Association study between polymorphisms of *CD28*, *CTLA4* and *ICOS* and non-segmental vitiligo in a Korean population

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Abstract. CD28 molecule (CD28), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and inducible T-cell co-stimulator (ICOS) are important regulators of the immune system. Vitiligo, a common autoimmune skin disorder, is characterized by a loss of melanocytes that results in cutaneous white patches. The aim of the present study was to determine whether or not polymorphisms of the *CD28*, *CTLA4* and *ICOS* genes are associated with non-segmental vitiligo in a Korean population. To determine the relationships between *CD28*, *CTLA4* and *ICOS* genes and vitiligo, four single nucleotide polymorphisms (SNPs) associated with the *CD28* gene [rs1879877 (promoter, -1198), rs3181097 (promoter, -1059), rs2140148 (intron 1) and rs3116494 (intron 2)], two SNPs associated with the *CTLA4* gene [rs231777 (intron 1) and rs231779 (intron 1)] and five SNPs associated with the *ICOS* gene [rs4270326 (intron 3), rs11571314 (intron 3), rs10183087 (3' untranslated region; UTR), rs4404254 (3'UTR) and rs1559931 (3'UTR)] were selected. Two hundred and thirty-one patients with non-segmental vitiligo (NSV) and 405 healthy controls were enrolled. Genotyping was performed using the restriction fragment length polymorphism technique and direct sequencing. SNPStats, Haploview 4.2 and SPSS 18.0 were used to conduct the analyses. Significant differences were noted between *CTLA4* ($p<0.05$) and NSV, but not *CD28* and *ICOS* ($p>0.05$). However, these associations disappeared after Bonferroni correction. The *CD28*, *CTLA4* and *ICOS* genes may not be associated with NSV.

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Introduction

Vitiligo is an acquired autoimmune skin disorder that is characterized by a loss of epidermal melanocytes (1). The prevalence of vitiligo is approximately 1% in the US and 0.1-2% worldwide (1). Vitiligo usually occurs in childhood or young adulthood, with a peak onset between 10 and 30 years of age (1). The pathogenesis of vitiligo is not completely understood.

CD28 molecule (CD28), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and inducible T-cell co-stimulator (ICOS) are important regulators of the immune system. The *CD28*, *CTLA4* and *ICOS* genes lie in a 300-kb region on chromosome 2q33 (2). CD28 is a cell surface molecule present on most peripheral T-cells (2). CD28 provides co-stimulatory signals to T-cells that prevent the induction of cell death, and promotes interleukin 2 production and clonal expansion (3). CTLA4 is a member of the immunoglobulin superfamily and is a co-stimulatory molecule expressed by activated T-cells (4). CTLA4 transmits an inhibitory signal to T-cells, whereas CD28 transmits a stimulatory signal (4). ICOS is a receptor belonging to the same family as CD28 and CTLA4, known to regulate T-lymphocyte activation in immune responses. ICOS and CD28 enhance T-cell function for effective antigen-specific immune responses, whereas CTLA4 counterbalances CD28-mediated signals and thus prevents overstimulation of the lymphoid system (5). CTLA4 is associated with several autoimmune diseases, including autoimmune thyroid disease, Graves' disease and Hashimoto's thyroiditis (6). The *CD28*, *CTLA4* and *ICOS* genes, which are involved in T-cell regulation, may be candidate genes in autoimmune diseases.

In this study, we determined whether or not single nucleotide polymorphisms (SNPs) in the *CD28*, *CTLA4* and *ICOS* genes are associated with increased susceptibility to non-segmental vitiligo (NSV) in a Korean population.

Materials and methods

Patients and controls. Patients with NSV were recruited among dermatologic outpatients seeking treatment at the Kyung Hee University Medical Center in Seoul, Republic of Korea. Two hundred and thirty-one NSV patients [95 males and 136 females; mean age ± standard deviation (SD), 38.7±18.2 years] and 405 healthy controls (171 males and 234 females; mean age ± SD,

Table I. Clinical characteristics of the NSV patients and control subjects.

	NSV	Controls
Male/female, n	95/136	171/234
Age (mean age ± SD, years)	38.7±18.2	39.2±9.8
Onset age, n		
Childhood (≤18 years)	80	
Adulthood (>18 years)	151	
Autoimmune diseases, n		
With	6	
Without	225	
Family history, n		
With	29	
Without	202	

NSV, non-segmental vitiligo.

39.2±9.8 years) were enrolled in the present study (Table I). NSV is an acquired chronic pigmentation disorder characterized by white patches, often with a symmetric distribution, which usually increases in size over time (1). The NSV patients were stratified into three groups according to the following clinical characteristics: i) child (age ≤18 years) and adult onset (age >18 years); ii) association with other autoimmune diseases; and iii) family history (parents, sons, daughters, brothers and sisters) of vitiligo. The control subjects were recruited among participants in a general health check-up program after confirming that they had no clinical evidence of vitiligo or any other diseases. The Institutional Review Board of Kyunghee University Hospital approved the present study, and all participants provided written informed consent.

SNP selection and genotyping. We selected 11 SNPs within the *CD28*, *CTLA4* and *ICOS* genes, and determined whether or not these SNPs were associated with vitiligo. Four SNPs of the *CD28* gene [rs1879877 (promoter, -1198), rs3181097 (promoter, -1059), rs2140148 (intron 1) and rs3116494 (intron 2)], two SNPs of the *CTLA4* gene [rs231777 (intron 1) and rs231779 (intron 1)] and five SNPs of the *ICOS* gene [rs4270326 (intron 3), rs11571314 (intron 3), rs10183087 (3' untranslated region; UTR), rs4404254 (3'UTR) and rs1559931 (3'UTR)] were selected based on data gathered from public SNP databases (http://www.ncbi.nlm.nih.gov/SNP/ and http://hapmap.ncbi.nlm.nih.gov) and previous studies (7-9). Coding SNPs of the *CD28*, *CTLA4* and *ICOS* genes with heterozygosities <0.1 were excluded (*CD28* gene, rs41272649, rs35290181 and rs75899942; *CTLA4* gene, rs16840275; and *ICOS* gene, rs76778263). Other coding SNPs of the *CD28*, *CTLA4* and *ICOS* genes were also excluded because of non-detected heterozygosity.

Genomic DNA was prepared from peripheral blood using a genomic DNA isolation kit (Roche, Indianapolis, IN, USA). The Roche DNA Extraction kit simplifies isolation of DNA from blood with a fast spin-column. DNA binds specifically to the Roche silica-gel membrane, while contaminants pass through. The DNA was stored at -20˚C for further study. Genotypes of *CD28*, *CTLA4* and *ICOS* SNPs were determined using a restriction fragment length polymorphism (RFLP) technique and direct sequencing analyses.

Statistical analysis. Chi-square tests were used to assess Hardy-Weinberg equilibrium (HWE). Genetic data were analyzed using SNPStats (http://bioinfo.iconcologia.net/index.php?module=Snpstats), Helixtree (Golden Helix Inc., Bozeman, MT, USA), SNPAnalyzer (Istech Inc., Goyang, Korea) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Calculation of linkage disequilibrium (LD) among the *CD28*, *CTLA4* and *ICOS* polymorphisms was performed using Haploview 4.2. Haplotypes of LD were determined by the Gabriel method (10). Multiple logistic regression models (co-dominant, dominant and recessive models) were used to determine the odds ratio (OR), 95% confidence interval (CI) and p-value, controlling for age and gender as co-variables (11,12). The significance level was set at 0.05.

Results

Clinical characteristics of the NSV patients. Eighty and 151 patients had child and adult onset NSV, respectively. Six patients had other autoimmune diseases and 225 patients had no other autoimmune diseases. Twenty-nine patients had family histories of NSV and 202 patients had no family histories of NSV (Table I).

Association study between polymorphisms of CD28, CTLA4 and ICOS and NSV in a Korean population. We investigated whether *CD28*, *CTLA4* and *ICOS* polymorphisms are asssociated with NSV in a sample of Korean patients and controls. No significant deviations from HWE were detected for *CD28*, *CTLA4* and *ICOS* polymorphisms in the control group (data not shown). The genotype frequencies of *CD28*, *CTLA4* and *ICOS* polymorphisms in NSV patients were compared to those of healthy control subjects by logistic regression models. Multiple logistic regression analyses were performed, while controlling for age and gender as co-variables in three models (co-dominant, dominant and recessive models).

The allele and genotype frequencies of *CD28*, *CTLA4* and *ICOS* polymorphisms are shown in Tables II and III. The allele and genotype frequencies of the *CD28* and *ICOS* polymorphisms in the NSV patients were similar to the control subjects. However, the allele frequencies of the *CTLA4* polymorphism (rs231777) were shown to differ between the NSV patients and the control subjects (p=0.041, OR=1.40, 95% CI 1.01-1.94). The T allele frequency of rs231777 was shown to have a higher rate in the NSV patients than in the control subjects (16.2 vs. 12.1%) (Table II). However, there was no significant difference after Bonferroni correction. The genotype frequencies of the *CTLA4* polymorphisms (rs231777 and rs231779) were also shown to be associated with NSV [rs231777, p=0.041, OR=1.49, 95% CI 1.01-2.20 in co-dominant model 1 (C/C vs. C/T), and p=0.040, OR=1.48, 95% CI 1.02-2.14 in the dominant model (C/C vs. C/T and T/T); rs231779, p=0.005, OR=1.64, 95% CI 1.16-2.30 in co-dominant model 1 (T/T vs. T/C), and p=0.009, OR=1.54, 95% CI 1.11-2.14 in the dominant model (T/T vs. T/C and C/C), respectively] (Table III). However, there was no significant difference after Bonferroni correction.

Table II. Allele frequencies of polymorphisms of *CD28*, *CTLA4* and *ICOS* genes in NSV patients and control subjects.

Gene	SNP	Allele	NSV	Control	OR (95% CI)	p-value
			n (%)	n (%)		
CD28	rs1879877	A	280 (60.6)	491 (61.4)	1	
	-1198	C	182 (39.4)	309 (38.6)	1.03 (0.82-1.31)	0.787
	rs3181097	A	235 (50.9)	403 (50.0)	1	
	-1059	G	227 (49.1)	403 (50.0)	0.97 (0.77-1.21)	0.767
	rs2140148	T	418 (90.5)	724 (89.4)	1	
	Intron 1	G	44 (9.5)	86 (10.6)	0.89 (0.60-1.30)	0.536
	rs3116494	A	431 (93.3)	743 (91.7)	1	
	Intron 2	G	31 (6.7)	67 (8.3)	0.80 (0.51-1.24)	0.316
CTLA4	rs231777	C	387 (83.8)	710 (87.9)	1	
	Intron 1	T	75 (16.2)	98 (12.1)	**1.40 (1.01-1.94)**	**0.041**
	rs231779	T	310 (67.1)	575 (72.1)	1	
	Intron 1	C	152 (32.9)	223 (27.9)	1.26 (0.99-1.62)	0.064
ICOS	rs4270326	C	400 (86.6)	708 (88.1)	1	
	Intron 3	G	62 (13.4)	96 (11.9)	1.14 (0.81-1.61)	0.443
	rs11571314	A	382 (82.7)	674 (84.3)	1	
	Intron 3	G	80 (17.3)	126 (15.8)	1.12 (0.82-1.52)	0.469
	rs10183087	A	381 (82.8)	660 (84.2)	1	
	3'UTR	C	79 (17.2)	124 (15.8)	1.10 (0.81-1.50)	0.532
	rs4404254	T	381 (82.8)	664 (83.6)	1	
	3'UTR	C	79 (17.2)	130 (16.4)	1.06 (0.78-1.44)	0.714
	rs1559931	G	383 (82.9)	663 (84.1)	1	
	3'UTR	A	79 (17.1)	125 (15.9)	1.10 (0.80-1.49)	0.568

NSV, non-segmental vitiligo; CD28, CD28 molecule; CTLA4, cytotoxic T-lymphocyte-associated protein 4; ICOS, inducible T-cell co-stimulator; SNP, singe nucleotide polymorphism; UTR, untranslated region; OR, odds ratio; CI, confidence interval. n, number of subjects. p-values were calculated from logistic regression analyses. Bold numbers indicate significant associations.

Haploview 4.2 was used to evaluate LD and haplotypes among the polymorphisms of the *CD28*, *CTLA4* and *ICOS* genes. Three LD blocks were observed among 11 SNPs of the *CD28*, *CTLA4* and *ICOS* genes by the Gabriel method (data not shown). The haplotypes were constructed in block 1 of *CD28*, block 2 of *CTLA4* and block 3 of *ICOS*. These haplotypes included the following SNPs: block 1 (rs1879877, rs3181097 and rs2140148); block 2 (rs231777 and rs231779); and block 3 rs4270326, rs11571314, rs10183087, rs4404254 and rs1559931). The haplotype (rs231777 and rs231779) of the *CLTA4* gene displayed a difference between the NSV patients and the control subjects (TC, χ^2=4.069, p=0.0437; Table IV).

We also evaluated differences in three promoter SNPs according to clinical parameters of NSV. No significant differences were shown in allele, genotype or haplotype frequencies for polymorphisms based on the age of disease onset, a family history or co-existing autoimmune diseases.

Discussion

In the present study, we examined whether or not polymorphisms of the *CD28*, *CTLA4* and *ICOS* genes are associated with patients with NSV. The causes of vitiligo remain under debate, but it is generally accepted to be an acquired disorder (13). Genetic linkage and association studies have implicated a number of different susceptibility genes, such as glutathione S-transferase (*GST*), NLR family genes, pyrin domain containing 1 (*NLRP1*) and tyrosinase (*TYR)* (14-18). Liu *et al* reported that individuals in a sample of Chinese with mutant alleles of the GST gene were at high risk for vitiligo.

We also observed a significant association in null alleles of the *GSTM1* gene (p<0.001, OR=2.048, 95% CI 1.529-2.743) in a previous study (14). Jin *et al* reported that a polymorphism of the *NLRP1* gene contributed to vitiligo susceptibility. In a genome-wide association study, a variant of the *TYR* gene was found to be associated with autoimmunity susceptibility in generalized vitiligo in a European population (15).

The *CTLA4* polymorphisms previously investigated in autoimmune diseases include +49A/G (rs231775) and microsatellite polymorphisms. A microsatellite polymorphism (106-bp) located in exon 3 of the *CTLA4* gene is related to autoimmune diseases, such as Grave's disease (20). A SNP (rs231779) in intron 1 of *CTLA4* was significantly associated with Grave's disease in a sample of Chinese Han individuals (16). The Thr17Ala polymorphism of *CTLA4* was shown to be a genetic marker of autoimmune Addison's disease in a meta-analysis of European studies (17). An association between *CTLA4* and vitiligo has been controversial. Several studies have also reported that

Table III. Genotype frequencies of the polymorphisms of *CD28*, *CTLA4* and *ICOS* genes in the NSV patients and control subjects.

Gene	SNP	Genotype	NSV	Control	Model	OR (95% CI)	p-value
			n(%)	n (%)			
CD28	rs1879877	A/A	84 (36.4)	147 (36.8)	Co-dominant1	1.00 (0.70-1.42)	0.978
	-1198	A/C	112 (48.5)	197 (49.2)	Co-dominant2	1.11 (0.67-1.84)	0.725
		C/C	35 (15.2)	56 (14.0)	Dominant	1.02 (0.73-1.43)	0.910
					Recessive	1.11 (0.70-1.76)	0.660
	rs3181097	A/A	55 (23.8)	100 (24.8)	Co-dominant1	1.13 (0.76-1.68)	0.577
	-1059	A/G	125 (54.1)	203 (50.4)	Co-dominant2	0.94 (0.58-1.51)	0.753
		G/G	51 (22.1)	100 (24.8)	Dominant	1.07 (0.73-1.56)	0.740
					Recessive	0.86 (0.59-1.27)	0.450
	rs2140148	T/T	191 (82.7)	325 (80.2)	Co-dominant1	0.83 (0.54-1.28)	0.396
	Intron 1	T/G	36 (15.6)	74 (18.3)	Co-dominant2	1.14 (0.32-4.11)	0.847
		G/G	4 (1.7)	6 (1.5)	Dominant	0.85 (0.56-1.30)	0.450
					Recessive	1.18 (0.33-4.23)	0.800
	rs3116494	A/A	200 (86.6)	344 (84.9)	Co-dominant1	0.97 (0.60-1.56)	0.898
	Intron 2	A/G	31 (13.4)	55 (13.6)	Co-dominant2	0.00 (0.00-NA)	
		G/G	0 (0.0)	6 (1.5)	Dominant	0.87 (0.55-1.40)	0.570
					Recessive	0.00 (0.00-NA)	0.020
CTLA4	rs231777	C/C	164 (71.0)	317 (78.5)	Co-dominant1	**1.49 (1.01-2.20)**	**0.041**
	Intron 1	T/C	59 (25.5)	76 (18.8)	Co-dominant2	1.41 (0.55-3.57)	0.473
		T/T	8 (3.5)	11 (2.7)	Dominant	**1.48 (1.02-2.14)**	**0.040**
					Recessive	1.28 (0.51-3.24)	0.600
	rs231779	T/T	98 (42.4)	212 (53.1)	Co-dominant1	**1.64 (1.16-2.30)**	**0.005**
	Intron 1	T/C	114 (49.4)	151 (37.8)	Co-dominant2	1.15 (0.62-2.10)	0.668
		C/C	19 (8.2)	36 (9.0)	Dominant	**1.54 (1.11-2.14)**	**0.009**
					Recessive	0.91 (0.51-1.62)	0.740
ICOS	rs4270326	C/C	174 (75.3)	312 (77.6)	Co-dominant1	1.11 (0.75-1.64)	0.602
	Intron 3	G/C	52 (22.5)	84 (20.9)	Co-dominant2	1.51 (0.45-5.02)	0.512
		G/G	5 (2.2)	6 (1.5)	Dominant	1.14 (0.78-1.66)	0.510
					Recessive	1.47 (0.44-4.89)	0.530
	rs11571314	A/A	161 (69.7)	286 (71.5)	Co-dominant1	1.04 (0.72-1.51)	0.817
	Intron 3	A/G	60 (26.0)	102 (25.5)	Co-dominant2	1.48 (0.63-3.51)	0.372
		G/G	10 (4.3)	12 (3.0)	Dominant	1.09 (0.76-1.55)	0.640
					Recessive	1.47 (0.62-3.45)	0.390
	rs10183087	A/A	161 (70.0)	280 (71.4)	Co-dominant1	1.02 (0.70-1.49)	0.893
	3'UTR	A/C	59 (25.6)	100 (25.5)	Co-dominant2	1.45 (0.61-3.44)	0.398
		C/C	10 (4.3)	12 (3.1)	Dominant	1.07 (0.75-1.53)	0.710
					Recessive	1.44 (0.61-3.39)	0.410
	rs4404254	T/T	161 (70.0)	280 (70.5)	Co-dominant1	0.99 (0.68-1.43)	0.944
	3'UTR	T/C	59 (25.6)	104 (26.2)	Co-dominant2	1.34 (0.57-3.13)	0.501
		C/C	10 (4.3)	13 (3.3)	Dominant	1.02 (0.72-1.46)	0.890
					Recessive	1.35 (0.58-3.12)	0.490
	rs1559931	G/G	162 (70.1)	280 (71.1)	Co-dominant1	0.99 (0.68-1.44)	0.958
	3'UTR	A/G	59 (25.5)	103 (26.1)	Co-dominant2	1.57 (0.65-3.79)	0.313
		A/A	10 (4.3)	11 (2.8)	Dominant	1.04 (0.73-1.49)	0.810
					Recessive	1.58 (0.66-3.78)	0.310

NSV, non-segmental vitiligo; CD28, CD28 molecule; CTLA4, cytotoxic T-lymphocyte-associated protein 4; ICOS, inducible T-cell co-stimulator; SNP, singe nucleotide polymorphism; UTR, untranslated region; OR, odds ratio; CI, confidence interval; NA, not applicable. p-values were calculated from logistic regression analyses. Bold numbers indicate significant associations.

CTLA4 polymorphisms were associated with vitiligo (18,19). For example, the GG genotype and G allele of the +49A/G (rs231775) SNP of *CTLA4* was observed at a higher frequency in vitiligo patients compared to control subjects (20). However, Deeba *et al* showed that there is no association between the CTLA-4 A49G gene polymorphism and vitiligo in a southern

Table IV. Haplotype frequencies of polymorphisms of *CD28*, *CTLA4* and *ICOS* genes in the NSV patients and control subjects.

Haplotype	Frequency	NSV		Control		Chi-square	p-value
		+	-	+	-		
Block 1							
AAT	0.504	235	227	404	402	0.065	0.7988
CGT	0.390	182	280	312	494	0.058	0.8089
AGG	0.103	44	418	86	720	0.419	0.5173
Block 2							
CT	0.703	310	152	579.9	224.1	3.548	0.0596
CC	0.160	77	385	126.1	677.9	0.210	0.6472
TC	0.137	75	387	98.0	706.0	4.069	**0.0437**
Block 3							
CAATG	0.835	382	80	662	126	0.372	0.5419
GGCCA	0.124	61	401	94	694	0.436	0.5093
CGCCA	0.039	18	444	31	757	0.001	0.9734

Haplotype of block 1 consists of rs1879877, rs3181097 and rs2140148. Haplotype of block 2 comprises of rs231777 and rs231779. Haplotype of block 3 consists of rs4270326, rs11571314, rs10183087, rs4404254 and rs1559931. NSV, non-segmental vitiligo. Bold number indicates significant associations.

Indian population (21). In the present study, *CTLA4* SNPs (rs231777 and rs231779) were also shown to have no association with NSV. We also did not observe a significant association between NSV and the *CD28* and *ICOS* genes.

We calculated the required sample size for sufficient statistical power using a genetic power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html). In this study, the sample powers of our SNPs were 0.931 for rs231777 (α=0.05; genotype relative risk, 2-fold; number of cases for 80% power, 153) and 0.985 for rs231779 (α=0.05; genotype relative risk, 2-fold; number of cases for 80% power, 105). Therefore, our results have statistical confidence.

In conclusion, we investigated whether SNPs of the *CD28*, *CTLA4* and *ICOS* genes are related to NSV in a sample of Korean individuals; we observed no significant associations between *CD28*, *CTLA4* and *ICOS* and NSV in the Korean population.

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