



Comparative genomic hybridization and amplotyping by arbitrarily primed PCR in stage A B-CLL

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Abstract

Cytogenetic analysis is useful in the diagnosis and to assess prognosis of B-cell chronic lymphocytic leukemia (B-CLL). However, successful cytogenetics by standard techniques has been hindered by the low in vitro mitotic activity of the malignant B-cell population. Fluorescence in situ hybridization (FISH) has become a useful tool, but it does not provide an overall view of the aberrations. To overcome this hurdle, two DNA-based techniques have been tested in the present study: comparative genomic hybridization (CGH) and amplotyping by arbitrarily primed PCR (AP-PCR). Comparative genomic hybridization resolution depends upon the 400-bands of the human standard karyotype. AP-PCR allows detection of allelic losses and gains in tumor cells by PCR fingerprinting, thus its resolution is at the molecular level. Both techniques were performed in 23 patients with stage A B-CLL at diagnosis. The results were compared with FISH. The sensitivity of AP-PCR was greater than CGH (62% vs 43%). The use of CGH combined with AP-PCR allowed to detect genetic abnormalities in 79% (15/19) of patients in whom G-banding was not informative, providing a global view of the aberrations in a sole experiment. This study shows that combining these two methods with FISH, makes possible a more precise genetic characterization of patients with B-CLL. © 2001 Elsevier Science Inc. All rights reserved.

1. Introduction

The pathogenic events in chronic B-cell lymphocytic leukemia (B-CLL), the most common form of adult leukemia in Western countries, remains poorly understood. B-CLL is a malignancy of self-reactive, activated CD5+ B cells devoted to the production of polyreactive natural autoantibodies [1]. The clinical course of this disease is extremely variable; some patients survive only a few months whereas others have stable disease greater than 10 years [2]. Rai and Binet clinical staging systems are useful prognostic tools. However, they do not distinguish which patients in early stages of the disease will progress. Other prognostic parameters, e.g., short lymphocyte doubling time and/or dif-

fuse bone marrow infiltration are known risk factors for progressive disease in early-stage CLL [2,3]. Although patients lacking these features have a low risk for disease progression, 10–15% of such cases will still progress within 5 years of diagnosis, emphasizing the need for additional prognostic factors in early CLL [3,4].

Genetic studies have provided useful information in understanding the pathogenesis of CLL, and some aberrations may also have a prognostic impact [4–10]. In CLL, accurate and successful cytogenetic analysis has been hindered by the low in vitro mitotic activity of the critical B-cell population with metaphases arising from normal T-cells being frequent. Thus, G-banding analysis is informative in only a minority of cases. Although FISH has become a useful tool and, in some studies, showing to be informative in up to 80% of cases [10], it does not provide a global view of the chromosomal aberrations. Two molecular techniques

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that are not cell-growth based, comparative genomic hybridization (CGH) and amplotyping by arbitrarily primed PCR (AP-PCR), have been recently introduced and applied to study genetic abnormalities in human malignancies. CGH allows tumor genomes to be tested for the presence of chromosomal unbalances without the need of metaphases and only requires small numbers of tumor cells to prepare DNA [11]. The resolution of this technique depends upon the 400-bands of the human standard karyotype. AP-PCR is a molecular karyotyping (amplotype) approach which under controlled conditions allows to estimate quantitative changes in DNA content (allelic losses and gains) in tumor cells relative to normal tissue from the same cancer patients [12].

This study aimed to investigate the value of CGH and AP-PCR to detect chromosomal abnormalities in B-CLL and compares their sensitivity with FISH. Both techniques were performed in diagnostic samples from 23 patients with stage A B-CLL, the majority (19 cases) with normal karyotype or not available metaphases. B

2. Materials and methods

Twenty-three patients with stage A B-CLL from whom DNA was available at diagnosis were studied. All patients showed lymphocytosis with morphological and immunophenotypical features consistent with B-CLL (CD5+, CD19+, CD23+). The definition of atypical morphology was based on the FAB classification of CLL. Table 1 summarizes the main disease features. Five patients had disease progression: two died due to the disease-related cause, and three required treatment because of progressive disease.

Cytogenetic studies by conventional methods were carried out in bone marrow cells. Two cultures were established from each sample: one with a B-cell mitogen (pokeweed) and one without mitogens. Clonal abnormalities were described according to the ISCN [13]. Out of 23, 10 showed a normal karyotype, 9 were failures and 4 had an

abnormal karyotype and were used as positive controls (Table 2). The presence of monosomy Y was not considered as abnormality (cases 5 and 6).

CGH was performed according to Kallioniemi et al. [11] with some modifications, as previously described [14].

AP-PCRs were performed in 15 μ l of reaction containing 40, 60, or 80 ng of genomic DNA, 1 μ M of an arbitrary primer, 1 unit of Taq DNA polymerase (Perkin-Elmer/Cetus), 125 μ M each of four dNTPs, 10 mM Tris HCl (pH 8.3), 1.5 μ Ci of [α - 32 P]dCTP (3000 Ci/mmol), 50 mM KCl, 4.5 mM MgCl₂, and 0.1% gelatin. Amplification was performed in a PTC 100 thermal cycler (MJ Research) for the first five cycles under low-stringency conditions (at 94°C for 30 s, 50°C for 1 min, and 72°C for 1.5 min), and then for 25 cycles under high-stringency conditions (at 94°C for 15 s, 60°C for 15 s, and 72°C for 1 min). PCR products were subjected to electrophoresis in a 5.5% denaturing polyacrylamide gel. The MCG1 and BLUE arbitrary primers used in this study had been previously described [12].

FISH was performed in 16 cases in which fixed cells were available. The presence of trisomy 12 was assessed by using a probe specific for the centromeric region of chromosome 12, D12Z3. To identify 13q14 deletions we used the LSI D13S25 probe (Vysis, Downers Grove, IL, USA). At least 200 cells were evaluated in each case.

3. Results and discussion

The proportion of patients with stage A B-CLL and chromosomal changes increased from 17% (4/23 cases) detected by conventional G-banding to 43% (10 cases) detected by CGH, and 62% (15 cases) by AP-PCR. The use of CGH combined with AP-PCR allowed to detect genetic abnormalities in 79% of cases (15/19) with apparently normal or unsuccessful karyotype (Table 2). As has been reported in previous studies using FISH, in our series losses of 13q and gains of chromosome 12 were the most frequent changes both by CGH and by AP-PCR [1,4–10]. These results were confirmed by FISH. Besides, these DNA-based techniques allowed to detect several new abnormalities in addition to other recurrent changes.

3.1. CGH analysis

CGH showed a number of recurrent unbalances as follows: loss of chromosomal material on 13q (four cases), 6q (two cases), and 5q (two cases); and gain of chromosomal material on 12q (three cases, two with trisomy 12) and of the chromosomes 17 and 19 (two cases each). Other unbalances of chromosomal regions detected in single cases were: loss of 4q, 7p, 11q, 14q, 15q, 17p, 18q, and Xq, and gain on 1p and 3q. Detailed data of CGH results compared to cytogenetics and AP-PCR is shown in Table 2. Examples of most significant changes are shown in Fig. 1.

CGH was more sensitive than G-banding to detect gains of chromosome 12. Three cases with gains on chromosome

Table 1
Clinical features of 23 B-CLL patients at diagnosis

Age median (range)	66 (42–87)
Sex	
Male	18
Female	5
Stage Binet/Rai	
A/0	14
A/I	9
No. of lymphocytes in blood	
$\leq 30.000/\text{mm}^3$	20
$> 30.000/\text{mm}^3$	3
Morphology	
Typical	21
Atypical	2
Histopathological findings	
Nondiffuse infiltration of BM	20
Diffuse infiltration of BM	3
Clinical course	
Stable	18
Progression/exitus	5

Table 2

Results of CGH and AP-PCR analysis in 23 patients with B chronic lymphoid leukemia at diagnosis

Case	G-banding analysis	CGH		AP-PCR	
		Gains	Losses	Gains	Losses
1	46,XY[6]/48,XY,t(2;14)(p13;q32),-10,+3mar[20]	12q11~q21	17p11~pter	Not evaluable	
2	47,XX,+12[30]	12		12,13q14	N
3	46,XY,dup(13)(q12q13)[32]	17,19	5q15~q23,13q21~q31	N	N
4	46,XY[6]/46,XY,del(6)(q14q21)[16]/45,idem,-Y[24]		6q14~q23	N	N
5	46,XY/45,X,-Y	12	14q21~q31	12	N
6	46,XY/45,X,-Y	N		12	N
7	46,XY	1p33~p36,17,19	4q23~q32,6q13~q24,13q21~q32,18q12~q22,Xq21	9q22	N
8	46,XX	N		N	N
9	46,XY	N		12,13q31	N
10	46,XY	N		Not evaluable	
11	46,XY	N	N	N	13q14
12	46,XX	N	N	N	2,13q14
13	46,XY	N	N	N	9q22
14	46,XY	N	N	N	N
15	46,XY	N	N	N	N
16	Not evaluable	3q26~29	15q26	N	N
17	Not evaluable		5q15~q22,7p13~p21,13q14~q22	8q24	7,13q14
18	Not evaluable		13q14~q21	N	11,13q14
19	Not evaluable		11q14~q23	9q22	11
20	Not evaluable	N		N	13q14
21	Not evaluable	N		11	N
22	Not evaluable	N		12	13q14
23	Not evaluable	N		12	N

N: Normal

12 were identified by CGH, while only one of them had trisomy 12 by G-banding. In two of these patients gains were complete trisomy 12. The third patient had a partial trisomy with gain on 12q11~q21 (Fig. 1), a region containing the *MDM-2* (murine double minute 2) gene, which has been implicated as a relevant target within the trisomy. *MDM-2* encodes an oncoprotein that operates in an autoregulatory feedback loop with the tumor suppressor protein p53. Amplification of the *MDM-2* gene leads to over-expression of the protein that inactivates p53. Over-expression of *MDM-2* RNA and of several *MDM-2* proteins have been detected in CLL. Findings in our patient support the importance of this specific region in B-CLL [1,15,16].

Deletions or translocations of chromosome 13 are the most common structural abnormalities in B-CLL. Molecular studies have demonstrated a high incidence of genetic loss, which is frequently homozygous, telomeric to the *RBI* gene, indicating the site of a potential tumor suppressor gene involved in the pathogenesis of CLL [17,18]. In our study, del(13q) was the most common aberration detected by CGH (four cases), although 13q14 band was only involved in two of them. Deletion of chromosome 13 was missed by conventional cytogenetics in all samples.

CGH detected loss of chromosomal material on 5q, 7p, 11q, 13q (two cases), and 15q in 8 cases with normal or unsuccessful karyotype. Two patients had multiple aberrations (cases 5 and 7), one together with +12 and another with del(13q). Furthermore, CGH revealed some additional abnormalities in 2 patients with an abnormal G-banded kary-

otype. In case 1, the chromosomal origin of a marker chromosome could be probably matched with the gain of chromosome 12 (12q11~q21) detected by CGH. In the other (case 3), G-banding detected dup(13q), while CGH also showed an interstitial deletion on 5q and 13q and gains of the whole chromosomes 17 and 19. FISH confirmed the del(13q). Although the duplication on chromosome 13 could be material from chromosome 17 or 19, unresolved chromosomal aberrations, such as marker chromosomes, could not completely explain the differences between CGH and karyotyping. A likely explanation for the discrepancies is that the methods analyze different cell populations [19].

Three previous CGH studies have been published on characterization of the genetic changes in CLL [6,20,21] and only two compared CGH with G-banding results [6,20]. Bentz et al. [20] found aberrations in 50% of normal or not evaluable cases (9/18) whereas Karhu et al. [6] documented concordant results between karyotyping and CGH, with only one abnormal CGH of 13 patients with normal G-banding. Our study shows that CGH is useful to detect genetic abnormalities in cases in which G-banding did not provide information.

3.2. AP-PCR analysis

This is the first study in samples from patients with CLL using AP-PCR. The use of primers MCG1 and BLUE generates a total number of 45 bands with known chromosomal localization, representing the entire human genome with the

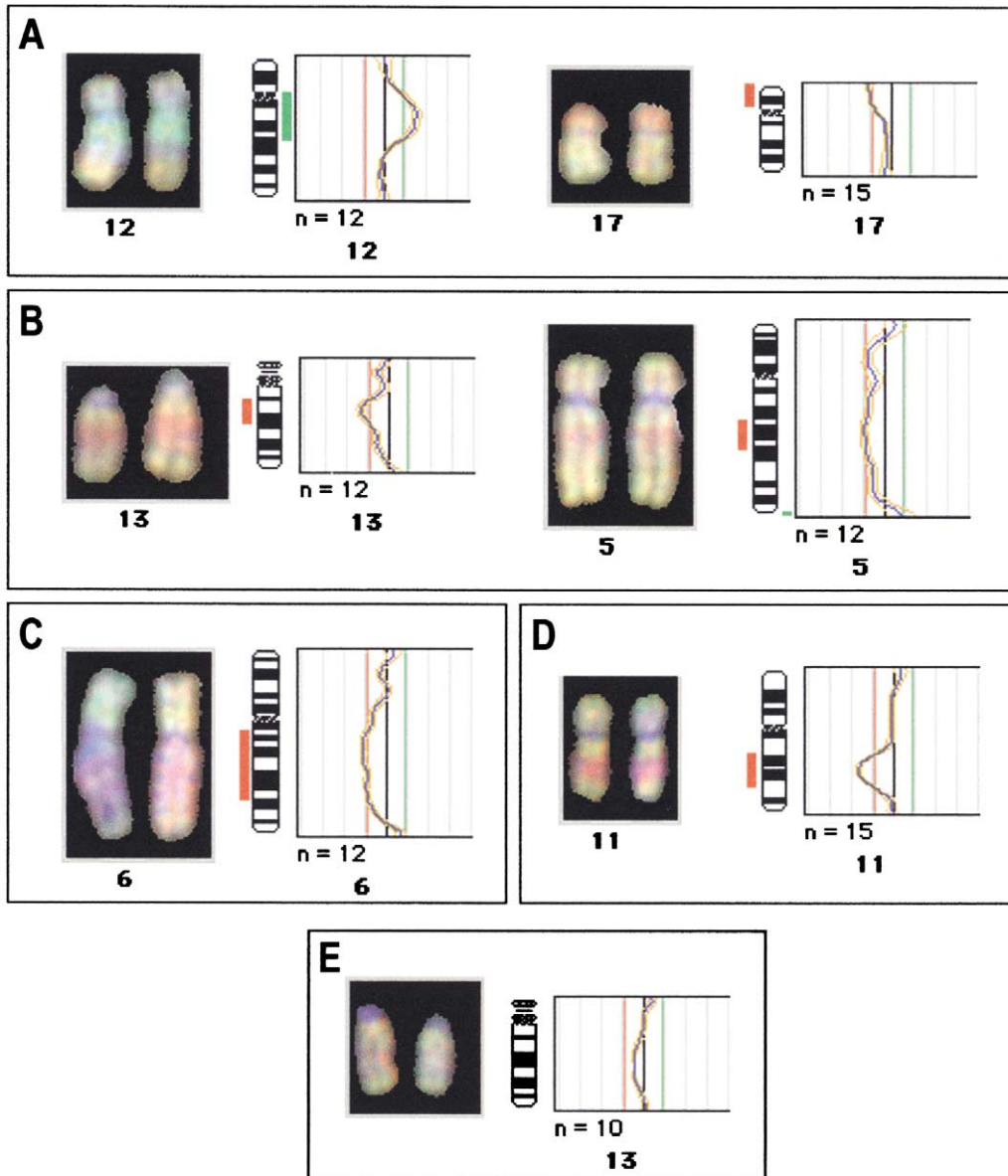


Fig. 1. CGH analysis of B-CLL. Partial karyotypes (left) and corresponding ratio profiles (right) observed in five cases. (A) Case 1 showed an amplified region on chromosome 12 at band 12q11~q21 and loss of 17p. (B) Interstitial deletions in chromosomes 5 and 13 detected in case 17. (C) Loss of chromosomal material on 6q in case 4. (D) $\text{del}(11)(q14q22)$ detected as a single change in case 19. (E) Partial karyotype observed in case 12. Loss of 13q was found to be not significant by the software using the threshold 0.8/1.2.

exception of chromosomes 19, 21 and Y [12]. Table 2 summarizes the genetic alterations detected after the amplotype analysis using these two arbitrary primers. A representative result of these experiments is shown in Fig. 2, which highlights the regions of the amplotype gel containing bands exhibiting consistent changes in these leukemia samples. Determination of losses and gains was carried out by visual inspection and densitometric analyses [12]. The interpretation of chromosome losses and gains was based by comparing leukemic DNA fingerprints with several DNA preparations from normal tissues, and with the previous accumulative DNA data from many normal tissues. Inten-

sity changes of some bands such as band I (Fig. 2) were not considered for the analysis because of their polymorphic nature in the human population. However, determination of losses or gains was possible with the fingerprint bands that showed no major fluctuations in their intensities among these normal tissue DNA preparations.

Amplotyping analysis in our series allowed detection of genomic changes in over two-thirds (15/23) of patients. The most recurrent alterations were also loss of chromosome 13 sequences in 6 cases (26%) and gain of chromosome 12 sequences in 6 cases (26%). MCG1 band J was responsible for the detection of common losses of chromosome 13 se-

quences (with the exception of case 1 (Table 2). In contrast, no losses were detected of band E from the AP-PCR fingerprints with the same MCG1 primer (data not shown). Sub-chromosomal localization of these two bands using Gene-Bridge 4 Radiation Hybrid panels indicated that band E is located at 13q31~q32 and band J is more centromeric (13q14.1~q14.3). Therefore, band J, but not band E is located in the region close to the common region of loss identified for CLL, as mentioned. Other recurrent changes were allelic losses of chromosomes 11 (cases 18 and 19), and gains of 9q22 sequences (cases 7 and 19) (Table 2).

AP-PCR was more informative than CGH for detecting genetic changes in B-CLL (62% vs 43% of abnormal cases, respectively) (Table 2). This might be because the amplo-type is a PCR-based technique that is able to detect alterations in chromosomal regions, which are too small to be detected by CGH. The sensitivity of the CGH analysis depends on the size of the gain or loss, and on the contamination of the sample with normal cells [22,23]. The resolution for the detection of high-copy number amplifications using CGH has been defined around 2 Mbp. However, only deletions to a size of 10–12 Mbp can be detected based on a diagnostic threshold value of 0.8, if the vast majority of cells carried the deletion [21]. Interestingly, we detected del(13q)

in four patients (cases 11, 12, 20, and 22) using AP-PCR and these results were confirmed by FISH. These patients had also loss on 13q by CGH, although the software did not detect this aberration as significant using the threshold 0.8/1.2 (Fig. 1E). The power of AP-PCR is limited because it does not cover the entire genome. This limitation can be in principle overcome by the use of additional arbitrary primers yielding amplo-types with a higher density of informative chromosomal loci. The discrepancies between the findings in both techniques indicate the need to be used in a complementary way. Although CGH is less sensitive than AP-PCR, the technique is easier and it offers a more complete scope of genetic changes in the whole genome of the tumor.

In conclusion, the application of CGH and AP-PCR provides new information on gains and losses of chromosomal regions in B-CLL that can be especially useful in cases in which conventional cytogenetics do not provide data on karyotypic abnormalities. In our series, CGH and AP-PCR allowed for the characterization of the abnormalities in 79% of cases with normal or not evaluable karyotype. Our study using DNA-based techniques confirms that gains of chromosome 12 and loss of 13q, as detected by FISH, are the most common abnormalities, and that in several cases these aberrations are not the sole abnormality in the karyotype.

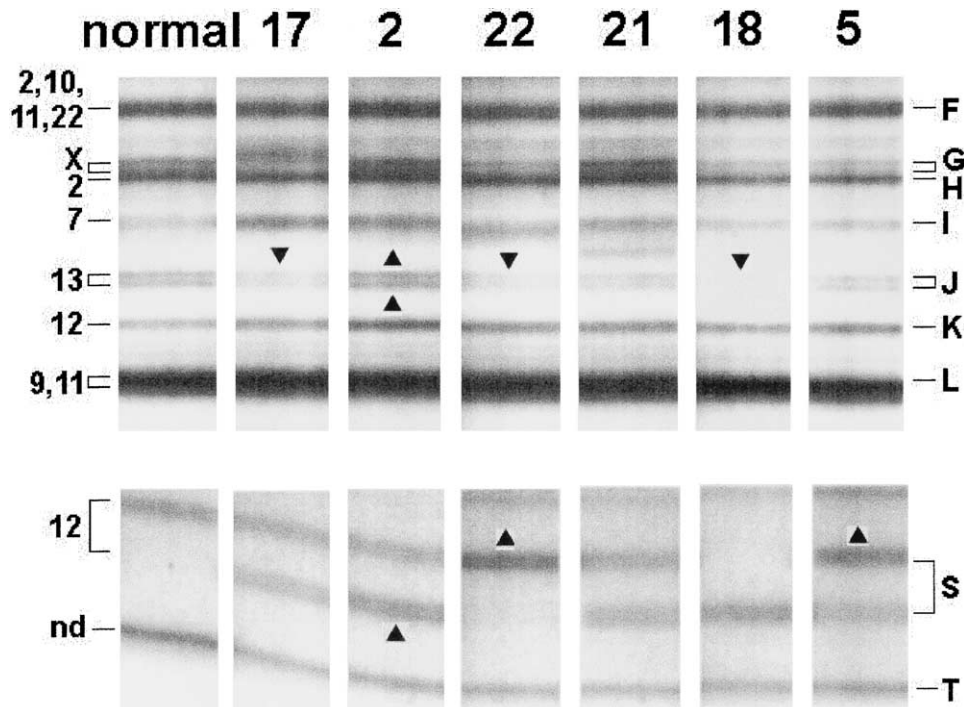


Fig. 2. AP-PCR DNA fingerprinting analysis of B-CLL. Patient identification number is indicated at the top. DNA from normal colon tissue was used as a diploid control (see text). Two lanes were loaded from each case corresponding to AP-PCR reactions with 40 and 60 ng of genomic DNA. Numbers at the left indicate the chromosomal origin of the bands named by letters at the right. Changes in the intensity of band G indicate genetic dosage of chromosome X, which identifies the gender of the patients. BAND I from chromosome 7 is polymorphic in the human population, affecting to the primer annealing site, and, therefore, in the absence of matching normal tissue, changes of this band cannot be considered for karyotyping purposes. Cases 17, 18, and 22 show loss of band J at 13q14. Band S on chromosome 12 is represented by two polymorphic alleles with different length. Cases 2 and 5 are heterozygous for this polymorphism and show gain of the lower allele in case 2 and gain of the upper allele in case 5. These gains can be detected also in band K from the Blue primer derived from chromosome 12.

Both CGH and AP-PCR might also be useful in the detection of complex karyotypes, which seem to have a clinical impact [4], and could contribute to the future discovery of more specific genetic markers for B-CLL. Other studies in a larger number of patients are required to determine recurrent abnormalities with prognostic significance.

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