Follicular Lymphoma grade 3B. A separate entity?
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CHAPTER 5

Array CGH reveals a very high frequency of deletions of the long arm of chromosome 6 in testicular Lymphoma

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Abstract

Despite the fact that numerous studies have been performed on diffuse large B-cell lymphoma (DLBCL), only few have concerned extranodal lymphomas occurring in the testis. We performed a cytogenetic and molecular study of 17 testicular Non-Hodgkin lymphomas (NHL) of which 14 were proven primary DLBCL of the testis. Cytogenetic analysis revealed in 8 out of 11 evaluable cases a structural abnormality of the long arm of chromosome 6, with deletion or addition of material of unknown origin, and with breakpoints spanning the region 6q12-6q23. The cytogenetic findings were confirmed by fluorescent in situ hybridization (FISH) with a chromosome 6 painting probe. Using array based-CGH on 16 evaluable cases, including 5 cases not tested by cytogenetics or FISH, 14 (88%) showed chromosome 6q deletions. We identified two regions of minimal deletion (RMD), at 104Mb-113Mb (6q16.3-q21) and 137.5Mb-138.8Mb (6q23.3), respectively. In one case we observed a 2.7Mb homozygous deletion ranging from 135.3Mb to 138.0Mb that partly overlapped with the RMD at 6q23.3. Our study indicates that 6q deletions play a major pathogenetic role in DLBCL of the testis and that many of these deletions are part of unbalanced translocations.
Introduction

Non-Hodgkin lymphomas (NHL) localised to the testes are relatively rare (Shahab and Doll, 1999). Apart from the extremely rare follicular lymphomas that occur in children or adolescents and that have a very favourable prognosis (Pileri et al., 2002), they usually occur as a primary and occasionally as a secondary testicular diffuse large B-cell lymphoma (DLBCL). Primary testicular DLBCL are clinically aggressive lymphomas with a relatively poor prognosis, which is in contrast with other primary extranodal DLBCLs, e.g., of the stomach and skin (Zucca et al., 2003). Testicular DLBCL also show a specific pattern of metastasis, e.g., to extranodal sites such as the contralateral testis and the central nervous system (CNS) (Moller et al., 1994; Fonseca et al., 2000; Hasselblom et al., 2004). Both the testis and CNS are considered as immune-privileged sites, in which immune responses occur in a manner that differ from other sites or do not occur at all (Riemersma et al., 2000, 2005).

Genetic alterations in DLBCL often comprise complex abnormalities, including translocations, trisomies, amplifications and deletions. A common aberration concerns deletion of the long arm of chromosome 6. Several studies showed that 6q deletions are more frequent in DLBCL with an activated B cell type gene expression profile (40% of the cases) than in DLBCL with a germinal center cell type of gene expression profile (20%) and that they are very frequent (50-60%) in DLBCL of the CNS (Rickert et al., 1999; Weber et al., 2000; Bea et al., 2005). In general, deletion of 6q is considered a secondary chromosomal abnormality in lymphomas. In some cases, however, a primary pathogenetic role of 6q deletion is suggested by the observation that it occurs as the sole cytogenetic abnormality. Moreover, the deletions may harbour prognostic value as markers of poor outcome (Offit et al., 1991).

Using combined cytogenetic analysis, FISH and loss of heterozygosity (LOH) studies in a variety of lymphoma subtypes, three regions of minimal molecular deletion (RMD) were identified within the region 6q21-6q27 (Gaidano et al., 1992; Offit et al., 1993, 1994; Zhang et al., 1997; Merup et al., 1998; Jackson et al., 2000).

Chromosome 6 aberrations have not been studied in detail in testicular lymphomas (Lambrechts et al., 1995). In the present study, we describe 6q abnormalities in a series of 17 NHL of the testis, 14 representing primary DLBCL of the testis.
Materials and Methods

Patients
Seventeen tumor samples were collected during standard diagnostic procedures over a period of about 20 years and reviewed by one of the pathologists (P.K.) before inclusion in this study. Except for one case (#12), all tumours were classified as DLBCL according to the WHO classification (Jaffe et al., 2001). Analysis of clinical data revealed that in 14 cases (82%) the tumour was a primary testicular DLBCL (Table 2). Case #12 represented a testicular relapse of an aggressive lymphoma with morphologic features of Burkitt lymphoma, but genetic analysis was incompatible with this diagnosis and suggested a variant of DLBCL or a progressed follicular lymphoma (no breakpoint at 8q24, breakpoint at 18q21 indicative of a t(14;18)(q32;21) see Table 1). Four cases (#3,#4,#5,#9) have been reported before (Lambrechts et al., 1995).

Cytogenetic Methods
Cytogenetic investigation using standard cytogenetic techniques was carried out on 12 cases from which fresh material was available for cell culture. Karyotypes were reported in accordance with ISCN 1995 (ISCN, 1995).

Fluorescence in Situ Hybridization
Metaphase spreads from the 11 cases for which cytogenetic analysis was successful were subjected to fluorescence in situ hybridization (FISH), using standard protocols. A whole chromosome 6 paint (WCP6) was used to verify the chromosome 6 aberrations that were identified by cytogenetics and to reveal hidden chromosome 6 material in the marker chromosomes (Table 1). To characterize the 3q27 breakpoints observed in 4 cases with respect to the BCL6 gene, we performed a segregation FISH assay using PAC 165121 (green) and BAC RP11-137K3 (red) (BACPAC resource, Children’s Hospital Oakland, CA, USA) (Bosga-Bouwer et al., 2005).

Array based Comparative Genomic Hybridization
Samples were analyzed with an array that contained 340 chromosome 6-specific clones. These clones were selected from the 1-Mb BAC collection obtained from Dr. Nigel Carter (Wellcome Trust Sanger Institute, Cambridge, UK) (Fiegler et al., 2003), and from the Human BAC Resource Consortium_1 Set (Dr. Pieter de Jong, Children’s Hospital Oakland Research Institute, Oakland, CA, USA), with a small selection of additional BACs to fill a few remaining gaps. 445 subtelomere-specific clones selected from our subtelomere array (Kok et al., 2005) were
included on the array to give an impression of the overall level of genomic instability of the testis lymphoma samples (Westra et al., 2005). In order to map the clones with respect to the sequence of the human genome we used the May 2004 reference sequence of the human genome (UCSC version hg17) based on NCBI Build 35 and produced by the International Human Genome Sequencing Consortium. Detailed information on the BAC/PAC set of our subtelomeric array is available upon request (k.kok@medgen.UMCG.nl). Detailed array design, hybridization, image analysis and processing were performed according to recently described protocols (Tchinda et al., 2004; Kok et al., 2005).

Results
Cytogenetics and FISH
The cytogenetic findings of 12 testicular lymphomas are summarized in Table 1. Various clonal numerical or structural aberrations, like balanced translocations, isochromosomes and deletions were found. In 4 cases (#1,#4,#9,#10) an aberration affecting the 3q27 region was observed. Three of these 4 cases could be further studied by FISH and in two cases (#1 and #9) a BCL6 breakpoint was detected.

The most striking finding was the high frequency of structural abnormalities at the long arm of chromosome 6, as seen in 7 of the 9 informative cases. Four cases showed a deletion and 3 cases addition of unknown material. The smallest region of overlap for the breakpoints was between bands 6q15 and 6q21. In addition, case #9, which was near-tetraploid by karyotyping, had lost two chromosomes 6. Case #11, also a near tetraploid tumor, contained five unaltered chromosomes 6.
Array CGH reveals a very high frequency of 6q deletions in TL.

Table 1: Cytogenetic Results on 12 Testicular Lymphomas.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Karyotype:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46-47, X.-Y., del(1)(q41),ins(1:?)(q21:?),+?3,add(6)(q13),+7,add(14)(q32),-19, +[r][cp5] / 46,XY[1]</td>
</tr>
<tr>
<td>2</td>
<td>46,XY[9]</td>
</tr>
<tr>
<td>3</td>
<td>75-99&lt;4n&gt;,XXYY,+Y,der(1)(t(1;11)(q32;q13)x2,-2,-2,-5,-5,add(6)(q?15),add(6)(q?21), add(7)(q22)x2,i(7)(q10),-8,add(8)(q24)x2,-9,-9,-9,-10,-11,del(11)(q13q21),-12,-13, -14,-15,-16,-17,-17,-17,del(18)(q12q23)x2,-19,-19,-20,-22,+der(?)(t(5:?)(q13:?))x2, +der(?)(t(17:?)(q21:?))x2,+13mar[cp10]</td>
</tr>
<tr>
<td>5</td>
<td>45-50,dup(X)(p21p22),-Y,del(6)(q273),del(6)(q15),+8,t(11;14)(p11;q11), +12,+13,del(14)(q31),+18[cp12]</td>
</tr>
<tr>
<td>6</td>
<td>Failure</td>
</tr>
<tr>
<td>7</td>
<td>46,XY[3]</td>
</tr>
<tr>
<td>8</td>
<td>86-89,XXYY,+X,add(1)(p11),del(2)(p11),-3,-4,del(6)(q15q21)x3,+7,t(7;19)(q11;q13.1), +8,-10,-11,-12,-13,-15,-15,-16,-17,-17,-18,-19,add(19)(p13.1), der(19)(t(7;19)(q11;q13.1)),-20,-22,+mar1x2,+mar2x2,+mar3[cp10]</td>
</tr>
<tr>
<td>11</td>
<td>101-104,add(X)(q271)x2,YY,+Y,add(2)(p1?3)x2,+add(2)(p1?3)x2,+6,add(7)(q22)x2, +add(7)(q22),-8,-8,+10,+14,-15,+16,+19,+20,+21,+der(?)(t2;?)(p12:?), +der(?)(t8:?)q11.2:?)x2[cp10] /46,XY[2]</td>
</tr>
<tr>
<td>12</td>
<td>37,XY,+X,+5,del(6)(q1q2),add(7)(p11),dic(9;12)(p271;q22),+10,ins(11:?)(q21:?), +12,add(13)(q22),-14,add(14)(q32),add(18)(q21),+20,+21,+der(?)(t1:?)q12:?), +r,+mar1,+mar2,+mar3[10] /46,XY[10]</td>
</tr>
</tbody>
</table>
Metaphase spreads from 11 of the 12 cases were further studied by FISH and hybridized with a WCP6 probe. The analysis was informative in 6 cases (Table 2). Although a chromosome paint does not allow to map breakpoints, a partial loss of 6q or substitution of 6q by material of unknown origin (addition) can be determined in most cases. In two cases (#1, #3) the chromosome 6 paint revealed addition of other material at 6q, leading to a net loss of 6q material, and therefore supporting the abnormalities seen by cytogenetic analysis. In cases #8 and #12 FISH analysis also showed addition of material of unknown origin on 6q, again leading to a net loss of 6q, whereas cytogenetics suggested a small interstitial deletion. Additionally, in case #8, one of the marker chromosomes contained chromosome 6 material. Only case 4 with a del(6)(q15q16) did not reveal any abnormality with FISH, suggesting that this might represent a simple interstitial deletion. The loss of 2 chromosomes 6 in case #9 with a near-tetraploid tumor was confirmed by FISH. In case 7 with a normal karyotype, the FISH assay did also not show any abnormality. Two out of 8 cases with a cytogenetic aberration on 6q (#2 and #5) were inconclusive due to failure of the FISH experiments.
Table 2: Summary of the Cytogenetic and Molecular Results on 17 Testicular lymphomas.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>P / S</th>
<th>Cytogenetics:</th>
<th>FISH</th>
<th>array-CGH gap in chr. 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P</td>
<td>add(6)(q13)</td>
<td>add(6)(q) 6x2</td>
<td>60 --&gt; 170 (qter)</td>
</tr>
<tr>
<td>2</td>
<td>?</td>
<td>normal</td>
<td>failure 6x2</td>
<td>no loss of 6q material</td>
</tr>
<tr>
<td>3</td>
<td>P</td>
<td>add(6)(q15)/add(6)(q21)</td>
<td>add(6)(q)x2</td>
<td>no more material available</td>
</tr>
<tr>
<td>4</td>
<td>P</td>
<td>del(6)(q15q16)</td>
<td>6x2</td>
<td>104 --&gt; 123</td>
</tr>
<tr>
<td>5</td>
<td>P</td>
<td>del(6)(q15)/del(6)(q23)</td>
<td>failure 6x2</td>
<td>60 --&gt; 143 + homoz.del.135 --&gt; 137</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
<td>failure</td>
<td>no material 6x2</td>
<td>60 --&gt; 170 (qter)</td>
</tr>
<tr>
<td>7</td>
<td>P</td>
<td>normal</td>
<td>6x2</td>
<td>97 --&gt; 170 (qter)</td>
</tr>
<tr>
<td>8</td>
<td>P</td>
<td>del(6)(q15q21)x3</td>
<td>add(6)(q)x3 60 --&gt; 170 (qter)</td>
<td>102 --&gt; 168 + 102--150(x2)</td>
</tr>
<tr>
<td>9</td>
<td>P</td>
<td>(4n),-6,-6,+10~20 mar</td>
<td>6x2</td>
<td>pter --&gt; qter</td>
</tr>
<tr>
<td>10</td>
<td>P</td>
<td>add(6)(q12)</td>
<td>failure 6x2</td>
<td>76 --&gt; 139</td>
</tr>
<tr>
<td>11</td>
<td>P</td>
<td>(4n), +6</td>
<td>6x5</td>
<td>137 --&gt; 138</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>del(6)(q1q2)</td>
<td>add(6)(p/q)x2 36 --&gt; 161</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>82 --&gt; 112; 131 --&gt; 138; 143 --&gt; 146</td>
</tr>
<tr>
<td>14</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>157 --&gt; 161</td>
</tr>
<tr>
<td>15</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>no loss of 6q material</td>
</tr>
<tr>
<td>16</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>60 --&gt; 170 (qter)</td>
</tr>
<tr>
<td>17</td>
<td>P</td>
<td>-</td>
<td>-</td>
<td>93 --&gt; 116; 138 --&gt; 149</td>
</tr>
</tbody>
</table>

a P = primary testicular lymphoma; S = secondary testicular lymphoma; case 2, no data available; case 6, primary CNS lymphoma; case 12, primary Burkitt lymphoma.
b Fluorescence in situ hybridisation with wc6p probe.
c For details, see Fig. 1.
Array based Comparative Genomic Hybridization

Eleven of the 12 cases, and 5 other primary DLBCL cases of the testis without cytogenetic data, were subjected to array-based Comparative Genomic Hybridization (aCGH). Fourteen of these 16 cases (88%) showed 6q deletions, ranging from deletions of the entire long arm of chromosome 6 (cases #1, #6, #9, and #16) to a small interstitial deletion of 2.2 Mb (case #11). From the 2 cases that had a normal karyotype (cases #2 and #7), one showed a deletion at 6q (#7) and the other tumor (#2) showed no loss of chromosome 6 material (Table 2). In the latter case, whole genome aCGH showed duplications for chromosome 7, 8q and 19q, indicating that the sample analyzed contained a sufficient number of tumor cells (data not shown).

Comparison of the deleted regions revealed two different regions of overlapping deletions; one segment ranged from 104Mb to 113Mb, corresponding to chromosome bands 6q16.3-q21, and was deleted in 12 out of 14 cases. The second segment ranged from 137.5Mb to 138.8Mb, corresponding to chromosome band 6q23.3, and was deleted in 12 out of 14 cases (Fig.1). A homozygous deletion, ranging from 135.3Mb-138.0Mb was observed in case #5.

Three of the 16 cases studied by aCGH were near-tetraploid cases. In case 11 we saw a small interstitial deletion within 6q against a seemingly normal background. In case 9 aCGH revealed a diminished ratio for all 6q specific clones indicating loss of the entire chromosomes 6. In case 8 aCGH revealed 4 levels of ratios, most probably indicating regions for which 1, 2, 3, or 4 copies were present (Fig.1).
Array CGH reveals a very high frequency of 6q deletions in TL

Figure 1
The array-CGH results in 14 testicular DLBCL cases with 6q deletions. The centromere of chromosome 6 is located around 60 Mb. Black bars indicate the deleted regions for each case. Case numbers are indicated at the right side. For case #8 the increasing thickness of the bars corresponds with the loss of increasing number of alleles. For case #5 the additional small bar indicates the position of the homozygous deletion. The vertical grey bars indicate the position of the minimal region of deletion.
Discussion

A group of 17 testicular lymphomas, of which 14 were proven primary DLBCL of the testis, was analysed with a combination of chromosome banding analysis, FISH and aCGH. Karyotyping revealed complex karyotypes with multiple numerical and structural abnormalities in 9 cases. The apparent normal karyotypes seen in two cases may have been caused by preferential culturing and harvesting of normal cells instead of tumor cells. Indeed, for one of these two cases, aCGH analysis did reveal several copy number changes.

Aberrations in the 3q27 region were observed in 4 cases, which is consistent with a frequency of 3q27 abnormalities in approximately 30% of the DLBCLs (Bastard et al., 1994). We cannot comment on the true incidence of \textit{BCL6} breakpoints in testicular DLBCL, since we applied FISH on only four cases (2 out of 4 showing a breakpoint in or nearby the \textit{BCL6} gene).

Chromosome banding analysis revealed seven cases with structural abnormalities of chromosome 6 resulting in a loss of sequences from 6q in 4 cases and addition of foreign material in 3 cases, whereas one case with a near-tetraploid karyotype lost two chromosomes 6. The chromosome 6 paint helped to identify marker chromosomes, or chromosomes with an unidentified centromere that contained unrecognized chromosome-6 material, as illustrated in case 8. Moreover, the interstitial deletions of chromosome 6 identified by karyotyping in cases 8 and 12 in fact turned out to be derivative copies of chromosome 6 with a terminal deletion and addition of material of unknown origin to bands 6q15 and 6q1, respectively. This indicates that almost all 6q deletions found in our lymphomas were the result of unbalanced chromosomal translocations instead of terminal or interstitial deletions.

Combining all three methods, we found 6q deletions in 15 out of 17 cases (88%) in the lymphomas located in the testis and in 13 of the 14 primary DLBCL of the testis (93%), which is much higher than reported for other DLBCL, even if only DLBCL with an activated B-cell-like (ABC) type of gene expression are considered. Using conventional CGH and comparison with array based gene expression data in 224 cases of DLBCL, Bea et al. (2005) described loss of 6q16 in 22% and loss of 6q21-q22 in 26% of the cases, both abnormalities being more frequent in ABC than in germinal center B-cell-like (GCB) type of DLBCL. Loss of 6q21 was also associated with a loss of the T-cell expression signature in these lymphomas, suggesting that one or more genes involved in immune interaction with T cells were deleted.

The frequency in testicular lymphomas is also much higher than the 47-62% previously reported in DLBCL of the CNS (Rickert et al., 1999; Weber et al.,
Array CGH reveals a very high frequency of 6q deletions in TL

2000; Boonstra et al., 2003). Of note in our series of 16 lymphomas evaluable by array CGH, we included 5 primary DLBCL of the testis for which no cytogenetic data were available (cases 13-17) and in 4 out of these 5 cases (80%) we detected a 6q deletion, indicating that the high incidence of 6q deletions is not biased by selection of cases with cytogenetically proven 6q alterations.

Two commonly deleted regions were identified by karyotype analysis, one at 6q15-q21 and one at 6q23-q24. By applying aCGH, we could further reduce these two regions to 6q16.3-q21 and 6q23.3, respectively. These two RMDs do not overlap with the commonly deleted 6q25-q27 region as described in intermediate grade B cell lymphomas, but they may be related to the commonly deleted region in 6q21-23, described in primary DLBCL of the CNS (Weber et al., 2000), ALL and immunoblastic lymphoma (Offit et al., 1993).

Both the testis and CNS are immuneprivileged sites and DLBCL of these sites share major biological and clinical features including a relatively poor outcome, which might be related to the deletions at the long arm of chromosome 6. The 9-Mb RMD at 104Mb-113Mb (6q16.3-q21) contains 53 RefSeq genes (http://genome.ucsc.edu), none of which is an immediate candidate tumor suppressor gene in lymphomas. The distal 1-Mb RMD covering the 137.5Mb-138.8Mb segment of 6q23.3 contains only 8 RefSeq genes (http://genome.ucsc.edu). A 2.7-Mb homozygous deletion detected in one case partly overlapped with this segment. The overlap is approximately 0.5 Mb, and contains only three RefSeq genes, IL22RA2 and IFNGR1, and OLIG3. A possible role of a decreased or absent IL22RA2 and/or OLIG3 expression in the development of testicular lymphoma is unclear. IFNGR1 encodes the ligand-binding chain (alpha) of the heterodimeric gamma interferon receptor, which is involved in the regulation of expression of HLA class II genes. HLA class II downregulation is frequently found in testicular DLBCL and CNS DLBCL (Riemersma et al., 2000). Interestingly, in DLBCL arising at non-immuneprivileged sites, downregulation of HLA class II is associated with a very poor prognosis (Rimsza et al., 2004). Another gene that might be of interest, IL20RA, was deleted in 10 out of 16 cases, including a homozygous deletion in case #5

In conclusion, DLBCL of the testis are characterized by very frequent loss of parts of chromosome arm 6q. We identified two deletion hot spots on the long arm of chromosome 6, mapping at 6q16.3-q21 and 6q23.3, respectively. The very high frequency of these events suggest that functional loss of one or more genes within these regions may be crucial in the pathogenesis of testicular lymphomas.
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