Recurrent numerical aberrations of *JAK2* and deregulation of the JAK2-STAT cascade in lymphomas

Cecile Meier[1], Sylvia Hoeller[1], Caroline Bourgau[1], Petra Hirschmann[1], Juerg Schwaller[2], Philip Went[1], Stefano A Pileri[3], Andreas Reiter[4], Stephan Dirnhofer[1] and Alexandar Tzankov[1]

[1]*Department of Pathology, University of Basel, Basel, Switzerland;* [2]*Department of Research, University of Basel, Basel, Switzerland;* [3]*Department of Hematology and Oncological Sciences 'L. and A. Seràgnoli', Haematopathology Section, Bologna University School of Medicine, St Orsola Hospital, Bologna, Italy and* [4]*Department of Hematology and Oncology, University of Heidelberg Faculty of Clinical Medicine Mannheim, Mannheim, Germany*

The Janus kinase 2 (JAK2)-signal transducers and activators of transcription (STAT) pathway plays an important role in hematological malignancies. Mutations and translocations of the *JAK*2 gene, mapped at *9p24*, lead to constitutive activation of JAK2 and its downstream targets. The presence of *JAK2* mutations in lymphomas has been addressed in larger cohorts, but there are little systemic data on numerical and structural *JAK2* aberrations in lymphoid neoplasms. To study the molecular epidemiology of these aberrations and the consecutive activation of the JAK2-STAT pathway in lymphomas, we examined 527 cases, covering the most common entities, in a tissue microarray by fluorescent *in situ* hybridization with breakable *JAK2* probes, and immunohistochemistry for phosphorylated JAK2 (pJAK2) and its preferred downstream pSTAT3 and pSTAT5. *9p24* gains were detected in 6/17 (35%) primary mediastinal B-cell lymphomas (PMBCLs), 25/77 (33%) Hodgkin's lymphomas (HLs), 3/16 (19%) angioimmunoblastic T-cell lymphomas (AILTs) and 1/5 ALK1$^+$ anaplastic large cell lymphomas (ALCLs); breaks were observed only in three cases. pJAK2 expression was most prevalent in PMBCL, peripheral T-cell lymphomas and HL. pSTAT3 predominated in ALCLs, HLs, AILTs, PMBCLs and peripheral T-cell lymphomas. pSTAT5 expression was detected frequently in follicular lymphomas, diffuse large B-cell lymphomas and AILTs. *9p24* gains correlated with increased proportions of tumor cells expressing pJAK2 (*P* = 0.002) and pSTAT3 (*P* = 0.001). In follicular lymphomas, concomitant expression of pJAK2 and pSTAT5 was linked to better prognosis, whereas expression of pSTAT3 in nongerminal center-like diffuse large B-cell lymphomas could identify a patient group with an inferior outcome. Our findings stress that despite the rarity of activating *JAK2* mutations in lymphomas, *JAK2* is recurrently targeted by numerical, and rarely by structural, genetic aberrations in distinct lymphoma subtypes and that JAK2-STAT pathway may play a role in lymphomagenesis.

Genetic alterations of tyrosine kinases play an important oncogenic role.[1] The V617F and the less common K539L mutations of the Janus kinase 2 (*JAK2*) gene have been shown to be key pathogenic players in chronic myeloproliferative diseases.[2–4] Recently, translocations involving the *JAK2* locus such as t(9;12)(p24;p23), t(8;9)(p24;q11.2), t(3;9) (q21;p24) and t(8;9)(p22;p24) were suggested to be of oncogenic importance in various hematological neoplasms, such as acute lymphoblastic and myelogenous leukemias, atypical chronic myeloid leukemias, myelodysplastic/myeloproliferative diseases and peripheral T-cell lymphomas.[5–11] Gains of *JAK2* gene dosage, particularly due to trisomy *9p24*, which is recurrent in classical Hodgkin's lymphoma

(cHL) and primary mediastinal B-cell lymphoma (PMBCL),[12] are probably also efficient mechanisms of JAK2 activation.

Janus kinases are important components of the receptor-mediated signal transduction of growth factors, hormones and cytokines.[13] A specific phosphotyrosine domain in these receptors works in association with a phosphorylated JAK (pJAK) as binding ligand for the SH2 domain of signal transducers and activators of transcription (STAT). In turn, STAT become phosphorylated, dimerize and subsequently migrate to the nucleus to induce transcription of target genes.[14] STAT are regulated by protein inhibitors of activated STAT (PIAS), phosphotyrosine phosphatases and suppressors of cytokine signaling (SOCS).[13] STAT3 and, particularly, STAT5 are the preferred downstream targets of phosphorylated JAK2 (pJAK2).[1,12] Both STAT3 and STAT5 have been shown to be of functional importance in lymphomas.[15] Constitutive STAT3 activation is a highly consistent finding in ALK1^{+} anaplastic large cell lymphomas (ALCLs)[16,17] and, recently, STAT3 was shown to be an important prognostic factor in activated B-cell-like diffuse large B-cell lymphomas (DLBCLs).[18] The JAK2-STAT pathways seem to be of particular oncogenic significance in cHL and PMBCL, as they are targeted by multiple genetic aberrations (JAK2 locus gains, point mutations and deletions of SOCS1).[19–21]

Although the presence of JAK2 mutations in lymphomas has been addressed in larger cohorts,[22,23] there are little systemic data on the presence of numerical and structural aberrations of JAK2. Therefore, to study the molecular epidemiology of the latter and the consecutive activation of the JAK2-STAT pathways in lymphomas, we examined 527 cases by fluorescent *in situ* hybridization (FISH) with breakable JAK2 probes and immunohistochemistry for pJAK2 and the active phosphorylated forms of its preferred downstream targets STAT3 and STAT5 (pSTAT3 and pSTAT5).

Materials and methods

Case Selection

Reactive lymph nodes ($n = 10$) removed by chance within nononcological surgical procedures served as reference (control) cases. For tissue microarray (TMA) construction, mature B- and T-cell lymphoma and HL cases ($n = 514$), including three nodular lymphocyte-predominant HLs (NLPHLs), diagnosed between 1974 and 2001 and reclassified according to the World Health Organization criteria[24] were collected from the archives of the Institutes of Pathology at the University Hospitals of Basel and Innsbruck and the Unit of Hematopathology at the University of Bologna. In addition, eight ALK1^{+} ALCLs and five T-lymphoblastic lymphomas (T-LBLs) were examined on conventional material. Paraffin blocks were selected on the basis of

availability and preservation. Retrieval of tissue and clinical data was carried out according to the regulations of the local institutional review boards and data safety laws. Clinical and follow-up data were obtained by chart reviews. Treatment regimens, follow-up data and definitions of response, relapse and treatment failures are detailed in previous reports.[25–27]

Tissue Microarray Construction

The TMA were constructed and validated as described elsewhere.[25–28] One (peripheral T-cell lymphomas (PTCLs) and angioimmunoblastic T-cell lymphomas (AILTs)), two (DLBCLs, follicular lymphomas (FLs), small lymphocytic lymphomas/chronic lymphocytic leukemias (SLLs/CLLs) and HLs) or three (PMBCLs) cores from every sample were arrayed. In cases of FL, neoplastic germinal centers were sampled. To obtain reliable results on the PTCL/AILT TMA, each marker was examined on slides from the two constructed array replicates.

Fluorescent *In Situ* Hybridization

Bacterial artificial chromosome (BAC) clones for the 5′ and 3′ regions of JAK2 (RP11-3H3, RP11-2302 and RP11-28A9, RP11-60G18) were identified from http://www.ensembl.org and obtained from the Sanger Institute (Cambridge, UK). The BAC clones were grown in selective media and DNA was extracted according to standard protocols. Dual-color FISH was performed according to standard procedures; RP11-3H3 and RP11-2302 were labeled with Biotin (cat. no. 18427-015 BioNick Labeling System from Invitrogen, Lucerne, Switzerland) and RP11-28A9 and RP11-60G18 were labeled with Digoxigenin (cat. no. 11558706910 Digoxigenin-11-dUTP from Roche, Basel, Switzerland and cat. no. 18160-010 Nick System from Invitrogen). The sections were further processed with a paraffin pretreatment reagent kit (Abbott/Vysis, Baar, Switzerland), and hybridization was performed as described in Vysis' protocol. Denaturation lasted for 10 min at 73°C, and BAC clones mix was incubated overnight at 37°C in Hybrite (from Vysis). Staining was performed using the Fluorescent Antibody Enhancer Set for Digoxigenin Detection (Roche, cat. no. 1768506) and Texas-Red-Avidin and Biotinylated Anti-Avidin (A-2006 and Ba-0300 from Biologo, Kronshagen, Germany). Finally, the slides were counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole in antifade solution. FISH signals were visualized on Zeiss fluorescence microscope equipped with double-band pass filters for simultaneous visualization of green and orange signals. Cases were considered evaluable for FISH if at least 200 tumor cell nuclei/core or, in cases of HL, at least 200 small nuclei/core, corresponding to the background lymphocytic infiltrate, and at least two giant

Table 1 Antibodies, antigen retrieval procedures and detection systems

Antibody	Source	Clone	No.	Dilution	Retrieval	Detection
pJAK2	Cell signaling	C80C3	mAb3776	1:40	EDTA buffer, pH8, MW 100°C/30′	AEC+avidin–biotin blocking
pSTAT3	Cell signaling	D3A7	mAb9145	1:50	TEC buffer, pH8, MW 100°C/30′	DAB+avidin–biotin blocking
pSTAT5	Cell signaling	C11C5	mAb9359	1:50	Citrate buffer, pH6, MW 100°C/30′	DAB+avidin–biotin blocking

AEC: aminoethylcarbazole; DAB: diaminobenzidine.

nuclei/core representing Hodgkin and Reed–Sternberg cells (HRSCs) or, in NLPHL, lymphocytic and histiocytic (L&H) cells, displayed positive signals. Results for HL cases with <5 pathological cells on the TMA were validated on conventional sections. High-level amplification was defined as the presence of either more than 10 gene signals or tight clusters of at least 5 gene signals. Polysomies and low-level amplifications of *9p24* (termed together as 'gains') were defined as the presence of tumor cell nuclei with three or more signals exceeding the mean plus three standard deviations of tri-/polysome nuclei in the reference cases. This cutoff score best discriminated between stochastic aberrations in control cases and aberrations in lymphomas as suggested by comparison of the *9p24* gains' distribution box-plots of both groups. Any detectable *JAK2* split was recorded. Validation of all cases with detectable *JAK2* splits on the TMA was performed by comparison of the TMA results with conventional full-tissue sections, and only cases with splits in ≥5% tumor cells were considered of potential relevance. To assess the reproducibility of the FISH data, comparison between the results gained by two observers (CM and AT) in 80 cases was performed in a blinded manner and analyzed using the *K* statistics.

Immunohistochemistry

The primary antibodies were diluted in a 1% solution of bovine serum albumin in phosphate-buffered saline (pH 7.4) and incubated overnight at 4°C; primary antibody sources, dilutions and antigen retrieval conditions are listed in Table 1. Bound secondary antibodies were visualized using the streptavidin–biotin–peroxidase detection system with either diaminobenzidine or aminoethylcarbazole as chromogens. The sensitivity of the applied pJAK2 and pSTAT5 antibodies was tested in 10 cases of myeloproliferative disorders with high *JAK2* mutated/wild-type allelic ratios as well as in 10 cases of myeloid disorders without *JAK2* mutations (myeloproliferative disorders and acute leukemias). The sensitivity of the pSTAT5 antibody was also tested in cell culture pellets of the *BCR-ABL1*-positive erythroleukemia cell line K562 with known constitutive STAT5 phosphorylation. For negative control experiments, species- and class-matched nonrelevant primary antibodies were used. Cases

on the TMA were considered evaluable when at least 50% of the arrayed core was available for morphological analysis and contained unequivocal tumor cells and, in cases of HL, at least two HRSCs or L&H cells, as well as positive internal controls stained as expected. Results for HL cases with <5 pathological cells on the TMA were validated on conventional sections. In AILT, regions with higher amounts of PD-1$^+$ tumor cells (assessed on sequential sections) were analyzed. Only nuclear expression of pSTAT was considered. As intensity varied between cases due to different tissue preservation methods, only the relative proportion (percentage) rather than intensity of positively stained tumor cells was considered. This proportion was assessed as the mean of the positively stained tumor cells from all tumor cells on the TMA cores. The mean expression of the respective markers in the reference lymph nodes plus three standard deviations were used as cutoffs to define lymphoma cases with relevant expression (positive cases). To assess the reproducibility of the immunohistochemical data, comparison between the results of two observers (CB and AT) in all T-cell lymphomas, HLs and PMBCLs, as well as in one-third of all other disease entity samples, was performed in a blinded manner and assessed using the *K* statistics. When possible, DLBCLs were immunophenotypically classified as germinal center B-cell-like or nongerminal center-like as reported previously.[25,26]

Statistics

Statistical analysis including data description was done using the Statistical Package of Social Sciences version 15.0 for Windows (SPSS, Chicago, IL, USA). Incomplete data were represented by empty spots in the primary SPSS table and were not excluded from the tests done. Interobserver agreement for FISH and immunohistochemical analyses was assessed using the *K* statistics, a *K*-value of ≥0.75 implying excellent agreement. The Spearman's test was used to analyze relationships between markers. The Kruskal–Wallis test was applied to assess mean differences between groups. The prognostic performance of the variables and determination of optimal cutoff values of continuous variables was established by receiver-operating-characteristic (ROC) curves plotting sensitivity *vs* 1-specificity. The optimal cutoff point was calculated using Youden's
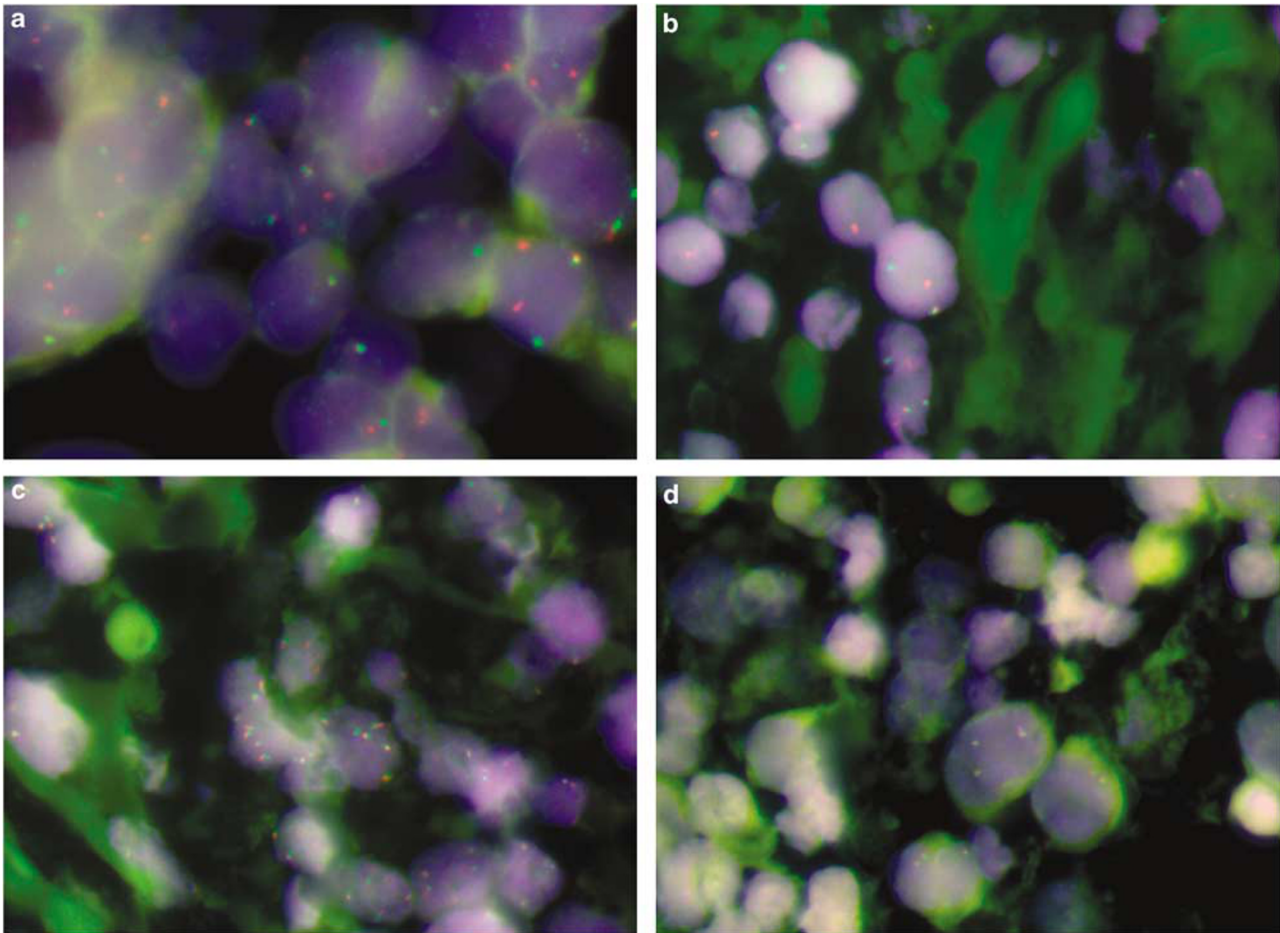
Figure 1 (**a**) Split red and green FISH signals with the RP11-3H3 and RP11-2302 (red) and RP11-28A9 and RP11-60G18 (green) BAC probes for the 5′ and 3′ regions of *JAK2* in the index case (positive control) of erythroid preleukemia with t(8;9)(p22;p24),[7] 1000 × . (**b**) Split FISH signals in a case of angiommunoblatic T-cell lymphoma. Note green autofluorescent prominent vascularization in the background, 630 × . (**c**) Multiple FISH signals in primary mediastinal B-cell lymphoma tumor cells. Note green autofluorescent scrlerosing bundles on the left side, 630 × . (**d**) Three FISH signals in a giant nucleus of a classical Hodgkin lymphoma, 630 × .

index (Y), denoting Y = sensitivity + specificity–1, as this method can determine the optimal cutoff value with the highest sensitivity and specificity.[29] The results from the ROC analysis were considered in every disease entity for overall survival (OS) and disease-specific survival (DSS). Applying the cutoff values calculated by the ROC/Y, OS and DSS were then analyzed by the Kaplan–Meier method and compared by the log-rank method. Statistical significance was defined as $P < 0.05$; two-sided tests were used throughout.

Results

Fluorescent *In Situ* Hybridization

Fluorescent *in situ* hybridization analysis was successful in 198 TMA cases (39%), in 5 ALCL cases (63%) and 2 T-LBL cases (40%), and in all 10 reference cases. FISH-related problems, such as hybridization failure and unspecific or weak fluorescence, were responsible for the majority of the nonanalyzable cases; 82 arrayed cases were nonanalyzable due to too few cells, empty or noninformative spots or in, cases of HL, lack of identifiable HRSCs or L&H cells in the array cores. Eight HL cases with successful hybridization contained < 5 HRSCs on the array spots, so that the obtained results (2 cases with *9p24* gains, 6 disome cases) were validated on conventional sections, which did not lead to change in interpretation. Interobserver reproducibility of the FISH result was good (K = 0.88). Rarely (0.5 ± 1%), cells with gains at *9p24* were observed in the control cases. Thus, considering our definition of gains and practicability, a cutoff value of ≥5% cells with three or more FISH signals was chosen to classify tumor cases positive for *9p24* gains. The number of FISH signals in lymphomas ranged between 1 and 6 per cell (mean 3.3), being higher in HLs, PMBCLs and AILTs (Figure 1a–c). Systematic data on the distribution of cases with *9p24* gains and *JAK2* breaks are shown in Table 2. According to our criteria, no high level amplifications of the *JAK2* gene were detected in

Table 2 Observed frequencies and quantitative data on lymphomas with numerical and structural aberrations in the *JAK2* locus

Disease	N	N cases with 9p24 gains in >5% of cells (%)	Mean % ± s.d. of cells with >3 FISH signals in cases with 9p24 gains	JAK2 breaks
CLL	5	0 (0)		
DLBCL	39	2 (5)	9 ± 4	
Transformed DLBCL	7	1 (14)	12	
FL	19	0 (0)		
MCL	4	0 (0)		
cHL	77	25 (32)	34 ± 32	1
NLPHL	3	2 (75)	6 ± 1	
PMBCL	17	6 (35)	19 ± 15	
PTCL	11	0 (0)		
AILT	16	3 (19)	28 ± 21	1
ALCL	5	1 (20)	10	1
T-LBL	2	0 (0)		
Control cases	10	0 (0)		
P-value		P<0.0001[a]	0.293[b]	

Mean proportions of lymphoma nuclei with ≥3 FISH signals were calculated for cases considered to have *9p24* gains according to our definition (presence of tumor cell nuclei with three or more signals, exceeding the mean rate plus three standard deviations of tri-/polysome nuclei in the reference cases).
[a]Spearman's test.
[b]Kruskal–Wallis test.

this series. Split FISH signals in ≥5% tumor cells were observed in each one cHL, ALCL and AILT cases (Figure 1d). Our attempt to detect *PCM1-JAK2* cDNA by reverse transcription-PCR using RNA extracted from the original archival material failed.

Immunohistochemistry

The number of evaluable TMA cases varied for the different markers, being highest for pSTAT5 (480/514, 93%) and lowest for pJAK2 (442/514, 86%), and, with respect to disease entities, highest in CLL/SLL (up to 97%, 38 of 39 cases) and lowest in nodular sclerosis cHL and PMBCL (down to 70%, 50 of 70 cases and 32 of 50, respectively). The analysis failure was mainly due to TMA technology short-falls, such as too few cells, empty or noninformative spots or, in cases of HL, lack of identifiable HRSCs or L&H cells in the samples. Interobserver reproducibility of the immunohistochemical result was good, with the lowest *K* for pSTAT5 of 0.82 and highest for pSTAT3 of 0.98.

In controls, pJAK2 was detectable in the cytoplasm of centroblasts (1–5%) and marginal zone lymphocytes (1%) as well as in higher proportions in endothelial and dendritic cells (Figure 2a). Nuclear pSTAT3 was seen in follicular cells (1–5%), in lymphocytes surrounding the germinal centers (2%) and in plasmocytoid/monocytoid lymphocytes (up to 20%), corresponding to its important role in terminal B-cell differentiation,[30] as well as in endothelial and vascular smooth muscle cells (Figure 2b). Nuclear pSTAT5 was detectable in lymphocytes surrounding the germinal centers (1–5%) (Figure 2c). In this regard, a subpopulation of germinal center B cells that is characterized by

pSTAT5 expression with an activated centrocyte phenotype has been identified recently.[31] Nuclear expression of pSTAT5 was detected in all megakaryocytes of myeloproliferative disorders with high *JAK2* mutated/wild-type ratios, in all nuclei of the K562 pellets, and in erythroid precursors of myeloid disorders without *JAK2* mutations (Figure 2d and e).

Lymphomas showed only cytoplasmic expression of pJAK2, which was prevalent in a significant number of cases and tumor cells of PMBCL, PTCL and HL (Figure 3a and b). pSTAT3 predominated in cHL, AILT and DLBCL (Figure 3c); as expected,[16,17] all ALCLs expressed pSTAT3. Nuclear pSTAT5 expression was most commonly detected in intra- and perifollicular lymphocytes of FL and in large blasts of DLBCL (Figure 3d). The HRSCs of only two cHL cases showed nuclear expression of pSTAT5 (Figure 3e). The quantitative results are shown in Table 3. Considering the cases with *JAK2* breaks, the one cHL case expressed both studied pSTAT, whereas the AILT and the ALCL cases expressed pSTAT3.

Correlation Analysis

9p24 gains correlated with increased proportions of tumor cells staining positive for pJAK2 (*P* = 0.002, correlation coefficient 0.233) and pSTAT3 (*P* = 0.001, correlation coefficient 0.264). Considering the different entities stratified for *9p24* gains, cHL, PMBCL and AILT with *9p24* gains had a slightly increased proportion of cases expressing pSTAT3 (15/24 *vs* 21/48, 4/6 *vs* 4/11 and 3/3 *vs* 4/13, respectively), whereas expression of pSTAT3 in both ALCLs (the one with *JAK2* break and the one with *9p24* gain) was similar to that in the cases without
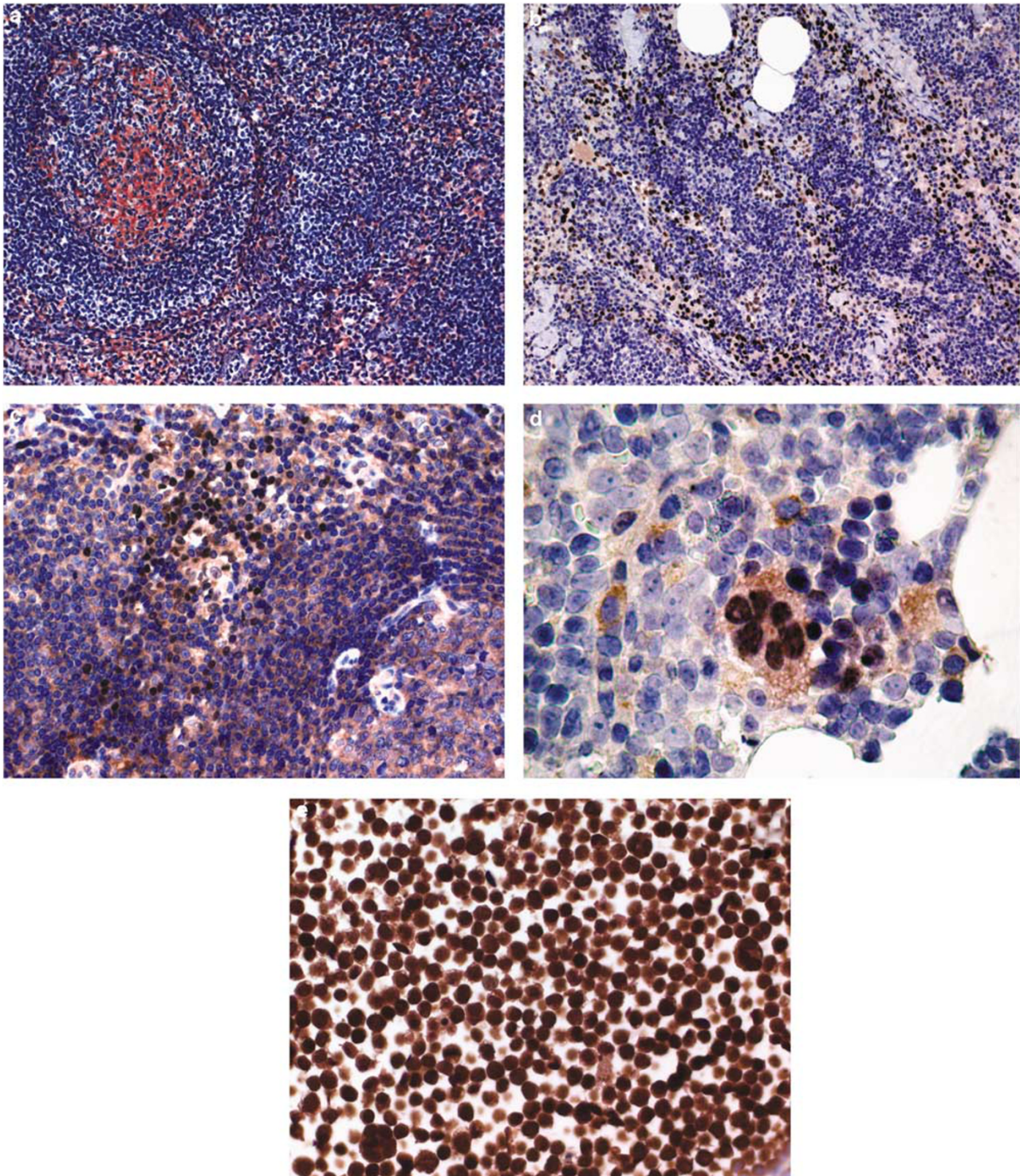
Figure 2 (a) Expression of pJAK2 in follicular dendritic cells, endothelia and isolated lymphocytes of a reactive lymph node. Immunoperoxidase stain, 50×. (b) Expression of pSTAT3 in plasmocytoid/monocytoid sinus-associated lymphocytes of a reactive lymph node. Immunoperoxidase stain, 50×. (c) Expression of pSTAT5 in marginal zone lymphocytes surrounding a germinal center of a reactive lymph node. Immunoperoxidase stain, 200×. (d) Nuclear expression of pSTAT5 in a megakaryocyte of a polycythaemia vera patient with known high allelic *JAK2* mutated/wild-type ratio. Immunoperoxidase stain, 400×. (e) Striking nuclear positivity for pSTAT5 in K562 cell pellets. Immunoperoxidase stain, 300×.

9p24 abnormalities. The mean expression and the number of pSTAT3-positive cases was higher in nongerminal center-like DLBCL 18 (22/34 in non-germinal center-like DLBCL compared with 10/33 in germinal center B-cell-like DLBCLs, *P* = 0.005). Expression of both studied pSTAT correlated with
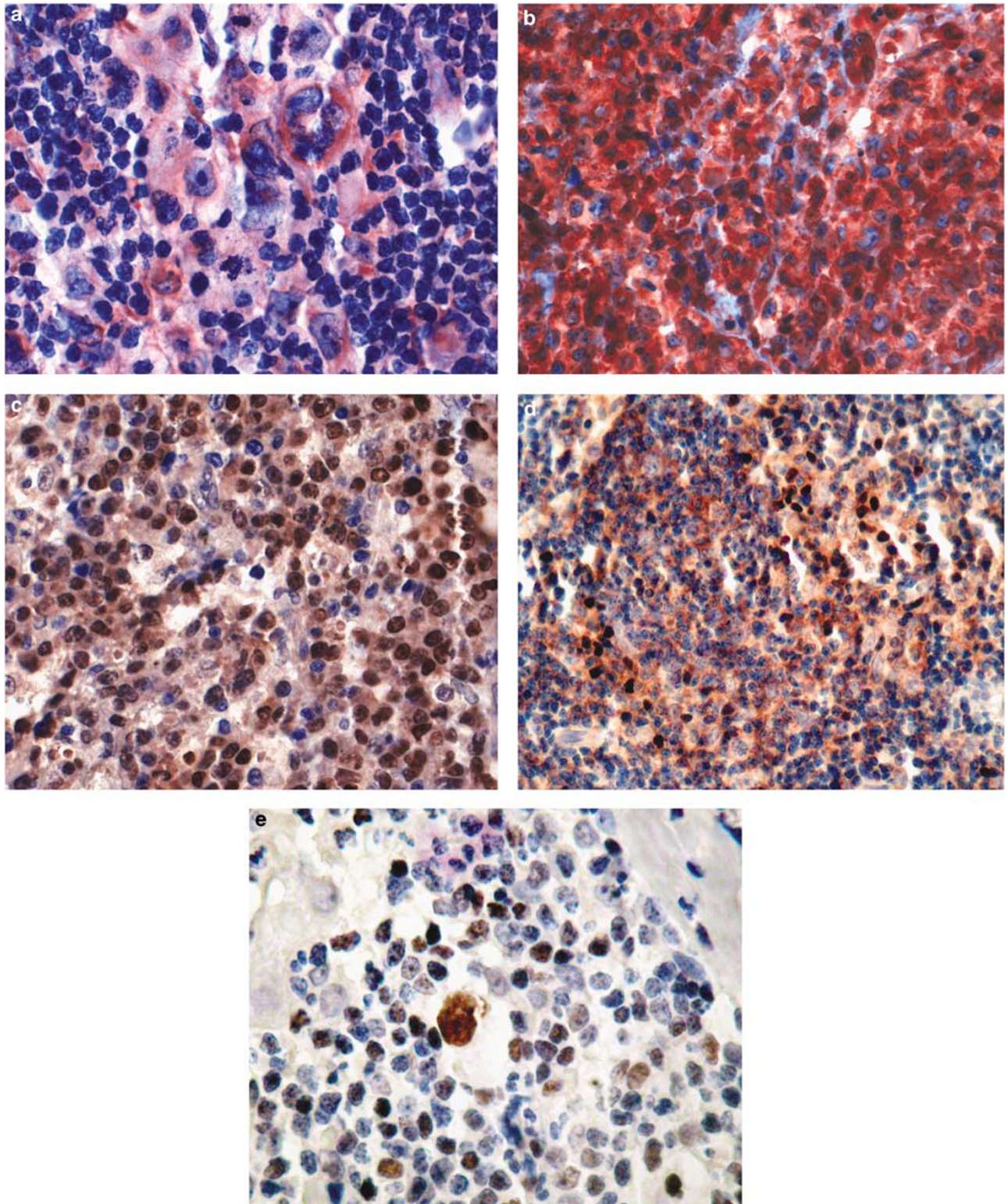
Figure 3 (**a**) Expression of pJAK2 in a Reed–Sternberg cell. Note negative surrounding lymphocytes. Immunoperoxidase stain, 400 × . (**b**) Expression of pJAK2 in almost all tumor cells of a primary mediastinal B-cell lymphoma. Immunoperoxidase stain, 400 × . (**c**) Expression of pSTAT3 in a diffuse large B-cell lymphoma. Immunoperoxidase stain, 400 × . (**d**) Expression of pSTAT5 in scattered centrocytoid cells at the periphery of a neoplastic follicle of follicular lymphoma. Immunoperoxidase stain, 200 × . (**e**) Expression of pSTAT5 in a Hodgkin cell of one of the two positive classical Hodgkin's lymphoma cases. Immunoperoxidase stain, 400 × .

Table 3 Quantitative data of the expression of pJAK2, pSTAT3 and pSTAT5 in lymphomas

Disease	pJAK2	pSTAT3	pSTAT5
	N cases expressing the respective marker above the cutoff[a]/N evaluable (%)		
	Mean % ± s.d. positive tumor cells in cases expressing the respective marker above the cutoff		
CLL	14/36 (39)	7/36 (19)	16/38 (42)
	12 ± 6	17 ± 7	8 ± 5
DLBCL	63/100 (63)	41/109 (38)	41/107 (38)
	16 ± 16	37 ± 25	17 ± 14
Transformed DLBCL	9/13 (69)	3/15 (25)	2/17 (14)
	13 ± 10	30 ± 26	5 ± 0
FL	41/80 (51)	28/79 (35)	42/85 (50)
	12 ± 10	18 ± 9	11 ± 6
MCL	3/17 (18)	2/18 (1)	6/18 (33)
	12 ± 8	18 ± 16	15 ± 6
cHL, HRSC	42/115 (36)	59/118 (50)	2/127 (2)
	49 ± 29	75 ± 28	32 ± 35
NLPHL, L&H cells	1/3 (33)	1/4 (25)	0/4 (0)
	50	70	
PMBCL	29/32 (91)	9/39 (23)	0/41 (0)
	56 ± 33	34 ± 23	
PTCL	10/15 (67)	4/17 (23)	1/17 (6)
	28 ± 23	26 ± 16	5
AILT	10/21 (48)	10/25 (40)	4/25 (16)
	25 ± 21	28 ± 20	33 ± 23
ALCL	2/7 (30)	8/8 (100)	1/8 (13)
	11 ± 6	49 ± 24	5
T-LBL	0/5 (0)	0/5 (0)	1/5 (20)
			5
Control cases	6/10 (60)	10/10 (100)	8/10 (80)
	2 ± 2	4 ± 4	1 ± 5
P-values[b]	<0.0001	<0.0001	<0.0001

[a]Cutoff to consider a case positive was the mean expression plus three standard deviations in the reference cases.
[b]Kruskal–Wallis test.

Table 4 Determination of optimal cutoff levels considering overall survival (OS) and disease-specific survival (DSS) for the different markers applying receiver operating characteristic (ROC) curves

Marker	Disease	Survival	P-value[a]	Cutoff	Specificity	Sensitivity	P-value[b]
pJAK2	FL	OS	0.04	3.125%	67%	67%	0.032
pSTAT5	FL	DSS (OS)	0.039	3.75%	83%	45%	0.077
pSTAT3	*non-GC DLBCL*	*DSS (OS)*	*0.073*	*12.5%*	*100%*	*37%*	*0.054*
pSTAT5[c]	*DLBCL*	*OS*	*0.181*	*16.25%*	*97%*	*19%*	*0.031*

The *P*-values in the ROC columns delineate the discriminatory power of the suggested cutoff. The prognostic significance of the markers was finally tested after dichotomization by the Kaplan–Meier test and the log-rank method. Factors in which values >cutoff had adverse prognostic effects are italicized. If a factor had analogous effects on DSS and OS, only DSS was shown and OS was mentioned in brackets.
[a]ROC curves.
[b]Log-rank method.
[c]Trends toward negative prognostic effects for pSTAT5 expression remained in both germinal center B-cell-like and nongerminal center-like DLBCL phenotypic groups, but the statistical significance got lost.

the presence of SOCS1 in tumor cells (data not shown) only in cases without *9p24* gains. In FLs, the number of tumor-infiltrating regulatory T cells, assessed by the expression of FOXP3,[25] significantly correlated with the expression of both pSTAT3 and pSTAT5 (correlation coefficients 0.325 and 0.397, respectively). In cHLs, Epstein–Barr virus association correlated only with increased expression of pJAK2 in bystander cells (*P* = 0.01, correlation coefficient 0.238).

Prognostic Significance

The prognostic significance of *9p24* gains as well as of pJAK2 and its downstream pSTAT3 and pSTAT5 was analyzed by ROC curves. Statistically significant results, calculated cutoffs and their sensitivity and specificity as well as the respective *P*-values from the log-rank test are listed in Table 4. Interestingly, better survival in FL was strongly associated with concomitant expression of both JAK2 and
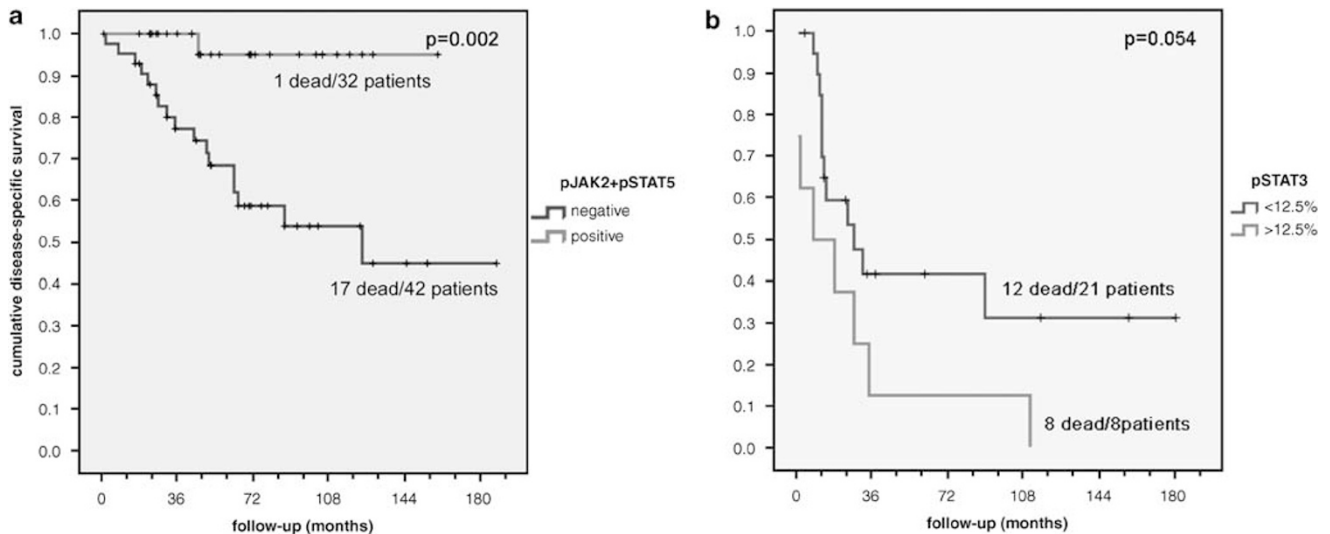
Figure 4 (**a**) Disease-specific survival in follicular lymphoma with regard to the amount of JAK2 and pSTAT5 coexpressing intrafollicular lymphocytes. (**b**) Disease-specific survival in nongerminal center-like diffuse large B-cell lymphomas with regard to the amount of pSTAT3-expressing tumor cells. The cutoffs for variable dichotomization were calculated by receiver-operating-characteristic (ROC) curves (see Materials and methods).

pSTAT5 (1 dead patient/32 cases, mean survival 154 months, median not reached) compared with patients without expression of JAK2 and/or pSTAT5 (17 dead patients/42 cases, mean survival 115 months, median survival 124 months, $P = 0.002$; Figure 4a). Nongerminal center-like DLBCL expressing pSTAT3 above the ROC-determined prognostic cutoff of 12.5% positive tumor cells (pSTAT3-positive cases) had a worse prognosis, with 8/8 lymphoma-related deaths (mean survival 25, median survival 8 months) compared with 12/21 in pSTAT3-negative cases[18] (mean survival 74, median survival 27 months, $P = 0.054$; Figure 4b). Presence of *9p24* gains did not influence survival. The one cHL patient with breaks involving the *JAK2* locus was still alive after 78 follow-up months; there were no follow-up data for the ALCL and AILT patients with breaks involving the *JAK2* locus.

Discussion

The JAK2-STAT pathway is known to play an important role in hematological malignancies.[1,13,15] Mutations and translocations of the *JAK2* gene, mapped at *9p24*, lead to constitutive activation of the JAK2 kinase and its downstream targets in myeloid malignancies,[2–12] but little is known about the molecular epidemiology of numerical and structural *JAK2* aberrations in lymphoid malignancies. Our FISH data confirm and substantially extend data from previous studies on numerical and structural *JAK2* gene aberrations in lymphomas by taking advantage of a validated lymphoma TMA in a large cohort of cases covering a broad spectrum of disease entities currently recognized by the WHO. Moreover, we addressed the potential functionality

of the *JAK2* aberrations by analyzing the amount of pJAK2 and its downstream targets pSTAT3 and pSTAT5 in our TMA collective. One underperformance of our study was the high number of inconclusive TMA cases for FISH analysis (61%), which was slightly higher than the expected number of noninformative cases on TMA. This was primarily related to poor tissue preservation of the original archival material, particularly those cases diagnosed before 1995. Nevertheless, the numerical and structural status of the *JAK2* gene could be determined in 198 lymphoma cases, which is the largest population studied thus far.

Considering numerical changes at *9p24*, we found gains in 35% of PMBCLs, 32% of cHL and, although assessed in a small number of cases, in 19% of AILTs and 10% of ALCLs. Considering the former two entities, our data fit with previously reported frequencies in PMBCLs and cHL and are supported by data from gene expression profiling, pointing to the close relationship of these entities at the molecular level.[12,19,32,33] In cHL, the JAK2-STAT cascade is constitutively maintained at an active high level by multiple mechanisms, including stimulatory loops through interleukin (IL)-13 and its receptor IL13Rα1, gene dosage effect of the gained *JAK2* locus at *9p24* in over 30% of cHL cases (present data) and aberrations of *SOCS1*.[19,21,34] These multiple mechanisms of JAK2-STAT activation in cHL point to its important oncogenic role. The essential nature of this pathway is emphasized by the fact that STAT3 downregulation in a HRSC line induces apoptosis.[35] This 'oncogenic addiction' of HL to the JAK2-pSTAT3 cascade makes it an intriguingly targetable pathway. Considering the other disease entities, a significant new finding in our study was the high frequency of *9p24* gains in

AILT, observed previously only in not nearly specified mature T-cell lymphomas.[36] Finally, the observed increased *JAK2* gene dosage in our disease collectives were probably functional, as cases with *9p24* gains showed (i) higher amounts of pJAK2, (ii) higher pSTAT3 and (iii) decreased amount of SOCS1 (data not shown). Compared with the relatively high frequencies of numerical alterations, we rarely observed structural alterations of the *JAK2* gene (each one cHL, ALCL and AILT cases with breaks). Interestingly, a cHL case with a t(4;9)(q21;p24) leading to a *SEC31A–JAK2* fusion has been reported recently.[37]

Apart from their adherence to *9p24* gains, the amount and distribution of the studied pSTAT varied between the disease entities. Similar to others, we observed preferential expression of pSTAT3 in ALCL,[16,17] HL[35] (but not in NLPHL) and nongerminal center DLBCL.[18] Our PTCLs also preferentially expressed pSTAT3. As in ALK1+ ALCL, NPM-ALK constitutively activates STAT3 by increased protein phosphorylation and increased transcription/stabilization of *STAT3* mRNA and ALCLs overexpress JAK3,[16] our observed high predominance of pSTAT3, despite low expression of pJAK2 was expected. Nevertheless, one ALK1+ ALCL showed *9p24* gain and one case showed a break involving the *JAK2* locus, the meaning of which cannot be deduced from these single observations, but may point, similarly to the multiple mechanisms of JAK2-STAT activation in cHL, toward the essentiality of JAK-STAT pathways in ALK1+ ALCLs. The important nature of STAT3 in ALK1+ ALCL is emphasized by the fact that STAT3 downregulation in ALCL cell lines induces apoptosis.[17] This 'oncogenic addiction' of ALCL to pSTAT3 and the predominance of pSTAT3 in other lymphomas, such as HL (see above), nongerminal center DLBCL and PTCL, substantiates its oncogenic capacity as well as its potential as a target for molecular approaches,[14,38] for example, STAT3 antisense molecules, such as ISIS 345794. Finally, our evidence points toward a probable minor, if any, role of JAK2-STAT members in T-LBL.

One variation of pSTAT distribution in our collective and previously reported data,[21,34] which we would like to address, was the very low amount of HL cases with nuclear expression of pSTAT5. Importantly, we tested the sensitivity of the applied antibody in myeloproliferative disorders with high *JAK2* mutated/wild-type allelic ratios and cell culture pellets of K562 cells with known constitutive STAT5 phosphorylation. When analyzing the TMA, we paid attention to the presence of internal positive controls in the analyzed cases (positively staining endothelial cell nuclei), and our arrays also included unequivocal cases with nuclear pSTAT5 expression in higher proportions of the tumor cells (external control equivalents). All these results highlighted the reliability of our pSTAT5 staining. Thus, some differences between our and previous studies could be related to the use of polyclonal antibodies by the latter, or to general problems related to phosphorylation status-specific antibodies.[39]

Taking into account ROC-determined cutoff values of the respective markers, another noteworthy finding of our study was the identification of the favorable prognostic value of the concomitant pJAK2 and pSTAT5 expression in FL. One of the several possible explanations for this prognostic effect of the concomitant pJAK2 and pSTAT5 expression could be sought in the known ongoing immunological cross talk in a particular group of FL with better prognosis,[40] immunological cross talk being mediated by members of the STAT family at the intracellular site. Indeed, gene expression of, for example, *IL7* receptor and *STAT4* is part of the favorable immune response 1 signature of FL.[40] In our FL collective, the expression of pSTAT5 correlated with higher amounts of tumor-infiltrating regulatory T cells, which are also known to be associated with favorable outcomes.[25] Finally, we confirmed the prognostic importance of pSTAT3 in activated B-cell-like DLBCL, which was recently observed by others,[18] as our own nongerminal center DLBCL expressing pSTAT3 had also an inferior disease-specific survival, again indicating the oncogenic potential of pSTAT3.

Taken together, our and previous findings stress that despite the rarity of activating *JAK2* mutations in lymphomas,[22,23] *JAK2* is recurrently targeted by numerical, and very rarely by structural, genetic aberrations in distinct lymphoma subtypes, such as cHL, PMBCL and AILT, and that these *JAK2* aberrations are very likely to be functional. In addition, in FL, pJAK2 and pSTAT5 expression is linked to better prognosis, whereas expression of pSTAT3 in nongerminal center-like DLBCL can identify a patient group with an inferior outcome. Our molecular epidemiology and prognosis data might be of practical importance when selecting collectives for future JAK2 small molecular inhibitor trials.

Disclosure/conflict of interest

Nothing to declare, no potential conflicting interests.

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