The t(14;18) Is Associated with Germinal Center-derived Diffuse Large B-Cell Lymphoma and Is a Strong Predictor of Outcome

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INTRODUCTION

The t(14;18)(q32;q21) is considered to be the major pathogenetic mechanism in FL because of the deregulation of BCL2. The translocation is also demonstrated in up to 30% of cases of DLBCL (1, 2). The significance of the t(14;18) in DLBCL is unclear and is additionally complicated because a proportion are likely to represent cases with an underlying FL, either occult disease or previously detected. Excluding known transformed FL, the translocation has been demonstrated in 18–20% of patients (2, 3). BCL2 protein is also expressed in a number of cases of DLBCL, irrespective of the presence of the t(14;18); (3–5).

The prognostic significance of the t(14;18) in DLBCL is controversial. Some studies have shown no effect (4–8), whereas others have shown an increased incidence of relapse (2), decreased responsiveness to therapy or shorter survival (9–11), or a correlation with extensive disease (5). In some studies, poor outcome (3–5, 12) or resistance to treatment (7) has been attributed to expression of the BCL2 protein, rather than the presence of the translocation.

These inconsistencies may be partly explained by variations in methodology used to detect the translocation in previous studies. Using PCR strategies for the MBR and mcr alone, ~25% of breakpoints are not detected (13). In addition, the use of paraffin-embedded tissue results in poor quality DNA. The reported incidence of the t(14;18) as detected by MBR and mcr PCR in paraffin tissue is highly variable, with a maximum of 47% of cases of FL positive for the t(14;18) using this methodology.

To attempt to clarify these uncertainties, we have used the Vysis LSI IgH/BCL2 probe set in an interphase FISH assay, which is applicable to paraffin tissue. These probes span the IgH and BCL2 genes entirely, and therefore, this method has the advantage that all breakpoints can be demonstrated. We have used this paraffin FISH assay to retrospectively determine the incidence of the t(14;18) in nodal DLBCL and correlate this with BCL2 expression, a GC immunophenotype and patient outcome.

In conclusion, the t(14;18) is common in DLBCLs, particularly in GC-type DLBCLs, where the presence of the translocation has a poor prognostic effect. BCL2 protein expression defines a group of non-GC DLBCL patients with a poor prognosis.
MATERIALS AND METHODS

Sample Selection and Clinical Information. Formalin-fixed, paraffin wax-embedded lymph nodes from 137 presentation nodal DLBCLs were investigated. Patients were selected only on the basis of availability of clinical information and histological material. Diagnoses were confirmed by pathologic review using the diagnostic criteria defined in the Revised European-American Classification of Lymphoid Neoplasms/WHO classification. All patients were newly presenting, with no previous history of FL or other hematological malignancy, were previously untreated, and received standard primary anthracycline-containing combination chemotherapy (predominantly CHOP) with curative intent.

The median follow up was 28.5 months (range, 0–165 months), and median OS was 39.4 months. Fifty-seven patients had an intermediate risk IPI with a median OS of 14.3 months, with 35% OS at 5 years. Eight patients were high risk and had a median survival of 2.2 months. In the low IPI groups (53 patients), the median survival has not yet been reached, but OS at 5 years was 68%.

Immunohistochemistry. All cases with available paraffin blocks were stained for BCL2 and BCL6 proteins using microwave antigen retrieval and standard immunocytochemistry and CD10 using Tyramine Signal Amplification. Cases were classed as BCL2 expressing if the protein was detected in >50% of tumor cells, and a GC phenotype was defined by the coexpression of CD10 and BCL6, as described previously (15). This immunophenotype was used to classify cases as GC or non-GC type.

FISH Analysis. FISH for the t(14;18) was performed on all cases using the Vysis LSI IgH Spectrum Green/LSI BCL2 Spectrum Orange probe set (32-191018; Vysis, Inc.).

Validation of FISH Assay. This FISH assay has been validated by the analysis of a series of typical FL cases and comparison with a gold standard multiplex PCR technique capable of detecting the majority of known breakpoints (16, 17). Twenty-eight paired frozen and fixed cases of FL and 5 reactive controls were analyzed. FISH produced an accurate result in all cases. FISH was compared with conventional G-banding in a proportion of cases that had cytogenetic results, and concordant results were observed in each case (data not shown).

FISH on Whole Nuclei Extracted from Paraffin-embedded Tissue. Thirty-five-μm thick paraffin sections were de-waxed in xylene and rehydrated through graded alcohols to water. The sections were digested at 37°C in 0.1 M Tris, 0.07 M NaCl buffer (pH 7.1), containing 0.025% Protease XXIV (Sigma P8038) and 0.1% NP40. Digested nuclei were washed in PBS and resuspended in 3:1 methanol:acetic acid, and the nuclear suspension was dropped by pipette onto aminopropyltriethoxy-silane-coated microscope slides (method as described (19, 20)).

FISH was carried out according to the product datasheet using a Vysis Hybrite hybridization system. An additional 90°C predenaturation in hybridization buffer was used to optimize the technique for paraffin nuclei. Denaturation was 73°C for 5 min, and hybridization was at 37°C for 16–24 h. Posthybridization washes were 2 × 2 min in 0.4× SSC/0.3× NP40 at 73°C. 4',6-Diamidino-2-phenylindole was used as the counterstain.

Interpretation of FISH Results. At least 100 intact, individual nuclei were assessed, using the Zeiss Axioplan II microscope. Representative images were captured via a monochrome digital camera using Metasystems ISIS software.

Cases were defined as normal if there were two green (IgH) and two red (BCL2) signals. A t(14;18) was defined when there were extra signals of both the BCL2 and the IgH probes, along with at least two colocalized signals. The presence of additional signals of either or both probes was also noted.

Cases with additional BCL2 signals that were not accounted for as part of the translocation were additionally investigated using the CEP18 (D18Z1) alpha satellite SpectrumAqua probe (32-131018; Vysis, Inc.) to control for aneuploidy of chromosome 18.

Statistical Analysis. The χ² test was used to examine relationships between variables. OS time was calculated from the date of diagnosis until death or date of last follow-up. Survival curves were estimated by the Kaplan-Meier method, using the log-rank test to analyze the statistical differences between the groups. Analyses were carried out using Microsoft Excel and SSCP software.

RESULTS

Incidence of the t(14;18) in DLBCL. Eighteen of 137 (13%) cases of de novo DLBCL were positive for the t(14;18) using FISH (Fig. 1a). The t(14;18) was more commonly associated with GC-type DLBCL: positive in 14 of 47 (30%) cases, compared with 4 of 89 (4.5%) in the non-GC group (Pearson’s χ² = 28.4; P < 0.0001). In the non-GC group with a demonstrable translocation, CD10 was positive in 3 of 4 cases, and BCL6 was positive in the case that was CD10 negative. One t(14;18)-negative case was not classified according to GC status because of the lack of available material. All cases with a translocation expressed the BCL2 protein. None of the BCL2-negative cases had a demonstrable translocation (Pearson’s χ² value = 157.0, P < 0.0001). Forty cases expressed BCL2 protein in the absence of a t(14;18), and of these, 14 (35%) cases were GC-type and 26 (65%) were non-GC DLBCLs. The results are summarized in Table 1.

Demonstration of Alternative Patterns of FISH Signals. Multiple (≥3) BCL2/IgH fusion signals (Fig. 1b) were seen in 3 of 18 (17%) t(14;18) positive de novo DLBCL cases. Extra signals of BCL2 alone (n = 10), IgH alone (n = 14), or both (n = 17; Fig. 1c) were demonstrated in the absence of fusion signals in an additional 41 cases. Using an alpha satellite 18 probe in combination with the BCL2/IgH probe set, 3 of 10 cases with extra signals of BCL2 alone were classified as trisomy 18. The remainder had additional copies of the BCL2 gene.
probe that exceeded the number of copies of the chromosome. In at least 3 of these cases, the signals were numerous and clustered (Fig. 1d), a pattern that is suggestive of gene amplification. BCL2 protein expression was demonstrated in all cases with additional copies of BCL2, and 5 of 4 expressed a GC phenotype. In all cases with extra signals of BCL2 and IgH, the additional BCL2 signals were attributable to additional copies of the chromosome.

The Presence of the t(14;18) Has an Adverse Effect on OS in de Novo DLBCL. The median OS of de novo DLBCL with a t(14;18), BCL2 expression in the absence of a translocation, and BCL2-negative cases was 12.5 and 19.8 months and median survival not yet reached, with 5-year OS of 15.41, and 58%, respectively ($P = 0.005$; Fig. 2a). There was no association between the presence of a t(14;18) or BCL2 protein expression and the IPI. Bone marrow involvement was present in 14 of 89 cases investigated and was more common in 33.3% of the BCL2-positive, t(14;18)− cases and 25% of the t(14;18)+ cases, compared with 7.5% of the BCL2-negative cases (Pearson’s $x^2$ value $= 7.0$, $P = 0.03$). Ten of 14 cases were marrow infiltration with DLBCL, and 4 had evidence of low-level involvement with FL. Forty-eight patients did not have a staging bone marrow. IPI, GC phenotype, BCL2 expression, and t(14;18) all maintained a significant effect on OS in multivariate Cox regression analysis (Table 2).

Cases with extra copies of the BCL2 gene had a similar outcome to the cases with a t(14;18) (median OS, 15.0 months compared with 12.5 months), and cases classified as aneuploid had a median OS of 28 months.

The t(14;18) Is a Strong Predictor of Outcome in GC-type DLBCL. In the de novo GC-type DLBCL patients, 14 of 47 (30%) had a t(14;18) and expressed BCL2 protein, 14 of 47 (30%) expressed BCL2 in the absence of a translocation, and 19 of 47 cases (40%) were negative for both the translocation and BCL2 protein. GC-type DLBCL patients with a t(14;18) had a significantly adverse median OS (12.3 months) compared with those patients without the translocation (2-year survivals were 29 and 63%, respectively; $P = 0.006$). Patients expressing BCL2 protein in the absence of a translocation had a similar outcome to those without BCL2 expression (Fig. 2b).

BCL2 Protein Expression Predicts Outcome of the Non-GC DLBCL Group. The median OS of the non-GC DLBCLs with BCL2 protein expression was 15.0 months but was not reached in the BCL2-negative group ($P = 0.02$; Fig. 2c). OS at 2 years was 64 and 38%, respectively. Only 4 cases had a t(14;18) in the non-GC series, and these patients had a median OS of 12.5.

DISCUSSION

In this study, we have examined the incidence and significance of the t(14;18) in nodal DLBCL. We consider the FISH assay described to be the most appropriate technique for this purpose because it is superior to an optimized multiplex PCR technique (16, 17) and is applicable to paraffin-embedded material (18, 19). In addition, the Vysis LSI IgH Spectrum Green/BCL2 Spectrum Orange probe set used in this study consists of a 1.5-Mb locus-specific IgH probe spanning the entire IgH gene, and a 750-kb BCL2 probe spanning the entire BCL2 gene, ensuring that all known breakpoints can be demonstrated. Furthermore, the definition of a translocation requires both probe splitting and colocalization, minimizing the risk of false positives.

The overall incidence of the t(14;18) in de novo nodal DLBCL was 13% in this study using FISH analysis. The t(14;18) was almost exclusively associated with GC-type DLBCL.
positive in 30% of cases, defined here by the coexpression of CD10 and BCL6 protein. An association between the t(14;18) and CD10 (21) and BCL6 expression (22) has been reported and suggested to represent a FL origin. Recent studies have demonstrated that the t(14;18) occurs exclusively in cases with a GC gene expression profile (23, 24). These cases were also shown to express BCL6 and CD10 by immunocytochemistry (23). In this study, the t(14;18) was also demonstrated in 4 non-GC DLBCL cases. CD10 was positive in the absence of BCL6 in 3 of 4, and BCL6 was positive in the case that was CD10 negative. The expression of one of the GC markers in these cases raises the possibility that an additional mutational or translocation event has resulted in loss of expression of one of the antigens, and it is possible that these cases would have been classified as GC type had gene expression analysis been used.

Taken together, these data suggest that the t(14;18) is an important event in the pathogenesis of GC-type DLBCL. High levels of mutation combined with the high proliferation rate associated with normal GC B cells renders these cells highly susceptible to translocations and favors the theory that the t(14;18) can occur during the GC phase of B-cell differentiation.

The t(14;18) was highly correlated with BCL2 protein expression. BCL2 protein is not expressed in normal GC B cells, and expression of the protein in GC-type DLBCL can therefore be explained as a direct effect of the translocation (25). Some studies have reported BCL2 negativity in significant numbers of cases with a detectable t(14;18; 3–5, 23). These differences may reflect discrepancies in antigen retrieval techniques, resulting in false negative BCL2 staining. An alternative explanation is that genetic events developing during tumor progression may abrogate the need for BCL2 (23). In this study and others (3, 4, 26), a number of cases expressed BCL2 protein in the absence of a demonstrable t(14;18), suggesting that the translocation is not the primary abnormality in all GC-derived tumors. A number of these can be accounted for by the presence of BCL2 gene amplification (26–28), which occurs independently of the t(14;18) and also results in overexpression of the protein (26). In this study, 7 cases had extra copies of the BCL2 gene, suggestive of gene amplification, and 3 of these were GC-DLBCL. In the remaining cases of GC-type DLBCL, expression of BCL2 in the absence of abnormalities of the gene is likely to be because of activation of other pathways such as nuclear factor κB (29).

A number of cases had extra copies of both IgH and BCL2, without fusion signals, suggestive of aneuploidy. Of particular interest was the demonstration of additional BCL2/IgH fusions in a small proportion of cases of de novo DLBCL. In a series of transformed FLs, over half of the cases had multiple fusions (data not shown), suggesting that duplication of the BCL2/IgH fusion gene may be a mechanism of disease transformation.

Of the non-GC DLBCLs, only 34% of cases expressed BCL2 protein in ≥50% of the tumor cells. Non-GC-DLBCLs are thought to be post-GC derived, based on the observation that they have mutated immunoglobulin genes without ongoing somatic mutations (30). By analogy with the normal post-GC B cell, constitutive expression of BCL2 protein would be expected in the majority of these cases. A subset of monocytoid B cells has been shown to be post-GC derived but completely devoid of BCL2 expression (31). An alternative explanation is that BCL2 may have been lost by mutation or deletion of the gene.

### Table 1  Characteristics of the cases of DLBCL investigated for the t(14;18) by FISH

A total of 137 de novo DLBCLs were classified according to their GC status defined by expression of BCL6 protein and CD10. BCL2 protein expression and the t(14;18), as detected by interphase FISH on paraffin-extracted nuclei, were significantly associated with a GC phenotype.

<table>
<thead>
<tr>
<th>BCL2/t(14;18) status</th>
<th>Overall n = 137</th>
<th>BCL2−, t(14;18)− n = 79 (58%)</th>
<th>BCL2+, t(14;18)− n = 40 (29%)</th>
<th>BCL2+, t(14;18)+ n = 18 (13%)</th>
<th>χ² significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC type</td>
<td>47</td>
<td>19 (40%)</td>
<td>14 (30%)</td>
<td>14 (30%)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Non-GC</td>
<td>89</td>
<td>59 (66%)</td>
<td>26 (29%)</td>
<td>4 (5%)</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0–1)</td>
<td>53</td>
<td>32 (60%)</td>
<td>16 (30%)</td>
<td>5 (10%)</td>
<td>P = 0.8</td>
</tr>
<tr>
<td>Intermediate (2–3)</td>
<td>57</td>
<td>31 (54%)</td>
<td>16 (28%)</td>
<td>10 (18%)</td>
<td></td>
</tr>
<tr>
<td>High (4–5)</td>
<td>8</td>
<td>5 (62%)</td>
<td>2 (25%)</td>
<td>1 (13%)</td>
<td></td>
</tr>
<tr>
<td>N/A</td>
<td>19</td>
<td>11</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>75</td>
<td>49 (65%)</td>
<td>18 (24%)</td>
<td>8 (11%)</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>Involved</td>
<td>14</td>
<td>4 (29%)</td>
<td>6 (42%)</td>
<td>4 (29%)</td>
<td></td>
</tr>
<tr>
<td>Not done</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* The t(14;18) was not detected in any of the BCL2 protein negative cases.

* N/A, not applicable.

* Ten of 14 cases were marrow infiltration with DLBCL, and the remaining 4 cases had low-level involvement with FL. There was no association between the type of marrow involvement and the presence of BCL2 abnormalities (data not shown).
with FL, suggesting that some newly presenting DLBCL patients may have evidence of underlying occult FL. Bone marrow involvement is a common feature of FL, and the relatively high frequency of infiltration in t(14;18)-positive patients highlights similarities between FL and DLBCL. The apparent additive adverse prognostic effect of the translocation compared with cases expressing BCL2 protein in the absence of a t(14;18) may be explained by the enhanced rate of transcription (32) and level of expression of BCL2 mRNA (33, 34) and protein (25) as a result of the translocation, producing a quantitative biological effect.

A number of previous studies have failed to demonstrate any impact on survival as a result of BCL2 gene rearrangement (3–8); however, these studies used standard MBR and mcr PCR techniques that do not detect all known translocation breakpoints. In addition, the poor quality of DNA extracted from paraffin tissue makes meaningful retrospective large-scale studies difficult.

The adverse prognostic effect of the t(14;18) was particularly significant when GC-type DLBCL was considered separately. It has been shown that DLBCL can be subdivided into GC and non-GC subtypes and that GC-type DLBCLs have a favorable prognosis (15, 24, 25). The prognostic effect of the t(14;18) has not previously been investigated within the favorable subgroup of GC-DLBCL, however, a proportion of cases with a GC-gene expression profile is associated with a poor outcome. Using genetic abnormalities, other than BCL2 rearrangement, identified by DNA microarray analysis, 24% of patients with a GC profile were in the poor risk group, with 34 of 115 GC-DLBCL patients dead in <2 years (24). The results of this study suggest that the t(14;18) negates the beneficial prognostic effect of the presence of a GC phenotype. It is therefore possible that the poor risk GC-DLBCL patients identified by gene expression analysis (24) did have BCL2 rearrangements (detected in 23% of cases; Ref. 24), which would has an additive adverse prognostic effect and identifies a group of patients with a very poor outcome. b, OS of GC-type de novo DLBCL classified according to BCL2 and t(14;18) status. Kaplan-Meier analysis demonstrating that although BCL2 expression is a poor prognostic feature, the t(14;18)

Table 2  Cox regression analysis of OS

<table>
<thead>
<tr>
<th>Regression coefficient (B)</th>
<th>Exp(B)</th>
<th>SE</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC phenotype</td>
<td>-0.3197</td>
<td>0.7264</td>
<td>0.1110</td>
</tr>
<tr>
<td>BCL2 status</td>
<td>0.7044</td>
<td>2.0226</td>
<td>0.3108</td>
</tr>
<tr>
<td>t(14;18)</td>
<td>0.1861</td>
<td>1.2046</td>
<td>0.0870</td>
</tr>
<tr>
<td>IPI</td>
<td>1.5576</td>
<td>4.7473</td>
<td>0.2993</td>
</tr>
</tbody>
</table>

Fig. 2  a, OS of the de novo DLBCL patients classified according to their BCL2 and t(14;18) status. Kaplan-Meier analysis demonstrating that although BCL2 expression is a poor prognostic feature, the t(14;18)
also account for the poor outcome. In the absence of a detectable translocation, the OS of GC-type DLBCL patients was favorable, regardless of BCL2 protein expression. We therefore propose that the outcome of GC-type DLBCL patients should be interpreted in the context of abnormalities of the BCL2 gene, and demonstration of the t(14;18) should be included as a routine diagnostic test in these cases. In contrast, the t(14:18) was rarely detected in non-GC DLBCLs, and BCL2 protein expression alone identified a group of patients with a very poor outcome. This suggests that the GC and non-GC types of DLBCL have distinct pathophysiology and additionally supports the rationale for the subclassification of DLBCL into GC and non-GC types.

In conclusion, this study has demonstrated the presence of the t(14:18) in a significant proportion of de novo DLBCL. All patients with the translocation expressed the BCL2 protein. The translocation was demonstrated almost exclusively in the GC subtype of DLBCL and was associated with an adverse prognostic effect. The outcome of patients in the non-GC group could be stratified by the expression of BCL2 protein, and these results support the subclassification of DLBCL into GC and non-GC type. The outcome of all patients with nodal DLBCL should be stratified by the analysis of their biological variables, including the presence of the t(14:18).

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REFERENCES

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