JOURNAL OF CLINICAL ONCOLOGY UNDERSTANDING THE PATHWAY

Transcription Factor STAT3 As a Prognostic Marker and Therapeutic Target in Cancer

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In the early 1990s, researchers identified a mechanism by which cytokines interacting with cell surface receptors could modulate gene expression in the nucleus. Using elegant genetic and molecular approaches, it was found that activation of these receptors leads to phosphorylation of a family of proteins now called STATs that then shuttle into the nucleus, bind to specific sequences in the regulatory region of target genes, and alter their expression[1,2] (Fig 1). STAT3, in particular, was found to mediate the acute phase response seen with inflammation and stress that is often mediated by interleukin-6 (IL-6) and other proinflammatory cytokines.[3] Genes regulated by STAT3 control critical cellular processes such as proliferation, survival, pluripotency, invasion, and angiogenesis.

Given the nature of these genes and their effect on cellular behavior, it is not surprising that physiologic STAT signaling is tightly regulated. When a cell is exposed to a cytokine like IL-6, STAT3 becomes phosphorylated and activated for only a matter of minutes. However, shortly after the discovery of physiologic STAT signaling, it was appreciated that in many human cancers abnormal continuous activation of STAT3 and other STATs was occurring.[4,5] Initially, this was observed in cancers characterized by an activated mutated tyrosine kinase such as Bcr-Abl or epidermal growth factor receptor.[6-8] However, the prevalence of STAT3 activation in human cancers far exceeds that of known activated kinases. For example, in breast cancer or prostate cancer, more than 70% of primary tumors display constitutive STAT3 activation.[9] In many of these cases, soluble factors such as IL-6 are released from stromal cells or from the tumor cells themselves driving activation of this pathway.[10]

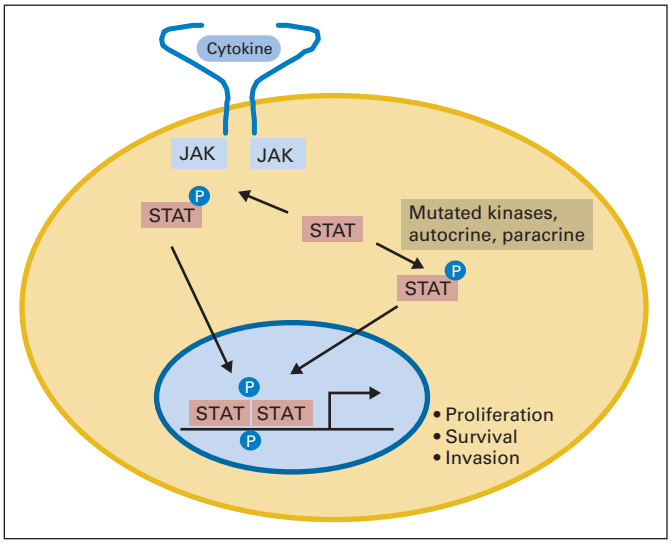

Fig 1. Physiologic and pathologic STAT signaling. STATs are transcription factors that, under basal conditions, remain inactive in the cytoplasm. Cytokine-mediated activation of Jak family kinases leads to tyrosine phosphorylation of STATs, after which they translocate into the nucleus, bind to specific regulatory regions of genes, and activate transcription. Many STAT target genes control critical cellular processes such as proliferation, survival, and invasion. Physiologic STAT activation is rapid and transient. In cancer, through the action of mutated kinases or autocrine or paracrine mechanisms, STATs become activated constitutively, thereby driving continued expression of genes that then underlie malignant cellular behavior. P, phosphate.

Recent evidence from a number of studies,[11-13] including the elegant analysis by Huang et al[14] in this issue of the *Journal of Clinical Oncology*, shows how our increased insight into STAT signaling can provide prognostic information for patients with cancer. The authors asked the important question of whether activation of STAT3 has prognostic significance in patients with diffuse large B-cell lymphoma (DLBCL) treated with the standard first-line therapy of rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. They performed immunohistochemistry (IHC) using an antibody that specifically recognizes the phosphorylated activated form of STAT3 and quantitated the frequency and intensity of staining in $CD20^+$ cells from biopsies. In their cohort, 30% of germinal center B-cell–like and 47% of activated B-cell–like DLBCL samples were positive for STAT3 activation. Their key finding was that the detection of STAT3 phosphorylation was associated with inferior survival in the entire cohort and in the activated B-cell–like subgroup. Although there was a similar trend in the germinal center B-cell–like group, the differences did not reach statistical significance.

Although phosphorylation of STAT3 is a marker for activation of this transcription factor, the function of STAT3 is to regulate gene expression in the nucleus. Thus, the authors asked whether it might be possible to define a gene expression pattern reflecting activated STAT3 and whether this would also carry prognostic information. Using a clever genetic strategy, they identified an 11-gene signature of STAT3 activation. They found that this signature also had strong prognostic weight in this patient population. This finding is important for several reasons. First, it supports the notion that STAT3 phosphorylation is not merely a marker of activation of some tangential signaling pathway, but rather that the activation of the transcriptional function of STAT3 is likely the important component. This is reinforced by the

finding that among the 11 genes in the signature are several that have already been suggested to have a role in cancer pathogenesis, and altered expression of these genes may underlie the inferior outcome of these patients. In addition, although IHC is available in many more clinical laboratories than gene expression analysis (at least currently), for technical reasons, gene expression analysis may be a more robust indicator of functional STAT3 activation. In particular, false-negative results can occur in IHC as a result of inadequate antigen retrieval or dephosphorylation of STAT3 from the release of phosphatases in the tissue between biopsy and fixation. Thus, gene expression analyses may one day supplant IHC for detecting activation of transcription factors such as STAT3.

Finally, the question that patients with cancer and their oncologists are most interested in is whether any of this information can be used not just to provide information about prognosis but to improve it. Because STAT3 itself is the mediator of the gene expression changes underlying malignant cellular behavior and it is a convergence point of many oncogenic pathways, might it be a good target for therapy? Although cancer cells are often addicted to activation of STAT3, normal cells are fairly tolerant of loss of its function, likely reflecting redundancies in normal signal transduction. Thus, STAT3 inhibitors have the potential for a high therapeutic index.[15] Furthermore, resistance to targeted therapies often arises from activation of an alternate signaling pathway, many of which also converge on STATs. This suggests that inhibition of these proteins may forestall resistance. In recent years, a number of clever strategies have been used to target STAT3, some of which have entered clinical trials.[16] STAT3 inhibitors may be effective as single agents, and they may also sensitize cells to other therapeutic modalities, including immune-based approaches.[17,18]

There are still many areas of spirited investigation regarding STATs in cancer pathogenesis. For example, in some tumor types, STAT3 activation has been associated with a favorable prognosis.[19-21] This can reflect the fact that depending on the epigenetic milieu of a cell, STATs can activate (or repress) distinct patterns of genes, which may alter the balance between oncogenicity and tumor suppression.[22] In addition, more than one STAT family member, such as STAT3 and STAT5, can be activated in the same tumor, and this can mediate disparate effects.[23,24]

The finding that STAT3 activation is associated with the prognosis of patients with DLBCL and other cancers reinforces the focus on transcription factors as focal points in the pathogenesis of tumors driven by a range of oncogenic events. During the next few years, additional insights into the biologic function of STAT3 in cancer will undoubtedly be revealed. Most exciting is the likelihood that we will be able to enhance the treatment of our patients based on this knowledge.

AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Activation of the STAT3 Signaling Pathway Is Associated With Poor Survival in Diffuse Large B-Cell Lymphoma Treated With R-CHOP

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ABSTRACT

Purpose
We previously reported that constitutive STAT3 activation is a prominent feature of the activated B-cell subtype of diffuse large B-cell lymphomas (ABC-DLBCL). In this study, we investigated whether STAT3 activation can risk stratify patients with DLBCL.

Patients and Methods
By an immunohistochemical method, we investigated phosphotyrosine STAT3 (PY-STAT3) expression from 185 patients with DLBCL treated with R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone). Cell line-based siRNA experiments were also performed to generate an 11-gene, PY-STAT3 activation signature, which was used to study a previously published cohort of 222 patients with DLBCL. The STAT3 activation status determined by these two methods and by *STAT3* mRNA levels were then correlated with survival.

Results
PY-STAT3 was detected in 37% of DLBCL and enriched in ABC-DLBCL cases ($P = .03$). PY-STAT3 positivity significantly correlated with poor overall survival (OS; $P = .01$) and event-free survival (EFS; $P = .006$). Similar observations were made for high levels of *STAT3* mRNA. In multivariable analysis, PY-STAT3 status ($P = .02$), International Prognostic Index ($P = .02$), and BCL2 expression ($P = .046$) were independent prognosticators of OS in this cohort. Among the cell-of-origin subgroups, PY-STAT3 was associated with poor EFS among non–germinal center B-cell DLBCL cases only ($P = .027$). Similarly, the 11-gene STAT3 activation signature correlated with poor survival in the entire DLBCL cohort (OS, $P < .001$; EFS, $P < .001$) as well as the ABC-DLBCL subgroup (OS, $P = .029$; EFS, $P = .025$).

Conclusion
STAT3 activation correlated with poor survival in patients with DLBCL treated with R-CHOP, especially those with tumors of the ABC-DLBCL subtype.

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 30% to 40% of newly diagnosed non-Hodgkin lymphoma.[1] The addition of rituximab (R-) to standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy regimens results in improved overall survival (OS) by 10% to 15%.[2] Nevertheless, a substantial number of patients still die as a result of the disease, highlighting the need for improved DLBCL prognostication and better therapy. DLBCL is a biologically and clinically heterogeneous disease, which is explained, at least in part, by the diversity in its normal cellular counterparts and transforming pathways.[3] On the basis of gene expression similarities to either normal germinal center (GC) B cells or activated peripheral blood B cells, DLBCL can be divided into two main subgroups: germinal center B-cell-like (GCB) DLBCL and activated B-cell-like (ABC) DLBCL.[4,5] In this cell-of-origin (COO) classification, GCB-DLCBL represents transformed GC centroblasts that are BCL6 high and lack features of B-cell activation. In comparison, ABC-DLBCLs,

likely correspond to activated centrocytes and/or preplasmablasts[3] and are characterized by constitutively activated NF-κB as well as JAK/STAT3 activation in many but not all cases.[6-8] It is also well documented that patients with GCB-DLCBL generally have a better prognosis than do patients with ABC-DLBCL in both the CHOP[9] and R-CHOP eras.[10-13] It is therefore important to identify new biomarkers that can risk-stratify ABC-DLBCL for the development and application of novel targeted therapies.

In normal cells, STAT3 activation is a transient and tightly controlled process because of rapid activation and feedback inactivation of growth factor/cytokine receptor signaling.[14] In many types of solid tumors, aberrant activation of upstream tyrosine kinases leads to constitutive activation of JAK/STAT3 signaling, which in turn promotes tumor cell growth, survival, angiogenesis, and metastasis.[15] Through inflammatory mediators in the tumor microenvironment, tumor cells with activated STAT3 can also evade immune surveillance by inhibiting antitumor immune responses.[15] In lymphoid malignancies, a pathogenic role of STAT3 has been shown in multiple myeloma, Hodgkin lymphoma, anaplastic large T-cell lymphoma, and, recently, in ABC-DLBCL.[7,8,16-19] Three mechanisms have been described to account for persistent STAT3 activation in ABC-DLBCL. First, constitutive NF-κB activation leads to production of interleukin (IL)-6 and IL-10, both of which are STAT3-activating cytokines.[8] In addition, 29% of ABC-DLBCLs express mutated MYD88 (L265P), which triggers cell signaling along the IRAK1/4-NF-κB axis as well as the JAK/STAT3 axis.[20] Finally, high expression of HDAC3 in ABC-DLBCL promotes STAT3 activity by modulating acetylation and subcellular localization.[21] An oncogenic role of STAT3 in ABC-DLBCL has been shown by studies using cell culture systems[8-9,22] and mouse xenograft models.[22] However, the prognostic significance of STAT3 activation has not been thoroughly evaluated in patients with DLBCL. In a recent report involving a small cohort of DLBCL cases, strong nuclear staining for STAT3 correlated with poor survival of patients treated with CHOP.[23] Herein, we report a retrospective analysis of a large cohort of patients with DLBCL treated with R-CHOP. This study was designed to test the specific hypothesis that constitutive STAT3 activation can be used as a biomarker for poor prognosis in R-CHOP–treated DLBCL.

PATIENTS AND METHODS

Patient Information

The sources of patient-derived material and data are summarized in the Data Supplement. The primary patient cohort included 309 patients with de novo DLBCL who received R-CHOP treatment. Among these patients, 87 were treated at the University of Nebraska Medical Center (UNMC), whereas the remaining 222 cases were treated at other Lymphoma/Leukemia Molecular Profiling Project (LLMPP)–affiliated institutions.[13] A subset of the primary cohort (n = 185) comprising the 87 UNMC cases and 98 cases from the LLMPP R-CHOP series were studied for PY-STAT3 expression using immunohistochemistry (IHC). Validation cohort 1 is a LLMPP CHOP series with 181 patients[13] and validation cohort 2 is an R-CHOP cohort consisting of 65 patients with DLBCL treated at the British Columbia Cancer Agency, Vancouver, Canada.[24] Clinical features of the patients were retrieved either from the clinical database of Department of Pathology and Microbiology at UNMC or from the Gene Expression Omnibus (GEO) database. This study was approved by the institutional review board of UNMC.

Tissue Microarray and IHC Analysis

Tissue microarray (TMA) construction, IHC procedure, and scoring criteria for CD10, BCL6, Mum1, GCET1, FoxP1, and BCL2 have been previously published.[12,25,26] The GCB/non-GCB subgroup status was determined using the algorithm of Choi et al for the 87 cases from UNMC.[25] For the LLMPP R-CHOP series and the two validation cohorts, subgroup classification was based on gene expression profiling (GEP) classifier.[9] Double immunostaining for PY-STAT3 and CD20 was performed on a subset of the primary cohort (n = 185, as described above) to evaluate tumor cell-specific PY-STAT3 expression (Data Supplement). The percentage of positive cells and the intensity of PY-STAT3 staining were independently scored. A four-tiered scale (negative, 0; weak, 3; medium, 6; strong, 9) was used to grade the staining intensity in tumor cells compared with reactive T-lymphocytes, which have strong PY-STAT3 expression (score 9). A 10-tiered scale (10% to 100%) was used to score the percentage of PY-STAT3 positive tumor cells. The product of the intensity and percentage of positive cells was used as the case score with a value ≥ 15 considered positive (eg, ≥ 50% positive tumor cells with an intensity of 3, or 25% positive cells with an intensity of 6).

STAT3 Knock-Down and GEP Analysis

STAT3 knock-down in DLBCL cell lines (Data Supplement), GEP data analysis, and development and validation of the 11-gene PY-STAT3 signature are described in the Data Supplement.

Table 1. Clinical Features of Patients With DLBCL According to PY-STAT3 Expression

	PY-STAT3 Expression				
	Negative (n = 116)		Positive (n = 69)		
Clinical Feature	No.	%	No.	%	*P*
Age, years					
Median	63		66		
Range	19.6-87.2		23.6-89.2		
< 60	58	50	27	39	.20
≥ 60	58	50	42	61	
Gender					
Male	67	58	35	49	.44
Female	49	42	34	51	
Karnofsky performance score					
> 70	98	88	58	87	1.0
≤ 70	14	12	9	13	
Ann Arbor stage					
I/II	60	54	27	42	.17
III/IV	52	46	38	58	
Extranodal sites, No.					
< 2	99	88	55	82	.42
≥ 2	14	12	12	18	
Serum lactate dehydrogenase					
Normal	71	64	33	54	.27
Elevated	40	36	28	46	
International Prognostic Index risk group					
Low (0-2)	88	80	41	67	.09
High (3-5)	22	20	20	32	
DLBCL subtypes					
GCB	67	58	29	40	.03
ABC/non-GCB	41	35	37	54	
Nonclassifiable	8	7	3	6	

NOTE. χ^2 test was used to compare the distribution of clinical features between PY-STAT3 negative and positive cases.

Abbreivations: ABC, activated B-cell-like; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B-cell-like.

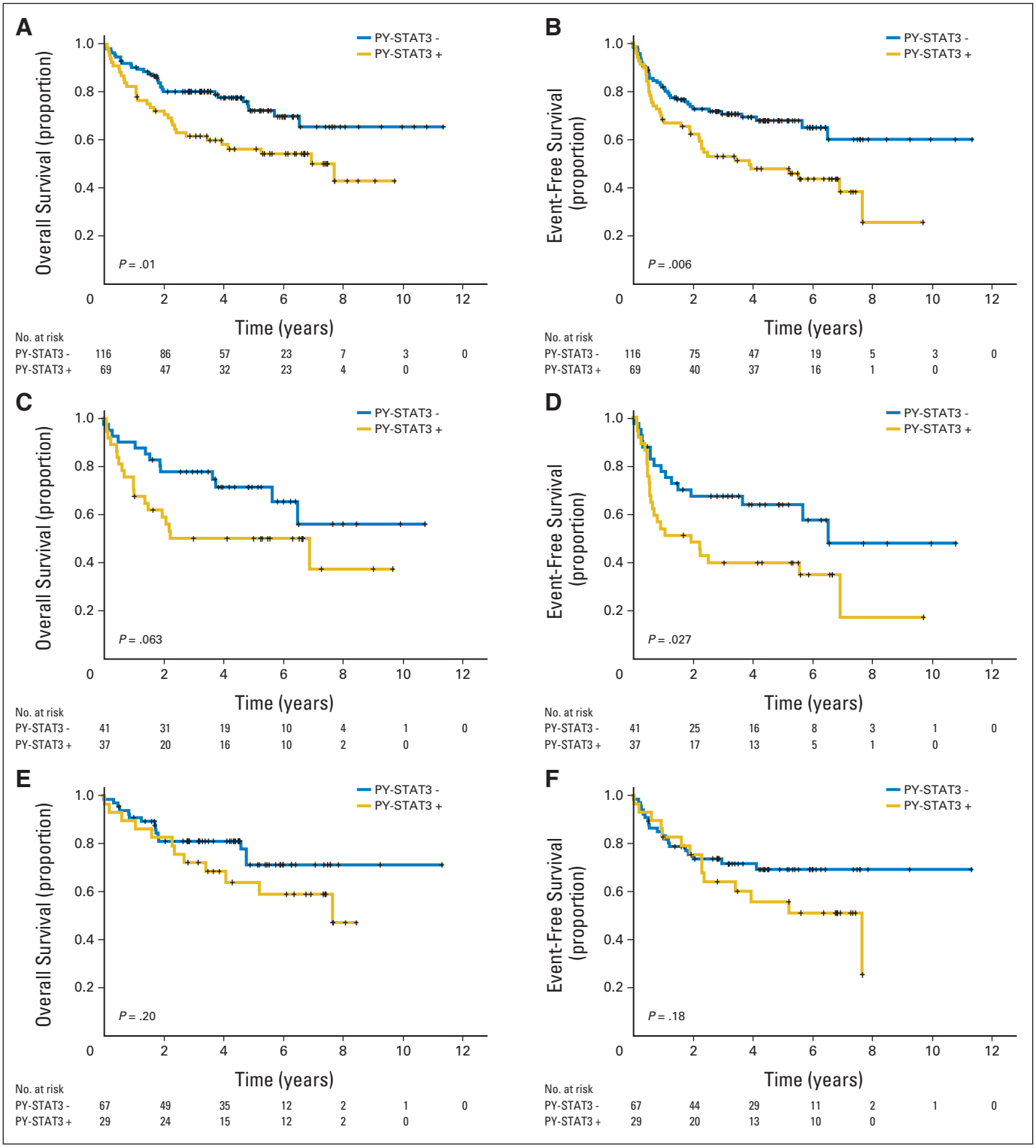

Fig 1. STAT3 activation is associated with poor survival in patients with diffuse large B-cell lymphoma (DLBCL) treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). (A) Overall survival (OS) and (B) event-free survival (EFS) of patients with DLBCL by PY-STAT3 expression. (C) OS and (D) EFS of patients with activated B-cell/non–germinal center B-cell-like (non-GCB) DLBCL by PY-STAT3 expression. (E) OS and (F) EFS of patients with GCB-DLBCL by PY-STAT3 expression.

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Statistical Analysis

Clinical and pathologic characteristics of the patients were compared by χ^2 test. The Kaplan-Meier method was used to estimate the overall survival (OS) and event-free survival (EFS) distributions, with the log-rank test performed to compare the survival curves. Univariable and multivariable Cox proportional hazards regression models were used to evaluate proposed prognostic factors. Concordance between PY-STAT3 IHC scores and the PY-STAT3 signature expression was examined by Spearman's rank correlation test. All reported *P* values are two-sided, and those less than .05 were considered statistically significant. SAS software (SAS Institute, Inc., Cary, NC) was used in all analyses.

RESULTS

PY-STAT3 Positivity Is Preferentially Associated With ABC-DLBCL

We measured tumor cell-specific PY-STAT3 activation in a subset of our primary cohort by IHC (n = 185). According to our scoring criteria, 69 (37%) cases were positive. The baseline clinical features were not significantly different between the PY-STAT3–positive and -negative cases (Table 1). However, the ABC/non-GCB subgroup contained significantly more PY-STAT3–positive cases than did the GCB-subgroup (47% [37 of 78] *v* 30% [29 of 96]; $P = .03$). Consistent with our previous reports on two different cohorts,[7,8] high *STAT3* mRNA expression also occurred preferentially in the ABC subgroup (Data Supplement).

PY-STAT3 Activation Is Associated With Poor Survival in DLBCL

As expected, the IPI and COO classifiers were prognostic for OS and EFS in the entire cohort (Data Supplement). PY-STAT3–positive cases had inferior survival compared to that of negative cases in the entire cohort (n = 185; Figs 1A and 1B) as well as in the ABC/non-GCB subgroup (Figs 1C and 1D), but not among the GCB-DLBCL cases (Figs 1E and 1F). Multivariable analysis showed that PY-STAT3 positivity had prognostic significance for both OS and EFS independent of IPI and BCL2 expression status (Table 2). In univariable analysis, PY-STAT3 status was a significant prognosticator in the entire cohort (for both OS and EFS) as well as in the ABC/non-GCB subgroup (for EFS), but not in the GCB subgroup (Table 2). As we previously reported,[26] the prognostic value of BCL2 expression was restricted to the GCB subgroup. These results further support the observation based on Kaplan-Meier estimates and indicate that STAT3 activation identifies a subset of patients with DLBCL, particularly among the ABC/non-GCB subgroup, who are at high risk when treated with R-CHOP.

We also examined the utility of combining PY-STAT3 and BCL2 expression.[26] Interestingly, BCL2 and PY-STAT3 double negative patients showed an exceedingly favorable prognosis (OS, $P < .001$; EFS, $P < .001$, estimated 5-year and 10-year OS of 91%) compared with those positive for either one or both markers (Data Supplement). We

Table 2. Multivariable and Univariable Analyses by Cox Proportional Hazard Model

Survival	Covariates	HR	95% CI	*P*
Multivariable analysis for IPI, PY-STAT3, and BCL2 as covariates in the entire cohort (n = 309)				
OS	IPI (high: 3-5 *v* low: 0-2)	2.3	1.1 to 4.7	.02
	PY-STAT3 (pos. *v* neg.)	2.3	1.1 to 4.6	.02
	BCL2 (pos. *v* neg.)	2.1	1.01 to 4.5	.046
EFS	IPI (high: 3-5 *v* low: 0-2)	1.9	0.97 to 3.7	.06
	PY-STAT3 (pos. *v* neg.)	2.2	1.1 to 4.1	.02
	BCL2 (pos. *v* neg.)	1.8	0.94 to 3.5	.08
Univariable analysis for each covariate in the entire cohort (n = 309)				
OS	Subtype (ABC *v* GCB)	2.1	1.4 to 3.2	< .01
	IPI (high: 3-5 *v* low: 0-2)	2.7	1.8 to 4.2	< .01
	PY-STAT3 (pos. *v* neg.)	1.9	1.2 to 3.2	.01
	BCL2 (pos. *v* neg.)	2.1	1.3 to 3.6	< .01
EFS	Subtype (ABC *v* GCB)	2.3	1.6 to 3.3	< .01
	IPI (high: 3-5 *v* low: 0-2)	2.5	1.7 to 3.6	< .01
	PY-STAT3 (pos. *v* neg.)	1.9	1.2 to 2.9	< .01
	BCL2 (pos. *v* neg.)	2.1	1.3 to 3.4	< .01
Univariable analysis for each covariate in the GCB subgroup (n = 153)				
OS	IPI (high: 3-5 *v* low: 0-2)	3.4	1.7 to 6.8	< .01
	PY-STAT3 (pos. *v* neg.)	1.6	0.8 to 3.5	.2
	BCL2 (pos. *v* neg.)	2.9	1.01 to 8.4	.047
EFS	IPI (high: 3-5 *v* low: 0-2)	3.2	1.7 to 5.9	< .01
	PY-STAT3 (pos. *v* neg.)	1.6	0.8 to 3.2	.18
	BCL2 (pos. *v* neg.)	4.8	1.8 to 12.8	< .01
Univariable analysis for each covariate in the ABC/non-GCB subgroup (n = 125)				
OS	IPI (high: 3-5 *v* low: 0-2)	1.9	1.1 to 3.2	.02
	PY-STAT3 (pos. *v* neg.)	1.9	0.95 to 3.9	.07
	BCL2 (pos. *v* neg.)	1.5	0.75 to 2.9	.25
EFS	IPI (high: 3-5 *v* low: 0-2)	1.7	1.0 to 2.7	.04
	PY-STAT3 (pos. *v* neg.)	2	1.07 to 3.8	.03
	BCL2 (pos. *v* neg.)	1.1	0.63 to 2.1	.66

Abbreviations: ABC, activated B-cell-like; EFS, event-free survival; GCB, germinal center B-cell-like; HR, hazard ratio; IPI, International Prognostic Index; OS, overall survival.

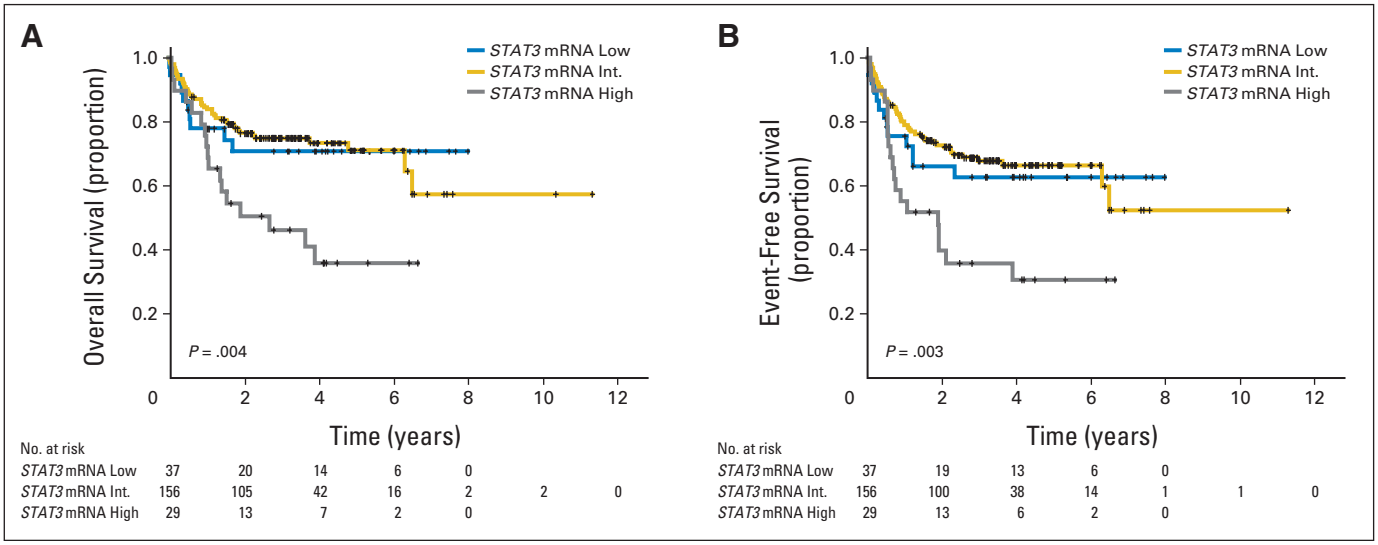

Fig 2. High *STAT3* mRNA expression is associated with poor survival in patients with diffuse large B-cell lymphoma (DLBCL) treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). (A) Overall survival and (B) event-free survival of patients with DLBCL by the expression levels of *STAT3* mRNA. Int, intermediate.

obtained similar findings when combining *BCL2* and *STAT3* mRNA expression. Furthermore, the combined PY-STAT3 and BCL2 status was associated with survival outcome only in the GCB- but not the ABC-DLBCL subgroups (Data Supplement). This subgroup specificity likely reflects a dominant prognostic impact by BCL2 in the GCB-DLBCL subgroup (Table 2).[26]

High STAT3 *mRNA Is Associated With Poor Survival in DLBCL*

We also examined the prognostic value of *STAT3* mRNA expression in the LLMPP R-CHOP series (n = 222). Using the average intensity of the three *STAT3* probesets, we divided the cases into three subgroups: low (< mean – one standard deviation [SD], n = 37), high (> mean + one SD, n = 29), and intermediate (the remaining cases, n = 156). Clinical characteristics of the patients in these three groups were not significantly different; however, the subgroup with the highest levels of *STAT3* mRNA was enriched in ABC-DLBCL subtype and cases stained positive for PY-STAT3 (Data Supplement), likely reflecting the fact that STAT3 is a positively auto-regulated gene.[27] Similar to our observations on PY-STAT3, cases with high *STAT3* mRNA had significantly worse prognosis (OS, $P = .004$; EFS, $P = .003$; Fig 2). However, *STAT3* mRNA was not a significant prognosticator when the cohort was divided into COO subgroups.

Development of a GEP-based PY-STAT3 Signature for DLBCL Prognostication

To further evaluate the prognostic significance of PY-STAT3, we constructed a GEP-based PY-STAT3 signature using differential gene expression on STAT3 knockdown in ABC-DLBCL cell lines, presence of at least one STAT3 binding site in the promoter region of putative target genes, and differential expression between PY-STAT3–positive and -negative DLBCL tumors (*t*-test $P < .05$; fold change > 2). This analysis leads to a set of 265 candidate genes (347 probe sets) that correlated with STAT3 activation status in DLBCL. Known STAT3 target genes such as *CD48*, *CD96*, *IRF1*, *IL10*, *BCL3*, and *IL2RB* were highly expressed in the PY-STAT3–positive tumors,[28-30] whereas the PY-STAT3–negative tumors expressed high levels of *RAC1*, *MAPK1* and *AKT2* (Fig 3).

Next, to enrich for genes with greatest prognostic values, we used a semi-supervised prediction algorithm.[31,32] The OS response from the LLMPP R-CHOP cohort (n = 222) was used at this step. Using a significance level ≤ .05, we obtained a list of 36 genes and subsequently filtered them for concordance expression in patient and cell line GEP data (Data Supplement). This five-step procedure finally produced an 11-gene PY-STAT3 signature (Fig 3). In the subsequent prognostic analyses, the averaged expression level of the 11 member genes is used as the signature score to correlate with DLBCL survival risk.

Three types of tests were performed to confirm that this GEP signature can reliably report PY-STAT3 activity. First, we validated four of the 11 genes by quantitative reverse transcriptase polymerase chain reaction in two ABC-DLBCL cell lines treated with STAT3 siRNA (Data Supplement). Second, applying Spearman's correlation test to the 98 cases for which both the IHC score and GEP data were available (Data Supplement), we established that the 11-gene signature strongly correlated with the IHC-based PY-STAT3 status in the entire cohort (coefficient $r = 0.47$; $P < .001$) as well as in the ABC ($r = 0.55$; $P < .001$) and GCB ($r = 0.47$; $P = .001$) subgroups. Third, after dividing three DLBCL cohorts (including primary cohort and two independent validation cohorts) into quartile subgroups using the signature expression, we demonstrate that ABC-DLBCL cases are significantly enriched in the intermediate/high and high quartile groups in all three cohorts (Data Supplement).

Eleven-Gene PY-STAT3 Signature Is Associated With Poor Survival in R-CHOP–Treated DLBCL

First, we compared the survival response of the quartile subgroups among the LLMPP R-CHOP cohort. As shown in Figures 4A and 4B, we detected significant survival differences among these quartile subgroups with 5-year OS rates at 84%, 81%, 57%, and 48%, and 5-year EFS rates at 81%, 77%, 51%, and 40% for quartiles 1, 2, 3, and

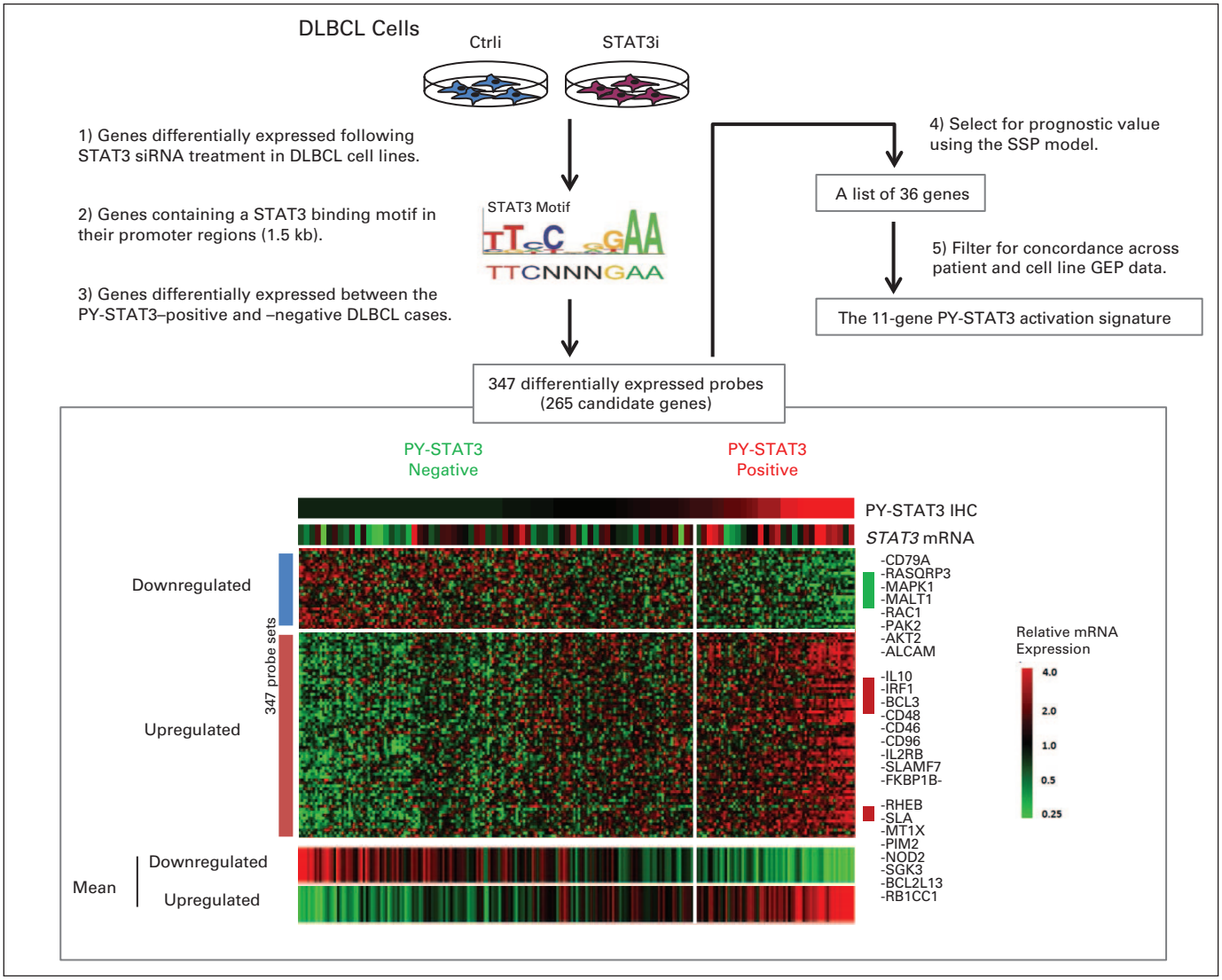

Fig 3. Development of a PY-STAT3–based gene signature to predict the survival of patients with diffuse large B-cell lymphoma (DLBCL) treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). Detailed description of the procedure is provided in the Data Supplement. The heat map shows the differential expression pattern of *STAT3* candidate genes between PY-STAT3–positive and –negative cases. PY-STAT3 immunohistochemistry (IHC) scores and *STAT3* mRNA levels are illustrated in the top of the heat map. Green and red colors in the heat map indicate relatively low and high gene expression, respectively, as indicated. GEP, gene expression profiling; SSP, semi-supervised prediction.

4, respectively. Among the ABC-DLBCL cases, those in the first quartile (lowest expression) had a more favorable outcome than did those in the other three quartiles (Figs 4C and 4D). In comparison, such quartile-associated survival differences were not detected among the GCB-DLBCL cases (not shown).

Since the LLMPP R-CHOP cohort was also used to develop the PY-STAT3 signature, it is important to confirm the validity of this 11-gene model using independent cohorts. In the absence of another large-scale R-CHOP cohort that features complete and publically accessible GEP and treatment response data, we turned to the LLMPP CHOP cohort[13] and a small R-CHOP series[24] for validation analyses (Data Supplement). The clinical features of these two cohorts are comparable with our primary cohort with the exception of serum lactate dehydrogenase, which was elevated more often in the CHOP series than in our primary R-CHOP cohort (Data Supplement). We used two validation approaches. First, survival analysis showed that high expression of the 11-gene signature was associated with poor OS in the LLMPP CHOP cohort (Data Supplement; n = 181). A similar trend was observed in the small R-CHOP series; however, the prognostic difference did not reach statistical significance, likely because of the small cohort size (Data Supplement; n = 65). Second, we conducted a calibration analysis to directly compare model-predicted and observed survival probability.[33] As shown in the Data Supplement, this analysis revealed good agreement at multiple time points between prediction by the 11-gene signature and observed OS risk in the intermediate and low-risk groups in the small R-CHOP cohort and in the high-risk group in the LLMPP CHOP cohort. Given the known survival differences between CHOP and R-CHOP regimens, results from this validation analysis suggest to us that the prognostic value of the 11-gene PY-STAT3 signature is likely applicable to the general setting of DLBCL treated with CHOP-containing therapies.

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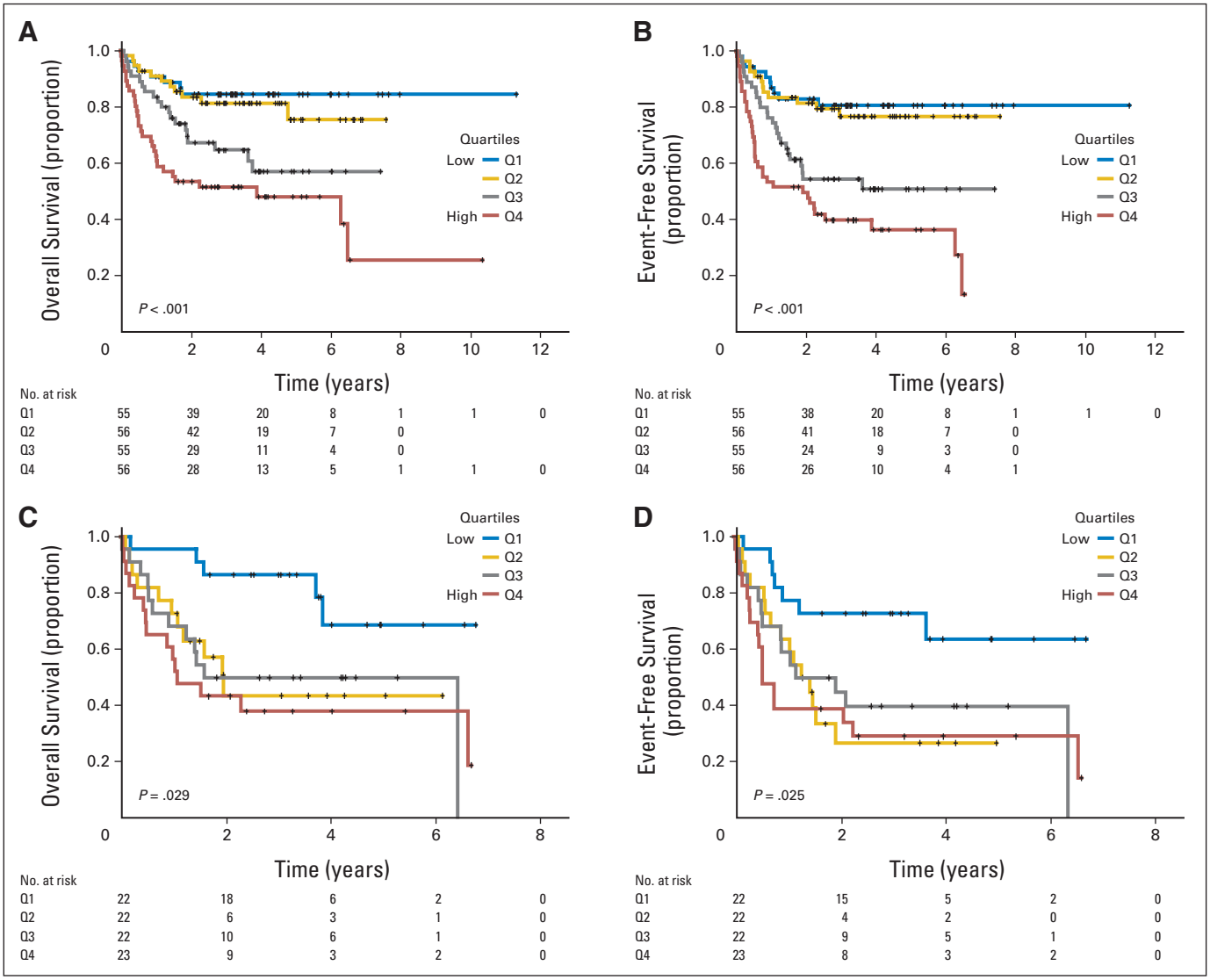

Fig 4. The 11-gene PY-STAT3 signature was predictive of survival of patients with diffuse large B-cell lymphoma (DLBCL) in the entire cohort (A, B) and in the activated B-cell DLBCL subgroup (C, D). Overall and event-free survival are shown using the expression quartiles (Q) of the 11-gene signature.

DISCUSSION

In this retrospective analysis, we demonstrated that PY-STAT3 activation is prognostic for poor survival in patients with DLBCL treated with R-CHOP, the current standard of care for DLBCL. The prognostic significance of PY-STAT3 is independent of International Prognostic Index and BCL2 expression, and particularly strong among the ABC-DLBCL subgroup. Our results also demonstrated the prognostic value of combining PY-STAT3/BCL2 expression for all patients with DLBCL and GCB-DLBCL. Furthermore, we report an 11-gene signature that can be used to track STAT3 activation status and correlate with poor outcome of patients with DLBCL treated with either CHOP or R-CHOP. To the best of our knowledge, this represents the largest and most comprehensive study to date demonstrating the prognostic significance of STAT3 activation in patients with DLBCL.

STAT3 activation determined by IHC is reportedly associated with poor prognosis in many cancers[34-36]; however, a recent study failed to identify prognostic value for PY-STAT3 in DLBCL.[37] Compared with that report, many more R-CHOP cases were examined in this study (185 *v* 38). More importantly, we used CD20/PY-STAT3 double staining to determine tumor-specific STAT3 activation, thus avoiding interference from stromal cells and reactive T-lymphocytes.[13] Admittedly, although we have validated the 11-gene PY-STAT3 signature using two independent cohorts, it is important to further test and extend its prognostic utility in prospective cohorts. Tracking activated STAT3 by a GEP-based signature is relatively new and has not been appreciated in clinical oncology. Yet, a GEP-based prognosticator, once fully validated, can be further developed using emerging technologies such as diagnostic gene chips at the point of care.

Before this report, two GEP-based DLBCL prognostic models have been reported by the LLMPP consortium, namely the bivariate

GCB/ABC model[4,5] and the trivariate model derived from the GCB/ABC, stromal-1, and stromal-2 GEP signatures.[13] Interestingly, although our 11-gene signature is a much simpler univariate predictor, its predictive power is quite comparable to the trivariate model. One possible explanation comes from the fact that STAT3 activation within the tumor cells is a comprehensive readout of both the intrinsic genetic alterations and the tumor-microenvironment cross-talk. It is worth noting that GEP-based PY-STAT3 positivity reported here is much more common than any single reported driver mutation in DLBCL, implying that STAT3 activation is a shared downstream oncogenic event that can be triggered by different genetic alterations. In addition, the genes that define the ABC-DLBCL signature were expressed at equivalent levels in subgroups of different PY-STAT3 status, suggesting that STAT3 activation is regulated independently from the defining features of the ABC-DLBCL subgroup.

The PY-STAT3 signature reported here may provide useful insights into the role of STAT3 in oncogenesis and possibly also therapeutic response in DLBCL. Six of the 11 genes in the signature have been functionally studied and five of them have been implicated in cancer. HSD17B4 is a dehydrogenase involved in peroxisomal fatty acid beta-oxidation, and its overexpression was prognostic in prostate cancer.[38] SLC2A13 encodes a H^+-myoinositol transporter that serves as a marker for cancer stem cells in oral squamous cell carcinoma.[39] Aberrant expression of MT1X has been observed in several kinds of carcinomas and was correlated with poor therapeutic response.[40,41] RHEB is a key regulator in the PI3K/Akt/mTOR pathway, which directly activates mTORC1 activity.[42] Cell type-specific oncogenic activity has been shown for RHEB, especially in the context of PTEN haploinsufficiency.[43] This is particularly interesting in light of our previous report that PTEN loss occurs in 11% of GCB-DLBCL.[44] Finally, ZNF420 encodes the KRAB-type zinc finger protein Apak, which has been implicated in DNA damage response.[45]

Our finding in this report also has therapeutic implications regarding the potential utility of targeting STAT3 directly. We and others have previously reported that blocking JAK/STAT3 activation in ABC-DLBCL cell lines can significantly reduce cell proliferation and survival.[8,22] Our results, when combined with these previous observations, further strengthen the rationale of targeting STAT3 directly for treatment of DLBCL, especially the ABC-DLBCL subtype.

In summary, we have demonstrated in this study that STAT3 activation status either using on an IHC method or an 11-gene GEP signature is correlated with poor prognosis of patients with DLBCL treated with the R-CHOP regimen. Our findings support the hypothesis that targeting the STAT3 signaling pathway, either with monotherapy or in combination with R-CHOP, may improve the survival of patients with DLBCL carrying PY-STAT3 positive tumors. Finally, none of the 11 genes in the PY-STAT3 signature have been previously shown to be a transcriptional target of STAT3 in DLBCL, and their potential contributions to lymphoma pathobiology await future investigation.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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