Chromosomal Imbalances in Lymphoid Tumors of the Orbit

Gro Oddveig Ness,¹ Helle Lybæk,¹ Jarle Arnes,² and Eyvind Rødahl³

PURPOSE. To identify chromosomal gains and losses in lymphoid tumors of the orbit and to examine whether such abnormalities are related to orbital presentation, disease severity, or risk for recurrent disease.

METHODS. Biopsy specimens from 26 patients were examined by histomorphologic and immunohistochemical analysis. Lymphomas were classified according to the Revised European-American Lymphoma Classification. Chromosomal imbalances were detected by high-resolution comparative genomic hybridization (CGH). Clinical data were obtained by retrospective evaluation of medical records.

RESULTS. Chromosomal imbalances were detected in 0 of 6 patients with idiopathic orbital inflammation, 0 of 2 with benign reactive lymphoid hyperplasia, 3 of 3 with highly malignant diffuse large B-cell lymphoma, 4 of 10 with marginal zone B-cell lymphoma, 0 of 1 with chronic lymphocytic leukemia, and 1 of 4 with immunocytoma. Among the low-grade malignancies, chromosomal imbalances were seen in 1 of 9 at stage IAE, 2 of 3 at stage IIE, and 2 of 3 at stage IVE. Chromosomal imbalances were observed in all primary tumors from the five patients that later developed recurrent disease. In 14 of 23 imbalances with intrachromosomal breaks outside the centromere region, the breaks were present at bands with known fragile sites. No chromosomal imbalances specific for orbital presentation were detected.

CONCLUSIONS. Chromosomal imbalances were seen mainly in orbital lymphomas that were either highly malignant or at an advanced stage. CGH analysis of orbital lymphomas could be prognostically relevant, but further studies are required to confirm this notion. (Invest Ophthalmol Vis Sci. 2002;43:9-14)

Lymphoid tumors of the orbit include a spectrum of lymphoproliferative disorders ranging from benign, reactive lymphoid hyperplasia to malignant lymphoma. Lymphomas are the most common entity of malignant orbital tumors and represent approximately 10% of orbital tumors in larger series.¹ Most orbital lymphomas are non-Hodgkin lymphomas. Approximately two of three of these are marginal zone B-cell lymphomas (MZBCLs), whereas follicle center lymphomas and diffuse large B-cell lymphomas (DLBCLs) constitute approximately 10% each.² Immunocytomas and chronic lymphatic leukemia (CLL) lymphomas are less common. Lymphomas of the ocular adnexa (i.e., the conjunctiva, orbit, and lacrimal gland) represent approximately 8% of extranodal lymphomas.³

Cytogenetic studies of non-Hodgkin lymphomas have revealed a number of chromosomal translocations. Well-known examples include t(8;14)(q24;q32) in Burkitt lymphoma,⁴ t(14;18)(q32;q21) in follicle center lymphoma,⁵ t(11;14)(q13;q32) in mantle cell lymphoma,⁶ and t(3;14)(q27;q32) in DLBCLs.⁷ In these translocations, an oncogene (MYC, BCL2, BCL1, and BCL6, respectively) is translocated to the vicinity of an Ig heavy-chain gene, causing an increased expression of the oncogene.

In contrast to the frequent detection of translocations, chromosomal gains or losses are less commonly seen in non-Hodgkin lymphomas with conventional karyotyping.⁸ A number of chromosomal imbalances have been observed, however, in lymphomas of different subtypes using comparative genomic hybridization (CGH). CGH is a genome-wide screening procedure for chromosomal gains or losses, but it does not detect balanced translocations.⁹ With this method, tumor DNA is used directly for analysis. No cultivation of cells is necessary, and the selection of subclones of tumor cells is therefore avoided.

Studies of chromosomal imbalances are of interest, because chromosomal gains or losses may indicate the location of oncogenes or tumor-suppressor genes, respectively. The presence of chromosomal aneuploidy in lymphomas can be important for prognosis.¹⁰,¹¹ Although a wide range of aberrations have been observed in lymphomas, some are more frequently seen within individual subtypes, indicating that CGH analysis could be relevant for the classification of lymphomas.

Very few orbital lymphomas and no benign lymphoid lesions of the orbit have been examined for chromosomal abnormality.¹² Conventional banding analysis revealed trisomy 3 and 7 in one patient with orbital mucosa-associated lymphoid tissue (MALT) lymphoma¹³ (in the Revised European-American Lymphoma [REAL] classification,¹⁴ MALT lymphomas are included among the MZBCLs), whereas in three other patients with MZBCLs, t(11;18)(q21;q21), trisomy 3 and t(14;18)(q32; q21), and hypotetraploidy, respectively, were detected.¹⁵ Fluorescence in situ hybridization (FISH) analysis with centromere-specific probes revealed trisomy 3 in two of five and trisomy 18 in one of five cases of MALT lymphoma.¹⁵ No chromosomal abnormalities were detected in a single case of orbital MZBCL examined by CGH analysis.¹⁶

In the present study, we analyzed different lymphoid tumors of the orbit for the presence of chromosomal aberrations by comparative genomic hybridization, using both fresh-frozen and archival paraffin-embedded specimens. In addition, we examined cases of idiopathic orbital inflammation that appeared as a space-occupying mass. Such lesions are usually not included among the lymphoproliferative disorders,¹⁷ but analysis for chromosomal aneuploidy is of interest, because cytogenetic abnormalities have been described in other tumors with an inflammatory component.¹⁸

We observed a variety of chromosomal gains or losses in 8 of 18 lymphomas, whereas no abnormalities were detected in the 8 benign lesions. Except for one case, the lymphomas with chromosomal imbalances were either highly malignant or at an advanced stage. All primary tumors from the five patients in

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TABLE 1. Clinical Data for Patients with Orbital Lymphomas

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Stage</th>
<th>Organ Involvement</th>
<th>Treatment/Response†</th>
<th>Disease-Free/Overall Survival (mo)</th>
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<tr>
<td>1</td>
<td>F</td>
<td>59</td>
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<td>IAE</td>
<td>L orbit</td>
<td>Irradiation/CR</td>
<td>18/18</td>
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<td>IAE</td>
<td>L orbit</td>
<td>Irradiation/CR</td>
<td>19/19</td>
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<td>IAE</td>
<td>L orbit</td>
<td>Excision+irradiation/CR</td>
<td>28/28</td>
</tr>
<tr>
<td>4</td>
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<td>IAE</td>
<td>R orbit</td>
<td>Excision/CR</td>
<td>42/42</td>
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<td>54</td>
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<td>IAE</td>
<td>L orbit</td>
<td>Excision+irradiation/CR</td>
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</tr>
<tr>
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<td>IAE</td>
<td>R orbit</td>
<td>Irradiation/CR</td>
<td>121/121</td>
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<td>IIE</td>
<td>L+R orbit</td>
<td>Irradiation/CR</td>
<td>78/78</td>
</tr>
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<td>IIE</td>
<td>L orbit+R lower lid</td>
<td>Excision+irradiation/CR</td>
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<td>IVE</td>
<td>L+R orbit+parotid gland+lymph node</td>
<td>CNOP/CR</td>
<td>14/25‡</td>
</tr>
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<td>L+R orbit+lymph node</td>
<td>Chlorambucil+prednison/PR</td>
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<td>IAE</td>
<td>L orbit</td>
<td>Irradiation/CR</td>
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<td>IVE</td>
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</tr>
<tr>
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<td>CHOP+intrathecal</td>
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</tr>
<tr>
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<td>45</td>
<td>DLBCL</td>
<td>IAE</td>
<td>L orbit</td>
<td>CHOP/CR</td>
<td>22/22</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>76</td>
<td>DLBCL</td>
<td>IIE</td>
<td>R orbit+lymph nodes</td>
<td>CHOP/PR</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Ann Arbor stage (I–IV).
† PR, partial remission; CR, complete remission; CNOP, cyclophosphamide, mixantrone, vincristine, prednisone; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone.
‡ Recurrent disease.

whom recurrent disease developed later showed chromosomal aberrations.

MATERIALS AND METHODS

Patients
Tumor samples were obtained from 26 patients who underwent biopsy at the Department of Ophthalmology, Haukeland University Hospital between 1986 and 2001. The protocol of the study adhered to the tenets of the Declaration of Helsinki.

Inflammatory lesions were present in six patients. Four had lymphoid infiltrates (two in the lacrimal gland, one in the posterior part of the orbit, and one in the medial part of the orbit), and two had granulomatous infiltrates (one in the lacrimal gland and one in the lower temporal part of the orbit). Reactive lymphoid hyperplasia was present in two patients.

Among the 18 patients with lymphoma, one had previously been treated for mediastinal lymphoma (patient 13), whereas primary tumors were seen in the remaining patients (Table 1). The median follow-up time for these patients was 29 months (range, 2–150 months). Three patients (patients 9, 11, and 15) died during the follow-up period of causes other than lymphoma. No lymphoma-related deaths were observed.

The biopsy specimens were processed for routine histomorphologic examination, using hematoxylin and eosin, as well as periodic acid–Schiff staining. For immunohistochemical examination, we used an avidin-biotin complex (ABC) technique with antibodies reactive in an automated slide processing system (ChemMate; Dako, Glostrup, Denmark). All specimens were reviewed as part of the present study, and the lymphoma diagnoses were according to the REAL classification of lymphoid neoplasms. The samples processed for CGH analysis contained more than 50% tumor cells. The samples were coded before DNA extraction, and the identity of the specimens remained unknown until the CGH analysis had been completed.

DNA Extraction
DNA was isolated from fresh-frozen tissue in 14 cases and from archival, paraffin-embedded material in 12 cases. The fresh-frozen samples were treated with proteinase K (Qiagen, Hilden, Germany) and DNA was isolated by phenol–chloroform extraction using standard procedures. The paraffin-embedded material was cut into 10-μm sections. Approximately 10 to 15 sections were deparaffinized in 2-mL tubes (Nalge Nunc, Naperville, IL; 2 × 1.5 mL xylene for 10 minutes each and 1 × 1.5 mL 100% ethanol for 10 minutes with centrifugation at 3500 rpm for 10 minutes; Eppendorf centrifuge; Brinkman Instruments, Westbury, NY). After they were air dried at room temperature, samples were suspended in 0.7 mL DNA extraction buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 8], 0.5 mM EDTA [pH 8], 1% sodium dodecyl sulfate, and 0.5 mg/mL proteinase K) and incubated at 58°C overnight. If digestion was not complete, additional proteinase K was added, and the samples were incubated at 58°C for another 24 hours. DNA was extracted by a standard phenol–chloroform procedure until the water phase was clear. After a final extraction with chloroform, NaCl was added to a final concentration of 0.2 M, and the DNA was precipitated with ethanol at −20°C. The samples were centrifuged at 14,000 rpm for 20 minutes, the pellet washed once with 70% ethanol, air dried, and then suspended in 10 mM Tris-HCl and 1 mM EDTA (pH 8.0). The quality and the concentration of the DNA were determined by agarose gel electrophoresis.

Comparative Genomic Hybridization
Metaphases from normal peripheral blood lymphocytes were prepared according to standard procedures, using phytohemagglutinin (PHA) for stimulation of the lymphocytes and methotrexate for synchronization of the cell cycle. Slides with metaphase spreads were postfixied in 1% formaldehyde in phosphate-buffered saline (pH 7.4) for 5 minutes at 4°C, dehydrated in an ethanol series (70%, 85%, and 100%) and stored at −20°C before hybridization.

CGH was performed essentially as described by Kallioniemi et al. Normal metaphases were denatured in 70% formamide and 2× SSC (pH 7.0) for 2 minutes at 70°C and dehydrated in an ethanol series. Normal male or female DNA was used as reference DNA after labeling with Texas red-5-dUTP (NEN Life Science Products, Inc., Boston, MA) using nick translation. Tumor DNA was labeled with fluorescein-isothiocyanate (FITC)-12-dUTP (NEN Life Science Products). Genomic DNA was digested to fragment lengths of 300 to 2000 bp. Labeled test DNA (800 ng) and normal reference DNA (800 ng), together with
Digital Image Analysis

For CGH analysis, image capturing and processing were performed on a digital image system (CytoVision System, ver. 2.7; High Resolution CGH analysis; Applied Imaging, Newcastle, UK). In each case, 15 to 20 metaphases were collected using an epifluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) and a charge-coupled device (CCD) camera interfaced to the workstation (CytoVision Station). The green (tumor DNA)-to-red (normal reference DNA) fluorescence ratio along the length of chromosomes was calculated. Detection of aberrations was performed by dynamic standard reference intervals as described by Kirchhoff et al.20,21 Briefly, the dynamic standard reference interval is based on an average of 17 normal cases. The mean ratio profile of the 99.5% (paraffin-embedded specimens) or 99.9% (frozen samples) confidence interval of each case was compared with the 99.5% or 99.9% confidence interval, respectively, based on the normal cases. The dynamic standard intervals are wide at regions known to produce unreliable CGH profiles. The dynamic standard reference interval was scaled automatically to fit each test case. To reduce the risk of false-positive results, DNA from all tumors was hybridized twice to both sex-matched and mismatched DNA, and only the reproducible aberrations were included. The DNA from fresh-frozen tissue produced narrow confidence intervals, and 99.9% confidence intervals could be used in the analysis of these samples, thus increasing the specificity.

RESULTS

CGH Analysis

CGH analysis was accomplished using DNA from fresh-frozen and paraffin-embedded material. Several small regions of chromosomal gains and losses were detected (Table 2). No chromosomal imbalances were observed in inflammatory lesions or in cases with reactive lymphoid hyperplasia. In lymphomas, CGH analysis revealed chromosomal imbalances in 8 cases, 4 of 10 MZBCLs, 1 of 4 immunocytomas, and 3 of 3 DLBCLs (Table 1; Fig. 1). Among the MZBCLs, gains at 3q and losses at 7q were observed in two tumors, whereas gains at 11q and losses at 6q occurred in two DLBCLs. No high-level amplifications were detected. Of the 23 chromosomal imbalances that involved intrachromosomal breaks outside the centromere, 14 (7 gains and 7 losses) were localized in chromosomal regions with known aphidicholin-inducible fragile sites.22

Correlation of CGH Karyotype to Clinical Status

Among the low-grade malignant lymphomas at stage IE, only one of nine showed any chromosomal abnormalities. In this case, a loss at 4p15-p16 was detected. Of the six cases with advanced stages of disease, chromosomal abnormalities were detected in four: two of three at stage IIE and two of three at stage IVE. In MZBCL, the primary tumors from the two patients with stage IV disease showed the highest number of imbalances. Recurrent disease was observed in four patients with low-grade malignant lymphomas. A local recurrence was seen in patient 10, whereas patients 8, 9, and 14 had recurrent disease that occurred at extraorbital sites. Chromosomal imbalances were detected in the primary tumor in all four patients in whom recurrent disease developed.

All DLBCLs were classified as highly malignant. The lowest number of chromosomal imbalances was detected in the primary tumor at stage IIE (patient 18). The highest number of imbalances was seen in the primary tumor of patient 16. This patient developed recurrent disease in the right gluteal region 6 months after the diagnosis of the primary tumor.

DISCUSSION

CGH

CGH analysis has been used to examine DNA copy aberrations in a variety of tumors. In many cases, it is difficult to compare data from different studies, because the protocols and software used for the CGH analysis may vary. In virtually all former CGH studies, chromosomal aneuploidy has been identified using “fixed thresholds”—that is, gains or losses have been annotated to regions showing green or red fluorescence intensity above a given level. The threshold chosen, however, varies among individual studies.

To improve sensitivity, a procedure based on standard reference intervals has recently been introduced.20 In this method, the confidence interval of the CGH profile from the tumor sample is compared with that obtained from a series of samples of normal DNA. Conventional CGH has a detection limit above 10 Mb. With the high-resolution analysis, deletions in the 3- to 10-Mb range can be detected.21 The use of this procedure was necessary to detect small deletions such as 7q31 in two of the MZBCLs.

Marginal Zone B-Cell Lymphomas

Very few CGH-studies of MZBCLs have been reported. In a series of 25 cases, Dierlamm et al.16 observed chromosomal imbalances in 80%. The disease was at stage II or more in 22 of the 25 patients. In our series, chromosomal imbalances were seen only in 40% of the MZBCLs, but were present in three of four tumors at stage IIE or IVE. The lower proportion of tumors with detectable chromosomal imbalances reported in this
study could have been due to the higher number of patients with disease at stage IAE.

Some, but not all, of the abnormalities seen in our cases of MZBCL have been reported previously. Gains at 1q and 3q with minimal common regions at 1q25-q31, 3q21-q23, and 3q25-q29 are frequently detected by CGH analysis.\textsuperscript{16} Losses at 7q31 have been observed in 40% of splenic MZBCLs with PCR analysis of microsatellite markers.\textsuperscript{23} Trisomy 8 has been detected with conventional banding techniques in follicular center lymphomas,\textsuperscript{24} but has not been reported previously in MZBCLs. Gains of 2q with a break at 2q33 were seen in one recurrent case of MZBCL in the series of Dierlamm et al.\textsuperscript{16} Losses at 4p and 4q are rare observations in non-Hodgkin lymphomas.\textsuperscript{8} Loss of chromosome 14 has been detected by CGH analysis in 1 of 45 cases of mantle cell lymphomas,\textsuperscript{11} but has not been reported previously in MZBCLs. Loss of 1p was seen in 1 of 25 cases of MZBCL.\textsuperscript{16}

**Immunocytoma**

No CGH analysis of immunocytomas has been reported previously. The gains at chromosomes 3 and 18 are common abnormalities in MZBCLs,\textsuperscript{16} whereas losses at 11p14 and 21q21 are unusual in non-Hodgkin lymphomas in general.\textsuperscript{8}

**Diffuse Large B-Cell Lymphomas**

Previous CGH studies of DLBCLs have revealed chromosomal abnormalities in more than 90% of the tumors examined.\textsuperscript{10,25,26} Frequently, the karyotype is complex, with five to six aberrations detected on average in each case. In two of three of the DLBCLs, gains at 11q and losses at 6q were observed. CGH analysis of central nervous system (CNS) DLBCL has revealed gains at 11q, 16p, 18q and losses at 6q as frequent findings.\textsuperscript{26,27} In a study of DLBCLs with nodal, splenic and extranodal manifestations, Monni et al.\textsuperscript{25} observed gains at chromosomes X (41%), 1q (38%), 7 (31%), 3 (24%), and 6p, 11, 12, and 18 (21% each), and losses at 6q (38%), X (21%), 1p (14%), and 8p (10%). Gains at chromosome 8, with a minimal common region at 8q23-24.2, were observed in 35% of recurrent tumors compared with only 6% of primary tumors.\textsuperscript{25} Deletions at 6q appear to be most frequently seen in the 6q16-q21 region with a 2-Mb region at 6q21 as the minimal common region.\textsuperscript{28} Involvement of 19q13 is of interest because of the presence of the $BCL3$ gene at 19q13.1. Conventional cytogenetic analysis has revealed translocations at 19q13 both in Hodgkin disease and in non-Hodgkin lymphomas.\textsuperscript{29,30}

Amplifications of 2p are observed in 5% to 10% of the cases,\textsuperscript{10,25,31} more frequent in recurrent than in primary tumors.\textsuperscript{25} Gains at 2p with amplification of the REL gene have been associated with extranodal presentation.\textsuperscript{31} Breaks at 3p14 are of interest, because they may disrupt the tumor suppressor gene $FHIT$.\textsuperscript{32} The most common abnormality is a loss of the $FHIT$ gene, but in some non-Hodgkin lymphomas, breaks at 3p14 have been observed with subsequent gains in the distal part of 3p.\textsuperscript{16}

**Fragile Sites**

The association of intrachromosomal breaks with fragile sites in non-Hodgkin lymphomas has also been observed by other investigators.\textsuperscript{11,33} Fragile sites are thought to contribute to chromosomal instability in tumor cells and have been associated with deletions, amplifications, and translocations.\textsuperscript{22} Fragile sites may represent areas of late-replicating DNA and could be destabilized as a result of increased replication of the tumor cells. They are also targets for mutations.\textsuperscript{34,35} A possible mechanism for fragile sites in the generation of amplified chromosomal units is the initiation of breakage-fusion-bridge cycles.\textsuperscript{36} Breaks at fragile sites may interrupt tumor-suppressor
The CGH karyotype of the DLBCLs was different from that of the MZBCLs and the immunocytoma. Considerable variation in the karyotype was also observed among tumors from the same subgroup. The only abnormalities that were common to more than one tumor were gains at 3q and losses at 7q31 in two of four MZBCLs and gains at 11q and losses at 6q in two of three DLBCLs. Although such imbalances are among the most frequently observed abnormalities in larger series of these tumors, they are present in only half of the tumors examined. They could define a subset of MZBCLs or DCBCL, but their relevance as genetic markers in lymphoma classification remains to be determined.

Studies of gastrointestinal DLBCLs and mantle cell lymphoma indicate that patients with tumors that have a less complex molecular cytogenetic karyotype (no aberrations or one aberration) have a significantly higher probability of survival than patients having tumors with two or more aberrations. All five patients in our series in whom recurrent disease developed had primary tumors with chromosomal abnormalities. More patients and longer follow-up time are needed, however, to determine whether CGH analysis is prognostically relevant in patients with orbital lymphomas.

Chromosomal Imbalances in Lymphoid Tumors


The sequence of luminance values (cd/m²) in Figure 1 is incorrect. The correct values should be, from the top down: 79, 68, 64, 59, 54, 49, 44, 40, 35, 32, 24, 0.

The online version of this article was corrected on December 11, 2001.