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ORIGINAL ARTICLE: RESEARCH

Polymorphisms in *TNF* and *IFNG* are associated with clinical characteristics of aplastic anemia in Argentinean population

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Abstract

The impaired hematopoiesis in acquired aplastic anemia (AA) results from immune-mediated mechanisms. We characterized polymorphisms implicated in controlling type-1 cytokine production in 69 patients with AA. Our data suggest that the studied polymorphisms are not associated with susceptibility in the overall AA population. However, the presence of the higher expressing *TNF* − 308A allele was associated with younger age ($p = 0.0297$) and more profound neutropenia ($p = 0.0312$), and over-represented in patients with very severe AA ($p = 0.0168$). The higher producing *IFNG* 12 CA-repeat allele showed strong linkage disequilibrium with the + 874T allele, and was associated with a lower hemoglobin level ($p = 0.0351$). Also, the presence of at least one higher expressing variant was more frequent among patients responding to immunosuppressive treatment ($p = 0.0519$). Our findings suggest that the presence of higher expressing variants of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in AA patient genotypes could be related to clinical parameters, disease severity and therapy outcomes.

Keywords: Tumor necrosis factor-α, *TNF*, interferon-γ, *IFNG*, polymorphisms, aplastic anemia

Introduction

Acquired aplastic anemia (AA) is a marrow failure syndrome with an incidence of 1–2 patients per 1 000 000 per year, characterized by peripheral blood pancytopenia and bone marrow hypoplasia. Many pathogenic mechanisms have been proposed to account for bone marrow failure, including hematopoietic stem/progenitor cell deficiency, abnormal hematopoietic microenvironment and immunity disorders [1–4]. In most cases, AA is an autoimmune disease in which the impaired hematopoiesis results from immune-mediated mechanisms. This model is supported by a number of *in vivo* and *in vitro* observations, including the response of patients to immunosuppressive treatment (IST). Cytotoxic T lymphocytes, T helper (Th) 1 lymphocytes and their cytokine products such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and Fas-induced apoptosis are considered the main effector mechanisms of immune-mediated suppression of hematopoiesis in marrow failure syndromes [5–8]. The combination of antithymocyte/antilymphocyte globulin (ATG/ALG), which lyses lymphocytes, and cyclosporine (CsA), which blocks T-cell function, is the first-line treatment for patients lacking human leukocyte antigen (HLA)-identical related donors. This IST option produces a response rate of around 70%, which appears to reflect the immune pathophysiology of AA [9–11].

A genetic predisposition is recognized in many autoimmune diseases. It is well established that highly polymorphic genes of the major histocompatibility complex (MHC) and various cytokines and cytokine receptors may influence both genetic susceptibility and resistance to several autoimmune diseases, including acquired bone marrow failure [12–15]. In several cytokine genes, polymorphisms that are located at regulatory regions or promoter regions can cause variable cytokine expression. The *TNF* gene contains functional single polymorphisms (SNPs) localized in the promoter region. Among them the most studied is − 308 G/A SNP, and the − 308A allele has been linked to higher expression of TNF-α [16]. Intron 1 of the *IFNG* gene contains

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two polymorphisms which could have functional consequences for gene transcription: + 874 A/T SNP and + 875 CA microsatellite. The + 874T allele and the 12 CA-repeat allele have been associated with increased IFN-γ production [17,18]. It has been documented that some patients with acquired marrow failure syndromes have a higher frequency of polymorphisms associated with higher production of pro-inflammatory cytokines such as TNF-α and IFN-γ [15,19–23]. Also, several published studies have described associations between polymorphisms that modify the activity of cytokines and clinical manifestations in myelodysplastic syndromes [21,24]. In AA, polymorphisms affecting cytokine expression have been associated with response to IST [22,25]. However, as far as we know, there are no previous reports that relate the presence of these polymorphisms to other clinical characteristics in this pathology.

To test the hypothesis that genetic factors may be linked to an increased susceptibility to AA, we investigated the − 308 G/A SNP of the *TNF* gene and + 874 A/T SNP and + 875 CA microsatellite of the *IFNG* gene. In addition, we analyzed whether the presence of the higher producing variants of these cytokines are associated with clinical parameters, severity of the disease and response of patients with AA to IST.

Materials and methods

Patients

This was a multicenter retrospective analysis of 69 patients with AA diagnosed from March 1987 to February 2013. Clinicians from the participating institutions completed a standard registration form for each patient detailing the clinical and hematological features at presentation and during follow-up. Diagnosis of AA and disease severity were established by bone marrow biopsy and peripheral blood counts according to criteria defined by Camitta *et al.* [26] and the International Agranulocytosis and Aplastic Anemia Study group [27]. Only patients lacking dysplasia in the hematopoietic series with normal or non-informative cytogenetics at diagnosis were included. Patients with congenital AA or positive Ham test and/or paroxysmal nocturnal hemoglobinuria (PNH) clone > 1.5% were excluded. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki of 1975, as revised in 2008. Informed consent was obtained from all patients to be included in the study. Table I shows clinical characteristics of the patients with AA. In addition, 120 normal blood donor samples were studied as the control population.

All patients eligible for evaluation to response to IST were treated with a combination of ATG/ALG and CsA. Response to IST was evaluated at 6 months after receiving therapy. Complete response (CR) was defined as a normal hemoglobin level according to age, neutrophil count > 1500/μL and platelet count > 100 000/μL. Partial response (PR) was defined as transfusion independence, neutrophil count > 500/μL, platelet count > 20 000/μL and hemoglobin level > 8.0 g/dL in patients with severe or very severe AA, and neutrophil count > 1000/μL, platelet count > 30 000/μL and hemoglobin level > 8.0 g/dL in patients with moderate AA [28]. Overall response was defined as CR or PR.

Table I. Clinical characteristics of patients with AA ($n = 69$).

Characteristic	n (%)
Age (years)	
Median	16.0
Mean	23.0
Range	2–74
Gender	
Males/females	34/35
Classified according to severity	
vsAA	14 (20)
sAA	41 (60)
mAA	9 (13)
ND	5 (7)
Initial treatment	
HLA-identical related HSCT	8 (11.5)
IST*	49 (71)
Other therapies†	8 (11.5)
ND	4 (6)
Eligible for evaluation at 6 months from IST (ATG/ALG plus CsA), $n = 38$	
Response to IST	
Yes	26 (68.4)
No	12 (31.6)

vsAA, very severe aplastic anemia; sAA, severe AA; mAA, moderate AA; ND, not determined; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplant; IST, immunosuppressive therapy; ATG/ALG, antithymocyte/antilymphocyte globulin; CsA, cyclosporine.
*Five patients were treated with HSCT after failed IST.
†Supportive care, androgens (oxymetholone) and/or hematopoietic growth factors among others.

Genotyping of cytokine polymorphisms

Genomic DNA from peripheral blood or bone marrow samples from patients and healthy donors were obtained by the standard phenol–chloroform and ethanol precipitation method.

TNF − 308 G/A SNP (rs1800629) was analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). Briefly, a 107 bp fragment of the *TNF* gene was amplified by PCR, under standard conditions, using primers: 5′-AGGCAATAGGTTTTGAGGGCCA T-3′ (forward) and 5′-TCCTCCCTGCTCCGATTCCG-3′ (reverse). The PCR products were digested with *Nco*I (Fermentas, Tecnolab, Argentina) overnight at 37°C. RFLP products were resolved on non-denaturing 12% (w/v) polyacrylamide gel electrophoresis (PAGE) (3% cross-linked) and visualized using silver staining [29].

IFNG + 874 A/T SNP (rs2430561) was studied by allele-specific PCR, under standard conditions. The primer sequences were as follows: generic primer 5′-TCAACAAA GCTGATACTCCA-3′, allele A-specific primer 5′-TTCTTACAACACAAAATCAAATCA-3′ and allele T-specific primer 5′-TTCTTACAACACAAAATCAAATCT-3′ (264 bp) [18]. A 632 bp fragment of the beta globin gene was amplified as internal control using primers: 5′-ATACAATGTATCATGCCTCTTTGCACC-3′ (forward) and 5′-GTATTTTCC CAAGGTTTGAACTAGCTC-3′ (reverse). The PCR products were monitored by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide. [AQ4]

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IFNG + 875 CA microsatellite (rs3138557) was detected as previously described [19]. The microsatellite region was amplified by PCR using two primers, 5′-GCTGTCATAA TAATATTCAGAC-3′ (forward) and 5′-CGAGCTTTAAAAGAT AGTTCC-3′ (reverse). [AQ5] PCR products (119-131 pb) were separated on a non-denaturing 12% (w/v) PAGE (5.3% cross-linked) and revealed by silver staining.

The obtained genotypes for each cytokine polymorphism were confirmed by automatic sequence analysis.

Statistical analysis

Descriptive statistics were reported as absolute frequencies and percentages for qualitative data and mean ± standard deviation (SD) or median with range for quantitative data. Comparison of genotypic and allelic frequencies between patients and controls, or between responders and non-responders to IST, was performed by χ^2 or Fisher's exact test. Differences in clinical parameters at diagnosis between patients regarding genotype were analyzed using *t*-test or Mann–Whitney test. *p*-Values < 0.05 were considered statistically significant. Data were analyzed using SPSS software version 17.00 (SPSS, Chicago, IL) and InfoStat 2008 version (Grupo InfoStat, Universidad Nacional de Córdoba, Argentina). Pairwise linkage disequilibrium (D′ value) between SNP (rs2430561) and microsatellite (rs3138557) were estimated according to Maynard [30].

Results

Distribution of cytokine polymorphisms

The distribution of allelic and genotypic profiles in patients with AA and controls for *TNF* − 308 G/A SNP and *IFNG* + 875 CA microsatellite is summarized in Table II. Genotypes were grouped according to the presence of *TNF* − 308A allele associated with a higher expression of this cytokine. There was no significant difference in allelic and genotypic frequencies between patients with AA and controls (Table II). Regarding the analysis of *IFNG* + 875 CA polymorphism, seven microsatellite variants were observed, with no significant differences in the distribution of genotypic and allelic frequencies when comparing patients versus controls (Supplementary Table I available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.966707). Genotypes were grouped according to the presence of the higher producing repeat as follows: homozygous 12/12 CA, heterozygous 12/non-12 CA and non-12/non-12 CA. Based on our data, there was no significant difference between patients with AA and the control population with respect to genotypic and allelic frequencies (Table II). The observed results for + 874 A/T SNP (data not shown) were similar to those obtained for + 875 CA microsatellite, and we found strong linkage disequilibrium between both 12 CA-repeat and + 874T functional alleles (D′ = 0.99).

Association of polymorphisms with clinical parameters and response to IST

Table III provides a summary of the clinical characteristics at diagnosis of patients with AA regarding genotypes for the *TNF* − 308 G/A SNP and *IFNG* + 875 CA microsatellite. The presence of the higher expressing *TNF* − 308A allele in our series was associated with younger age (12 vs. 17 years; $p = 0.0297$) and reduced neutrophil count (320 vs. 520/μL; $p = 0.0312$) at diagnosis. Also, G/A + A/A genotype was over-represented in patients classified as having very severe AA (4/7, 57% vs. 10/57, 17.5%; $p = 0.0168$, odds radio: 6.266), which is consistent with the observed association with more profound neutropenia. Regarding *IFNG* polymorphisms, analysis of the + 875 CA microsatellite revealed that the presence of the 12 CA-repeat allele in the genotype of our AA population was associated with a lower hemoglobin level (6.7 vs. 7.9 g/dL; $p = 0.0351$) at diagnosis. The other analyzed parameters were not statistically different (Table III). Similar associations with clinical characteristics were obtained for the *IFNG* + 874 A/T SNP (data not shown).

In addition, we evaluated the response to IST in patients with AA who received a combination of ATG/ALG and CsA as initial treatment. For this analysis we grouped patients with at least one variant associated with higher expression of cytokines (*TNF* − 308A allele and/or *IFNG* 12 CA-repeat allele) in their genotype. We found that this group of patients showed a higher frequency of individuals who achieved an overall response at 6 months, with borderline statistical difference (21/27, 78% vs. 5/11, 45%; $p = 0.0519$).

Discussion

The understanding of the pathogenesis of AA includes altered cellular immunity, gradual destruction of the hematopoietic stem cells and an abnormal hematopoietic microenvironment, which can lead to bone marrow failure [1,3]. However, the manner in which the target antigen

Table II. Genotypic and allelic frequencies of *TNF* − 308 G/A SNP and *IFNG* + 875 CA microsatellite in patients with AA and controls.

Genotype	Patients with AA, *n* = 69 (%)	Controls, *n* = 120 (%)	*p*-Value	Allele	Patients with AA, *n* = 138 (%)	Controls, *n* = 240 (%)	*p*-Value
TNF − 308 G/A SNP							
G/G (low)	62 (90)	104 (87)	0.6460*	G	131 (95)	222 (92.5)	0.3992*
G/A + A/A (high)	7 (10)	16 (13)		A	7 (5)	18 (7.5)	
IFNG + 875 CA microsatellite							
12/12 CA (high)	6 (9)	18 (15)	0.2236†	12 CA	47 (34)	83 (35)	1.0000†
12/non-12 CA (intermediate)	35 (51)	47 (39)		Non-12 CA	91 (66)	157 (65)	
Non-12/non-12 CA (low)	28 (40)	55 (46)					

SNP, single nucleotide polymorphism; AA, aplastic anemia.
*Fisher's exact test.
†χ^2 test.

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Table III. Clinical data at diagnosis of patients with AA regarding genotypes for *TNF* − 308 G/A SNP and *IFNG* + 875 CA microsatellite.

	TNF − 308 G/A SNP			*IFNG* + 875 CA microsatellite		
Genotype	G/A + A/A (high)	G/G (low)	*p*-Value	12/12 CA (high) + 12/non-12 CA (intermediate)	Non-12/non-12 CA (low)	*p*-Value
Age (years)						
Median (range)	12 (2–22)	17 (2–74)	0.0297*	17 (2–70)	15 (2–74)	0.3630*
Gender						
Male/female	2/5	32/30	0.2477†	21/20	13/15	0.6959†
Hemoglobin (g/dL)						
Mean ± SD	7.8 ± 1.6	7.1 ± 2.2	0.4284‡	6.7 ± 2.1	7.9 ± 2.0	0.0351‡
Platelet count/μL						
Median (range)	11 500 (3000–54 000)	11 150 (1000–68 000)	0.9738*	11 000 (1000–65 000)	11 650 (2300–68 000)	0.4422*
Neutrophil count/μL						
Median (range)	320 (45–770)	520 (0–2340)	0.0312*	420 (0–2080)	560 (77–2340)	0.3318*
Reticulocytes (%)						
Median (range)	0.5 (0–0.9)	0.5 (0–2.8)	0.6191*	0.45 (0–2.8)	0.55 (0–2.2)	0.3785*
Severity, *n* (%)						
vsAA	4 (29)	10 (71)	0.0168†, odds ratio: 6.266	5 (44)	6 (56)	0.6603†
sAA	3 (6)§	38 (94)§		22 (61)	18 (39)	
mAA	0 (0)§	9 (100)§		7 (57)	4 (43)	

AA, aplastic anemia; SNP, single nucleotide polymorphism; SD, standard deviation; vsAA, very severe AA; sAA, severe AA; mAA, moderate AA.
*Mann–Whitney test.
†Fisher's exact test or χ^2 test.
‡*t*-test.
§sAA and mAA were combined for χ^2 calculations.

triggers an immune response remains unknown. The disorder and dysfunction of T cell subsets and the abnormal regulation of cytokines might be key factors in the development of AA [6,8,31]. The immune effector mechanisms involved in AA and other marrow failure syndromes mainly include, in addition to direct cell-mediated killing, release of Th1 cytokines with inhibitory activity on hematopoietic progenitors such as IFN-γ and TNF-α [5–8].

It is well established that various immunogenic factors may impart an important predisposition to increased and/or reduced immune responses. A group of immunogenic factors that are becoming of great interest for the understanding of several autoimmune diseases are represented by cytokine functional polymorphisms [12–14]. An association with susceptibility or with specific clinical manifestations has been described for several acquired marrow failure syndromes [15,19–24]. In order to determine possible influences of pro-inflammatory cytokine polymorphisms on susceptibility, clinical characteristics and response to IST, we studied the *TNF* −308 G/A, *IFNG* +874 A/T and +875 CA microsatellite polymorphisms, which affect cytokine expression, in a cohort of Argentinean patients with AA.

In this study, the genotypes that confer higher TNF-α and IFN-γ production were not over-represented in our AA population. The obtained result for *TNF* −308 G/A SNP was in agreement with prior reports [20,22,23], consistent with an apparent homogeneity of the observed frequencies for *TNF* −308 G/A alleles among different ethnic control groups [32,33]. Although other studies described a relationship between AA and *IFNG* polymorphisms [15,19,20], the discrepancies may be explained by factors as the selection criteria, population size and ethnicity. Frequencies of higher expressing *IFNG* +874T and 12 CA-repeat alleles seem to be population-specific, being lower in normal controls from Japan (9%) and China (17%), and higher in Europe (45–50%) [19,32–37]. In addition, Serio *et al.* found that the genotype of higher IFN-γ production seems to be related to the presence of the PNH clone in patients with AA [23], and our series did not include patients with AA with a PNH clone higher than 1.5%.

We found an association between the presence of higher producing variants and clinical parameters in our AA series. The presence of the higher expressing *TNF* −308A allele was associated with younger age and more profound neutropenia in patients with AA at diagnosis. Genotype related to higher production of TNF-α was over-represented in patients classified as having very severe AA, which is consistent with the observed association with reduced neutrophil counts (very severe criterion is defined as a neutrophil count <200/μL). Also, analysis of the *INFG* +875 CA microsatellite revealed that the higher producing 12 CA-repeat allele was associated with a lower hemoglobin level in patients with AA at diagnosis. As far as we know, this is the first study to relate these polymorphisms with clinical characteristics in AA. Our findings are consistent with the notion that the immunoregulatory cytokine polymorphisms may act as a genetic modifier of disease severity in this pathology.

Several reports have suggested that either the CA repeat sequence itself has a regulatory function or there is an allelic linkage between the CA repeat and a functional SNP, which would account for the differences in IFN-γ production [17]. The +874 A/T SNP coincides with a putative binding site of transcription factor nuclear factor-κB (NF-κB) [38] that can lead to functional consequences for *IFNG* gene transcription. Pravica *et al.* reported preferential binding of NF-κB with the +874T allele as an absolute correlation between the presence of both this allele and the 12 CA-repeat allele [18]. In the present study, we found only nine out of 189 individuals without this correlation, supporting a strong

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linkage disequilibrium (D′ = 0.99) between both functional alleles. As expected, due to this strong correlation, the analysis of these *IFNG* polymorphisms showed similar results, not only in genotypic and allelic frequencies but also in association with clinical parameters.

In addition, we found that the presence of at least one variant associated with higher expression of these pro-inflammatory cytokines was more frequent among patients who achieved an overall response to IST at 6 months. Our results are in agreement with prior reports that related either the presence of *TNF* −308A allele [25] or polymorphisms in *IFNG* and *TGFB1* genes [22] to a higher response rate to IST. The current recommendations are that if a matched unrelated donor can be found quickly, then transplant could be considered a potential first-line option in those younger patients who lack a matched sibling donor [10]. Thus, genotyping of cytokine polymorphisms, which represents a minimum workload, may provide information for making a decision to proceed with ATG-based IST or an upfront transplant from a matched unrelated donor.

In conclusion, our data suggest that the studied polymorphisms in *TNF* and *IFNG* genes are not associated with susceptibility to AA in our population. Nevertheless, the presence of higher expressing variants in AA patient genotypes could be related to clinical parameters, severity of the disease and IST outcomes. These findings are consistent with the concept that polymorphisms affecting cytokine expression may act as a genetic modifier of AA severity. Further studies are needed to confirm that cytokine polymorphism profiles may help to identify patients who will benefit from IST.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Supplementary Table I showing genotypic and allelic frequencies of *IFNG* + 875 CA microsatellite.

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Supplementary material for Bestach Y. et al. Polymorphisms in TNF and IFNG are associated with clinical characteristics of aplastic anemia in Argentinean population. *Leuk Lymphoma* 2014; doi:10.3109/10428194.2014.966707.

Supplementary Table I. Genotypic and allelic frequencies of *IFNG* + 875 CA microsatellite in AA patients and controls.

Genotype	AA patients n = 69 (%)	Controls n = 120 (%)	*P-value*	Allele	AA patients n = 138 (%)	Controls n = 240 (%)	*P-value*
11/12 CA*	0 (0,0)	1 (0,8)					
12/12 CA	6 (8,7)	18 (15,0)		11 CA*	0 (0,0)	1 (0,4)	
12/13 CA	23 (33,3)	28 (23,3)					
12/14 CA	2 (2,9)	4 (3,3)		12 CA	47 (34,1)	83 (34,6)	
12/15 CA	8 (11,6)	13 (10,8)		13 CA	60 (43,5)	95 (39,6)	
12/16 CA*	1 (1,4)	0 (0,0)					
12/17 CA*	1 (1,4)	1 (0,8)	*p* = 0.1537	14 CA	5 (3,6)	18 (7,5)	*p* = 0.6399
13/13 CA	11 (15,9)	23 (19,2)		15 CA	24 (17,4)	41 (17,1)	
13/14 CA	1 (1,4)	9 (7,5)					
13/15 CA	14 (20,3)	12 (10,0)		16 CA*	1 (0,7)	0 (0,0)	
14/15 CA	2 (2,9)	4 (3,3)		17 CA*	1 (0,7)	2 (0,8)	
14/17 CA*	0 (0,0)	1 (0,8)					
15/15 CA	0 (0,0)	6 (5,0)					

*Less frequent genotypes and alleles were combined for chi-square calculations.

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