Expression of Myc, but not pSTAT3, is an adverse prognostic factor for diffuse large B-cell lymphoma treated with epratuzumab/R-CHOP

Mamta Gupta,1 Matthew J. Maurer,2 Linda E. Wellik,1 Mark E. Law,3 Jing Jing Han,1 Nazan Oksan,4 Ivana N. Micallef,1 Ahmet Dogan,3 and Thomas E. Witzig1

1Division of Hematology, Department of Internal Medicine, 2Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, and 3Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN; and 4Department of Pathology, Ege University, Izmir, Turkey

STAT3 regulates cell growth by up-regulating downstream targets, such as Myc. The frequency of phosphorylated STAT3 (pSTAT3) and Myc expression and their prognostic relevance is unknown within diffuse large B-cell lymphoma (DLBCL) germinal center B-cell (GCB) and non-GCB subtypes. pSTAT3 and Myc were studied by immunohistochemistry (IHC) on tumors from 40 DLBCL patients uniformly treated on a clinical trial of epratuzumab/rituximab-CHOP. A total of 35% of cases were pSTAT3-positive, and pSTAT3 positivity was more frequent in the non-GCB (P = .06) type but did not correlate with event-free survival (EFS). Myc expression was observed in 50% of cases and was more frequent in non-GCB type (P = .07). Myc-positive cases had inferior EFS in all patients, including the GCB and pSTAT3-positive cases, were more likely to express Myc (P = .06). Myc translocations involving the major breakpoint regions were found in 10% (3 of 29) of cases, and all 3 cases were GCB and had an inferior EFS (P = .09). pSTAT3, but not Myc expression, was correlated with elevated pretreatment serum cytokines, such as IL-10 (P = .05), G-CSF (P = .03), and TNF-α (P = .04). pSTAT3 IHC in DLBCL tumors has the potential to identify patients for STAT3 pathway-directed therapy; Myc IHC is a potential marker for inferior EFS in GCB patients. (Blood. 2012; 120(22):4400-4406)

Introduction

The most significant advance in the treatment of diffuse large B-cell lymphoma (DLBCL) over the past 15 years has been the addition of rituximab to standard CHOP chemotherapy (R-CHOP).1-3 We recently demonstrated in a phase 2 study (N0489; www.clinicaltrials.gov, #NCT00301821) that the anti-CD22 monoclonal antibody epratuzumab can be successfully used.4 Tissue microarrays were constructed using triplicate 0.6-mm cores from paraffin-embedded tissue blocks and included 10 nonmalignant tonsil controls. In some cases, only unstained slides were available. This resulted in not all studies being performed on all 40 cases. For Western blotting studies, frozen DLBCL tumor cells (n = 7) and normal tonsils (n = 10) were obtained through the University of Iowa/Mayo Lymphoma SPORE Biospecimens Core. Normal B cells were isolated using CD19 microbeads (Miltenyi Biotec, San Diego, CA). Normal B cells were isolated using a magnetic cell sorting protocol (Miltenyi Biotec, San Diego, CA).

Methods

Patients and biopsies

Paraffin-embedded tumors from 40 patients participating in N0489 were used.4 Tissue microarrays were constructed using triplicate 0.6-mm cores from paraffin-embedded tissue blocks and included 10 nonmalignant tonsil controls. In some cases, only unstained slides were available. This resulted in not all studies being performed on all 40 cases. For Western blotting studies, frozen DLBCL tumor cells (n = 7) and normal tonsils (n = 10) were obtained through the University of Iowa/Mayo Lymphoma SPORE Biospecimens Core. Normal B cells were isolated using CD19 microbeads (Miltenyi Biotec).
BLOODCL molecular subtyping

DLBCL cases (n = 38) were classified into GCB or non-GCB molecular type based on the Hans algorithm applied to paraffin-embedded tumor samples.20

IHC for pSTAT3/tSTAT3 and Myc expression

The tissue microarray from DLBCL tumors was stained with antibodies to Myc, tSTAT3, or pSTAT3. Paraffin-embedded sections from the human DLBCL cell lines DHL2 and DHL6 were used to validate the pSTAT3 stain. Furthermore, paraffin-embedded sections from DHL2, Ly3, Ly10, Ly1, Ly19, and DHL6 were used to validate Myc stain. IgG antibody was used as a negative control for pSTAT3 and Myc IHC staining. Briefly, slides were dried, deparaffinized, and endogenous peroxidase activity was quenched by incubation of sections in a 50% solution of 3% hydrogen peroxide and absolute methanol. Antigen retrieval was performed in a steamer for 30 minutes using 1 mM EDTA and pH 8.0 for Myc and citrate at pH 6.1 for pSTAT3 (Cell Signaling Technologies; 9145). The slides were placed on a DakoCytomation autostainer with the following autostainer protocol: antibodies to pSTAT3 or tSTAT3 (Cell Signaling Technologies) or Myc (Epitomics; 1472-1); Dako advance detection system, and substrate. The slides were removed from the autostainer, rinsed, and counterstained with hematoxylin. The slides were reviewed and scored by the study hematopathologist (A.D.) without knowledge of patient outcome. The expression of Myc and pSTAT3 was assessed semiquantitatively as follows: negative < 10%, 10%-30% (+, low), 30%-80% (+, intermediate), and > 80% (+++, high). A 30% cut off was chosen for pSTAT3 and Myc positivity based on this cut-point being commonly used in clinical practice and in studies such as Hans et al.21

Western blotting for pSTAT3 and tSTAT3

A total of 5 × 10⁶ cells from tumor cells were washed, lysed in RIPA buffer, and blotted with tyrosine-phosphorylated and unphosphorylated STAT3 antibody (Cell Signaling Technologies; 9138 and 9139). Lysates from and blotted with tyrosine-phosphorylated and unphosphorylated STAT3 and tSTAT3. Levels of pSTAT3/tSTAT3 and Myc were measured by immunoblotting as previously described.21 For cytokine experiments, DHL2 cell line was serum-starved overnight with 0.5% BSA and then treated with 50 ng/mL of IL-10 or G-CSF or TNF-α (PeproTech). Cells were then collected and lysates were subjected to Western blot analysis for pSTAT3.

FISH for MYC translocations

MYC translocations were detected by interphase FISH on paraffin-embedded DLBCL tissues (n = 29) using dual-color break-apart probe for Myc as described previously.22,25

Serum cytokine analysis

Multiplex ELISA (30-plex) was performed as previously described24 on available pretreatment patient serum (n = 32) and correlated with Myc and pSTAT3 results from paired tumor biopsies.

Statistical analysis

Event-free survival (EFS) was defined as the time from diagnosis until progression, re-treatment, or death of any cause. Patients without an event were censored at last known follow-up. Cox proportional hazard models and Kaplan-Meier curves were used to assess the association of EFS with pSTAT3 and Myc. Associations between cytokines and pSTAT3 status were assessed individually using nonparametric Wilcoxon rank-sum tests.

Results

 Constitutive STAT3 activity in untreated DLBCL tumors

DLBCL tumor samples were stained for detection of nuclear pSTAT3 expression (Figure 1A). Using a threshold of ≥ 30% of tumor nuclei staining positive, 35% (14 of 40) of patient tumors were pSTAT3⁺ (Figure 1A-B). An additional 17% (7 of 40) had between 10%-30% pSTAT3⁺ cells. The pSTAT3 staining was verified in paraffin-embedded sections from DLBCL cell lines previously demonstrated to be pSTAT3 positive or negative27 (supplemental Figure 1A, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). We also assessed the total STAT3 (tSTAT3) expression and found that 80% (32 of 40) of the patients were tSTAT3⁺ (Figure 1A). Nonmalignant tonsil tissues (n = 10) were positive for tSTAT3 in all cases; all but 1 case were pSTAT3 negative (data not shown).

We also analyzed the STAT3 phosphorylation by Western blot in DLBCL samples (n = 7). Three patient samples were strongly pSTAT3 positive, 2 were weakly positive, and the other 2 were negative. All 7 tumors were positive for tSTAT3 (Figure 1C). Normal CD19 B cell controls were positive for tSTAT3 but negative for pSTAT3. DHL2 was used as a positive control for pSTAT3 expression (Figure 1D). Overall, these data demonstrate that pSTAT3 is frequently expressed in a subset of DLBCL tumors.

Expression of Myc protein by IHC in untreated DLBCL

We have shown that JAK2 inhibition through the JAK2 specific inhibitor TG101348 inhibits Myc expression without affecting expression of other STAT3 downstream genes.24 Because Myc is downstream of STAT3 in the signal pathway, we next examined Myc protein expression by IHC. Twenty-four of the same DLBCL tumors used for pSTAT3 expression were stained and 50% (12 of
Table 1. Correlation of pSTAT3 and Myc expression by IHC with clinicopathologic characteristics of DLBCL patients treated on the N0489 ER-CHOP clinical trial

<table>
<thead>
<tr>
<th>Parameter</th>
<th>pSTAT3 IHC</th>
<th>c-Myc IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLBCL (n = 24), n (%)</td>
<td>STAT3− (n = 12), n (%)</td>
</tr>
<tr>
<td>Age ≥ 60 y</td>
<td>17 (43)</td>
<td>12 (46)</td>
</tr>
<tr>
<td>B symptoms present</td>
<td>20 (50)</td>
<td>14 (54)</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>30 (75)</td>
<td>23 (88)</td>
</tr>
<tr>
<td>Performance status ≥ 2</td>
<td>5 (13)</td>
<td>3 (12)</td>
</tr>
<tr>
<td>EN sites ≥ 2</td>
<td>7 (18)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>31 (78)</td>
<td>20 (77)</td>
</tr>
<tr>
<td>Bulky disease ≥ 10 cm</td>
<td>6 (15)</td>
<td>5 (19)</td>
</tr>
<tr>
<td>IPI ≥ 2</td>
<td>24 (60)</td>
<td>15 (58)</td>
</tr>
<tr>
<td>IPI 3-5</td>
<td>16 (40)</td>
<td>11 (42)</td>
</tr>
</tbody>
</table>

Myc translocations in untreated DLBCL patients

Using a break-apart probe for the Myc gene, MYC translocations in the major breakpoint regions were found in 10% (3 of 29) of cases (Figure 2D). When Myc FISH was correlated with Myc IHC in the 24 DLBCL cases that had both techniques performed, all 3 Myc translocation cases were GCB by IHC, 2 were strongly positive for Myc, and 1 was negative (Figure 2E). Among the 21 MYC FISH negative cases, 10 were Myc positive by IHC (Figure 2E). These data suggest that Myc expression in lymphoma is not only controlled by genetic events, such as translocations, but also by other signaling pathways, such as STAT3. Overall, these data confirm the Yoon et al findings that MYC translocation predicts poor prognosis, especially in GCB DLBCL.28

Prognostic impact of Myc, pSTAT3 and Myc translocations in DLBCL treated with ER-CHOP

Although the dataset was small, the fact that this was a uniformly treated group of patients treated on a clinical trial using R-CHOP–based treatment, we explored whether there was a correlation between tumor pSTAT3 or Myc expression and patient characteristics and outcome. pSTAT3 expression was correlated with an elevated serum LDH (P = 0.007; Table 1). Neither Myc nor pSTAT3 tumor expression correlated with other clinical or pathologic features (Table 1). Survival analysis revealed a trend toward shorter EFS for DLBCL patients whose tumors expressed Myc protein by IHC (P = .2) or had a Myc translocation (P = .09) by FISH (Figure 3A-B); pSTAT3 expression status did not predict EFS (P = .9; Figure 3C).

Expression of Myc in GCB and non-GCB DLBCL

By use of the Hans method, we classified DLBCL tumors (n = 23) into GCB (n = 17) and non-GCB (n = 6). Within the GCB group, 10 cases were Myc negative (10 of 17; 59%) and 7 cases were Myc positive (7 of 17; 41%). Among the non-GCB group, 83% (5/6) were Myc positive (Figure 4A). These data clearly suggest a trend of higher Myc positivity in the non-GCB DLBCL group (P = .07). Myc-positive cases had a clear trend of inferior EFS in both GCB (P = .2) and in non-GCB (P = .5) groups (Figure 4B-C).

Distribution of pSTAT3 in GCB and non-GCB DLBCL

The distribution of pSTAT3 (n = 38) expression was also evaluated in the GCB (n = 26) and non-GCB (n = 12) DLBCL. In the GCB group, 27% (7 of 26) were pSTAT3 positive compared with 58% (7 of 12) in the non-GCB group (Figure 5A). Thus, there was a...
A clear trend toward higher pSTAT3 positivity in non-GCB DLBCL tumors \( (P < 0.06) \); however, pSTAT3 status did not correlate with EFS in either GCB or non-GCB DLBCL patients (Figure 5B-C).

Correlation of pSTAT3 and Myc expression with serum cytokines in ER-CHOP–treated DLBCL

Previous data from our laboratory demonstrated that IL-10 (but not G-CSF and TNF-\( \alpha \)) was able to activate STAT3 tyrosine phosphorylation in the lymphoma cell line (Figure 6B).

**Discussion**

Constitutively activated STAT3 regulates tumor growth and invasion by affecting the expression of genes related to tumor cell survival.\(^{12,29}\) Studies in renal, squamous, and gastric cancers have found that the expression of pSTAT3 in these tumors was associated with an inferior prognosis.\(^{30,31}\) In lymphoma, it has been reported that ABC DLBCL lines are positive for pSTAT3, whereas GCB DLCBL lines are pSTAT3 negative.\(^{14,15}\) To date, there have been no studies relating pSTAT3 to survival parameters in DLBCL. We investigated pSTAT3 expression by IHC in primary tumor samples from 40 patients with DLBCL all treated on the same ER-CHOP clinical trial and found that 35\% of tumors were pSTAT3 positive. Non-GCB tumors by IHC were more likely to be positive for pSTAT3. ABC-DLBCL lymphomas treated with R-CHOP typically have an inferior EFS and OS compared with GCB-type tumors.\(^{6,32,33}\) In this study, pSTAT3, although more frequent in non-GCB tumors, did not correlate with EFS possibly because of the small numbers of patients in this study or the fact that ER-CHOP has potentially improved the EFS of the non-GCB type DLBCL.

A number of studies have evaluated translocations of MYC with immunoglobulin genes in Burkitt lymphoma and DLBCL.\(^{16}\) Although the incidence of MYC translocation in DLBCL is only 5\%-10\%, these cases have an unfavorable prognosis.\(^{17,34}\) To date, there have been no DLBCL studies evaluating the prognostic importance of the expression of Myc protein by IHC because of the lack of a good antibody for Myc IHC. We showed that Myc protein expression by IHC is observed in 50\% of DLBCL cases by IHC methodology; that Myc expression was frequent in the non-GCB group; and that Myc positive cases had an inferior prognosis, even if they were GCB tumors. Genetic translocations involving MYC defines a small group of DLBCL patients with a poor prognosis.\(^{18}\) MYC translocation by FISH was observed in 10.4\% of cases in this study, and all of them were in the GCB group and all were Myc-positive by IHC. A total of 45\% of the pSTAT3\(^{+}\) patients were also Myc-positive by IHC, suggesting the involvement of STAT3 signaling in these cases of Myc expression. Our study suggests that Myc by IHC is also a poor prognostic factor, even in patients treated with R-CHOP–based regimens, such as ER-CHOP. Potentially more important, our results suggest that Myc expression is an adverse prognostic factor within the GCB-group, a group that traditionally is thought of as having a good prognosis. The mechanisms of high Myc expression in DLBCL tumors without MYC translocation are not entirely clear at this time. One potential mechanism is that STAT3 signaling may be involved in the Myc expression. Indeed, about half of the Myc-positive tumors were pSTAT3\(^{+}\) by IHC using our criteria. A second potential mechanism is regulation of Myc protein stability. This has been shown to be important in breast cancer cells where Myc protein stability is regulated by phosphorylation at threonine 58 (T58) and serine 62 (S62), which coordinates with the scaffold protein Axin 1.\(^{35}\) Whether this mechanism exists in the lymphoma cells will require further studies.

Cytokines are low-molecular weight proteins that regulate several signaling pathways. The STAT3 pathway is activated with a variety of cytokines, which may or may not be secreted by tumors.
We have seen significant elevation of certain serum cytokines in the DLBCL patients compared with normal controls. Among several elevated cytokines, only IL-10, G-CSF, and TNF-α were significantly correlated with pSTAT3 expression. These cytokines have the potential to be important serum biomarkers for DLBCL. The strengths of this study are the use of a well-characterized and uniformly treated patient population, the patients were all untreated, the regimen was R-CHOP-based, and all tissues were reviewed by the same hematopathologist. Overall, our data provide evidence that overexpression of pSTAT3 and Myc is common in DLBCL. These biomarkers have potential as prognostic factors in the case of Myc and as a tool for selecting therapy for pSTAT3. Myc may be especially useful to further identify an adverse group of DLBCL patients within the otherwise favorable GCB tumor group. The availability of JAK/STAT and STAT-specific inhibitors provides the rationale to incorporate pSTAT3 staining in tumors from patients who are participating in these trials to learn if this biomarker can predict response. The primary limitation of our

![Figure 4. Myc expression and prognosis within DLBCL molecular subtypes.](image)

- **A** Myc expression by IHC in GCB and non-GCB subtypes.
- **B-C** EFS by Myc positivity in ER-CHOP–treated GCB and non-GCB DLBCL patients.

We have seen significant elevation of certain serum cytokines in the DLBCL patients compared with normal controls. Among several elevated cytokines, only IL-10, G-CSF, and TNF-α were significantly correlated with pSTAT3 expression. These cytokines have the potential to be important serum biomarkers for DLBCL. The strengths of this study are the use of a well-characterized and uniformly treated patient population, the patients were all untreated, the regimen was R-CHOP-based, and all tissues were reviewed by the same hematopathologist. Overall, our data provide evidence that overexpression of pSTAT3 and Myc is common in DLBCL. These biomarkers have potential as prognostic factors in the case of Myc and as a tool for selecting therapy for pSTAT3. Myc may be especially useful to further identify an adverse group of DLBCL patients within the otherwise favorable GCB tumor group. The availability of JAK/STAT and STAT-specific inhibitors provides the rationale to incorporate pSTAT3 staining in tumors from patients who are participating in these trials to learn if this biomarker can predict response. The primary limitation of our

![Figure 5. pSTAT3 expression and prognosis within DLBCL molecular subtypes.](image)

- **A** Correlation of pSTAT3 with GCB and non-GCB DLBCL (n = 38) subtypes.
- **B-C** EFS analysis by pSTAT3 positivity in ER-CHOP–treated GCB and non-GCB DLBCL patients.
study was that the number of cases studied was small, limiting the statistical correlations with Myc FISH and clinical outcome. The study does provide rationale to study Myc and pSTAT3 by FISH and IHC in large prospective trials of DLBCL to confirm and extend our initial findings.

Acknowledgments

This work was supported in part by the University of Iowa/Mayo Clinic Lymphoma Specialized Program of Research Excellence (Career Development Award P50 CA097274; M.G.), Goodwin Foundation Pilot award (M.G.), the North Central Cancer Treatment Group (CA25224; and Biospecimen Resource Grant CA114740), and the Predolin Foundation Award. This work is supported in part by R01-CA127433 to T.E.W.

Authorship

Contribution: M.G. designed the research, analyzed and interpreted data, made the figures, and wrote the paper; M.J.M. performed all the statistical analysis; M.E.L. performed the FISH assay; L.E.W. performed pSTAT3 and Myc IHC in large prospective trials of DLBCL to confirm and extend our initial findings. Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Mamta Gupta, PhD, Division of Hematology, Mayo Clinic, College of Medicine, 200 First St SW, Rochester, MN 55905; e-mail: gupta.mamta@mayo.edu.

References


Expression of Myc, but not pSTAT3, is an adverse prognostic factor for diffuse large B-cell lymphoma treated with epratuzumab/R-CHOP

Mamta Gupta, Matthew J. Maurer, Linda E. Wellik, Mark E. Law, Jing Jing Han, Nazan Ozsan, Ivana N. Micallef, Ahmet Dogan and Thomas E. Witzig

Updated information and services can be found at:
http://www.bloodjournal.org/content/120/22/4400.full.html

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml