High Incidence of Chromosomal Imbalances and Gene Amplifications in the Classical Follicular Variant of Follicle Center Lymphoma

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The classical follicular variant of follicle center lymphoma (FCL-fo) is associated with the chromosomal translocation t(14;18)(q32;q21). However, the sole presence of this translocation is not sufficient for malignant transformation, as demonstrated by experiments in a transgenic mouse model. Most of the secondary changes, which play a central role in tumor development and progression and which are presumed to be of prognostic value, are gains and losses of chromosomal material. We analyzed 28 FCL-fo patients using comparative genomic hybridization (CGH). The most frequent imbalances were gains on chromosomes X, 7, 8, 12, and 18 as well as losses of material on chromosome arm 6q. For chromosomes X, 8, 12, and 18, the CGH data allowed further narrowing of the relevant sub-regions. In addition, novel high-level DNA amplifications were identified in five instances mapping to chromosome bands 1p36, 6p21, 8q24 (2 patients), and 12q13-14. Previously, such amplifications have been identified very rarely in lymphomas. In the 2 patients with amplifications mapping to chromosomal band 8q24, involvement of the MYC proto-oncogene in the amplification unit was demonstrated by Southern blot analysis. These data provide further entry points for studies to identify genes relevant for tumor progression in FCL-fo.

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PATIENTS AND METHODS

Patient samples. Tissue samples of 28 patients (14 men and 14 women; age, 26 to 84 years; median, 56 years) drawn from our

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files of freshly frozen lymphoma tissue were selected for classical histology of FCL-I0. Both paraffin sections and frozen sections of the samples were reviewed before CGH analysis. The proportion of tumor cells in each sample was at least 50%. A sclerotic reaction was inconsistently present and was an important feature in only a minority of patients. In 19 of the 28 patients, an IgH-BCL2 fusion was present as demonstrated by polymerase chain reaction (in 15 patients, the breakpoint was located within the major breakpoint cluster region of the BCL2 gene and in 3 patients within the minor breakpoint cluster region). In the remaining 9 patients, no IgH-BCL2 fusion was detected.

**Comparative genomic hybridization.** Genomic DNA was prepared from tumor tissue as described using protein kinase K digestion and phenol–chloroform extraction. CGH was performed as reported previously. Briefly, normal human genomic DNA (control DNA) was labeled with digoxigenin-11-deoxyuridine triphosphate (dUTP; Boehringer Mannheim, Mannheim, Germany) and tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim) by a standard nick translation reaction. One microgram of labeled tumor DNA, 1 μg of differentially labeled control DNA and 70 μg of human Cot1-DNA (BRL Life Sciences, Gaithersburg, MD) were cohybridized to slides with metaphase cells prepared from blood of a healthy donor. After hybridization for 2 to 3 days and posthybridization washes, control and test DNAs were detected via rhodamine and fluorescein isothiocyanate (FITC), respectively. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI), resulting in a Q banding–like pattern that was used for chromosome identification.

**Digital image analysis.** Image analysis was performed as described previously. Images were acquired using an epifluorescence microscope (Axioplan Zeiss, Jena, Germany) equipped with a cooled CCD camera (Photometrics, Tucson, AZ). Ratio profiles of the fluorescence intensities of tumor-DNAs and control-DNAs were calculated for each individual chromosome using a dedicated software. For each patient, the mean ratio profiles of between 4 and 10 metaphase cells were computed. Ratio values of 1.25 and 0.75 were defined as upper and lower thresholds for the identification of chromosomal imbalances. These values have been used in several studies comparing CGH results with data obtained by other cytogenetic methods and have proven to provide robust criteria for the diagnosis of chromosomal gains and losses. Overrepresentations were diagnosed as high-level amplifications when the fluorescence intensity values exceeded 2.0 or when the FITC fluorescence showed strong focal signals and the corresponding ratio profile was beyond the diagnostic threshold for overrepresentation. For the assignment of these high-level amplifications to chromosomal bands, the signal intensities were compared to the DAPI banding on individual chromosomes. The extension of other imbalanced regions was assessed by the comparison of the fluorescence ratio profiles with the corresponding regions in chromosome ideograms. Interphase cytogenetics. In 16 instances, imbalances identified by CGH were further analyzed using interphase cytogenetics. For these experiments, the following DNA probes were used: repetitive probes specific for the centromeric regions of chromosomes 7 (D7Z1), 12 (D12Z3), and X (DXZ1, all obtained from Oncor Sciences, Gaithersburg, MD); “cos-p16,” a contig of eight cosmid clones covering 250 kb of the region containing the CDKN2 gene on chromosome 9p21.22 YAC probe 96bs67 mapping to chromosome band 6q21 (kindly provided by P. Bray-Ward and D. Ward, Yale University, New Haven, CT) and cosmid probe cos-myc72 mapping to chromosome band 8q24 as well as cosmid probe 1p36. For hybridization, nuclei were isolated from frozen tissue samples as described. In each patient, at least 200 cells were evaluated for the presence of hybridization signals with the respective DNA probes.

**Southern blot hybridization.** Southern blot analysis was performed as described. A 400-base pair MYC-specific probe (Oncogene Science, Uniondale, NY) was labeled by random priming using digoxigenin-11-dUTP (Boehringer Mannheim). Hybridized sequences were detected via alkaline phosphatase conjugated to an antidigoxigenin-specific antibody (Boehringer Mannheim). For control hybridizations, the genomic fragment gMHC-1-D from the cardiac β-myosin heavy-chain gene, MYH7, located on chromosome band 14q12–q13, was used. The degree of MYC amplification was determined by densitometric measurements of the hybridization bands obtained with the two DNA probes.

**RESULTS**

**Comparative genomic hybridization.** In 19 of the 28 patients, chromosomal gains or losses were identified by CGH analysis. An example of a hybridization experiment is shown in Fig 1A through C. Overrepresentations of chromosomal material were more frequent than underrepresentations (46 gains vs 13 losses). The most frequent aberrations were gains on chromosomes X (7 patients), 7 (6 patients), 12 (5 patients), 8q (4 patients), and 18 (4 patients), as well as losses on the long arm of chromosome 6 (6 patients). A synopsis of all chromosomal imbalances is shown in Fig 2. High-level DNA amplifications were identified in 4 patients: nos. 7 (chromosomal band 8q24), 14 (bands 8q24 and 12q13), 16 (band 6p21), and 21 (band 1p36). For each patient with high-level amplification, examples of the relevant chromosomes hybridized with the tumor DNA are shown in Fig 1D.

On each of the most frequently affected chromosomes, regional consensus areas were delineated by CGH analysis (see Figs 2 and 3). On chromosome X, the whole chromosome was overrepresented in 4 patients, whereas in 3 patients (no. 7, 17, and 19) only the short arm was affected. One of the patients with aberrations of chromosome 7 had only a partial gain of this chromosome extending from 7pter to 7q21 (patient no. 11). On chromosome 12, a high-level DNA amplification mapping to chromosome band 12q13–q14 pinpointed a possible consensus region (patient no. 14). Similarly, 2 of the 4 patients with gains on chromosome 8q exhibited high-level amplifications of chromosomal band 8q24 (patients no. 7 and 14). On chromosome 18, the commonly overrepresented region mapped to bands 18q21–q22 (defined by patients 2 and 16). For chromosome 6, two consensus regions of deletion were delineated on the long arm, one mapping to 6q13–q16 (defined by patients no. 3 and 16) and one to 6q23–qter (defined by patients no. 6 and 14).

**Interphase cytogenetics.** Based on the availability of sufficient material and suitable DNA probes, the CGH findings were tested using interphase cytogenetics. The results of these experiments are shown in Table I. In 9 patients (no. 1, 3, 4, 7, 8, 11, 16, 17, and 27) chromosomal imbalances were identified by CGH and the results were confirmed by interphase cytogenetic analysis. In four instances, the ratio profiles exhibited a clear shift toward overrepresentation (chromosome 12 in patient no. 4, chro-
Fig 1. Comparative genomic hybridization in follicle center lymphoma. (A and B) Normal metaphase spread hybridized with DNA from patient no. 21 detected via FITC (A, green) and a control DNA detected via rhodamine (B, red). In comparison to the control DNA, strong band-like hybridization signals are seen on the short arms of chromosome 1. Weaker staining of the short arms of chromosome 17 is also clearly visible. The more intense staining pattern of the long arms of chromosomes 16 and 17 is less obvious by visual inspection. (C) FITC to rhodamine fluorescence intensity ratio image of the same metaphase spread as in A and B. Chromosomal regions overrepresented in the tumor genome are displayed in green, whereas underrepresented regions are shown in red. (D) Representative FITC images of the five DNA amplifications identified in 4 patients. The chromosome number is given below the respective images. Beside each FITC image, an ideogram and the average ratio profile for each chromosome is shown. The ratios of FITC/rhodamine fluorescence are plotted along the ideograms. The central line indicates a ratio value of 1.0, right and left lines represent the diagnostic thresholds for overrepresentation and underrepresentation, respectively. (E) Average rate of amplification of MYC-specific sequences versus control sequences shown by densitometric evaluation of Southern blot analysis in patients no. 7 and 14.

Amplification of the MYC proto-oncogene. In one patient (no. 7), a high-level DNA amplification mapping to chromosome band 8q24 was identified. In another patient (no. 14), strong focal signals were seen at chromosomal band 8q24; however, the ratio profile did not exceed the diagnostic threshold for overrepresentation (see Fig 1D). This is the band, where the MYC proto-oncogene is localized, that plays...
a major role in the tumorigenesis of other types of NHL. To test whether the MYC gene is amplified in patients no. 7 and 14, we performed Southern blot analysis of genomic DNA from these tumors using the MYC-specific probe. For internal standardization, a probe for the cardiac β-myosin heavy chain was hybridized to the same filters. The ratio of the MYC- and MYH-7–specific signals showed a sevenfold and fourfold amplification of the MYC gene in patients no. 7 and 14, respectively, proving that MYC is part of the amplicons in these patients.
Fig 3. Average ratio profiles of chromosomes X, 6, 8, 12, and 18. Although the ratio profile did not exceed the diagnostic threshold for overrepresentation in this patient, a strong band-like hybridization signal was seen on chromosome 8 (see Fig 1D). Southern blot hybridization revealed a fourfold amplification of the MYC proto-oncogene in patient 14. In these patients, the ratio profiles were clearly shifted toward overrepresentation or underrepresentation. Interphase cytogenetic analysis showed underrepresentation of the terminal part of chromosome 6q and overrepresentation of chromosome 12 and the terminal part of chromosome 8 in significant proportions of cells (see Table 1).

**DISCUSSION**

FCL is closely associated with the chromosomal translocation t(14;18)(q32;q21). In spite of this close relationship, the sole presence of the t(14;18) is not sufficient for tumor development, and cells carrying this aberration have been identified in a significant proportion of healthy probands. This implies that additional genetic changes are crucial for the development of FCL. In some patients, these may be submicroscopic, such as point mutations or microdeletions, and thus not detectable by cytogenetic analysis. However, in the databank of the *Catalog of Chromosome Aberrations in Cancer*, 320 of 355 t(14;18)-positive lymphomas exhibited secondary chromosome aberrations. A recently published compilation of these data indicated that the pathogenetically essential molecular consequences of the secondary changes most likely are microscopically detectable genomic imbalances, i.e., gains or losses of genetic material, rather than rearrangements of specific genes.

In this light, molecular cytogenetic analysis of FCL-f0, which is the most frequent variant of FCL, by the novel technique of CGH seems a promising approach. Although this method does not detect any balanced aberrations such as translocations or inversions, it has proven to be a powerful tool for the detection of chromosomal gains and losses in tumor samples (see, eg, Bentz et al, Schröck et al, Isola
tions \[three versus two copies\]), the sizes of the imbalanced deletions \[ie, one copy of specific sequences in the tumor\]

"Therefore, the respective changes should be present in at least 50% of regions have to be in the range of 10 to 15 Mbp. Furthermore, genome versus two copies in the control DNA\] or duplications of our evaluation criteria.

Regarding the interpretation of CGH data, two important limitations of current protocols have to be considered: for the reliable detection of single-copy losses and gains, \(eg,\) with data obtained by interphase cytogenetics. CGH findings of this chromosome, one signal was seen in the majority of cells. This indicates that the centromere was not part of the overrepresented portion of chromosome X in this patient.

et al,30 Speicher et al,31 Kallioniemi et al,32 and Joos et al).33 Regarding the limitations of current protocols have to be considered: for the reliable detection of single-copy losses and gains, \(eg,\) deletions \(ie, one copy of specific sequences in the tumor genome versus two copies in the control DNA\] or duplications \(three versus two copies\), the sizes of the imbalanced regions have to be in the range of 10 to 15 Mbp. Furthermore, the respective changes should be present in at least 50% of the cell sample used for DNA extraction. Therefore, the proportion of tumor cells was carefully checked in the material used in the present study (see Patients and Methods). In contrast, CGH is particularly sensitive for the detection of high-level DNA amplifications. Amplification units as small as 90 kbp have been easily identified using this technique.16

Using CGH, we identified numerous gains and losses, as well as five amplification sites in 28 patients with FCL-fo. The most frequent aberrations were gains on chromosomes 7, 8, 12, and 18 as well as losses on the long arm of chromosome 6. In 11 patients, CGH findings were compared with data obtained by interphase cytogenetics. CGH findings were always confirmed, further substantiating the accuracy of our evaluation criteria.

Although these most frequent imbalances were identified before as recurrent secondary chromosome aberrations in t(14;18)-positive lymphomas,32 for chromosomes X, 8, 12, and 18, our CGH data allowed further narrowing of the relevant subregions. This was based on (1) comparison of the overrepresented regions resulting in delineation of the overlapping subregion (see Fig 2), namely the short arm of chromosome X, and band q21-q22 of chromosome 18 containing the BCL2 and FVT1 genes; or (2) the presence of a high-level DNA amplification on chromosomes 8 (band 8q24) and 12 (band 12q13-14). Such commonly overrepresented regions can be used as entry points for the identification of genes relevant for tumor progression in patients carrying a specific secondary aberration.

In contrast to previous studies in NHL using other cytogenetic techniques, a high incidence of high-level DNA amplifications (5 amplified regions in 4 of the 28 patients) was identified. Using banding techniques, hallmarks for gene amplification \(ie, double minute chromosomes or homogeneously staining regions\) were identified only in 19 out of more than 3,000 cases of NHL.28 The DNA amplifications of our series mapped to four different regions: 1p36, 6p21, 8q24 (2 patients), and 12q13-q14. Several candidate genes are located on these chromosomal bands, eg, the FGR protooncogene and CDC2L1, a tyrosine kinase interfering with the cell cycle on band 1p36, proto-oncogenes NRSLS3, PBX2, and PIM1 on band 6p21 and MDM2 as well as proto-oncogenes ERBB3, GLI1, and SAS on chromosomal band 12q13.24

In 2 patients of this study, high-level DNA amplifications mapping to band 8q24 were present. This is the location of the MYC proto-oncogene. A synergy of activated MYC and BCL2 genes was demonstrated in double transgenic mice.35 These animals developed rapidly growing lymphomas with an immature phenotype. Therefore MYC was further analyzed in these patients using Southern blot analysis. In both patients, amplification of the MYC gene was present. In a previous study, amplification of this gene in a patient with a chronic B-cell leukemia was demonstrated. These data suggest that activation of MYC via a gene amplification is not a rare event in low-grade lymphoproliferative disorders.

### Table 1. Interphase Cytogenetic Data of 11 Cases With the Follicular Variant of Follicle Center Lymphoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>CGH-Karyotype*</th>
<th>Interphase Cytogeneticsf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>rev ish XX</td>
<td>DXZ1/2 signals (62)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>rev ish enh(12), dim(6q13q16)</td>
<td>D12Z3/3 signals: (62.5)</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>rev ish enh(X), enh(12)</td>
<td>D1Z1/- signals: (62)</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>rev ish enh(X), enh(12)</td>
<td>D1Z2/3 signals: (43)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>rev ish enh(Xp7.12, enh1X,7p22q21,12)</td>
<td>D1Z1/1 signal: (97)</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,7,11q,13q22q34,21q22), dim(8p22q23,16q)</td>
<td>D7Z1/3 signals: (74)</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>rev ish enh(X,7p22q21,12)</td>
<td>D7Z1/3 signals: (69)</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>rev ish enh(1q,8q25q21,7,11q,13q22q34,21q22), dim(8p22q23,16q)</td>
<td>D7Z1/3 signals: (74)</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>D7Z1/3 signals: (75)</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>D1Z2/3 signals: (60)</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>IgH,-/3signals: (35)</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>cosp16/1 signal: (31)</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>966b7/1 signal: (62.5)</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>cos-myc7/2 signals: (70)</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>rev ish enh(1q,8q25q21,12)</td>
<td>966b7/1 signal: (28)</td>
</tr>
</tbody>
</table>

*CGH karyotypes are listed according to the ISCN nomenclature30; CGH findings in italics indicate cases, in which the ratio profiles exhibited a clear shift toward overrepresentation; however, the diagnostic threshold values were not exceeded. Bold CGH data represent aberrations that were confirmed by interphase cytogenetic analysis.16

†In this column, the locus or probe name, the number of hybridization signals and the percentage of cells exhibiting the respective number of signals is listed as follows: probe name/number of signals with percentage in parentheses.

‡In patient 7, the short arm of chromosome X was overrepresented. With an alphoid DNA probe mapping to centromere specific sequences of this chromosome, one signal was seen in the majority of cells. This indicates that the centromere was not part of the overrepresented portion of chromosome X in this patient.
To date, two other studies investigating a series of patients with lymphoproliferative disorders by CGH have been published. To similar the study in FCL-fo, in both series, high-level DNA amplifications were identified. Taken together, such amplifications were found in 12 instances in a total of 82 patients. In addition to the high sensitivity with respect to the detection of gene amplifications, CGH points to the chromosomal localization of the amplified sequences. This provides important information for the identification of the relevant genes involved in the amplification unit. For 5 of the 12 high-level DNA amplifications, involvement of specific proto-oncogenes in the amplification unit could be shown. In another study based on CGH data of a patient with diffuse large cell lymphoma, a high incidence of REL gene amplifications was identified in this type of lymphoma. Thus, gene amplifications appear to be much more important for tumor progression than previously assumed both in high-grade and low-grade NHL.

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