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Multiplex ligation-dependent probe amplification for detection of genomic alterations in chronic lymphocytic leukaemia

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Summary

Chronic lymphocytic leukaemia (CLL) is the commonest form of leukaemia in adults in Western countries. We performed multiplex ligation-dependent probe amplification (MLPA) analysis in 50 CLL patients to identify multiple genomic CLL-specific targets, including genes located at 13q14, 17p13 (*TP53*), 11q23 (*ATM*) and chromosome 12, and compared the results with those obtained with fluorescence *in situ* hybridization (FISH). There was a good correlation between MLPA and FISH results, as most alterations (89%) were detected by both techniques. Only three cases with a low percentage (<25%) of cells carrying the alterations were not detected by MLPA. On the other hand, as MLPA uses multiple probes it identified intragenic or small alterations undetected by FISH in three cases. MLPA also detected alterations in 8q24 (*MYC*) and 6q25–26. In summary, unlike interphase FISH, MLPA enabled the simultaneous analysis of many samples with automated data processing at a low cost. Therefore, the combination of robust multiplexing and high throughput makes MLPA a useful technique for the analysis of genomic alterations in CLL.

Keywords: chronic lymphocytic leukaemia, genomic alterations, multiplex ligation-dependent probe amplification, fluorescence *in situ* hybridization, deletions.

Chronic lymphocytic leukaemia (CLL), the commonest form of leukaemia in adults in Western countries, is characterized by the accumulation of $CD5^+$ B lymphocytes in peripheral blood and bone marrow and is a genetically heterogeneous disease (Chiorazzi *et al*, 2005). The development of fluorescence *in situ* hybridization (FISH) enabled observation of genomic aberrations in non-dividing CLL cells in more than 80% of cases (Dohner *et al*, 2000). The most frequent genetic alterations are deletions in 13q14 (50–60%), 17p13 (*TP53*) (5–10%), and 11q22–q23 (*ATM*) (10–20%) and trisomy of chromosome 12 (10–20%) (Dohner *et al*, 2000; Seiler *et al*, 2006; Caporaso *et al*, 2007; Cotter & Auer, 2007). Studies using FISH analysis of CLL cells have demonstrated that deletions of *TP53* or *ATM*, identify CLL patients with a more aggressive course (Dohner *et al*, 2000; Seiler *et al*, 2006) and reduced progression-free survival (Grever *et al*, 2007). The deletion in 13q probably involves two microRNAs, *MIRN15A* and *MIRN16-1*, located at 13q14.3, which negatively regulate Bcl-2 at post-transcriptional level (Calin & Croce, 2006).

Fluorescence *in situ* hybridization uses labeled probes directed to known targets and has a higher resolution than standard cytogenetics. However, it is unlikely to determine small or intragenic deletions. In clinical practice, FISH is performed for 13q14, *TP53*, *ATM* and chromosome 12, but the procedure is laborious, time-consuming and costly. FISH is performed at diagnosis but generally not during clinical follow-up, although recent data indicate that repeating FISH analysis may benefit therapy (Shanafelt *et al*, 2006). Throughput in FISH also remains low compared with other molecular genetic techniques. Finally, although many other chromosomal alterations have been described in CLL using various methodologies (Bentz *et al*, 1995; Odero *et al*, 2001; Novak *et al*, 2002; Stilgenbauer *et al*, 2002; Schwaenen *et al*, 2004; Dicker *et al*, 2006; Mayr *et al*, 2006), they are not usually analyzed in clinical practice.

Recently, a new method has been described for the measurement of gene copy number: multiplex ligation-dependent probe amplification (MLPA) (Schouten *et al*, 2002).

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MLPA relies on the comparative quantitation of specifically bound probes that are amplified by polymerase chain reaction (PCR) with universal primers, and allows simultaneous processing of multiple samples. This method has proven to be accurate and reliable for identifying deletions, duplications, and amplifications in several diseases (Sellner & Taylor, 2004).

In previous work we used MLPA analysis to identify genomic alterations in a few samples of CLL (Coll-Mulet *et al*, 2006). In the present study, we performed MLPA analysis in 50 CLL patients, using two kits that allow the simultaneous identification of 55 genomic CLL-specific targets, and compared the results of the analysis with FISH data.

Materials and methods

Patients with CLL and cell isolation

Fifty peripheral blood samples from patients with CLL were studied (Table SI). CLL was diagnosed according to standard clinical and laboratory criteria. Samples were obtained from the Hospital de Bellvitge, Barcelona, Spain. Written informed consent was obtained from all patients in accordance with the procedures of the Hospital de Bellvitge Ethical Committee. Mononuclear cells were isolated from peripheral blood samples by centrifugation on a Ficoll-Hypaque (Seromed, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany). B ($CD19^+$) cell purification was performed in all cases with less than 80% of lymphocytes in whole blood, by using RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies). Blood samples or isolated mononuclear cells were incubated with RosetteSep Human B Cell Enrichment Cocktail at 50 μl/ml for 20 min prior to centrifugation on a Ficoll-Hypaque, according to the manufacturer's protocol.

Analysis of cell purity by flow cytometry

The percentage of B cells was measured by CD19/CD3 as previously described (Coll-Mulet *et al*, 2006). 5×10^5 cells were washed in phosphate-buffered saline (PBS), and incubated in 50 μl binding-buffer with allophycocyanin (APC)–conjugated anti-CD3 and phycoerythrin (PE)–conjugated anti-CD19 (Becton Dickinson, Franklin Lakes, NJ) for 10 min in the dark, according to the manufacturer's protocol. Data were analyzed using Cell Quest software (Becton Dickinson, Mountain View, CA).

Fluorescence in situ hybridization (FISH) and karyotype

Fluorescent-labeled DNA probes were used in interphase cytogenetic analyses. Locus-specific probes (LSI P53/SpectrumOrange, LSI ATM/SpectrumGreen, LSI 13S319/SpectrumOrange, LSI 13q34/SpectrumAqua) were used to determine loss of these genetic regions within interphase nuclei. Trisomy 12 was detected in interphase nuclei using a chromosomal centromere enumeration probe (CEP) labeled with SpectrumGreen. These five probes are packaged together in a commercially available kit (Vysis Chronic Lymphocytic Leukaemia Multicolor Kit) and were used in accordance with the manufacturer's specifications. Cells were fixed with fresh fixative before placement onto slides. Probe mixture was applied directly to slides. These slides were denaturated at 74°C for 2 min and incubated overnight at 37°C. Slides were then washed with 0·4 × saline sodium citrate (SSC)-0·3% nonidet P-40 (NP-40) at 73 ± 1°C for 2 min and 2 × SSC-0·1% NP-40 at ambient temperature for 1 min. 4′,6-diamidino-2-phenylindole (DAPI) II counterstain (10 μl) was applied to the target area. Slides were stored at −20°C in the dark. Two hundred nuclei were analyzed per each probe, using a NIKON fluorescent microscope. Cut off levels used were 5% for CEP 12 and 7% for LSI probes. Karyotype analysis was performed in all samples.

Multiplex ligation-dependent probe amplification (MLPA) for genomic alterations

DNA was isolated according to the manufacturer's protocol with High Pure PCR template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA was analyzed by MLPA using SALSA MLPA kit P037 and P038 from MRC Holland (Amsterdam, The Netherlands) for the simultaneous detection of 55 target sequences, allowing determination of the status of *TP53* (17p13; eight probes), *RB1*/*DLEU*/*MIRN15A-MIRN16-1* region on 13q14 (12 probes), *ATM* on 11q23 (seven probes), *PTEN* on 10q23 (two probes), *MYCN* on 2p24 (three probes), the 8q24 *MYC* region (three probes), the 6q25–26 region (three probes), *CDKN2A* and *CDKN2B* on 9p21 (two probes) as well as the presence of trisomy 12 (10 probes) and trisomy 19 (five probes). Briefly, DNA (200 ng) was annealed overnight at 60°C to the MLPA probe mix. Annealed oligonucleotides were ligated by adding Ligase-65 (MRC-Holland, Amsterdam), and incubated at 54°C for 15 min. Ligation products were amplified by PCR (35 cycles, 30 s at 95°C, 30 s at 60°C and 1 min at 72°C) with one unlabeled and one 6-carboxyfluorescein (FAM)-labeled primer. The final PCR fragments were separated by capillary electrophoresis on a 48-capillary ABI-Prism 3730 Genetic Analyzer (Applied Biosystems/Hitachi, Foster City, CA). Peak area and height were measured using GeneScan analysis software (Applied Biosystems). Data were analyzed with Coffalyser Software (MRC-Holland) using DNA from six healthy donors as controls, and the relative copy number (RCN) values were obtained (Slater *et al*, 2004). During the set up of the method 10 samples were analyzed twice independently and the RCN values obtained were very similar (data not shown).

To compare the results obtained with MLPA and FISH, RCN values were calculated for each technique. For MLPA, RCN values corresponded to values obtained after normalization of peaks with controls. Values between 0·8 and 1·2 were

considered normal. Values below 0·8 or above 1·2 indicated an altered signal, and biallelic deletions should result in RCN values below 0·5 for those probes corresponding to the double deleted region. For each region, mean RCN values from altered peaks were calculated. Altered genomic regions had at least two consecutive probes outside the normal range. For FISH, RCN values were obtained from percentages of nuclei showing the alteration. RCN values of 1 represented two copies of the target sequence in the sample. The RCN values for the six samples from healthy donors were always between 0·8 and 1·2.

Results

Chromosomal alterations detected by MLPA and FISH in CLL

Fifty samples from CLL patients were analyzed for chromosomal abnormalities by FISH and by MLPA with SALSA P037 and SALSA P038 kits (Table I). Both MLPA kits contain probes for regions commonly studied by FISH in CLL, i.e. deletions in 13q14, *ATM*, and *TP53*, and trisomy 12. These kits also contain probes for other regions, such as 2p24, 6q25–26, 8q24, 9p21, 10q23 and chromosome 19, which may also be involved in the clinical course of the disease (Dohner *et al*, 2000; Novak *et al*, 2002; Stilgenbauer *et al*, 2002; Schwaenen *et al*, 2004). For each sample, results from both kits were combined and arranged in figures to represent the order in the chromosome (Fig 1).

Samples with less than 80% of lymphocytes in whole blood were purified, as detailed in Materials and Methods. This diminished the interfering effect from non-leukaemia cells, and the results became clearer after purification (Fig S1). Some cases with low percentages of cells carrying the alteration were detected by MLPA (Patients 3, 26, 27, 32, and 34) (Table I).

Relative copy number values for MLPA and FISH results were calculated, as detailed in Materials and Methods. A total of 54 alterations for 13q14, *ATM*, *TP53*, or chromosome 12, were detected using both techniques (Table I). MLPA and FISH data were in agreement in 89% of cases (48/54), and the failure rate was 5·6% (3/54) for both MLPA and FISH. As shown in Table I and summarized in Table SII, detection of alterations was similar using either MLPA or FISH, with the exception of the six cases detailed below.

Deletions detected in 13q14, ATM and TP53

Deletions in 13q14 were detected by MLPA in 30 of the 32 cases detected by FISH (Table I and summarized in Table II). The only cases that MLPA did not detect were Patients 21 and 44, with a percentage of cells carrying the deletion of only 20% and 16%, respectively; these cases were detected by FISH. However, in Patient 21, some consecutive probes gave slightly lower signals (RCN values of 0·86, 0·80, 0·80, and 0·86), suggesting the presence of the alteration. MLPA detected biallelic deletions in 13q14 also detected by FISH in

Table I. Alterations detected by MLPA or FISH in CLL.

		FISH		MLPA
Patient	Alteration	%	RCN	RCN
1	13q-	83	0·59	0·60
2	13q-	53	0·74	0·73
	17p-	86	0·57	0·58
3	11q-	15	0·93	0·75
	13q-	68	0·66	0·70
4	11q-	90	0·55	0·57
	13q-	0; 95*	0·05	0·05†
5	13q-	92	0·54	0·59
6	11q-	98	0·51	0·52
	13q-	100	0·50	0·51
7	normal			
8	11q-	87	0·52	0·55
	13q-	90	0·55	0·55
9	13q-	14; 17*	0·76	0·25†
10	17p-	43	0·79	0·68
11	13q-	55	0·73	0·67
12	11q-	75	0·63	0·49
	13q-	70; 10*	0·55	0·42†
13	6q-	NA		0·58
14	13q-	28	0·86	0·62
15	normal			
16	13q-	90	0·55	0·06†
17	normal			
18	13q-	38	0·81	0·52
19	13q-	57; 33*	0·39	0·04†
	+12‡	0	1	1·34
20	11q-‡	0	1	0·64
	+12	21	1·11	1·23
21	11q-‡	0	1	0·57
	13q-‡	20	0·90	0·86
	+8q	NA		1·34
22	13q-	9; 6*	0·90	0·58
23	normal			
24	normal			
25	+12	20	1·10	1·59
26	13q-	7	0·97	0·77
27	11q-	10	0·95	0·52
	13q-	18	0·91	0·76
	+8q	NA		1·44
28	normal			
29	17p-‡	24	0·88	0·86
30	11q-	75	0·63	0·57
31	13q-	63	0·69	0·70
32	13q-	14	0·93	0·74
33	13q-	59	0·71	0·69
34	13q-	17	0·92	0·72
	17p-	25	0·88	0·74
35	11q-	73	0·64	0·64
	+12	73	1·37	1·39
36	13q-	60	0·70	0·65
37	13q-	54; 21*	0·52	0·42†
38	13q-	75	0·66	0·56
	17p-	61	0·68	0·59
39	13q-	44	0·78	0·66

Table I. (Continued).

		FISH		MLPA
Patient	Alteration	%	RCN	RCN
40	17p-	94	0·53	0·57
41	13q-	97	0·52	0·20†
42	normal			
43	normal			
44	13q-‡	16	0·92	0·99
45	13q-	56	0·72	0·48
	17p-	34	0·83	0·79
46	13q-	56	0·72	0·69
47	+12	NA§		1·63
48	13q-	97	0·52	0·68
49	normal			
50	13q-	11; 11*	0·87	0·73

FISH RCN values were calculated from percentages of altered cells, as explained in Materials and Methods: values different from 1 indicate the proportion of allelic alteration detected (RCN > 1 indicates amplification; RCN < 1 indicates deletion). MLPA RCN values correspond to mean values from altered signal probes. Cut-off levels of 0·8 and 1·2 were used (RCN > 1·2 indicates amplification; RCN < 0·8 indicates deletion). When biallelic deletions are present, the percentages of cells carrying monoallelic and biallelic alterations are shown.
*Percentages of cells carrying the biallelic alteration.
†Mean RCN values were calculated from probes corresponding to the double deleted region.
‡Discordant cases.
§FISH failed and the alteration was confirmed by karyotype.
NA, not available.

five cases (Patients 4, 9, 12, 19, and 37). Discordant cases were found in four patients: (a) in Patients 16 and 41, a biallelic deletion was detected by MLPA, but not by FISH; and (b) in two patients the biallelic deletion was detected by FISH, but not by MLPA: Patient 22 (with only 9% and 6% of cells with monoallelic and biallelic alteration, respectively) and Patient 50 (with only 11% and 11% of cells with monoallelic and biallelic alteration, respectively). MLPA also mapped the region by using several small probes instead of one large one. Figure 2 shows four different cases: (a) Patient 23 with normal 13q status; (b) Patient 18 with 38% of cells with monoallelic 13q14 deletion (detected by FISH); (c) Patient 19 with 57% of cells with monoallelic and 33% with biallelic 13q14 deletion (detected by FISH); and (d) in Patient 16, MLPA detected a small biallelic deletion which was not detected by FISH.

Deletions in *ATM* were detected by MLPA in 10 cases (Table I and summarized in Table II). MLPA detected the deletions in *ATM* that were detected by FISH (Patients 3, 4, 6, 8, 12, 27, 30, and 35). Moreover, MLPA detected deletions that were not detected by FISH in two cases of *ATM* deletion (Patients 20 and 21). Mean RCN values for the altered probes were 0·64 and 0·57 for Patients 20 and 21, respectively. Figure 3 shows the pattern of altered MLPA probes suggesting a small or intragenic deletion.

Deletions in *TP53* were detected by MLPA in six of the seven cases detected by FISH (Table I and summarized in Table II). MLPA could not detect a deletion in *TP53* in one case (Patient 29) with only 24% of cells carrying the deletion, which was detected by FISH. However, in this case, some consecutive probes gave slightly lower signals (RCN values of 0·85, 0·78, and 0·81), suggesting the presence of the alteration.

Amplifications detected in chromosome 12

Amplifications in chromosome 12 were detected by MLPA in five cases (Table I and summarized in Table II). MLPA

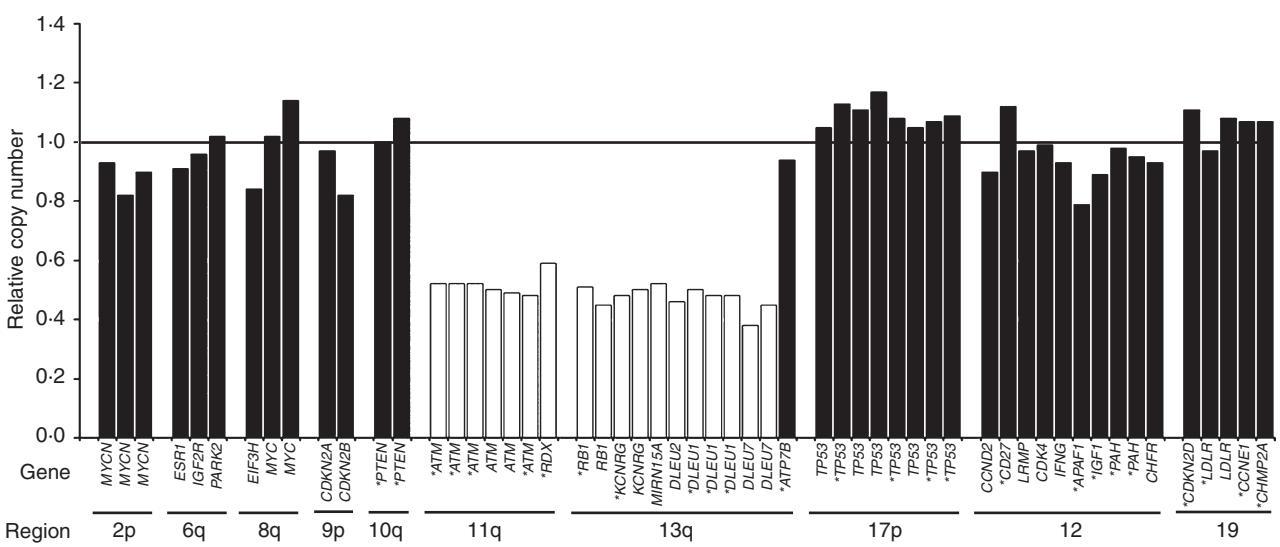

Fig 1. Histogram of RCN for CLL-specific targets. MLPA was performed on DNA from Patient 6. White bars indicate altered sequences from genes in the deleted region. Black bars indicate unaltered sequences. CLL MLPA P037 and P038 (marked with *) were used and data were combined so that consecutive probes in the chromosome are shown contiguously in the figure.

Table II. Frequency of genomic alterations detected for each gene and technique.

Genomic alteration	Total cases (%)*	FISH cases (%)	MLPA cases (%)
11q-	10 (19)	8 (15)	10 (19)
13q-	32 (59)	32 (59)	30 (56)
17p-	7 (13)	7 (13)	6 (11)
+12	5 (9)	4† (7)	5 (9)
6q	1	NA	1
8q	2	NA	2

*Total cases include cases detected by FISH or by MLPA.
†FISH failed in one +12 detection, which was confirmed by karyotype.
NA, not applicable.

detected the amplifications in chromosome 12 that were detected by FISH or karyotype (Patients 20, 25, 35 and 47). Moreover, MLPA detected amplifications that were not detected by FISH in Patient 19, since chromosome 12 alterations were not restricted to trisomies. Some of the probes used by MLPA for chromosome 12 detection were distant from the centromere, and amplifications that did not involve the centromeric region were detected; this was the case in Patient 19 with a 12q arm amplification (Fig 4A). As FISH used a centromeric probe it was unable to detect this amplification, which was confirmed by karyotype, showing a der(6)t(6;12)(q25;q12) (Fig 4B). Similar amplifications in chromosome 12 have been described elsewhere (Dierlamm *et al*, 1997; Merup *et al*, 1997; Chena *et al*, 2000). Interestingly, the four samples with amplified chromosome 12 presented some probes (CDK4, IFNG, APAF1, and IGF1)

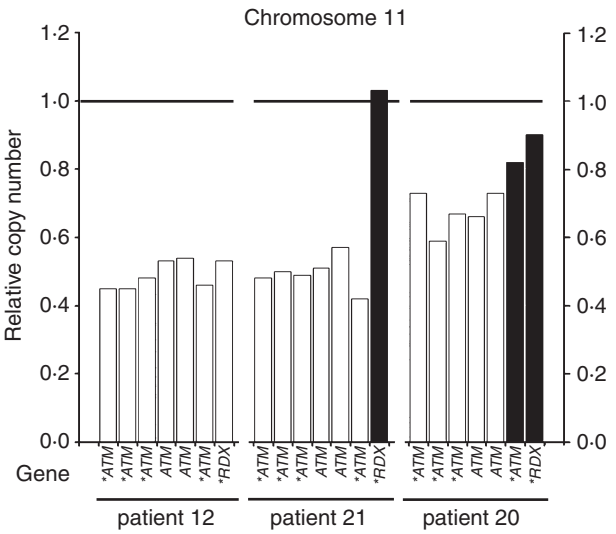

Fig 3. *ATM* alterations analyzed by MLPA in CLL. Histogram of RCN for 11q23 alterations in three different patients (Patients 12, 21, and 20), showing deletion in the *ATM* region. White bars indicate altered sequences. *Indicates probes contained in P038 kit.

which had lower RCN values than the rest of the probes (Fig 4A and data not shown).

6q and 8q alterations

Furthermore, MLPA detected deletions in 6q25–26 and amplifications in 8q24 (*MYC*), which were not included in the FISH kits used. In these cases, three consecutive probes showed altered signals: RCN mean values were 0·58 for 6q25–

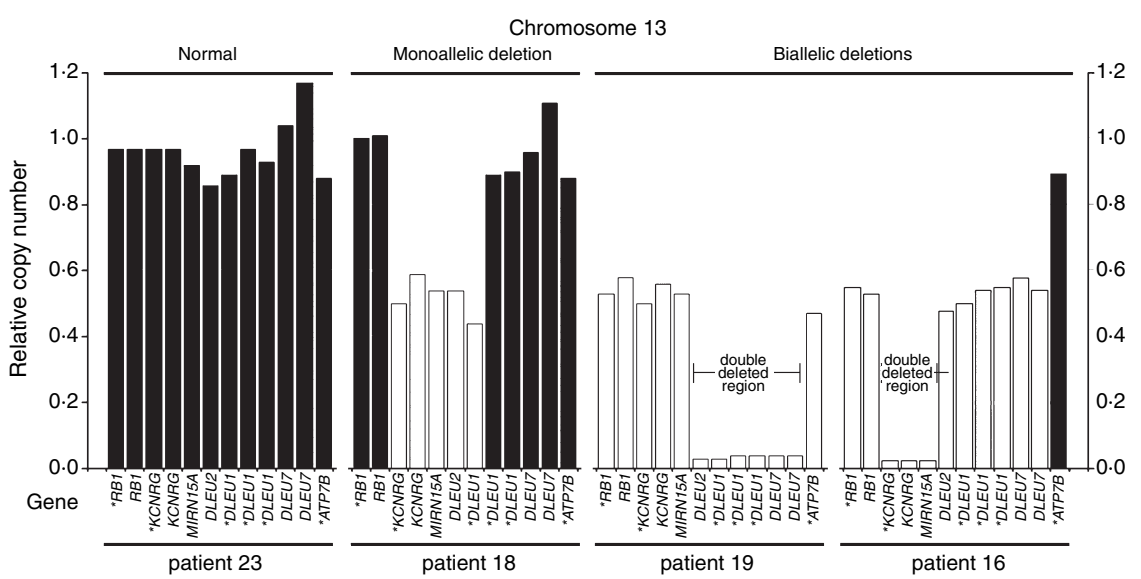

Fig 2. 13q14 alterations analyzed by MLPA in CLL. Histogram of RCN for 13q14 alterations in four different patients (Patients 23, 18, 19, and 16), showing different states of deletion. White bars indicate altered sequences. *Indicates probes contained in P038 kit.

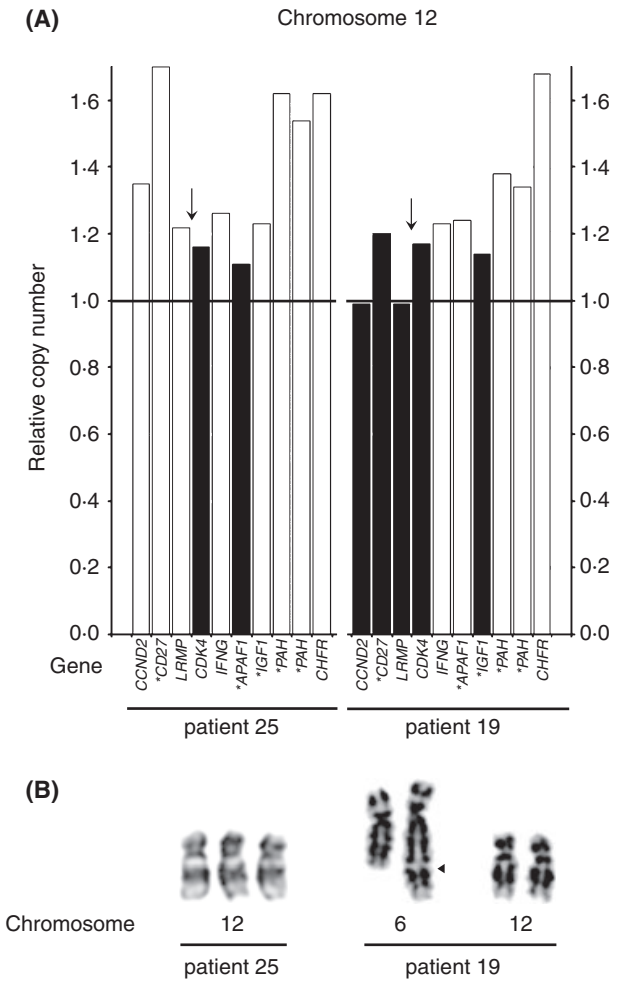

Fig 4. Chromosome 12 alterations analyzed by MLPA in CLL. (A) Histogram of RCN for chromosome 12 alterations in two different patients (Patients 25 and 19) showing different states of amplification. The arrow indicates the region where the centromere is located. White bars indicate altered sequences. *Indicates probes contained in P038 kit. (B) Karyotype images: left (Patient 25), chromosome 12 trisomy; right (Patient 19), der(6)t(6;12)(q25;q12).

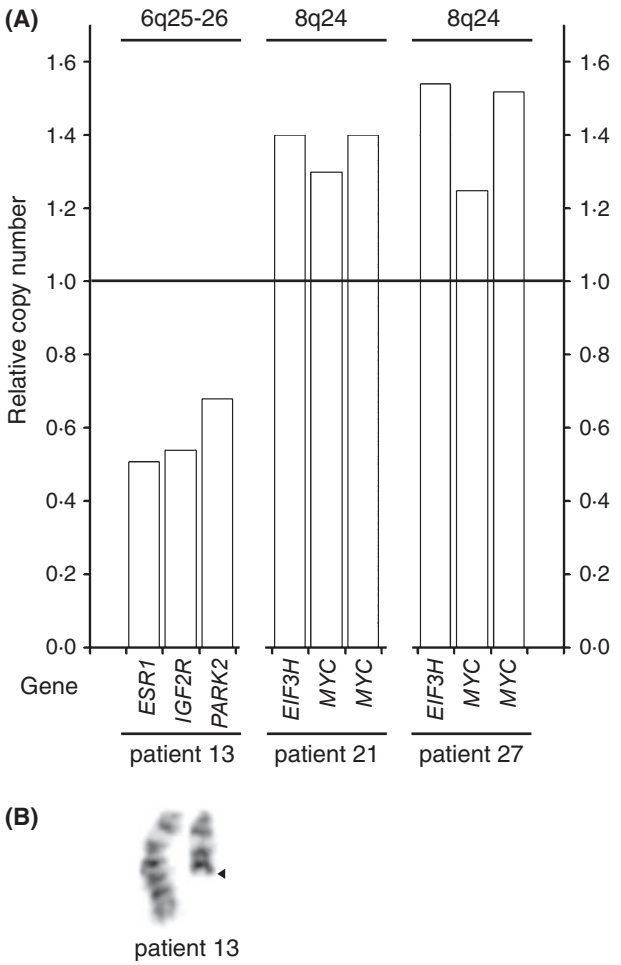

Fig 5. Detection of 6q25–26 and 8q24 alterations by MLPA in CLL. (A) Histogram of RCN for 6q or 8q alterations on three different patients (Patients 13, 21, and 27) showing deletion or amplification of the corresponding region. White bars indicate altered sequences. *Indicates probes contained in P038 kit. (B) Karyotype image: 6q deletion (Patient 13).

26 deletion (Patient 13), and 1·34 and 1·44 for 8q24 amplification (Patients 21 and 27, respectively) (Table I and Fig 5A). Deletions in 6q25–26 were also detected in the karyotype (Fig 5B).

Discussion

This report described the use of MLPA to detect genomic alterations in CLL DNA samples. Cytogenetic aberrations detected by interphase FISH have been shown to be independent prognostic factors in CLL (Dohner *et al*, 2000). However, current FISH analysis underestimates the complexity and heterogeneity of chromosomal aberrations, because (a) it only detects deletions or amplifications of fragments of DNA larger than 20–50 kb (Dohner *et al*, 1999), and (b) in clinical practice a small number of probes (usually four) are used, due to their high cost. The use of MLPA allows the analysis of more than 40 different small (~50 pb) DNA sequences in a single reaction.

There was a good correlation between the MLPA and FISH results, as most of the alterations were detected by both techniques. There were a few cases of discordance, which can be separated in to two groups. In the first group, the low percentages of cells carrying the alterations hindered MLPA detection. MLPA is restricted more than FISH by the percentage of the leukemic clone carrying the aberration of malignant B-cells. Only three cases, all with less than 25% of altered cells, determined by FISH, could not be identified as altered by MLPA. However, some consecutive probes in two of these cases had slightly lowered RCN values, suggesting a low percentage of cells carrying the alteration. Surprisingly, MLPA detected alterations in samples with apparently low percentages of cells carrying the alterations detected by FISH. This

may have been due to the possible contamination of the FISH sample with non-leukemic cells. When both techniques were used with high purity B cells, the correlation of the values was enhanced in most of the samples tested.

In the second group, FISH was not able to detect certain specific alterations that were detected by MLPA. The use of multiple probes permitted the identification of intragenic or small alterations not detected by FISH. First, double deleted regions were identified at the 13q14 locus. MLPA mapped the double deleted region at the 13q14 locus more precisely than FISH, allowing better classification of risk stratification groups. Second, intragenic deletions were identified in *ATM* in the 11q region. The sensitivity of FISH and MLPA is affected by the size of the probes. Short unique sequence oligonucleotides are much less likely to give false-negative results than the much larger hybridization probes. The FISH probe for 11q22–q23 region is a large probe of ~500 kb, and its resolution in the case of small deletions is impaired. As shown by the signal pattern of MLPA, the region deleted in both cases seemed to be too small for the FISH probe. Third, a partial trisomy was identified in chromosome 12. Interestingly, the probes for APAF1 showed no aberrant amplification in cases with trisomy 12 or amplification of the 12q arm. This could mean that there are alterations, mutations or even deletions in this gene in the new aberrant chromosome (or 12q arm). Though this point needs further investigation, it is conceivable that cancer cells eliminate this essential component of the apoptotic mitochondrial pathway (Yamamoto *et al*, 2000; Soengas *et al*, 2001; Sturm *et al*, 2006). Thus, the use of multiple probes for the identification of the alterations not only reduces the false positive and false negative results, but also shows MLPA to be a powerful tool for the identification of intragenic or small alterations.

Multiplex ligation-dependent probe amplification also detected less frequent alterations. In addition to the alterations commonly assessed by FISH kits, MLPA takes advantage of the simultaneous analysis of 55 genomic sequences and is able to assess alterations in other chromosomes. In our series, we found alterations in 6q25–26 and 8q24. The gene located at 6q25–26 that is involved in the pathogenesis of CLL is still unknown (Merup *et al*, 1998). *MYC* is located at 8q24 and alterations in *MYC* in CLL have been previously reported (Rimokh *et al*, 1991; Wang *et al*, 1991; Vallat *et al*, 2003; Kienle *et al*, 2005). Moreover, high expression of *MYC* has been associated with resistance to DNA-damage induced apoptosis in CLL (Vallat *et al*, 2003).

Multiplex ligation-dependent probe amplification can detect alterations detected by FISH and other less frequent alterations as well. It can also detect small alterations and map the abnormal region, or even the gene, due to the use of various small (~50 bp) probes. This enhanced resolution and the capability for mapping the extent of the duplication or deletion makes MLPA a particularly useful technique in genetic analysis. Moreover, archived DNA samples can be used for MLPA analysis, expanding the scope of this technique to include rapid retrospective diagnostic analyses. MLPA kits can be enhanced by including new probes as new genes involved in the disease are discovered. Together, these features overcome some of the limitations of FISH analysis, allowing small alterations, and even specific altered regions, to play a role in the risk stratification assessment and pathogenesis studies. Another MLPA kit (SALSA P006) containing probes for some tumor-associated genes, including *RB1*, *TP53* and *ATM*, was tested in CLL (Buijs *et al*, 2006). Though not focused on genes that are altered in CLL, it was reported to be useful in the genomic profiling of CLL. However, as this kit uses a low number of probes for the detection of some alterations, it is unlikely to map and detect small or intragenic alterations. Furthermore, as deletions in 13q14 do not necessary involve *RB1*, the percentage of samples with 13q14 deletion was underestimated. Recently, quantitative multiplex PCR of short fluorescent fragment (QMPSF) has been compared with FISH showing a good correlation (Bastard *et al*, 2007). In this study, only one or two probes were used to detect each alteration, thus making underestimation of alterations more probable. Our data demonstrate the advantage of using several probes for the characterization of the key regions in CLL. Although this study used both kits (P037 and P038), either of them is sufficient to analyze the four regions (13q14, *ATM*, *TP53*, and chromosome 12) routinely screened by FISH in CLL, as these regions are covered by several probes in both kits.

In summary, MLPA permits simultaneous analysis of many samples with automated data processing at a low cost per sample. Therefore, the combination of robust multiplexing and high throughput makes MLPA a useful technique for analysis of genomic alterations in CLL.

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Competing interests statement

The authors declare that they have no competing financial interests, neither have received financial support from MRC Holland.

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Supplementary material

The following supplementary material is available for this article online:

Fig S1. MLPA detection following purification of $CD19^+$ CLL cells. Histograms correspond to mononuclear cells isolated from peripheral blood samples before (left) and after (right) $CD19^+$ purification from patients 9 and 11. Although results from non-purified cells showed a shift in some contiguous probes, detection was clearer when using purified (>90%) $CD19^+$ cells.

Table SI. Characteristics of CLL Patients.

Table SII. Alterations detected by MLPA and FISH.

The material is available as part of the online article from:

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