

Comparison of somatic STAT3 and STAT5b gene mutations between Felty's syndrome and T-cell large granular lymphocytic leukemia with rheumatoid arthritis

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Abstract

Objectives Approximately 15% of patients with T-cell large granular lymphocytic leukemia (T-LGLL) have rheumatoid arthritis (RA). RA-associated T-LGLL with low large granular lymphocyte counts (aleukemic presentation) and Felty's syndrome (FS) have indistinguishable clinical presentations. These disorders are distinguished by T-cell clonality which is observed in T-LGLL but not in FS. Activating somatic mutations in the signal transducer and activator of transcription 3 (STAT3) and 5 (STAT5b) genes are involved in T-LGLL pathogenesis; however, the prevalence of these mutations in FS is unknown.

Methods Based on the rearrangements of T-cell receptor (TCR) gamma and beta genes according to the BIOMED-2 protocol, we examined T-cell clonality in 81 patients with RA and unexplained neutropenia. We stratified these patients by the presence or absence of T-cell clonality, respectively, into 2 groups: RA-associated T-LGLL (56 patients) and FS (25 patients). Allele-specific TaqMan Real-Time polymerase chain reaction assay was employed to detect point somatic mutations in STAT3 and STAT5b genes in each group.

Results Mutations of the STAT3 gene were detected in none of the 24 cases of FS and in 22 of 56 cases of RA-associated T-LGLL (39%) ($p < 0.001$). No mutation of the STAT5b gene was detected in any of the patients in each group.

Conclusions Although further data are needed, our results suggest that activating somatic mutations in STAT3 and STAT5b genes are not involved in the pathogenesis of FS.

Introduction

Currently, Felty's syndrome (FS) is considered an uncommon subset of seropositive rheumatoid arthritis (RA).[1] Persistent unexplained neutropenia (absolute neutrophil counts below $1.5-2.0 \times 10^9/L$) is a mandatory criterion for suggesting FS in a patient with RA. Although splenomegaly was one of the triads originally described by Felty, later studies showed that spleen size does not correlate with neutropenia and that patients with RA and neutropenia are like patients with the full triad.[2-4] At present, splenomegaly is not an absolute diagnostic requirement for diagnosing FS.[5, 6] FS is a clinical diagnosis and there is no specific single diagnostic test to confirm or exclude it; therefore, FS is essentially a diagnosis of exclusion. The pathogenesis of FS is unknown.

Large granular lymphocytic leukemia (LGLL) is a rare chronic lymphoproliferative disorder characterized by the expansion of immunophenotypically distinct, clonal, large granular lymphocytes (LGLs). In most cases, LGLL has an immunophenotype of cytotoxic T-lymphocytes (CD3+/CD8+) that co-express NK-cell lineage-associated antigens CD16 and/or CD57, and diminished or absent expression of pan T-cell markers CD5 and/or CD7.[7, 8] Approximately 15% of patients with T-cell LGLL (T-LGLL) have RA [9, 10]; on the contrary, clonal expansion of LGLs was detected in 3.6% of patients with RA.[11] T-LGLL is usually diagnosed 10-15 years after the manifestation of RA.[5] A typical manifestation of T-LGLL is neutropenia and splenomegaly detected in up to 84% and 50% of patients, respectively.[12] Recent studies on the

lymphocytes from patients with T-LGLL have shown activating somatic mutations in signal transducer and activator of transcription 3 (*STAT3*) and 5 (*STAT5b*) genes in 27–72% [13–16] and 2% [17] of all cases, respectively.

T-LGLL with RA resembles FS in many aspects and some researchers hypothesize that RA-associated T-LGLL and FS are part of a single disease process with a common pathogenic mechanism.[5, 18, 19] However, the prevalence of *STAT3* and *STAT5b* mutation status in FS is unknown. In this study, we stratified 81 patients with RA and unexplained neutropenia into 2 groups based on the presence and absence of T-cell clonality (RA-associated T-LGLL and FS), and then examined *STAT3* and *STAT5b* gene mutations in both groups. We also present the clinical and laboratory characteristics of 25 patients with FS and discuss their differential diagnosis with RA-associated T-LGLL.

Patients And Methods

We diagnosed FS if all three criteria were met: (i) RA diagnosed according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria [20]; (ii) neutropenia (absolute neutrophil counts $< 1.5 \times 10^9/L$) without an alternative explanation such as drug-induced suppression; (iii) absence of T-cell clonality.

Peripheral blood (PB) smears for LGL counting were re-examined in 18 cases. Bone marrow aspiration with differentiated cell counts was performed in 14 cases and in 11 of these, a bone marrow biopsy was also performed. The collected clinical data included patient age, sex, presence of splenomegaly, RA duration, titers of rheumatoid factor (RF), antibodies against cyclic citrullinated peptides (anti-CCP), antibodies against mutated citrullinated vimentin (anti-MCV), maximal Disease Activity Score derivative for 28 joints calculated using C-reactive protein (max.DAS28-CRP) during follow-up, erosive arthritis, and associated autoimmune diseases.

Flow cytometric analysis

A four-color flow cytometric analysis was performed on peripheral blood (16 cases), peripheral blood and bone marrow (2 cases), and bone marrow (1 case) specimens. Lymphocytes were gated using CD45 versus side scatter dot plots. Cells were stained with a panel of fluorescence-labeled monoclonal antibodies including CD3, CD4, CD5, CD7, CD8, CD16, CD19, CD56, and CD57. Flow cytometry analysis was performed on a BD FACSCanto™ II (Becton Dickinson, San Jose, CA, USA) using FCS express Version 3 (De Novo Software, Los Angeles, CA) software.

Immunohistochemical studies

Immunohistochemical studies were carried out for 6 cases using sections of decalcified paraffin-embedded bone marrow biopsy specimens. The following antibodies were used at the dilutions suggested by the manufacturers: CD3 (Polyclonal, Dako), CD4 (clone 4B12, Dako), CD8 (clone C8/144B, Dako), CD20 (clone L26, Dako), CD56 (clone 123C3, Dako), CD57 (clone TB01, Dako), granzyme B (clone

GrB-7, Dako), and TIA1 (clone 2G9, Immunotech, France). After dewaxing and heat-induced antigen retrieval, immunostaining was performed on an Autostainer Link 48 (Dako, Denmark) according to the manufacturer's instructions. All immunostained samples were counterstained with hematoxylin.

Evaluation of STAT3 and STAT5b gene mutations and T-cell clonality

STAT3 and *STAT5b* gene mutations and T-cell clonality were examined using genomic DNA extracted from blood (17 patients), blood and bone marrow (5 patients), bone marrow (1 patient), blood and spleen (1 patient), and blood, bone marrow and spleen tissue (1 patient) samples.

Evaluation of T-cell clonality was based on the rearrangements of the T-cell receptor (TCR) gamma ($V\gamma-J\gamma$) and TCR beta ($V\beta-J\beta$, $D\beta-J\beta$) genes. T-cell clonality assays were performed according to the BIOMED-2 standardized protocol.[21] Polymerase chain reaction (PCR) was carried out using an automated DNA Engine thermocycler (BioRad, Hercules, USA), and fragments were detected using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA); the data were analyzed using GeneMapper software version 4.0 (Applied Biosystems, Foster City, CA).

Allele-specific (AS) TaqMan Real-Time PCR assays were employed to determine the somatic point mutations in *STAT3* (p.Y640F; p.N647I; p.D661V; p.D661Y; p.D661H; p.D661N) and *STAT5b* (p.N642H) genes. DNA (200–400 ng) was added to 25 μ L of the reaction mixture containing 10 pmol of WT (wild type)-specific or MT (mutated type)-specific forward primer, 10 pmol of common reverse primer, and 7.5 pmol of the fluorescent probe. AS-PCR was then performed in triplicate (3 WT + 3 MT) using a StepOne Real-Time PCR System (Applied Biosystems, USA). PCR conditions included preliminary denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. A mixture of DNA from healthy donors was used as a negative control. Samples with mutations confirmed by Sanger sequencing were used as positive controls. The primer and probe sequences are shown in Supplement 1. As a comparison group, we tested the *STAT3* and *STAT5b* mutational status in 56 patients with T-LGLL in the context of RA.

Statistical analysis

Fisher's two-tailed test was used to compare the frequency of *STAT3* mutations between the groups of patients with FS and RA-associated T-LGLL.

Results

We stratified 81 patients with RA and neutropenia according to the presence and absence of T-cell clonality, respectively, into 2 groups: RA-associated T-LGLL (56 patients) and FS (25 patients). The clinical and biological characteristics of 25 FS patients are presented in Table 1 and in Supplementary Table 1. Seven of the 25 (28%) patients also had a concomitant autoimmune condition: 6 patients had Sjogren syndrome (SS) and one patient had SS and autoimmune thyroiditis. Nineteen of the 25 (76%) patients were females. The median age at FS diagnosis was 54 years (range, 30–79 years). The median duration

of RA prior to FS diagnosis was 7 years (range, 0–35 years). The median maximum RA activity over the observation period, estimated by DAS28, was 4.9 (range, 2.1–7.01). Erosive arthritis at FS diagnosis was detected in 16 of 22 (73%) of patients. RF was positive in 23 of 25 (92%) patients; however, 2 of these 23 RF-positive patients had low RF levels (less than 2 normal ranges). The median RF level was 332.7 IU/mL (range, 9.5–12900.0 IU/mL). Anti-CCP was positive in 23 of 24 (96%) patients. Anti-MCV was positive in all 11 patients examined. In all 4 RF-negative and weakly positive patients, the anti-CCP and anti-MCV titers were highly positive. The median neutrophil count was $0.496 \times 10^9/L$ with a range of 0.052–1224.0 $\times 10^9/L$.

Table 1
Characteristics of patients with Felty's syndrome

<i>Clinical and biological features</i>	
Number of patients	25
No. female/no. male	19/6
Age (y) at FS diagnosis, median (range)	54 (30–79)
Duration (y) of RA before FS diagnosis, median (range)	7 (0–35)
RA disease activity (max. DAS28), median (range)	4.9 (2.1–7.01)
Erosive arthritis	73% (16/23)
RF positive	92% (23/25)
Anti-CCP positive	96% (23/24)
Anti-MCV positive	100% (11/11)
Splenomegaly	83% (19/23)
Associated autoimmune diseases	28% (7/25)
<i>STAT3</i> mutation positive	0% (0/24)
<i>STAT5b</i> mutation positive	0% (0/24)
<i>Hematologic features</i>	
Leukocytes ($\times 10^9/L$), median (range)	1900 (1100–3400)
Neutrophils ($\times 10^9/L$), median (range)	0.496 (0.052–1224.0)
Lymphocyte ($\times 10^9/L$), median (range)	1.092 (0.42–2.32)
LGLs ($\times 10^9/L$), median (range)	0.413 (0.117–1.036)

A neutrophil count $< 0.5 \times 10^9/L$ was observed in 13 of 25 (52%) patients. None of the patients with FS in our cohort had lymphocytosis. The absolute number of lymphocytes in PB ranged from 0.42 to $2.32 \times 10^9/L$ with a median of 1.092. LGL counts $< 0.4 \times 10^9/L$ and $> 0.4 \times 10^9/L$ were observed in 8 (44%) and 10 (56%) of 18 cases, respectively, but did not exceed $2.0 \times 10^9/L$ in any of the cases.

The results of flow cytometric immunophenotyping of lymphocytes performed in 19 patients are summarized in Table 2. A weakening or absence of CD5 pan T-cell antigen expression on cytotoxic (CD3+/CD8+) T-lymphocytes was the most commonly identified phenotypic abnormality in 13 of 18 (72%) cases. CD16, CD56, and CD57 expression on cytotoxic T-lymphocytes was found in 1 of 15 (7%), 1 of 16 (6%), and 10 of 18 (56%) cases, respectively.

Table 2

Immunophenotypic characteristics of cytotoxic (CD3+/CD8+) T-lymphocytes in patients with Felty's syndrome

Case No.	Specimen type	CD57+	CD5low/ -	CD16+	CD56+	Lymphocyte counts with an aberrant immunophenotype in blood (in microliters)
1.	PB	+	+	+	+	409
2.	PB	-	+	-	ND	214
3.	PB	-	-	-	-	0
4.	BM	+	ND	-	-	ND
5.	PB	+	+	-	-	250
6.	PB	-	-	-	-	0
7.	PB	+	+	-	-	107
8.	PB	+	+	-	-	229
9.	PB	+	+	-	-	129
10.	PB	-	-	ND	-	0
11.	PB	+	-	-	-	98
12.	PB	ND	+	ND	ND	450
13.	PB	-	-	-	-	0
14.	PB and BM	+	+	-	-	202
15.	PB	-	+	-	ND	118
16.	PB	+	+	ND	-	389
17.	PB	-	+	-	-	239
18.	PB and BM	+	+	-	-	223
19.	PB	-	+	ND	-	102
Summary	18 PB; 3BM	56% (10/18) pos.	72% (13/18) pos.	7% (1/15) pos.	6% (1/16) pos.	median 165.5 (range, 0-450)
PB, peripheral blood; BM, bone marrow; -, express less than 10% of CD3 + CD8 + lymphocytes; +, express more than 10% of CD3 + CD8 + lymphocytes; pos., positive; ND, not done						

Normal or hypercellular bone marrow resulting from myeloid hyperplasia was revealed in all cases. There were no signs of myelodysplasia. The number of lymphocytes in the bone marrow was not elevated and comprised 3.8–18.4% of nucleated cells. In 11 of 14 patients bone marrow aspirate differential counts showed a significant reduction in segmented neutrophils, and in 1 patient, they showed a reduction in band and segmented neutrophils. However, in 2 patients, the bone marrow aspirate differential count was normal despite detection of PB neutrophils. A bone marrow immunohistochemical study in 2 of 6 cases showed interstitial clusters and/or linear arrays of intravascular CD8+/granzyme B + lymphocytes.

Splenomegaly was observed in 19 of 23 (83%) patients. Of these, splenectomy was performed in 2 patients. In both cases, the neutrophil count returned to normal values after the splenectomy and remained so during the entire follow-up (43 and 67 months, respectively). Mutations in the *STAT3* gene were not detected in any of the 24 cases with FS, whereas in the group with T-LGLL in the setting of RA, *STAT3* gene mutations were found in 22 of 56 patients (39%) ($p < 0.001$). No *STAT5b* gene mutation was detected in any of the 24 patients with FS and the 56 patients with RA-associated T-LGLL.

Discussion

Historically, LGLL could be readily recognized by reviewing a PB smear. An LGL count of more than $2 \times 10^9/L$ (normal LGL count in PB: $0.2-0.4 \times 10^9/L$) lasting > 6 months, was considered a criterion for determining this disease.[12, 22] For T-LGLL, the current diagnostic requirements have lowered this threshold to > 0.4 or $0.5 \times 10^9/L$ provided that a clonal T-LGL population is found with an appropriate clinical context.[23–25] Recent studies have shown that 49% of patients with T-LGLL have no absolute lymphocytosis and 36% of patients have blood LGLs $< 1 \times 10^9/L$. [9] As the clinical manifestations of RA-associated T-LGLL are often identical to those in which one would suspect an FS, it may be difficult to differentiate RA-associated T-LGLL with a low LGL count ($0.4-2.0 \times 10^9/L$) from FS. Moreover, expansion of LGLs can be detected in patients with FS.[26–28] RA-associated T-LGLL and FS can be distinguished by T-cell clonality determined by assessing the TCR gene rearrangements present in T-LGLL but not in FS. [1, 5, 29] However, there is considerable discussion regarding the significance of dominant T-cell clones as a hallmark of T-cell malignancy because small populations of clonally expanded T-LGLs are revealed in healthy individuals and in an exuberant reactive response.[30–34] Considering that the difference between RA-associated T-LGLL and FS often depends on a single test with well-known gray areas in interpretation and limitations,[35] it is necessary look for additional distinctions. We can use mutations in *STAT3* and *STAT5b* genes as molecular markers for T-LGLL diagnostics,[36] but their prevalence in FS and their diagnostic value for differential diagnosis between FS and RA-associated T-LGLL are unclear. In this study, we did not detect *STAT3* mutations in any of the 24 cases with FS, as opposed to 22 of 56 patients with RA-associated T-LGLL. Further, no *STAT5b* mutation was detected in any FS or RA-associated T-LGLL patient in our cohort.

Savola et al. examined *STAT3* and *STAT5b* mutations in 14 patients with RA and neutropenia.[37] Similar to our patient cohort, they did not find any *STAT5b* mutations. However, in contrast to our results, they identified *STAT3* mutations in 6 of 14 (43%) patients. We believe that difference between outcomes

obtained by Savola et al. and our study can be attributed to different methods of assessing T-cell clonality and the patient selection criteria. We tested T-cell clonality based on the rearrangement of gamma and beta chain-encoding genes by a PCR-based assay, whereas Savola et al. studied the clonality of T cells by flow cytometry using a V β kit, covering only 70% of the V β T-cell repertoire. In contrast to Savola et al., we did not include patients with T-cellular clonality in the FS group.

Female prevalence, age at FS diagnosis, and duration of RA prior to FS diagnosis in our series were comparable to the results found in literature.[4, 5, 38] Overall, in our patient cohort, RA was of moderate activity, even though RA is typically severe in patients with FS. All our patients were seropositive: RF+/anti-CCP+/anti-MCV + or RF-/anti-CCP+/anti-MCV+. Splenomegaly ranging from massive to detectable only based on abdominal imaging modalities, was detected in 83% of patients. SS was diagnosed in our study in 28% of patients, which is significantly less than in the FS patient cohorts reported by other authors: 48% (Sienknecht et al.), 69% (Barnes et al.), and 53% (Campion et al.).[2, 3, 39]

In our study, low count expansion of LGLs ($0.4-2.0 \times 10^9/L$) in PB was detected in 56% of cases, but did not exceed $2.0 \times 10^9/L$ and the bone marrow aspirate differential count showed no increase in lymphocytes. Flow cytometric immunophenotyping studies play an important role in the diagnosis of T-LGLs. The expression of CD57 and CD16 antigens, one or both of which are detected in the vast majority of T-LGLL cases, was found on cytotoxic T-lymphocytes in only 56% and 7% of our cases, respectively. In contrast, aberrant expression of CD5 was the most common finding in our patient group. This abnormality is frequently associated with T-LGLL, but is also found in T-cell reactive expansion.[41, 42] Bone marrow involvement is present in at least 75% of T-LGLL cases, although it is often subtle and difficult to detect. Specific criteria have been proposed for the diagnosis of T-LGLL in bone marrow sections using immunohistochemistry.[42; 43] However, as reported by Burks et al., there are probably no distinctive features in bone marrow biopsies that would separate T-LGLL from FS.[5] In 2 patients with FS in our cohort, immunohistochemical studies also revealed that bone marrow infiltration by cytotoxic T-lymphocytes was indistinguishable from T-LGLL lesions.

The pathogenesis of neutropenia in FS has not yet been fully studied and seems to be multifactorial. In 12 of 14 cases in our study, the bone marrow aspirate differential count fit into the expected consequence of peripheral destruction/sequestration of neutrophils. Although the role of splenic sequestration/destruction in neutropenia pathogenesis is not supported by all studies,[5] splenectomy produces a long-term hematologic response in 80% of patients with FS.[44] We also observed persistent recovery of neutrophil levels after splenectomy in 2 of our patients with FS and massive splenomegaly.

Limitations

We are aware of some limitations concerning our study design. Due to the retrospective design of the study, some of the data were incomplete. Additionally, we used of allele-specific TaqMan real-time PCR rather than Sanger sequencing to detect somatic point mutations in *STAT3* and *STAT5b* genes. A set of primers for most common mutations in *STAT3* and *STAT5b* genes was developed. Even though this

approach provides much higher sensitivity compared to Sanger sequencing, some rare mutations, not covered by the developed primers, could not be identified.

Conclusion

In conclusion, we did not detect *STAT3* and *STAT5b* gene mutations in any of the 24 FS cases in this study, whereas *STAT3* gene mutations were found in 22 of 56 patients (39%) with T-LGLL in RA-associated T-LGLL ($p < 0.001$). Although further data are required, our results suggest that identifying *STAT3* mutations in patients with clinical pattern resembling FS may be a counterargument to make this diagnosis and that careful patient evaluation is required to exclude RA-associated T-LGLL.

Abbreviations

FS: Felty's syndrome; RA:rheumatoid arthritis; T-LGLL:T-cell large granular lymphocytic leukemia; LGLs:large granular lymphocytes; STAT:signal transducer and activator of transcription; RF:rheumatoid factor; anti-CCP:antibodies against cyclic citrullinated peptides; anti-MCV:antibodies against mutated citrullinated vimentin; DAS28:Disease Activity Score derivative for 28 joints; CD:cluster of differentiation; TCR:T-cell receptor; PCR:polymerase chain reaction; AS:allele-specific; WT:wild type; MT:mutated type; PB:peripheral blood; BM:bone marrow; ND:not done

Declarations

Acknowledgments and affiliations

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Authors' contributions

V.R.G. substantially contributed to the conception of the work, acquisition, analysis, and interpretation of data; made a draft of the work; approved the final version of the work for publication; and agreed to be responsible for all aspects of the work. Y.V.S., N.A.K., V.I.V., N.A.P., N.V.R., and A.B.S. substantially contributed to the acquisition, analysis, and interpretation of data for the work; critically revised the work for important intellectual content; approved the final version of the work for publication; and agreed to be responsible for all aspects of the work.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

The study was approved by the V.A. Nasonova Research Institute of Rheumatology Ethics Committee (protocol #17 on the 20-02-2020) and was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2008. Written informed consent was given by all participants included in the study.

Consent for publication

Not applicable.

Competing interest statement

The authors declare that they have no conflicts of interest regarding the publication of this study.

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