,521,202	G	GWAS	2296/5014	0.062/0.040	2.25×10 ⁻⁷	1.53(1.31-1.79)
					Replication	2594/4414	0.058/0.056	4.98×10 ⁻¹	1.05(0.91-1.22)
					Combined	4895/9428	0.060/0.048	3.99×10 ⁻⁵	1.25(1.13-1.40)
6p25	EXOC2/IRF4	rs4959270	402,748	A	GWAS	2296/5171	0.445/0.494	1.23×10 ⁻⁷	0.82(0.77-0.88)
					Replication	2361/4372	0.466/0.469	6.34×10 ⁻¹	0.98(0.91-1.05)
					Combined	4662/9554	0.456/0.483	2.16×10 ⁻⁵	0.90(0.85-0.94)