RESEARCH ARTICLE

# Multiplex Ligation-Dependent Probe Amplification and Fluorescence In Situ Hybridization to Detect Chromosomal Abnormalities in Chronic Lymphocytic Leukemia: A Comparative Study

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Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease characterized by recurrent chromosomal aberrations of prognostic significance. We aimed to evaluate the potential of the multiplex ligation-dependent probe amplification (MLPA) assay to detect genomic alterations in CLL. Highly purified (>90%) peripheral mononuclear CD19+ cell populations from 100 untreated CLL patients (pts) in early stage disease (Binet stage A) were included in this study. All samples were investigated by fluorescence in situ hybridization (FISH) for the presence of trisomy 12 and 17p13.1, 11q22.3, and 13q14.3 deletions. For MPLA analysis, DNA was amplified by means of two commercially available probes sets allowing the simultaneous screening of 56 genomic sequences. Overall, a high degree of concordance (95%) between MPLA and FISH results was found, if the abnormal clone was present in more than 30% of the leukemic cell population. The use of multiple MPLA probes allowed the fine-mapping of the 13q14 deletion and the identification of intragenic or small alterations undetected by FISH. Moreover, additional alterations in 2p24 (*MYCN*) (3 pts), 8q24 (*MYC*) (1 pt), 9p21 (*CDKN2A2B*) (1 pt), 1q21 (*LMNA*) (1 pt), and 6q25-26 (1 pt) regions not covered by a standard FISH assay were detected and all confirmed by FISH. Our data extend previously limited evidence that MLPA may represent a useful technique for the characterization of well-known lesions as well as the investigation of additional genomic changes in CLL. 

## INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a genetically heterogeneous disease with a variable clinical course ranging from months to decades (Chiorazzi et al., 2005; Dighiero and Hamblin, 2008). The prognostic impact of several biological risk factors, including mutation status of the heavy chain variable regions of immunoglobulin genes ($IgV_H$) (Hamblin et al., 1999), the expression of the 70-kDa zeta-chain T-cell receptor-associated protein kinase (ZAP-70), and CD38 cell surface antigen (Damle et al., 1999; Wiestner et al., 2003) has been intensively evaluated. However, these markers alone do not identify all patients with a high-risk profile. Genomic aberrations have been identified by fluorescence in situ

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hybridization (FISH) in about 80% of CLLs; the most frequent include deletions in 13q14 (50–60%) followed by trisomy 12 (15–25%), 11q22.3 (*ATM*) (10–20%), and 17p13 (*TP53*) (5–10%) deletions (Döhner et al., 2000; Seiler et al., 2006; Hallek et al., 2008). A hierarchical association between these cytogenetic abnormalities and the clinical course of the disease has been demonstrated: 17p13 and 11q23 deletions are associated with the shortest overall survival, followed by trisomy 12 and the normal karyotype, whereas 13q14 deletion as the sole abnormality is associated with the best prognosis (Juliusson et al., 1990; Döhner et al., 2000; Dewald et al., 2003; Reddy, 2006; Seiler et al., 2006). Although FISH is the most common technique used to detect these abnormalities (Döhner et al., 2000), it is well known that it cannot identify smaller alterations detectable by more complex technologies such as high-resolution CGH or SNP arrays (Pfeifer et al., 2007; Kujawski et al., 2008; Grubor et al., 2009). Recently, a new method, called multiplex ligation-dependent probe amplification (MLPA), has been developed for the detection of DNA copy number changes (Schouten et al., 2002). MLPA is a polymerase chain reaction (PCR)-based technique that can quantify up to 45 different genomic targets simultaneously in a single experiment through amplification of specific hybridizing probes. Notably, MLPA is a fast, relatively inexpensive and easy method allowing a large numbers of samples to be processed simultaneously. Since its development, it has proved to be accurate and reliable for the detection of deletions, duplications, and amplifications in several types of cancers (Koolen et al., 2004; Buijs et al., 2006; Jeuken et al., 2006; Scarciolla et al., 2006, 2007) also including CLL (Coll-Mulet et al., 2008; Stevens-Kroef et al., 2009; Abdool et al., 2010; Al Zaabi et al., 2010). Concerning CLL, a few studies have been reported thus far but additional investigations are required to validate the sensitivity and specificity of the MLPA approach in a large series of highly purified CLL patients.

In this study, we compare MLPA and FISH as methods to investigate prognostic cytogenetic markers in highly purified (>90%) peripheral mononuclear CD19+ cells from a panel of 100 untreated CLLs in early stage disease (Binet stage A). As the commercially available MLPA probe sets specifically used for CLL analysis allow the simultaneous investigation of 56 genomic sequences, we applied the MLPA method to search for additional copy number alterations in genes not commonly assessed by FISH. Furthermore, any additional chromosomal alteration, detected by MLPA, was validated by FISH, using a panel of specific BAC clones. Finally, the use of multiple probes for the detection of alterations allowed us to map the extent of genomic lesions, in particular the 13q14 deletion.

## MATERIALS AND METHODS

### Patients and Material

Highly purified (>90%) peripheral mononuclear CD19+ cell populations from 20 healthy donors (10 males and 10 females; median age 34 years; range 27–38) and 100 CLL untreated patients in early stage disease (Binet stage A) were included in this study. The inclusion criteria were a diagnosis of typical CLL based on morphological and phenotypic analyses (i.e., the coexpression of CD19, CD5, and CD23 and weak SmIg, the monotypical expression of κ or λ light chains by neoplastic cells). All blood samples were collected within 1 year after diagnosis during diagnostic procedures from patients belonging to a database from a collaborative Italian study (Morabito et al., 2009). The study group included 63 males and 37 females; the mean and median ages at diagnosis were 63 and 64 years, respectively (range 29–86). In accordance with institutional guidelines, all patients provided their informed consent for the study. No conventional cytogenetic (G-banding) analyses were available.

### Sample Preparation, Immunophenotype Determination, and Prognostic Markers

Peripheral blood mononuclear cells from CLL patients were isolated by Ficoll-Hypaque (Seromed, Biochrom KG, Berlin, Germany) density-gradient centrifugation. If CLL cells were <90%, T cells, NK cells, and monocytes were removed by CD3, CD56, CD16, and CD14 monoclonal antibody (mAb) treatment (Becton Dickinson, Sunnyvale, CA) followed by magnetic bead separation (Goat Anti-Mouse IgG Dynabeads, Dynal Biotech ASA, Oslo, Norway) (Cutrona et al., 2008). The proportion of CD5/CD19/CD23 triple positive B cells in the suspension was determined by direct immunofluorescence performed using a FACS-sort flow cytometer (BD Biosciences, San Josè, CA) with antibodies for CD19 FITC/PE, CD23 PE, and CD5 Cy-Chrome (BD Biosciences). CD38-

positive leukemic cells were measured by triple staining with CD19 FITC, CD38 PE, and CD5 Cy-Chrome (Becton Dickinson). The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) as previously described (Cutrona et al., 2008). ZAP-70 was determined by flow-cytometry with a ZAP-70 FITC (clone 2F3.2, Upstate, Lake Placid, NY) or an isotype control mAb (mouse IgG2a FITC Becton Dickinson) (Cutrona et al., 2008; Morabito et al., 2009). A 30% cutoff was used for both CD38 and ZAP-70 expression. IgVH gene usage and mutational status was determined as previously described and a cutoff of 2% was used to distinguish mutated and unmutated patients.

### Interphase FISH (I-FISH)

The 100 CLL patients were investigated by I-FISH for the most common genomic aberrations described in CLL, including trisomy 12 and deletions at the 17p13.1, 11q22.3, and 13q14.3 loci. All of the probes are commercially available (Vysis, Downers Grove, IL) (Fabris et al., 2008). The cutoff points for positive values (mean +3 SDs) assessed on peripheral mononuclear cells from 10 control subjects were 3.4, 3.4, 1.7, and 3.8% for trisomy 12 and monosomies of *TP53*, *ATM*, and *D13S25*, respectively.

The validation of MLPA findings was performed using a panel of specific BAC clones selected using the University of California Santa Cruz (UCSC) Genome Browser Database (http://genome.ucsc.edu/). The panel of probes selected for the MLPA validation was tested on normal human metaphase cells to verify the absence of cross-hybridization. Two hundred interphase nuclei were analyzed for each probe set.

### MLPA Analysis

DNA was extracted by a commercial kit (Promega, Madison, WI) and analyzed by MLPA using two sets of probes SALSA MLPA kit P037 and P038 (MRC Holland Amsterdam, The Netherlands). As described in Supporting Information Table S1, both MLPA kits contain 37 target sequence probes for regions commonly studied by FISH in CLL, that is, deletions in 13q14, 11q22.3, and 17p13.1 and trisomy 12 and 19 probes specific of regions reported to be involved in the disease: that is, 2p24, 6q25-26, 8q24, 9p21, 10q23, and chromosome 19. Twenty-four control probes located in genomic regions not involved in CLL disease are also included. The reaction phases, including hybridization, ligation, and PCR amplification, were performed according to the manufacturer's instructions on the Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA). In each experiment, at least one healthy blood donor was included as control. Briefly, for hybridization, 5 μl of genomic DNA (150–250 ng) was denatured at 98°C for 5 min, followed by addition of the probe mix. Samples were incubated at 95°C for 1 min and then annealed for 16–18 hr at 60°C. In the ligation phase, the annealed oligonucleotides were incubated with Ligase-65 (MRC-Holland) at 54°C for 15 min. In the third step, ligation products were amplified by PCR (35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec). Finally, samples were electrophoresed using an ABI 3130 DNA analyzer (Applied Biosystems), with Liz 500 size standard and GeneMapper ID software v3.2 (Applied Biosystems).

MLPA results were first visually analyzed and failed samples were discarded and reanalyzed as specified in the manufacturer's instructions. Results of peak areas were exported from GeneMapper to an Excel spreadsheet (Microsoft, www.microsoft.com). The relative peak area (RPA) of each probe was obtained by dividing the single peak area by the sum of peak areas of all internal control probes. The probe RPA ratio (RPR) was generated dividing each RPA value to the mean value of corresponding RPAs from the 20 healthy individual samples. To establish the presence of loss/gain we set the RPR value for disomic locus between 0.8 and 1.2, and for a gain, monoallelic deletion, or biallelic deletion between 1.3 and 1.5, 0.45 and 0.65, or $<0.4$, respectively. When the RPR values resulted different from the above criteria, the analysis was considered invalid and was repeated.

## RESULTS

### Biological and Molecular Features of CLL Patients Included in the Study

The chromosomal alterations detected by FISH and MLPA as well as the main biological characteristics of the 100 CLL patients included in the study are shown in Supporting Information Table S2. ZAP-70, CD38, and $IgV_H$ mutational analyses were performed in 98 cases. Forty-nine patients had unmutated $IgV_H$ genes; ZAP-70 and CD38 were positive in 41 and 39 cases, respectively.

TABLE 1. Frequency of Recurring Cytogenetic Abnormalities Detected by FISH in CLL Patients

| I-FISH analyses | Frequency |
|---|---|
| Normal | 19 |
| Abnormal | 81 |
| Isolated abnormalities: | 68 |
| del(11)(q22.3) | 8 |
| del13qx1 (13qx2) (13qx2/13qx1) | 29 (6) (2) |
| del13qx1 (*RB1*) | 1 |
| del13qx1 (*RB1*: 13qx2) | 1 |
| del(17)(p13) | 2 |
| +12 | 19 |
| Multiple cytogenetic abnormalities: | 13 |
| del13qx1; del(11)(q22.3) | 6 |
| del13qx1; del(17)(p13) | 4 |
| del13qx2; del(17)(p13) | 1 |
| del13qx1; del(17)(p13); del(11)(q22.3) | 1 |
| del13qx1; +12 | 1 |

Note: 13qx1, monoallelic deletion; 13qx2, biallelic deletion; 13qx2/13qx1, monoallelic and biallelic deletions.

TABLE 2. Frequency of Recurring Cytogenetic Abnormalities Detected by MLPA in CLL Patients

| MLPA | Frequency |
|---|---|
| Normal | 21 |
| Abnormal | 79 |
| Isolated abnormalities: | 66 |
| del(11)(q22.3) | 5 |
| 13qx1 (13qx2) | 24 (12) |
| 13qx1 (*RB1*:13qx2) | 1 |
| 13qx1 (*RB1*) | 1 |
| del(17)(p13) | 3 |
| +12 | 19 |
| dup(2)(p24) | 1 |
| Multiple cytogenetic abnormalities: | 13 |
| del(11)(q22.3); del13qx1 | 5 |
| del(11)(q22.3); del13qx1; del(17)(p13) | 1 |
| del(11)(q22.3); del(6)(q25-26) | 1 |
| del(11)(q22.3); dup(2)(p24) | 1 |
| del13qx1; del(17)(p13); dup(2)(p24) | 1 |
| del (9)(p21); dup(8)(q24) | 1 |
| del13qx1; del(17)(p13) | 1 |
| del13q14(*RB1*); del(1)(q21) | 1 |
| del13qx1; +12 | 1 |

Note: 13qx1, monoallelic deletion; 13qx2, biallelic deletion.

### Chromosomal Aberrations Detected by MLPA and FISH

Concerning the investigated FISH panel probes, at least one abnormality was found in 81/100 cases. The most recurrent FISH abnormality was del(13)(q14), detected in 52 cases followed by trisomy 12 (20 pts), del(11)(q22.3) (15 pts), and del(17)(p13) (8 pts). The 13q14 deletion was found as a sole abnormality in 39 patients; in the remaining cases, it was combined with trisomy 12 (one pt), 17p13 (6 pts), or 11q22.3 deletions (7 pts). The 13q deletion was found as a monoallelic deletion (13qx1) in 43 cases, whereas the presence of biallelic deletion (13qx2) was detected in 9 cases; two of these showed concomitant monoallelic deletion (13qx1/13qx2) (Table 1 and Supporting Information Table S2). All 13q14 deleted CLLs were also hybridized using the *RB1* specific clone RP11-305D15 (data not shown). FISH analysis documented a loss of the *RB1* gene in 25/52 (48%) cases with 13q14 deletion. Monoallelic loss of the *RB1* was detected in 23 cases and associated in two patients (#60 and #97) with a biallelic deletion of the 13q14 FISH probe (*D13S25*). The remaining two samples (#25 and #46) showed a *RB1* biallelic losses associated, respectively, with a biallelic or monoallelic deletion of the *D13S25* probe.

To evaluate the potential of the MLPA approach for the identification of prognostically relevant abnormalities in CLL, we compared MLPA to FISH data from our panel. Deletions of 13q14, 17p13, and 11q23 were detected by MLPA in 48/52, 6/8, and 13/15 cases detected by FISH, respectively, whereas trisomy 12 was observed by MLPA in all positive FISH cases (Table 2). In all of the seven cases with nonoverlapping results, the most valid explanation is the low percentage of leukemic cells carrying the lesion: in fact patients #95 and #96 harbored the 11q23 deletion in 20% and 21% of cells, respectively; patients #97 and #99 showed the 17p13 deletion in 25% and 28% of cells. Instead, the four patients (#81, #82, #85, and #99) with 13q14 deletion carried the lesion in 25, 21, 27, and 27% of cells, respectively (Table 3). Furthermore, the MLPA approach provided additional information, detecting the 13q14 alteration in five patients (#41, #89, #94, #96, and #98; Supporting Information Tables S3 and S4). Specifically, MLPA analysis revealed a partial 13qx2 deletion in three cases (#89, #96, and #98) with a monoallelic pattern by FISH and in two cases (#41 and #94) having a mixed 13qx1/13qx2 pattern detected by FISH.

### Characterization of the 13q Deletion by MLPA Approach

The use of multiple probes makes MLPA a useful tool for the mapping of the abnormal regions as well as for the identification of intragenic or small alterations. Concerning the 13q14 region, the MLPA approach showed that 13q deletions varied significantly in size, ranging from a minimum of 82 Kb (#80) to a maximum of 3.6

Mb (#8, #19, #33, #42, #44, #60, #61, #62, #78, #86, and #98; Fig. 1 and Supporting Information Tables S3 and S4). In 26 of the 48 cases showing 13q14 deletion detected by MLPA, the *RB1* gene was included in the affected segment. Two of the 26 samples showed a biallelic loss of the *RB1* locus by MPLA characterized by a partial homozygous deletion (#25) or a small intragenic *RB1* deletion on the second allele (#46) (Supporting Information Table S4). All these findings were confirmed by FISH, with the exception of patients #46 and #23 who showed small monoallelic deletions only involving one of the MLPA *RB1* probes (kit P037) located telomerically (37 Kb) to *RB1* FISH (Fig. 1).

With regards to the *miR-15a/16-1* cluster, we found a concomitant loss of one copy in 33 of the 35 patients with a 13q14 monoallelic deletion; the remaining two cases (#1 and #80) showed a 13q14 deletion telomeric or centromeric to the cluster, respectively. A loss of both copies of the miRNA cluster was found in 11 out of 13 patients with a biallelic 13q14 deletion: the remaining two patients showed a 13q14 deletion in the second allele located telomerically (#89) or centromerically (#46) to the cluster (Fig. 1 and Supporting Information Tables S3 and S4).

TABLE 3. Genomic Abnormalities Detected by I-FISH and not Identified by MLPA

| Patient | I-FISH (% of abnormal cells) | MLPA |
|---|---|---|
| #81 | del(17)(p13) (97) + del13q14x1 (25) | del(17)(p13) |
| #82 | del13qx1 (21) | normal |
| #85 | del13qx1 (27) | normal |
| #95 | del(11)(q22.3) (20) | normal |
| #96 | del(11)(q22.3) (21) + del13qx1 (96) | del13q14x2 |
| #97 | del(17)(p13) (25) + del13q14x2 (98) | del13q14x2 |
| #99 | del(17)(p13) (28) + del13q14x1 (27) | normal |

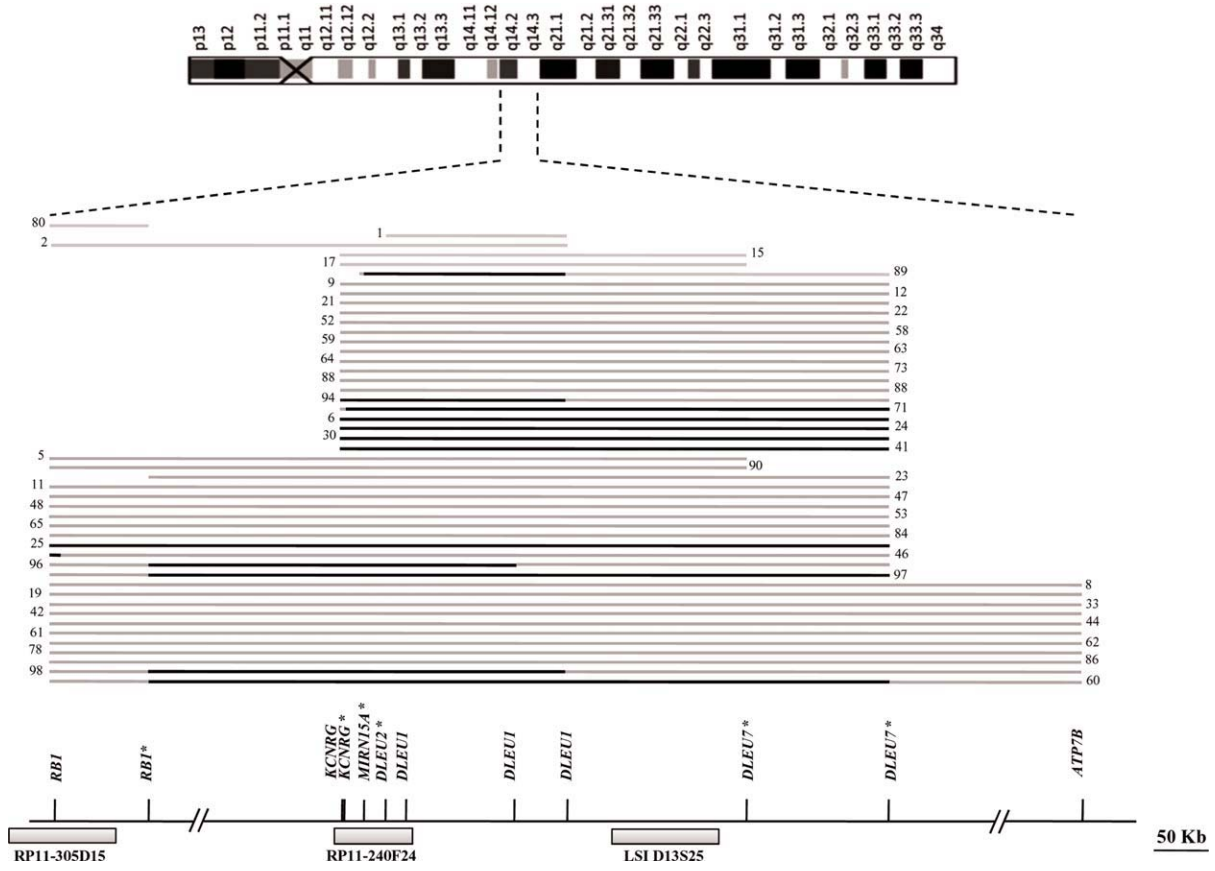


Figure 1. Chromosome 13 deletion pattern in 48 CLLs detected by MLPA analysis. 13qx1 and 13qx2 deletions are indicated by gray and black lines, respectively. The localization of 12 MLPA probes along with FISH probes are also displayed. * Indicates probes contained in the MLPA P037 kit.

### Identification of Additional Genomics Abnormalities by MPLA assay and FISH Validation

As the MLPA assay included probes targeting genomic aberrations not included in the FISH probe panel, additional gains or losses were detected using this approach (Table 4 and Fig. 2). Gains of 2p24 (*MYCN* locus) and 8q24 (*MYC* locus) were found in three (#13, #50, and #61) and one (#37) patient, respectively. In all of the gained 2p cases RPR mean values (1.3–1.5) obtained by three 2p24 consecutive probes (also including one or all of the 2p13-21 referenced probes) suggested a gain involving most of the short arm of chromosome 2: the same RPR mean values for only two 8q24 consecutive probes was found in case #37. We also detected a loss at 9p21 (*CDKN2A2B*), 1q21 (*LMNA*), and 6q25-26 regions in cases #37, #80, and #75, respectively. In these cases two, one or three consecutive probes, respectively, exhibited abnormal RPR mean values ranging between 0.45 and 0.65. No abnormalities involving 10q23 (*PTEN*), 18q21, or chromosome 19 were detected in our panel.

Genomic alterations detected by MLPA were further validated by FISH analysis using a panel of specific BAC clones corresponding to the MLPA probes having altered values. A co-hybridization using specific clones encompassing the altered locus and specific alpha-satellite as control probe for ploidy status was performed and MLPA data were confirmed in all samples. Specifically, RP11-480N14, RP11-440N18, CTD-2503E5, RP11-450E24, and RP11-35P22 BAC clones were used to investigate 2p24 and 8q24 gains, and 9p21, 6q25-26, and *LMNA* losses, respectively. Finally, a specific BAC clone RP11-240F24 was used to validate the occurrence of biallelic segments involving the *miR-15a/16-1* cluster and flanking monoallelic segments. MLPA data were confirmed in all of the six investigated samples (#60, #89, #94, #96, #97, and #98).

TABLE 4. Additional Abnormalities Detected by MLPA and Not Identified by I-FISH

| Patient | I-FISH (% of abnormal cells) | MLPA |
|---|---|---|
| #13 | normal | dup(2)(p24) |
| #37 | normal | del(9)(p21)+dup(8)(q24) |
| #50 | del(11)(q22.3) | del(11)(q22.3) + dup(2)(p24) |
| #61 | del13qx1 + del(17)(p13) | del13qx1 + del(17)(p13) + dup(2)(p24) |
| #75 | del(11)(q22.3) | del(11)(q22.3) + del(6)(q25-26) |
| #80 | del13q14(*RB1*) | del13q14(*RB1*) + del(1)(q21) |

## DISCUSSION

Chromosomal aberrations used for CLL risk stratification are commonly investigated by

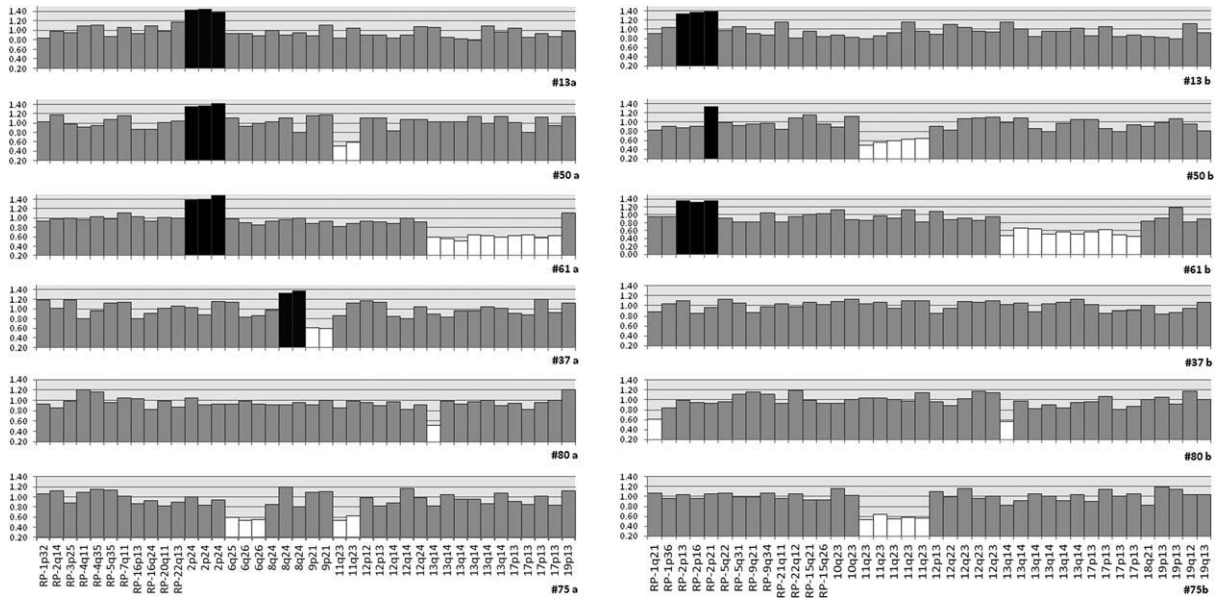


Figure 2. Histogram of relative peak ratio (RPR) values for CLL specific targets and reference probes. 2p24 (*MYCN*) and 8q24 (*MYC*) gains were found in three (#13, #50, and #61) and one (#37) sample, respectively. Case #37 also showed 9p21 (*CDKN2A2B*) deletion. Losses at 1q21 (*LMNA*) and 6q25-26 regions were detected in cases #80 and #75, respectively. White and black bars represent deleted and gained sequences, respectively. a and b indicate MLPA P037 and P038 kits, respectively.

I-FISH using individual commercial probes. Our study evaluates the use of MLPA, a quantitative PCR method, as a feasible alternative to FISH for the detection of cytogenetic aberrations. A series of 100 highly purified untreated CLLs in early stage disease (Binet stage A) were selected on the basis of the distribution of cytogenetic lesions as assessed by FISH and showing a proportion of cells carrying the alteration, ranging from 20 to 100%. The whole panel of patients was also investigated for the major described biological risk factors such IgVH gene mutational status and CD38 and ZAP-70 expression levels.

Overall, our data concerning the occurrence of common alterations (trisomy 12 and 13q14, 11q23, and 17p13 deletions) involved in CLL, showed a high degree of concordance between MPLA and FISH. In fact, nonoverlapping results between these two approaches were found in a relatively small number of cases, ~10% of our panel, which can be distinguished in two groups. In the first group, including seven patients, the low proportion of cells carrying the alteration, ranging from 21 to 28%, could explain the discrepancy. These cases were excluded from the reliability analyses since, these results are to be expected considering MLPA limitations in the identification of genomic alterations in pathological samples containing <50% of tumor cells. However, our data obtained from enriched neoplastic B cells allowed us to determine abnormalities even in DNA samples containing a percentage of tumor cells, which was significantly lower than the 50% sensitivity threshold (~30%). A similar reduced threshold was reported by Coll-Mulet et al., (2008) on a cohort of purified peripheral mononuclear CD19+ cells from 50 CLL cases investigated by MPLA. In contrast, a lower sensitivity was observed when DNA was extracted from whole blood, as described by Al Zaabi et al., (2010), although recently a protocol was developed to improve the MLPA assay in CLLs not subjected to B-cell purification (Abdool et al., 2010). In the second group, including five patients, MLPA provided additional results in the detection of 13q14 alterations. In three samples (#89, #96, and #98), MLPA identified partial 13q14×2 deletions as opposed to the 13qx1 deletions found by FISH. However, as the *D13S25* FISH probe maps between the most telomeric *DLEU1* and the most centromeirc *DLEU7* MLPA probes (Fig. 1), FISH was in agreement with the *DLEU1* and *DLEU7* RPR values found for these patients. Finally, the MPLA limitation in the investigation of a single cell may explain the failure to detect the concomitant 13qx1 and 13qx2 deletion pattern observed by FISH in the remaining two cases (#41 and #94).

Based on the limited number of probes currently used by FISH in diagnostic procedures, the extension of genomic aberrations is not generally investigated. The availability of multiple probes in the MLPA assay not only may help to identify small alterations, thus reducing false negative results, but it may also provide information about the size of the lesions. In particular, with the MPLA assay we were able to characterize better the extension of the 13q14 deletion that is known to be heterogeneous in size, as we and others previously reported using SNP arrays (Ouillette et al., 2008; Mosca et al., 2010). Of the 48 CLLs with 13q deletion detected by MLPA, 26 showed *RB1* deletion. *RB1* is a critical regulator of cell cycle progression and genomic stability, and its mono or biallelic loss may contribute to leukemogenesis in CLL (Hernando et al., 2004). Among these patients, MLPA analysis identified in particular, two cases with small *RB1* single intragenic deletions not identified by FISH and a case showing a *RB1* monoallelic deletion. Our approach also provided information concerning the genomic status of the *miR-15a/16-1* locus located at 13q14 right upstream of the FISH probe. Previous reports showed a down-regulation of the miRNAcluster in patients with 13q14 deletion suggesting its potential pathogenetic role in the disease (Calin et al., 2002, 2005). Nevertheless, in line with what others and we have recently reported using SNP arrays, there are a few CLL cases in which 13q14 deletions were not associated with the deletion of the *miR-15a/16-1* locus (Fulci et al., 2007; Ouillette et al., 2008). In our MPLA analysis, a monoallelic 13q14 deletion not involving the cluster was found in two cases, whereas the retention of one copy of the cluster was detected in two additional cases showing a biallelic 13q14 deletion.

MLPA allowed the identification of additional genetic alterations in CLL that are not included in the standard FISH analyses. In particular, we described gains of 2p involving the *MYCN* gene (3% in our series) that is in agreement with previous data (Pfeifer et al., 2007). Although a higher frequency observed in CLL patients in more advanced stages has been reported (Schwaenen et al., 2004; Pfeifer et al., 2007), gain of the *MYC* on 8q24 and loss of the 6q25–q26 region were found in only one patient. Extracopies of *MYC* gene have been described in CLL, with an

incidence of 5% (Chapiro et al., 2010); alterations and overexpression of *MYC* have been associated with resistance to DNA-damage induced apoptosis (Döhner et al., 2000). Loss of the 6q25–q26 region occurring in 6–7% of CLLs has been reported to produce an intermediate clinical outcome (Coll-Mulet and Gil, 2009), but thus far the gene involved in the pathogenesis of CLL is unknown (Döhner et al., 2000; Cuneo et al., 2004). In our study, MLPA also detected deletions at 9p21 and 1q21 corresponding to *CDKN2A2B* and *LMNA* (lamin A/C) genes, respectively. *CDKN2A2B* deletion at 9p21, previously described in CLL using Southern blot analysis (Haidar et al., 1995; Pinyol et al., 1998), were detected in only one patient, a finding in line with a recent study involving a series of 63 untreated CLL patients in early stage disease (Coll-Mulet and Gil, 2009). The Lamin A/C gene, found deleted in only one patient in our series, has been described to be inactivated by hypermethylation in nodal diffuse large B-cell lymphoma (Agrelo et al., 2005). However, Forsterova et al., (2010) reported that lamin A/C gene was unmethylated in all of the CLL samples analyzed. Therefore, this finding remains a controversial issue prompting for future investigation of lamin A/C expression/methylation in larger prospective cohort of CLL.

Notably, some of the alterations (2p and 8q24 gains or 9p21 deletion) were found as the sole abnormality in only two patients, suggesting that they could be considered secondary changes to alterations commonly associated with CLL, as also suggested by Al Zaabi et al., (2010).

This study extends previous evidence that MLPA analysis is a valid means of identifying recurrent genomic aberrations as well as other less frequent alterations in CLL. Although the MPLA assay is limited in the identification of alterations occurring in small leukemic subclones, we showed that sensitivity could be enhanced by pre-enrichment of the B-cell population. Furthermore, differently from the few previously published studies (Al Zaabi et al., 2010; Coll-Mulet et al., 2008; Buijs et al., 2009), we performed an independent validation by FISH of additional altered regions detected by MLPA. This validation allowed us to define one probe exceeding the normal range as threshold sufficient to define the alteration of the considered region. In conclusion, our data indicate that MLPA can be used in association with FISH analysis in CLL based on its high success rate in detecting alterations by simultaneous screening of 56 genomic sequences. The use of multiple probes also permitted the identification of small alterations not detected by FISH studies as well as the mapping of the abnormal regions, thus providing an important contribution to the identification of prognostically relevant risk groups.

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