Prevalence of *STAT3* mutations in patients with rheumatoid arthritis-associated T-cell large granular lymphocytic leukaemia and Felty syndrome

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Abstract

Objective

Neutropenia is a key presentation of Felty syndrome (FS) and rheumatoid arthritis (RA)-associated T-cell large granular lymphocytic (T-LGL) leukaemia. Clonal rearrangement of T-cell receptor (TCR) gene supports the diagnosis of T-LGL leukaemia but not FS. Mutations in the signal transducer and activator of transcription 3 (STAT3) gene are highly specific for T-LGL leukaemia, but their prevalence in FS remains poorly clarified.

Methods

The study included 100 patients with RA and unexplained neutropenia. TCR rearrangements were examined in blood (100 cases), bone marrow (47 cases), and spleen (12 cases) using the BIOMED-2 protocol. Patients were stratified into RA-associated T-LGL leukaemia cohort if a clonal TCR rearrangement was identified in any of the tested patient samples, and into FS cohort in other cases. Mutations in the STAT3 were examined using next-generation sequencing (NGS) technology in blood (100 cases), bone marrow (37 cases), and spleen (7 cases).

Results

STAT3 mutations were identified in 71% (49/69) patients with RA-associated T-LGL leukaemia and in 10% (3/31) patients with FS ($p=4.7\times10^{-8}$). Three samples from the RA-associated T-LGL leukaemia cohort and 5 samples from the FS cohort had STAT3 mutations in the absence of clonal TCR rearrangement.

Conclusion

The results suggest that STAT3 mutations are significantly less common in FS than in RA-associated T-LGL leukaemia. Moreover, NGS can detect clones undetectable by fragment analysis. We speculate that in patients with RA and neutropenia, the detection of STAT3 mutations can point to T-LGL leukaemia even in the absence of clonal TCR rearrangement.

Key words

STAT3 mutation, T-cell large granular lymphocyte leukaemia, Felty syndrome, rheumatoid arthritis, next-generation sequencing

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Introduction

In the setting of rheumatoid arthritis (RA), neutropenia is the key clinical presentation of both Felty syndrome (FS) and T-cell large granular lymphocytic (T-LGL) leukaemia. The formal approach to the diagnosis of FS based on the presence of a combination of RA and neutropenia, which is sometimes used, can lead to undiagnosed T-LGL leukaemia.

Detection of monoclonal rearrangement of T-cell receptor (*TCR*) genes in a patient with RA and neutropenia supports the diagnosis of T-LGL leukaemia but not FS (1-3). However, the polymerase chain reaction (PCR)-based analysis of *TCR* gene rearrangements has a maximal sensitivity of 5–10% (4, 5), and consequently, in patients with a low tumour burden, detection of the clonal peak may be questionable, or it may not be detected against a polyclonal background. In addition, due to the expansion of reactive cytotoxic T-lymphocytes in patients with RA, immunohistochemical staining of bone marrow biopsy specimens does not allow a reliable distinction between T-LGL leukaemia and FS (2).

On the other hand, mutations in the signal transducer and activator of transcription 3 (*STAT3*) gene are highly specific, under appropriate clinical conditions, for T-LGL leukaemia and can be detected even in cases with a low tumour burden (6). Previously, using allele-specific PCR assays for the detection of canonical *STAT3* mutations, we have shown that *STAT3* mutations are not characteristic of FS as opposed to RA-associated T-LGL leukaemia (7). The aim of the present study was to evaluate the incidence of *STAT3* mutations in expanded cohorts of patients with FS and RA-associated T-LGL leukaemia using the next-generation sequencing (NGS) technology, which covers a wider range of *STAT3* mutations.

Materials and methods

The inclusion criteria were: 1) age over 18 years, 2) diagnosis of RA confirmed according to the 2010 American College of Rheumatology/European League Against Rheumatism criteria (8), 3) absolute neutrophil count less than 1.5×10^9/L persisting for at least 21 days after withdrawal of all drugs that could cause a decrease in neutrophil counts, and 4) the availability of tissue specimens for evaluation of T-cell clonality and *STAT3* mutations. Patients with haematological malignancies other than T-LGL leukaemia were excluded from the analysis.

A total of 100 patients met the criteria and were included in the retrospective analysis.

Evaluation of T-cell clonality

T-cell clonality was examined using genomic deoxyribonucleic acid (DNA) extracted from blood (100 cases), bone marrow (47 cases), and spleen tissue (12 cases). The evaluation of T-cell clonality based on the rearrangements of the *TCR gamma* (Vγ-Jγ) and *TCR beta* (Vβ-Jβ, Dβ-Jβ) genes was performed in all cases. T-cell clonality assays were performed according to the BIOMED-2 protocol (4). PCR amplification 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 analysed using GeneMapper software v. 4.0 (Applied Biosystems, Foster City, CA). A patient was regarded as having T-cell clonality if monoclonal rearrangement of the *TCR gamma* and/or *TCR beta* chain genes was detected.

Evaluation of STAT3 mutations

STAT3 mutations were examined using genomic DNA extracted from 100 peripheral blood samples (31 cases from the FS cohort and 69 cases from the T-LGL leukaemia cohort) and 37 bone marrow samples (13 cases from the FS cohort and 24 cases from the T-LGL leukaemia cohort). The quality of DNA extracted from spleen tissue allowed the study of *STAT3* mutations in seven cases: one patient from the FS cohort and six from the T-LGL leukaemia cohort.

Mutations in the linker domain (exon 19) and Src homology 2 (SH2) domain (exons 20 and 21) of the *STAT3* gene were identified using NGS. Appropriate DNA regions were amplified using primers to exon 19–20 (product length

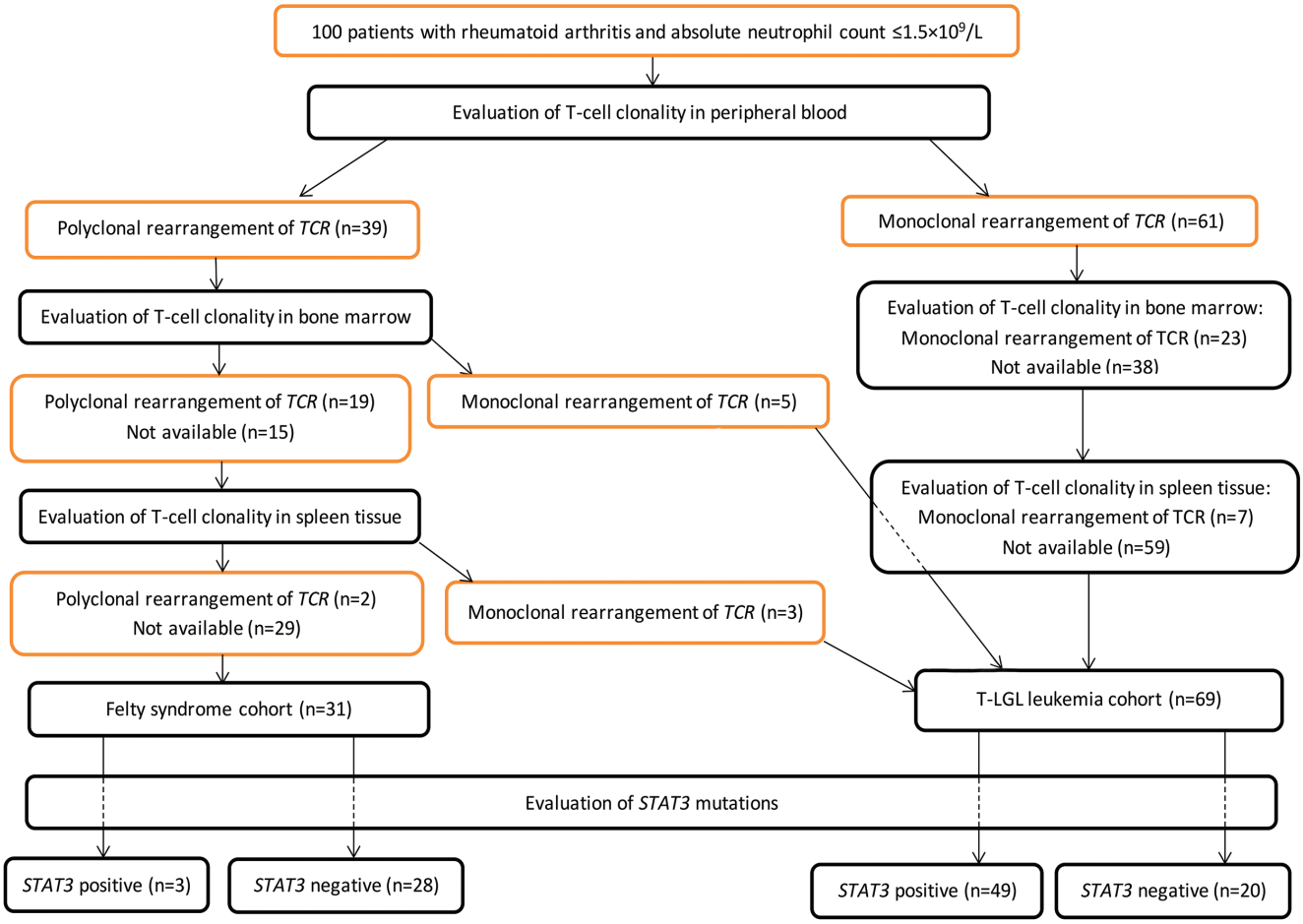

Fig. 1. Flowchart of stratification of patients.

502 bp) and exon 21 (product length 522 bp), as described previously (6). Following the manufacturer's instructions, amplified DNA fragments were converted into sequencing libraries using Nextera XT DNA Library Prep and Nextera XT Index Kit v2 (Illumina, USA). Nucleotide sequences were analysed on a MiSeq sequencer (Illumina, USA) using MiSeq Reagents Kit v2 – 300 cycles (Illumina, USA). Raw data filtering, removal of accessory sequences, mapping of reads, and searching for variants were performed using the Trimmomatic, BWA, SAMtools, and VarDict (9-12). Usually, 2000–5000 reads were obtained for each target, and variant allele frequency (VAF) for reporting variants was set to 0.5% and more. Cases with VAF less than 1.5% were considered positive for the *STAT3* mutation if the mutation was observed in the repeated analysis of the sample and/or in another sample from the same patient. Discovered variants were annotated with ANNOVAR utility using COSMIC, ClinVar, refGene, and snp138 open databases (13-17).

The study was approved by the Ethics Committee of V.A. Nasonova Research Institute of Rheumatology (protocol no. 11 on the 04-08-2022). All patients signed an informed consent for the scientific use of their data.

Statistical analysis

Categorical data were presented as numbers and percentages. A two-sample test for equality of proportions with continuity correction was used for analysis. Overall survival was estimated using the Kaplan-Meier method and was compared using the Gehan-Wilcoxon test.

Results

Grouping patients into the RA-associated T-LGL leukaemia cohort was based on the presence of *TCR* monoclonal rearrangement in any of the examined patient samples (Fig. 1).

Monoclonal *TCR* rearrangement in peripheral blood was detected in 61 (61%) of 100 patients, and in the remaining 39 patients, *TCR* rearrangement was polyclonal. In 24 of these 39 patients, we also had the opportunity to evaluate T-cell clonality in the bone marrow. Monoclonal *TCR* rearrangement in the bone marrow was detected in five patients, and polyclonal *TCR* rearrangement was observed in the remaining 19 patients. Of these 19 patients with polyclonal *TCR* rearrangement in the blood and bone marrow, *TCR* rearrangement in spleen tissue was examined in five patients: two patients had polyclonal *TCR* rearrangement, and three patients had monoclonal *TCR* rearrangement.

Of the 61 patients with monoclonal *TCR* rearrangement in the blood, we had the opportunity to examine T-cell clonality in the bone marrow in 23 patients and spleen tissue in five patients. All samples showed monoclonal *TCR* rearrangement. In two more cases with pol-

Table I. Results of an investigation of *STAT3* mutations by NGS in cohorts of patients with RA-associated T-LGL leukaemia and Felty syndrome

	Peripheral blood		Bone marrow		Spleen	
	Percentage of positive cases	Number of positive cases / total number of samples tested	Percentage of positive cases	Number of positive cases / total number of samples tested	Percentage of positive cases	Number of positive cases / total number of samples tested
RA-associated T-LGL leukaemia	67	46/69	54	13/24	100	6/6
Felty syndrome	10	3/31	15	2/13	0	0/1

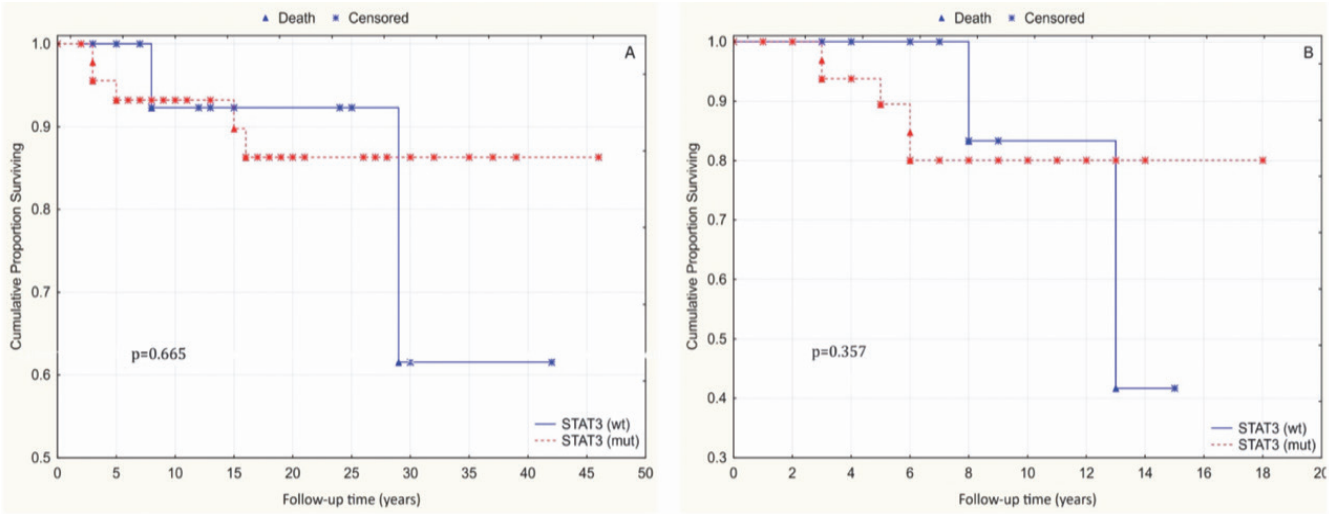

Fig. 2. A. Overall survival from the onset of RA in patients with (n=49) and without (n=20) *STAT3* mutations. **B.** Overall survival from the manifestation of T-LGL leukaemia in *STAT3*-positive patients (n=49) compared with patients without *STAT3* mutations (*STAT3* wildtype) (n=20).

yclonal TCR rearrangement in the blood and monoclonal rearrangement in the bone marrow, examination of the spleen tissue also revealed monoclonal TCR rearrangement. The pattern of clonal rearrangement was identical in different samples from the same patient.

In total, monoclonal *TCR* rearrangement was detected in at least one sample (blood, bone marrow, or spleen tissue) in 69 (69%) of 100 patients, and these patients were stratified into the T-LGL leukaemia cohort (Supplementary Table S1). In eight of these 69 patients, polyclonal *TCR* rearrangement was detected in the blood, and they were assigned to the T-LGL leukaemia cohort based on the detection of T-cell clonality in their bone marrow and/or spleen tissue.

In 31 (31%) of the 100 patients, T-cell clonality was not detected in any of the samples examined; therefore, these patients were stratified into the FS cohort (Suppl. Table S2).

Of the 69 patients with RA-associated T-LGL leukaemia, *STAT3* mutations were found in peripheral blood in 46 (67%) patients, in bone marrow in 13 (54%) of 24 patients, and in spleen tissue in all six patients studied (Table I and Suppl. Table S1). In total, *STAT3* mutations were detected in 49 (71%) of 69 patients with RA-associated T-LGL leukaemia in at least one of the samples (blood, bone marrow, or spleen) tested. Forty-one patients had one mutation, five patients had double, and three patients had triple mutations in the *STAT3* gene. Mutational hot spots included Y640F in 19 cases, D661Y in 12 cases, N647I and S614R in 10 cases each, Y657_K658insY in three cases, D661V and G618R in two cases each, K658R and S614G in one case each. Of the 65 *STAT3* mutated samples, 30 (46%) had variant allele frequency (VAF) ≤5%.

We found no statistically significant difference in overall survival both from the beginning of RA (Fig. 2A) and from the manifestation of T-LGL leukaemia (Fig. 2B) between *STAT3* positive (n=49) and *STAT3* negative patients (n=20).

In the FS cohort, the *STAT3* mutations were identified only in three (10%) of 31 patients (Table I and Suppl. Table S2). One of these three patients had one *STAT3* mutation, another had three *STAT3* mutations, and the third had four *STAT3* mutations. Mutational hotspots included D661Y in three cases, Y640F in two cases, and N647I, G618R, and D661V in one case each

In summary, we found the statistically significant difference (p=4.7×10^{-8}) in the frequency of *STAT3* mutations in patients with RA-associated T-LGL leukaemia (71% [49 of 69 patients]) and FS (10% [3 of 31 patients]).

The clinical and laboratory data of 11 diagnostically challenging cases are shown in Tables II and III. In eight patients from the T-LGL leukaemia cohort (Table II, cases 1–8), we did not detect *TCR* clonal rearrangement in the peripheral blood. Therefore, these patients could have been initially assigned to the FS cohort. However, the detection of monoclonal *TCR* rearrangement in the bone marrow in five of these eight patients (Table II, cases 4–8) allowed us to include them in the T-LGL leukaemia

Table II. *TCR* gene rearrangements and *STAT3* mutations in 11 diagnostically challenging patients.

Patient no./ Sex/Age* (y)	Diagnosis	*TCR* rearrangement			*STAT3* mutation			Mutational hotspots
		PB	BM	Spleen	PB	BM	Spleen	
1 / F / 69	RA-associated T-LGL leukaemia	Poly	Poly	Mono	–	ND	ND	–
2 / F / 58	RA-associated T-LGL leukaemia	Poly	Poly	Mono	–	+	+	S614R
3 / M / 39	RA-associated T-LGL leukaemia	Poly	Poly	Mono	–	–	ND	–
4 / M / 76	RA-associated T-LGL leukaemia	Poly	Mono	Mono	+	+	+	S614R; Y640F; N647I
5 / M /42	RA-associated T-LGL leukaemia	Poly	Mono	Mono	–	+	+	Y640F
6 / M / 71	RA-associated T-LGL leukaemia	Poly	Mono	ND	–	–	ND	–
7 / M / 52	RA-associated T-LGL leukaemia	Poly	Mono	ND	+	+	ND	Y640F; D661Y
8 / F/ 61	RA-associated T-LGL leukaemia	Poly	Mono	ND	–	–	ND	–
9 / F / 46	FS	Poly	ND	ND	+	ND	ND	D661Y
10 / M / 66	FS	Poly	Poly	ND	+	+	ND	Y640F; N647I; D661Y
11 / F / 52	FS	Poly	Poly	ND	+	+	ND	G618R; Y640F; D661Y; D661V

* At the time of detection neutropenia and/or splenomegaly.
y: years; RA: rheumatoid arthritis; T-LGL leukaemia: T cells large granular lymphocytic leukaemia; FS: Felty syndrome; *TCR*: T cell receptor gene; Mono: monoclonal *TCR* gene rearrangement; Poly: polyclonal *TCR* gene rearrangement; *STAT3*: signal transducer and activator of transcription 3 gene; +: present; –: absent; ND: no data; PB: peripheral blood; BM: bone marrow.

Table III. The clinical and laboratory data of 11 diagnostically challenging patients.

Patient no.	Erosive arthritis	RF / anti-CCP	Spleen	Absolute lymphocyte count ($\times 10^9$/L)	Absolute LGL count ($\times 10^9$/L)	Percentage of lymphocytes in the BM	Immunophenotype
1	+	+ / +	Severe splenomegaly	0.460	ND	5.2	Spleen (IHC): CD3+, CD4–, CD5–, CD8–, CD16+, CD43+, CD56–, CD57–, TIA1+
2	+	+ / +	Severe splenomegaly	0.476	0.063	18.4	Spleen (FC): CD3+, CD4–, CD5–, CD8–, CD16+, CD56–, CD57–
3	ND	+ / +	Severe splenomegaly	0.490	0.196	22.6	Spleen (IHC): CD3+, CD8+, Granzime B+
4	+	+ / +	Severe splenomegaly	1.936	0.660	16.2	BM (FC): CD3+, CD4–, CD5–, CD7+, CD8–, CD16–, CD56–, CD57–. Spleen (IHC): CD3+, CD4–, CD5–, CD8–, TIA1+, Granzyme B+.
5	–	+ / +	Severe splenomegaly	0.931	ND	7.8	Spleen (IHC): CD3+, CD4–, CD5–, CD8–, TIA1+
6	+	+ / +	Moderate splenomegaly	1.342	0.440	17.0	BM (FC): CD3+, CD5low, CD8+, CD16–, CD57–
7	+	+ / +	Severe splenomegaly	0.770	0.297	20.2	BM (IHC): CD3+, CD4–, CD8+, CD57–, TIA1+, Granzyme B+
8	+	+ / +	Moderate splenomegaly	1.782	0.484	17.8	BM (FC): CD3+, CD5low, CD8low, CD16–
9	+	+ / +	Normal	1.768	ND	ND	ND
10	+	+ / +	Moderate splenomegaly	0.528	0.240	20.8	PB (FC): CD3+, CD5low, CD8+, CD16–, CD57+
11	–	– / +	Moderate splenomegaly	1.376	0.448	22.8	PB and BM (FC): CD3+, CD5low, CD8+, CD16–, CD57+

RF: rheumatoid factor; anti-CCP: antibodies against cyclic citrullinated peptides; PB: peripheral blood; BM: bone marrow; FC: flow cytometric analysis; IHC: immunohistochemistry analysis; +: present; –: absent; ND: no data.

cohort. Three patients (Table II, cases 1–3) posed a special diagnostic challenge as they showed polyclonal *TCR* rearrangement in both blood and bone marrow, and only the detection of monoclonal *TCR* rearrangement in spleen tissue made it possible to classify them in the T-LGL leukaemia cohort.

Three samples (Table II; in cases 2, 4, and 7) from the RA-associated T-LGL leukaemia cohort and five samples (Table II; in cases 9–11) from the FS cohort showed discordance between the absence of *TCR* clonal rearrangement and the presence of the *STAT3* mutation. VAF was less than 5% in all these samples.

Discussion

T-LGL leukaemia is a rare chronic lymphoproliferative disorder characterised by the expansion of clonal, immunophenotypically distinct, cytotoxic T-cells that are larger than most circulating lymphocytes and have moderate to abundant weakly basophilic cytoplasm

containing azurophilic granules (18). The typical manifestations of T-LGL leukaemia are LGL lymphocytosis (≥2 × 10^9/L), neutropenia, and splenomegaly (19). A molecular hallmark of T-LGL leukaemia is activating mutations in the *STAT3* gene (19, 20). An intriguing feature of T-LGL leukaemia is its association with autoimmune disorders, especially RA. According to extensive studies, 3–28% of patients with T-LGL leukaemia have concomitant RA (18, 21). The reasons why T-LGL leukaemia is more often associated with RA than with other autoimmune diseases are not clear. Deregulation of Janus kinase/signal transducer and activator of transcription nuclear factor kappa β signal pathways, playing an important role in the pathogenesis of both RA (22-25) and T-LGL leukaemia (26, 27), may be responsible for the frequent association of these diseases.

Chronic antigenic stimulation, initially leading to poly- or oligoclonal T-LGLs expansion, is thought to be the trigger event underlying T-LGL leukaemia (28-30). The proliferation of T-LGLs is supported by several cytokines, including platelet-derived growth factor, interleukin (IL)-2, IL-6, IL-12, IL-15, and IL-18 (28), which also play a critical role in the pathogenesis of RA (31).

The presence of *STAT3* mutations points toward neoplastic transformation of the initially reactive T-LGL proliferation (6). The initiating event that causes the *STAT3* mutations in antigen-driven T-LGLs remains undiscovered. *STAT3* mutations are most often localised in exons 20 and 21 encoding the SH2 domain, which causes the activation of the *STAT3* protein and ultimately, the expression of anti-apoptotic genes *MCL-1* or *BCL-2* (27).

Activating mutations in *STAT3* are not pathognomonic for T-LGL leukaemia and can be detected in various non-haematological and haematological malignancies (32). However, in an appropriate clinical context, the detection of *STAT3* mutations is highly specific for T-LGL leukaemia (6, 32-35). The incidence of *STAT3* mutations in patients with T-LGL leukaemia varies from 27% to 72%, as reported by different researchers (6, 33-35). In our cohort of patients with RA-associated T-LGL leukaemia, *STAT3* mutations were detected in 71% of patients. Such a high frequency may be due to two reasons. First, we included cases with low VAF, and second, patients with RA are more likely to have *STAT3* mutated status of T-LGL leukaemia, as shown in several studies (6, 35). It should be noted that *STAT3* gene mutations can also occur outside the SH2 domain, but the frequency of such cases is low (3.8%) (36).

In the absence of LGL lymphocytosis, cases of RA-associated T-LGL leukaemia manifesting neutropenia (with or without splenomegaly) present a special diagnostic challenge because they are clinically indistinguishable from cases of FS. FS is an uncommon variant of RA characterised by neutropenia with absolute neutrophil counts below 1.5–2.0 × 10^9/L and splenomegaly, although the updated diagnostic criteria do not consider splenomegaly an absolute requirement for FS diagnosis (1, 37, 38).

Some researchers hypothesise that FS and RA-associated T-LGL leukaemia are part of a single disease process (2, 39, 40). Savola *et al*. found that the frequency of *STAT3* mutations in patients with FS is 43% (6 of 14 patients) (41), which was comparable to the *STAT3* mutation frequency reported by some researchers in patients with T-LGL leukaemia (6, 30). However, contrary to the present study, Savola *et al*. analysed the clonality of T cells by flow cytometry using a Vβ kit, covering only 70% of the Vβ T-cell repertoire, and included patients with T-cell clonality in the FS cohort. If we combine patients with FS and RA-associated T-LGL leukaemia in the present study, the 52% (52 of 100 cases) frequency of *STAT3* mutations in the pooled cohort is consistent (p=0.72) with that reported by Savola *et al*. (41).

The pathophysiological mechanisms leading to neutropenia in FS and T-LGL leukaemia are not completely elucidated and are probably multifactorial. In FS, the destruction of neutrophils due to exposure to anti-neutrophils antibodies is believed to be the main cause (2, 42). Various autoantibodies, which may contribute to neutropenia development, have been found in the serum of patients with FS with higher titres or with higher frequencies than those in patients with RA but without FS (43-45). In contrast, Fas ligand-mediated apoptosis of mature neutrophils or myeloid precursors appears critical for the development of neutropenia in T-LGL leukaemia (2, 46). Recently, a pathogenic link between *STAT3* activation, inhibition of miR-146b expression, and increased Fas ligand production has been identified (47, 48). Refining the diagnosis in patients with RA is important not only from an academic point of view but also to objectively evaluate the effectiveness of new therapeutic approaches.

Using the NGS method, we found *STAT3* mutations in only three (10%) of 31 patients who were assigned to the FS cohort based on the absence of *TCR* monoclonal rearrangement in the samples studied. In one of these three patients, we had the opportunity to study *TCR* rearrangement only in the blood sample, whereas, in the other two patients, it was studied in both blood and bone marrow samples. It is possible that in the unexamined tissue samples (bone marrow and/or spleen) of these patients a monoclonal *TCR* rearrangement could be detected, and then these cases would be classified as T-LGL leukaemia. As an argument, one could mention the absence of clonal rearrangement of *TCR* in the blood in eight (12%) of the 69 patients from the RA-associated T-LGL leukaemia cohort. However, in five of these patients, T-cell clonality was detected in the bone marrow, and in the other three patients, it was detected only in the spleen (the splenic variant of T-LGL leukaemia). Moreover, in three patients from the RA-associated T-LGL leukaemia cohort, *STAT3* mutation was detected in samples with polyclonal *TCR* rearrangement (Table II; case no. 2 [bone marrow] and cases no. 4 and no. 7 [peripheral blood]). This is most likely due to the greater sensitivity of the NGS method in detecting a tumour clone with *STAT3* mutation compared with fragment analysis. In addition, BIOMED-2 primer sets do not cover all possible *TCR* rearrangements, and, hence a small number of T-LGL leukae-

mias may be overlooked in the analysis (4, 5). Given the above, the three patients in the FS cohort, in whom *STAT3* mutations were found but no T-cell clonality was detected in the analysed samples, may have T-LGL leukaemia.

Conclusions

The present study has demonstrated that *STAT3* mutations are significantly more common in RA-associated T-LGL leukaemia than in FS. In addition, the results suggest that NGS can detect *STAT3*-mutated clones that are not detected using fragment analysis of PCR products of *TCR* gene rearrangements.

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