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Improved Detection of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia by Integrating the Results of Conventional Cytogenetics Using CpG-Oligodeoxynucleotide/ Interleuken-2 Mitogen Stimulation and Fluorescence in Situ Hybridization Techniques

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ABSTRACT

Background: Cytogenetic abnormalities are important prognostic parameters in chronic lymphocytic leukemia (CLL) patients. Because traditional mitogens are not always effective mitotic stimulants, usually, only interphases Fluorescence in Situ Hybridization (FISH) is done for diagnosis and follow-up of patients. Recently, CpG-oligodeoxynucleotide (ODN) stimulation has shown more effectiveness than standard mitogens in CLL cells.

Objectives: Our study aimed to test the effectiveness of CpG-ODN/IL-2 as a mitotic stimulant for CLL cells in comparison with other traditional ones and verify the consistency of detected cytogenetic abnormalities between different cultures. In addition we aimed to assess if doing interphase FISH analysis in parallel improved the rate of abnormalities detection in our cohort study.

Material and Methods: Our study included 60 CLL patients referred for routine cytogenetic and FISH analysis during disease evolution. Parallel cultures of peripheral blood were done adding either Lipopolysaccharide (LPS), B-cell mitogen 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or a combination of CpG-ODN and interleukin-2 (IL-2). Cytogenetic analysis was done on all cases cultured using CpG-ODN/IL-2, TPA and LPS. Simultaneously FISH was done for 40/60 cases using the following probes: 13q14.3/13q34, ATM/CEP11, IgH14q32, CEP12, 6q21/ SEC63, p53 (17p13)/SE17.

Results: Success rate in cultures was 86.6% using CpG-ODN/IL-2 mitogen, 48.3% using TPA and 40% using LPS. Clonal abnormalities were observed in 33/52 cases (63.46%) cultured using CpG-ODN/IL-2; the aberrant clones were present consistently in TPA and/or LPS cultures when successful. Out of these, 7 cases (21.2%) showed clonal abnormality with normal FISH, and 5 cases (15.15%) showed clonal abnormality with positivity only for 13q14.3 deletion. On the other hand, performing FISH allowed the detection of abnormalities (deletion 13q14.3) invisible by conventional cytogenetic analysis in 8/40 (20%) cases.

Conclusion: our results confirm that CpG-ODN/IL-2 stimulation increases the detection rate of chromosomal abnormalities and complexity in CLL compared with TPA and LPS. Furthermore, applying the used FISH panel, improved abnormalities detection rate. However, neither conventional karyotyping nor FISH detected all aberrations, demonstrating the indispensability of both techniques.

Key Words: *Chronic lymphocytic leukemia – CpG-oligodeoxunucleotide/IL-2 – Fluorescence in situ hybridization – Karyotyping*

INTRODUCTION

Assessment of cytogenetic aberrations has been shown to represent an independent predictor of prognosis in B-cell chronic lymphocytic leukemia (B-CLL) **[1-4]**. However the standard used mitogenic stimulants as 12-O-tetradecanoyl -phorbol-13-acetate (TPA) and Lipopolysaccharides (LPS) reveal cytogenetic abnormalities in only ~50% of cases **[1]** which has limited the application of metaphase cytogenetics in CLL. On the other hand, fluorescence in situ hybridization (FISH) has been widely applied in CLL increasing the rate of detected abnormalities up to 80% **[2,5-9]**. Nonetheless, the results provided are only informative about the genomic regions for which the FISH probes are designed neither detecting other abnormalities nor complex karyotyping. CLL is a clonal disease in which lymphocytes have a low mitotic index. Therefore,

CD40 ligand-induced cell cycle stimulation has been tried increasing the rate of abnormalities detection to 89% **[10]**. However, it was not widely used due to its difficult applicability in routine analysis. More recently CpG–oligodeoxynucleotide (ODN) stimulation has been used in combination with IL-2 increasing the rate of abnormalities detection up to 80% **[3,11-14]**, a percentage nearly equivalent to that obtained in interphase FISH with the added advantage of having a global view of cytogenetic abnormalities and/or complexities. CpG-ODNs are synthetic or bacterial short single strands of DNA, in which the CpG motifs are not methylated. They enter the B-cells and stimulate response to cytokines through Toll-like receptor-9 mediation **[15-18]**. Some studies addressed performing comparative cytogenetic analysis using different mitogenic stimulants: TPA and CpG-ODN/IL-2 **[13,14,19]**. The study conducted by Put et al. **[14]** and Muthusamy et al. **[20]**, addressed the relevance of combining both cytogenetic analysis and interphase FISH although in the latter the mitogenic stimulant was different. Therefore we decided to perform cytogenetic analysis for CLL using these different mitogenic stimulants in parallel with interphase FISH to establish CpG/IL-2 stimulation as a more effective method for conventional Karyotyping and to further confirm the importance of integrating both the results of conventional karyotyping with interphase FISH for more accurate detection of chromosomal abnormalities in CLL.

MATERIAL AND METHODS

Patients:

The study was performed on 60 patients including 43 males and 17 females with an age range of 36-83 and a median of 58.5 years at the time of presentation. The protocol was approved by the IRB of "Sapienza" University of Rome and all patients gave their written informed consent to the blood collection and to the biologic analyses included in the present study according to the Declaration of Helsinki. The diagnosis of CLL was based on the presence of more than 5000 lymphocytes/μL in the peripheral blood that expressed a conventional CLL immunophenotype (CD5/CD20$^+$, CD23$^+$, CD79a$^+$, FMC7$^-$, weak CD22$^+$, weak sIg$^+$, CD79b weak/$^-$, CD10$^-$) **[21]**.

Conventional cytogenetic analysis:

Cell separation:

Peripheral blood samples were drawn on heparin. Samples were separated on Ficoll-Hypaque (CEDARLANE CL5015), and centrifuged. Mononuclear layer was retrieved, washed, centrifuged and cell pellet was obtained. Cell count was adjusted to 10-20X10^6 cells in 5-10ml of medium according to the mitogen added (as explained below).

Mitogens:

For every sample 3 separate cultures were done with LPS, TPA, and CpG-ODN/IL-2 added as follows:

- Culture 1: 20X10^6 cells/10ml of culture medium with 400ul LPS [40ug/ml] (Sigma-Aldrich # L 2654).
- Culture 2: 20X10^6 cells/10ml of culture medium with 50ul TPA [50ng/ml] (Sigma # P8 139).
- Culture 3: 10X10^6 cells/5ml of culture medium with 100ul of CpG-ODN DSP30 [2nmol/ml] (Roche Diagnostics # 232622 DSP30) and 100ul IL-2 [100U/ml] (Roche # 10 799 068 001).

Samples were incubated for 72 hr at 37ºC and 5% CO2 in a fully humidified atmosphere. Colchicin 50ul was added [10ug/ml] (Gibco, Invitrogen Corporation #15210-40) then incubated for 3 hrs in the same conditions. The cells were treated with hypotonic solution, centrifuged, and the resulting pellet was fixed and washed in methanol/acetic acid (3:1). Cells were re-suspended in fixative and dropped on slides. Karyotypes were examined after the G banding technique and described according to International System for Human Cytogenetic Nomenclature (ISCN 2009) **[22]**.

Interphase FISH analysis:

Interphase FISH was performed on nuclei preparations of CpG-ODN/IL-2, TPA, or LPS cultures depending on quality of nuclei on slides preparation. The following commercially available probes were used to investigate loci commonly involved in CLL: 6q21 [SEC63] (Kreatech # KBI-10105), 11q22-23 [LSI ATM, CEP 11 Probe] (Vysis/Abbott # 30-231059), centromere 12 [CEP 12] (Vysis/Abbott # 30-

170012), 13q14.3/13q34 [LSI D13S319 (13q14.3) Spectrum Orange/ LSI 13q34 Spectrum Green Probe] (Vysis/Abbott # 30-231061), 14q32 [LSI IGH Dual Color, Break Apart Rearrangement Probe] (Vysis/Abbott # 30-191019), and p53 (17p13) /SE 17 (Kreatech # KBI-1-112). FISH analyses were performed according to the manufacturer's protocols and as previously reported **[23]**. In all of the analyzed CLL cases, at least two hundred interphase round nuclei with well-delineated fluorescent spots were counted; positive cases were defined as having ≥% the cut-off of nuclei displaying the investigated abnormality which differed with the probe used: ≥5% for 6q21, ≥10% for 11q22-23 and 13q14, ≥0% for centromere 12, ≥4.6% for 14q32, and ≥20% for p53 (17p13).

RESULTS

Success rate in cultures using CpG-ODN/IL-2 mitogen was 86.6%. Failure occurred in 8 cases. A minimum of 20 metaphases were cytogenetically analyzed in them. Failure occurred in 31 cases after TPA (success rate of 48.3%) and in 36 cases after LPS cultures (success rate of 40%). Failure was defined as less than 10 metaphases in the culture under consideration, whereas success was defined as 10 or more metaphases or the presence of a clonal abnormality. Clonal abnormalities were observed in 33/52 cases (63.46%) cultured using CpG-ODN/IL-2; the aberrant clones were present consistently in TPA and/or LPS cultures when successful (Table 1) (Figs. 1,2).

Table (1): Clonal abnormalities detected by conventional cytogenetic analysis in 33/52 chronic lymphocytic leukemia patients.

Case number	Translocation
Case 1	46,XY,add(5)(p13) **[4]**/46,XY**[16]***
Case 4	46,XY,der(15)t(3;15)(q25;q24)**[7]**/46,XY**[13]***
Case 5	46,XX,del(17)(p10)**[7]**/46,XX**[13]**
Case 8	48,XX,+12,+19**[20]**
Case 13	47,XX,+12 **[6]**/46,XX**[14]**
Case 15	46,XY,der(16)t(14;16)(q32;q23)**[11]**/46,XY**[9]***
Case 16	Complex Karyotype**
Case 17	46,XY,del(7)(q21.2q31)**[14]**/46,idem,del(11)(q31)**[2]**/46,XY**[4]**
Case 19	46,XY,add(22)(p12)**[14]**/46,XY**[6]***
Case 21	46,XY,del(6)(q13q25)**[12]**/46,XY,add(18)(p11.2)**[3]**/46,XY**[5]****
Case 22	47,XY,+12**[20]**
Case 23	46,XY,del(6)(q13q25)**[6]**/46,XY**[14]***
Case 24	46,XY,del(11)(q22.3),del(16)(q?)**[3]**/46,idem,del(13)(q14q21)**[1]**/46,XY,del(13)(q14q21)**[4]**/46,XY,del(16)(q?)**[2]**/46,XY**[10]**
Case 25	44,XY,del(1)(p34),-8,-9,-10,del(11)(p11.2),-13,-16,-17,+4mar**[2]**/46,XY**[18]****
Case 27	45,XX,-4,del(11)(q21),der(17)t(4;17)(q13;p11.2)**[20]**
Case 28	46,XX,del(11)(q23)**[3]**/46,XX,del(11)(q13)**[1]**/46,XX**[16]***
Case 29	47,XY,+12**[3]**/46,XY**[17]**
Case 31	46,XX,-17,+mar**[4]**/46,XX **[16]****
Case 33	46,XY,del(13)(q14q21)**[10]**/46,XY,del(13)(q14q21)X2**[1]**/46,XY**[9]**
Case 34	46,XY,-19,+mar**[5]**/46,XY**[15]****
Case 35	47,XY,+12**[12]**/47,idem,del(11)(q22)**[6]**/46,XY**[2]**
Case 36	46,XY,del(11)(q22)**[18]**/45,XY,-11**[1]**/46,XY**[1]**
Case 38	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**
Case 39	44,XY,del(1)(q42),der(4)t(4;17)(p13;q21),add(8)(p11.2),del(14)(q32),-15,add(17)(p13.1),-17[**15]**/46,XY,del(1)(q42),del(14)(q32)**[2]**/46,XY**[3]**
Case 40	46,XY,t(1;7)(q21;p22),del(2)(p13),del(11)(q22),add(14)(q32)**[2]**/46,XY,del(11)(q22)[**2]**/46,X **[16]**
Case 41	46,XY,der(17)t(9;17)(q22;p13.2)**[3]**/47,XY,+12[**2]**/46,XY**[15]**
Case 42	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**
Case 44	Complex Karyotype
Case 46	46,XY,del(7)(q22),add(7)(q32).ishdel(7)(q22),t(7;?)(q32;q?)**[3]**/46,X **[17]**
Case 48	46,XY,del(11)(q23),del(13)(q14q21)**[1]**/46,XY,del(11)(q23)**[1]**/46,XY,del(13)(q14q21)**[3]**/46,XY**[15]**
Case 49	46,XY,del(11)(q23)**[5]**/46,XY,del(6)(q13q25),del(11)(q23)**[12]**/46,XY**[3]**
Case 50	47,XX,+mar**[2]**/47,idem,del(9)(q12)**[2]**/46,XX**[16]***
Case 52	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**

* Cases with a normal panel of interphase FISH analysis. ** Cases with clonal abnormality only for 13q14.3 deletion.

Table (2): Abnormalities detected by interphase FISH analysis in 40/60 of chronic lymphocytic leukemia patients.

Case No.	13q14/13q43	17p13 (p53)	11q22/23 (ATM)	14q32 (IgH)	CEP 12	6q21
Case 3	20%*					
Case 5	19%	90%				
Case 8					50%	
Case 9	40%/50%*/**					
Case 10	20%*					
Case 13					42%	
Case 14	15%*					
Case 16	93%					
Case 17	45% /27%**					
Case 18	60% /7%*/**					
Case 20	30%*					
Case 21	82%/12%**					
Case 22					66%	
Case 24	87%		90%			
Case 25	85%					
Case 26	25%*					
Case 27	90%	30%	80%			
Case 29					23%	
Case 31	20%/33%**					
Case 32	35%*					
Case 33	64%/16%**					
Case 34	85%/5%**					
Case 35			14%		42%	
Case 36			77% /9%***	5%		
Case 38	53% /16%**					
Case 39		33%	14%			

* Cases where 13q14 deletion was not detected cytogenetically.
** Double deletion of the 13q14 locus.
*** Monosomy of chromosome 11.

Interphase FISH analysis was done for 40 cases. A full probe panel of the six commonly involved regions (mentioned above) was applied. One or more abnormalities were detected in 26 cases (65%) (Table 2, Fig. 3). Out of the 33 cases showing clonal abnormalities, 7 (21.2%) had a negative panel of interphase FISH. In cases 23 and 28 (as indicated in Table 1) deletion of chromosome 6 and chromosome 11 were detected respectively. In these 2 cases FISH failed to detect the specific abnormality which was visible at the cytogenetic level. In addition, in 3/7 of these cases whole chromosome painting was done for specific chromosomes to further clarify the results (as indicated in Table 1): For case 4 whole chromosome painting was done for chromosome 3 (Kreatech # pKBI-30003G) (Fig. 4), for case 15 whole chromosome painting was done for chromosome16 (Kreatech # pKBI-30016G) and for case 19 whole chromosome painting was done for chromosome 22 (Kreatech # pKBI-30020R). In addition, 5 cases (15.15%) showed clonal abnormality with positivity only for 13q14 deletion (Fig. 5), of those for case 21 (as indicated in Table 1) whole chromosome painting was done for chromosome 18 (Kreatech # pKBI-30018R) and CEP 18 (Vysis/Abbott # 05J08-028) was also done for further specification of the karyotyping. On the other hand 8/40 cases (20%) showed 13q14 deletion which was invisible by conventional cytogenetic analysis.

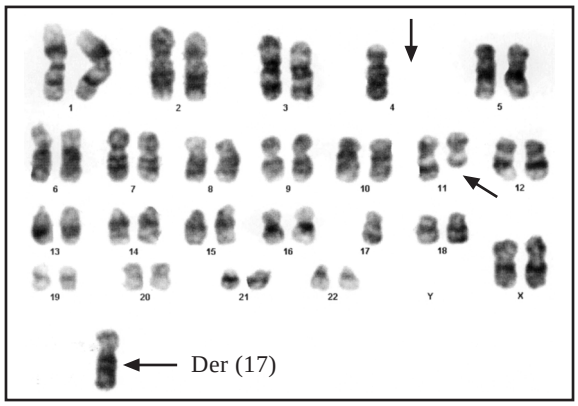

Fig. (1): Case 27: 45,XX,-4,del(11)(q21),der(17)t(4;17)(q13;p11.2)**[20]**.

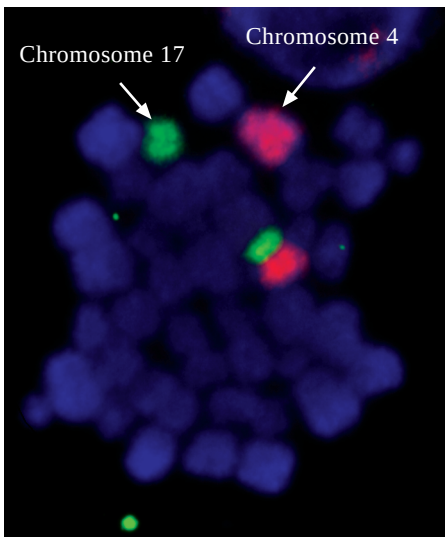

Fig. (2): Case 27: showing der(17)t(4;17)(q13;p11.2) (WCP 4Red, 17Green).

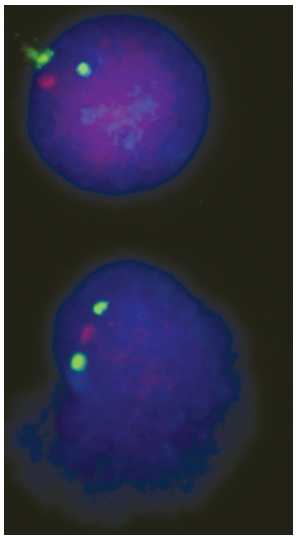

Fig. (3): Case 36: showing ATM 11q22.3 deletion [LSI ATM Red, CEP 11 Green probe].

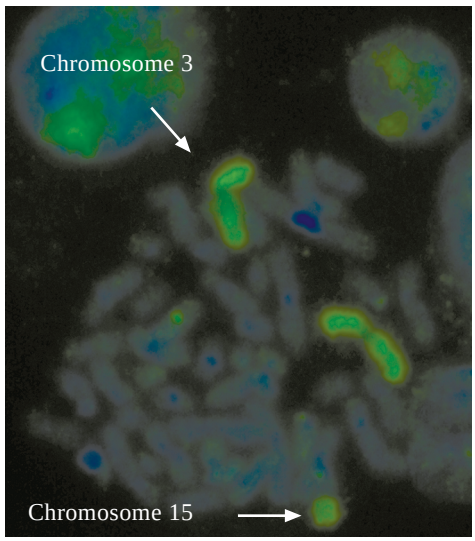

Fig. (4): Case 4: painting of chromosome 3 showing der (15)t(3;15)(q25;q24)(WCP 3 Green).

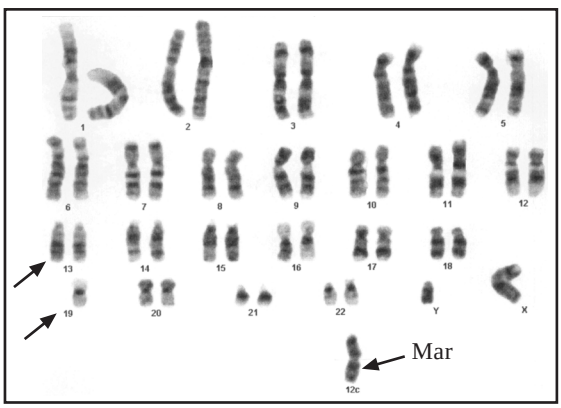

Fig. (5): Case 34: 46,XY,-19,+mar**[5]**/46,XY**[15]**.

DISCUSSION

Cytogenetic abnormalities are considered major prognostic parameters for predicting response to treatment and survival of CLL patients. The heterogeneous biology of the CLL cells, as well as their poor in vitro mitotic index pose a great challenge for obtaining reproducible and reliable abnormal metaphases. Attempts to improve culture conditions to increase the cycling status of the CLL cells for efficient cytogenetic analysis have been investigated. Several B-cell mitogens/stimulatory agents including pokeweed mitogen (PWM), phorbol 12-myristate13-acetate (PMA), TPA and LPS have been applied and used as a standard method in many cytogenetic laboratories. Although each of these mitogens has its own advantages and disadvantages, recently, the use of novel im-

mune-stimulatory agents including CpG ODNs, CD40 ligand, and cytokines such as IL-2, and IL-15 were evaluated **[3,10,11,13,24-26]**. Introduced culture methods with CpG-ODN/IL-2 improved proliferation capacity of CLL cells and yielded detection rates of cytogenetic aberrations comparable with interphase FISH, i.e., 81-83% **[11,12]**. Furthermore, the reproducibility of CpG-ODN/IL-2 stimulation for detecting chromosomally abnormal clones has been established in five laboratories **[19]**. In our study, the overall detection rate of chromosomal aberrations was (86.6%) when stimulated with CpG-ODN/IL-2; this represents a significant improvement over both TPA and LPS cultures which showed a success rate of 48.3% and 40% respectively. The success rate of CpG-ODN/IL-2 is comparable with some studies **[11,13]**, and is higher than others **[14,19]**. The difference was explained by the different methodology in which the cells were not separated; a hypothesis which has been proposed by the authors although it was not ascertained **[11,13]**. In addition, the higher success rate of CpG-ODN/IL-2 as a mitogen in comparison with both TPA and LPS which we report is comparable with some studies **[14,19]** and different from another **[13]**. The latter reported a higher success rate with TPA although they reported a higher proportion of metaphase abnormalities detected using CpG-ODN/IL-2. The variability in the results could be attributed to the slightly different methodologies as each laboratory has its own standardized techniques. In addition we cannot discard the biological heterogeneity of the disease itself and the proliferation capacity of the CLL cells even under standardized conditions. Furthermore, in our study the clonal abnormalities detected were the same among the cultures with CpG-ODN/IL-2 and both TPA and LPS when successful. This finding differed from others who found differences in abnormalities detected and their frequency (an issue which was not found in our study) between different cultures **[13,14,19]**. This could be attributed to the small cohort enrolled in our study compounded by the absolute low success rate with TPA and LPS, a fact which rendered it difficult to fully investigate this point. Interphase FISH identified abnormalities in 65% of cases. In 20% of the cases, FISH detected a 13q14 deletion which was invisible by conventional cytogenetic analysis, an aberration which is often cytogenetically cryptic. Conversely, in 21.2%, FISH failed to detect any abnormality despite abnormal conventional cytogenetics. Hence, the combination of conventional cytogenetics and FISH could increase the detection rate of clonal abnormalities. Of note, the abnormalities observed by conventional cytogenetics and FISH, using classical CLL probe panels, were not always overlapping. In case 23 and 28 (as indicated in Table 1) deletion of chromosome 6 and chromosome 11 were detected respectively. In these 2 cases FISH failed to detect the specific abnormality which was visible at the cytogenetic level. As previously reported this may reflect a selective proliferative response of the CLL clone or inappropriate location of the FISH probe **[14]**. This illustrates the need for a combined routine analysis, including both techniques. Another finding that was observed while doing FISH analysis is that 5 cases showed clonal abnormality with positivity only for 13q14 deletion. Although CLL with 13q deletion as the sole cytogenetic abnormality (del13q-only) usually has good prognosis, more aggressive clinical courses are documented for del13q-only CLL carrying higher percentages of 13q deleted nuclei. Moreover, deletion at 13q of different sizes has been described; its prognostic significance was unknown **[27-30]**. Interestingly in 4/5 cases the percentage of the 13q deleted nuclei was >70%, the cut off % documented by a multi-centric as a bad prognostic parameter. However, the probe we used for detecting 13q14 deletions did not encompass the DLEU2/MIR15A/MIR16-1 and RB1 loci which according to this study influenced the prognosis where cases with larger deletions involving the RB1 locus had poor prognosis even with a low percentage of deleted nuclei rendering it an independent prognostic parameter; this fact might explain our remaining case with a lower percentage of deleted nuclei and cytogenetic clonal abnormalities. This further emphasizes the importance of performing conventional cytogenetics in addition to FISH.

In conclusion, we confirm that the detection rate of clonal chromosomal abnormalities is superior after CpG/IL-2 stimulation compared with TPA and LPS. The combination of the three culture techniques does not result in an increase of the detection rate or in the detection of different clonal abnormalities. Therefore, CpG/IL-2 should be preferred for routine conventional cytogenetic analysis of CLL. However,

the lack of detection of differences in clonal abnormalities among cultures using different mitogens as previously reported, could possibly be attributed to our small cohort, the low success rate of TPA and LPS cultures (although comparable with others), or to the biological heterogeneity of the response of CLL cells in our routine settings. Finally, neither conventional cytogenetics nor CLL-specific FISH detected all aberrations, further confirming the complementary nature of both techniques.

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