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Genetic variations in Toll-like receptors (TLRs 3/7/8) are associated with systemic lupus erythematosus in a Taiwanese population

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Toll-like receptors (TLRs), as innate immunity sensors, play critical roles in immune responses. Six SNPs of *TLR3*, *TLR7*, and *TLR8* were genotyped to determine their associations with systemic lupus erythematosus (SLE) and clinical manifestations of SLE. TLR7 SNP rs3853839 was independently associated with SLE susceptibility in females (G *vs.* C: $p = 0.0051$). TLR7 rs3853839-G (G *vs.* C: $p = 0.0100$) and TLR8 rs3764880-G (recessive model: $p = 0.0173$; additive model: $p = 0.0161$) were associated with pericardial effusion in females relative to healthy females. Anti-SSA positive cases were more likely to have the dominant TLR7 rs179010-T allele than normal controls ($p = 0.0435$). TLR3 rs3775296-T was associated with photosensitivity ($p = 0.0020$) and anemia ($p = 0.0082$). The "G-G" haplotype of TLR7 rs3853839 and TLR8 rs3764880 increased risk of SLE in females (age adjusted $p = 0.0032$). These findings suggest that TLR variations that modify gene expression affect risk for SLE susceptibility, clinical phenotype development, and production of autoantibodies.

System lupus erythematosus (SLE) is a systemic autoimmune disease characterized by widespread loss of immune tolerance to self-antigens. Genetic and environmental factors contribute to the development of SLE, and patients typically experience alternating periods of flare-up and remission[1]. The generation of numerous autoantibodies that react with self-nuclear and -cytoplasmic antigens is associated with the dysfunction of multiple organ systems[2–5]. The genetic transmission and patterns of inheritance of SLE have not yet been elucidated. In particular, it has been difficult to identify specific genetic polymorphisms associated with SLE due to the presence of polygenic inheritance, extensive genetic heterogeneity, the small size of most genetic studies, and the low disease prevalence[6,7]. Genome-wide association studies (GWASs) of various populations have identified several common immune response pathways and the presence of genetic variants in some ethnic groups that are involved in the pathogenesis of SLE[8–13]. However, genetic dissection of SLE and other autoimmune diseases is difficult because these are complex diseases that involve alterations of multiple biologic pathways[14]. The advent of modern genomics and the availability of new technologies have made it possible to fine-map candidate genes for SLE and other specific diseases based on knowledge of gene map position and functional relevance[15–18]. Such studies may help to identify the roles of candidate genes for SLE and the roles of different genes in the expression of different clinical manifestations[4,19,20].

Previous studies have indicated that innate pattern recognition receptors, such as Toll-like receptors (TLRs), play important roles in the development of autoimmunity. TLR proteins are localized on the cell surface or in endosomes, and play critical roles in innate immune responses against different pathogens[21,22]. Internalized nucleic acid immune complexes act as endogenous ligands that activate intracellular TLRs, and these initiate several signaling pathways that lead to increased production of type I interferons (IFNs) in plasmacytoid dendritic

Table 1 | Clinical characteristics of the SLE study population (n = 795)

Clinical manifestation	Total (N = 795)	Male (N = 68)	Female (N = 727)
		Mean ± SD	
Age	30.71 ± 11.62	31.46 ± 12.52	30.64 ± 11.56
		N/evaluable cases (%)	
Oral ulcer	207/795 (26.04%)	13/68 (19.12%)	194/727 (26.69%)
Arthritis	496/795 (62.39%)	38/68 (55.88%)	458/727 (63.00%)
Malar rash	443/795 (55.72%)	37/68 (54.41%)	406/727 (55.85%)
Discoid rash	150/795 (18.87%)	16/68 (23.53%)	134/727 (18.43%)
Photosensitivity	180/795 (22.64%)	13/68 (19.12%)	167/727 (22.97%)
Pleural effusion	154/795 (19.37%)	12/68 (17.65%)	142/727 (19.53%)
Pericardial effusion	96/795 (12.08%)	13/68 (19.12%)	83/727 (11.42%)
Ascites	42/795 (5.28%)	3/68 (4.41%)	39/727 (5.36%)
Nephritis	451/795 (56.73%)	45/68 (66.18%)	406/727 (55.85%)
Neuropsychiatric manifestations	130/795 (16.35%)	10/68 (14.71%)	120/727 (16.51%)
Leukopenia (WBC count < 3500/uL)	451/795 (56.73%)	41/68 (60.29%)	410/727 (56.40%)
Anemia (Hb < 9 g/dL)	247/795 (31.07%)	11/68 (16.18%)	236/727 (32.46%)
Thrombocytopenia (plat count < 10^5/uL)	208/795 (26.16%)	23/68 (33.82%)	185/727 (25.45%)
Complement depressed	615/782 (78.64%)	53/66 (80.30%)	562/716 (78.49%)
Anti-dsDNA	590/777 (75.93%)	52/67 (77.61%)	538/710 (75.77%)
Anti-Sm	245/646 (37.93%)	27/60 (45.00%)	218/586 (37.20%)
Anti-RNP	279/645 (43.26%)	24/59 (40.68%)	255/586 (43.52%)
Anti-SSA	346/535 (64.67%)	28/45 (62.22%)	318/490 (64.90%)
Anti-SSB	139/535 (25.98%)	8/45 (17.78%)	131/490 (26.73%)
Anti-cardiolipin IgG	178/628 (28.34%)	12/50 (24.00%)	166/578 (28.72%)
Anti-cardiolipin IgM	52/577 (9.01%)	4/47 (8.51%)	48/530 (9.06%)

cells (pDCs)[22–26]. Increased production of type I IFNs increases apoptosis, neutrophil cell death *via* neutrophil extracellular trap (NETosis), innate immune signaling, and viral infection-induced autoimmunity[27–29]. Aberrant stimulation of the innate immune system through intracellular TLRs may lead to hyperactive immune responses and contribute to the pathogenesis of SLE[30–32].

TLR3, TLR7, and TLR8 are primarily associated with endosomal membranes and they recognize microbial nucleic acids. TLR3 binds to viral double-stranded RNA (dsRNA), and induces antiviral immune responses by promoting the production of type I IFN and pro-inflammatory cytokines. TLR7 binds to single-stranded RNA (ssRNA) from RNA viruses, and triggers pDCs to produce type I IFN. TLR8 is phylogenetically related to TLR7 and also mediates recognition of viral ssRNA. Thus, these 3 TLRs are responsible for pathogen clearance, antigen recognition, and induction of cytokine production[30,33,34].

Genetic and hormonal factors appear to partially explain the female predominance of SLE[35]. In particular, TLR7 and TLR8 are on the X chromosome (Xp22.2) and play critical roles in innate immunity and inflammatory responses[30]. Studies of animal models indicated that TLR7 gene dosage significantly affected the hyperactivity of B cells[36–41]. Mice with a Y-linked autoimmune accelerator (Yaa) that carries an extra copy of TLR7 develop autoimmunity to RNA-associated autoantigens, but mice with a low TLR7 copy number require additional susceptibility loci to develop autoimmunity[39,40]. In humans, males with Klinefelter syndrome carry an extra X chromosome (47, XXY) and are more likely to develop SLE; females with Turner syndrome lack one X chromosome (45, X) and are less likely to develop SLE[42,43]. In addition, a study of a Mexican population indicated that increased TLR7 copy number correlated with TLR7 mRNA levels and susceptibility to childhood-onset SLE[44] although TLR7 copy number variations (CNVs) are infrequent in human SLE[45]. Other studies indicated that increased expression of TLRs in peripheral blood mononuclear cells and lymphocytes led increased IFN-α expression in SLE patients[46–48].

Previous research indicated that a functional TLR7 SNP (rs3853839-G > C) affects TLR7 expression by modulation of microRNA-3148 (miR-3148)[49], and that two other intron SNPs (rs5935436 and rs179010) were associated with increased SLE susceptibility[45,49]. The TLR8 SNP rs3764880 is a functional polymorphism that affects TLR8 transcription and translations of TLR8 isoforms, which leads to the activation difference of NF-κB, is also associated with SLE susceptibility[45,49–52]. A study of cell cultures indicated that TLR3 rs3775296 (in the promoter) and rs3775291 (in exon 4) affected TLR3 cell surface expression and localization, and subsequently influenced NF-κB cascade induction although the effect of these mutations has not been assessed in clinical studies[53]. The present study examined the associations of the above mentioned 6 SNPs (3 in TLR7, 1 in TLR8, 2 in TLR3) with SLE and with specific clinical manifestations of SLE.

Results

We investigated the role of 6 SNPs in the susceptibility to SLE (rs3775296 and rs3775291 from TLR3; rs5935436, rs179010, and rs3853839 from TLR7; and rs3764880 from TLR8) by examination of 795 SLE patients (68 males and 727 females) and 1162 healthy controls (513 males and 649 females). The average age of cases was 30.71 years (SD = 11.62, range: 8 to 77 years), 8.55% were males (31.46 ± 12.52 years-old), and 91.45% were females (30.64 ± 11.56 years-old). The healthy controls had a mean age of 40.24 years (SD = 10.88, range 18–64 years), with similar average ages of males and females (40.26 ± 9.26 years *vs.* 40.23 ± 12.02 years). The age difference of SLE patients and healthy controls was statistically significant for both males and females, so we adjusted for age in the subsequent association analysis. Table 1 shows the clinical characteristics of the 795 SLE patients, with independent statistical analyses for males and females. In the female cases and controls, there were no deviations from HWE in the six candidate SNPs.

Table 2 shows the single-locus associations between the six candidate SNPs and susceptibility to SLE. In males, there were no significant case-control associations after the false discovery rate (FDR) correction, possibly due to the small sample size. In females, there was a significant allelic association between SLE and rs3853839 in TLR7 (P_{FDR} = 0.013, OR = 1.38, 95% CI = 1.12–1.69). Moreover, the risk allele G had a recessive effect in females (GG *vs.* GC + CC: P_{FDR} = 0.017, OR = 1.44, 95% CI = 1.14–1.83), suggesting that rs3853839 plays a role in development of SLE. We also evaluated the independent contributions of the 6 candidate SNPs (adjusted for

Table 2 | Association of TLR SNPs with SLE in the study population (n = 795)

				Frequency		Allelic Association		Genotypic Association: Additive		Genotypic Association: Dominant		Genotypic Association: Recessive	
SNP	Position	Sample	Risk Allele	SLE (N = 795)	Normal (N = 1162)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)
TLR3													
rs3775291	Chr4	All	G	1036 (65.16%)	1496 (64.37%)	0.358	1.08 (0.92–1.26)	0.359	1.08 (0.92–1.25)	0.357	0.86 (0.63–1.18)	0.060	1.23 (0.99–1.52)
	187004074	Male	A	48 (35.29%)	352 (34.31%)	0.999	1 (0.68–1.48)	0.999	1 (0.67–1.49)	0.703	0.9 (0.53–1.54)	0.558	1.27 (0.58–2.77)
		Female	G	948 (65.2%)	822 (63.33%)	0.305	1.09 (0.92–1.29)	0.309	1.09 (0.92–1.29)	0.453	0.88 (0.62–1.23)	0.056	1.25 (1–1.58)
rs3775296	Chr4	All	T	401 (25.22%)	585 (25.17%)	0.860	0.98 (0.83–1.17)	0.859	0.98 (0.83–1.17)	0.469	0.92 (0.75–1.14)	0.295	1.26 (0.82–1.95)
	186997767	Male	G	108 (79.41%)	756 (73.68%)	0.254	1.31 (0.83–2.06)	0.268	1.29 (0.83–2.01)	0.901	0.94 (0.34–2.59)	0.143	1.52 (0.87–2.64)
		Female	T	373 (25.65%)	315 (24.27%)	0.727	1.03 (0.86–1.24)	0.724	1.03 (0.86–1.25)	0.925	0.99 (0.79–1.24)	0.270	1.32 (0.81–2.15)
TLR7													
rs3853839	ChrX	All	G	1259 (82.77%)	1405 (77.67%)	0.001	1.35 (1.13–1.62)						
	12907658	Male	G	57 (85.07%)	389 (76.13%)	0.126	1.76 (0.86–3.64)						
		Female	G	1202 (82.67%)	1016 (78.27%)	0.002	1.38 (1.12–1.69)	0.002	1.38 (1.12–1.69)	0.133	1.58 (0.87–2.87)	0.003	1.44 (1.14–1.83)
rs5935436	ChrX	All	T	64 (4.2%)	65 (3.59%)	0.308	1.22 (0.83–1.77)						
	12883891	Male	C	66 (97.06%)	494 (96.3%)	0.927	0.93 (0.2–4.3)						
		Female	T	62 (4.26%)	46 (3.54%)	0.219	1.3 (0.86–1.97)	0.219	1.30 (0.86–1.98)	0.269	1.27 (0.83–1.95)	0.980	N/A
rs179010	ChrX	All	T	565 (37.12%)	612 (33.81%)	0.097	1.14 (0.98–1.32)						
	12902885	Male	T	26 (38.24%)	165 (32.23%)	0.295	1.34 (0.78–2.32)						
		Female	T	539 (37.07%)	447 (34.44%)	0.257	1.1 (0.93–1.3)	0.265	1.1 (0.93–1.3)	0.365	1.11 (0.88–1.4)	0.349	1.17 (0.84–1.64)
TLR8													
rs3764880	ChrX	All	G	1286 (84.55%)	1444 (79.73%)	0.033	1.23 (1.02–1.5)						
	12924826	Male	G	58 (86.57%)	377 (73.49%)	0.054	2.09 (0.99–4.41)						
		Female	G	1228 (84.46%)	1067 (82.2%)	0.296	1.12 (0.9–1.39)	0.296	1.12 (0.9–1.39)	0.781	1.1 (0.55–2.2)	0.272	1.15 (0.9–1.47)

[9]For all samples, the test p-value was adjusted by age and sex; for the males and females, the test p-value was adjusted by age respectively.

Table 3 | Associations of TLR SNPs with SLE clinical phenotypes and autoantibodies in male SLE patients

SNP	Clinical Association (Sample 1 vs. Sample 2)	Sample Size Ratio	Alleles[a]	RAF[b] in Sample 1	RAF[b] in Sample 2	P[c]	P_{FDR}[d]	OR (95%CI)
TLR3								
rs3775291	Complement depressed+ vs. Complement depressed-	53 : 15	G/A	36 (67.92%)	8 (53.33%)	0.0465	0.2790	2.47 (1.03–5.91)
rs3775296	None							
TLR7								
rs3853839	Arthritis+ vs. Normal	37 : 511	G/C	34 (91.89%)	389 (76.13%)	0.0273	0.0546	3.55 (1.07–11.76)
	Leukopenia+ vs. Normal	40 : 511	G/C	36 (90.00%)	389 (76.13%)	0.0442	0.1004	2.82 (0.98–8.08)
	Thrombocytopenia+ vs. Normal	23 : 511	G/C	22 (95.65%)	389 (76.13%)	0.0296	0.1118	6.90 (0.92–51.72)
rs5935436	None							
rs179010	Oral ulcer+ vs. Normal	13 : 512	T/C	8 (61.54%)	165 (32.23%)	0.0264	0.1056	3.36 (1.08–10.43)
	Malar rash+ vs. Malar rash−	36 : 31	C/T	32 (88.89%)	26 (83.87%)	0.0377	0.1509	2.88 (1.05–7.92)
	Pleural effusion+ vs. Pleural effusion−	12 : 56	C/T	11 (91.67%)	31 (55.36%)	0.0188	0.0753	8.87 (1.07–73.45)
TLR8								
rs3764880	Arthritis+ vs. Normal	37 : 513	G/A	34 (91.89%)	377 (73.49%)	0.0129	0.0514	4.09 (1.24–13.53)
	Malar rash+ vs. Normal	36 : 513	G/A	32 (88.89%)	377 (73.49%)	0.0404	0.1618	2.89 (1.00–8.32)
	Photosensitivity+ vs. Normal	12 : 513	G/A	12 (100%)	377 (73.49%)	0.0383	0.1530	8.66 (0.51–147.60)
	Pleural effusion+ vs. Normal	12 : 513	G/A	12 (100%)	377 (73.49%)	0.0383	0.1530	8.66 (0.51–147.60)
	Anti-RNP+ vs. Normal	24 : 521	G/A	23 (95.83%)	383 (73.51%)	0.0141	0.0565	8.30 (1.11–62.05)

[a]The former allele is the risk allele.
[b]RAF: Risk allele frequency.
[c]The p-value was calculated from chi-square tests or Fisher's exact tests when appropriate.
[d]P_{FDR}: The p-value was adjusted after the FDR correction.

age) to SLE risk in females, and performed a multivariate logistic regression analysis that included the significant SNPs with the same genetic models. The rs3853839 SNP was the only significant susceptibility marker among the 6 examined SNPs (G *vs.* C: $p = 0.0051$, OR = 1.42, 95% CI = 1.11–1.82).

TLR SNP polymorphisms affected SLE phenotype and production of autoantibodies. Patients with SLE are present with heterogeneous clinical features, and have significant variations in the severity, nature, and spectrum of clinical involvement. Thus, we examined the effect of TLR SNP polymorphisms on SLE clinical parameters and phenotypes. Based on the clinical characteristics of males and females (Table 1), we initially performed two comparisons: *(i)* allele frequencies of SLE patients with each characteristic ("+" in Tables S1–S3) and SLE patients without the characteristic ("−" in Tables S1–S3); and *(ii)* allele frequencies of SLE patients with each characteristic and the normal controls ("normal" in Tables S1–S3). In males, there were no significant associations of the 6 candidate SNPs with SLE clinical manifestations after the FDR correction (Table 3, with more details in Tables S1 and S3). Again, this may result from the small sample size.

In females, TLR7 rs3853839 G risk allele was associated with several clinical manifestations of SLE (Table 4). The comparison of phenotype-positive cases and normal controls indicated significant associations of this allele with oral ulcer, arthritis, malar rash, photosensitivity, pericardial effusion, depressed complement, anti-dsDNA, anti-Sm, and anti-SSA ($P_{FDR} < 0.05$ for all comparisons). Multivariate logistic regression analysis of the independent association of each clinical characteristic with rs3853839 indicated that only pericardial effusion was significantly associated with the TLR7 rs3853839 G risk allele. In particular, cases with pericardial effusion were more likely to have the G risk allele than normal controls ($p = 0.0100$, OR = 2.82, 95% CI = 1.28–6.19). Table S2 shows that anti-SSA was associated with the rs179010 T risk allele (T *vs.* C: $P_{FDR} = 0.0366$, OR = 1.47, 95% CI = 1.11–1.94; CT + TT *vs.* CC: $P_{FDR} = 0.0037$, OR = 1.93, 95% CI = 1.33–2.81). Multivariate logistic regression analysis also showed that cases who were anti-SSA positive were more likely to carry the dominant rs179010 T risk allele than normal controls ($p = 0.0435$, OR = 1.33, 95% CI = 1.01–1.75), but cases who were anti-SSA negative had the opposite tendency ($p = 0.0299$, OR = 0.69, 95% CI = 0.49–0.96).

Although rs3764880 in TLR 8 was not associated with SLE susceptibility, the risk allele G was associated with oral ulcer with significant additive effects ($P_{FDR} = 0.0232$: OR = 1.66, 95% CI = 1.18–2.35, G *vs.* A: $P_{FDR} = 0.0300$, OR = 1.65, 95% CI = 1.17–2.32). However, incorporation of clinical variables with nominal p values below 0.05 into the multivariate logistic regression model indicated that pericardial effusion was the only clinical characteristic associated with rs3764880. In particular, pericardial effusion-positive cases were more likely to carry the rs3764880 G risk allele than normal controls, either with a recessive effect ($p = 0.0173$, OR = 2.91, 95% CI = 1.21–7.02) or with an additive effect ($p = 0.0161$, OR = 2.96, 95% CI = 1.22–7.14).

Analysis of the association of TLR3 SNPs with SLE clinical characteristics indicated no significant associations for males (Tables 3 and S3). In females, comparison of phenotype-positive cases with phenotype-negative cases and of phenotype-positive cases with normal controls indicated positive associations of rs3775296 with anemia (Table 4, TT *vs.* GG + GT: $P_{FDR} = 0.0244$, OR = 2.41, 95% CI = 1.33–4.39; TT *vs.* GG + GT: $P_{FDR} = 0.0373$, OR = 2.11, 95% CI = 1.23–3.65, respectively). Multivariate logistic regression analysis indicated that photosensitivity-negative cases and anemia-negative cases were associated with recessive rs3775296 T risk allele relative to normal controls (photosensitivity: $p = 0.0020$, OR = 2.40, 95% CI = 1.38–4.19; anemia: $p = 0.0082$, OR = 0.44, 95% CI = 0.24–0.81).

TLR haplotypes were associated with SLE susceptibility in females. In Figure 1, pair-wise LD measures r^2 and D' for the four TLR7 and TLR8 SNPs rs3853839, rs5935436, rs179010 and rs3764880 in the female healthy controls were presented. We estimated the frequencies of haplotypes formed by these 4 SNPs in females to determine the associations of different haplotypes with susceptibility to SLE. Table 5 shows the 4 inferred haplotypes that had estimated frequencies more than 5%. Performance of haplotype-trait association tests in female SLE patients and healthy controls indicated that the "C-C-C-G" haplotype protected against development of SLE (5.78% in cases *vs.* 8.55% in controls; permutation p-value = 0.0033, OR = 0.60, 95% CI = 0.44–0.82 after age adjustment). In Table 6 shows the estimates for haplotypes from rs3853839 and rs3764880 SNPs only. These results show that the effect of "C-G" on SLE susceptibility remained significant when we controlled for age (7.08% in cases *vs.* 8.39% in controls; permutation

Table 4 | Associations of candidate SNPs with SLE clinical phenotypes and autoantibodies in female SLE patients

SNP	Clinical Association (Sample 1 vs. Sample 2)	Sample Size Ratio	Genotypic Model	RAF[a] in Sample 1	RAF[a] in Sample 2	P[b]	P_{FDR}[c]	OR (95%CI)
TLR3								
rs3775291	Anti-dsDNA+ vs. Normal	538 : 649	GG vs. AA + AG	715 (66.45%)	822 (63.33%)	0.0469	0.1408	1.26 (1.00–1.59)
	Anti-SSB+ vs. Normal	131 : 649	Additive	183 (69.85%)	1132 (63.88%)	0.0429	0.1288	1.35 (1.01–1.80)
rs3775296	Photosensitivity+ vs. Photosensitivity−	167 : 560	GG + GT vs. TT	257 (76.95%)	824 (73.57%)	0.0244	0.1465	3.30 (1.17–9.30)
	Pleural effusion+ vs. Pleural effusion−	142 : 585	Additive	87 (30.63%)	286 (24.44%)	0.0317	0.1902	1.37 (1.03–1.83)
	Pleural effusion+ vs. Normal	142 : 649	Additive	87 (30.63%)	315 (24.27%)	0.0238	0.0713	1.40 (1.05–1.86)
	Anemia+ vs. Anemia−	236 : 491	TT vs. GG + GT	132 (27.97%)	241 (24.54%)	0.0041	0.0244	2.41 (1.33–4.39)
	Anemia+ vs. Normal	236 : 649	TT vs. GG + GT	394 (83.47%)	1067 (82.20%)	0.0075	0.0373	2.11 (1.23–3.65)
TLR7								
rs3853839	Oral ulcer+ vs. Normal	194 : 649	GG vs. CC + CG	327 (84.28%)	1016 (78.27%)	0.0065	0.0196	1.62 (1.15–2.30)
	Arthritis+ vs. Normal	458 : 649	GG vs. CC + CG	767 (83.73%)	1016 (78.27%)	0.0012	0.0074	1.52 (1.18–1.96)
	Malar rash+ vs. Normal	406 : 649	GG vs. CC + CG	674 (83.00%)	1016 (78.27%)	0.0061	0.0364	1.45 (1.11–1.88)
	Photosensitivity+ vs. Normal	167 : 649	Additive	285 (85.33%)	1016 (78.27%)	0.0042	0.0251	1.63 (1.17–2.28)
	Pleural effusion+ vs. Normal	142 : 649	Additive	237 (83.45%)	1016 (78.27%)	0.0498	0.0997	1.41 (1.00–1.99)
	Pericardial effusion+ vs. Pericardial effusion−	83 : 644	Additive	147 (88.55%)	1055 (81.91%)	0.0363	0.2179	1.70 (1.04–2.79)
	Pericardial effusion+ vs. Normal	83 : 649	Additive	147 (88.55%)	1016 (78.27%)	0.0022	0.0134	2.19 (1.33–3.61)
	Nephritis+ vs. Normal	406 : 649	GG vs. CC + CG	667 (82.14%)	1016 (78.27%)	0.0196	0.1174	1.37 (1.05–1.77)
	Leukopenia+ vs. Normal	410 : 649	GG vs. CC + CG	677 (82.56%)	1016 (78.27%)	0.0145	0.0723	1.38 (1.07–1.80)
	Anemia+ vs. Normal	236 : 649	Additive	390 (82.63%)	1016 (78.27%)	0.0440	0.1141	1.33 (1.01–1.74)
	Complement depressed+ vs. Normal	562 : 649	GG vs. CC + CG	937 (83.36%)	1036 (78.48%)	0.0013	0.0075	1.48 (1.17–1.88)
	Anti-dsDNA+ vs. Normal	538 : 649	GG vs. CC + CG	895 (83.18%)	1016 (78.27%)	0.0019	0.0116	1.47 (1.15–1.87)
	Anti-Sm+ vs. Normal	218 : 649	GG vs. CC + CG	366 (83.94%)	1016 (78.27%)	0.0032	0.0161	1.65 (1.19–2.31)
	Anti-RNP+ vs. Anti-RNP−	255 : 472	CC vs. CG + GG	89 (17.45%)	163 (17.27%)	0.0330	0.1972	2.91 (1.10–7.73)
	Anti-RNP+ vs. Normal	255 : 649	GG vs. CC + CG	421 (82.55%)	1016 (78.27%)	0.0090	0.0537	1.51 (1.11–2.06)
	Anti-SSA+ vs. Normal	318 : 649	GG vs. CC + CG	528 (83.02%)	1016 (78.27%)	0.0050	0.0248	1.51 (1.13–2.01)
	Anti-SSB+ vs. Normal	131 : 649	GG vs. CC + CG	217 (82.82%)	1016 (78.27%)	0.0299	0.0746	1.57 (1.05–2.36)
rs5935436	Neuropsychiatric manifestations+ vs. Neuropsychiatric manifestations−	120 : 607	Additive	16 (6.67%)	46 (3.79%)	0.0498	0.2990	1.79 (1.00–3.19)
	Neuropsychiatric manifestations+ vs. Normal	120 : 649	Additive	16 (6.67%)	46 (3.54%)	0.0260	0.1559	1.96 (1.09–3.54)
rs179010	Discoid rash+ vs. Discoid rash−	134 : 593	TT vs. CC + CT	106 (39.55%)	433 (36.51%)	0.0198	0.1189	1.77 (1.10–2.85)
	Discoid rash+ vs. Normal	134 : 649	TT vs. CC + CT	106 (39.55%)	447 (34.44%)	0.0113	0.0680	1.85 (1.15–2.98)
	Pleural effusion+ vs. Normal	142 : 649	Additive	119 (41.90%)	447 (34.44%)	0.0198	0.0713	1.36 (1.05–1.76)
	Pericardial effusion+ vs. Normal	83 : 649	CT + TT vs. CC	71 (42.77%)	447 (34.44%)	0.0344	0.1119	1.70 (1.04–2.76)
	Anti-SSA+ vs. Anti-SSA−	318 : 409	CT + TT vs. CC	252 (39.62%)	287 (35.09%)	0.0006	0.0037	1.93 (1.33–2.81)
	Anti-SSA+ vs. Normal	318 : 649	Additive	252 (39.62%)	447 (34.44%)	0.0280	0.0840	1.24 (1.02–1.51)
TLR8								
rs3764880	Oral ulcer+ vs. Oral ulcer−	194 : 533	Additive	343 (88.40%)	885 (83.02%)	0.0136	0.0816	1.55 (1.10–2.20)
	Oral ulcer+ vs. Normal	194 : 649	Additive	343 (88.40%)	1067 (82.20%)	0.0039	0.0232	1.66 (1.18–2.35)
	Arthritis+ vs. Arthritis−	458 : 269	GG vs. AA + AG	787 (85.92%)	441 (81.97%)	0.0228	0.1369	1.47 (1.06–2.03)
	Arthritis+ vs. Normal	458 : 649	GG vs. AA + AG	787 (85.92%)	1067 (82.20%)	0.0108	0.0325	1.41 (1.08–1.84)
	Pericardial effusion+ vs. Normal	83 : 649	GG vs. AA + AG	145 (87.35%)	1067 (82.20%)	0.0446	0.1105	1.75 (1.02–3.02)
	Anti-SSB+ vs. Normal	131 : 649	Additive	231 (88.17%)	1067 (82.20%)	0.0184	0.1105	1.63 (1.09–2.44)

[a]RAF: Risk allele frequency.
[b]The p-value was calculated from chi-square tests or Fisher's exact tests when appropriate.
[c]P_{FDR}: The p-value was adjusted after the FDR correction.

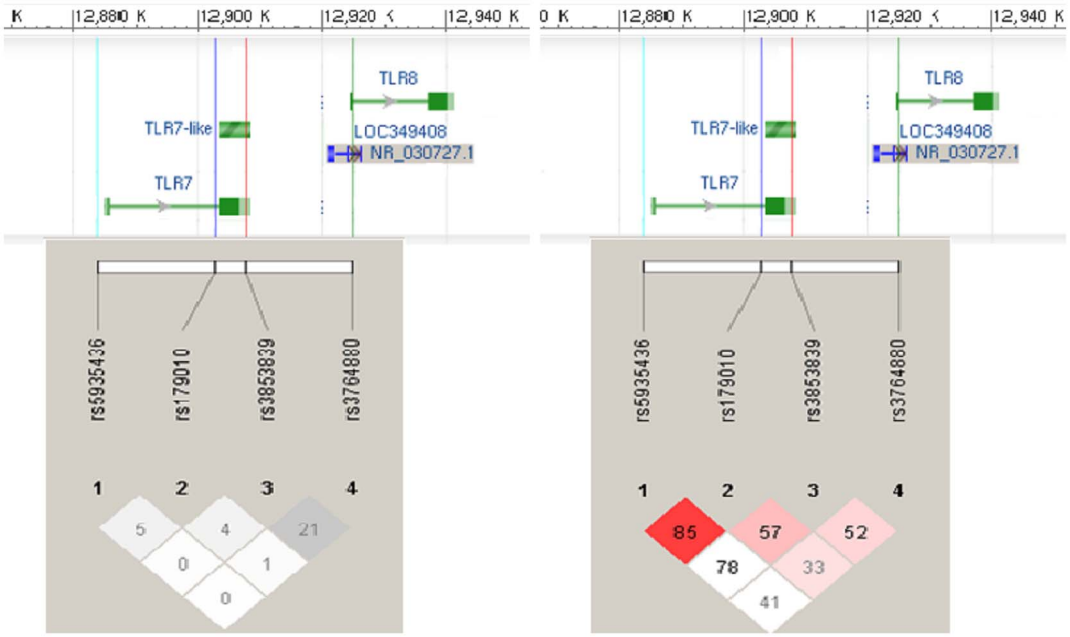

Figure 1 | Pair-wise linkage disequilibrium patterns with r^2 (left side) and D′ (right side) measures of the four SNPs in *TLR7* and *TLR8* at Xp22.3 for 649 healthy female controls.

p-value = 0.0073, OR = 0.62, 95% CI = 0.46–0.83). Nevertheless, females with "G-G" haplotypes of rs3853839 and rs3764880 were significantly more likely to develop SLE (age adjusted *p*-value = 0.0032, OR = 1.32, 95% CI = 1.10–1.60).

Discussion

Previous research with large samples indicated that the functional TLR7 SNP rs3853839-G > C was significantly associated with SLE in East Asians, especially in males[45]. The current study identified the rs3853839-G allele of TLR7 as the main susceptibility marker in female SLE patients from Taiwan based on single-locus and multivariate logistic regression analyses. This confirms the role of this SNP in the pathogenesis of SLE. The genetic effect of TLR7 on SLE appears to vary among different ethnic groups[45,54–56]. In particular, a previous study indicated that the TLR7 intronic SNPs rs179019 and rs179010 were associated with SLE, independent of the 3′ UTR SNP rs3853839 in Japanese females[54]. Other research indicated that the TLR7 SNP rs179008 was not associated with SLE in a European population, but was significantly associated with SLE in Brazilians[55,56]. Recently, Deng et al. conducted a large trans-ancestral fine-mapping of European Americans, African Americans, and Amerindian/Hispanics and identified rs3853839-G as the only genetic risk variant for SLE in the TLR7-TLR8 region, although they did not confirm the male specific association. Notably, rs3853839-G appears to increase risk for SLE in different populations, although there are different frequencies in different ethnic groups. This highlights the critical role of elevated TLR7 expression in the pathogenesis of SLE, which in this case is mediated by slower mRNA degradation due to miR-3148 expression[49].

TLR7 and TLR8 contribute to antigen recognition and antibody production in the pathogenesis of SLE. TLR ligands, which are present in viruses and virus-like particles (VLPs), can directly stimulate B cells, and B cell responses are integrated with dual antigen-specific B cell receptor (BCR) and TLR engagement[57,58]. B cells with up-regulated MYD88 expression become more responsive to TLR ligands and promote class-switch recombination[59]. The inactivation or overexpression of genes that encode TLRs or of molecules that alter TLR signaling provides a bridge between the innate and adaptive immune systems, and this is critical to the presence of B cells defects in the pathogenesis of SLE[60,61]. Moreover, the recognition of endogenous RNA-containing antigens by TLR7/3 may trigger autoreactive B cells in the germinal center, and this is accompanied by the suppression of T regulatory cells, leading to disruption of self-tolerance[62–64]. In this regard, numerous innate and adaptive immunity related genes involving IFN-alpha mediated signature pathway as well as T and B cells activation signaling pathways participate in the SLE pathogenesis[65]. The present study found that TLR7 rs3853839 G risk allele was associated with several clinical manifestations of SLE, including oral ulcer, arthritis, malar rash, photosensitivity, pericardial effusion, depressed complement, and anti-dsDNA, anti-Sm, and anti-SSA autoantibodies. The TLR7 rs179010 T risk allele was also associated with anti-SSA autoantibodies. These findings suggest that TLR7 may play a key role in autoantibody production because it increases B-cell sensitivity to RNA-containing autoantigens in the development of systemic autoimmunity. However, given the limited sample sizes of our stratified phenotype groups, this finding requires replication by larger future studies.

Table 5 | Haplotype analysis of TLR7 and TLR8 SNPs in female SLE patients and healthy controls

Haplotype (rs3853839-rs5935436-rs179010-rs3764880)	Estimated Frequency (%)			Permutation	Logistic regression		Logistic regression adjusted by age	
	All	SLE	Normal	P*	P	OR (95% CI)	P	OR (95% CI)
G-C-C-G	44.00	45.32	42.53	0.1571	0.1405	1.12 (0.96–1.30)	0.0849	1.15 (0.98–1.36)
G-C-T-G	27.47	28.27	26.58	0.2137	0.3229	1.09 (0.92–1.29)	0.6663	1.04 (0.87–1.25)
C-C-C-A	10.54	9.56	11.63	0.0742	0.0768	0.80 (0.63–1.02)	0.1279	0.82 (0.63–1.06)
C-C-C-G	7.09	5.78	8.55	0.0033	0.0049	0.66 (0.49–0.88)	0.0016	0.60 (0.44–0.82)

*p-values for estimated haplotypes were generated from 10,000 permutations using the expectation-maximization (EM) algorithm.

Table 6 | Haplotype analysis of the TLR7 SNP rs3853839 and the TLR8 SNP rs3764880 in female SLE patients and healthy controls

Estimated Haplotype (rs3853839-rs3764880)	Estimated Frequency (%)			Permutation	Logistic regression		Logistic regression adjusted by age	
	All	SLE	Normal	P*	P	OR (95% CI)	P	OR (95% CI)
G-G	75.00	77.37	72.34	0.0015	0.0024	1.31 (1.10–1.55)	0.0032	1.32 (1.10–1.60)
C-A	11.01	10.25	11.86	0.2837	0.1756	0.85 (0.67–1.08)	0.2996	0.87 (0.68–1.13)
C-G	8.39	7.08	9.86	0.0073	0.0090	0.70 (0.53–0.91)	0.0014	0.62 (0.46–0.83)
G-A	5.60	5.30	5.93	0.3297	0.4677	0.89 (0.64–1.23)	0.7796	0.95 (0.67–1.35)

*p-values for estimated haplotypes were generated from 10,000 permutations using the EM algorithm.

We also observed that the non-synonymous TLR8 SNP rs3764880-G allele was a risk factor for oral ulcer and pericardial effusion, with significant additive effects. Interestingly, previous research indicated that the TLR8 rs3764880-G allele protected against tissue damage in active tuberculosis and predicted a slower disease course in patients with HIV infections[52,66]. The *TLR8* SNP rs3764880 alters the ATG start codon of TLR8 isoform B into a GTG. A methionine at position 4 of isoform B is used as the start codon for the *TLR8*-rs3764880G allele, resulting in a truncated TLR8 isoform B with a shorter signal peptide (1038 residues for the *TLR8*-rs3764880G allele *vs.* 1041 residues for the *TLR8*-rs3764880A allele). Immune cells carrying the *TLR8*-rs3764880G allele had augmented TNFα-responses, but decreased translation of truncated TLR8 isoform B and NF-κB production relative to those carrying the *TLR8*-rs3764880A allele[50,66]. Therefore, TLR8 appears to have important roles in autoimmune diseases and in response to infections.

Previous research indicated that TLR7 and TLR8 have closely related functions in immune responses. We observed that the TLR7 and TLR8 SNP haplotype rs5935436-C/rs179010-C/rs3853839-C/rs3764880-G protected against development of SLE, but the effect of rs3853839-C/rs3764880-G on SLE susceptibility remained significant when controlled for age. On the other hand, females with G-G haplotypes of rs3853839 and rs3764880 were significantly more likely to develop SLE. These results indicate that TLR8 may play a regulatory role in TLR7 function in innate immunity, similar to that documented in mice[67].

TLR3 recognizes dsRNA, and its elevated level in human SLE peripheral blood cells suggests that it may play a key role in IFN signature gene activation[22,46–48]. In addition, previous research indicated that TLR3 aggravated lupus nephritis in lupus-prone mice[68]. We observed that TLR3 rs3775296-T risk allele had recessive effects on photosensitivity and anemia-negative SLE patients. Thus, our data indicate that TLR3 may play a role in the development of different SLE phenotypes, and in antiviral responses that trigger expression of pro-inflammatory genes.

In conclusion, the present study confirmed that certain functional TLR7 and TLR8 variations, particularly TLR7 rs3853839-G > C, modify gene expression and increase the risk for SLE, development of certain SLE phenotypes, and production of autoantibodies. These results suggest that TLR7/8 genetic variations are potential biomarkers for prediction of SLE phenotypes, and also have implications for the development of therapeutic measures that may prevent various pathological conditions that are characteristic of SLE.

Methods

Characteristics of the study populations. All patients were recruited from the clinics of Chang Gung Memorial Hospital, and rheumatology specialists confirmed that all patients fulfilled the 1982 and 1997 American College of Rheumatology (ACR) diagnostic criteria for SLE[69]. For the purpose of this study, healthy controls were selected following a questionnaire to assure that they did not have any autoimmune disease phenotypes. This study was approved by the ethics committees of Chang Gung Memorial Hospital, and all patients provided written informed consent according to the Declaration of Helsinki.

Genomic DNA extraction. Genomic DNA was extracted from EDTA-anticoagulated peripheral blood using the Purgene DNA isolation kit as described previously[70].

SNPs genotyping assays. Validated Applied Biosystem TaqMan SNP assays were used for genotype determination of TLRs according the vendor's instructions (Life Technologies, Grand Island, NY). The TaqMan allele discrimination assays were performed on an ABI ViiA 7 Real-time System (Life Technology) and probes were labeled with a fluorescent dye (FAM and VIC).

Statistical analysis. The functional candidate gene approach was used to perform a case-control association study by examination of 795 SLE patients (68 males and 727 females) and 1162 healthy controls (513 males and 649 females). The Hardy-Weinberg equilibrium (HWE) was examined for the 6 selected SNPs using chi-square tests in females with and without SLE. Differences of allele frequencies in each of the 6 SNPs were separately assessed in males and females to investigate the single-locus associations. Additionally, for females, the significance of differences in genotype frequencies were also evaluated, and dominant and recessive models were tested for each SNP. The *p*-values, odds ratios (ORs), and 95% confidence intervals (CIs) were then calculated based on the risk allele identified. TLR 8 and TLR7 are located on the X chromosome (Xp22.2), so female and male data were analyzed separately. Meta-analysis with generation of meta *p*-values using the Cochran-Mantel-Haenzsel (CMH) method was used to assess the allelic associations of the 6 SNPs in all samples.

We initially stratified clinical phenotypes according to diagnostic criteria to investigate the association of each SNP with SLE clinical manifestations. SLE patients with the phenotype under investigation were classed as "+" cases, those without this phenotype as "−" cases, and healthy controls as "normal". Then, allele frequencies were compared for "+" cases and "−" cases, and for "+" cases and "normal" controls. In females, additional analyses also considered additive, dominant, and recessive effects for the risk allele of each SNP in order to assess genotype-phenotype associations. To investigate the independent association of SLE clinical characteristics with the six SNPs, multivariate logistic regression analysis was subsequently carried out to identify the independent statistical association of significant phenotypes (identified above) with the six SNPs. The additive, dominant and recessive allele effects for each SNP were modeled as the response variable and the three categories of individuals: "+" cases, "−" cases and "Normal" pertaining to each clinical characteristic were used as the independent variables.

Linkage disequilibrium (LD) patterns of neighboring SNPs on the same chromosome were analyzed by Haploview 4.2 (Broad Institute, Cambridge, MA, USA; http://www.broad.mit.edu/mpg/haploview). Haplotype information was inferred and frequencies were estimated using the HAPLOTYPE procedure in SAS 9.2 (SAS Institute, Cary, NC). Differences in haplotype frequencies were assessed for SLE cases and controls were separately assessed in males and females. The permutation (N = 10,000) *p*-values of each haplotype were calculated using the expectation-maximization (EM) algorithm, conditional on the other haplotypes, to evaluate the independent association of each category of haplotypes. Benjamini and Hochberg's linear step-up method in the SAS MULTTEST procedure was used to account for multiple testing[71]. The False Discovery Rate (FDR)-adjusted *p*-values are defined in a step-up fashion, with less conservative multipliers and control. A corrected *p*-value (P_{FDR}) less than 0.05 indicated statistical significance.

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Author contributions

C.M.W. and J.Y.C. performed study design, manuscript preparation and coordination. S.W.C. performed statistical analysis, data interpretation and manuscript preparation. T.C.C. participated in the statistical analysis. Y.J.J., J.C.L., H.H.H. and P.Y. participated in sample acquisition and data interpretation. J.W. conceived of the study, participated in its design, and helped to draft the manuscript. All authors reviewed the manuscript.

Additional information

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