Chromosomal Imbalances in Diffuse Large B-Cell Lymphoma Detected by Comparative Genomic Hybridization

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Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma. In contrast to many other hematological malignancies, no chromosomal abnormalities with a diagnostic or prognostic value have been identified in DLBCL. Numerical chromosomal imbalances were characterized by comparative genomic hybridization (CGH) performed on 54 DLBCL tumors from a total of 40 patients. The clonal relatedness was demonstrated in 9 of 11 pairs of matched diagnostic tumors and their relapses as determined by *IGH* gene rearrangement analysis and/or the CGH profiles. Furthermore, immunohistochemical expression analyses of BCL2 and BCL6/LAZ3 were performed on all cases. Copy number changes were detected in 94% of the diagnostic tumor samples and in all of the relapses. Chromosomal losses in diagnostic tumors were preferentially observed at 8p22-pter (29%), 1p34-pter (26%), 6q23-qter (20%), 17p12-pter (17%) and 22q (17%), 9p23-pter (14%), whereas gains were mainly seen in Xq25–26 (43%), 13q22 (26%), 12cen-q14 (20%), 3q24–25 (11%), 7 (11%), and 18q12–21 (11%). Loss of 22q was significantly more commonly seen in the diagnostic tumor samples with more advanced clinical stage in other words, Stage III–IV compared with Stage I–II, and band 18q21 was significantly more often gained in relapses as compared to diagnostic tumors. None of the recurrent alterations were detected as a single abnormality, suggesting that other genetic lesions below the detection level of CGH may be the initiating event in the tumorigenesis of DLBCL. However, the distribution of CGH alterations support the idea of a progression of genetic events where loss of 8p and 9p and gain of 3q, 13q, and 18q would represent relatively early events because they were distributed in tumors with only two abnormalities.

KEY WORDS: Chromosome, CGH, Clonality DLBCL.

Non-Hodgkin lymphomas (NHL) constitute a heterogeneous group of lymphoid neoplasms that display different morphological, immunological, cytogenetic, and molecular genetic features. The revised European-American classification of lymphoma (1) takes all these features into consideration for the distinction of different lymphoma entities. Lymphomas have been associated with a wide range of recurrent chromosomal abnormalities. Some of these can occur as a single cytogenetic alteration and may sometimes be recurrent in a lymphoma entity, such as the t(14;18)(q32;q21), which is found in 85 to 90% of follicular lymphomas. The occurrence of distinct chromosomal changes in specific histopathological subtypes of NHL has improved the understanding of the genetic basis of lymphomagenesis (2).

Diffuse large B-cell lymphomas (DLBCL) constitute approximately 40% of all lymphomas and are typically characterized by an aggressive behavior in

TABLE 1. Clinical Data of the 40 Cases of Diffuse Large B-Cell Lymphomas

Case		Age at Diagnosis	Sex (M/F)	Clinical Stage*	Tumors Analyzed	
No.	ID				Diagnostic	Relapse
1	A	21	M	IVB	+	−
2	B	44	F	IIIA	+	−
3	C	57	M	IAE	+	−
4	D	80	M	IIIB	+	−
5	E	49	M	IVB	+	−
6	F	42	M	IA	+	−
7	229	62	F	IA	+	−
8	239	72	M	IVA	+	−
9	433	64	F	IA	+	−
10	542	45	M	IA	+	−
11	877	72	M	IAE	+	−
12	878	46	F	IIIB	+	−
13	228	54	M	IIIA	+	−
14	K	71	M	IVB	+	−
15	L	74	F	IVA	+	−
16	M	38	F	IVB	+	−
17	74	71	F	IAE	+	−
18	222	71	M	IVB	+	−
19	790	75	M	IVB	+	−
20	661	47	M	IIB	+	−
21	413	62	M	IIIB	+	−
22	288	54	M	IIA	+	−
23	577	48	M	IVA	+	−
24	657	56	F	IV	+	−
25	852	44	F	IVB	+	−
26	G	67	M	IIIA	+	+
27	H	60	F	IIB	+	+
28	I	52	M	IVA	+	+
29	N	25	M	IIIB	+	+
30	S	47	M	IIA	+	+
31	612	63	M	IAE	+	+
32	J	65	M	IVA	+	+
33	454	69	F	IIIB	+	+
34	425	65	F	IVA	+	+
35	O	50	M	IA	+	+
36	R	54	M	IVB	−	+
37	471	61	M	IIIB	−	+
38	574	66	M	IA	−	+
39	836	69	M	IIAE	−	+
40	633	64	M	IAE	−	+

Clinical staging based on the involvement of one lymph node (I), >1 lymph node on the same side of the diaphragm (II), >1 lymph node on both sides of the diaphragm (III), and disease with bone marrow infiltration (IV).

M/F = male/female; A refers to A-symptoms and B to B-symptoms; E = extra nodular neoplasia.

which less than half of the patients are cured (3). Despite intensified treatment with chemotherapy and radiotherapy, no real improvement of the clinical handling and survival have been achieved in the last 20 years. The clinical presentation and the course of the disease are extremely heterogeneous. Information regarding the chromosomal alterations found in DLBCL has come from regular cytogenetics (2, 4) as well as from analyses using comparative genomic hybridization (CGH) (5). Gains were preferentially identified for chromosomes X, 3, 7, 11, 12, and 18, whereas losses involved chromosomes X, 4, 6, and 8. Translocations involving the *BCL6/LAZ3* gene at 3q27 and *BCL2* at 18q21 are found in 25 to 40% and 25 to 50% of the cases, respectively (6). The most common translocations, t(3;14) and t(14;18), appear in 20% and 30% of the cases, respectively

(7). In lymphomas with these translocations, *BCL6* and *BCL2* expression is deregulated as a result of their juxtaposition to the *IGH* gene at 14q32 (8). Increased expression of *BCL2* has also been shown to result from gene amplification (9). Genetic alterations involving the *BCL6* gene are mainly associated with DLBCL, and in addition to the t(3;14), translocations between *BCL6* and a variety of chromosomes have been observed (10). *BCL6* is usually expressed in normal B- and CD4-positive T-cells in the germinal center (GC), and helps to control the GC function (11–13). However, it is still not known whether *BCL6* overexpression provides survival benefit for the DLBCL cells.

So far, the genetic mechanisms behind the clinical heterogeneity of DLBCL are poorly understood. To approach this question, we have analyzed a large panel of diagnostic tumor samples and relapses of DLBCL with regard to the distribution of unbalanced chromosomal aberrations that are possibly involved in tumor progression as well as their clonal relationship.

MATERIALS AND METHODS

Patients and Tumor Specimens

A total of 54 DLBCL specimens were obtained from 40 patients, 28 men and 12 women, diagnosed between 1985 and 1998 (Table 1). The patients were identified from the files of the Departments of Pathology at the Uppsala University Hospital, and the Umeå University Hospital, Sweden. For 10 cases, tumor material was available from both the diagnostic sample of DLBCL (D) and from subsequent relapses (R). Frozen tumor biopsies were available for all patients, and paraffin-embedded tumor material was available from all tumors except 11D, 13D, 19D, 20D, 25D, 34R1, 34R2, and 40R2. All tumors were reevaluated by histopathological analyses performed by two of the authors (CS and GR) and were confirmed to be DLBCL according to the revised European-American lymphoma classification (1). The term *diagnostic tumor* refers to the first sample obtained from each of the 40 patients which at histopathological examination was classified as DLBCL.

Comparative Genomic Hybridization

High molecular weight DNA was prepared from fresh frozen tumor tissue using a standard protocol for phenol–chloroform extraction, and the quality and concentrations of the samples were verified by agarose gel electrophoresis. CGH was performed as previously described (14, 15) with minor modifications. In brief, tumor DNA samples were labeled with fluorescein isothiocyanate (FITC)- 12-dUTP
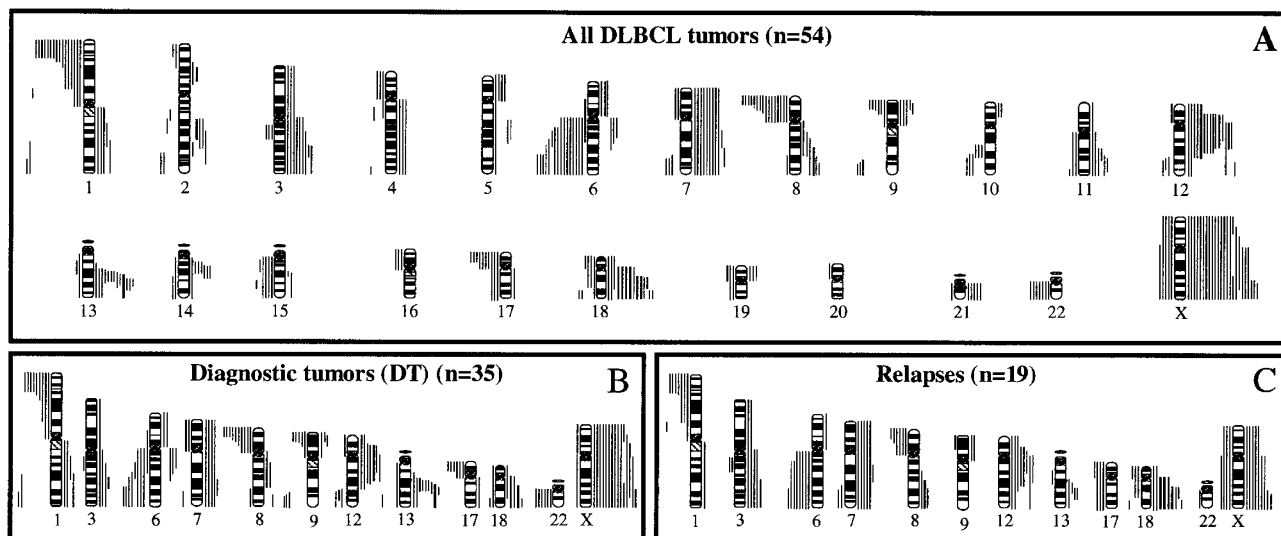
FIGURE 1. Copy number changes in 54 diffuse large B-cell lymphomas (DLBCL) from 40 patients. Each line represents one alteration detected in one tumor, with losses indicated to the *left* and gains to the *right* of the ideograms. *Thick lines* indicate high-level amplifications. **A,** all comparative genomic hybridization alterations detected in the entire material. The most commonly involved chromosomes in (**B**) diagnostic tumors, (**C**) relapses. n, the total number of tumors analyzed in each category.

(Dupont, Boston, MA) by nick translation. Equivalent amounts (300–500 ng) of tumor and normal reference DNA labeled with Texas Red (Vysis, Inc.) were mixed together with unlabeled Cot-1-DNA (Vysis, Inc.), denatured and applied to slides with denatured metaphases of normal lymphocytes (Vysis, Inc.). After hybridization at 37° C for 48 h, the slides were washed in 0.4× standard saline citrate (SSC)/0.3% Nonidet P-40 at 74 ± 1° C for 2 minutes, in 2× SSC/0.1% Nonidet P-40 at room temperature for 2 minutes, and in sterile autoclaved water for 2 minutes. After air drying, slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vysis, Inc; 125 ng/mL). Two control experiments were performed in which DNA from a healthy male donor was hybridized against normal reference male DNA, and DNA from a breast cancer cell-line (MPE600, Vysis, Inc) was hybridized against normal reference female DNA.

In the subsequent digital image analyses, 8 to 11 three-color digital images (DAPI, FITC, and Texas Red fluorescence) were captured from each hybridization using an Axioplan 2 epifluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) and Sensys charge-coupled equipped (CCD) camera (Photometrics) interfaced to an IPLab Spectrum 10 workstation (Signal Analytics Corp., Vienna, VA). Relative DNA sequence copy number changes were detected by analyzing the hybridization intensities of tumor and normal DNA along the length of all chromosomes in each metaphase spread. The absolute fluorescence intensities were normalized so that the average green to red ratio of all chromosomes in each metaphase was 1.0. The final results were plotted as a series of green to red ratio profiles

and corresponding standard deviation for each human chromosome from p- to q-telomere. At least 12 ratio profiles were averaged for each chromosome (except for X and Y) to reduce noise. Green to red ratios >1.2 were considered as gains and ratios less than 0.8 as losses. The cut-off values were based on the average value from the whole material. Overrepresentation was considered as high-level amplifications when the ratio exceeded 2.0. Heterochromatic regions in the centromeric and paracentromeric parts of the chromosomes, the short arm of the acrocentric chromosomes, the Y chromosome, as well as all regions next to the telomeres were not included in the evaluation. As an internal control of the CGH methodology, six of the tumor samples were hybridized and analyzed on two different occasions, giving concordant results in all cases.

Analyses of Clonal Relationship

The consensus primers used for detection of *IGH*-rearrangements and the PCR conditions have been described previously (16, 17). To distinguish monoclonal PCR products from polyclonal, single-stranded conformation polymorphism (SSCP), analysis was performed as reported elsewhere (18). In one case for which a clonal PCR product was lacking in the diagnostic tumor (Case 33), further analyses were carried out using Southern blotting and hybridization with a J_H probe to determine clonality of the *IGH* gene as previously detailed (19). Cases with changed SSCP pattern at relapse, but displaying one or several identical bands in common with the diagnostic tumor, were considered as clonally related, whereas divergent banding pattern be-

TABLE 2. Findings by CGH and Immunohistochemistry in the 54 Diffuse Large B-cell Lymphoma samples Analyzed in the Study

Tumor	No. of Changes	CGH Alterations*	Immunohistochemistry BCL2	BCL6
1D	12	+Xp21-qter,−1p34-pter,+2q22-32,+4q,+5q14-22,+6c-q16,+8q13-22,−9q33-qter,−12q23-qter,+13q21-22,+14q13-22,−22q	0	0
2D	7	+1q23-31,+7,+12pter-q21,+13q22-31,+14q13-22,+18q,−22q	2	2
3D	7	−1p32-pter,+5,+6p,−6q,+11q,+13q14-qter,−19p	2	1
4D	12	−1p34-pter,+2q22-32,+3q23-25,+4q,+5q14-23,+6c-q22,+9p,+13q21-22,−15q22-24,−16p,−19,−22q	0	1
5D	7	+Xp21-pter,+Xq23-qter,+1q,+12p11.2-q21,+13q21-qter,+18q22-qter,+21q	2	1
6D	3	+X,+14q13-22,−16p	1	1
7D	4	+X,−1q41-qter,−2q33-qter,−17p	2	1
8D	0		1	0
9D	3	−6q21-qter,−8p22-pter,−9p23-pter	0	2
10D	8	+X,+2p13-21,+2q32-33,−3q13.1-21,−6q23-qter,−7p,+9p23-pter,+12p12-q21	1	2
11D	4	+X,−7p,−8p21-pter,+19p	lm	lm
12D	4	−6q,+11q14-qter,−17p12-pter,−22q	0	1
13D	7	+1q31-qter,−2p23-qter,−4q13-21,−8p12-pter,+11q22-23,**+12p11-q21**,−18q22-qter	lm	lm
14D	2	+12q24,**+13q21-qter**	1	0
15D	3	−2p16-pter,−9p,+12p12-q14	1	2
16D	5	−6q15-22,+7q31-34,−8p,+15q21,+18pter-q21	0	0
17D	2	**+2p13-16,+2q22-24**	0	2
18D	14	−2q14-21,+2q24-35,+6c-q21,−6q22-qter,−8p,−8q23-qter,−9q34,+9p21−23,+11q14−22,−11q24-qter,+13q21-31,−17p12-pter,−18,−19	0	1
19D	5	+X,+7,−9p21-pter,+11,−17p12-pter	lm	lm
20D	1	+8cen-q22	lm	lm
21D	2	−8p22-pter,−9p23-pter	1	2
22D	2	−1q31-qter,+3	0	1
23D	3	+Xp11.3-q26,−8p22-pter,−9p22-pter	1	1
24D	7	+3q13.3-qter,−4p,+4q,−12p,−13q,−17p,+17q	2	0
25D	0		lm	lm
26D	7	+Xq24-qter,−1p,+3q,+5p,+8q24-qter,−15q,**+18q12-qter**	2	0
26R	6	+Xq24-qter,−1p,+5p,+8q23-qter,+14q,**+18q12-qter**	2	0
27D	4	+X,−1p31-pter,−9q32-qter,−12q22-24	0	1
27R	4	−X,−6q,+12q,+18q22-qter	0	1
28D	7	+X,−1p34-pter,−8p21-pter,+12c-q21,+13q21-22,+14q12-21,−22q	0	0
28R	7	+Xq,+3,−6q,−8p21-pter,+12c-q21,+13q21-22,+18q12-qter	0	0
29D	3	+X,−12q22-qter,−22q	0	0
29R	1	−X	0	0
30D	4	+Xq,−1p34-pter,−8p,+13q21-22	0	1
30R	6	+Xq,−1p34-pter,+3q24-qter,−6q14-qter,−8p,+18q12-21	2	2
31D	3	+X,−7q32-qter,−14q22-qter	0	0
31R	9	+Xq25-qter,−2q22-24,+5p,−7q31-qter,−14q,+15q21-qter,−17p,−18p,−20p	1	0
32D	11	−1p,−2q33-qter,−4pter-q21,−6p21-q21,+7,+10p12-pter,−10q23-qter,−11q,+12pter-q21,+14q12-21,+21q	2	0
32R	10	+X,−1p32-pter,+7,+10p12-pter,−10q23-qter,−11q,+12p12-q21,+14q12-21,+21q,−22q	2	2
33D	5	−X,+1q,−4p,+6p,−6q23-qter	2	0
33R	4	−X,+1q,+6p,−6q23-qter	0	0
34D	8	+X,−1p21-pter,−6q,+7,−10q22-23,−15q,−16p,−17p	2	2
34R1	11	+X,−1p21-pter,−3q13.1-21,−6q,+7,−10q22-23,−15q,−17,−18pter-q21,+19p,+21q	lm	lm
34R2	11	+X,−1p21-pter,−3q13.1-21,−6q,+7,−10q22-23,−15q,−17,−18pter-q21,+19p,+21q	lm	lm
35D	2	−8p,**+18q**	2	1
35R1	2	−8p,**+18q**	2	1
35R2	5	−1p34-pter,−6q14-qter,−8p,+9p,**+18q**	2	1
35R3	4	−1p34-pter,−8p,+9p,**+18q**	2	1
36R	3	−9p21-pter,+12,+13q21-qter	2	1
37R	5	+X,+7,**+8q22-qter,+12pter-q21**,−18q22-qter	2	2
38R	4	−Xq,−6q,+7,+12p12-q21	0	2
39R	13	+X,+3,−4q34-qter,+7q21-q22,−7q32-qter,−8p22-pter,+8q21-qter,−10q24-qter,+11q22-qter,−15q12-q15,−17,+18,−21q	0	1
40R1	6	−1p13-p21,+3q,+12,−13q13-q21,+13q22-q32,+18	2	0
40R2	3	+3q,+12,+13q22-q32	lm	lm

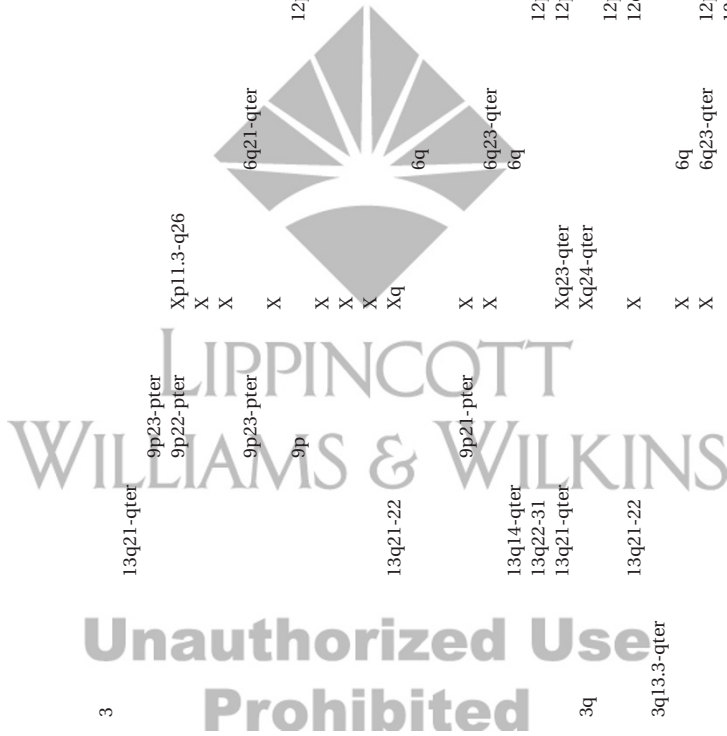* Bold style indicates region with high level amplification.

D = diagnostic tumor; R = relapse; lm = lack of material; classes from immunohistochemistry results concerning BCL2 and BCL6: 0 < 20%, 1 = 20–80%, 2 > 80% of cells positively stained.

tween diagnostic tumor sample and relapse indicated separate tumor clones. To confirm the SSCP finding that Tumors 29D and 29R were not clonally related, the PCR products from these samples were sequenced directly using an auto- mated ABI PRISM 377 sequencer (Perkin Elmer– Cetus, Norwalk, CT). The sequences were then aligned with *IGH* sequences retrieved from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/igblast/).

TABLE 3. The Most Frequent CGH Alterations in the Diagnostic Tumors in Relation to the Total Number of Alterations in the Diffuse Large B-cell Lymphomas Analyzed

Case	No. of Alterations	8p22-pter Loss	18q12-21 Gain	3q24-25 Gain	13q22 Gain	9p23-pter Loss	Xq25-26 Gain	6q23-qter Loss	12c-q14 Gain	17p12-pter Loss	1p34-pter Loss	22q Loss	7 Gain
25	0												
8	0												
20	1												
35	2	8p	18q										
17	2												
22	2			3									
14	2				13q21-qter								
21	2	8p22-pter				9p23-pter							
23	3	8p22-pter				9p22-pter	Xp11.3-q26						
6	3						X						
29	3						X						
9	3	8p22-pter				9p23-pter	X						
31	3							6q21-qter					
15	3					9p			12p12-q14	17p			
7	4						X						
11	4	8p21-pter					X						
27	4						X						
30	4	8p			13q21-22		Xq	6q					
12	4									17p12-pter	1p34-pter	22q	
16	5	8p	18pter-q21			9p21-pter							
19	5									17p12-pter			7
33	5						X	6q23-qter					
3	7		18q		13q14-qter			6q					
2	7			3q	13q22-31		Xq23-qter		12pter-q21		1p32-pter	22q	
5	7				13q21-qter		Xq24-qter		12p11.2-q21				7
26	7		18q12-qter										
13	7	8p12-pter							12p11-q21				
28	7	8p21-23					X		12c-q21		1p	22q	
24	7			3q13.3-qter									
34	8						X	6q		17p	1p34-pter		
10	8						X	6q23-qter		17p	1p21-pter		7
32	11						Xp21-qter				1p		
1	12			3q23-25	13q21-22				12p12-q21		1p34-pter	22q	7
4	12				13q21-22				12pter-q21		1p34-pter	22q	7
18	14	8p			13q21-31			6q22-qter		17p12-pter		22q	

TABLE 4. The Most Frequent CGH Alterations in Diagnostic Tumors in Relation to Relapses

Alteration	Diagnostic Tumors	Relapses*	P Value
Gain Xq25-26	15/35 (43%)	9/15 (60%)	ns
Loss 8p22-pter	10/35 (29%)	4/15 (27%)	ns
Loss 1p34-pter	9/35 (26%)	5/15 (33%)	ns
Gain 13q22	9/35 (26%)	3/15 (20%)	ns
Loss 6q23-qter	7/35 (20%)	7/15 (47%)	ns
Loss 22q	6/35 (17%)	1/15 (7%)	ns
Gain 12c-q14	7/35 (20%)	6/15 (40%)	ns
Loss 17p12-pter	6/35 (17%)	3/15 (20%)	ns
Loss 9p23-pter	5/35 (14%)	1/15 (7%)	ns
Gain 3q24-25	4/35 (11%)	4/15 (27%)	ns
Gain 7	4/35 (11%)	5/15 (33%)	ns
Gain 18q12-21	4/35 (11%)	6/15 (40%)	$P < .02$
Mean number of CGH alterations	5.1	6.3	ns

* Refers to 15 cases with in total 19 tumors. Each CGH alteration in subsequent relapses from the same case were counted once.

ns = not significant.

TABLE 5. The Most Frequent CGH Alterations in Diagnostic Tumors of Clinical Stage I–II in Relation to Clinical Stage III–IV

Alteration	Clinical Stage I–II	Clinical Stage III–IV	P Value
Gain Xq25-26	7/13 (54%)	9/22 (41%)	ns
Loss 8p22-pter	4/13 (31%)	6/22 (27%)	ns
Loss 1p34-pter	2/13 (15%)	6/22 (27%)	ns
Gain 13q22	2/13 (15%)	7/22 (32%)	ns
Loss 6q23-qter	3/13 (23%)	4/22 (18%)	ns
Loss 22q	0/13 (0%)	6/22 (27%)	$p < .04$
Gain 12c-q14	1/13 (8%)	6/22 (27%)	ns
Loss 17p12-pter	1/13 (8%)	5/22 (23%)	ns
Loss 9p23-pter	1/13 (8%)	4/22 (18%)	ns
Gain 3q24-25	1/13 (8%)	3/22 (14%)	ns
Gain 7	0/13 (0%)	4/22 (18%)	ns
Gain 18q12-21	1/13 (8%)	3/22 (14%)	ns
Mean number of CGH Alterations	3.6	6.0	$p < .05$

ns = not significant.

TABLE 6. Estimation of Clonal Relatedness based on *IGH*-Rearrangements and CGH

Tumors	*IGH*-Rearrangements	CGH
26D + 26R	Yes	Yes
27D + 27R	nc	No
28D + 28R	Yes	Yes
29D + 29R	No	No
30D + 30R	nc	Yes
31D + 31R	Yes	Yes
32D + 32R	nc	Yes
33D + 33R	Yes	Yes
34D + 34R	Yes	Yes
35D + 35R1-3	Yes	Yes
40R1 + 40R2	Yes	Yes

D = diagnostic tumor; R = relapse; nc = not conclusive.

Immunohistochemistry

Paraffin-embedded sections (4 μm) from 46 DLBCL tumors were deparaffinized and rehydrated. BCL2 and BCL6 antigen retrieval was achieved by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) (20) and 10 mM EDTA buffer (pH 8.0)

(21) separately, and treated within a pressure cooker (Biocare Medical, Walnut Creek, CA) at 103 kPa/15 psi for 7 minutes. Staining was carried out in an automated immunostainer (Ventana 320ES, Ventana Medical Systems, Tucson, AZ) using Ventana basic 3,3'-diaminobenzidine detection kit (Ventana Medical Systems), according to the recommendations of the manufacturer. Briefly, sections were incubated with murine anti-BCL2 (clone 124, DAKO, Glostrup, Denmark) (22) or murine anti-BCL6 (clone PG-B6p, DAKO) (23) monoclonal antibodies (diluted 1/20) for 28 minutes. Immunoreactivity was visualized with avidin-labeled HRPO complex and 3,3 diaminobenzidine followed by counterstaining with Mayer's hematoxylin (Ventana medical systems). All incubations were performed at 37° C. Incubation with murine IgG$_1$ sera (20 μg/mL, DAKO) was used as a negative control, and BCL2 and BCL6 staining of sections of a reactive tonsil as a positive control. The proportion of positively stained cells was estimated on sections from each tissue biopsy. The results were divided into three classes: Class 0, with <20% stained cells; Class 1, with 20 to 80% stained cells; and Class 2, with >80% stained cells. Eight of the 54 tumors (11D, 13D, 19D, 20D, 25D, 34R1, 34R2, and 40R2) could not be analyzed because of lack of material.

Statistical Analyses

Differences in proportions of CGH alterations were tested with the χ^2 test. The distribution of the number of CGH alterations in different subgroups was investigated with the t test. Probabilities of <0.05 were considered statistically significant. The statistical analyses were performed using the Statistica 5.0 software.

RESULTS

Overview of CGH Alterations in DLBCL

The CGH karyotypes of each tumor are detailed in Table 2 and the distribution of the detected gains and losses is illustrated in Figure 1A. Copy number changes were identified in 52 of the 54 tumors analyzed (96%), and gains were more commonly seen than losses. Overall, the subchromosomal regions most frequently gained were Xq25–26 (44%), 12cen-q14 (28%), 13q22 (24%), and 18q12–21 (22%), whereas losses preferentially involved 1p34-pter (30%), 8p22-pter (30%), and 6q23-qter (28%) (Fig. 1A). None of the recurrent alterations were detected as a single abnormality. High-level amplifications were recorded in 10 tumors from six patients (Table 2). The regions 2p13–16 and 2q22–24 as well as 8q22-qter and 12pter-q21 were coexisting as high-level amplifications in one tumor each, whereas
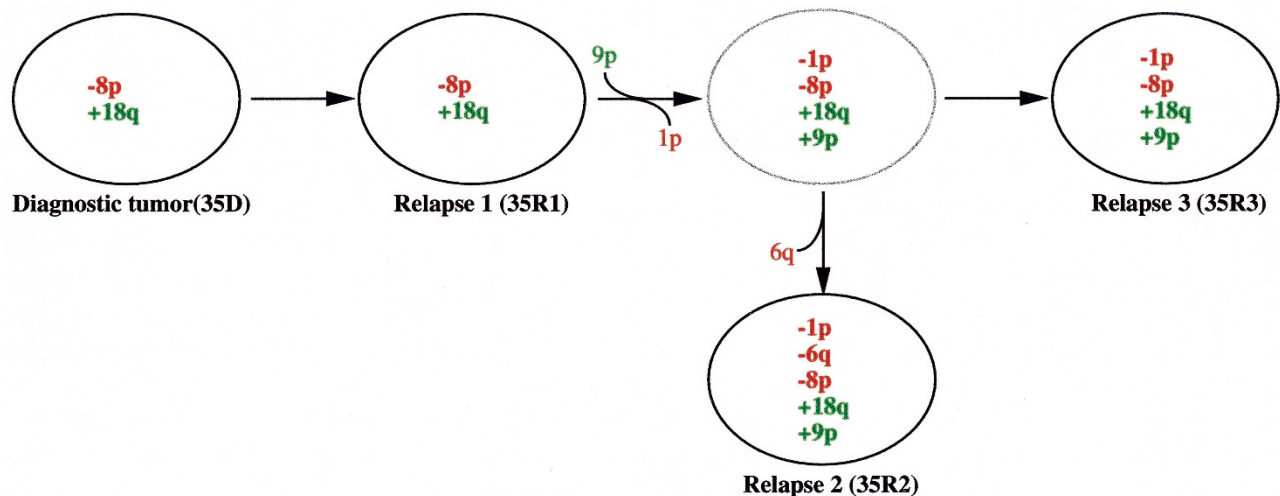
FIGURE 2. Tumor progression in Case 35 based on changes detected by comparative genomic hybridization. The model includes the results from the diagnostic tumor (35D) and the three subsequent relapses (35R1-R3), as well as a hypothetical intermediate tumor (***dotted circle***). A plus (+) indicates chromosomal gains, and a minus (−) denotes losses.

regions 12p11-q21, 13q21-qter, 18q12-qter, and 18q appeared as single high-level amplifications in four cases. The high-level amplifications involving 18q were identified in two diagnostic tumors and were maintained in the subsequent relapses (Table 2).

Regions from 12 different chromosomes were found to be frequently aberrant in the diagnostic tumors analyzed (Fig. 1B) and in relapses (Fig. 1C). In the diagnostic tumors, commonly gained regions were Xq25–26 (43%), 13q22 (26%), 12cen-q14 (20%), 3q24–25 (11%), 7 (11%), and 18q12–21 (11%), and losses were mainly detected at 8p22-pter (29%), 1p34-pter (26%), 6q23-qter, (20%), 17p12-pter (17%), 22q (17%), and 9p23-pter (14%). In the re-lapses, gains were mainly observed at Xq25–26 (60%), 12c-q14 (40%), 18q12–21(40%), 7 (33%), 3q24–25 (27%), and 13q22 (20%), whereas losses mainly encompassed 6q23-qter (47%), 1p34-pter (33%), 8p22-pter (27%), and 17p12-pter (20%). Fur-thermore, losses of 9p23-pter and 22q was only found in 5% of the relapses, whereas a loss of Xq appeared in 21% of the relapses and only in 3% of the diagnostic tumors.

In the individual diagnostic tumors the pattern of CGH alterations varied depending on the total number of detected alterations (Table 3). Loss of 8p and 9p and gain of 3q, 13q, and 18q were detected in tumors with only two abnormalities. The most common alteration, that is, gain of Xq, was only seen in tumors with three or more alterations. Sim-ilarly, loss of 1p, 6q, 17p, and 22q and gain of 7 and 12q were detected in cases with three or more al-terations only. Taken together, these findings would suggest a progression of genetic events in which they are early but not initiating events in DLBCL tumorigenesis. None of the common alter-ations were detected as a single abnormality.

CGH Alterations in Diagnostic Tumors *Versus* Relapses

The total number of CGH alterations was similar between diagnostic tumors and relapses, and the mean number of alterations in the two groups was 5.1 and 6.3, respectively. When the most frequent alterations in the 35 diagnostic tumors (1D-35D) were compared with the aberrations identified in the 19 relapses (26R-40R) from the 15 cases with one or more relapses (Table 4), a gain of 18q12–21 was significantly more often detected in relapses ($P < .02$). In these analyses, CGH alterations occurring in more than one relapse (34R1–2, 35R1–3, and 40R1–2) were counted only once.

CGH Alterations in Relation to Clinical Stage and Sex

Possible associations between the total number of CGH alterations and most frequent CGH alter-ations were evaluated in relation to clinical stage, sex, and their presence in diagnostic tumors and relapses. Firstly, the diagnostic tumors were or-dered according to increasing clinical stage. This revealed that the total number of CGH alterations was significantly higher among the 22 cases with the most advanced clinical stages (III–IV), com-pared with the 18 tumors of Stages I and II, and the mean number of CGH alterations in the two groups was 6.0 and 3.6, respectively ($P < .05$) (Table 5). Loss of chromosome 22q was significantly more common in cases with more advanced clinical stage ($P < .04$) (Table 5). Notably, all commonly occur-ring aberrations except gain of 3q and 7, and loss of 22q were detected already in tumors of Clinical Stage I. Thus, the majority of CGH abnormalities occur early in the clinical progression (not shown).

No difference in total number CGH alterations was observed between diagnostic tumors from male ($n = 28$) compared with female ($n = 12$) cases. However, when the most commonly involved regions were taken into account, a gain of 13q showed a tendency to be more frequent in male cases (not shown).

Analyses of Clonal Relatedness

In 11 cases (Cases 26–35 and 40), multiple DLBCL tumors were obtained and the sequence of CGH alterations were analyzed in subsequent relapses. The clonal relationship between the diagnostic tumor and relapses was also evaluated based on the patterns of *IGH*-rearrangement (Table 6). For three of the 11 cases (27, 30, and 32), the analyses of *IGH* rearrangement were not conclusive. In seven cases (26, 28, 31, 33, 34, 35, and 40) the CGH profile was very similar in matched tumors, and the *IGH* rearrangement analyses showed that subsequent tumors had identical *IGH* rearrangements. The relapses thus seemed to be derived from the same clone as the diagnostic tumor sample. In Case 30, the diagnostic tumor and the subsequent relapse are also likely to be related based on the similarity of the CGH profiles.

For Case 35, three subsequent relapses were studied in addition to the diagnostic tumor, and based on the CGH alterations identified, a scheme for the tumor progression could be suggested (Fig. 2). However, in two cases (Cases 27 and 29), there were profound differences in the CGH profiles between the diagnostic tumor and relapse, and in Case 29 also a different *IGH*-rearrangement in the relapse could be confirmed, thus indicating that the relapses in these cases were not clonally related to the diagnostic tumors (Table 6). In both of these cases the time interval between diagnosis and relapse was approximately 1 year.

Immunohistochemistry and CGH

Thirty of the analyzed samples were diagnostic tumors, and 16 were relapses (Table 2). No significant association was observed between a gain of 3q27 and the level of BCL6 expression. A high-level amplification of 18q21 was recorded in six of the tumors (26D, 26R, 35D, 35R1, 35R2, and 35R3), and each sample showed a Class 2 expression of BCL2 (Table 2). In nine of the analyzed tumors (six diagnostic tumors and three relapses) with Class 2 expression of BCL2 we could not, by CGH, detect a gain of 18q (Table 2).

DISCUSSION

In this study, we identified several frequently occurring CGH alterations in DLBCL. In agreement with previous studies (5, 24–26), recurrent changes were gains of X, 3q, 7, 12q, and 18q, whereas 6q, and 17p were frequently lost. In addition, previously not-reported losses of 1p, 8p, 9p, and 22q and gain of 13q were identified as frequent. There was a tendency toward an increased number of changes in relapses compared with diagnostic tumors, and chromosomal imbalances were identified in 94% of the diagnostic tumor samples and in all off the relapses.

The concordance between chromosomal changes in DLBCL identified by G-banding and the alterations detected by CGH is in general good (24). However, the cytogenetic evidence, such as double-minutes (DM) or homogeneously staining regions (HSR), for CGH-detected high-level amplifications is scarce (24). This higher sensitivity to detect an increase of genetic material could be explained by the fact that the CGH technique scans the DNA of the entire tumor including all cellular subclones. Banding analysis requires cell culturing that is known to favor growth of certain cell populations (27), whereby cells carrying DM may remain undetected because they lack functional centromeres. Twenty percent of the tumors in this series exhibiting a chromosomal gain carried a high-level amplification. The most likely cytogenetic mechanism behind these amplifications is DM, because DM is the main cytogenetic manifestation of gene amplification in noncultured cells taken directly from patients (28), such as the tumor biopsies used in this study.

In agreement with a previous CGH study on DLBCL (5), high-level amplifications were most frequently found at the distal part of the long arm of chromosome 18 with a minimal region of 18q12–21. In the present study, all cases with high-level amplification of 18q21 also showed Class 2 expression of *BCL2*. This suggests that gene amplification, in these cases, could be the mechanism for increased expression of BCL2; however, it does not exclude the possibility of a coexisting *BCL2* involving translocation such as the t(14;18), which also increases the gene expression. The (14;18)-translocation is the most likely mechanism behind high BCL2 expression in those tumors lacking a gain of 18q21. The gain of minimal region 18q12–21 was significantly more common in relapses than in diagnostic tumors ($P < .02$); however, there was no significant difference concerning the BCL2 expression between diagnostic tumors and relapses carrying a gain of 18q12–21. An increased level of BCL2 expression has been reported to appear as an early event in tumor progression (29) and an up-regulation of BCL2 as a result of a t(14;18) is considered to be the primary event in follicular B-cell lymphomas (30). Thus other genes than *BCL2* located in this chromosomal region might be involved in the progression of DLBCL.

Loss of 22q was significantly more common in cases with clinically more advanced stage of the disease (Stages III–IV compared with Stages I–II; $P < .04$), and one possible explanation for this finding may be loss of the *G22P1* gene at 22q13 that is encoding for the Ku70 protein. Ku70 heterodimerizes with Ku80 to form a protein kinase that is essential in both the repair of double-stranded DNA breaks (DSBs) and in the V(D)J recombination (31–33). Tumor cells with a deficient mechanism for DSB repair would result in cells with more chromosomal imbalances and a more advanced disease. However, >600 genes are located to the long arm of chromosome 22 (J. Dumanski, personal communication). Some have been excluded to play an important role in the DLBCL tumorigenesis, such as the cell cycle regulator gene *CHK2* (34).

None of the recurrent alterations were detected as a single abnormality (Table 3), which implies that other genetic lesions below the detection level of CGH may be involved in the initiation of DLBCL. It has recently been suggested that an aberrant somatic hypermutation mechanism may play an important role for the pathogenesis in these neoplasias (35). Such dysregulated hypermutation machinery would increase the occurrence of chromosomal aberrations as well as it would result in mutations of numerous genes with consequences analogous to defects in DNA mismatch repair in colon cancer (36). Our results of relatively spread CGH events support the concept about an aberrant somatic hypermutation mechanism in DLBCL that also would explain the clinical heterogeneity of the disease.

Earlier studies on lymphoma have shown that disease progression could either be clonally related or represent clonally unrelated neoplasms (37, 38). Clonally unrelated relapses are uncommon but have been reported (37, 38). Treatment may induce changes that are responsible for the formation of new neoplasms in patients. Two of the 11 cases (27 and 29) analyzed with multiple DLBCL tumors had relapses that did not seem to be clonally related to the diagnostic tumor sample. The time interval between the diagnostic tumor and relapse was in both cases short, which makes therapy-induced lymphomas less likely. From the CGH analysis of Case 35, we could follow the development from the diagnostic tumor to the third relapse, and we observed that the tumor progression was not cytogenetically linear (Fig. 2). From this, we propose that even if patients are in complete remission clinically, the original lymphoma-causing clone may give rise to one or more subclones that did not respond to the given treatment. To irradiate these dormant clones, new complementary treatment should be used in addition to traditional therapies.

The distribution of CGH alterations supports the idea of a progression of genetic events in which loss of 8p and 9p and gain of 3q, 13q, and 18q would represent relatively early events because they were distributed in tumors with the lowest number of abnormalities. However, none of the recurrent alterations were detected as a single abnormality, indicating that none of these represent the initiating event in the tumorigenesis of DLBCL. These could constitute balanced rearrangements or discrete alterations that would remain undetected by CGH. In conclusion, the clinical and histopathological diversity of DLBCL is paralleled by a similarly complex genetic background of primary events. Some of the recurrent alterations identified in our study are expected to be involved in tumor progression, thus providing possible new genetic markers of prognostic significance.

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Book Review

This is a multi-authored book published in a series sponsored by the American Cancer Society. It deals with the recent advances in the pathology, epidemiology, molecular biology, clinical presentations, and management of malignant lymphomas. Clinical aspects of lymphoma diagnosis and therapeutics form the backbone of the book, but adequate space is reserved for the discussion of pathology and molecular biology and other facets of neoplastic diseases of the lymphopoietic system.

This book is aimed primarily at clinical oncologists in general and oncologic hematologists in particular. Topics such as staging of lymphomas and Hodgkin disease, prognostic factors, chemotherapy, transplantation, and radiation therapy as well as immunotherapy are dealt with in detail. Recommendations for treatment are based on contributors' personal experiences supported by data from recent literature. The text is supplemented with numerous summary tables and graphs illustrating the treatment outcomes. The references are up-to date and many are from 2000 and even 2001.

The pathologic features of various lymphomas are illustrated in color photographs. Many of these photographs are of substandard quality and essentially uninterpretable, which is a pity. A CD-ROM containing the entire text and all of the pictures is included with the book, but the illustrations on the disc are not any better than those in the book.

Like the other books in this ACS series, this book will be used mostly by clinicians. Nevertheless, pathologists interested in lymphomas and Hodgkin disease also will find enough valuable material between its covers.

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