Identification of CCND3 and BYSL as Candidate Targets for the 6p21 Amplification in Diffuse Large B-Cell Lymphoma

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Abstract

Purpose: Increases in gene dosage through DNA amplification represents a common feature of many tumors and can result in the up-regulation of tumor-promoting genes. Our recent genome-wide, array-based comparative genomic hybridization analysis of 66 cases of diffuse large B-cell lymphoma found that genomic gain of 6p21 was observed in as many as 17 cases, including 14 cases with low-level copy number gain and three cases with high-level copy number gains (amplifications).

Experimental Design and Results: To identify the target gene(s) for 6p21 amplification, we constructed a detailed amplicon map at the region of genomic amplification with the aid of high-resolution contig array-based comparative genomic hybridization glass slides, consisting of contiguously ordered bacterial artificial chromosome/P1-derived artificial chromosome clones covering 3 Mb throughout the 6p21 amplification region. Alignment of the amplifications identified a minimally overlapping 800 kb segment containing 15 genes. Quantitative expression analysis of the genes from both patient samples and the SUDHL9 cell line revealed that CCND3 and BYSL (1.9 kb telomeric to the CCND3 gene locus) are the targets of 6p21 genomic gain/amplification.

Conclusions: Although it is known that t(6;14)(p21;q32) induces aberrant overexpression of CCND3 in B-cell malignancies, we were able to show that CCND3, which encodes the cyclin D family member protein that controls the G1-S phase of cell cycle regulation, can also be a target of genomic gain/amplification. Overexpression of CCND3 through genomic amplification is likely to lead to aberrant cell cycle control, although the precise biological role of BYSL with respect to tumorigenesis remains to be determined.

Deregulation of oncogenes via genomic amplifications is a common occurrence in various tumors, including malignant lymphomas. Previous studies have reported several candidate genes of genomic amplification in malignant lymphomas, such as 2p15 amplification with REL overexpression (primary large B-cell lymphoma of the gastrointestinal tract, Hodgkin lymphoma; refs. 1–3), 9p24 amplification with REL overexpression (primary mediastinal B-cell lymphoma, Hodgkin lymphoma; refs. 4–6), and 10p12 amplification with BMI-1 overexpression (mantle cell lymphoma; ref. 7). Very recently, we showed that CCND3 and/or CCND3 (1.9 kb telomeric to the CCND3 gene locus) are the targets of 6p21 amplification.

To identify the target gene(s) for 6p21 amplification, we constructed a detailed amplicon map at the region of genomic amplification with the aid of high-resolution contig array-based comparative genomic hybridization glass slides, consisting of contiguously ordered bacterial artificial chromosome/P1-derived artificial chromosome clones covering 3 Mb throughout the 6p21 amplification region. Alignment of the amplifications identified a minimally overlapping 800 kb segment containing 15 genes. Quantitative expression analysis of the genes from both patient samples and the SUDHL9 cell line revealed that CCND3 and BYSL (1.9 kb telomeric to the CCND3 gene locus) are the targets of 6p21 genomic gain/amplification.

Conclusions: Although it is known that t(6;14)(p21;q32) induces aberrant overexpression of CCND3 in B-cell malignancies, we were able to show that CCND3, which encodes the cyclin D family member protein that controls the G1-S phase of cell cycle regulation, can also be a target of genomic gain/amplification. Overexpression of CCND3 through genomic amplification is likely to lead to aberrant cell cycle control, although the precise biological role of BYSL with respect to tumorigenesis remains to be determined.

Our recent array-based comparative genomic hybridization (array CGH) study of diffuse large B-cell lymphoma (DLBCL) has identified recurrent high-level genomic aberrations as 1q31-q32, 2p15, 6p21, 9p24, 11q22-q24, 13q31, and 18q21 (9). Of these genomic alterations, the various cytogenetic abnormalities of chromosome band 6p21 in mature B-cell malignancies include translocations and amplifications. t(6;14)(p21;q32) has been previously reported in a variety of B-cell malignancies, such as DLBCL and splenic marginal zone lymphomas, and it has been shown that deregulation of CCND3 is a result of this translocation (10).

Although recurrent amplifications of 6p21 have been detected and described in B-cell lymphomas, such as follicular cell lymphoma, mantle cell lymphoma, and DLBCL (11–13), no detailed studies have been conducted of the gene(s) responsible for the amplification. To further identify these target gene(s) in DLBCL, we did the “contig” array CGH using

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glass slides on which contiguously ordered bacterial artificial chromosome/P1-derived artificial chromosome (BAC/PAC) clones were spotted throughout 3 Mb of the 6p21 genome.

Materials and Methods

Tumor samples and B-cell lymphoma cell lines. Data of genomic gains and losses region of 66 DLBCL cases have been reported previously (9). The cell lines used in the study presented here were SUDHL9 (Southwestern University: diffuse large B-cell lymphoma cell line), SP49 (mantle cell lymphoma cell line; ref. 14), and OCI-LY8 (immunoblastic B-cell lymphoma; ref. 15). These cell lines were maintained in RPMI 1640 supplemented with 10% FCS at 37°C (immunoblastic B-cell lymphoma; ref. 15). These cell lines were

DNAs and RNA samples. DNA was extracted with a standard phenol-chloroform method from lymphoma specimens of tumors and of SUDHL9, SP49, and OCI-LY8. Normal DNA was prepared from peripheral blood lymphocytes of healthy male donors. Total RNA was extracted with the standardized guanidium isothiocyanate and cesium chloride method from human placenta and normal lung as well as from SUDHL9, SP49, and OCI-LY8.

Fluorescence in situ hybridization and comparative genomic hybridization analyses. Fluorescence in situ hybridization (FISH) and CGH were done as described elsewhere (8).

Genome-wide array-based comparative genomic hybridization. DNA preparation, labeling, array fabrication, and hybridization were done as described elsewhere (8, 9, 16). Briefly, the array consisted of 2,088 BAC and PAC clones, covering the human genome at a 1.5 Mb resolution, from chromosome/P1-derived artificial chromosome (BAC/PAC) Resource Center at the Children’s Hospital Oakland Research Institute in Oakland, CA (http://bacpac.chori.org). The thresholds for the log2 ratio of gains and losses were set at log2 ratios of +0.2 and −0.2, respectively. High-level gain (amplification) was defined as log2 ratio ≥+1 and low-level copy number gain was defined as +0.2 ≤ log2 ratio < +1.0 (8).

Contig array-based comparative genomic hybridization. Twenty-five BAC/PACs of 6p21 were isolated from their bacterial cultures with the relevant antibiotics and extracted with a plasmid mini kit (Qiagen, Valencia, CA). The exact location of each clone was determined by standard FISH analysis. Degenerate oligonucleotide primed PCR (17) was done on the DNA of BAC/PAC clones as described before (8). Degenerate oligonucleotide primed PCR products were dissolved in 30 µL of TE buffer [100 mM Tris-HCl and 1 mM EDTA (pH 7.5)], and 10 µL of Solution I (Takara Bio, Inc., Tokyo, Japan) was added to each of the products, which were then spotted in triplicate onto the Hubble-activated slides (Takara Bio) using the Stampman Arrayer (Nippon Laser and Electronics Lab, Nagoya, Japan) with a split pin. Slides were fixed in 0.2% SDS for 2 minutes and in 0.3 N NaOH for 5 minutes, then dehydrated with 100% cold ethanol for 3 minutes, and finally air dried. DNA preparation, labeling, array fabrication, and hybridization were done according to the method described previously (8, 9, 18, 19).

Image scanning. The Agilent Micro Array Scanner (Agilent Technologies, Palo Alto, CA) was used for scanning analysis. The array images thus acquired were analyzed with the Genepix Pro 4.1 package (GenePix Pro 4.1 (Axon Instruments, Inc., Foster City, CA).

Reverse transcription-PCR analysis for screening of candidate genes. Human placenta, normal lung, SUDHL9, SP49, and OCI-LY8 were subjected to reverse transcription-PCR (RT-PCR) analysis, whereas SuperScriptII (Life Technologies, Division of Life Technologies, Inc., Gaithersburg, MD) was used for cDNA driven from human placenta and normal lung. Each 5 µg of total RNA was reverse-transcribed into cDNA dissolved in 40 µL of distilled water. RT-PCR was done for 25 genes using the specific corresponding primers. Gene names and accession numbers were as follows: FOXP4 (NM_138457), MDF1 (NM_005586), TFE3 (NM_001672), PGC1α (NM_002630), BRS3 (NM_006563), Cof499 (NM_013397), USP49 (NM_004275), BSYL (NM_004053), CCND3 (NM_001760), TBX21 (NM_138572), LOC389389 (XM_371820), GIGA1A (NM_000409), GIGA1B (NM_002998), MRPS10 (NM_018141), TRECER (AF297872, AL096814), Cof3315 (NM_015255), RDS (NM_003222), TBCB (NM_003192), KIAA0420 (XM_166479, XP_166479), RPL7L1 (NM_198486), PTCRA (NM_138296), TNRC5 (NM_006586), LOC389390 (XM_374167, XP_374167), GNM (AI014777), and PEX16 (NM_000287). Each primer was designed so that the Tm was between 55°C and 60°C. Amplifications were done on a Thermal Cycler (Perkin-Elmer Corporation, Norwalk, CT), and RT-PCR was done with the touchdown PCR method. The reactions comprised 10 cycles of denaturation (94°C, 0.5 minutes), annealing (63°C, 0.5 minutes, 1°C decrease per two cycles), and extension (72°C, 2.5 minutes), followed by 35 cycles of denaturation (94°C, 0.5 minute), annealing (58°C, 0.5 minute), and extension (72°C, 2.5 minutes), and a final extension of 5 minutes at 72°C. The annealing temperature of the reaction ranged from 63°C to 58°C. RT-PCR was also done under different conditions by changing the annealing temperature from 65°C to 60°C or from 60°C to 55°C. If no PCR products were obtained, we designed new primer sets to confirm the negativity of genes. All PCR products were separated by electrophoresis and purified using the QIA quick Gel Extraction kit (Qiagen). Direct sequence determination with the same primers used for nested-PCR was done with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis. The Mann-Whitney test was done for detecting significance in expression levels of CCND3 and BSYL between groups with and without 6p21 genomic amplifications. All the statistical analyses were conducted with the STATA version 8 statistical package (StataCorp, College Station, TX).

Results

Recent high-level amplification at 6p21 in diffuse large B-cell lymphoma. The array CGH analysis at a resolution of 1.5 Mb throughout the whole genome showed that 26 of 66 DLBCL cases had copy number gains on chromosome 6p (Fig. 1). Seventeen of the 26 cases included 6p21 gain, with 3 of
the 17 cases showing genomic amplification (log₂ ratio >1) at PAC, RP5-973N23. As shown in Fig. 2, partial genomic profiles of three individual tumors (D906, D648, and D912) and the SUDHL9 cell line showed that each of the highest peaks was detected at PAC, RP5-973N23, indicating that the peaks may be biologically significant. FISH analysis using PAC, RP5-973N23 as the probe confirmed strong genomic amplification at 6p21 in SUDHL9 (>15 copies; Fig. 3). The amplification-overlapping region of the three tumors (D906, D648, and D912) and SUDHL9 could be clearly defined to the restricted region between BAC, RP11-552E20 (40.3 Mb) and PAC, RP5-895C5 (43.5 Mb) at 6p21.

**Determination of amplicon core by contig array comparative genomic hybridization.** We speculated the target genes of the high copy number gains of 6p21 were within the 3 Mb region between 40.3 Mb (BAC, RP11-552E20) and 43.5 Mb (PAC, RP5-895C5). To specify the alterations of 6p21 in greater detail, we constructed high-resolution contig array glass slides containing 25 BAC/PAC clones, which were contiguously placed throughout the 3 Mb region at the 6p21 genome. Contig array CGH was conducted for the SUDHL9 cell line and three tumors (D906, D648, and D912), for which the genome-wide array CGH showed high copy number gains at 6p21. The analysis was also conducted for OCI-LY8 and SP49 cell lines that did not show genomic amplification at chromosome 6. Mixed partial individual genomic profiles of 6p21 for D906, D648, D912, SUDHL9, and OCI-LY8 are shown in Fig. 4. It was found that the “amplicon core” region of 6p21 was narrower than could be expected from data obtained from the genome-wide array CGH. Contig array CGH showed that the minimal common region (amplicon core region) of the three tumors and SUDHL9 was 800 kb in length and ranged from BAC, RP11-328M4 (41.6 Mb) to PAC, RP1-139D8 (42.3 Mb). The amplicon core contained 15 genes and it was speculated that candidate genes of 6p21 amplification were located within this region.

**Reverse transcription-PCR analysis of the genes within the 6p21 amplification region of SUDHL9.** The contig array CGH for SUDHL9 showed that 25 known genes reside within the 6p21 amplification region (2.5 Mb in length), which includes the “amplicon core.” Fifteen of these genes lie within the amplicon core, whereas the other 10 are localized centromerically to it (Fig. 4). RT-PCR using human placenta, normal lung, SUDHL9, SP49, and OCI-LY8 cell lines was used to screen for expressions of these 25 genes. The genes and primers used for RT-PCR and the results are shown in Supplemental Table S1. The expected sizes of all products obtained by RT-PCR were confirmed by electrophoresis. Expression of 13 genes (FOXP4, TFEB, FRS3, USP49, BYSL, CCND3, TBN, MRPS10, TRERF1, TBCC, KIAA0240, PTCRA, and TNRC5) could be detected in SUDHL9 but not of the other 12 genes. MDF1 and GUGA1A did not result in any PCR products in the five RNA samples studied, and although the RT-PCR of MDF1 and GUGA1A was done under different conditions by changing the annealing temperature from 65°C to 60°C or from 60°C to 55°C, and with different primer pairs to detect these genes, no bands were detected. We, therefore, concluded that the expression levels of these two genes were undetectable and excluded them for further expression analysis.

**Northern blot analysis for screening gene expressions.** Because expressions of the 13 genes were confirmed in SUDHL9 by RT-PCR analysis, we next did Northern blotting for quantitative...
analyses of gene expression in five samples (human placenta, normal lung, SUDHL9, SP49, and OCI-LY8). Expression levels of BYSL, CCND3, TBN, TBCC, and KIAA0240 in SUDHL9 were on average 1.5 to 4 times higher than in human placenta, normal lung, SP49, and OCI-LY8 (Table 1). However, expressions of the other eight genes did not show good correlation with the level of genomic amplification. The possible candidate genes for 6p21 amplification were thus BYSL, CCND3, TBN, TBCC, and KIAA0240. To examine gene expressions of other hematologic malignancies, we conducted Northern blot analysis of these five genes for a variety of hematologic malignant cell lines that did not feature 6p21 amplification. These cell lines comprised five B-cell lymphomas, three T-cell lymphomas, one multiple myeloma, and two acute myeloid leukemias. Expression levels

Fig. 2. Individual partial genomic profiles of chromosome 6p for three patient samples (D906, D648, and D912) and the SUDHL9 cell line. Horizontal lines, megabase from 6p telomere to centromere; vertical lines, log2 ratio. Each spot is contiguously ordered from p telomere to centromere with, on average, 1.5 Mb resolution. The threshold for gain and loss was defined as the log2 ratio of +0.2 and −0.2, respectively. A, D906; B, D648; C, D912; D, SUDHL9. Vertical thick arrow, highest peak at 6p21. Log2 ratios: D906, 1.7; D648, 1.1; D912, 1.1; SUDHL9, 2.6.

Fig. 3. CGH (A) and FISH (B) data of a patient sample (D906) and SUDHL9. A. Conventional CGH. Conventional CGH accurately shows amplification at 6p21 in D906. Conventional CGH of SUDHL9 also shows genomic amplification at 6p21-qtel but fails to detect the amplicon at 6p21. B. FISH. RP5-973N23 (red signal) was the probe used for FISH analysis of the SUDHL9 cell line. Arrows, 6p21 locus.
of BYSL, CCND3, TBN, TBCC, and KIAA0240 in the SUDHL9 cell line were again higher than in other cell lines (Fig. 5A).

**Candidate gene identification by Northern blot analysis.** Five patient samples, three of which possessed 6p21 amplifications, were subjected to Northern blot analysis. The expression level of BYSL in two patients (D906 and D912) with 6p21 amplifications was, on average, 1.7 times higher than that in patients without the amplification (Fig. 5B). Similarly, the

**Table 1. Northern blot analysis of 6p21 candidate genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene size (kb)</th>
<th>Probe size (bp)</th>
<th>Human placenta</th>
<th>Lung</th>
<th>SUDHL9</th>
<th>OCI-LY8</th>
<th>SP49</th>
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<td>FOXP4</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>TFEB</td>
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<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>USP49</td>
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<td>440</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
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<td>+</td>
<td>+++</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
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<td>2,000</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>TBN</td>
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<td>–</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>MRPS10</td>
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<td>TBCC</td>
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<td>200</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>+/–</td>
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<td>KIAA0240</td>
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<td>–</td>
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</table>

Note: The strength of the signal within each blot is represented from strongest (+++) to undetected (–). The mRNA expression level for each sample was normalized on the basis of the corresponding β-actin expression. Gene expression was normalized relative to β-actin (gene expression/β-actin): –, gene/β-actin ≤ 0.25; –/+), 0.25 ≤ gene/β-actin < 0.75; +, 0.75 ≤ gene/β-actin < 1.25; ++, 1.25 ≤ gene/β-actin < 1.75; ++++, gene/β-actin ≥ 1.75.

Abbreviation: ND, not done.
CCND3 expression level in two patients (D648 and D912) with 6p21 amplifications was on average 2.5 times higher than that in patients without the amplification. On the other hand, the expression level of TBN, TBCC, and KIAA0240 did not differ between patients with or without 6p21 amplification. This suggested that the most likely candidate genes for 6p21 amplification are BYSL and CCND3. CCND3 and BYSL mRNA expression is higher in the SUDHL9 cell line than in patient cases. This might account for the higher log2 ratio of BAC RP5-973N23 in SUDHL9 (log2 ratio = 2.6), whereas the log2 ratio of patient cases was lower than the cell line (1< log2 ratio < 2).

Quantitative real-time reverse transcription-PCR for CCND3 and BYSL. Quantitative real-time reverse transcription-PCR analysis of CCND3 and BYSL was then done on 20 patient samples. As shown in Fig. 5C, the 20 DLBCL cases were divided into three groups with amplification (3 cases), low copy number gains (7 cases), and no copy number changes (10 cases). Samples derived from D648 and D912 with 6p21 amplification showed overexpression of CCND3. These two cases also showed overexpression of BYSL. The expression level of both CCND3 and BYSL in cases that had shown low or high copy number gains (10 cases) was significantly higher than in cases without 6p21 gain (10 cases; CCND3, P = 0.0343; BYSL, P = 0.0082). These results lead us to conclude that the target genes of gain/amplification at 6p21 are BYSL and CCND3.

Discussion

Genomic amplification has been observed in a variety of tumors and represents one aberrant molecular pathway by which gene expression is constitutively enhanced beyond the level of physiologically normal variation. It can be expected that the “driver” genes are located at the narrow region with the highest level of copy number changes as shown in our study. Our strategy for the identification of target genes was to use analyses combining genome-wide (1.5 Mb resolutions throughout the genome) and contig array CGH (3 Mb in length at 6p21). Chromosome 6p may harbor several candidate oncogenes responsible for chromosome 6p gain/amplification, such as IRF4 for 6p25 amplification and E2F3, DEK, and RBKIN/KIF13A that are associated with 6p22.3 gain. We were able to investigate candidate targets using three tumors and one cell
line that showed genomic amplification at the 6p21 region. We constructed a detailed 3 Mb physical map of the 6p21 amplicon, which included the 800 kb amplicon core. The structure of the 6p21 amplicon could be mapped in detail, and the number of copies throughout the amplified region was accurately estimated. The approach used by us and described here proved useful in characterizing amplified genomic regions of a wide variety of tumors, not only DLBCL. This strategy was also used in a previous study of ours in which we detected the aberrant expression of FHIT that had originated from a 3p14 small deletion in DLBCL (18).

Quantitative expression analyses showed that BYSL (21) and CCND3 (22) are target genes of the amplicon core at 6p21, whereas CCND3 is the translocation target of t(6;14) (p21.1;q32.3) in B-cell lymphoma (10). Moreover, BYSL and CCND3 are located near each other. CCND3 is centromeric to BYSL. Finally, the amplicon core includes the breakpoint of 6p21 translocation, and both CCND3 and BYSL are generally telomeric to this breakpoint, indicating that both genes could be the targets for 6p21 chromosome translocation.

Although it has been widely speculated that the CCND3 is the target gene for 6p21 genomic gain/amplification, no detailed investigations have been reported. The findings in the present report may, therefore, constitute the first evidence that CCND3 is in fact the target for genomic gain/amplification in malignant lymphomas. Because CCND3 is the cyclin D family member protein that controls the G1-S phase of cell cycle regulation, overexpression of CCND3 through genomic amplification is likely to lead to aberrant cell cycle control and may contribute to tumorigenesis (23). Although BYSL is known as a bystin-like gene that mediates cell adhesion between trophoblasts and endometrial epithelial cells through its interaction with trophinin, tastin, and cytokeratin (21), the link between BYSL and tumorigenesis remains to be determined. It is likely that overexpression of BYSL results from its close proximity to CCND3. Interestingly, a similar co-overexpression pattern of two closely located genes has been reported in oral cancer cell lines by Huang et al. (24). They showed that the TAOS1 gene, which is located ~12 kb distal to the CCND1 gene, is co-overexpressed with CCND1 with 11q13 amplification. Similarly, coexpression of EMS1 with CCND1 with 11q13 genomic amplification has been detected in several solid tumors (25–29). Although EMS1 is known as an oncogene, it is not known whether it is associated with TAOS1-related tumorigenesis as in the case of BYSL.

Finally, we investigated whether 6p21 gain/amplification of DLBCL was reflected in the clinical data. Four cases with 6p21 gain could be subjected to gene expression clustering (19). These four cases were evenly distributed into activated B-cell-like and germinal center B-cell-like types. We found that 6p21 gain was frequently found in younger patients (<60 years, \( P = 0.02 \)) but no significance was found for other prognostic factors, such as lactate dehydrogenase, performance status, and stage. Additional studies will be needed to confirm these observations in larger series of patients.

In summary, although it is known that t(6;14)(p21;q32) induces aberrant overexpression of CCND3 in B-cell malignancies, we were able to show that CCND3 can be also a target of genomic gain/amplification. Overexpression of CCND3 through genomic gain/amplification is likely to lead to aberrant cell cycle control, although the precise biological role of BYSL with respect to tumorigenesis remains unknown. Further biological studies are needed to determine the tumorigenic function of these candidate genes in DLBCL.

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References


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