High-level DNA amplifications are common genetic aberrations in B-cell neoplasms

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Abstract

Gene amplification is one of the molecular mechanisms resulting in the up-regulation of gene expression. In non-Hodgkin's lymphomas, such gene amplifications have been identified rarely. Using comparative genomic hybridization, a technique that has proven to be very sensitive for the detection of high-level DNA amplifications, we analyzed 108 cases of B-cell neoplasms (42 chronic B-cell leukemias, 5 mantle cell lymphomas, and 61 aggressive B-cell lymphomas). Twenty-four high-level amplifications were identified in 13% of the patients and mapped to 15 different genomic regions. Regions most frequently amplified were bands Xq26-28, 2p23-24, and 2p14-16 as well as 18q21 (three times each). Amplification of several proto-oncogenes and a cell cycle control gene (N-MYC (two cases), BCL2, CCND2, and GLI) located within the amplified regions was demonstrated by Southern blot analysis or fluorescence in situ hybridization to interphase nuclei of tumor cells. These data demonstrate that gene amplifications in B-cell neoplasms are much more frequent than previously assumed. The identification of highly amplified DNA regions and genes included in the amplicons provides important information for further analyses of genetic events involved in lymphomagenesis.
Short Communication

High-Level DNA Amplifications Are Common Genetic Aberrations in B-Cell Neoplasms

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From the Medizinische Klinik und Poliklinik V,* Universität Heidelberg, and Deutsches Krebsforschungszentrum,† Heidelberg; Medizinische Klinik,‡ Universität des Saarlands, Homburg; Pathologisches Institut der Universität Würzburg; Würzburg, and Pathologisches Institut der Universität Ulm,§ Ulm, Germany

Gene amplification is one of the molecular mechanisms resulting in the up-regulation of gene expression. In non-Hodgkin's lymphomas, such gene amplifications have been identified rarely. Using comparative genomic hybridization, a technique that has proven to be very sensitive for the detection of high-level DNA amplifications, we analyzed 108 cases of B-cell neoplasms (42 chronic B-cell leukemias, 5 mantle cell lymphomas, and 61 aggressive B-cell lymphomas). Twenty-four high-level amplifications were identified in 13% of the patients and mapped to 15 different genomic regions. Regions most frequently amplified were bands Xq26–28, 2p23–24, and 2p14–16 as well as 18q21 (three times each). Amplification of several proto-oncogenes and a cell cycle control gene (N-MYC (two cases), BCL2, CCND2, and GLI) located within the amplified regions was demonstrated by Southern blot analysis or fluorescence in situ hybridization to interphase nuclei of tumor cells. These data demonstrate that gene amplifications in B-cell neoplasms are much more frequent than previously assumed. The identification of highly amplified DNA regions and genes included in the amplicons provides important information for further analyses of genetic events involved in lymphogenesis. (Am J Pathol 1997, 151:335–342)

Materials and Methods

Tumor Specimens

Tumor samples of 108 patients with B-cell neoplasms classified according to the REAL classification were analyzed by CGH. For 15 cases of Burkitt's lymphoma (all previously untreated), 5 cases of mantle cell lymphoma (3 previously treated), and 42 cases of chronic B-lymphocytic leukemia (B-CLL; 15 previously treated), frozen tumor material was used. In 46 patients with aggressive lymphomas other than Burkitt's lymphoma (all previously untreated), only limited amounts of paraffin-embedded

Alterations of proto-oncogenes play a key role in the development of malignant tumors. One of the mechanisms for activating proto-oncogenes is gene amplification resulting in an enhanced expression of the corresponding gene product (for review see Ref. 1). Clinically, gene amplifications often have been associated with a more aggressive tumor phenotype and a shorter overall survival in several tumor types. Whereas gene amplification is known to occur frequently in many solid tumors, eg, in neuroblastoma or in breast cancer, in non-Hodgkin's lymphoma (NHL) it has been considered a rare event. Using banding techniques, the cytogenetic hallmarks of DNA amplification, homogeneously staining regions or double minute chromosomes, have been reported in only 19 of more than 3500 NHL cases. Whereas recent studies using the technique of comparative genomic hybridization (CGH) in small groups of patients suggested a much higher frequency of high-level DNA amplifications, CGH data led to the identification of REL amplifications in diffuse large-cell lymphomas and primary mediastinal B-cell lymphomas. These findings underline the usefulness of CGH for the detection of high-level DNA amplifications.

In the present study, we applied CGH to 108 cases with B-cell neoplasms to identify and map high-level DNA amplifications. The chromosomal map positions of the amplified sequences were used as entry points to study the involvement of specific genes by Southern blot analysis and fluorescence in situ hybridization (FISH).

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tissue samples were available for DNA isolation and subsequent CGH experiments. Histopathological diagnoses of the 14 cases with high-level DNA amplifications are listed in Table 1.

### Comparative Genomic Hybridization

Genomic DNA was prepared from fresh tumor tissue as described using proteinase K digestion and phenol-chloroform extraction. From paraffin sections, genomic DNA was isolated according to the method described by Isola and co-workers. In one sample (AMP11), a universal polymerase chain reaction protocol was necessary to obtain a sufficient amount of DNA. CGH was performed as reported previously. Briefly, tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), and normal human control DNA was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by a standard nick translation reaction. One microgram of biotin-labeled tumor DNA, one microgram of digoxigenin-labeled control DNA, and seventy micrograms of human Cot-1-DNA (BRL Life Sciences, Gaithersburg, MD) were co-hybridized to slides with metaphase cells prepared from blood of a healthy donor. After hybridization for 2 to 3 days and post-hybridization washes, control and test DNAs were detected via rhodamine and fluorescein isothiocyanate (FITC), respectively. For identification, chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

### Digital Image Analysis

Digital image analysis was performed using an epifluorescence microscope (Axioplan, Zeiss, Jena, Germany) and the commercially available image analysis systems ISIS (MetaSystems, Altusussheim, Germany) or CYTOSION (Applied Imaging, Sunderland, UK). Sensitivity and specificity of both systems were validated by applying a series of cases with well defined abnormalities to the systems. Furthermore, in the 42 cases with DLL, CGH data were compared with interphase cytogenetics of at least five chromosomal regions for each case, proving the high accuracy of these evaluation software programs (manuscript in preparation). Ratio values of 1.25 and 0.75, which have been proven to provide robust criteria for diagnosing over- and underrepresentations, were used as upper and lower thresholds for the identification of chromosomal imbalances. Overrepresentations were considered as high-level amplifications when the fluorescence ratio values exceeded 2.0 or when the FITC fluorescence showed strong focal signals and the corresponding ratio profile was diagnostic for overrepresentation. Assignment of highly amplified sequences to chromosomal bands was performed by comparison of

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### Table 1. CGH Data of B-Cell Neoplasms with High-Level DNA Amplifications

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>High-level amplification</th>
<th>Low-level changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP1</td>
<td>DLC-B</td>
<td>amp(Xq26-28), amp(12q13-14)</td>
<td>enh(5q23q31,6p,7q11q22), dim(1p21p31,6q,9p2p24,13q21q31)</td>
</tr>
<tr>
<td>AMP2</td>
<td>FCL, Iv</td>
<td>amp(18q21-22)</td>
<td>enh(3q11q27,4p,4q12q21,7p,7q11q22,11,12), dim(6q)</td>
</tr>
<tr>
<td>AMP3</td>
<td>FCL, Iv</td>
<td>amp(14q21-24)</td>
<td>enh(Xp21p22,Xq21q28,2p13p21,6p22p35,7p15p21,18q11q22)</td>
</tr>
<tr>
<td>AMP4</td>
<td>FCL, Iv</td>
<td>amp(Xq21-24), amp(3q12-13)</td>
<td>enh(7p13p21,7q21q36,9p2p24,11), dim(9q12q21,17p)</td>
</tr>
<tr>
<td>AMP5</td>
<td>FCL, Iv</td>
<td>amp(2p22-24)</td>
<td>dim(6q16q21)</td>
</tr>
<tr>
<td>AMP6</td>
<td>FCL, Iv</td>
<td>amp(19q13)</td>
<td></td>
</tr>
<tr>
<td>AMP7</td>
<td>DLC-B</td>
<td>amp(Xp11-21)</td>
<td></td>
</tr>
<tr>
<td>AMP8</td>
<td>FCL, Iv</td>
<td>amp(2p13-16)</td>
<td>enh(1q21q42,7q11q21,16,19,X)</td>
</tr>
<tr>
<td>AMP9</td>
<td>FCL, Iv</td>
<td>amp(2p14-16)</td>
<td>enh(1q24q44,5q13q23,7p,13q14q32,14q13q32)</td>
</tr>
<tr>
<td>AMP10*</td>
<td>MCL</td>
<td>amp(Xq26-28)</td>
<td>dim(8p2p23,10p13p15,13q13q34)</td>
</tr>
<tr>
<td>AMP11</td>
<td>BL</td>
<td>amp(2p23-25)</td>
<td>-</td>
</tr>
<tr>
<td>AMP12</td>
<td>FCL, Iv</td>
<td>amp(2p14-16)</td>
<td>enh(3q25q29)</td>
</tr>
<tr>
<td>AMP13</td>
<td>B-CLL</td>
<td>amp(14q33-32)</td>
<td>enh(X,8), dim(2q36q37)</td>
</tr>
<tr>
<td>AMP14*</td>
<td>MCL</td>
<td>amp(Xq27-28)</td>
<td>enh(3q21q29,12q), dim(13q14q34)</td>
</tr>
</tbody>
</table>

DLC-B, diffuse large B-cell lymphoma; FCL, Iv, follicle center lymphoma, large cell variant; MCL, mantle cell lymphoma; BL, Burkitt’s lymphoma; B-CLL, chronic B-lymphocytic leukemia. CGH data are presented according to the ISCN: High-level amplification (amp), high-level DNA amplification (amp), enhanced signal intensity (enh), diminished signal intensity (dim), and loss of chromosomal material (loss).

*Only cases AMP10 and AMP14 have been treated before molecular cytogenetic analysis.

In this case, degenerate oligonucleotide-primed (DOP—PCR) was necessary before CGH to obtain a sufficient amount of genomic DNA. Due to inconsistencies in repeated experiments, low-level copy number changes were not scored in this case. However, the high-level amplification mapping to bands 2p23-25 was detected in each experiment. This finding was also confirmed by the demonstration of a greater than 100-fold amplification of the N-MYC proto-oncogene in this case (see also Figure 3B).
signal intensities and DAPI banding on individual chromosomes.

Fluorescence in Situ Hybridization

FISH analysis of interphase nuclei was performed using the following probes: the cosmid probe “GLI” and the aliphoid satellite probe D12Z3 (both obtained from Oncor, Gaithersburg, MD) were used to investigate the amplification of the GLI proto-oncogene. For characterization of an amplification unit at 14q32, probes cos-C, containing the C, constant gene segment of the immunoglobulin heavy chain (IgH) locus23 and YAC Y6 containing variable region gene segments of the IgH locus22–24 were used. For evaluation of FISH experiments, at least 200 nuclei were analyzed. Criteria for gene amplification were either the presence of a tight cluster of signals in addition to one or more normal hybridization spots or a signal ratio of ≤4 of the respective gene probe relative to a simultaneously hybridized probe for the centromeric region of the same chromosome (see also Ref. 25). In all cases with amplifications diagnosed by FISH, either of these criteria was fulfilled in at least 25% of interphase nuclei.

Southern Blot Analysis

Southern blot analysis was performed using standard procedures.14 Briefly, 8 μg of genomic DNA was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nylon membranes (Boehringer Mannheim). The membranes were hybridized with the following DNA probes labeled by random priming with [32P]dCTP: a 2.8-kb BCL2-specific probe (Oncor), a 1.0-kb N-MYC-specific probe (Oncor), and pXcD2, a cyclin D2 (CCND2)-specific cDNA probe26 (kindly provided by Drs. P. Jansen-Dürr, Deutsches Krebsforschungszentrum, Heidelberg, Germany, and B. Henglein, INSERM U 75, Paris, France). Probe pMDM2A, a 600-bp fragment specific for MDM2, and a 534-bp nucleotide probe for CDK4 were kindly provided by Dr. G. Reifenberger, University of Düsseldorf, Germany.27 For control hybridizations, the genomic fragment gMHIC-1-D from the cardiac β-myosin heavy chain gene MYH7, located on chromosome band 14q12-1q3, was used.29 Determination of gene dosage was performed by densitometric measurements of the hybridization bands obtained with the target probe and MYH7. For normalization of hybridization signals, an equal amount of normal human lymphocyte DNA was included in each experiment.

Results

Comparative Genomic Hybridization

Using CGH, 24 high-level DNA amplifications were identified and mapped to 15 different genomic regions in 14 of the 108 NHLs (13% of the cases). In these amplified regions, there was either a fluorescence ratio value greater than 2.0 or a strong focal FITC signal with a corresponding ratio profile diagnostic for overrepresen-

tation (see also Materials and Methods). The chromosomal localizations of the highly amplified DNA sequences are summarized in Figure 1. Apart from the high-level amplifications, the most frequent chromosomal imbalances in these cases were gains (characterized by fluorescence ratio values >1.25 without fulfilling the criteria for a high-level amplification) on chromosomes 7 (six cases) and X (three cases) and on the long arm of chromosome 3 (three cases) as well as losses (defined by fluorescence ratio values <0.75) on chromosome arms 6q and 13q (three cases each). The complete CGH data of these 14 cases are summarized in Table 1.

High-level amplifications were more frequent in aggressive lymphomas (11 of 61 cases, 18%; whole series: 14 of 108, 13%). The most frequently affected genomic regions were chromosomal bands 18q21–22, 2p23–24, 2p14-p16, and Xq27-q28, which were highly amplified in three instances each. In two cases, amplifications mapping to 19q13 were observed. The other DNA amplifications were located at chromosomal bands Xp11–21, Xq21–24, 3q12–13, 3q26–29, 4q32–35, 8q23–24, 12q13–14, 14q21–24, 14q31–32, and 15q23–24. Among the 14 patients with high-level DNA amplifications, four exhibited more than one amplification site (see Table 1). A partial CGH karyotype of case AMP12 with four amplification sites is shown in Figure 2.

Southern Blot and FISH Analysis

To identify genes included in the amplicons, databases were screened for candidate genes in the regions exhibiting amplification sites. In five cases, genes included in the amplicons were identified (see Table 2). The proto-oncogene BCL2 was an obvious candidate gene for cases with amplification units mapping to band 18q21. Southern blot analysis of case AMP10, the only one of these cases for which sufficient frozen material was available, revealed a fivefold amplification of the BCL2 gene. High-level DNA amplifications mapping to bands 2p23–24 were observed in three cases (AMP5, AMP10, and AMP11). As this is the localization of the proto-oncogene N-MYC, Southern blot analysis with an N-MYC-specific probe was performed in the two cases for which DNA of sufficient quality was available. Densitometric evaluation of the N-MYC and control (MYH-7) signals revealed a greater than 100-fold and a 4-fold amplification of this gene in cases AMP11 and AMP10, respectively (see Figure 3B). In case AMP13, a high-level amplification was identified at 14q31–32. FISH analysis using probes cos-C and Y6 showed multiple co-localized cos-C and Y6 signals, indicating an amplification of DNA sequences derived from the IgH locus.

To identify amplified proto-oncogenes on chromosome 12, the gain of which is particularly frequent in B-cell neoplasms, we also investigated two cases, which were previously published by our group.6,8,30 A CLL case exhibited amplified DNA sequences mapping to two distinct loci on the short arm of chromosome 12, namely, bands 12p11–12 and 12p13 (case A, see Table 2), the latter of which contains the cyclin D2
gene (CCND2). Subsequent Southern blot analysis with a CCND2-specific probe showed a high copy number increase of CCND2, proving an amplification of this gene.

In addition to case AMP1, an amplification site mapping to bands 12q13–14 was also present in a follicle center lymphoma (case B). In this case, nuclei were available for FISH using a cosmid probe specific for the proto-oncogene GLI. In 25% of the nuclei, FISH revealed a high-level amplification of this gene. This was demonstrated by simultaneous hybridization of the GLI-specific probe and an alphoid probe specific for the centromeric and paracentromeric repetitive sequences of chromosome 12 (D12Z3) (see Figure 3A). In this case, additional Southern blot experiments were performed using probes for the candidate genes CDK4 and MDM2, which are in close proximity to GLI. In both experiments, normal copy numbers of these genes were demonstrated.
Discussion

In contrast to many other tumor types, gene amplifications were rarely identified in NHL. Based on banding analyses, only 19 of more than 3500 cases exhibited homogeneously staining regions or double minutes. Even in such cases, the identification of amplified genes was difficult, mainly because banding data did not provide any clues with regard to the genomic origin of the amplified sequences. In one series of nine patients with cytogenetic evidence for gene amplification, a panel of DNA probes for 23 proto-oncogenes and the multidrug resistance gene PGY1 was tested by Southern blot hybridization. In none of the cases was an amplification of one of these genes demonstrated. In contrast, CGH proved to be very sensitive for the detection of high-level DNA amplifications. In addition, the amplified sequences are mapped within the genome, allowing the rapid selection of candidate genes. Thus, the identification of amplified genes is greatly facilitated. In the present study, we analyzed a large number of patients with B-cell neoplasms and detected high-level DNA amplifications in 14 of 108 cases (whole series: 13%; aggressive lymphomas: 18%). The higher frequency of such amplifications in aggressive lymphomas as compared with indolent lymphomas may indicate a possible association with a more aggressive tumor phenotype.

On the short arm of chromosome 2, two different regions of amplification were identified, namely, bands 2p14-16 and 2p23-24. Recently, amplifications of the REL proto-oncogene mapping to bands 2p14-15 have been reported in aggressive lymphomas. In contrast, amplification of genes mapping to bands 2p23-24 have not been reported in NHL. A candidate gene located in this region is the N-MYC proto-oncogene, which is amplified and overexpressed in several tumor types such as neuroblastoma, retinoblastoma, and small-cell lung carcinoma. In transgenic mouse models, an association of N-MYC overexpression and lymphoma development was demonstrated; similar to C-MYC, N-MYC transgenic mice develop pre-B- and B-lymphoid malignancies. In two of the three NHL cases with amplifications mapping to 2p23-24, Southern blot analysis was performed, and an amplification of N-MYC was detected. These findings

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Amplified region(s)</th>
<th>Amplified gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP10</td>
<td>MCL</td>
<td>Xq26-28</td>
<td>N-MYC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2p23-24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3q26-29</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18q21-23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19q13</td>
<td>BCL2</td>
</tr>
<tr>
<td>AMP11</td>
<td>BL</td>
<td>2p23-25</td>
<td>N-MYC</td>
</tr>
<tr>
<td>AMP13</td>
<td>B-CLL</td>
<td>14q31-32</td>
<td>IGH</td>
</tr>
<tr>
<td>A*</td>
<td>B-CLL</td>
<td>12p11-12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12p13</td>
<td>CCND2</td>
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<tr>
<td>B†</td>
<td>FCL, slv</td>
<td>8p24</td>
<td>C-MYC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12q13-14</td>
<td>GLI</td>
</tr>
</tbody>
</table>

MCL, mantle cell lymphoma; BL, Burkitt’s lymphoma; B-CLL, chronic B-lymphocytic leukemia; FCL, slv, follicle center lymphoma, small and large cell variant.

*Case 22 in Bentz et al.*

†Case 14 in Bentz et al.; C-MYC amplification has already been reported in this publication.
further substantiate a possible role for N-MYC deregulation in lymphomagenesis.

Another frequently affected region was located on the long arm of chromosome 18 (bands 18q21–22). This coincides with the chromosomal localization of the BCL2 proto-oncogene, a gene that is known to inhibit apoptosis. Deregulation of BCL2 plays an important role in most follicle center lymphomas and in many cases of diffuse large-cell lymphoma. Amplification of this gene was reported recently in six cases of diffuse large-cell lymphoma. In the present study, a BCL2 amplification was also demonstrated in a case of mantle cell lymphoma.

In one case, a high-level DNA amplification mapping to bands 14q31–32 was identified. FISH analysis revealed an amplification of DNA sequences derived from the immunoglobulin heavy chain gene locus. Such an amplification of the IgH gene has been described before in a t(11;14)(q13;q32)-positive NHL cell line. Rearrangements involving the IgH gene are frequent in B-cell neoplasias. In these rearrangements, different proto-oncogenes are juxtaposed to regulatory sequences of the IgH gene. However, there is no clear evidence of an oncogenic potential of the IgH gene itself. Thus it remains unclear whether an increased gene dosage of the IgH gene is of any pathogenetic relevance. It cannot be excluded that a proto-oncogene in close proximity to this locus (eg, TCL1) might be the target of the amplification in this case.

Identification of amplified genes on chromosome 12 appeared particularly attractive, because an overrepresentation of this chromosome is a common cytogenetic finding in B-cell neoplasms. Gains of genetic material derived from the chromosomal region 12q13–14 were identified frequently both by banding analysis and by
There are several candidate genes mapping to 12q13-14 including *MDM2*, *CDK4*, and *GLI*. So far, no amplifications of genes mapping to this chromosomal region were demonstrated in NHL. FISH analysis of case B with follicle center lymphoma showed a high-level amplification of the *GLI* proto-oncogene, whereas a normal gene dosage was found for *CDK4* and *MDM2* by Southern blot analysis. This is in contrast to data in malignant gliomas and sarcomas, where *MDM2*, *CDK4*, or SAS belonged to the critical genomic region in tumors exhibiting 12q13-14 amplifications, whereas *GLI* was co-amplified in only a few cases.

On the short arm of chromosome 12, amplifications have been mapped to bands 12p12 and 12p13. In case A, an amplification of the *CCND2* gene was demonstrated. *CCND2* is a D-type cyclin implicated in the regulation of cell cycle progression. Amplifications of this gene were recently reported in two cases of aggressive NHL.

The other high-level DNA amplifications identified in this study were mapped to 10 chromosomal regions, 6 of which (3q12-13, 3q26-29, 4q32-35, 19q13, 14q21-24, and 15q22-24) have not been described before to contain amplification sites in NHL. Candidate genes located in these bands include *BCL3* (located at 19q13) and *BCL6* (located at 3q27), which are known to be deregulated in some types of B-cell neoplasms. This CGH study revealed a high incidence of high-level DNA amplifications in B-cell neoplasms and provided information that allowed the identification of amplified genes in several cases using a candidate gene approach. Although additional studies are required to elucidate the pathogenetic consequences of these amplifications, our findings indicate a possible role of proto-oncogenes in lymphomagenesis, alterations of which were demonstrated only rarely (*CCND2*) or not at all (*GLI* and *N-MYC*) in NHL before.

**Acknowledgments**

We gratefully acknowledge Professor Alfred C. Feller (University of Lübeck, Germany) for providing histopathological data on some of the NHL cases, Dr. Guido Reifenberger for providing DNA probes, and Dr. Andreas Plesch (MetaSystems, Altusheim, Germany) for support in the image analysis as well as Andrea Riefling and Magdalena Schlotter for excellent technical assistance.

**References**