Expression of the FOXP1 Transcription Factor Is Strongly Associated with Inferior Survival in Patients with Diffuse Large B-Cell Lymphoma

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ABSTRACT

Gene expression profiling studies have reported up-regulated mRNA expression of the FOXP1 forkhead transcription factor in response to normal B-cell activation and high expression in a poor prognosis subtype of diffuse large B-cell lymphoma (DLBCL). The purpose of this study was to investigate the prognostic importance of FOXP1 protein expression in an independent series of DLBCL.

First, the specificity of our FOXP1 monoclonal antibody was verified by confirming that it did not recognize the closely related FOXP2, FOXP3, or FOXP4 proteins. FOXP1 protein expression was then analyzed by immunohistochemistry using a DLBCL tissue microarray constructed from 101 previously untreated de novo cases from the British Columbia Cancer Agency. FOXP1 expression was scored as either positive (>30% positive nuclei) or negative (<30% positive nuclei).

The overall survival curves clearly showed that patients grouped as FOXP1-positive (40%) had a significantly decreased overall survival (\(P = 0.0001\)). FOXP1-positive patients had a median overall survival of 1.6 years compared with 12.2 years in FOXP1-negative cases. In addition, FOXP1-positive patients showed a clear trend to earlier progression in comparison to the FOXP1-negative patients. The analysis of FOXP1 expression within low, medium, and high International Prognostic Index groupings found that FOXP1-negative patients had better overall survival within each group indicating that FOXP1 expression has predictive value independent of the International Prognostic Index subgrouping, a finding that was confirmed in multivariate analysis. These initial results suggest that FOXP1 expression may be important in DLBCL pathogenesis.

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) accounts for 30% to 40% of all adult non-Hodgkin’s lymphomas and is heterogeneous in terms of its morphology and clinical features (1). The genetic abnormalities underlying DLBCL remain poorly understood, and in contrast to other lymphoma types (e.g., follicular lymphoma or Burkitt’s lymphoma), no single characteristic genetic alteration has been found. This heterogeneity may be partially caused by an aberrant somatic hypermutation mechanism that has been reported to target multiple proto-oncogene loci and to favor chromosomal translocations in >50% of DLBCLs (2).

Approximately 60% of patients with DLBCL relapse after conventional anthracycline-based combination of cyclophosphamide, Adriamycin, vincristine, bleomycin, and prednisolone type of treatment (3). A number of attempts to increase the survival rate have proven unsuccessful (4). To improve the outcome for these patients, clinicians need first to be able to identify high-risk patients at diagnosis and second to develop alternative strategies for treatment, such as therapeutic antibodies or novel chemotherapy combinations that can be effectively tested in clinical trials. The International Prognostic Index (IPI) is still the tool most routinely used for predicting the outcome for patients with DLBCL, but this alone cannot accurately predict treatment outcome for individual patients and only indirectly reflects tumor biology. There is, therefore, a need for additional methods or markers to identify high-risk patients at diagnosis and to improve the definition of specific gene expression pathways for novel therapeutic intervention.

In recent years, the introduction of microarray-based gene expression profiling techniques to study DLBCL has provided compelling evidence for the existence of distinct subtypes of this disease that are associated with different clinical outcomes (5–8). The most widely studied of these subtyping systems has been based on two molecularly distinct forms of DLBCL related to different stages of B-cell differentiation (5), although at least one more additional category, type 3, has subsequently been identified (6). In the two original DLBCL subtypes, one has a gene expression profile related to that of germinal center B cells and is called germinal center B-cell-like. The t(14;18) translocation, c-rel amplification, CD10 expression, and evidence of ongoing immunoglobulin somatic hypermutation...
are restricted to this group (6, 9, 10). This subgroup of patients has the highest 5-year survival rate (5). The second subtype expresses genes induced by in vitro activation of peripheral blood B cells and is referred to as the activated B-cell–like subgroup and has a significantly worse prognosis than the germinal center B-cell subgroup (5, 6, 8). Using a different statistical approach to the analysis of these data led to a more accurate prediction of subclass distinction (8). Many of the cases previously identified as type 3 were reclassified as germinal center B-cell, leaving only one sixth of the DLBCL cases as unclassified. Moreover, the Bayesian predictor was able to distinguish an outcome difference between germinal center B cell and activated B-cell–like DLBCL within the data published by Shipp et al. (7, 8).

Rosenwald et al. have used gene expression profiling to formulate a molecular predictor of survival that is a prognostic indicator independent of the IPI score (6). This is based on a combination of four different gene expression signatures (germinal center B cell, MHC class II, lymph node, and proliferation), together with BMP-6 mRNA expression, as there is no single gene whose expression accurately predicted the outcome for all patients (5, 6, 8). However, few centers performing routine lymphoma diagnosis have access to the outcome for all patients (5, 6, 8). However, few centers performing routine lymphoma diagnosis have access to microarray gene expression profiling technology. Additionally, most lymphoma diagnosis is done on paraffin embedded tissues, and fresh or frozen samples are often not available for RNA extraction. It is important, therefore, that reagents, such as antibodies for immunohistochemical analysis are developed to enable the routine assessment of the disease subtype markers identified by gene expression profiling studies. Immunohistochemical staining of a series of DLBCL cases as unclassified. Moreover, the Bayesian predictor was able to distinguish an outcome difference between germinal center B cell and activated B-cell–like DLBCL within the data published by Shipp et al. (7, 8).

We have previously described our cloning and identification of a novel winged helix transcription factor, FOXP1, using a monoclonal antibody (JC12) that detects its protein target in both frozen and routinely fixed tissue samples (12). Both FOXP1 mRNA and protein expression are widely deregulated in solid tumors and the FOXP1 protein, the reactivity of the antibody was tested on COS-1 cells expressing FOXP1 (encoded by plasmid pAB195; ref. 12) or the closely related FOXP2, FOXP3, or FOXP4 proteins. The FLAG-tagged FOXP3 cDNA was kindly provided by Dr. Mary Brunkow (Celltech, Bothell, WA). The FOXP2 and FOXP4 cDNAs were PCR amplified and cloned as described below.

### MATERIALS AND METHODS

**Validation of JC12 Antibody Specificity for FOXP1.** To confirm that the JC12 antibody was specifically reactive with the FOXP1 protein, the reactivity of the antibody was tested on COS-1 cells expressing FOXP1 (encoded by plasmid pAB195; ref. 12) or the closely related FOXP2, FOXP3, or FOXP4 proteins. The FLAG-tagged FOXP3 cDNA was kindly provided by Dr. Mary Brunkow (Celltech, Bothell, WA). The FOXP2 and FOXP4 cDNAs were PCR amplified and cloned as described below.

**PCR Amplification and Cloning of the FOXP2 cDNA.** A cDNA encoding full-length FOXP2 was amplified by PCR in a 50-μL reaction containing 1 μL of a 1:10 dilution of human fetal brain cDNA library (Clontech, Palo Alto, CA), oligonucleotide primers (forward 5′-CTCGGATTCTATGTG-CAGGAATCTGCGAGACAGACAATAAGC-3′ and reverse 5′-CTCGAATTCCTATTCCAGATCTCAGATAAAAAG-GTCTTTCTTC-3′, Invitrogen, Carlsbad, CA) at 0.5 μmol/L, deoxyxynucleotide triphosphates at 200 μmol/L, 1× reaction buffer, and 2.5 units of Pfu Turbo Polymerase (Stratagene, La Jolla, CA). The reaction mixture was heated to 95°C for 3 minutes followed by a ‘touch-down’ thermal cycling protocol: 94°C, 30 seconds; 62°C, 30 seconds; and 72°C, 5 minutes, with the annealing temperature reduced in subsequent rounds, as follows: 61°C, 60°C, 59°C, 58°C, 57°C, 56°C, 55°C, 55°C, 52°C, and 52°C; then maintained at 50°C for 26 cycles, with a final 10 minutes extension at 72°C. Following BamHI/EcoRI digestion and gel purification, the FOXP2 PCR product and pBluescript II KS+ (Stratagene) were ligated together and cloned using standard molecular biology methods (14). The FOXP2 cDNA insert was fully sequenced confirming that this encoded FOXP2 (Genbank accession no. AF337817). The polyglutamine region had one less Q than in the reported sequence and there was a single base pair change of T to C in the codon encoding amino acid 29. However, as the T-to-C substitution was silent and small naturally occurring variations in the length of the FOXP2 polyglutamine repeat have been reported (15), this cDNA clone was used for protein expression studies. The FOXP2 cDNA was excised from pBluescript using BamHI/EcoRI digestion and subcloned into pcDNA4/HisMax (Invitrogen) to create the pcDNA4/HisMax/FOXP2 eukaryotic expression construct.

**PCR Amplification and Cloning of the FOXP4 cDNA.** The FOXP4 cDNA was generated by PCR using the cDNA clone MGC:48953 (IMAGE:5527139, provided by the UK Human Genome Mapping Project Resource Centre) as template, with primers (forward 5′-CTGGGATTCCCATGTGGTG-GAATCTGCCCTCGG-3′ and reverse 5′-CTGAATTCTTCTAG-GACAGTTTCTTCCGGCAG-3′) at 0.5 μmol/L, and all other components as described above. The thermal cycler was programmed as follows: 95°C, 1 minute followed by 26 cycles of 95°C, 30 seconds; 60°C, 30 seconds; 72°C, 3 minutes followed by a final 10 minutes extension at 72°C. The PCR-generated FOXP4 fragment and pcDNA4/HisMax(C) were digested with BamHI and EcoRI, ligated together, and cloned to create the eukaryotic expression construct pcDNA4/HisMax/FOXp4. Plasmid DNA was prepared and the construct insert was fully sequenced.
Eukaryotic Expression of the FOXP Proteins. Plasmid DNA was prepared for transfection using the HiSpeed Plasmid Midi Kit (Qiagen, Valencia, CA). COS-1 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% FCS, 2 mmol/L l-glutamine, 50 units ml⁻¹ penicillin, and 50 μg ml⁻¹ streptomycin (Invitrogen), at 37°C in 5% CO₂. pAB195 (FOXP1), pcDNA4/HisMax/FOXP2, pcDNA4/HisMax/FOXP3, or vector alone were transfected into COS-1 cells using FuGENE 6 transfection reagent, following the protocol described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). Approximately 24 hours post-transfection, the cells were washed with sterile PBS and harvested by trypsinisation. Cytocentrifuge preparations were made for immunocytochemical staining and stored at −20°C (16).

Antibodies and Immunostaining of Frozen and Routinely Fixed Material. Production of the mouse monoclonal anti-FOXP1 antibody, JC12, has been described previously (12). Cytocentrifuge preparations of transfected COS-1 cells were fixed in ice-cold methanol for 10 minutes, then indirectly immunoenzymatically labeled using anti-Xpress antibody (Invitrogen) at 5.5 μg ml⁻¹, anti-FLAG M2 antibody (Sigma) at 10 μg ml⁻¹, or JC12 hybridoma supernatant diluted 1:10 in PBS/10% human serum. The DLBCL TMAs were dewaxed in citroclear (HD Supplies, Aylesbury, United Kingdom) and antigen retrieval was done by microwave pressure cooking for 3 minutes in PBS/10% human serum. The DLBCL TMAs were dewaxed in citroclear (HD Supplies, Aylesbury, United Kingdom) and antigen retrieval was done by microwave pressure cooking for 3 minutes at full pressure in 50 mmol/L Tris; 2 mmol/L EDTA (pH 9). The JC12 antibody was applied at a dilution of 1:80 in PBS/10% human serum. All primary antibodies were applied for 30 minutes at room temperature and antibody binding was detected using the ChemMate DAKO EnVision Detection Kit, Peroxidase/3,3’-diaminobenzidine as defined by the manufacturer (DAKO, Glostrup, Denmark).

Diffuse Large B-Cell Lymphoma Patient Characteristics and Tissue Microarray Construction. This project was reviewed and approved by the University of British Columbia, British Columbia Cancer Agency Research Ethics Board. All the specimens included in this study were retrospectively collected from cases that had presented to the British Columbia Cancer Agency with de novo newly diagnosed, previously untreated DLBCL. Cases were included in the current study if representative tumor tissue was available for creation of a TMA for FOXP1 protein immunohistochemical analysis, if classic cytogenetic analysis had been done on the original diagnostic biopsy and if complete clinical information including long-term follow-up was available. No discordant or transformed cases were included. Cases (n = 101) with formalin-fixed paraffin blocks and sufficient biopsy material to construct the TMA were selected. Of these, 88 were treated with combination of cyclophosphamide, adriamycin, vincristine, bleomycin, and prednisolone type of chemotherapy; five were too frail to treat and were managed with palliative chemotherapy and symptomatic measures; six received radiation only. The cases were gathered over a substantial period of time and not on a prospective basis and were thus managed with treatment regimens thought most likely to be curative at that time (except for the five given palliative treatment). Duplicate TMAs with cores of 0.6 mm were constructed using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). In addition, seven replicate cases and cores from reactive lymph nodes were included in each TMA block in order to improve the quality control assessment of staining. Five-micrometer-thick sections were cut from both TMA blocks and stained with H&E and antibody JC12 (anti-FOXP1). The H&E sections were used to verify adequate representation of diagnostic biopsies.

Fig. 1 The JC12 monoclonal antibody is not cross-reactive with the other FOXP proteins. COS-1 cells were transfected with either an empty vector or constructs encoding either FOXP1, FOXP2, FOXP3, or FOXP4 proteins. FOXP1 protein expression was confirmed using the JC12 antibody. Antibodies to the relevant epitope tags were used to confirm FOXP2 (anti-Xpress), FOXP3 (anti-FLAG M2), and FOXP4 (anti-Xpress) protein expression. All FOXP-COS transfectants were tested for reactivity with the JC12 antibody. JC12 reactivity was observed solely with the FOXP1 protein.
Scoring of the FOXP1 immunostaining was done as follows: failed, complete lack of nuclear staining of internal control small lymphocytes; 0, <10% staining of large tumoral B cells; 1, 10% to 30% of the large B cells with nuclear staining; 2, 31% to 50% nuclear staining of large B cells; and 3, >50% nuclear staining of large neoplastic B cells. Two cases showed an unusual cytoplasmic staining pattern and were not included in this analysis. For the purposes of this study, scores of 0 and 1 (<30% of the cells positive) were considered negative and scores of 2 and 3 (>30% of the cells staining) were considered positive for FOXP1 expression. Duplicate cores that did not have identical scores for FOXP1 staining were averaged.

Outcome Assessment and Statistical Analysis. Progression-free survival was defined as the time from diagnosis to the time of documented progression or, if a complete response had occurred after primary treatment, recurrence of lymphoma. Progression was defined as the regrowth of previously responding lesions or the appearance of disease at a new site. Overall survival was defined as the time from diagnosis to the time of death from any cause. Patients still alive were censored at the last known date of contact. Kaplan-Meier estimates of progression free survival and overall survival were determined and the univariate and multivariate significance of differences in actuarial survival evaluated by the log-rank and Cox regression methods employing SPSS for Windows, version 11.0. Only FOXP1 protein expression status and IPI groupings (0-1, 2-3, and 4-5) were included in the multivariate analysis.

RESULTS

Expression cloning was originally used to identify FOXP1 as the protein recognized by the JC12 monoclonal antibody (12). The epitope recognized by this antibody has not been precisely defined, although the cloning of an NH2-terminally truncated FOXP1 protein confirmed that it is not within the NH2-terminal 212 aa. Three additional members of the FOXP subfamily have now been identified, FOXP2, FOXP3 and FOXP4, and several regions of these protein show a substantial degree of sequence conservation, particularly in the COOH-terminal region containing the JC12 epitope. Before undertaking additional immunohistochemical staining studies it was important to investigate whether the JC12 antibody was specifically labeling the FOXP1 protein, by testing its ability to recognize the other FOXP proteins.

The JC12 Antibody Does Not Recognize the Closely Related FOXP2, FOXP3, or FOXP4 Proteins. The Xpress-tagged FOXP2 and FOXP4 proteins together with the FLAG-tagged FOXP3 protein were transiently expressed in COS-1 cells and their expression was confirmed by immunostaining with the relevant monoclonal antibody against the epitope tag (Fig. 1). Whereas there was some weak background nuclear JC12 staining of endogenous FOXP1 in COS-1 cells, the lack of additional staining comparable to the expression pattern observed with the anti-tag antibodies showed that the JC12 antibody recognized the FOXP1 protein and not the recombinant FOXP2, FOXP3, or FOXP4 proteins (Fig. 1).

FOXP1 Protein Expression in Biopsy Samples of Diffuse Large B-Cell Lymphoma Using the Tissue Microarray. In 101 cases of newly diagnosed DLBCL, there was sufficient material available in the formalin-fixed blocks of the original biopsy specimen to allow construction of two duplicate TMAs. After immunohistochemical labeling with the JC12 monoclonal antibody, to analyze FOXP1 protein expression, two cases showed exclusively cytoplasmic staining and these were excluded for the purpose of this study as no statistically significant conclusions concerning this observation could be
reached. The resulting 99 cases formed the study group for the analysis of FOXP1 expression in DLBCL. The scores for the FOXP1 expression in cases were as follows: 0, 44 patients; 1, 15 patients; 2, 14 patients; and 3, 26 patients. Thus, 59 cases (60%) having scores of 0 or 1 and <30% nuclear positivity were considered negative for FOXP1, and 40 (40%) having scores of 2 or 3 and >30% nuclear positivity were considered positive. Examples of the immunostaining of DLBCL cases from the TMA are illustrated in Fig. 2.

No Relationship was Identified Between FOXP1 Expression and Cytogenetic Data. Routine cytogenetic analysis was done on all the cases included on the TMA. There was no relationship identified between FOXP1 expression and the occurrence of rearrangement, gain or loss involving chromosome band 3p14.1, the site of the FOXP1 gene.

Diffuse Large B-Cell Lymphoma Patients with FOXP1 Protein Expression Have Shorter Overall Survival. Characteristics of the 99 cases for which nuclear FOXP1 expression data was obtained are shown in Table 1. Overall survival and progression free survival for the entire 99 patients were not unusual for a mixed group of patients with DLBCL: 5-year overall survival 57%, median overall survival 8.6 years (95% confidence interval, 4.8-12.4 years); 5 year progression free survival 50%, median progression free survival 4.6 years (95% confidence interval, 1.4-7.8 years). The corresponding outcomes for the FOXP1-negative and FOXP1-positive patients are shown in Table 1 and graphically in Figs. 3 and 4. The dramatic impact of FOXP1 protein expression status is evident. Median overall survival of the FOXP1-negative patients was 12.2 years whereas that of the FOXP1-positive cases was only 1.6 years. Likewise, the 5-year overall survival for the FOXP1-negative patients was 69% and for the FOXP1-positive patients, only 39% (Fig. 3). The progression-free survival curves show a similar trend with median values of 5.7 years for FOXP1-negative patients and 1.25 years for FOXP1-positive patients (Fig. 4).

FOXP1 Expression Is an Independent Prognostic Indicator in Multivariate Analysis. The data presented in Fig. 5 show the expected highly significant impact on overall survival for the three IPI subgroups. However, multivariate analysis indicates that FOXP1 protein expression exerts a strong independent impact even after the IPI subgrouping is taken into account.

![Overall survival for FOXP1-positive versus FOXP1-negative expression. Kaplan-Meier overall survival curves stratifying patients according to FOXP1 protein expression.](clincancers.aacrjournals.org)
consideration. The proportionality of hazards was verified and met for both variables. The results of the Cox regression multivariate analysis are shown in Table 2. These data can be seen graphically when the outcomes within each subgroup are examined (Fig. 6). Progression-free survival was also examined, comparing patients with FOXP1-negative and FOXP1-positive lymphomas broken down by IPI subgroups. Median progression-free survival in the IPI 0 to 1 subgroup had not been reached for either FOXP1-negative or FOXP1-positive cases; for IPI 2 to 3, not reached for the negative cases, 1.5 years for the positive cases; for IPI 4 to 5, 0.5 years for the negative cases and 0.3 years for the positive cases ($P = 0.05$ for the comparison pooled across the three strata).

**DISCUSSION**

The forkhead DNA binding domain that defines the forkhead/winged helix transcription factor family was identified in 1990 (17) in a Drosophila homeotic gene where a mutation caused two spike-head structures in embryos. This family has numerous members with diverse roles including the control of cellular differentiation and proliferation, pattern formation, signal transduction, and oncogenesis (reviewed in ref. 18). The FOXP subfamily members FOXP1, FOXP2, FOXP3, and FOXP4 are distinct from other members of the forkhead family in that they have a COOH-terminal forkhead domain together with a C2H2 zinc finger motif. Mutations in both FOXP2 and FOXP3 genes have associated these molecules with human disease (19–25).

The FOXP1 protein is widely expressed in normal tissues where it is predominantly expressed in the cell nucleus, a distribution that is consistent with its likely function as a transcription factor (12). The FOXP1 gene has been mapped to chromosome 3p14.1, a locus that shows frequent loss of heterozygosity in solid tumors (26) and both FOXP1 mRNA and protein expression are commonly affected in a range of solid tumors (12). A survey of 3p deletions in hematologic malignancies concluded that these were not common and when identified were more distal (3p25-3p26) than those seen in solid tumors (27). In the current study of DLBCL, there was no evidence of cytogenetic alterations at the 3p14.1 locus. It therefore seems likely that alternative mechanisms, such as somatic hypermutation, promoter methylation or other factors controlling transcriptional/translational regulation are largely responsible for the lack of FOXP1 mRNA and protein expression in some cases of DLBCL.

Here we show that FOXP1 protein expression has prognostic significance in patients with de novo DLBCL, treated at a single institution. The FOXP1 immunostaining results allowed scoring as either positive or negative and the overall survival curves clearly show the dramatic impact of FOXP1 expression status on outcome. FOXP1-positive patients had a median overall survival of 1.6 years compared with 12.2 years in FOXP1-negative cases. Ninety percent of patients received potentially curative combination of cyclophosphamide, adriamycin, vincristine, bleomycin, and prednisolone type of chemotherapy programs and both FOXP1-positive and FOXP1-negative patients were treated similarly. Thus, there is no reason to suspect that a treatment bias affected outcome and as this is a retrospective study there was no knowledge of FOXP1 status in determining the choice of treatment or patient management.

In addition, the FOXP1-positive patients showed a greater tendency toward earlier progression, compared with the FOXP1-negative patients. Interestingly, the differences between the overall and progression-free survival curves imply that once disease progression occurs despite primary treatment in the FOXP1-positive cases, subsequent treatment is ineffective and patients die quickly. In contrast, secondary treatment of the FOXP1-negative patients was more effective and prolonged survival after progression. Once again all patients were treated similarly being offered the current best known treatment including high dose chemotherapy and stem cell transplant if appropriate. Because treatment approaches were the same regardless of FOXP1 status (which could not have been known), there is no chance that differing approaches to treatment at diagnosis or relapse could account for the differences in outcome.

**Fig. 4** Progression-free survival for FOXP1-positive versus FOXP1-negative expression. Kaplan-Meier progression-free survival curves stratifying patients according to FOXP1 protein expression.

**Fig. 5** Overall survival for three IPI subgroups. Kaplan-Meier overall survival curves validating the prognostic value of the IPI subgroups in predicting the survival of patients within this case series.
A high IPI score is used routinely by clinicians to identify those patients most at risk from their disease. The analysis of FOXP1 expression within low, medium and high IPI groupings found that FOXP1-negative patients had better overall survival within each group, indicating that FOXP1 expression has predictive value independent of the IPI subgrouping, a finding that was confirmed in multivariate analysis.

The survival data presented here differ from the results obtained in a recent study of FOXP1 expression in 147 DLBCL cases by the Leukaemia and Lymphoma Molecular Profiling Project (11). In that study, expression of the FOXP1 protein was not significantly associated with either overall or event-free survival, and in contrast to the result predicted from the mRNA expression profiling data, FOXP1 protein was commonly expressed in the GC DLBCL subtype. Our confirmation that the JC12 antibody does not recognize other FOXP proteins suggests that recognition of other FOXP proteins by this reagent is not the explanation for this finding. One possible explanation for the discrepancy is that FOXP1 mRNA expression may not accurately reflect protein levels. Both studies investigated cases of de novo DLBCL that were predominantly treated using anthracycline-based chemotherapy, the JC12 monoclonal antibody was used in both studies and the same cutoff of 30% nuclear positivity was employed. The British Columbia Cancer Agency cases are from a single institution and, therefore, patient treatment/management and biopsy tissue fixation are likely to be less variable than in the larger Leukaemia and Lymphoma Molecular Profiling Project series which was obtained from six different institutions in five countries. An additional single center study has also recently reported that strong FOXP1 expression in DLBCL identifies patients with poor outcome (28). Results from independent retrospective studies of the prognostic significance of the expression of individual markers in DLBCL are frequently variable and it is unlikely that any one marker will be predictive of clinical outcome in all series. Immunohistochemical studies on the expression of BCL-6, CD10, BCL-2, and MUM1 have all found that in some DLBCL series there is an association with outcome whereas in others there is not (reviewed by ref. 11). In summary, we have studied the expression of the FOXP1 protein in an independent series of de novo DLBCL and shown this to be an important predictor of overall survival providing additional prognostic information to that obtained from the IPI score alone. The JC12 monoclonal antibody directed against the FOXP1 protein should facilitate further studies to determine the prognostic value of FOXP1 expression in other case series. Further biological studies are required to define the functional consequences of FOXP1 expression in both normal and malignant B cells. These will aim to identify genes whose expression is regulated by FOXP1 and to address whether FOXP1 expression or function may represent an additional therapeutic target for this high-risk group of patients that do not respond well to current treatment regimens.

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**Fig. 6** Overall survival within each IPI subgroup for FOXP1-positive versus FOXP1-negative expression. Kaplan-Meier overall survival curves showing the impact of FOXP1 protein expression on poor survival within each of the three IPI subgroups. P = 0.0005 for trend across all three IPI subgroups.
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