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No Evidence for the *JAK2* (V617F) or *JAK2* Exon 12 Mutations in Primary Mediastinal Large B-Cell Lymphoma

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Abstract

Dysregulated *JAK2* signaling has been shown to play a significant role in the pathogenesis of myeloproliferative disorders. Recently, our work comparing gene expression signatures of primary mediastinal large B-cell lymphomas (PMLBCL) versus nodal diffuse large B-cell lymphomas (DLBCL) revealed a relative increase in *JAK2* transcripts in the former suggesting a role for increased *JAK2* signaling in a subset of these tumors. Given the likelihood of increased *JAK2* signaling in PMLBCL, we sought to determine whether *JAK2* activating mutations were an alternative mechanism for increased *JAK2* signaling in untreated PMLBCLs. We performed amplification refractory mutation analysis for the *JAK2* (V617F) mutation and bi-directional sequencing for the recently described *JAK2* exon 12 mutations on genomic DNA isolated from a well characterized cohort of PMLBCLs. No evidence of the mutant *JAK2* (V617F) allele or *JAK2* exon 12 mutations was detected in 31 PMLBCL cases tested. Analysis using cell lines derived from PMLBCLs (n=1) and from the molecularly similar classical Hodgkin lymphoma (n=4) also failed to reveal involvement of this mutant *JAK2* allele. Taken together, these results suggest that *JAK2* signaling in PMLBCLs occurs by mechanisms distinct from *JAK2* (V617F) or *JAK2* exon 12 activating mutations.

INTRODUCTION

Primary mediastinal large B-cell lymphoma (PMLBCL) is a distinct subtype of diffuse large B-cell lymphoma with morphologic, immunohistochemical, and molecular similarities to classical-type Hodgkin lymphoma (cHL).¹ Patients are commonly young women in the third to fourth decade of life, presenting with Stage I or II disease with a bulky, anterior mediastinal mass and have an approximately 50% five-year survival.^{2, 3} Initial extension of the tumor into local adjacent structures typically portends a poor prognosis. While the clinical response to initial therapy is usually robust, patients may often relapse with distant involvement of multiple organ sites.

On histopathologic examination, the tumor cells typically appear as large pleomorphic centroblastic or immunoblastic cells with abundant eosinophilic cytoplasm, distinct cell margins, and prominent nucleoli.⁴ There is often abundant fibrosis similar to that seen in

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classical type nodular sclerosis Hodgkin lymphoma.⁴ Immunohistochemical studies performed on these tumors demonstrate the presence of B-cell markers, consistent with the putative origin of this tumor from thymic medullary B-cells, showing immunopositivity for CD19, CD20, CD22 and CD79a.⁵ Molecular studies performed for immunoglobulin receptor gene rearrangements often show clonal rearrangement, however, most characteristically, these tumors lack or have markedly diminished surface B-cell receptor and class I and II HLA molecule expression.⁶

The role for dysregulated signaling by Janus kinase 2 (JAK2) in PMLBCL has been suggested consistently in various studies. Chromosomal gains in the 9p24 locus have been identified in this tumor, implicating the involvement of *JAK2* which resides at that locus.^{7, 8} Specifically, chromosomal gains in 9p occur in up to 75% of PMLBCLs cases as compared to only 20% of typical diffuse large B-cell lymphomas.^{7, 9} Recent work has further shown constitutive STAT signaling in PMLBCLs which, together with JAK2, transmits cytokine induced signals from the extracellular matrix to the nucleus to effect gene transcription. Analysis of our previously generated gene expression microarray data showed a relative increased abundance of *JAK2* transcripts in a subset of PMLBCLs as compared to diffuse large B-cell lymphomas.¹ Taken together, these data suggest that dysregulated JAK2 signaling may play an important role in the pathogenesis of this entity; however, increased *JAK2* expression could not completely account for this potential increase in JAK2 signaling. To further examine the role of JAK2 in PMLBCL, particularly in those tumors from our gene expression analysis with minimally increased *JAK2* transcript levels, we sought to determine whether the common activating (V617F) mutation or the recently described activating mutations in exon 12 of *JAK2* are present in PMLBCL patient samples.

MATERIALS AND METHODS

Transcriptional Profiling Analysis

Transcript abundance data was obtained from our prior study on Affymetrix U133A and U133B oligonucleotide microarrays (Affymetrix, Santa Clara, CA) which was generated as previously described.¹ The raw gene expression values have been scaled in order to account for differences in global chip intensity. Statistical differences in the median levels of *JAK2* transcript abundance between nodal large cell lymphomas and PMLBCL was determined by use of the Mann-Whitney test.

JAK2 (V617F) Mutation Analysis

An amplification refractory mutation system (ARMS) PCR approach to assay for the common 1849G>T activating mutation of *JAK2* using a fluorescent capillary gel electrophoresis format and Gene Mapper software (Applied Biosystems, Foster City, CA) was developed based on a published report.¹⁰ The position of the forward outer primer was adjusted to optimize annealing temperatures (5'- CAGGCTTACACAGGGGTTTC -3'; $T_m = 60^\circ\text{C}$); no other significant change from the original protocol or primers was made.¹⁰ The adapted system was validated using ten cases of myeloproliferative disorders in which the *JAK2* mutational status was definitively known (gift of R. Levine and G. Gilliland, BWH). Genomic DNA was isolated from 34 untreated PMLBCL tumor specimens that were derived from mediastinal masses or contiguous nodal biopsies from our prior study as previously described.¹ PMLBCLs were identified using clinical criteria (predominant mediastinal mass with or without local extension and no extrathoracic disease) and pathologic features. The histopathology and immunophenotype of each PMLBCL was reviewed by expert hematopathologists to confirm the diagnosis. In addition, DNA was isolated from one PMLBCL (Karpas 1106) and four Hodgkin lymphoma cell lines (L428, Sup-HD1, HD-LM2, and HD-MY-Z).

All samples were amplified in a single tube PCR reaction with primers specific for both the wild-type and mutant *JAK2*, with the resulting amplicons distinguished by expected size using fluorescent capillary gel electrophoresis (wild-type and mutant amplicons were 229 and 297 base pairs respectively). An outer product of 471 bp size was used concomitantly as an internal control for the PCR. Fluorescent primers were used as shown (Figure 2). Detection of the wild-type and mutant *JAK2* amplicon from K562 and HEL cell lines served as negative and positive controls respectively. The human erythroleukemic cell line, HEL, is known to be a homozygous mutant for the activating *JAK2* (V617F) mutation. Control mixing experiments showed that the sensitivity of this assay for detecting the mutant amplicon in the background of the wild-type product was consistently reproducible to a dilution of 1:100 (data not shown).

***JAK2* Exon 12 Mutational Analysis**

Direct bi-directional sequencing of *JAK2* exon 12 was performed as described by Pietra et al.¹¹ on an ABI 3730 XL DNA sequencing platform. Briefly, primers were designed to amplify a 453 bp region containing the 128 bp of the exon 12 of *JAK2*. PCR was performed in a 50 µl reaction containing 100-300 ng of DNA from either PMLBCL or cell lines, 200 µM each deoxynucleotide triphosphate, 1.5 mM MgCl₂, 1.5 U of Taq Polymerase together with 1X PCR buffer (ABI) and 20 pmoles of each of the following primers:

- Forward 5'-CAAAGTTCAATGAGTTGACCCCTA-3'
- Reverse 5'-ACACAAGGTTGGCATATTTTCATAAG-3'.

Cycling conditions were 95°C for 10 min (one cycle); 95°C for 30 sec, 55°C for 20 sec, 72°C for 20 sec, for a total of 35 cycles, and 72°C for 7 minutes (one cycle). Computer-assisted sequence analysis was performed using SeqScape® software, version 2.5, (Applied Biosystems, Foster City, CA).

RESULTS

All PMLBCL specimens in our cohort demonstrated large atypical lymphoid cells with variable nuclear features, prominent nucleoli, well-demarcated cell borders, and interspersed, variable fibrosis. A more detailed analysis of relative *JAK2* transcript abundance in PMLBCL and nodal diffuse large B cell lymphomas was performed using gene expression microarray data generated in our prior studies (Figure 1).¹ Evaluation of the two probe sets for *JAK2* transcripts revealed a relative increase in *JAK2* expression in a subset of PMLBCLs (Figure 1A). However, it is clear that a proportion of PMLBCLs do not show increased *JAK2* expression at the transcriptional level. Further comparison of *JAK2* expression in PMLBCLs to diffuse large B-cell lymphomas showed a statistically significant increase in the median transcript abundance of *JAK2* transcripts in PMLBCLs versus DLBCL (Figure 1B). These findings pointed to the potential importance of *JAK2* in PMLBCLs as a group; however, differences in transcript levels among PMLBCLs suggested different mechanisms for *JAK2* signaling. We hypothesized that activating mutations in *JAK2* could be present in PMLBCL, particularly in cases with low levels of *JAK2* transcripts. To address this possibility, we devised an amplification refractory mutation analysis for the *JAK2* (V617F) mutation in a large cohort of PMLBCL primary tumors (Figure 2). Genomic DNA was isolated from 34 untreated PMLBCLs, and each specimen was amplified in conjunction with a blank specimen (water) and two cell line controls, K562 and HEL cells, containing the wild-type and mutant *JAK2* (V617F), respectively. K562 and HEL control cell lines demonstrated absence (K562) and presence (HEL) of the *JAK2* mutation as predicted (Figure 3). Control blanks were consistently negative in our analysis (data not shown). Three of the thirty-four specimens failed to amplify despite repeat testing and were excluded from further analysis. The remaining 31 cases amplified well and showed only evidence of the wild-type amplicon (Figure 3; Table 1). In some cases, the larger outer band did not amplify, most likely due to competition from the more efficient reaction producing the

smaller amplicon. This failure to amplify the larger outer product was not due to poor quality DNA, as we confirmed that each specimen could be amplified for the larger, outer reaction product in a separate PCR run using only the outer primers in the PCR mixture (data not shown). These results confirm that none of the PMLBCLs tested showed involvement of the common activating *JAK2* (V617F) mutation. Of note a PMLBCL cell line, Karpas 1106, also failed to show evidence of a *JAK2* (V617F) mutation (Table 1). We have recently provided evidence for a molecular relationship between PMLBCL and classical Nodular Sclerosis Hodgkin Lymphoma (HL).¹ These entities not only present in the same patient population and demonstrate overlapping histopathologic and immunophenotypic features, but appear molecularly similar particularly with regard to activation of the NF- κ B pathways and potentially dysregulated *JAK2* signaling.¹ To explore the possibility of this *JAK2* mutation being involved in the dysregulated signaling of nodular sclerosis Hodgkin lymphoma, four HL cell lines were also examined for the *JAK2* (V617F) mutation (Table 1).¹² Our studies showed no evidence of the mutant *JAK2* allele in the HL cell lines examined.

In light of our negative findings, we sought to assess whether other activating *JAK2* mutations aside from *JAK2* (V617F) might be present. Recent reports have implicated activating mutation in exon 12 of *JAK2* in a variety of chronic myeloproliferative disorders^{11,13}. We hypothesized that these mutations were also possible candidates for dysregulating *JAK2* signaling in PMLBCL. We therefore examined all of the patient PMLBCL samples and cell lines for the presence of exon 12 of *JAK2* mutations after PCR of this entire exonic region and bi-directional sequencing (Figure 3B and Table 1). We found only wildtype sequences in each amplicon confirming the absence of any known or novel *JAK2* exon 12 mutation.

DISCUSSION

Our previous work using gene expression analysis identified a relative increased abundance of *JAK2* transcripts in PMLBCLs compared to nodal type diffuse large B-cell lymphoma.¹ While there was an average relative two-fold increase in *JAK2* transcripts, not all cases showed increased transcripts, and we hypothesized that other mechanisms might be responsible for dysregulated *JAK* signaling in these cases. To determine whether the most common activating *JAK2* mutation [*JAK2* (V617F)] identified in chronic myeloproliferative disorders is also involved in PMLBCLs, we tested 31 cases of PMLBCLs and found no evidence of these mutations in this cohort using an amplification refractory mutation system (ARMS) approach. Similar results were seen with cell lines derived from PMLBCLs and Hodgkin lymphoma. Our results confirm the findings of other investigators who also showed no evidence of the *JAK2* (V617F) mutation in a separate cohort of specimens using an alternative method.¹⁴ Using a site specific restriction analysis approach, they examined DNA from 20 primary PMLBCL cases, 16 micro-dissected classical Hodgkin lymphoma, five cell lines derived from cHL, two from PMLBCLs, and saw no evidence of a *JAK2* mutation.¹⁴ This prior study did not evaluate for the presence of a *JAK2* exon 12 mutation. Based on recent reports of mutations in *JAK2* exon 12 resulting in *JAK2* signal dysregulation^{11, 13}, we expanded on this prior study to evaluate for the presence of *JAK2* exon 12 mutations. In our cases, we did not find any mutations in *JAK2* exon 12 using bi-directional sequencing. Taken together, these data suggest that other mechanisms for altered *JAK2* signaling aside from the common activating *JAK2* mutations are present in PMLBCL. Apart from *JAK2*, mutations in other regulatory molecules within the *JAK2* signaling pathway may result in dysregulated *JAK2* signaling.^{14, 15} Indeed, there is careful regulation of the *JAK*-*STAT* pathway as complex cues from the extracellular environment are processed to effect gene transcription and other complex cellular functions.¹⁶⁻¹⁸ One molecule, *SOCS1*, the suppressor of cytokine signaling which directly inhibits *JAK2*, may also result in increased *JAK2* activity if mutated, deleted, or if its expression is diminished, through promoter methylation.^{19, 20} Recent studies have identified several truncation mutations in *SOCS1* gene in PMLBCLs.¹⁵ These mutations result in increased *JAK2* effect

and constitutive STAT activation.¹⁵ SOCS mutations may, therefore, be present and involved in our cohort of cases, particularly in the cases where our gene expression analysis showed only minimally increased *JAK2* transcripts.¹ Further studies are in progress to evaluate this more fully.

Another possible explanation for the failure to observe a mutant *JAK2* (V617F) allele is a technical failure of detection due to allelic dilution.²¹ The 9p locus has been observed to be amplified frequently in PMLBCLs.⁷ While this amplification could be sufficient to drive dysregulated JAK-STAT signaling, if there was a coexisting activating *JAK2* mutation, its detection could be missed due to dilution by wildtype amplified alleles.²¹ While this is certainly one possibility, our assay was consistently sensitive to at least 1:100 dilution of the mutant into the wild-type allele in mixing studies, and reports on gene amplifications of 9p in PMLBCL appear to suggest only a minimally increased copy number.⁷ Allelic dilution therefore appears to be unlikely in this setting.

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A.

Primary Mediastinal Large B-Cell Lymphoma (M)

B.

JAK2 Transcript Abundance

Figure 1.

JAK2 mRNA transcript abundance in primary mediastinal large B cell lymphoma and nodal diffuse large B cell lymphoma. A) Each column is a primary mediastinal large B cell lymphoma sample and each row an independent probe set measuring *JAK2* transcript abundance. Red indicates high relative expression; blue, low expression. Color scale at the bottom indicates relative expression in standard deviations from the mean. B) The relative transcript abundance for *JAK2* using two probes (205842 and 205841) is compared between diffuse large B cell lymphoma and primary mediastinal large B cell lymphoma.

Figure 2.

Adapted schematic of ARMS PCR for *JAK2* (V617F) used herein.¹⁰ Arrows delineate position of primers for either the G (wild-type) or T (mutant) nucleotides. Green arrows and stars demonstrate respectively the position of fluorescent primers and their incorporation into the PCR products. Wild-type (229 bp), mutant (297 bp), and outer control (471 bp) amplicons may be generated in this single-tube PCR assay.

Figure 3.

A) Representative ARMS PCR results showing capillary gel electrophoresis of a representative PMLBCL sample (top) with control bands for the mutant (middle) and wild-type (bottom) *JAK2* amplicon. A blank control (water) consistently showed no peaks (not shown). B) Representative example of wild-type Exon 12 *JAK2* bi-directional sequencing. The boxed area highlights the locus within exon 12 of the prior reported mutations¹¹.

Table 1

Summary of results for ARMS-PCR for *JAK2* (V617F) and direct sequencing for *JAK2* exon 12 mutations in PMLBCL and cell lines

<u>MUTATION</u>		
Sample	<i>JAK2</i> (V617F)	<i>JAK2</i> Exon 12
Primary Mediastinal Large B-Cell Lymphoma tumors	0/31	0/31
Primary Mediastinal Large B-Cell Lymphoma cell line (Karpas 1106)	0/1	0/1
Classical Hodgkin Lymphoma cell lines (n=4)	0/4	0/4