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Rapid Communication

Indoleamine 2,3 dioxygenase gene polymorphisms correlate with CD8+ Treg impairment in systemic sclerosis

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ABSTRACT

Systemic sclerosis (SSc) is characterized by tissue fibrosis, vasculopathy and autoimmunity. Indoleamine 2,3 dioxygenase (IDO) plays a pivotal role in immunological tolerance modulating regulatory T cell (Treg) generation and function. Single nucleotide polymorphisms (SNPs) of IDO gene could impact on Treg function and predispose to autoimmunity. Here, the existence of an association between specific IDO SNPs and SSc was analyzed.

Five specific SNPs in IDO gene were searched in 31 SSc patients and 37 healthy controls by gene sequencing or restriction fragment length polymorphism. The function of both CD4+CD25+ and CD8+ Treg from SSc patients was analyzed by proliferation suppression assay.

SNP rs7820268 was statistically more frequent in SSc patients than in controls. Notably, SSc patients bearing the T allelic variant of the rs7820268 SNP showed impaired CD8+ Treg function.

Our unprecedented data show that a specific IDO gene SNP is associated with an autoimmune disease such as SSc.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by immune alterations, vasculopathy and fibrosis [1]. Although SSc pathogenesis is poorly defined, autoimmunity seems to play an important role as demonstrated by the presence of circulating autoantibodies and activated T cells infiltrating diseased tissues [1,2]. Genetic predisposition to the disease has been postulated on the basis of epidemiologic evidences and supported by the discovery of mutations/polymorphisms affecting different genes related to the connective tissue, the vascular system as well as the immune system [3]. Recently, the existence of an unbalance between Th17 pro-inflammatory responses and regulatory T lymphocyte (Treg) function has been highlighted, suggesting that the impairment of the Treg compartment could play role in SSc pathogenesis as already shown for other autoimmune diseases [4,5]. Among the mechanisms involved in Treg generation and function, the enzyme indoleamine 2,3-dioxygenase (IDO) is mainly at play. Indeed, IDO, leading to kynurenine production through tryptophan degradation, suppresses effector T cell function and favors the differentiation of Treg [6–11]. Genetic variants of the IDO gene may modify IDO activity [12] potentially predisposing to immune alterations and/or autoimmunity. Whether genetic variants of IDO gene may be present in SSc patients has not been investigated. Here, the association of specific single nucleotide polymorphisms (SNPs) of IDO gene with SSc was analyzed taking also into account Treg functional activity in individual SSc patients.

2. Material and methods

2.1. Patients and controls

Thirty-one patients affected with SSc (mean age 64 ± 11 years, female/male ratio 7/1) were enrolled at the Division of Internal Medicine and Clinical Immunology of the Department of Internal Medicine, University of Genoa, after giving their informed consent.

Diagnosis of SSc was made according to the American College of Rheumatology criteria [13] and median disease duration was 17 ± 13 years. Patients were subdivided as having limited (lSSc, 25 patients) or diffuse (dSSc, 6 patients) SSc and were under

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treatment with vasoactive (e.g. iloprost or dihydropyridine calcium channel blockers) but not with immunosuppressive drugs [14].

Thirty seven healthy subjects, age and sex matched with the SSc patients, were studied as controls.

The study was approved by the Ethical Committee of San Martino Hospital in Genoa.

2.2. Gene sequencing

Genomic DNA was extracted from 300 μl of peripheral blood in sodium citrate using the Puregene Dna purification system (GENTRA System, MN). Primers were designed to amplify the exons 1, 3 and 5 as well as the introns 5 and 6 (chromosome 8, 39777452-39777758; BLAST NCBI Genome Browser) of IDO gene (GenBank ID: NG_028155). Gene amplification was performed by polymerase chain reaction (PCR) using 1.5 nM Mg^{2+} concentration and different annealing temperatures (Table 1). PCR products of exons 1 and 5 as well as of introns 5 and 6 were then purified by UltraClean PCR clean up Sample Kit (QIAGEN, Hilden, Germany), sequenced using the ABI BigDye Terminator (Applied Biosystems, Foster City, CA), and then run on an ABI 3130XL Genetic Analyzer machine (Applied Biosystems, Foster City, CA). Chromatogram analysis was performed by Chromas Software (Technelysium Pty Ltd., Helensvale, Au). Each sample was compared with the IDO gene sequence present in NCBI database by Lasergene SeqMan Software (DNASTAR, Madison, USA).

2.3. Genotyping analysis by restriction fragment length polymorphism

The rs35099072 SNP in exon 3, located within the restriction site of HAEIII, was searched by restriction fragment length polymorphism. Ten to twenty micro litre of the corresponding PCR product were digested using the Fastdigest HaeIII restriction enzyme (FERMENTAS - Thermo/Fisher Scientific Company - Waltham, Massachusetts, USA) following manufacturer's recommendations. Allelic discrimination was performed running digested DNA on a 2% agarose electrophoresis.

2.4. Generation of CD8+ Treg

CD8+ Treg were generated as described [15]. Peripheral blood mononuclear cells (PBMC) were purified by centrifugation on Ficoll gradient. CD8+CD28− T cells were sorted using magnetic microbeads conjugated with specific monoclonal antibodies (mAb) (Life Technologies - Carlbad, USA). The purity of sorted cells was ⩾95% as demonstrated by flow cytometric analysis. Purified CD8+CD28− T lymphocytes were cultured in complete culture medium RPMI 1640 with 20 U/ml of IL2 (Proleukin, Eurocetus, BV) and 20 ng/ml of IL10 (R&D System, MN USA) for 7 days. At the end of the incubation the cells were used as suppressors in a proliferation suppression assay.

2.5. Purification of CD4+$CD25^{hi}$ Treg

$CD4+CD25^{hi}$ Treg were purified from PBMC by magnetic bead cell sorting using the $CD4+CD25^{hi}$ human regulatory T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of sorted cells was ⩾95% as demonstrated by flow cytometric analysis.

2.6. Immunofluorescence analyses

Phenotypes were analyzed by immunofluorescence incubating the cells (1×10^5 cells in 100 μl of PBS) with specific mAbs at 4 °C for 30 min in the dark. The following mAbs were used: phycoerythrin (PE) conjugated anti-CD127, allophycocianin (APC)-cyanin (Cy) 7-conjugated anti-CD3 (e-Biosciences, San Diego, CA), PE and fluorescein isothiocynate (FITC) conjugated anti-CD25 (Miltenyi, Bergisch Gladbach, Germany), Pe-Cy7-conjugated anti-CD8 (Biolegend, San Diego, CA), Peridin-Chlorophyll-protein complex (PerCP)-Cy5.5-conjugated anti-CD28 (eBioscience, San Diego, CA). Then the cells were analyzed by a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, New Jersey) using FACSDIVA software (BD Biosciences, Franklin Lakes, New Jersey).

2.7. Proliferation suppression assay

The suppressive activity of Treg was evaluated by monitoring the inhibition of dye dilution in PBMC stained with Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (5 μM) (Molecular Probes, Life Technologies, Carlbad, USA) before the test. Thereafter, the cells were pulsed with the anti-CD3 UCHT-1 mAb (5 μg/ml) (BD Bioscience, Franklin Lakes, New Jersey) and cultured for 5 days in 96 well flat bottomed plates (1×10^5 cells/well) in the presence (or not) of CD8+ or $CD4+CD25^{hi}$ Treg (1×10^5 cells/well). After washings the samples were analyzed by flow cytometer excluding dead cells from analysis by adding 7-amino-actinomycin D (7AAD) (BD Bioscience, Franklin Lakes, New Jersey) before acquisition.

2.8. Statistical analysis

Statistically significant differences between frequencies were assessed using the Chi-square test for binary variables. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego – California, USA).

3. Results

3.1. The frequency of rs7820268 (C6202T) SNP is increased in scleroderma patients compared with controls

The following SNPs of IDO gene, already described in the general population (Gene Sorter, NCBI Genome Browser), were comparatively analyzed in SSc patients and healthy controls: rs35059413 (C123T) in exon 1, rs35099072 (C4325T) in exon 3, rs61753677 (C6293T) in exon 5, rs7820268 (C6202T) in intron 5 and rs73619577 (C6982A) in intron 6.

Concerning the rs7820268 SNP, the genotypes carrying the T allelic variant (C/T + T/T), together considered, were significantly more frequent in SSc patients than in healthy donors (Table 2). Accordingly, the frequency of the T variant allele was significantly higher in the studied SSc population than in control group (Table 3).

Table 1
Primers and temperatures used for PCR amplifications.

Exon	Intron	Forward primer 5′-3′	Reverse primer 5′-3′	Annealing temperature (°C)
1		CTCACTGCCCCTGTGATAAAC	AGTTAACTTGGCCAGGTAAGG	56
3		AGAGTAGTCAGGAGGATT	TGCATGATGCAGTTATAATGC	52
5	5–6	TGTAATGCCTACTGAAGAAAC	CTTAAATTATTTTTTGGCTGAATTCAA	54

Table 2
Frequencies of genotypes carrying the rs7820268 SNP T allelic variant in SSc patients and controls.

SSc patients	Controls	Odds ratio	95% confidence intervals	P value
22/31	17/37	2.876	1.047-7.895	0.03

Chi-square test comparing the frequency of C/T + T/T genotypes in 31 SSc patients and 37 healthy controls was performed.

Table 3
Frequencies of rs7820268 SNP T allele in SSc patients and controls.

SSc patients	Controls	Odds ratio	95% confidence intervals	*P* value
27/62	18/74	2.400	1.155-4.985	0.01

Chi-square test comparing the frequency of the T allele in 31 SSc patients and 37 healthy controls was performed.

In our cohorts of scleroderma patients and healthy donors the rs35059413, rs35099072, rs61753677 and rs73619577 SNPs were not detected.

No statistically significant associations were detected between the frequency of rs7820268 (C6202T) SNP and independent variables such as age, sex, disease form, and specific clinical features typical of SSc, analyzed by a panel of clinical tests routinely applied in our center to SSc patients [16], including: frequency/intensity of Raynaud phenomenon as well microvessel abnormalities (measured by nailfold videocapillaroscopy), skin fibrosis (measured by mRodnan score and by plicometry) [17], lung alterations (analyzed by spirometry), esophagous abnormalities (analyzed by pH-metry and manometry), renal dysfunction (tested by dosing serum creatinine levels and measuring the intrarenal resistive index by duplex doppler sonography), heart disease (evaluated by echocardiography) (not shown).

3.2. Alteration of CD8+ Treg function is present in patients bearing the rs7820268 (C6202T) SNP

The suppressive functions of CD4+CD25^{hi} or CD8+ Treg were comparatively analyzed in SSc patients carrying or not the T allelic variant of the rs7820268 SNP.

A suppression activity $\geqslant$25% was considered as normal since comparable to that of healthy controls [4,18]. Impaired suppression activity of CD8+ Treg was observed in 5/11 (45%) of SSc patients carrying the T allelic variant, and in any of SSc patients with the corresponding C allelic variant (Fig. 1 and Table 4). Concerning this parameter, no differences were observed between homozygous and heterozygous SSc patients for the rs7820268 SNP T allele (not shown).

Table 4
CD8+ Treg suppression activity in SSc patients carrying or not the rs7820268 SNP T allele.

Patient #	rs7820268 SNP genotype	CD8+ Treg suppression activity (%)
2	C/C	65
3	C/C	91
7	C/C	85
9	C/C	75
10	T/T	51
12	C/T	0
14	C/T	30
15	C/C	56
18	C/T	88
19	T/T	78
21	C/T	15
22	C/C	83
24	C/T	79
25	C/C	58
26	T/T	0
27	C/T	75
28	C/C	80
29	C/T	0
30	C/C	70
31	C/T	17

No statistically significant difference of CD4+CD25^{hi} Treg function was detected between SSc patients carrying or not the T allelic variant (data not shown).

4. Discussion

The results of this study show that the T allelic variant of the IDO gene rs7820268 SNP: (a) is significantly more frequent in SSc patients than in healthy controls; (b) may be associated with impaired CD8+ Treg suppression activity.

IDO is highly involved in maintenance of immunologic homeostasis since its activation inhibit effector/inflammatory T cells and promote the differentiation and activation of Treg [6–11]. Interestingly, Treg alterations have been recently demonstrated in SSc patients [4] and IDO itself seems to be related to different pathological conditions such as autoimmunity, allergy, chronic inflammation and tumors [19–21].

Although polymorphisms of IDO gene have been observed in the general population [12], no data exist on their possible association with specific diseases. Here it is reported the unprecedented finding of an IDO gene SNP, rs7820268, that appears to be associated with SSc. Notably, the fact that a nucleotidic substitution,

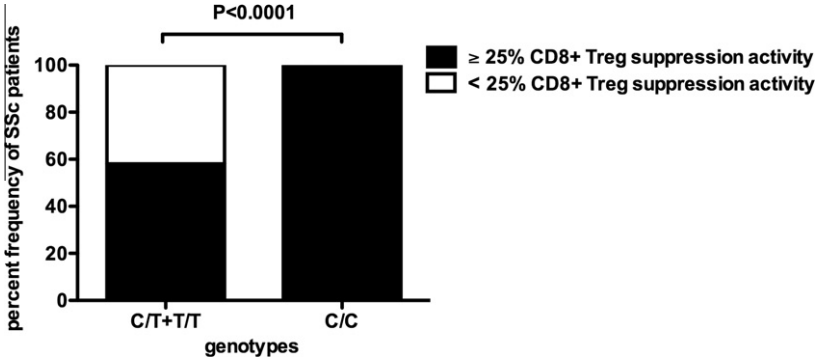

Fig. 1. Frequency of SSc patients carrying the rs7820268 T allelic variant showing altered CD8+ Treg function. Proliferation suppression assays were performed with CD8+ Treg from SSc patients carrying either the C allelic variant in homozygosis or the T allelic variant in homozygosis or in heterozygosis. A suppression activity $\geqslant$25% was considered as normal since comparable to that of healthy controls. Black boxes relate to patients with a $\geqslant$25% CD8+ Treg suppression activity; white boxes relate to patients with a <25% CD8+ Treg suppression activity. A Chi-square test analysis was performed and the statistically significant difference is indicated.

occurring at an intronic site, may vary structure and/or function of a gene product is not surprising since it may affect mRNA splicing or specific regulatory sequence(s) modulating gene expression [22].

When the SSc population was comparatively analyzed in relation to the presence of the T or the C rs7820268 SNP allelic variants, alterations of Treg function, targeting specifically the CD8+ Treg, were observed only in SSc patients carrying the T allelic variant. This finding suggests that this IDO allele might effectively impact on Treg-controlled immunological regulatory circuits. Interestingly, IDO is not expressed by the subpopulation of CD8+ Treg (namely, non-antigen specific CD8+ Treg [15]) on which we focused our study (our unpublished observation). Hence, it is likely that rs7820268 SNP affects IDO expression or function in cell populations different from Treg (i.e. in dendritic cells) and that such event may have an effect on the cell-to-cell crosstalk leading to CD8+ (but not CD4+CD25^{hi}) Treg generation. The validity of this hypothesis as well as the predictive/prognostic relevance of the analysis of rs7820268 SNP in SSc will be addressed by a new study involving a larger SSc population.

5. Author contribution

ST carried out the molecular genetic studies and contributed to data acquisition, data analysis and interpretation, and manuscript preparation; SN selected the patients for the study and contributed to data acquisition, data analysis and interpretation, and manuscript preparation; GC and FF contributed to molecular genetic studies, data analysis and interpretation; AP, FB, and FK performed the immunoassays; DF contributed to immunoassays and participated in the design of the study; MC participated in the study design and helped to draft the manuscript; GF participated in the study design, contributed to data analysis and interpretation, and wrote the manuscript.

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