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Advanced-stage Mycosis Fungoides. Role of STAT3, NFKB and NFAT pathways

Running head: STAT3 activation in MF

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Conflict of interest

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What's already known about this topic?

- Mycosis fungoides is characterized by a clonal expansion of T cells in the skin.
- The mechanisms controlling disease development and progression are not fully understood.

What does this study add?

- An association of NFAT and NFkB pathways was found, which could reflect a common mechanism of activation. These pathways were activated in early and advanced stages at the same level.
- STAT3 activation was associated to Large Cell Transformation and was more frequent in advanced stages.
- A genomic analysis of CTCL associated genes was performed. Nine mutations were detected.

What is the translational message?

- These results could have important implications in disease treatment in near future.

Abstract

Introduction. The malignant mechanisms that control the development of cutaneous T-cell lymphoma (CTCL) are beginning to be identified. Recent evidence suggests that disturbances in specific intracellular signalling pathways, such as RAS-MAPK, TCR-PLCG1-NFAT and JAK-STAT, may play an essential role in the pathogenesis of CTCL.

Material and methods. We collected 100 samples that were submitted for diagnosis of, or a second opinion regarding mycosis fungoides (MF) between 2001 and 2018, 80% of which were in the early clinical stages of the disease. Formalin-fixed paraffin-embedded tissues were used for histological review and to measure the expression by immunohistochemistry of the surrogate markers of activation of the TCR-PLCG1-NFAT, JAK-STAT and NFkB pathways. Folliculotropism and large-cell transformation were also examined.

Results. NFAT and NFkB markers showed a comparable activation status in early and advanced stages, while STAT3 activation was more frequent in advanced stages and was associated with large-cell transformation. Consistent with this observation, STAT3 activation occurred in parallel with MF progression in two initially MF-negative cases. A significant association of NFAT with NFkB markers was also found, reflecting a common mechanism of activation in the two pathways. Genomic studies identified nine mutations in seven genes known to play a potential role in tumorigenesis in T-cell leukaemia/lymphoma, such as *PLCG1*, *JAK3* and *STAT3*, which underlies the activation of these key cell-survival pathways. A higher mutational allele frequency was detected in advanced stages.

Conclusions. Our results show that STAT3 is activated in advanced cases and associated with large-cell transformation, while the activation of NFAT and NFkB is maintained throughout the disease. These findings could have important diagnostic and therapeutic implications.

Keywords: Mycosis Fungoides, STAT3, NFAT, NFkB, PLCG1

Introduction

Cutaneous T-cell lymphoma is defined as a non-Hodgkin lymphoma comprising a group of disorders characterized by clonal expansion of malignant T cells present in the skin¹. Mycosis fungoides (MF) is the most common type of CTCL, accounting for around 55% of them¹. The clinicopathological conditions of MF are broad, from single lesions to more generalized skin involvement with patches, plaques, tumours or erythroderma. Accurate staging, based on TNMB (tumour, node, metastasis, blood), is required because of its great prognostic value². Early stages (stage IA-IIA) have a good prognosis (52-100% 10-year overall survival) and a relatively low risk of disease progression (12-33% in 10 years). However, advanced stages (stage IIB-IVB) have a worse prognosis (<38% 10-year overall survival) and more than 58% of them progress². MF patients are usually clinically diagnosed based on the presentation of patches and/or plaques of indolent behaviour that cover the skin surface; these are defined as classic patch/plaque. Patients in early stages can be kept stable for several decades, with a 5-10% or 17-39% risk of progression to advanced stages in the first 10 years following diagnosis, depending on the proportion of the body surface involved (<10% and $\geq 10\%$, respectively)³.

The malignant mechanisms controlling disease development and progression are not fully understood, although some of them have been identified. Recent evidence suggests that disturbances in specific intracellular signalling pathways, such as RAS-MAPK, TCR-PLCG1-NFAT and JAK-STAT, among others, can play an essential role in the pathogenesis of CTCL⁴⁻⁶. Deregulated JAK/STAT signalling is involved in many types of cancer. In fact, somatically acquired genetic alterations of JAK or STAT genes that induce aberrant activation of downstream signalling, via STAT phosphorylation, have been reported in some human haematological malignancies, including CTCLs^{4, 7-13}.

This study aimed to investigate the activation status of these three main survival pathways — NFAT, NFκB and JAK-STAT— in a cohort of sequential MF patients by assessing the frequency, clinical relevance and interactions among them. To identify the possible genomic alterations contributing to the activation of these pathways, we performed targeted sequencing in a panel of T-cell lymphoma-associated genes.

Material and Methods

Clinical samples

Cases submitted for an MF diagnosis or a second opinion in different Institutions of the Spanish National Health System between 2001 and 2018 were included in the study. Diagnostic criteria were based on World Health Organization–European Organization for Research and Treatment of Cancer (WHO-EORTC) classification¹⁴. CTCL cases other than MF were excluded. Clinical stage was established on the basis of their TNMB classification¹⁵. This study was conducted in accordance with the Declaration of Helsinki and was supervised by the Ethical Committee of Hospital Universitario Marqués de Valdecilla, Santander, Spain.

We used only samples from skin biopsies. Formalin-fixed paraffin-embedded (FFPE) tissues were used for histological examination and DNA sequencing.

IHC/molecular studies were performed using the initial diagnostic samples. Survival analysis was performed since the date of the initial diagnosis of Mycosis Fungoides.

Immunohistochemistry

A panel of antibodies was used to quantify the levels of TCR-PLCG1-NFAT, NFkB and JAK-STAT expression in 112 samples from 99 patients. Folliculotropism and large-cell transformation (LCT) were also assessed¹⁵. Immunohistochemical (IHC) staining was performed on slides using the following antibodies: NFAT-C1, p-STAT1, p-STAT3, p-STAT5, p50, p52, p65, RelB (supplementary table 1). Each slide was reviewed by two independent pathologists, who examined the nuclear staining in each case. Specific thresholds are described in supplementary table 1. A positive staining was defined as a score higher than 1. IHC controls were provided by inflammatory cutaneous disorders. Each immunostained slide included positive controls. Negative controls were routinely used for each marker (Supplementary figure 1).

Targeted enrichment and sequencing

DNA was extracted from FFPE with High Pure FFPE DNA Isolation Kit under manufacturer' instructions (Roche Life Science). DNA was quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), with a range of 0.5-390 ng/μl (median 7.55 ng/μl). DNA quality was tested by qPCR using Infinium FFPE QC under manufacturer' instructions (Illumina, San Diego, CA, USA). Targeted enrichment of 16 genes known to play a potential role in tumorigenesis in T-cell leukaemia¹⁶ was performed by Truseq Custom Amplicon Low Input (Illumina, San Diego, CA, USA) (Supplementary table 2). 20-100ng were utilised to perform Truseq enrichment. Samples with $\Delta Cq > 6$ were discarded to Truseq enrichment. We also carried out adaptor-anchored PCR-based targeted enrichment in frequently mutated regions of *PLCG1*, *JAK1* and *JAK3* (supplementary table 3) for Truseq validation (supplementary table 4 and supplementary figure 2).

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Variants were called using MiSeq Reporter, employing the default settings, and were visually inspected within Integrative Genomics Viewer (www.broadinstitute.org/igv/). Variants were annotated with Variant Effect Predictor (GRCh37, <http://grch37.ensembl.org/Tools/VEP>). Known SNPs with an allelic frequency greater than 1% in public databases (dbSNP138, 1000 Genomes Project, Exome Sequencing Project, Exome Aggregation Consortium) were filtered out.

Statistical analysis

The chi-square or Fisher exact test was used, as appropriate, to determine associations between the presence and absence of markers. Overall survival (OS) was taken to be the period between the initial diagnosis and the date of death from any cause, or of last contact in the case of living patients. Progression-free survival (PFS) was measured as the period between the initial diagnosis and the date of death from any cause, or of progression of the lymphoma disease to a higher clinical stage, or of the last contact for living patients with no disease progression. Kaplan–Meier survival analyses were carried out for OS and PFS using the log-rank test to examine differences between groups. A multivariate Cox regression model was also derived. Estimates were considered statistically significant for two-tailed values of $p < 0.05$. All analyses were carried out using SPSS for Windows version 15 (Chicago, IL).

Results

Activation of STAT3 in advanced stages of MF

The initial series included 106 patients and 119 samples. After case-by-case revision, six patients were excluded because finally they were judged to correspond to other CTCL

subtypes. Clinical characteristics of the 100 MF patients are summarized in table 1. The median follow-up time was 55 months. We dichotomised the patients into early (stage IA, IB and IIA) or advanced (stage IIB, III, IVA1, IVA2 and IVB) clinical stages. At the time of initial diagnosis, this series consisted mainly of early stages (78.2%). Most of the patients presented with classic patch/plaque (78.2%). Advanced-stage patients (21.8%) were characterized by classic patches/plaques (68.8%), erythroderma (50%), tumour skin lesions (37.5%), and extracutaneous lesions (visceral, blood and bone marrow involvement; 12.5%, 12.5% and 6.25%, respectively). At the time of data analysis, 12 patients had died, 58 patients were censored by their last appointment, eight patients had been lost to follow-up, and clinical data could not be obtained from 22 patients. Early-stage patients showed better OS and PFS than those in advanced stages ($p=0.025$ and $p=0.057$, respectively; Fig 1).

Immunohistochemical analysis of the TCR-PLCG1-NFAT, NFkB and JAK-STAT pathways was performed in 111 samples, taking only nuclear staining into account. We also analysed large cell transformation and folliculotropism. We observed different ranges of activation of the assessed markers of NFAT (27%), NFkB (25.9%-50.5%) and STAT (11.7%-25.5%) (table 2 and supplementary figure 3a). Regarding the relationship between the presence of pairs of markers, we found an association between pSTAT3 and large cell transformation ($p<0.001$; supplementary figure 3b), and a relationship between the expression of the NFAT and NFkB pathway markers ($p\leq 0.017$, supplementary figure 3b). No differences were found when comparing all patients and those with follow up (data not shown).

When patients were grouped by clinical stage, only pSTAT3 expression showed an association with advanced stages ($p<0.001$; table 2b). Analysis of the histological variables showed that large cell transformation was associated with shorter OS and PFS ($p=0.018$ and $p=0.007$, respectively; Fig 2). Patients with activated STAT3 also showed shorter OS and PFS, although the differences were not statistically significant ($p=0.147$ and $p=0.187$, respectively; Fig 2).

None of the other markers showed differences in OS and PFS between positive and negative markers, except for pSTAT1 ($p=0.001$ and $p=0.007$, for OS and PFS, respectively) (supplementary figure 4). The multivariate analysis showed that age at diagnosis and activation of STAT1 were independent predictors of OS, while large-cell transformation was independently associated with the risk of progression (table 3).

In four cases, paired samples were available for initial and progressed stages. Strikingly, three out of these four patients showed expression of pSTAT3 only in the more advanced sample (Supplementary figure 5). The other patient that progressed showed STAT1/5 but not STAT3 expression (Fig 3). Other three patients with parallel samples that did not progress showed no expression of STAT3 in the second sample.

Mutational profile and activation of pathways in MF patients

After checking quality and quantity, 73% of the patients included in the study were sequenced. We detected nine mutations in seven genes from eight patients (table 4a). Among them, the recurrent mutations p.S345F and p.A573V in *PLCG1* and *JAK3*, respectively, were found in advanced-stage patients. Samples with *PLCG1* mutations had concurrently activated NFAT, NFκB and STAT (pSTAT3) pathways. We also found mutations in *TNFRSF1B*, *CCR4*, *IDH2*, *TET2* and *STAT3*. Regarding *JAK3* mutations, the sample harbouring p.Q507P, which was predicted to be benign, did not show pSTAT3 staining. Conversely, cases with p.A573V and p.R70H (predicted to be deleterious) did show STAT3 activation.

Discussion

The molecular mechanisms controlling the development of MF are not well understood. Several pathways and aberrant molecular findings have been associated with CTCL genesis or progression, but specific markers of progression have not previously been confirmed. Most of the molecular studies of this disease have considered advanced-stage MF and SS patients^{4, 8, 11-13}; only the studies conducted by Tracey and McGirt included early-stage patients^{13, 17}.

Our study concentrated on a series of patients, mainly represented by early stages with classic patches/plaques, in whom we immunohistochemically tested activation markers of three of the possible pathways involved in MF pathogenesis/progression, such NFAT, NFkB and JAK-STAT pathways^{4,18}. Approximately one-quarter of the patients showed activation of the NFAT pathway, with no significant differences between early and advanced stages. This proportion of activated cases was similar to that observed in our previous study in a smaller, independent series of advanced-stage cases⁴. Vaqué and collaborators showed that the use of calcineurin inhibitors was able to decrease NFAT signalling, reducing cell proliferation and viability⁴. Following these findings, a proof of concept clinical trial was conceived in which early-stage MF patients were treated with a topical calcineurin inhibitor, pimecrolimus (PimTo-MF, EudraCT Number: 2014-001377-14).

The NFkB pathway is involved in proliferation, survival/apoptosis and cytokine production in T cells. In MF and SS, NFkB can be activated upon TCR¹⁹ and TNF signalling pathway activation, and by structural alterations in the pathway components^{8, 11, 12, 20}. Recently, it has been shown that FKBP51 and TAK1 proteins are involved in NFkB activation in CTCL^{21, 22}. We detected variable nuclear expression level of NFkB subunits (p50, p65, p52, RelB). p50 and p52 were both detected in T cell nuclei in around 50% of cases, while p65 and RelB were found in 25% of them. We found an association between the expression of NFAT and the four NFkB markers (supplementary figure 3b), which suggests the presence of a common upstream activator of

both pathways, possibly due to the activation of T-cell receptor signalling. We did not find any differences in NFκB marker expression between early and advanced stages.

STAT3 activation is associated with tumour progression in different types of cancer, including CTCL^{5, 9, 18, 23-25}. Analysis of tissue samples has already shown that STAT3 activation occurs frequently in MF patients^{18, 23}. Here we have found that expression of pSTAT3, a marker of STAT3 activation, is associated with advanced stages, confirming previous observations by Sommer et al in eight patients¹⁸. Consistent with these observations, we also found large-cell transformation to be an independent predictor of progression, as previously described²⁶. Although we did not find any statistically significant differences in OS and PFS between positive and negative pSTAT3 groups, the high proportion of advanced MF stages with increased expression pSTAT3 suggests this may be a marker of MF progression.

STAT1 activation proved to be an independent predictor of OS. However, since this is a relatively rare alteration in our series, larger studies should be carried out to confirm this result.

The use of JAK inhibitors, such as ruxolitinib, has been proposed to treat CTCL patients with an altered JAK/STAT pathway since it is known to reduce STAT signalling, cell viability and DNA-synthesis⁹.

From a genomic perspective, we used targeted sequencing to analyse 16 genes that are frequently mutated in T-cell lymphomas¹⁶. We also performed targeted sequencing of hotspot regions in *PLCG1*, *JAK1* and *JAK3* genes, in two independent rounds of library preparation and sequencing. Finally, we were able to detect nine mutations (table 4). It is worth noting that early-stage biopsies showed a low mutational frequency (0.5-11.2%), whereas advanced-stage biopsies had a higher mutation frequency (>20%). The mutational data generated here are in line with those of McGirt's study, which detected mutations only in about 1% of variant allelic frequency in a few early-stage cases¹³. This apparent low mutational load could be due to low

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number of tumour cells present in these lesions. A more sensitive strategy to detect genetic changes present in small subpopulations could be used in future to characterize this type of lymphoma.

Some of the mutated genes in this series are involved in the activation of the analysed pathways: i) *PLCG1* in NFAT and NFκB, ii) *TNFRSF1B* in NFκB, and iii) *JAK1*, *JAK3*, *STAT3*, *STAT5* in JAK-STAT. *PLCG1* p.S345F has been previously associated with NFAT activation⁴. We only detected one patient bearing this mutation, and who exhibited STAT3 and NFκB, but not NFAT, activation. We detected a sample carrying a previously unreported mutation, *PLCG1* p.K713N, in which the three pathways were concurrently activated. We observed that p.A573V (*JAK3*) and p.R70H (*STAT3*) were associated with activation of the JAK-STAT pathway, but p.Q507P (*JAK3*) was not. The first mutation had been reported as being present in early stages and at low frequency¹³ and in the Hut-78 cell line¹⁶. A previous study by our group showed that STAT3 is not constitutively activated in Hut-78 under basal conditions, suggesting that this mutation may require an additional contribution, perhaps mediated through cytokines/interleukins, to activate the pathway²⁷. Interestingly, the coincidence of *PLCG1* mutations and activation of STAT3 deserves special consideration, since it might constitute a novel mechanism for activating this pathway (work in preparation).

Analysis of consecutive biopsies in the same patients could theoretically confirm the role of STAT activation in MF progression (Fig 3 and supplementary figure 5). Strikingly, the mutation p.A573V (*JAK3*; table 4) was found in a progression biopsy (sample 1-B, stage IIB), but we did not find it in the first early-stage diagnostic biopsy (sample 1-A, stage IA-IB). This patient showed no activation of any marker at diagnosis but STAT activation (STAT3 and STAT5) and large-cell transformation were present in the tumour lesion. Patient 107 developed tumour a lesion showing activation of STAT1 and STAT5, compared with the previous plaque phase biopsy (Fig 3). Another patient who developed tumours showed STAT3 activation when the

disease progressed (sample 10-B, stage IIB). In this case, we found no mutation in the JAK/STAT pathway, although we did detect mutations in *PLCG1* and *TET2*. Finally, patient 8, who developed erythroderma, showed activation of STAT3 in the progression biopsy but had no detectable mutation representative early or advanced samples.

Besides the accumulation of activating mutations by the malignant T cells, other genetic or environmental events could drive to a constitutive STAT3 activation. *Staphylococcus aureus* has been reported to be common in MF and a primary cause of death in advanced disease^{28,29}, triggering STAT3 activation in primary malignant T cells³⁰. Recurrent chromosomal alterations in STAT3/5³¹ and deletion³² or hypermethylation³³ of JAK-STAT signaling inhibitors have also been reported in MF.

In summary, this study evaluates three important survival pathways involved in T-cell lymphomas and sets out future directions for possible therapeutic options that could block the progression of the disease. The activation of STAT3 is associated with large-cell transformation, which is known to be a risk factor for progression³⁴. For this reason, pSTAT3 could constitute a biomarker of advanced stages in MF and therefore be useful as a predictive marker for targeted therapy.

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Conflict of interest

Miguel Piris has received lecture fees and Advisory Committee fees from the following companies: Janssen, Takeda, Cellgene, Kyowa Kirin and Gilead. Research developed at the lymphoma laboratory at both IDIVAL and the FJD is receiving grants from the following companies: Kura, Takeda and Gilead.

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Figure legends

Figure 1. Kaplan-Meier curves for early and advanced clinical stages. OS and PFS are expressed in months.

Figure 2. Kaplan-Meier curves for the main markers found associated to advanced clinical stages. OS and PFS are expressed in months.

Figure 3. Expression of immunomarkers in FFPE samples of a MF patient (patient 107) with lesions in plaque phase who developed tumour lesions. Images were taken at 40x. The NFAT pathway was not activated neither in plaque nor in tumour lesion. A cytoplasmic staining was only detected. The JAK-STAT pathway was only activated in tumour lesion, through phosphorylation of STAT1 and STAT5. In the plaque phase, STAT1 was shown to be activated in keratinocytes but not in tumoral T cells. Regarding the NFkB pathway, it highlights the strong staining of p52 in the tumour. The NFkB subunits p50, p65 and RelB increased their activation in the tumour lesion.

Table 1. Clinical and demographic characteristics of the patients included in the study at the time of biopsy.

All			Early stages		Advanced stages	
Age at MF diagnosis - years						
Median	64		63		66	
Range	12-87		12-87		38-87	
Gender - n (%)						
	n	%				
Male	47	61.0	36	59.0	11	68.8
Female	30	39.0	25	41.0	5	31.3
Clinical stage at diagnosis						
Early (IA-IIA)	61	79.2	61	100.0	0	0.0
Advanced (IIB-IVB)	17	20.8	0	0.0	17	100.0
NA	22	-				
MF variant at diagnosis						
Classical patch/plaque	61	78.2	57	93.4	4	25.0
Follicular MF	5	6.4	4	6.6	1	6.3
Erythrodermic	5	6.4	0	0.0	5	31.3
Tumour	7	9.0	0	0.0	6	37.5
NA	22	-				

p>0.05

p>0.05

NA: Not available

Table 2. Relationships between IHC markers and clinical stage. The first biopsy (diagnostic biopsy) and the clinical stage at diagnosis were taken into account for Chi-square calculations. Positive staining was established as a IHC score higher than 1 (supplementary table 1).

			Clinical stage					p (χ 2)
	All samples		Early stage		Advanced stage			
	n	%	n	%	n	%		
NFAT	30/111	27.03	13/59	22.0	6/17	35.3	0.342	
pSTAT1	11/94	11.70	3/56	5.4	3/13	23.1	0.076	
pSTAT3	28/110	25.45	6/58	10.3	9/17	52.9	<0,001	
pSTAT5	82/93	88.17	9/58	15.5	4/17	23.5	0.475	
p50	28/110	25.45	31/59	52.5	8/17	47.1	0.690	
p65	87/115	75.65	27/57	47.4	9/17	52.9	0.502	
p52	23/110	20.91	11/57	19.3	5/17	29.4	0.687	
RelB	55/78	70.51	12/58	20.7	6/17	35.3	0.332	
Large Cell Transformation	56/111	50.45	14/59	23.7	8/17	47.1	0.075	
Folliculotropism	59/115	51.30	10/59	16.9	3/17	17.6	1.000	

Table 3. Multivariate Cox analysis to identify independent predictors of Overall survival and Progression free survival.

	n (%)	Overall survival			Progression free survival		
		HR	95% CI	p value	HR	95% CI	p value
Male	37 (63)	9.099	0.389 - 212.924	0.170	4.559	0.663 - 31.338	0.123
Age ≥ 60 years	30 (48)	10.838	1.133 - 103.660	0.039	2.447	0.628 - 9.533	0.197
Advanced stage	13 (21)	0.271	0.017 - 4.185	0.350	0.624	0.081 - 4.815	0.651
LCT >25%	16 (25)	7.215	0.701 - 74.266	0.097	4.625	1.028 - 20.819	0.046
NFAT (+) ¹	15 (25)	2.310	0.458 - 11.652	0.310	1.427	0.355 - 5.730	0.616
pSTAT1 (+) ¹	5 (8)	10.417	1.139 - 95.288	0.038	5.282	0.955 - 29.230	0.057
pSTAT3 (+) ¹	10 (17)	6.193	0.627 - 61.206	0.119	3.127	0.570 - 17.151	0.189
p50 (+) ¹	33 (56)	4.339	0.420 - 44.859	0.218	4.115	0.754 - 22.460	0.102

LCT: Large cell transformation

¹ Positive marker (+) was tabulated as a IHC score higher than 1 (supplementary table 1).

Table 4. Mutational landscape and phenotype-genotype association. a) List of variants found in 95 samples. b) Phenotype-genotype association of mutations and expression markers.

a)														
Patient	Clinical stage	Chr	Start	Ref	Alt	Gene	Protein position	Amino acids	%VAF	SIFT	PolyPhen	Condel	Validated	Methodology
40	IA-IB	1	12266817	G	A	TNFRSF1B	376	G/R	11.17%	deleterious(0)	probably_damaging(1)	deleterious(0.945)	Yes	TSCA A+B
42	NA	3	32995059	G	T	CCR4	49	V/F	28.11%	deleterious(0)	probably_damaging(0.999)	deleterious(0.935)	Yes	TSCA A+B
10	IB	4	106197557	T	C	TET2	1964	Y/H	25.30%	tolerated(0.07)	probably_damaging(0.999)	deleterious(0.809)	Yes	Targeted seq
15	IA-IB	15	90631877	C	T	IDH2	159	R/H	0.56%	deleterious_low_confidence(0)	possibly_damaging(0.66)	deleterious(0.677)	Yes	TSCA & targeted seq
61	IB	17	40498651	C	T	STAT3	70	R/H	22.34%	deleterious(0)	probably_damaging(0.988)	deleterious(0.882)	Yes	TSCA A+B
1	IVB	19	17948006	G	A	JAK3	573	A/V	2.63%	deleterious(0.05)	probably_damaging(0.959)	deleterious(0.748)	Yes	Targeted seq
11	IA-IB	19	17949121	T	G	JAK3	507	Q/P	4.01%	tolerated(0.34)	benign(0.146)	neutral(0.036)	Yes	Targeted seq
10	IB	20	39792584	C	T	PLCG1	345	S/F	23.26%	tolerated(0.06)	probably_damaging(0.988)	deleterious(0.760)	Yes	TSCA & targeted seq
19	IA-IB	20	39795337	G	T	PLCG1	713	K/N	2.69%	deleterious(0)	probably_damaging(0.992)	deleterious(0.892)	Yes	Targeted seq
TSCA A+B: Truseq Custom Amplicon, both strands														
Targeted seq: PCR-based targeted enrichment														
TSCA & targeted seq: Truseq Custom Amplicon (one strand) and PCR-based targeted enrichment														

b)

Patient	Clinical stage	Gene	Protein position	Amino acids	NFAT	pSTAT1	pSTAT3	pSTAT5	p50	p65	p52	RELB
40	IA-IB	TNFRSF1B	376	G/R								
42	NA	CCR4	49	V/F								
10	IIB	TET2	1964	Y/H								
15	IA-IB	IDH2	159	R/H								
61	IIB	STAT3	70	R/H								
1	IVB	JAK3	573	A/V								
11	IA-IB	JAK3	507	Q/P								
10	IIB	PLCG1	345	S/F								
19	IA-IB	PLCG1	713	K/N