STAT3 mutations are present in aggressive B-cell lymphomas including a subset of diffuse large B-cell lymphomas with CD30 expression

Signal transducer and activator of transcription-3 (STAT3) is a dimerizing transcription factor and oncogene involved in cytokine growth signaling and cell survival pathways.[1] While inactivating *STAT3* mutations have been identified in hyper-IgE syndrome,[2] activating mutations are seen in specific T-cell and NK-cell malignancies such as T-cell large granular lymphocytic leukemia (T-LGL), chronic NK lymphoproliferative disorders, and CD30$^+$ T-cell lymphomas.[3-5] Though the frequencies and role of *STAT3* mutations in T- and NK-cell malignancies have been well-studied, the significance of *STAT3* mutations in B-cell malignancies is uncertain.

As such, we read with great interest the recent *Haematologica* paper by Couronne *et al.*[6] that studied the role of the *STAT3* activating mutation Y640F in an *in vitro* mouse model and demonstrated that expression of this mutated protein resulted in myeloproliferative neoplasms in mice. In addition, the mutational status of *STAT3* in numerous hematologic neoplasms was assessed and mutations were seen in 4 cases of T-cell lymphomas and 2 cases of diffuse large B-cell lymphoma (DLBCL). While these DLBCL cases were identified as mutated, the significance of such mutations in these cases of DLBCL remains unknown. Given these recent findings, we strove to further advance the understanding of *STAT3* mutations in B-cell malignancies by analyzing 143 B-cell lymphomas.

We sequenced exons 19-24 of *STAT3*, where all known activating mutations have been identified, and included cases of: diffuse large B-cell lymphoma not otherwise specified (DLBCL-NOS; n=48), follicular lymphoma (FL; n=24), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL; n=24), mantle cell lymphoma (MCL; n=14), marginal zone lymphoma (MZL; n=12), Burkitt lymphoma (n=10), primary mediastinal large B-cell lymphoma (PMLBL; n=4), classical Hodgkin lymphoma (CHL; n=1), Epstein Barr virus-positive diffuse large B-cell lymphoma of the elderly (EBV+ DLBCL; n=2), B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma (BCLU, DLBCL/B; n=3), and B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma (BCLU, DLBCL/CHL; n=1). Genomic DNA was isolated from May-Grunwald-Giemsa stained slides (n=7), frozen whole blood (n=11), or formalin fixed paraffin embedded (FFPE) tissues (n=125) using a Qiagen (Louisville, KY, USA) DNeasy Blood & Tissue Kit as previously described.[4]

Of these 143 lymphomas, STAT3 mutations were identified in 3 of 48 DLBCL, NOS cases (6%) and in 2 of 3 cases of BCLU, DLBCL/B (67%). Mutations were all localized to the SH2 domain of *STAT3*. In the cases of DLBCL, NOS that contained *STAT3* mutations, the histomorphological and immunophenotypic features were similar (Table 1 and Figure 1). Neoplastic cells showed anaplastic morphologies with high mitotic indices of more than 10 mitoses/5 high power fields (HPF) (Table 1). All cases were CD20 positive and CD30 expression was seen in a subset of the anaplastic cells (>20% of cells) in all 3 DLBCL. Anaplastic lymphoma kinase (ALK) protein expression was not detected by immunohistochemistry in these cases. Of all cases of DLBCL, NOS with CD30$^+$ malignant cells, patients with STAT3 mutations represented 17% of such cases (3 of 18 cases). No significant association with anatomic site, sex, or age was observed.

Of the 2 BCLU, DLBCL/B cases with *STAT3* mutations, one had a *MYC* translocation (case 4) whilst the other positive case (case 5) had both an *MYC* and *BCL-6* transloca-

Table 1. Clinicopathological data.

WHO classification (n=143)	STAT3 mutated
CLL/SLL (n=24)	0/24 (0%)
MZL (n=12)	0/12 (0%)
FL (n=24)	0/24 (0%)
MCL (n=14)	0/14 (0%)
BL (n=10)	0/10 (0%)
DLBCL, NOS (n=48)	3/48 (6%)
BCLU, DLBCL/B (n=3)	2/3 (67%)
EBV+ DLBCL (n=2)	0/2 (0%)
BCLU, DLBCL/CHL (n=1)	0/1 (0%)
PMLBL (n=4)	0/4 (0%)
CHL (n=1)	0/1 (0%)

Case	Type	STAT3 mutation	Age	Sex	Site	CD30	Anaplastic morphology	Mitotic rate (>10/5HPF)
1	DLBCL, NOS	E616G	91	F	Intra-nasal	Yes	Yes	Yes
2	DLBCL, NOS	G617R	61	M	Axillary LN	Yes	Yes	Yes
3	DLBCL, NOS	Y640F	18	M	Parotid gland	Yes	Yes	Yes
4	BCLU, DLBCL/B	S649L	26	F	Ovary	No	Yes§	Yes
5	BCLU, DLBCL/B	T663I	17	M	Groin	No	Yes§	Yes

CLL/SLL: chronic lymphocytic leukemia/small cell lymphoma; MZL: marginal zone lymphoma; FL: follicular lymphoma; MCL: mantle cell lymphoma; BL: Burkitt lymphoma; EBV+ DLBCL: Epstein Barr virus positive diffuse large B-cell lymphoma of the elderly; BCLU, DLBCL/CHL: B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma; PMLBL: primary mediastinal diffuse large B-cell lymphoma; CHL: classical Hodgkin lymphoma; DLBCL, NOS: diffuse large B-cell lymphoma not otherwise specified. B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. LN: lymph node; WHO: World Health Organization. §A subset of cells showed anaplastic morphology.

tion (Figure 1 and Table 1). Both cases had similar morphological features with high mitotic rates (>10 mitoses/5HPF) and a subset of anaplastic multinucleated large lymphoid cells; however, unlike mutated *STAT3* DLBCL, NOS, neither of these were CD30 positive.

We also assessed the protein expression of *STAT3* in the 3 cases of mutated DLBCL, NOS and 2 cases of mutated BCLU, DLBCL/B. In the cases of DLBCL, NOS, STAT3 was over-expressed in the nucleus of malignant cells in these mutated cases and visibly up-regulated in the $CD30^{+}$ cells

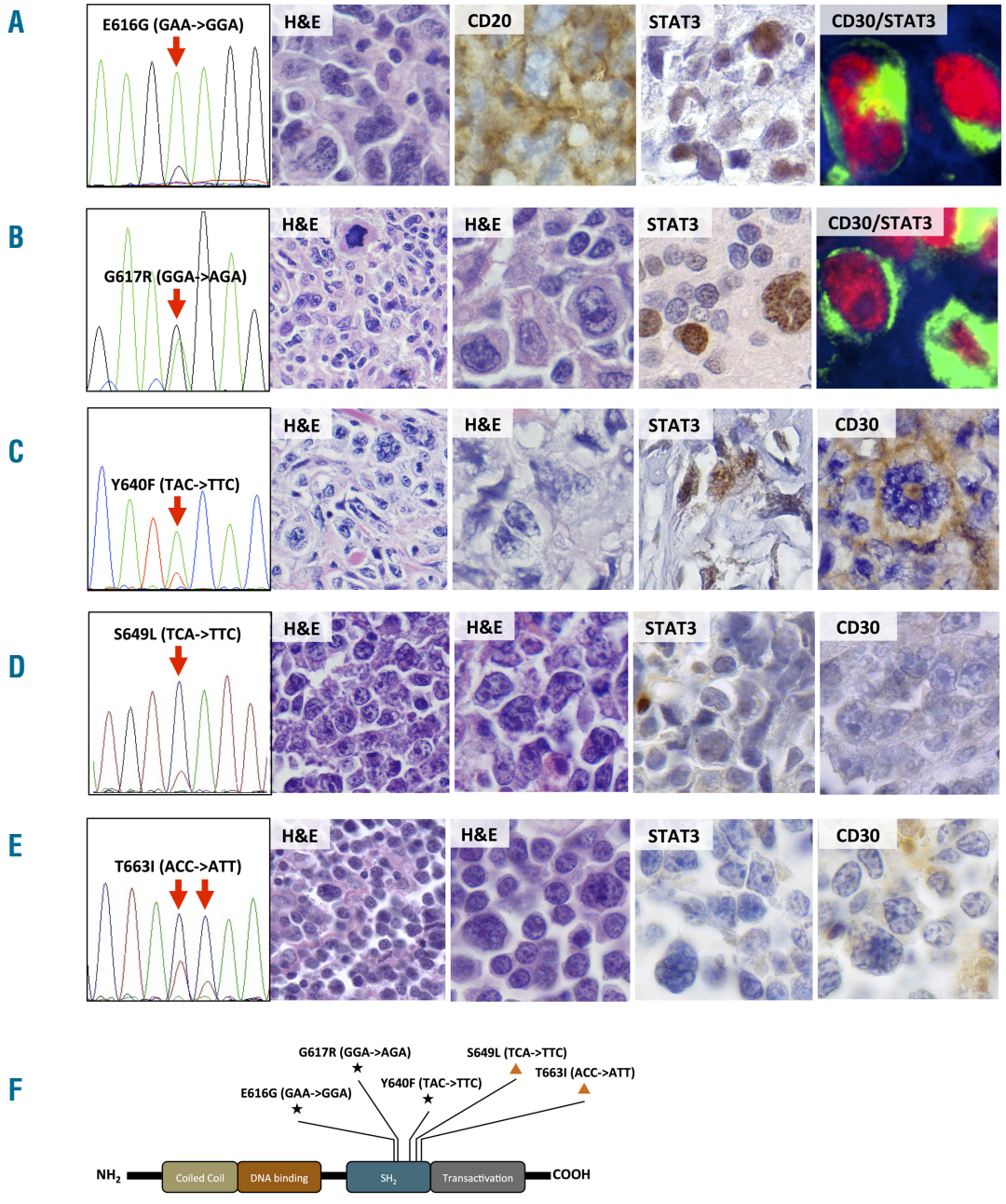

Figure 1. *STAT3* mutations in aggressive B-cell lymphomas. (A) Case 1: diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS) with Sanger sequencing of the E616G mutation shown in the first left panel and H&E and immunohistochemical and immunofluorescence images in following panels for this case. (B) Case 2: DLBCL, NOS with Sanger sequencing of the G617R mutation shown in the first left panel and H&E and immunohistochemical and immunofluorescence images in following panels for this case. (C) Case 3: DLBCL, NOS with Sanger sequencing of the Y640F mutation shown in the first left panel and H&E and immunohistochemical images in following panels for this case. (D) Case 4: B-cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt (BCLU, DLBCL/B) with Sanger sequencing of the S649L mutation shown in the first left panel and H&E and immunohistochemical images in following panels for this case. (E) Case 5: a case of BCLU, DLBCL/B with Sanger sequencing of the T663I mutation shown in the first left panel and H&E and immunohistochemical images in following panels for this case. (F) Location of mutations in 5 cases of aggressive B-cell lymphomas with *STAT3* mutations. Black stars denote cases of DLBCL, NOS while orange triangles indicate cases of BCLU, DLBCL/B. Src homology 2 domain (SH2). All H&E and immunohistochemical stained slide images (CD30 or STAT3) were taken at either 400X or 1000X. Immunofluorescence images were taken at 2000X with CD30 in green, STAT3 in red and DAPI nuclear staining in blue.

(Figure 1). However, in the STAT3 mutated BCLU, DLBCL/B, the protein was not over-expressed by immunohistochemistry. Furthermore, overexpression of STAT3 was not specific to B-cell lymphoma cases with *STAT3* mutations. Nuclear overexpression of *STAT3* was also seen in 14 of 39 other DLBCL, as well as 3 of 8 FL, 2 of 7 CLL/SLL, 2 of 10 Burkitt lymphomas (BL), and one of 5 MZL; 2 cases of PMLBL had no evidence for STAT3 overexpression. Interestingly, one case of classical Hodgkin lymphoma showed distinctive nuclear overexpression of STAT3 in malignant cells; malignant cells constituted more than 20% of the tumor, though STAT3 was not mutated in this case.

While Couronne *et al*. identified rare cases of DLBCL with *STAT3* mutations recently,[6] our results demonstrate a phenotypic-genotypic link between the mutational status of STAT3 and morphology of cells in these aggressive B-cell lymphomas. While only 3 of 48 of our cases of DLBCL, NOS were positive for a *STAT3* mutation (6% of DLBCL cases), the frequency of STAT3 mutations specifically in CD30$^+$ DLBCL (3 of 18 cases; 17%) is higher than by chance alone (P=0.047). In addition, though we were only able to sequence 3 cases of BCLU, DLBCL/B, 2 of these cases harbored *STAT3* SH2 domain mutations. Similarly to the cases of *STAT3* mutated DLBCL, NOS, in the 2 cases of *STAT3* mutated BCLU, DLBCL/B, these both contained a subset of anaplastic multinucleated cells; though CD30 and STAT3 proteins were not over-expressed. One possible explanation for the absence of STAT3 protein overexpression in mutated cases of BCLU, DLBCL/B, concerns the relationship between MYC and STAT3 transcriptional protein pathways. STAT3 is known to up-regulate MYC protein expression and it is possible that the increased activity of MYC in these cases with *MYC* translocations, results in a feed-back loop which suppresses visible overexpression of the STAT3 protein.[7-9] However, further *in vitro* experiments are necessary to test such a hypothesis. Finally, we have also demonstrated that these mutations are not seen in lower grade B-cell lymphomas such as CLL/SLL, FL and MZL.

In addition, STAT3 was also over-expressed in a subset of other B-cell malignancies without these mutations: 14 of 39 DLBCL, 3 of 8 cases of FL, 2 of 7 cases of CLL/SLL, and one of 5 cases of MZL. These findings are in agreement with the studies of other groups that have noted increased activation of STAT3 in a subset of B-cell lymphomas.[10-13] Why some cases of DLBCL show protein overexpression of STAT3 but harbor no STAT3 mutation is not entirely clear; however, it is certainly feasible that there are other mechanisms to increase STAT3 activity via upstream protein regulators.

In some studies, STAT3 overexpression has been correlated with poor prognosis.[10,14] Whether cases with *STAT3* DNA mutations, as seen here, are associated with poor prognosis is unclear, and larger patient cohorts are necessary to evaluate this hypothesis. However, it is interesting to note that of the 4 cases of PMLBL, a CD30$^+$ large B-cell lymphoma that responds favorably to chemotherapy, none had STAT3 mutations, and, in addition, the STAT3 protein was not over-expressed in 2 cases of PMLBL analyzed.

Finally, our results expand on the mechanistic relationship between STAT3 and morphological anaplasia which has been noted by others, including Chiarle *et al*.,[15] whereby STAT3 overexpression results in increased morphological anaplasia, and overall increased proliferation and cell survival.[15] In addition, given the recent development of STAT3 inhibitors and their current clinical testing, the significance of our findings and the potential therapeutic implications in these cases with *STAT3* activating mutations is an area for promising future research.

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