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Association of rs2294020 in the X-linked *CCDC22* with susceptibility to autoimmune diseases with focus on systemic lupus erythematosus

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Abstract

Autoimmune diseases often share common susceptibility genes. Most genetic variants associated with susceptibility to systemic lupus erythematosus are also associated with other autoimmune diseases. The X-linked variant rs2294020 is positioned in exon 7 of the *CCDC22* gene. The encoded protein functions in the regulation of NF-kB, a master regulator in immune response. The aim of this study is to investigate whether the rs2294020 polymorphism may be a general susceptibility factor for autoimmunity. We evaluated case-control association between the occurrence of rs2294020 and different autoimmune diseases, including new data for systemic lupus erythematosus and previous genome-wide association studies (GWAS) (though most did not analyse the X chromosome) of psoriasis, celiac disease, Crohn's disease, ulcerative colitis, multiple sclerosis, vitiligo, type-1 diabetes, rheumatoid arthritis, and ankylosing spondylitis. Cases from patients affected by amyotrophic lateral sclerosis and type-2 diabetes were also included in the study. We detected nominal significant associations of rs2294020 with systemic lupus erythematosus (additive model test: p=0.01), vitiligo (p =0.016), psoriasis (p =0.038), and in only one of two studies of multiple sclerosis (p =0.03). Our results suggest that rs2294020 is associated with the risk of several autoimmune diseases in European populations, specifically with diseases that present themselves, among else, in the skin.

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Keywords

systemic lupus erythematosus; autoimmune disease; CCDC22 gene; *FOXP3* gene; single nucleotide polymorphism

1. Introduction

Systemic lupus erythematosus (SLE) is a complex and systemic autoimmune disorder. It is characterized by abnormal T- and B-cell responses, occurrence of autoantibodies, and immune complex depositions [1]. The mechanisms underlying the etiopathogenesis of SLE remain elusive, but several genetic and environmental factors have been associated with the disease [2]. Genetic predisposition plays a determinant role in SLE onset and maintenance. Many genetic variants have been associated with SLE susceptibility, including those which are involved in NF-κB signalling [3,4]. NF-κB, a master regulator in immune response [5], is involved in several autoimmune diseases, including SLE [6,7].

The coiled-coil domain-containing 22 gene (CCDC22; gene ID: 50943) is located on the X-chromosome. The functions of this highly conserved encoded protein are still elusive. However, it has been shown that this protein may be involved in NF-κB activation, through its interaction with copper metabolism gene MURR1 domain (COMMD) proteins [8].

Since CCDC22 is correlated with NF-kB activation, we focused on the CCDC22 gene in order to investigate its potential role in the susceptibility to autoimmune disorders, including SLE. Focusing our attention on the putative 3'UTR region of *CCDC22* gene and on the basis of Ensembl linkage disequilibrium data (www.ensembl.org) (X:49236763–49256762; population: 1000GENOMES:phase 3 CEU), we identified the variant rs2294020 (bp position 49246763 - forward strand) as a tag polymorphism for this region with a minor allele frequency (MAF) >0.01. The rs2294020 variant, located on exon 7 of the coding *CCDC22* gene, is positioned in close proximity to the *FOXP3* gene in the complementary strand and within the putative 3'-UTR region of *FOXP3* (www.ensembl.org) [9]. Thus, the occurrence of rs2294020 may contribute to the impaired function of *FOXP3*.

rs2294020 has been investigated in juvenile idiopathic arthritis [10], hay fever [11], and alopecia areata [12]. In particular, Eastell et al [10], found no association between *FOXP3* rs2294020 polymorphism and juvenile idiopathic arthritis in a case-control association analysis of a UK population, although the authors did not rule out FOXP3 as a candidate gene for this disease, due to the low statistical power obtained in male enrolled patients. By using an additive effect-only logit model, rs2294020 has been found associated with hay fever in a German population. The occurrence of the SNP rs2294020 also slightly increased the risk for atopy [11]. Alopecia areata, an autoimmune disorder, has been shown to be associated with the rs2294020 polymorphism in an Italian population [12].

The aim of the present study was to investigate the association between the rs2294020 *CCDC22* SNP and susceptibility to SLE in a North Italian Caucasian population. Furthermore, we analyzed 14 GWAS datasets in order to reveal association between this SNP and susceptibility to other autoimmune diseases in individuals of European ancestry.

Such autoimmune diseases included psoriasis, celiac disease, Crohn's disease (CD), ulcerative colitis (UC), multiple sclerosis (MS), vitiligo, type-1 diabetes (T1D), rheumatoid arthritis (RA), and ankylosing spondylitis (AS). We also included datasets for amyotrophic lateral sclerosis (ALS) and type-2 diabetes (T2D), where etiopathogenetic autoimmune mechanisms have been suggested [13,14].

2. Materials and Methods

2.1 Subjects

The study, designed as case-control, was composed of 189 unrelated Italian patients with SLE (171 females and 18 males). 180 Healthy subjects (131 females and 49 males) were used as control individuals. All patients and healthy subjects were recruited from the Department of Internal Medicine (University of Milan, Italy). Patients were diagnosed according to the "1982 Revised Criteria for Classification of Systemic Lupus Erythematosus" [15].

Clinical manifestations included the occurrence of malar rash or discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorders, neurological disorders, hematological disorders (hemolytic anemia, leukopenia, thrombocytopenia), positive antinuclear antibodies (ANA), and immunological alterations (occurrence of anti-dsDNA, anti-Sm). All subjects gave informed consent for the study, which was approved by the local ethics committee. The clinical characteristics of the patients are summarized in table 1.

2.2 DNA Extraction and Genotyping

Genomic DNA was extracted from whole peripheral blood with a commercial DNA isolation kit (Nuclear Laser Medicine, Italy), using a salting out method. The candidate SNP rs2294020 was selected for genotyping in patients and control subjects. Genotype analysis was performed by high-resolution melting (HRM) analysis. The primer sequences (size: 182 bp) were:

FW: 5'- CTGCTTCCCCCGCCTTTTCT-3';

RV: 5'- GCCCTTAGGAGCACCAGTCTT-3'.

Polymerase chain reaction (PCR) was carried out in 25 μl reaction mixture: 12.5 μl 2×HRM PCR Master MIX, with EVAGreen® dye (Qiagen, Germany), 1 μl of genomic DNA (50 ng), 3.5 μl of primer mix (containing 0.7 μM of the forward and reverse primers), 8 μl water. Water was used as a negative control for PCR contamination. HRM analysis was performed on Rotor-Gene Q real time instrument (Qiagen). All the analyses were run according to the following conditions: 40 cycles of 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 10 seconds, and a melt from 65°C to 95°C at intervals (ramps) of 0.02°C/s. To ensure genotyping quality, positive control and template negative controls were included for each genotype in each run. The analysis of the result was carried out using Rotor-Gene 6000 software (Corbett Life Science, Australia).

In order to verify the efficiency of real time-PCR HRM approach, we performed a direct genotyping, by PCR-direct sequencing, on 20% of randomly chosen samples. The purified

samples were analyzed with the ABI PRISM BigDyeTM Terminator kit (Applied Biosystems, USA) on the automatic sequencer 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled using the ABI PrismDNA software 3.7 (Applied Biosystems). 100% concordance was obtained.

2.3 Datasets

As already described [16], GWA datasets were obtained from dbGaP and Wellcome Trust Case Control Consortium 1 or 2. Characteristics of these datasets are summarized in table 2.

2.4 Statistical analysis

Since the *CCDC22* gene is located on the X chromosome, we utilized XWAS: a specialized-software for analysis of the X chromosome in GWAS [37, 38], which is implemented on the basis of PLINK [39]. Single SNP association analysis in SLE patients study was performed with the use of Fisher's exact test. P values < 0.05 were considered statistically significant. Odds ratios (OR) and 95% confidence intervals (CI 95%) were calculated. Logistic association analysis was performed assuming additive and sex-stratified models (see below $MF_{comb\ Fish}$). Then, we tested whether the effect size was different between males and females. Moreover, dataset analysis was performed as described in Gao et al. [37]. Briefly, we used four tests: a) FM_{01}, assuming skewed X-inactivation; b) MF_{02}, assuming complete female X-inactivation; c) $MF_{comb\ Fish}$, male and female subjects are analyzed separately. Then, a combined value of significance was obtained from Fisher method. X-inactivation status is non influential; d) $MF_{Comb\ Stouffer}$, similar to $MF_{comb\ Fish}$, a combined value of significance was obtained from Stouffer method. This test accounts for different sample sizes between males and females. As only a single SNP has been considered in this studies, nominal p-values were considered, without correction for multiple hypothesis.

3. Results

To evaluate the association between the *CCDC22* rs2294020 SNP and systemic lupus erythematosus, we conducted a case-control study in an Italian Caucasian population. Moreover, we screened the SNP in 16 GWAS datasets of different autoimmune diseases in individuals of European ancestry. As reported in table 3, under the additive model, there was a significant association between the presence of the rs2294020 SNP and the susceptibility to SLE (OR: 1.59; CI: 1.10–2.30; p = 0.01). Furthermore, the effect sizes were different between the sexes (OR: 0.33; CI: 0.18–0.59; p < 0.01). Sex stratified analysis showed a borderline significant association between the presence of the SNP under study and susceptibility to SLE (Males OR: 1.43; female OR: 1.60, $p_{comb.\ Fisher} = 0.05$).

Furthermore, we analyzed the frequency distributions between different phenotypic clinical aspects (including age of disease onset, presence of malar rash or discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorders, neurological disorders, hematological disorders [hemolytic anemia, leukopenia, thrombocytopenia], positive antinuclear antibodies [ANA], and immunological alterations [occurrence of anti-dsDNA, anti-Sm]) and healthy control subjects. Such comparisons did not associate statistically to the susceptibility to the disease (data not shown).

Table 4 reports the GWAS results of the association between the rs2294020 SNP and susceptibility to 14 different autoimmune diseases, as well as to amyotrophic lateral sclerosis (ALS) and type-2 diabetes (T2D). In particular, patients affected by multiple sclerosis from database WT2 showed a significant association (MF01 = 0.034; $MF_{comb\ Stouffer} = 0.030$). However, these results were not replicated in the dbGaP dataset of multiple sclerosis patients. Both vitiligo datasets showed a significant association between the rs2294020 SNP and susceptibility to the disease ($MF_{01} = 0.018$ and 0.023, respectively). Furthermore, vitiligo GWAS1 showed significant *p* values of association ($MF_{02} = 0.013$; $MF_{comb\ Fisher} = 0.040$; $MF_{comb\ Stouffer} = 0.016$). Similarly, the psoriasis dataset shows a significant association between the occurrence of the SNP under study and the susceptibility to psoriasis ($MF_{02} = 0.027$; $MF_{01} = 0.039$; $MF_{comb\ Stouffer} = 0.038$).

4. Discussion

In the present case-control study, we hypothesized that *CCDC22* rs2294020 polymorphism might be involved in susceptibility to SLE in an Italian Caucasian population. Our data supports this hypothesis. Furthermore, we have shown that such a genetic variant may be associated with the susceptibility to other autoimmune diseases, such as vitiligo and psoriasis. A significant association between the rs2294020 SNP and multiple sclerosis was found only in the WT2 dataset. Discrepancies between the two MS datasets' results may be due to several factors. Genetic heterogeneity, environmental factors, interactions between genetic and environmental factors, and different geographic regions may significantly impact study replication [40,41].

The genetic variant rs2294020 is positioned on exon 7 of the coding *CCDC22* gene. Since this SNP is a synonymous variant for the *CCDC22* product, its effect may be due to its position in 3'UTR region of *FOXP3* and/or *CCDC22* gene itself (ensembl.org) [9].

Production of autoantibodies and breakdown of peripheral immune tolerance are critical players in the pathogenesis of SLE. Peripheral tolerance is controlled by regulatory T cells (Tregs), a heterogeneous population of T lymphocytes [42]. The *FOXP3* gene is mainly expressed in Tregs and encodes the transcription factor FOXP3 [43]. A reduced number or a functional impairment of Tregs may be the cause of predisposition to several autoimmune diseases [44], including SLE [45]. Many *FOXP3* gene polymorphisms have been shown to be associated with autoimmune diseases, including SLE [46–48]. Additionally, genetic variants associated with autoimmune diseases largely encompass functional sequences, such as enhancer and regulatory sequences, which can interact with specific transcription factors. About 90% of genetic variants, identified in twenty-one autoimmune diseases, are non-coding. Moreover, about 60% of these variants lie in immune cell enhancers [49].

Functional SNPs are potential regulatory variants which may affect transcription [50,51]. 3' untranslated regions (3'UTR) are considered to contribute to mRNA stability and localization, and translational efficiency of the gene [52]. Thus, alterations in the 3' UTR regions of *FOXP3* may result in a 90% decrease of protein expression, which in turn may induce impairment of Treg functions [53].

However, we cannot exclude a similar 3' UTR function for *CCDC22*. Impairment in CCDC22 expression, due to rs2204020 SNP located in the 3' UTR, may dysregulate NF-kB signalling [5], thus contributing to the inflammatory process in SLE. NF-kB is a master regulator of the immune response and its enhanced or inappropriate activation has been shown to be involved in several autoimmune diseases, including SLE [54,55]. In SLE, there is an increased production of autoantibodies by polyclonal activation of B cells, which may be maintained by the inducible transcription factor NF-kBp65 [56]. NF-kB therapeutic modulation, through ubiquitination and degradation of individual subunits of this transcription factor, may represent an attractive approach in combatting autoimmune diseases [55].

Finally, there is the possibility that these two genes could contribute independently to impaired Treg function. In fact, impaired Treg function could be achieved by defective expression of *FOXP3* gene, whose product is considered to be an essential key regulator for the induction and development of this cell subset. Alternatively, defective expression of *CCDC22* gene could be responsible for a dysregulated NF-kB signalling pathway, which is known to be a key regulator of FOXP3 expression [57].

Our data show that the rs2294020 genetic variant displays significant association mainly in autoimmune diseases involving the skin, i.e. systemic lupus erythematosus, psoriasis and vitiligo. Moreover, the occurrence of this SNP has been shown, by other authors, to be associated with susceptibility to alopecia aerata [12]. It would be interesting to investigate in the near future the possible pathogenetic mechanisms underlying these skin-related autoimmune disorders. These studies may focus on mechanisms involving plasmacytoid dendritic cells (pDCs), the most potent producers of type I IFN. Chronic pDC activation is known to contribute to the initiation of different autoimmune skin disorders, including systemic lupus erythematosus, psoriasis, vitiligo and alopecia aerata [58]. Furthermore, the occurrence of a feedback regulatory loop does exist between DCs and FOXP3+ Treg cells, which are essential for maintaining self tolerance [59]. An impaired function of FOXP3 may induce a loss of Treg cells, which, in turn, would increase DCs proliferation and skin infiltration, thus inducing the early phase of autoimmune skin diseases by type I IFN production.

In conclusion, our study suggests that the presence of the rs2294020 gene variant may be associated with susceptibility to SLE in a Caucasian Italian population. The rs2292040 SNP is shown to have a small to moderate effect size, like many other gene variants associated with different autoimmune diseases [60]. Furthermore, the occurrence of the rs2294020 SNP also seems to be associated with the susceptibility to vitiligo and psoriasis, thus suggesting that different autoimmune diseases may share common genetic factors. However, for genes that harbor this SNP, the mechanisms leading to autoimmunity remain unclear. The limitation of our study is the absence of functional analysis and gene-targeted assays for this genetic variant.

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Highlights

- rs2294020 in the X-linked *CCDC22* was tested for association with susceptibility to different autoimmune diseases
- Significant associations between this SNP and patients affected by systemic lupus erythematosus, vitiligo and psoriasis were identified
- rs2280883 may play an important role in the development of some autoimmune diseases, specifically with those that present themselves in the skin, among other places.

Table 1

Baseline demographic and clinical characteristics of SLE patients.

Sex (female)	171 (90.48%)
Age of disease onset (years), mean ± SEM	30.71± 0.85
Malar rash	124 (65.61%)
Discoid rash	38 (20.10%)
Photosensitivity	110 (58.20%)
Oral ulcers	13 (6.88%)
Arthritis	119 (62.96%)
Serositis	53 (28.04%)
Pleuritis	33 (17.46%)
Pericarditis	33 (17.46%)
Renal disorders *(*)*	60 (31.75%)
Neurological disorders *(**)*	22 (11.64%)
Hematological disorders	121 (64.02%)
Haemolytic anemia (with reticulocytosis)	27 (14.28%)
Leukopenia	94 (49.73%)
Lymphopenia	16 (8.46%)
Thrombocytopenia	48 (25.40%)
Immunological disorders *(***)*	164 (86.77%)
ANA positivity	154 (81.48%)

(*): Renal disorders include: a) persistent proteinuria greater than 0.5 grams per day or grater than 3 if quantitation not performed or b) presence of cellular casts (red cell, hemoglobin, granular, tubular, or mixed).

(**): Neurological disorders include: a) seizures or b) psychosis,; both manifestations in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance.

(***): Immunological disorders include: a) positive SE cell test or b) presence of anti-DNA antibody or c) anti-Sm antibody or d) false positive serologic test for syphilis known to be positive for at least 6 months and confirmed by T*reponema pallidum* immobilization or fluorescent treponemal antibody absorption test

Table 2

GWAS datasets.

Dataset	Disease	Source (accession no.)	Ref.	Cases (F/M ratio)	Controls (F/M ratio)
ALS Finland	Amyotrophic Lateral Sclerosis (ALS)	dbGaP (phs000344)	[17]	400 (1.02)	490 (3.76)
ALS Irish	Amyotrophic Lateral Sclerosis (ALS)	dbGaP (phs000127)	[18]	221 (0.86)	210 (0.87)
Celiac disease CIDR	Celiac disease	dbGaP (phs000274)	[19]	1576 (2.52)	504 (1.24)
MS Case Control	Multiple Sclerosis (MS)	dbGaP (phs000171)	[20]	943 (2.02)	851 (1.93)
MS WT2	Multiple Sclerosis (MS)	WT2	[21]	2666 (2.82)	1389 (0.98)
Vitiligo GWAS1	Vitiligo	dbgaP (phs000224)	[22]	1391 (2.38)	4521* (1.28)
Vitiligo GWAS2	Vitiligo	-	[23]	415 (1.88)	2552* (1.62)
CD NIDDK	Chron's disease (CD)	dbGaP (phs000130)	[24]	791 (1.09)	922 (1.02)
CDWT1	Crohn's disease (CD)	WT1	[25]	1592 (1.62)	1701 (0.84)
Psoriasis CASP	Psoriasis	dbGaP (phs000019)	[26]	1209 (1.06)	1271 (1.17)
T2D GENEVA	Type-2 Diabetes (T2D)	dbGaP (phs000091)	[27]	2515 (1.39)	2850 (1.40)
T2D WT1	Type-2 Diabetes (T2D)	WT1	[25]	1811 (0.72)	1668 (1.35)
T1D WT1	Type-1 Diabetes (T1D)	WT1	[25]	1867 (0.96)	1714 (0.82)
RA WT1	Rheumatoid Arthritis (RA)	WT1	[25]	1772 (3.00)	1709 (0.86)
AS WT2	Ankylosing Spondylitis (AS)	WT2	[28]	1472 (0.51)	1260 (0.89)
UC WT2	Ulcerative Colitis (UC)	WT2	[29]	2341 (1.04)	1699 (1.01)

*Controls were obtained from dbGaP: phs000206 [30,31], phs000168 [32], phs000138 [33], phs000125 [34], phs000092 [34–36].

Abbreviations: WT1 (2), Wellcome Trust Case Control Consortium 1 (2).

Table 3

Genotype analysis for *CCDC22* SNP rs2294020 in patients affected by SLE and healthy controls (MAF, Minor allele frequency; OR, odds ratio; L95, lower limit of confidence interval; U95, upper limit of confidence interval, *p*, $\chi 2$ test *p* value).

TEST	***MAF cases***	***MAF controls***	***OR***	***L95***	***U95***	***STAT***	***p***
Add	0.2806	0.1833	1.59	1.10	2.30	2.49	**0.01**
Sex	-	-	0.33	0.18	0.59	−3.66	**0.000024**
TEST			***OR M***	***OR F***	***p F***	***Fisher Chi squared***	$p_{Comb\ Fisher}$
SexStrat	-	-	1.43	1.60	0.01	9.36	**0.05**

Table 4

GWA dataset analysis for *CCDC22* SNP rs2294020 in patients affected by different autoimmune diseases, including myotrophic lateral sclerosis and type-2 diabetes (MAF, Minor allele frequency; for other abbreviations refer to text).

Disease	MAF cases	MAF controls	MF_{02}	MF_{01}	$MF_{comb\ Fisher}$	$MF_{comb\ Stouffer}$
Amyotrophic Lateral Sclerosis (Finland)	0.3449	0.3640	0.642	0.541	0.819	0.539
Amyotrophic Lateral Sclerosis (Irish)	0.2943	0.2743	0.501	0.596	0.781	0.579
Celiac disease	0.2723	0.2902	0.793	0.580	0.598	0.559
Multiple Sclerosis	0.2491	0.2681	0.140	0.191	0.303	0.206
Multiple Sclerosis (WT2)	0.2812	0.2712	0.084	**0.034**	0.090	**0.030**
Vitiligo (GWAS1)	0.2593	0.3075	**0.013**	**0.018**	**0.040**	**0.016**
Vitiligo (GWAS2)	0.2362	0.2787	**0.044**	**0.023**	0.071	0.051
Chron's disease (NIDDK)	0.2929	0.2679	0.658	0.438	0.470	0.417
Chron's disease (WT)	0.2684	0.2546	0.439	0.485	0.729	0.498
Psoriasis	0.2536	0.2846	**0.027**	**0.039**	0.077	**0.038**
Type-2 Diabetes (GENEVA)	0.2815	0.2733	0.503	0.375	0.671	0.381
Type-2 Diabetes (WT)	0.2843	0.2689	0.202	0.389	0.346	0.425
Type-1 Diabetes	0.2663	0.2674	0.255	0.390	0.466	0.445
Rheumatoid Arthritis	0.2581	0.2676	0.374	0.626	0.444	0.633
Ankylosing Spondylitis	0.2692	0.2706	0.211	0.685	0.024	0.625
Ulcerative Colitis	0.2751	0.2705	0.548	0.769	0.564	0.797