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Genetic Imbalances in Progressed B-Cell Chronic Lymphocytic Leukemia and Transformed Large-Cell Lymphoma (Richter's Syndrome)

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Chromosomal imbalances were examined by comparative genomic hybridization in 30 cases of B-cell chronic lymphocytic leukemia (CLL) at diagnosis, in sequential samples from 17 of these patients, and in 6 large B-cell lymphomas transformed from CLL [Richter's syndrome (RS)] with no available previous sample. The most common imbalances in CLL at diagnosis were gains in chromosome 12 (30%), and losses in chromosomes 13 (17%), 17p (17%), 8p (7%), 11q (7%), and 14q (7%). The analysis of sequential samples showed an increased number of chromosomal imbalances in 6 of 10 (60%) patients with clinical progression and in 2 patients with stable stage C disease. No karyotypic evolution was observed in four cases with stable stage A disease and in one RS clonally unrelated to the previous CLL. Gains of 2pter, and 7pter, and losses of 8p, 11q, and 17p were recurrent alterations associated with karyotype progression. RS showed a higher number of gains, losses, total alterations, and losses of 8p and chromosome 9 than CLL at diagnosis. 17p losses were associated with p53 gene mutations and with a significantly higher number of chromosomal imbalances than tumors with normal chromosome 17 profile. However, no relationship was observed between 9p deletions and $p16^{INK4a}$ gene alterations. Losses of 17p and an increased number of losses at diagnosis were significantly associated with a shorter survival. These findings indicate that CLL has frequent chromosomal imbalances, which may increase during the progression of the disease and transformation into large cell lymphoma. Genetic alterations detected by comparative genomic hybridization may also be of prognostic significance. ***(Am J Pathol 2002, 161:957–968)***

B-cell chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in adults and accounts for >30% of all leukemia cases in Europe and North America. CLL is characterized by clonal proliferation and accumulation of mature-appearing neoplastic B lymphocytes. Clonal chromosome aberrations are detected in ~40 to 50% of CLL cases by conventional cytogenetics and approximately half of the patients show single abnormalities. In contrast to other lymphoproliferative disorders, CLL has a very low frequency of chromosomal translocations involving immunoglobulin genes and the most frequent genetic abnormalities are losses of 13q and 11q, trisomy 12, and losses of 6q and 14q.[1–4] Fluorescence *in situ* hybridization (FISH) studies in interphase cells have shown the presence of cell clones carrying chromosomal aberrations in cases in which no abnormalities were found by banding analysis.[5] Using this technique, clonal aberrations can be detected in >80% of cases.[2] However, only a few chromosomal regions can be examined in a single experiment by interphase FISH. Conversely, comparative genomic hybridization (CGH) allows a rapid analysis of chromosomal imbalances within the tumor genome without the requirement of cell culturing and metaphase preparation.

The clinical course of CLL is variable, with some patients having a very short survival rate and others having a normal life span.[6] Different prognostic factors for survival have been described, including different types of chromosomal abnormalities.[2,6–8] Some genetic lesions may be involved in the development of CLLs whereas others contribute to disease progression. At diagnosis, CLL cells generally have relatively few detectable chro-

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Table 1. CGH, FISH, and *p53* Mutations Results in CLL at Diagnosis and Progression

Case	Time*	Follow-up	Sample	Diagnosis and stage	CGH results[3‡] Gains	CGH results[3‡] Losses
1a			PBL	CLL-A	8q22-q24.3, 12p13-q13	-
1b	6		PBL	CLL-A	8q22-q24.3, 12p13-q13	-
1c	17		PBL	CLL-C	**1q21-q25**, 8q22-q24.3, 12p13-q13	-
1d	39		PBL	CLL-C	**1q21-q25**, 8q22-q24.3, 12p13-q13	-
1e	52	56+	PBL	CLL-C	**1q21-q25**, 8q22-q24.3, 12p13-q13	-
2a			LN	CLL-A	-	11q14-q23
2b	15	39	LN	CLL-B	-	11q14-q23
3a			PBL	CLL-A	-	14q24-q32
3b	13		PBL	RS	-	14q24-q32
3c	13	95+	BM	RS	-	14q24-q32
4a			PBL	CLL-B	-	13q14-q34
4b	13	23	PBL	CLL-C	**2p16-p25, 7p21-p22**	**11q23-q25**, 13q14-q34
5a			PBL	CLL-A	-	-
5b	48	61+	LN	CLL-C	-	**7q22-q31**
6a			LN	CLL-C	-	8p21-p23, 11q23
6b	6	33	LN	RS	**2p16-p25, 7p**	**2p15-q24**, 8p21-p23, 11q23
7a			PBL	CLL-A	-	-
7b	87	72+	PBL	CLL-C	-	-
8a			PBL	CLL-A	3q13.3-q29, 15q23-q26	17p12-p13
8b	50	50	PBL	RS	3q13.3-q29, 15q23-q26	**8p**, 17p12-p13
9a			PBL	CLL-A	12	14q22-q32
9b	2		LN	CLL-A	12	14q22-q32
9c	2	11+	Colon	RS[†]	-	-
10a			PBL	CLL-A	-	-
10b	78	81+	PBL	CLL-C	-	-
11a			PBL	CLL-B	12	-
11b	20	25	PBL	CLL-C	12	17p
12a			PBL	CLL-A	-	13q14-q21
12b	79		PBL	CLL-A	-	13q14-q21
12c	98	98+	PBL	CLL-A	-	13q14-q21
13a			PBL	CLL-A	-	-
13b	108	130+	PBL	CLL-A	-	-
14a			PBL	CLL-A	12	-
14b	33	62+	PBL	CLL-A	12	-
15a			PBL	CLL-A	12	-
15b	108	118+	PBL	CLL-A	12	-
16a			PBL	CLL-C	-	-
16b	24	57	PBL	CLL-C	12	-
17a			PBL	CLL-C	-	17p12-p13
17b	21	27	PBL	CLL-C	12	**8p**, 17p12-p13
18		96+	PBL	CLL-A	-	13q14-q32
19		82+	PBL	CLL-A	17	-
20		29+	LN	CLL-A	12	-
21		92+	PBL	CLL-B	12	-
22		25+	PBL	CLL-B	-	-
23		25+	LN	CLL-B	12	-
24		98+	LN	CLL-B	12q	-
25		25	PBL	CLL-C	-	-
26		1	PBL	CLL-C	-	6p, 8p23-q11.2, 9, 17p12-p13
27		31	PBL	CLL-C	2p	13q21-q31, 17p12-p13
28		2	PBL	CLL-C	-	-
29		6+	LN	CLL-C	-	10q24-q26, 17p
30		55+	PBL	CLL-C	11q23-q25	1q, 13
31		38+	LN	RS	8q, 11q24-q25, 12q24, 13q33-q34, X	2q11.1-q35, 6p23, 8p, 9, 10q24, 13q11-q14, 15q11.1-q21, 15q25-q26, 17p13
32		38	LN	RS	12	13q21-q31, 17p12-p13
33		23	LN	RS	-	9p24-q33, 10q24-q26, 13q14-q21, 17p12-p13, Xq26-q28
34		1+	LN	RS	-	-
35		71+	LN	RS	1p36.3-q41, 11q13-q25, 12q15-q24.3	8p21-p23
36		64	LN	RS	17q	3p, 9p24-p22, 11p14-p13, 13q14-q21, 18q12-q23

(*Table continues*)

Table 1. *Continued*

FISH Results[§]					
ATM 11q22	12p11.1-q11	RB1 13q14	P53 17p13	*p53* Mutation[¶]	*p16* Deletion
		No		No	
				No	
	37%	No	No		
				No	
				No	
				No	
No	No	No	No	No	
				No	
69%	No	49%	No	No	
	No	No	53%	No	
				No	GL
				No	Del
				No	
No	No	46%	No	No	
				Mut	
				Mut	
				No	
				No	
				No	
	No			No	
No	No	73%	No	No	
				No	
				Mut	
	No			No	
No	No	57%	No	No	
No	No	64%	No	No	
				No	
	No		No	No	
	34%			No	
No	35%	76%	No	No	
	40%			No	
				No	
				No	
				No	
				Mut	
				Mut	
				No	
				No	
				No	
				No	
				No	
	64%	No	No	No	
				No	
				No	
				Mut	
				Mut	
				No	
				Mut	
	No			No	
				Mut	Del
				Mut	GL
	No			Mut	GL
				No	GL
				No	
				No	GL

*Time interval (in months) between samples. +, Alive.
†Case 9c was not clonally related to 9a and 9b.
‡Bold CGH data represent aberrations that were detected only in the sample obtained at progression.
§For details of probes see Materials and Methods section.
¶For detailed *p53* mutations see Table 2.
BM, bone marrow; LN, lymph node; PBL, peripheral blood lymphocytes; GL, germinal line status; Del, homozygous deletion.

mosomal alterations, but throughout time the cells may accumulate additional genetic changes, altering their biological and clinical behavior. Moreover, ~5 to 10% of patients with CLL develop a histological transformation into aggressive large B-cell lymphomas [Richter's syndrome (RS)]. The chromosomal changes associated with the disease progression and transformation to RS are not well known. A few studies using classical cytogenetics have indicated that the karyotype is relatively stable during the evolution of the disease.[9–11] On the other hand, the number of cytogenetic studies in well-characterized RS is scarce and no recurrent chromosomal abnormalities have been demonstrated. CGH is a sensitive technique that may reveal more chromosomal alterations than conventional cytogenetics and does not require metaphase preparations from tumor cells. Therefore, in this study we have examined a series of CLL at diagnosis, sequential samples of patients with stable and progressive disease, and RS using CGH to identify possible chromosomal imbalances that may play a role in the progression of this disease.

Materials and Methods

Patients

Thirty patients with CLL (21 males, 9 females; median age, 64 years) were examined at diagnosis before treatment (Binet's stage A, 15 cases; stage B, 6 cases; and stage C, 9 cases). Sequential samples during the evolution of the disease were available in 17 patients, 6 of which did not progress clinically and 11 that progressed to either a more advanced Binet's stage (seven cases), or transformed into a large B-cell lymphoma (RS) (four cases). In addition, six patients diagnosed with RS (2 males, four females; median age, 61 years) in which genomic DNA or frozen cells from the initial CLL were not available, were also examined (Table 1). To determine the clonal relationship between sequential samples, the CDRIII region of the immunoglobulin heavy chain (IgH) gene was amplified as previously described.[12] The amplified products were purified and sequenced using the cycle-sequencing BigDye terminator chemistry (Applied Biosystems, Foster City, CA). Sequencing reactions were run on a Perkin-Elmer ABI-377 automated sequencer (Perkin-Elmer, Emeryville, CA). All samples were analyzed by flow cytometry or immunohistochemistry and showed >60% of tumor cells. The main initial clinical features of the patients, including white blood cell, lymphocyte, and platelet counts; Rai and Binet stages; serum lactate dehydrogenase, serum albumin, and serum β2-microglobulin levels were recorded to determine the possible relationship with the genetic alterations. In addition, response to therapy and clinical outcome of patients were also evaluated.

DNA Extraction

High molecular weight DNA was extracted using standard Proteinase K/RNase treatment and phenol-chloroform extraction. Normal DNA was obtained from three male and one female healthy blood donors. DNA was diluted to a concentration of 40 to 60 ng/μL and 1 μL of each sample was analyzed in 0.8% agarose gel and stained with ethidium bromide to verify its quality and concentration.

CGH

Hybridization was performed as described previously.[13] Briefly, normal human genomic DNA (control DNA) was labeled with Spectrum Green-dUTP and tumor DNA with Spectrum Red-dUTP by nick translation using a commercial kit (Vysis, Downers Grove, IL). Control experiments in which the Red-dUTP and Green-dUTP fluorochromes were interchanged between normal and tumor were also performed in a subset of samples. Negative control experiments were performed using differentially labeled male *versus* male DNA and female *versus* female DNA. Subsequently, equal amounts of normal and tumor-labeled probes (600 ng) and 10 μg of Cot-1 DNA were co-precipitated using ethanol. Normal metaphase spreads (Vysis) were denatured and hybridized with the DNA mixture in a moist chamber for 2 to 3 days. Slides were washed according to the protocol supplied by the manufacturer. Chromosomes were counterstained with 4–6-diamino-2-phenylindole. Image acquisition, processing, and evaluation were performed as described previously.[13] Slides were analyzed using the Cytovision Ultra Workstation (Applied Imaging, Sunderland, UK).

FISH Analysis

FISH analysis was performed on fixed cultured peripheral blood samples and lymph node biopsies. Slides were stored for 24 hours at room temperature. After being dehydrated in ethanol series and air-dried, slides were denatured in 70% formamide solution at 72°C for 2 minutes. Probes were also denatured at 72°C for 5 to 10 minutes. Five μl of the mixture of probe solution were added to each slide and covered by a coverslip. The preparations were hybridized at 37°C overnight in a moist chamber. Posthybridization washing consisted of three changes of 10 minutes each with 50% formamide solution at 45°C and one change of 10 minutes with 2× standard saline citrate at 45°C and one last change of 5 minutes with 2× standard saline citrate/0.1 Nonidet P-40. FISH was performed with chromosome 12-specific α satellite DNA probe (CEP12, 12p11.1-q11, Spectrum Orange), and chromosome 17-specific α satellite DNA probe (CEP17, 17p11.1-q11.1, Spectrum Green), and locus-specific probes from 13q14 (LSI RB1, Spectrum Orange), 17p13 (LSI p53, Spectrum Orange), and 7q11.23 (Elastin Gene, Spectrum Orange) combined with 7q31 (control probe D7S486, D7S522, Spectrum Green) LSI Williams syndrome region probe. All these probes were obtained from Vysis.

The ATM gene locus was analyzed with the YAC clone 756a6 mapping to 11q22.3–11q23.1.[14] Slides were evaluated using fluorescence microscopy by two of the au-

Table 2. Correlation Between 17p Losses by CGH and *p53* Gene Mutations

Case	Diagnosis	17p Loss by CGH	Exon	Codon	Nucleotide	Amino acid
8a	CLL-A	Yes	4	76	GGA-GCA	Ala-Gly
8b	RS	Yes	4	76	GGA-GCA	Ala-Gly
11b	CLL-C	Yes	6	215	AGT-ATT	Ser-Ile
17a	CLL-C	Yes	8	264-265	Δ792-794	No frameshift
17b	CLL-C	Yes	8	264-265	Δ792-794	No frameshift
26	CLL-C	Yes	6	209	Δ626-627	Frameshift
27	CLL-C	Yes	5	179	CAT-CTT	His-Leu
29	CLL-C	Yes	5	136	CAA-GAA	Gln-Glu
31	RS	Yes	8	301	Δ902-906	Frameshift
32	RS	Yes	5	171	GAG-GGG	Glu-Gly
33	RS	Yes	8	306	CGA-TGA	STOP

thors independently of each other and without knowledge of any previous available CGH results. A minimum of 500 nuclei and 200 nuclei were analyzed for centromeric and locus-specific probes, respectively. False-positive rate indicating del(7)(q31), del(11)(q22), +12, del(13)(q14), and del(17)(p13) was assessed in 10 normal specimens. Chromosome gain was considered when the percentage of cells with trisomy was >5% and loss of chromosome when the abnormality was present in >15% of cells.

Molecular Studies

Mutational analysis of exons 4 to 8 of the p53 gene was performed in all patients (54 samples) (Tables 1 and 2). Individual exons were amplified by polymerase chain reaction using specific primers, and single-stranded conformational polymorphism analysis and direct sequencing were performed as previously described.[15]

Southern blot analysis of the $p16^{INK4a}$ gene was performed in eight samples from seven patients as previously described.[16] The probe was radiolabeled using a random primer DNA labeling kit (Amersham) with [α-^{32}P]-dCTP. To normalize the DNA loading, the blots were hybridized with a *β-actin* probe. Quantitative evaluation of the signals was performed with the Quantity-One software (version 4.0.1; Bio-Rad, Hercules, CA). Single-stranded conformational polymorphism analysis of exons 1α and 2 of $p16^{INK4a}$ gene was used to screen for gene mutations according to a previously described method.[16,17]

Statistical Analysis

Differences in CGH imbalances between CLL and RS, different CLL stages and stage C, as well as other initial and evolutive features of the patients, were assessed by means of the Fisher's exact test (two-tailed). The observed means of gains, losses, and total alterations were compared by using nonparametric tests (Mann-Whitney *U*-test). The number of CGH alterations in sequential samples of clinically progressed cases was compared by the Wilcoxon test. Survival times and censored waiting times measured from the date of diagnosis were plotted using Kaplan and Meier estimates.[18] Univariate analysis of differences in survival was tested by the log-rank method.[19] Multiple regression analysis of survival data were done using the Cox proportional hazards regression model.[20]

Results

DNA Imbalances in CLL at Diagnosis

DNA imbalances were observed in 22 of the 30 (73%) untreated CLLs at diagnosis. Chromosome losses (n = 20) were more frequent than gains (n = 15), and no high-level DNA amplifications were detected in these cases (Figure 1, Table 1). Cases with no chromosome imbalances were four CLL in stage A, one in stage B, and three in stage C. Single chromosome imbalances were detected in 14 of the 22 (64%) patients with CGH alterations. These single alterations consisted in gain of chromosome 12 (cases 11a, 14a, 15a, 20, 21, and 23), gain of 12q (case 24), gain of chromosome 17 (case 19), loss of 11q14-q23 (case 2a), loss of 13q14-q21 (cases 4a, 12a, and 18), loss of 14q24-q32 (case 3a), and loss of 17p12-p13 (case 17a). Recurrent chromosomal alterations consisted in gains of chromosome 12 (30%), with a minimal common region at 12q13, and losses of chromosome 13 (17%), 17p (17%), 8p (7%), 11q (7%), and 14q (7%) with minimal common regions in 13q14-q21, 17p12-p13, 8p21-p23, 11q22, and 14q24-q32, respectively.

In these series, the presence of chromosome 12 gains seemed mutually exclusive with 13q and 17p losses. Thus, chromosome 12 gains were detected in nine cases and 13q losses by CGH in five tumors, but none of these cases showed both alterations simultaneously by CGH. However, one patient (case 14) showed both abnormalities by FISH analysis. Chromosome 12 gains and 17p deletions were observed in nine and five cases, respectively, but none of them showed both alterations. Concomitant 13q loss and 17p loss were only observed in one patient (case 27).

DNA Imbalances in Sequential Samples

Sequential samples could be examined in 17 patients (40 samples) (Table 1). Six of these patients did not progress to a more advanced clinical stage or RS (cases 12 to 17). Analysis of the IgH gene confirmed identical clonal gene

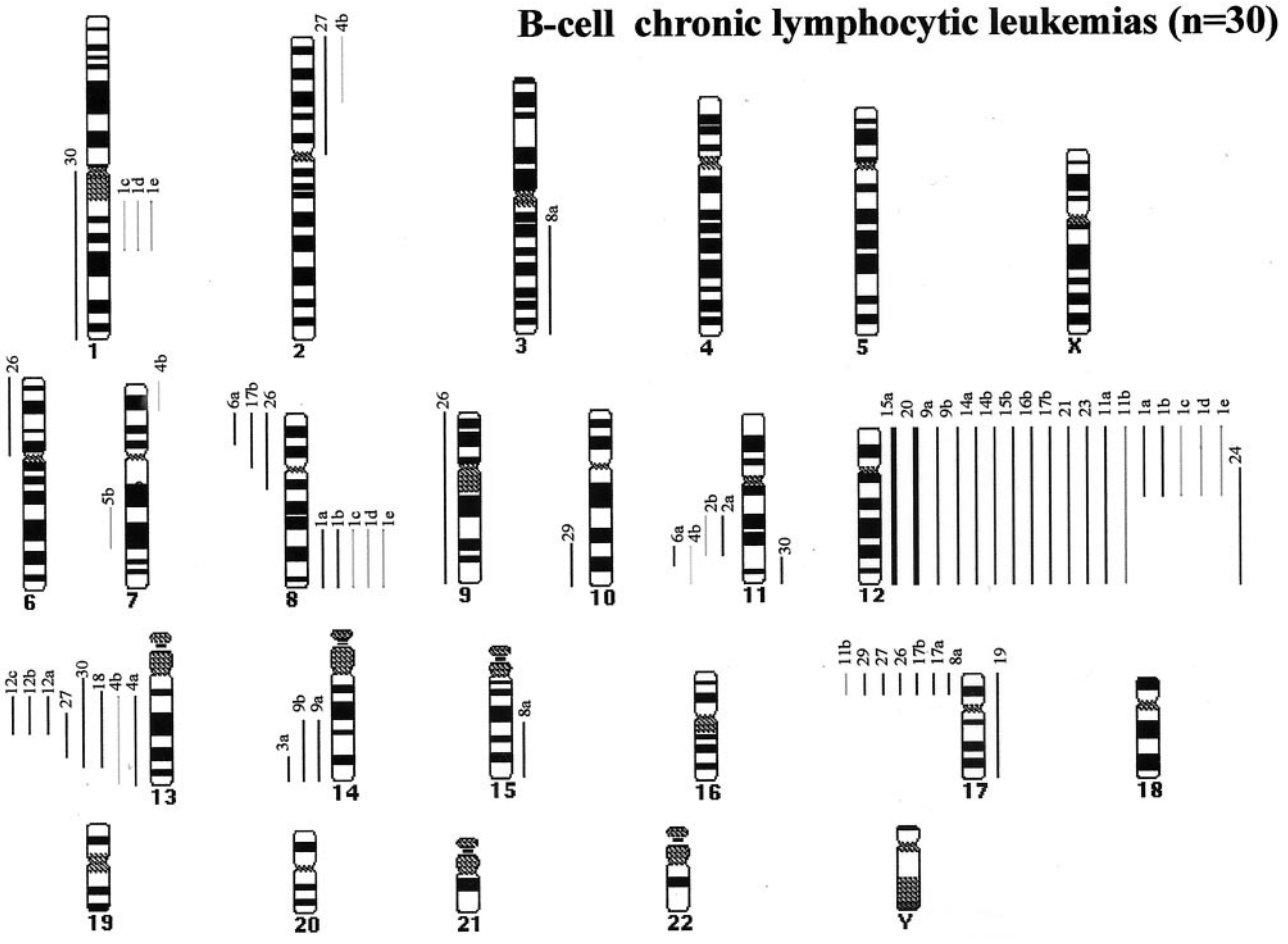

Figure 1. Summary of all DNA copy number changes detected by CGH in 30 patients with CLL, 13 patients have more than one sample, 7 of them had progressed to more advanced stages (**gray lines**). **Left**: **Lines** indicate loss of chromosomal material. **Right**: **Lines** indicate gain of chromosomal material. **Thick black bars** represent chromosomal gains exceeding 1.5 in a large chromosomal region. Each **line** represents a gained or lost region in a single sample. The most common gains involved chromosome 12 (30%), whereas the most frequent losses were detected in chromosomes 13 (17%), 17p (17%), 8p (7%), 11q (7%), and 14q (7%).

rearrangement in the sequential samples of all these patients. The median time of interval between samples was 65 months (range, 21 to 108 months) and the follow-up of the patients was 80 months (range, 27 to 130 months). The four cases in stage A (cases 12 to 15) showed the same chromosome alterations in the initial and sequential samples. However, the sequential sample of the two cases in stage C (cases 16 and 17) showed the same alterations detected in the initial samples and additional changes. The acquired change was a gain of chromosome 12 in both cases and a loss of 8p in one case.

Eleven patients (cases 1 to 11) progressed to a more advanced clinical stage (seven cases) or to an RS (four cases) (Figures 1 and 2, and Table 1). All of these cases had initial abnormal CGH profiles. The median time of interval between samples was 20 months (range, 2 to 87 months) and the follow-up of the patients was 50 months (range, 11 to 95 months). In 10 cases, analysis of the IgH gene showed identical clonal gene rearrangement in the sequential samples from the same patient. However, case 9 showed a different clonal band in the transformed large B-cell lymphoma and in two DNA samples of CLL. In this case, the initial peripheral blood and a subsequent lymph node biopsy diagnosed with CLL with the same clonal IgH rearrangement (samples 9a and 9b) showed a gain of chromosome 12 and a loss of chromosome 14q. However, no CGH alterations were observed in the large B-cell lymphoma showing a different IgH clonal rearrangement, confirming a *de novo* origin of this lymphoma (sample 9c) (Table 1). This case was excluded for the subsequent comparisons and analyses. In the 10 remaining cases, all CGH changes detected in the initial samples were also found in the sequential samples obtained at progression (Table 1). Furthermore, six (60%) of these cases showed additional changes in the progressed sample. The number of CGH alterations in the sequential samples of these 10 patients was compared by the Wilcoxon test. The number of CGH imbalances in the progressed stages (mean, 2.1 $\pm$ 0.5) was significantly higher than in initial stages (mean, 1.1 $\pm$ 0.3) ($P = 0.03$). The acquired imbalances were gains at 2p16-p25 and 7p21-p22 in two cases, and gain of 1q21-q25, and losses at 2p15-q24, 7q22-q31, 8p, 11q23-q25, and 17p in one case, respectively (Table 1). Losses of 8p, 11q, and 17p were also present in the initial sample of three additional patients who progressed into a more advanced stage.

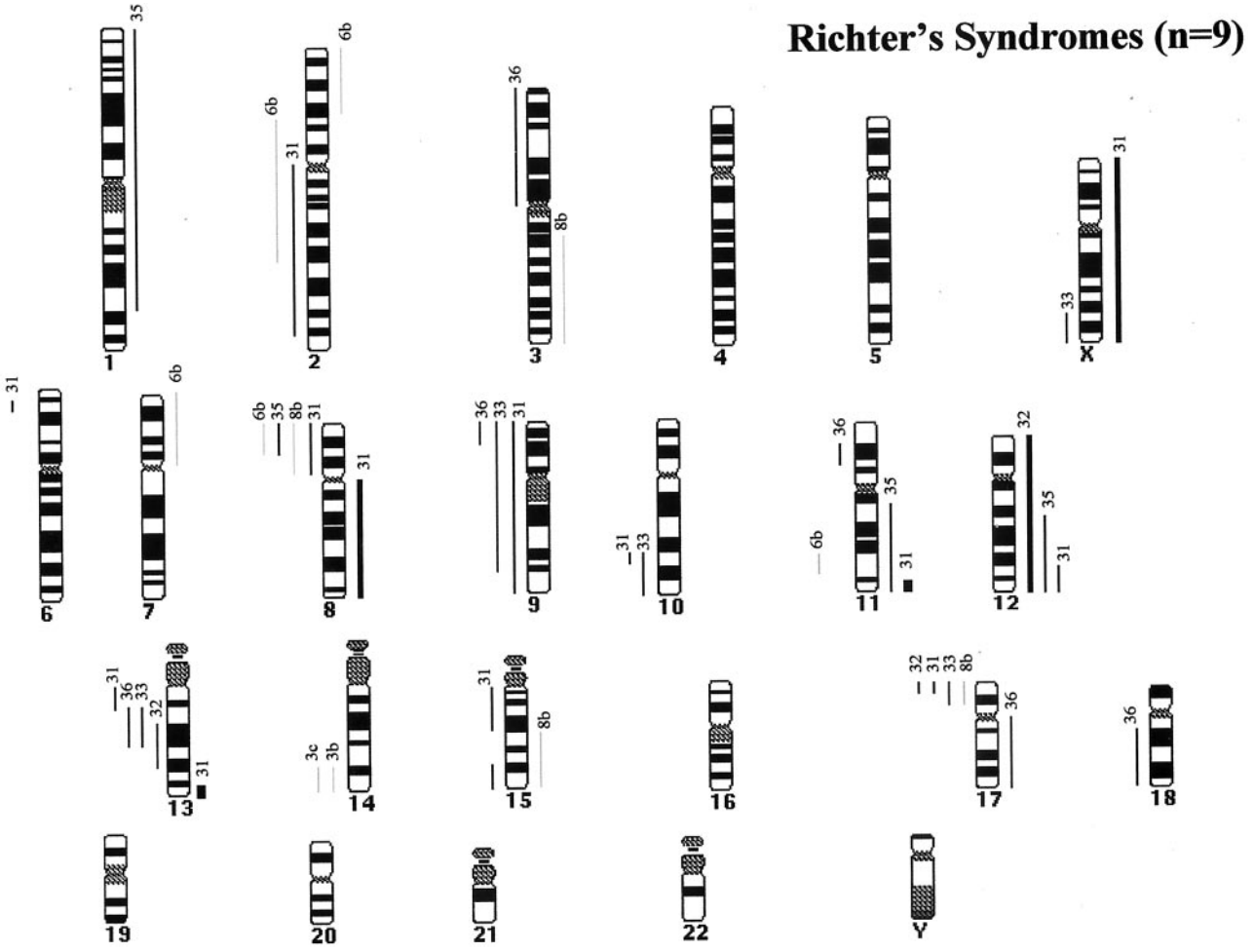

Figure 2. Summary of all DNA copy number changes detected by CGH in nine patients with RS. Case 9c was excluded. **Left**: **Lines** indicate loss of chromosomal material. **Right**: **Lines** indicate gain of chromosomal material. **Gray lines** indicate samples after progression. **Thick black bars** represent chromosomal gains exceeding 1.5 in a large chromosomal region. High-level DNA amplifications are represented as **squares**. Each **line** represents a gained or lost region in a single sample. The most common gains were gain of chromosome 12 (33%) and additionally gain of 11q (22%), high-level DNA amplifications in two different regions of the genome (11q25 and 13q34), and the most frequent losses involved chromosomes 13 (44%), 17p (44%), 8p (44%), and 9 (33%).

DNA Imbalances in RS

Chromosome imbalances were observed in eight of the nine (89%) cases of RS (Figure 2, Table 1). Similar to CLL, CGH losses ($n = 28$) were more frequent than gains ($n = 12$). All altered cases, except case 3 (samples 3b and 3c), showed multiple chromosomal imbalances. The case with the highest number of abnormalities (patient 31) showed 14 CGH alterations, including the only two high-level DNA amplifications observed in this study at 11q25 and 13q34. Almost all of these aberrations corresponded to partial chromosome losses. Case 3a with a single alteration had a loss of 14q24-q32. The most common imbalances in these patients were gains of chromosome 12 (33%) and 11q (22%), and losses involving chromosomes 8p (44%), 13 (44%), 17p (44%), and 9 (33%).

For the comparison of number of CGH imbalances and particular CGH alterations between CLLs and RS, we excluded the four previous CLLs that developed an RS (cases 3a, 6a, 8a, and 9a) and the *de novo* RS (9c) so that the data fulfilled the criteria for independent samples. Thus, chromosome imbalances were more frequent in RS (mean, 4.7 ± 4 per case) than in CLLs at diagnosis (mean, 1.2 ± 1 per case) ($P = 0.002$). RS had more chromosome gains (mean, 1.3 ± 1.2) than CLLs (mean, 0.5 ± 0.6) ($P = 0.044$). Similarly, chromosome losses were significantly higher in RS (mean, 3.1 ± 2.8) than in CLL (mean, 0.6 ± 1) ($P = 0.001$). Moreover, RS showed more frequently loss of 8p (44% *versus* 4%, $P = 0.01$), loss of chromosome 9 (33% *versus* 4%, $P = 0.04$), and a tendency to loss 17p (44% *versus* 15%, $P = 0.16$).

Comparison between CGH, FISH, and Molecular Studies

Interphase FISH analysis was performed in 13 cases (18 samples). This technique confirmed the CGH results showing imbalances of chromosomes 7q, 11q, 12, and 17p (Table 1). Loss of 11q was detected by FISH in the case in which a loss of 11q23-q25 was observed by CGH (case 4b, Figure 3A). A normal FISH pattern was observed in the remaining five samples with no 11q alterations by CGH. Similarly, there was a total concordance

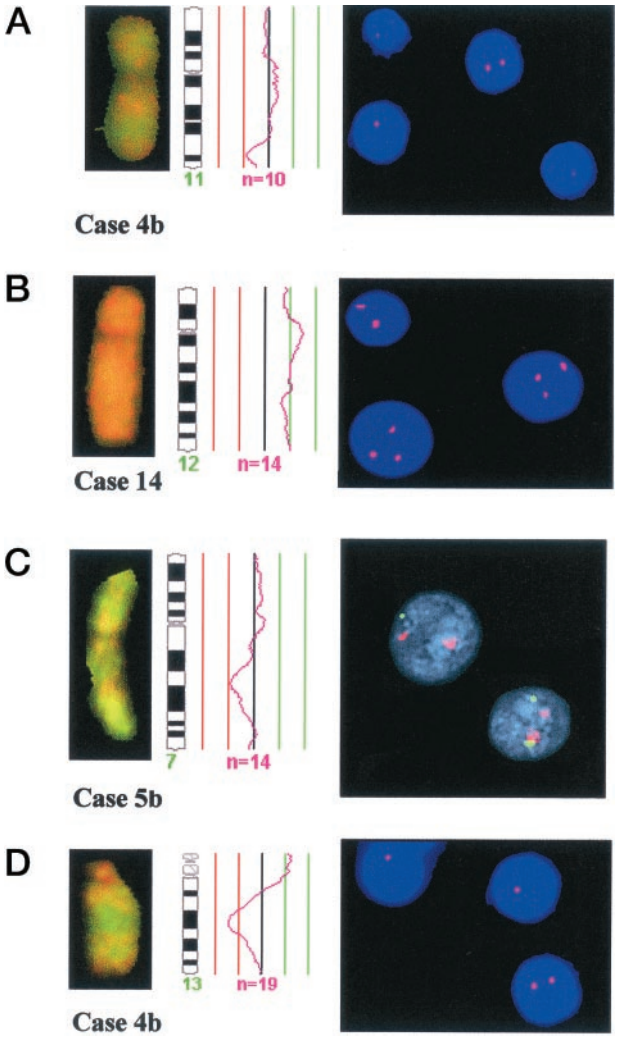

Figure 3. Individual representative examples of CGH digital images (**left**) and fluorescent ratio profiles (**right**) illustrating genomic alterations. **A:** Loss of 11q23-25 by CGH and FISH analysis with an ATM locus probe demonstrating loss of this locus. **B:** Gain of chromosomes 12 by CGH and by FISH analysis with a chromosome 12 centromeric-specific probe. **C:** Loss of 7q22-q31 by CGH (tumor DNA labeled with Spectrum-Green fluorochrome) and loss of 7q31 by FISH with 7q11.23 (Elastin Gene, Spectrum Orange) combined with 7q31 (control probe D7S486, D7S522, Spectrum Green) LSI Williams syndrome region probe. **D:** Loss of 13q14-q22 by CGH and loss by FISH analysis with a 13q14-specific probe.

between CGH results on chromosome 12 and the presence of trisomy 12 by FISH analysis in 13 patients, 4 of them presenting an extra chromosome 12 by both CGH and FISH (Figure 3B). There was also a good agreement between the CGH results on 17p and the 17p13 FISH results in nine cases. The only exception was one case (case 5b) with normal 17p by CGH and a loss of p53 in 53% of cells by FISH analysis. Additionally, case 5b with a loss of 7q22-q31 by CGH was hybridized with a 7q11.23-q31 probe confirming the loss of 7q31 in 25% of the cells (Figure 3C). On the other hand, five cases had losses of 13q by FISH analysis (cases 4b, 7b, 10b, 14b, 12a, and 12b). Two of them (cases 4b and 12a and b) showed a loss at 13q by CGH (Figure 3D), but the other three cases (cases 7b, 10b, and 14b) did not show any loss of chromosome 13 by CGH. Four additional samples showed normal chromosome 13 by both CGH and FISH analysis.

The status of the *p53* gene was studied by single-stranded conformational polymorphism analysis in all patients (Table 1). All cases with 17p losses by CGH showed an anomalous single-stranded conformational polymorphism pattern and were subsequently sequenced. The results are summarized in Table 2. Six point mutations and three microdeletions were detected in these cases, always associated with loss of the remaining allele. In cases 8 and 17, both sequential samples of the same patient showed the same mutation. In case 11, the loss of 17p was acquired in the progressed sample. The status of the p53 gene was also examined in the rest of the cases with normal chromosome 17 CGH profile (27 cases and 43 samples). No *p53* gene mutations were found in any of these cases, including case 5b with a loss of 53% of *p53,* by FISH analysis.

Patients with losses of chromosome 17p by CGH (n = 8) in the whole series of patients were associated with a significantly higher number of total chromosomal imbalances than in tumors with a normal chromosome 17 profile (n = 27) (mean, 4.5 ± 4 *versus* 1.2 ± 1.6, respectively; P = 0.02) and also with higher number of chromosomal losses (mean, 3.4 ± 2.6 *versus* 0.6 ± 1.1, respectively; $P < 0.001$). Interestingly, when the analysis was restricted to the CLL patients at diagnosis, excluding the RS patients, cases with 17p deletions (n = 5) were still associated with a significantly higher number of imbalances (mean, 2.6 ± 1.1) than cases with a normal chromosome 17 (n = 25) (mean, 0.9 ± 0.8) (P = 0.004) and higher number of losses (mean, 2 ± 1.2 *versus* 0.4 ± 0.6, respectively; P = 0.002).

The $p16^{INK4a}$ gene was examined by Southern blot in eight samples from seven patients. No correlation between this molecular study and chromosome 9p CGH profile was observed. A $p16^{INK4a}$ homozygous deletion was only detected in one of three cases with 9p losses by CGH (case 31). The other two cases with 9p losses (cases 33 and 36) showed a $p16^{INK4a}$ gene in germ line. On the other hand, a $p16^{INK4a}$ homozygous deletion was found in the Richter's transformation of case 6 (sample 6b), in which the CGH showed a normal 9p profile. Three additional cases (cases 8b, 32, and 34) and the initial CLL of case 6 (sample 6a) showed $p16^{INK4a}$ gene in germ line associated with a normal 9p CGH profile. No mutations of $p16^{INK4a}$ gene were found in any of these cases.

Clinical Significance of CGH Imbalances

The clinical significance of the recurrent CGH imbalances was analyzed in 30 CLL patients in whom the sample was examined at diagnosis. The 4-year survival of these patients was 66% (95% CI, 48 to 84%), with this being 92%, 62%, and 26% for stages A, B, and C, respectively (P = 0.001). Patients at early stages (A and B)

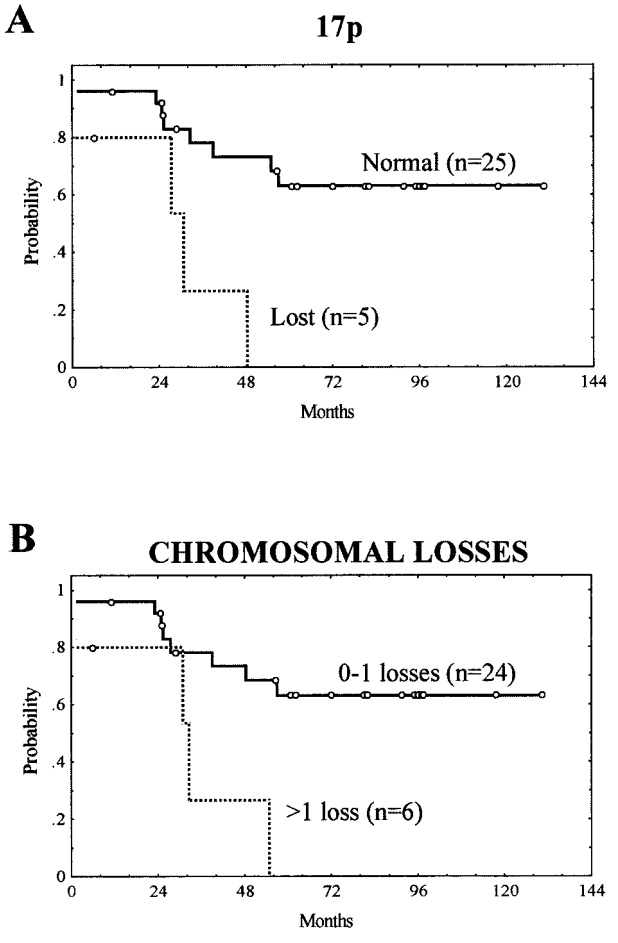

Figure 4. A: Survival curves of patients with CLLs according to 17p losses (normal 17p *versus* 17p deletion; $P = 0.02$). **B:** Survival curves of patients with CLLs according to increased number of chromosomal losses (≤1 *versus* >1 losses per case; $P = 0.03$).

presented trisomy 12 more frequently than those at stageC (42% *versus* 0%, respectively; $P = 0.03$). In addition, the total number of chromosomal losses was higher in stage C patients (mean, 1.4 ± 1.3) than in stages A and B (mean, 0.3 ± 0.5) ($P = 0.01$) and cases in stage C showed more frequently loss of 17p (44% *versus* 0%) ($P = 0.002$).

Patients with loss of 17p had shorter survival rates than cases with a normal chromosome 17 profile (4-year survival, 27% *versus* 73%) ($P = 0.02$) (Figure 4A). Lymphocyte counts $>20 \times 10^9$/L were associated with 17p losses ($P = 0.02$). In addition, the presence of chromosome losses was also associated with a poor outcome (≤1 *versus* >1 losses per case; 4-year survival, 27% *versus* 74%, respectively; $P = 0.03$) (Figure 4B). No other initial characteristics of the patients were related to the CGH alterations.

A Cox proportional-hazards analysis was performed with the 30 CLLs to analyze the relative prognostic weight of 17p loss and the number of losses for survival. In this analysis, only 17p loss retained predictive value (relative risk, 4.13; $P = 0.046$).

Discussion

In the present study we have analyzed by CGH a series of CLLs at diagnosis, multiple sequential samples during the evolution of the disease, and transformed large B-cell lymphomas evolved from CLL. Chromosomal imbalances were detected in 73% of CLLs, a higher number of genetic abnormalities than those detected by conventional cytogenetics, but similar to the findings using FISH with multiple DNA probes.[2] The total number of chromosomal losses and 17p deletions was significantly associated with shorter survival of these patients. Increasing number of chromosomal imbalances in sequential samples was associated with clinical progression and stage C disease. Transformed large B-cell lymphomas had a relatively similar pattern of CGH alterations to that of CLL. However, these tumors showed a significantly higher number of total chromosomal imbalances and losses, and specific deletions of 8p and chromosome 9.

Previous cytogenetic studies have identified gains and trisomy of chromosome 12, and losses of chromosomes 13q, 11q, 17p, 6q, and 14q as frequent genetic aberrations in CLL. Our study confirms most of these alterations as recurrent targets in CLL, but the frequency of the alterations was higher in the CGH analysis than in most cytogenetic studies. Furthermore, we have found certain recurrent imbalances, such as gains of 2p, 8q, and losses of 8p, 10q, and chromosome 9, not previously recognized by conventional cytogenetics. Other CGH studies have found similar chromosomal alterations in these tumors, although the frequency varies.[21–24] In our study, we observed a relatively high frequency of trisomy 12 and loss of 13q. In addition, other frequently gained (2p and 11q) and lost (8p, 10q, and chromosome 9) regions have not been found in other studies. The presence of trisomy 12 and 13q deletions in the same cell has been described as a rare event.[3,25] In our study, apparently only one patient had both abnormalities. Most of the individual CGH alterations detected in this series of CLLs have already been observed in other non-Hodgkin's lymphomas. Moreover, our results confirm the similarities between CGH alterations in CLL and mantle cell lymphoma (MCL), as gains of 8q and chromosome 12, and losses of chromosomes 13, 11q, 17p, and 9p that were also recurrent CGH alterations in MCL, although the frequency of these alterations in MCL was higher.[13,26] However, 3q gains and 14q losses were more frequent in MCLs and CLLs, respectively.

Genetic events underlying progression of CLLs into more advanced stages or RS are primarily unknown. Only a few studies using chromosomal banding have been reported in isolated or small series of patients with RS, and in some of these cases the sample analyzed did not correspond to the transformed lymphoma. These studies have identified frequent complex karyotypes but not clear recurrent anomalies.[27–30] In the present study using CGH, we have shown that RS has relatively similar chromosomal imbalances to those of CLL with frequent trisomy 12 and 13q losses. However, the transformed lymphomas had a significantly higher number of total imbalances, gains, and losses than CLL. Furthermore, RS

showed additional particular alterations, such as losses of 8p and chromosome 9. These findings suggest that although CLL and RS have a particular genetic profile different from other non-Hodgkin's lymphoma, the chromosomal alterations associated with RS transformation, including losses of 8p and chromosome 9, are relatively similar to those observed in other aggressive lymphomas. Although 17p deletion was more frequently found in RS than in CLL, this aberration was also relatively common in CLL patients in stage C at diagnosis.

Different studies analyzing genetic abnormalities in the progression of CLL have suggested that the karyotype is relatively stable during the evolution of the disease.[9–11] In contrast, Juliusson and colleagues[31] have identified karyotypic evolution in 15% of cases. Additionally, karyotypic evolution was significantly associated with progressive disease in two cases. However, all these studies have been performed using conventional cytogenetics. In this study, we have used CGH to examine the evolution of chromosomal imbalances in sequential samples of 17 patients, 6 with stable clinical disease and 11 who progressed to a more advanced stage or transformed into a large B-cell lymphoma. In 10 of the 11 cases with progressive disease we were able to confirm the clonal relationship between samples, whereas one case of large cell lymphoma was clonally unrelated to the original CLL. This relationship was also confirmed by the CGH analysis because the clonally related samples showed the same CGH imbalances in the initial and progressed samples. However, additional chromosomal aberrations were also observed in the progressed samples of six (60%) cases and in the two patients with stable stage C. In contrast four cases in stage A, studied at diagnosis and after a median follow-up of 103 months without clinical progression, maintained the same chromosome alterations. These findings suggest that clinical progression of CLL patients may be associated with an increasing number of chromosomal aberrations more frequently than it was initially observed by conventional cytogenetic studies. The recurrent alterations in patients with karyotypic evolution were gains of 2p and 7p and losses of 8p, 11q, and 17p. Interestingly, 8p deletions have been recently associated with blastoid variants of leukemic mantle cell lymphoma,[32] suggesting that losses of this region may be involved in aggressive transformation of these lymphomas.

We have also performed interphase FISH to directly assess the copy number of the *RB1*, *p53*, 7q31 loci and chromosome 12. The results obtained with the chromosome 12 and 7q probes confirmed CGH results. Only one case with no 17p loss by CGH showed a deletion of *p53* by FISH analysis. However, three of the cases with *RB* deletion by FISH did not show a 13q14 loss by CGH. This could be because of the small size of the deletions around *RB* locus that could not be detected by CGH technique, as it has been shown by other studies.[33]

Deletions in the short arm of chromosome 17 have been detected in 10 to 15% of CLL untreated patients and are associated with poor prognosis.[34] *p53* gene mutations have been observed in a similar proportion of these patients.[34–37] However, the relationship between the cytogenetic and molecular findings is not clear, because 17p deletions have not always been associated with *p53* mutations in CLL.[34] In our study, p53 gene mutations were detected in all cases with 17p losses by CGH. However, we were unable to detect a *p53* gene mutation in a case with normal 17p profile but with a loss of *p53* by FISH analysis. Interestingly, patients with 17p losses by CGH had a significantly higher number of CGH imbalances than cases with normal 17p profile, suggesting that *p53* inactivation may be involved in increasing chromosomal instability in these tumors. *p53* aberrations in CLL seem to occur more frequently in cases with no trisomy 12, and it has been proposed that these alterations may represent alternative pathways of progression.[37] The present CGH analysis seems to be concordant with this observation because only one case showed both abnormalities simultaneously.

Inactivation of the tumor suppressor gene *p16^{INK4a}* at 9p21 has frequently been found in aggressive and transformed non-Hodgkin's lymphomas.[17] In this study, losses of chromosome 9p were associated with Richter's transformation. However, the relationship between 9p losses and p16^{INK4a} inactivation in these tumors is not clear. In this study, we detected p16^{INK4a} alterations only in one of the three tumors with 9p losses, suggesting that additional gene targets may be present in this chromosomal region. On the other hand, we have also observed *p16^{INK4a}* gene homozygous deletion in an RS in which CGH analysis showed a normal chromosome 9p profile, indicating that inactivation of this gene may be associated with microdeletions beyond the sensitivity of CGH analysis.

Different cytogenetic studies, including chromosomal banding and FISH, have analyzed the prognostic significance of genetic alterations in CLL.[2,38,39] However, the possible relationship of CGH imbalances and survival in CLL patients has not been investigated. In agreement with previous cytogenetic studies, the CGH results reported in this study indicate that the complexity of the genetic alterations, and particularly the number of losses, are significantly associated with a shortened median survival. Furthermore, loss of 17p was associated with a poor prognosis.[2,4,34,37,39–41] The prognostic significance of trisomy 12 in CLL has been controversial. Initial studies suggested a greater tendency to disease progression and poor prognosis in patients with this alteration.[38,39] However, other series have not confirmed these observations.[2,4,42] In our study, the presence of trisomy 12, alone or in combination with other alterations, was not related to poor survival.

In conclusion, this study shows that CLL has frequent genetic alterations, which may increase with the disease progression. Transformation of CLL into large cell lymphomas is associated with higher number of genetic imbalances and specific chromosomal aberrations that may play a role in the pathogenesis of this progression. In addition, our findings also suggest that certain genetic alterations detected by CGH may be of prognostic significance in this disease.

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