The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile


Recently we have identified subgroups of de novo primary diffuse large B-cell lymphoma (DLBCL) based on complementary DNA microarray-generated gene expression profiles. To correlate the gene expression profiles with cytogenetic abnormalities in these DLBCLs, we examined the occurrence of the t(14;18)(q32;q21) in the 2 distinctive subgroups of DLBCL: one with the germinal center B-cell gene expression signature and the other with the activated B cell–like gene expression signature. The t(14;18) was detected in 7 of 35 cases (20%). All 7 t(14;18)-positive cases had a germinal center B-cell gene expression profile, representing 35% of the cases in this subgroup, and 6 of these 7 cases had very similar gene expression profiles. The expression of bcl-2 and bcl-6 proteins was not significantly different between the t(14;18)-positive and -negative cases, whereas CD10 was detected only in the group with the germinal center B-cell expression profile, and CD10 was most frequently expressed in the t(14;18)-positive cases. This study supports the validity of subdividing DLBCL into 2 major subgroups by gene expression profiling, with the t(14;18) being an important event in the pathogenesis of a subset of DLBCL arising from germinal center B cells. CD10 protein expression is useful in identifying cases of DLBCL with a germinal center B-cell gene expression profile and is often expressed in cases with the t(14;18).

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Detection of BCL2 gene rearrangement by polymerase chain reaction

Of 35 cases studied by FISH, 30 cases had high-molecular-weight DNA available for polymerase chain reaction (PCR) analysis. Amplification of the BCL2/IGH translocation at the major breakpoint region (mbr) and the minor cluster region (mcr) was performed as described by Sharp et al.\textsuperscript{[1]10} using an OmiGene thermocycler (Hybaid, Middlesex, United Kingdom). Positive controls consisted of DNA extracted from the human B-cell lymphoma line RL-7 for the mbr of BCL2, and DHL-16 for the mcr of BCL2. Negative controls consisted of sterile water instead of DNA, and DNA from normal peripheral blood mononuclear cells obtained from healthy volunteer donors. Standard precautions were taken to guard against PCR contamination, and amplified materials were analyzed in a laboratory physically separated from the one where unamplified materials were handled.\textsuperscript{[1]11}

Immunohistochemistry for CD10, bcl-2, and bcl-6

Formalin-fixed, paraffin-embedded 5-μm sections were stained with the avidin-biotin-peroxidase complex (ABC) method.\textsuperscript{[12]} Antigen retrieval was performed by incubation for 30 to 60 minutes in 10 mM citrate buffer (pH 6.0) in a 95°C water bath followed by 20 minutes of cooling at room temperature (32 cases from Nebraska), or using EDTA buffer at pH 8.0 for CD10 and bcl-6 (3 cases from Stanford). Antibodies to the following antigens were used: CD10 (clone 56C6, Ventana, Tucson, AZ, for cases from Nebraska; or Novocastra, Newcastle upon Tyne, United Kingdom, for cases from Stanford), bcl-2 (clone 24; Dako, Carpinteria, CA), and bcl-6 (polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, for cases from Nebraska; or PG-B6p, Dako, for cases from Stanford). A tumor was considered to be positive for expression of a specific antigen if more than 10% of the tumor cells showed appropriate immunoreactivity.

Statistical analysis

The Fisher exact test was used to compare the frequencies of the t(14;18) in the GCBL and ABL subgroups of DLBCL. This test was also used to compare the frequencies of CD10, bcl-2, and bcl-6 protein expression in the t(14;18)-positive and -negative subgroups of DLBCL. The survival analysis was performed on cases with and without t(14;18) using log-rank test. The Student $t$ test was used to compare the microarray measurements of gene expression levels between the t(14;18)-positive and -negative subgroups of DLBCL. Genes differentially expressed between the 2 groups were selected with a cutoff of $P < .05$. The ratio of specific gene expression in the t(14;18)-positive cases versus the t(14;18)-negative cases of the GCBL subgroup was calculated by dividing the mean fluorescence intensity of the t(14;18)-positive by the mean fluorescence intensity of the t(14;18)-negative GCBL cases. Agglomerative hierarchical clustering was performed using the CLUSTER program (M. Eisen: http://www.microarrays.org/software).\textsuperscript{[13]}

Results

Occurrence of the t(14;18) in DLBCL

To define the subset of DLBCL with the t(14;18), we applied interphase FISH to nuclei isolated from tumor tissues (Figure 1). Of the 35 cases studied, a t(14;18) was detected in 7 of 20 cases (35%) of GCBL-DLBCL but in none of 15 cases of ABL-DLBCL ($P = .01$, Fisher exact test; Figure 2). Using PCR, we found BCL2/IGH gene rearrangements in 3 of 5 t(14;18)-positive cases by FISH, which is similar to our detection rate in follicular lymphoma. None of the t(14;18)-negative cases by FISH had a BCL2/IGH rearrangement by PCR.

Patients with the t(14;18) included 6 women and 1 man ranging from 59 to 81 years of age at the time of diagnosis. There was no significant difference in overall survival when these 7 patients were compared with the t(14;18)-negative patients in the entire group ($P = .35$) or in the GCBL group ($P = .99$), but the number of cases studied is small.

Bcl-2, bcl-6, and CD10 protein expression

To further characterize the cases, we performed immunohistochemistry to determine the expression of bcl-2, bcl-6, CD10, and CD20 proteins. These results are summarized in Table 1. The frequencies of bcl-2 protein expression were not significantly different between the GCBL (15 of 20, 75%) and ABL (10 of 15, 67%) subgroups of DLBCL ($P = .46$). Four of 7 t(14;18)-positive cases (57%) and 21 of 28 t(14;18)-negative cases (75%) of DLBCL showed bcl-2 protein expression in the tumor cells. This finding indicates that the t(14;18) does not always lead to bcl-2 protein expression and that bcl-2 expression is often present in DLBCL in the absence of the t(14;18). Expression of bcl-6 protein was detected in 30 of 35 cases (86%) of DLBCL and showed no significant association with any subgroup. However, CD10 protein was detected in 11 of 20 cases (55%) in the GCBL subgroup, including 6 of 7 with the t(14;18) (86%), and none in the ABL subgroup ($P < .001$). Protein expression data for the 7 cases with the t(14;18) is
shown in Table 2. All 7 cases with the t(14;18) showed expression of bcl-6 protein, but not always detectable bcl-2 or CD10 protein by immunohistochemistry.

**Gene expression profile associated with the t(14;18) in DLBCL**

All 7 cases with the t(14;18) by FISH had the GC gene expression signature. Six of these cases, all positive for CD10 protein expression, had very similar gene expression patterns and formed a tight cluster together with normal GC B cells and the t(14;18)-positive cell line SUDHL-6 in the dendrogram (Figure 2). However, one t(14;18)-positive case (DLCL-0003), which was negative for CD10 and bcl-2 protein expression, was located outside of this tight cluster, indicating a substantial difference in its overall gene expression pattern. BCL2 gene expression was quite variable and generally low in GCBL-DLBCL as compared to the reference pool when hybridization to clones 1336385 and 342181 were examined (Figure 3). When the sequences of the 3 different bcl-2 cDNA clones (232714, 342181, 1336385) used for microarray analysis were examined, only clone 232714 was able to consistently detect the bcl-2 transcripts in t(14;18)-positive lymphomas and the t(14;18) lymphoma cell line SUDHL-6. This clone contained a cDNA fragment from the middle portion of BCL2 gene and can hybridize to truncated transcripts from cases with bcl-2 translocation at the mbr. When we examined BCL2 gene expression as indicated by cDNA clone 232714, we found that 6 of 7 t(14;18)-positive DLBCL cases show overexpression (the exception being the outlying case DLCL-0003). The case (DLCL-0018) with translocation at the mcr showed concordant results with all the clones. A group of t(14;18)-negative cases in both the GCBL and ABL groups also had high levels of bcl-2 transcripts, which are detected by all 3 cDNA clones as expected.

To search further for genes differentiating the t(14;18)-positive from t(14;18)-negative cases of GCBL-DLBCL, all genes on the Lymphochip (4026 clones) including GC B cell-associated genes were compared between the 7 cases with the t(14;18) and the 13 cases of GCBL-DLBCL without the t(14;18). We identified genes or expressed sequence tags (ESTs) represented by 403 cDNA clones showing significant differential expression \((P < .05)\) using the 2-tailed Student \(t\) test. Genes or ESTs represented by 251 clones were expressed at higher levels in the t(14;18)-positive cases, whereas other genes or ESTs represented by 152 clones were expressed at lower levels in the t(14;18)-positive cases (Figure 4). The 10 most differentially expressed genes are listed in Table 3.

**Discussion**

The t(14;18)(q32;q21) translocation is a characteristic feature of follicular lymphoma and is thought to be the initiating event in
These 7 cases clustered closely with each other, and with normal GC subgroup, wherein the frequency was 35% (7 of 20 cases). Interestingly, these cases occurred exclusively in the GCBL and detected the t(14;18) in 20% (7 of 35 cases) of DLBCL. The FISH assay has a higher sensitivity for the presence of the t(14;18) in a series of DLBCL using 2 techniques, FISH and PCR. The FISH assay has a higher sensitivity and detected the t(14;18) in 20% (7 of 35 cases) of DLBCL. These ratios are a measure of the relative gene expression of the experimental sample versus the reference standard and are depicted according to the color scale shown at the bottom, from a fluorescence ratio of 0.25 to 4 (-2 to +2 in log-base 2 units). GC B and GC centroblasts indicate GC B cells and GC centroblasts from tonsil; FL, follicular lymphoma. Note that normal GC cells have very low levels of bcl-2 expression. All follicular lymphoma cases, the t(14;18)-positive SUDHL-6 cell line, and 6 of the 7 DLBCL cases with the t(14;18) translocation have overexpression of bcl-2 when assessed with clone 232714, which detects bcl-2 mRNA truncated at the 3' end due to breakage at the mbr. The case DLCL-0018 has a breakpoint at the mcr by PCR assay and the intact message is detectable with all cDNA clones. The t(14;18)-negative DLBCLs that overexpress bcl-2 have bcl-2 mRNA detectable by clones other than 232714, indicating that the message is not truncated.

By analogy, DLBCL with the same translocation may be related to follicular lymphoma and the t(14;18) may also be important in its pathogenesis. In this study, we looked for the presence of the t(14;18) in a series of DLBCL using 2 techniques, FISH and PCR. The FISH assay has a higher sensitivity and detected the t(14;18) in 20% (7 of 35 cases) of DLBCL. Interestingly, these cases occurred exclusively in the GCBL subgroup, wherein the frequency was 35% (7 of 20 cases). Six of these 7 cases clustered closely with each other, and with normal GC cells and the t(14;18)-positive lymphoma cell line SUDHL-6 in the dendrogram, indicating very closely related gene expression profiles. The fact that these 6 cases shared a unique translocation and had gene expression profiles that were very similar to those of normal GC cells provides strong evidence that DLBCL with the t(14;18) is a distinctive subset within the GCBL subgroup. This observation also supports the validity of subdividing DLBCL into the GCBL and ABL subgroups, and indicates that tumors sharing an important genetic abnormality may also share a distinctive gene expression profile.

We also examined the messenger RNA (mRNA) and protein expression patterns of bcl-6 and CD10 because of their known association with normal and neoplastic GC B cells. The bcl-6 protein was expressed in all cases with the t(14;18), but it was also expressed in a high percentage of the remaining cases. CD10 was highly expressed at the mRNA level and frequently detected at the protein level in GCBL-DLBCL, but none of the cases of the ABL subgroup expressed CD10 by protein immunohistochemistry. These results suggest that CD10 protein expression is a specific, but not a very sensitive, marker for GCBL-DLBCL. Moreover, CD10 protein expression was demonstrated in a high percentage (86%) of the t(14;18)-positive cases.

Some of our t(14;18)-positive DLBCLs did not show detectable bcl-2 protein expression by immunohistochemistry, in keeping with previously published reports. Kramer et al reported that 12 of 25 cases of DLBCL with BCL2 gene rearrangement had low or absent expression of bcl-2 protein. In the study reported by Tang et al, only 2 of 15 cases of DLBCL with the BCL2 gene rearrangement were found to have a high level of bcl-2 protein expression. It

Figure 3. BCL2 gene expression in DLBCL. Each horizontal row represents a separate cDNA clone on the microarray and each vertical column a separate case. The results are represented as the ratios of the hybridization signals on each spot on the microarray generated by the experimental mRNA samples versus the reference mRNA sample. These ratios are a measure of the relative gene expression of the experimental sample versus the reference standard and are depicted according to the color scale shown at the bottom, from a fluorescence ratio of 0.25 to 4 (-2 to +2 in log-base 2 units). GC B and GC centroblasts indicate GC B cells and GC centroblasts from tonsil; FL, follicular lymphoma. Note that normal GC cells have very low levels of bcl-2 expression. All follicular lymphoma cases, the t(14;18)-positive SUDHL-6 cell line, and 6 of the 7 DLBCL cases with the t(14;18) translocation have overexpression of bcl-2 when assessed with clone 232714, which detects bcl-2 mRNA truncated at the 3' end due to breakage at the mbr. The case DLCL-0018 has a breakpoint at the mcr by PCR assay and the intact message is detectable with all cDNA clones. The t(14;18)-negative DLBCLs that overexpress bcl-2 have bcl-2 mRNA detectable by clones other than 232714, indicating that the message is not truncated.

Table 3. The 10 genes most differentially expressed between the t(14;18)-positive and t(14;18)-negative cases of GCBL-DLBCL.

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Ratio*</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Similar to ribosomal protein S5; clone = 1241660</td>
<td>2.4</td>
<td>.0001</td>
</tr>
<tr>
<td>Protein phosphatase 2, regulatory subunit A (PR 65), beta isoform; clone = 626746</td>
<td>2.1</td>
<td>.0001</td>
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<tr>
<td>EWS = RNA binding protein; clone = 1192958</td>
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<td>.0001</td>
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<td>Unknown; clone = 1241826</td>
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<td>Unknown; clone = 1241577</td>
<td>1.7</td>
<td>.0002</td>
</tr>
<tr>
<td>Lactate dehydrogenase B; clone = 624760</td>
<td>1.9</td>
<td>.0002</td>
</tr>
<tr>
<td>MP (inosine monophosphate) dehydrogenase 2; clone = 292008</td>
<td>2.2</td>
<td>.0003</td>
</tr>
<tr>
<td>Ribosomal protein L27; clone = 272185</td>
<td>1.9</td>
<td>.0003</td>
</tr>
<tr>
<td>Unknown; clone = 1340942</td>
<td>0.8</td>
<td>.0004</td>
</tr>
<tr>
<td>Unknown; clone = 1186027</td>
<td>0.7</td>
<td>.0005</td>
</tr>
</tbody>
</table>

*Ratio of mean levels of mRNA for each cDNA clone between the t(14;18)-positive and -negative cases.
†Probability based on Student t test.
is possible that bcl-2 expression is essential early in lymphomagenesis, but additional genetic abnormalities developing during tumor progression may abrogate the requirement for bcl-2. The mechanisms for dissociation between BCL2 gene rearrangement and bcl-2 protein expression are unclear. At the transcription level, bcl-2 expression was quite variable and showed no dependence on the presence of t(14;18). In t(14;18)-positive cases of DLCLB, bcl-2 transcripts were up-regulated in 6 of 7 cases with a probe that can detect bcl-2 mRNA in cases with BCL2 translocation at the mbr. Case DLCL-0003, which was negative for both bcl-2 and CD10 expression, showed a very low or undetectable level of bcl-2 transcripts with all cDNA clones. A group of t(14;18)-negative cases in both GCBL and ABL groups showed high levels of bcl-2 transcripts detected by all cDNA clones, indicating that alternative mechanisms up-regulating bcl-2 expression are operative in these cases. A set of genes differentially expressed between GCBL- DLCL with and without the t(14;18) can be identified by statistical analysis (Figure 4 and Table 3). However, due to the small number of cases compared in our study, these results are considered preliminary and further analysis in a larger study is necessary to identify differentially expressed genes at a high level of confidence.

How is primary DLBCL with a t(14;18) different from follicular lymphoma that typically has a t(14;18)? In this study, the t(14;18)-positive cases of DLBCBL were carefully reviewed and no follicular structures were seen morphologically or by immunostaining for follicular dendritic cells. Furthermore, none of these patients had a prior history of follicular lymphoma. Therefore, these cases are not follicular lymphomas misdiagnosed as DLBCL. The fact that primary DLBCLs with the t(14;18) have gene expression profiles similar to normal GC B cells suggests that the t(14;18) plays an important role in the pathogenesis of this subset of DLBCL, just as it does in follicular lymphoma. The t(14;18) is thought to occur as a low-frequency event during IGH gene rearrangement in normal B-cell maturation, resulting in abnormal bcl-2 overexpression, which allows the cells to escape apoptosis if they become GC B cells. However, the t(14;18) is not sufficient for neoplastic transformation. In fact, rare cells with the t(14;18) are detectable in tonsils, lymph nodes with follicular hyperplasia, and blood lymphocytes from healthy individuals. These GC B cells harboring the t(14;18) are able to undergo repeated cycles of clonal expansion when the appropriate antigen is encountered. Extensive proliferation introduces additional genetic changes that lead to neoplastic transformation. The different genetic abnormalities occurring during this phase may lead to follicular lymphoma or primary DLBCL. We illustrate this concept in Figure 5.

It is likely that a number of different genetic lesions can result in the development of DLBCL from a t(14;18)-positive GC B cell, and each of these genetic alterations may be associated with a unique gene expression signature. One could speculate that the outlying case DLCL-0003 with the t(14;18) was associated with a different transformational event than the other 6 cases that clustered tightly together. We believe that genetic and gene expression analysis of additional cases will allow us to define the critical genetic events that lead to neoplastic transformation of GC B cells.

Acknowledgments

We thank Grant Wu and Greg Cochran for technical assistance.

References


Erratum

In the article by Pinotti et al entitled “Residual factor VII activity and different hemorrhagic phenotypes in CRM+ factor VII deficiencies (Gly331Ser and Gly283Ser),” which appeared in the February 15, 2002, issue of Blood (Volume 99:1495-1497), 3 author names were misspelled. The correct spellings are Mirko Pinotti, Daniela Etro, and Guglielmo Mariani.
The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile