Research Article

Investigation of 13q14.3 Deletion by Cytogenetic Analysis and FISH Technique and miRNA-15a and miRNA-16-1 by Real Time PCR in Chronic Lymphocytic Leukemia

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Abstract

Background: The most frequent cytogenetic aberration is 13q14.3 deletion in Chronic Lymphocytic Leukemia (CLL). Hsa-miR-15a/hsa-miR-16-1 are tumor suppressor miRNAs encoded from 13q14.3 region. **Objectives:** The aim of this study was to investigate the 13q14.3 deletion using molecular and cytogenetic techniques and association with miRNA-15a/miRNA-16-1. **Materials & Methods:** We used peripheral blood samples of 30 CLL patients which were either induced and or non-induced with DSP30+IL-2 for determine 13q14.3 deletion by karyotyping and iFISH methods. Expression levels of hsa-miR-15a/miR-16-1 were measured using Quantitative Real Time PCR and compared with deletions. **Results:** 13q14.3 deletion was detected in 8.6% of cases by karyotyping and in 65% by iFISH. Mosaic forms (monoallelic+biallelic) were observed in 50% of cases. Besides determining common chromosome abnormalities such as add(2)(q37), t(2;7)(p11.2;q22), del(6)(q13q21), del(6)(q25), add(9)(q21), del(11)(q23), t(11;14)(q13;q32), del(13)(q11q12), del(13)(q12q14), add(14)(q23), del(14)(q23), t(14;19)(q32;q13.1), del(15)(q23), del(17)(p12), t(18;22)(q21;q11.2), add(21)(p13) and t(17;21)(q11.2;122), we also determined t(1;13)(q32;q34), inv(2)(p25q21), del(13)(q22q32), t(14;19)(q24;q13), dup(17)(q21q23), rob(21;21)(p13;p13) which have not been reported previously. Mitotic index data was found statistically significant and DSP30+IL-2 increased mitotic index by 2.5 folds. Association between decreased miR-16-1 expression and deletions was statistically significant. **Conclusion:** We suggest that cytogenetic and iFISH analyses are complementary and use of DSP30+IL-2 is effective in CLL. Decreased expression of hsa-miR-16-1 is remarkable.

Keywords: CLL; miR-15a/miR-16-1; 13q14.3 deletion; DSP30; iFISH; qRT-PCR

Introduction

Chronic Lymphocytic Leukemia (CLL) is characterized by the gradual accumulation of functionally immature, small, monoclonal CD5+ and CD23+ B cells most of which are non-proliferating cells arrested at G0/G1 phase of the cell cycle. [1] CLL comprises about 30% of all cases of adult leukemia in Western World affecting both sexes and chromosomal abnormalities are widely used in CLL to prognose, treat and follow the overall survival in patients. [2] These abnormalities include del(13)(q14), del(11)(q22), trisomy 12 and del(17)(p13) and they can be detected using conventional banding techniques. The most frequent abnormality is deletion of 13q14 which is associated with favorable prognosis. [3,4] Deletion of 13q14 is mostly monoallelic (in 76% of cases), but also it is detected in biallelic (24%) and mosaic forms by iFISH. [4,5]

Conventional karyotyping and Interphase Fluorescence *In Situ* Hybridization (iFISH) techniques are widely used to detect cytogenetic abnormalities seen in CLL. However, approximately 50% of patients with CLL can be analyzed by conventional karyotyping due to very low responsiveness of CLL cells to mitogenic stimuli *in vitro*. [3] Recently a new mitogen CpG-oligodinucleotide (CpD-ODN) called DSP30 was used in CLL cytogenetics which was reported to be the most effective CpG-ODN in stimulating human B cells. [6]

MicroRNAs (miRNAs) are 20–22 nucleotide long small noncoding RNAs which can bind to Untranslated Regions (UTRs) of target mRNAs resulting in translational repression or mRNA degradation. [7] DLEU2 gene encoding miR-15a and miR-16-1 are located within the deletion region of 13q14. Both miR-15a and miR-16-1 have been shown to exhibit tumor-suppressing activities by inducing apoptosis and inhibiting

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proliferation. Generally speaking, levels of both miRNAs are decreased due to deletion 13q14 but physical loss of 13q14 is not the only mechanism causing a decrease in miRNAs levels. Epigenetic regulations and defects in miRNA biogenesis can contribute to their dysregulation as well. [8,9] In this study, we investigated miR-15a and miR-16-1 levels by qRT-PCR and compared with 13q14.3 deletion detected by conventional karyotyping and iFISH techniques. We also investigated other possible cytogenetic abnormalities and effects of DSP30+IL-2 combination using conventional karyotyping techniques in patients with CLL.

Methods

Peripheral blood was collected from 30 CLL patients. All patients were followed up in division of hematology. 18 (60%) of patients are males and 12 (40%) are females. 15 healthy control subjects 9 (60%) males and 6 (40%) females) were also included in the study. Expression levels of miR-15a and miR-16-1 were measured using qRT-PCR, while deletion of 13q14 region was assessed using conventional cytogenetic and iFISH techniques. Informed consent was obtained from mall patients prior to the study and all study was conducted following declaration of Helsinki. The Ethical Committee of Istanbul University Cerrahpasa Medical Faculty approved the study (nr:83045809/604/01-01/114304).

Conventional cytogenetic analysis using DSP30+IL-2

Conventional karyotyping analysis was performed in all patients using standard protocols. Peripheral blood samples (~1 ml) of patients were added in three separate conical tubes and suspended in 2.5 ml of 1640 RPMI (Biochrome, Berlin, Germany). We added 10 µl of CpG-ODN DSP30 (TıbMolBiol, Berlin, Germany) with a concentration of 1µM and 50 µl IL-2 (Roche, Mannheim, Germany) with a concentration of 100 U/mL for both first and second tubes. Third tubes were used as control which does not include DSP30 and IL-2. All samples were cultured at 37°C for 72 h. 50 µl of colcemid (KaryoMax, Thermo Fisher Scientific, Waltham, MA, USA) (with a final concentration of 10 µg/ml) was added prior to harvesting of the cells.

Chromosome preparation and staining using G-Banding technique was performed following standard procedures in patients and control samples, as described elsewhere. [10] Karyotypes were scored according to the International System for Human Cytogenetic Nomenclature (ISCN) 2016. [11]

Mitotic index was scored by counting metaphases on a slide containing 1000 cells per mitosis in induced by DSP30 and non-induced patient samples and compared to the controls.

iFISH experiments

iFISH analyses were performed in interphase cells using 13q14.3 locus specific probe (Cytocell LSI-D13S319 Plus, Cambridge, UK) in induced samples by DSP30 of all patients. Cultured samples were re-suspended with fresh Carnoy fixative (3:1 methanol:acetic acid) for two hours at Room Temperature (RT), centrifuged, removed the supernatant, and then re-suspended in fresh fixative for a few more minutes before spreading and then CytocelliFISH protocol was performed. [12] Three hundred interphase nuclei were scored in all samples. Same protocols were applied for the control samples.

qRT-PCR experiments

miRNA was isolated using ExiqonmiRCURY RNA isolation kit (Exiqon A/S, Vedbaek, Denmark) following the manufacturer's instructions and cDNA was synthesized using miRCURY LNA™ microRNA PCR, Polyadenylation and cDNA synthesis kit II (Exiqon A/S, Denmark) with an initial amount of 10 ng of RNA. cDNA synthesis was performed in T100 Thermal Cycler System (Bio-rad Laboratories, Hercules, CA, USA) with following conditions: 42°C for 60 min, 95°C for 5 min and 4°C for cooling. The expression levels of miR-15a and miR-16-1 were determined with a quantitative system based on SYBR Green probe technology [miRCURY LNA™ microRNA PCR, ExiLENT SYBR® Green master mix (Exiqon S/A, Denmark)]. The assay was performed in a total volume of 10 µl and contained 5 µl 1xPCR master mix, 1 µl of each primer

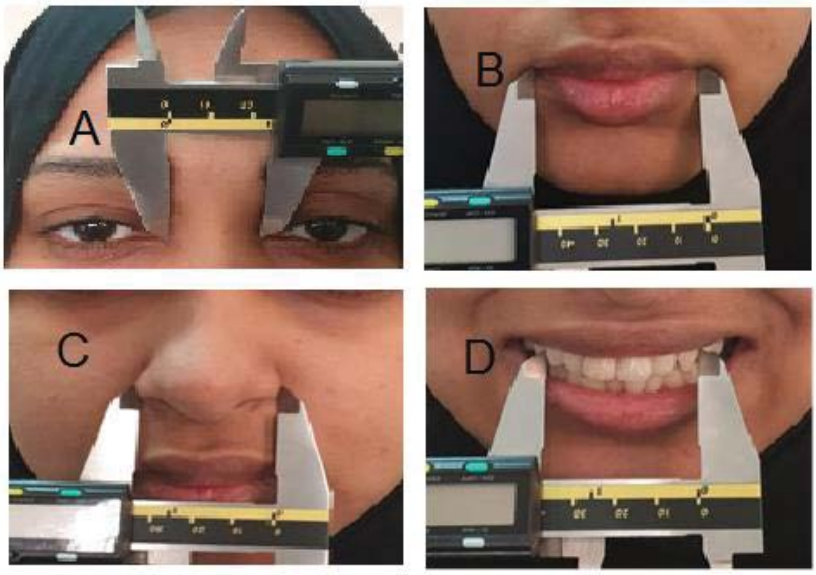

Figure 1: Facial measurements recorded in the present study (A: Intercanthal distance, B: Inter-commissural width, C: Inter-alar width, D: Maxillary inter-canine distance).

(miRCURY LNA PCR Primer mix for hsa-miR-15a-5p and hsa-miR-16-1-3p; Exiqon S/A, Denmark) and 4 µl of cDNA sample. The expression levels of miRNA-15a and miR-16-1 were measured in Light Cycler 1.5 System (Roche Diagnostics, Mannheim, Germany) under the following conditions: 95°C for 10 min initial denaturation, 40 cycles of 95°C 10 s and 60°C for 1 min for denaturation, 1 cycle of 95°C for 1min, 40°C for 2 min, 95°C for 1s for melting. Relative miRNA expression levels were calculated using the 2–ΔΔCT method. [13] The U6 housekeeping was used as a reference. All experiments were performed in triplicates.

Statistical analyses

Statistical analyses were performed with the SPSS 21 software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Wilcoxon test was used to confirmation of mitotic index in DSP30+IL-2 induced and non-induced samples and mitotic index differences between patient and control samples ($p<0.001$). Cut-off values of deletion types in patients and control samples were calculated using ROC analysis. Association between miRNAs (hsa-miR-15a, hsa-miR-16-1) expression levels and 13q14.3 deletion types were analyzed using Mann-Whitney U test. Significance value was determined as ($p<0.05$).

Results

In DSP30+IL-2 induced samples, the sufficient number of metaphases suitable for cytogenetics analysis was obtained in 23/30 (76.6%) of the patients and clonal chromosomal aberration were detected in 16/23 (69.5%) of the patients. Normal karyotype was found in 7/23 (30.4%) of the patients. 13q14.3 deletion was detected only in 2/23 (8.6%) of the patients with conventional karyotyping techniques. In DSP30+IL-2 non-induced samples metaphases suitable for cytogenetics analysis were observed in 7/30 (23.3%) of the patients and chromosomal aberrations were detected in 2/7 (28.5%) of the patients. 13q14.3 deletions was not observed in non-induced samples by conventional karyotyping. Cytogenetic aberrations of twenty-three successfully karyotyped patients are presented in Table 1. Also some different abnormalities we found in induced samples are shown in Figure 1.

Mitotic index was calculated as 10.48% in DSP30+IL-2 induced

Table 1: Conventional cytogenetic results in samples induced and non-induced with DSP30+IL-2.

Caseno.	DSP30+IL-2 induced	DSP30+IL-2 non-induced
1	-	-
2	-	-
3	46, XY(22)	-
4	46, XX(14)	-
5	41~46,XY, -Y(3),t(2;7)(p11.2;q22)(28),del(6)(q?q?) (9), 9qh+c(19),der(17)t(3;17)(q21;p11.2)(29),-21(5),+21(5),add(21)(p13)(6),rob(21;21)(p13;p13)(14),mar1(3)(cp31),/46,XY,9qh+c(2)	-
6	46, XY,del(17)(p12),dup(17)(q21q23)(3)/46~47,XY,+13,del(13)(q11q12)(cp2) /46,XY(4)	46, XY(4)
7	46,XX,der(13)(q12q14)del(3)(q22q32)(8)/38~46,XX,del(6)(q?q?) (12),der(13)del(13)(q12q14) del(13)(q22q32)(16),-17(4),t(17;21)(q11.2;q22)(2),der(21)t(17;21)(q11.2;q22)(2),-21(3),-22(3) (cp19) /46,XX(2)	-
8	40~45, XY, -20(cp4)/46, XY(19)	46, XY(3)
9	46, XY(8)	-
10	-	-
11	43~49,XY,+8(1),del(14)(q23)(21)(cp21)/92~98,XXY,+4(2) +8(1),+14(2) del(14)(q23)x2(4),+19(2) (cp4) /46,XY(2)	45, XY, del(14)(q23)(cp1)/ 46, XY(1)
12	46,XY,t(1;13)(q32;q34)(3),del(6)(q13q21)(2)(cp3)/ 46,XY(8)	-
13	46,XY(19)	46, XY(11)
14	-	-
15	42~46,XX,-10(3),t(11;14)(q13;q32)(10),-21(3)(cp12) /46,XX(9)	-
16	46,XX,del(11)(q23)(2)/46,XX(5)	-
17	39~46, XY, t(18;22)(q21;q11.2)(11), -21(3)(cp11) /46, XY(5)	-
18	-	-
19	47, XY, +12(22), add(14)(q32)(3), t(14;19)(q32;q13.1) (15),t(14;19)(q24;q13)(2)(cp22)	43~48, XY, +12(10), t(14;19) (q32;q13.1)(4),add(14)(q32) (3),t(14;19)(q24;q13)(1)(cp10)
20	41~46, XY, +12(20), -21(3)(cp21)/46, XY(2)	-
21	46, XX, dup(12)(q13q24)(2)/46, XX(24)	-
22	46, XX, add(2)(q37), del(6)(q25), del(15)(q23), +mar1(2)/ 46, XX, inv(2)(p25q21)(cp2)/46, XX(40)	46, XY(1)
23	46, XX(10)	-
24	42~45, XX, -21(cp4)/46, XX(16)	-
25	46, XY(16)	-
26	46, XX, +20(cp2)/46, XX(8)	-
27	46, XX(2)	-
28	-	-
29	-	-
30	46, XY, del(13)(q12q14)(10)/46, XY(7)	-

(-): No results are available due to absent cells and/or low mitotic index

samples and as 4.2% in non-induced samples whereas defined as 45% in control subjects. We identified that using DSP30 increased mitotic index by 2.5 folds. Difference in induced and non-induced samples was found statistically significant ($p<0.001$) [Table 2]. Metaphase plaques (number of metaphases) and cell density in induced and non-induced samples are shown in Figure 2.

13q14.3 deletion was detected in 2/23 (8.6%) of DSP30+IL-2 induced patients by conventional karyotyping whereas this deletion was detected by iFISH in 17/26 (65.3%) of the same patients. Monoallelic and mosaic deletions were determined in 17/26 (65.3%) and in 13/26 (50%) of the patients, respectively. Statistically; cut-off values, performed by ROC analyses ($p<0.05$) [Table 3], were calculated as 1.88% and 0.13% for monoallelic (2G/1R) and biallelic (2G) deletions, respectively.

iFISH images of mono and biallelic deletions at 13q14 and other chromosomal abnormalities except 13q14 that were detected in DSP30+IL-2 induced samples are shown in Figure 3 and Figure 4, respectively.

We showed that miRNA-15a expression was decreased in 13/30 (45.5%), increased in 15/30 (50%), not changed in 2/30 (6.6%) of the patients whereas miR-16-1 expression decreased in 15/30

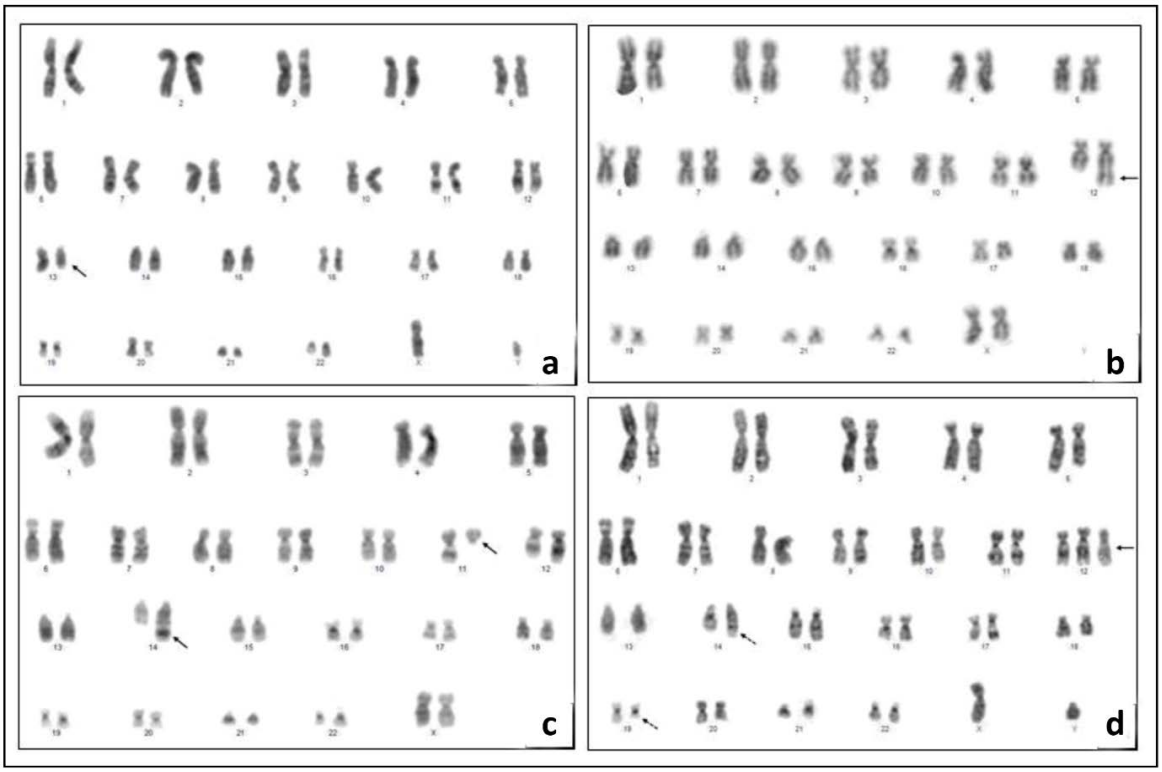

Figure 1: Karyogram samples including some chromosomal abnormalities in different patients. (a)46,XY,del(13)(q14.3), (b) 46,XX,dup(12)(q13q24), (c) 46,XX,t(11;14)(q13;q32), (d) 46,XY,+12,t(14;19)(q32;q13.1).

Table 2: Results of Mann-Whitney Test Statistic used for compare of mitotic index with using and non-using DSP30+IL-2 samples.

Induced with DSP30+IL-2	Non-induced with DSP30+IL-2	P value	
Patient Median (min-max)	3.5 (0-46.8)	0 (0-2.50)	<0.001

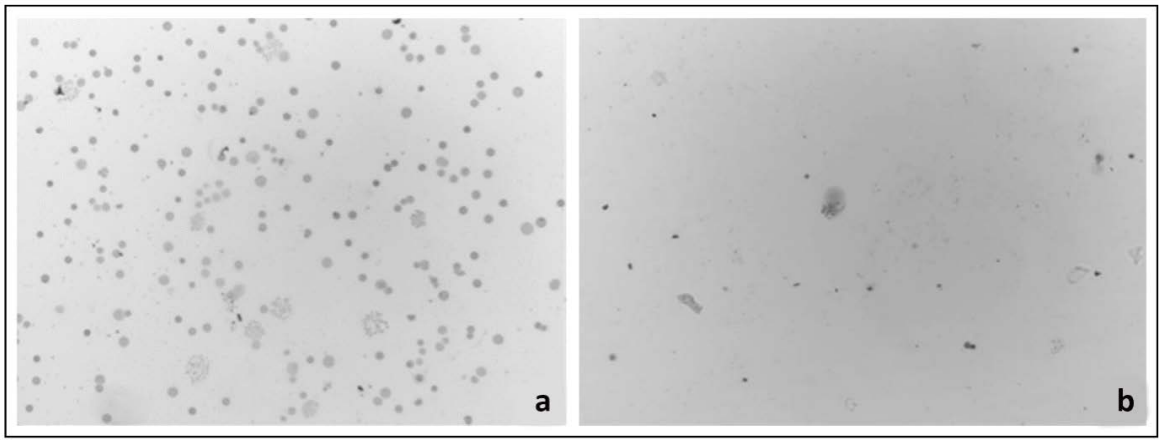

Figure 2: Images of metaphase plaques and cell density in different patients samples with (a) DSP30+IL-2, (b) without DSP30+IL-2.

(50%), increased in 14/30 (46.6%), not changed in 1/30 (3.3%) of the patients when compared to controls. Expression levels of both miRNAs were simultaneously decreased in 7/30 (23.3%) and increased in 8/30 (26.6%) of the patients. Hsa-miR-15a and hsa-miR-16-1 expression changes are shown in Table 4. Statistical analyses showed that changes were not significantly

Table 3: Results of ROC analysis for del(13q14.3).

Test result variable(s) (%)	Area	Std. Error	p value	95% Confidence interval
2G/2R (Normal)	0.314	0.077	0.014	0.164-0.465
2G/1R (Monoallelic D.)	0.674	0.076	0.022	0.523-0.823
1G/1R (Monosomy)	0.606	0.078	0.163	0.452-0.759
2G (Biallelic D.)	0.737	0.069	0.002	0.601-0.873

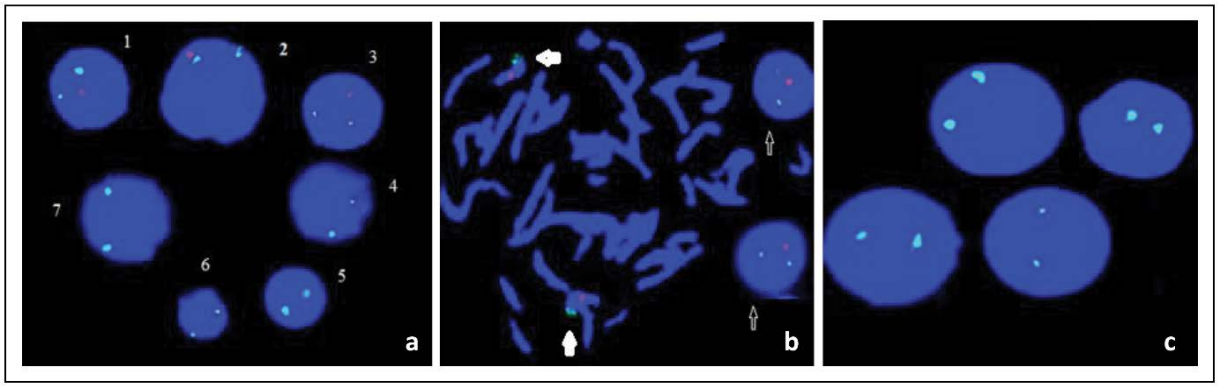

Figure 3: FISH images of monoallelic and biallelic deletions of 13q14.3 in different patient samples induced with DSP30+IL-2: (a) iFISH images of monoallelic (1, 2, 3) and biallelic (4, 5, 6, 7) 13q14.3 deletions, (b) Metaphase FISH (mFISH) images of normal cell and iFISH images of monoallelic deletion cells, (c) iFISH images of biallelic 13q14.3 deletions cells (Green signal:Control=13qter/13q34/Red signal: Deletion=13q14).

Table 4: Expression patterns of miR-15a and miR-16-1 in patients.

Case no	miR-15a	miR-16-1	Patient No	miR-15a	miR-16-1
1	decreased	decreased	16	increased	decreased
2	increased	increased	17	increased	increased
3	increased	increased	18	increased	increased
4	decreased	not change	19	decreased	decreased
5	increased	decreased	20	increased	increased
6	decreased	decreased	21	increased	increased
7	decreased	decreased	22	increased	decreased
8	decreased	decreased	23	not change	decreased
9	decreased	increased	24	increased	decreased
10	decreased	decreased	25	increased	increased
11	decreased	increased	26	decreased	increased
12	increased	decreased	27	decreased	decreased
13	increased	increased	28	decreased	increased
14	increased	increased	29	decreased	increased
15	increased	decreased	30	increased	decreased

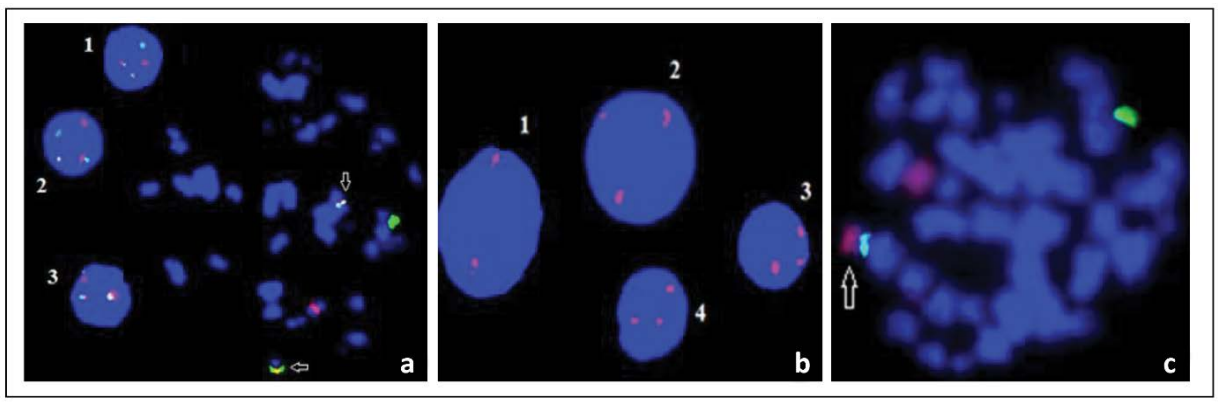

Figure 4: FISH images of different chromosomal abnormalites in DSP30+IL-2 induced samples: (a) iFISH(1,2,3) and mFISH images of t(11;14) (q13.3;q32.33) (Red Signal,11q13.3; Green Signal,14q32.33), (Yellow Signal, Fusion (t(11;14)(q13.3;q32.33)), (b) iFISH images of normal (1) and trisomy 12 (2,3,4) cells (Red Signal,Centromere:12/12p11.1-q11.1/D12Z3), (c) mFISH image of der(21)t(17;21) (Red signal, chr.17; Green Signal, chr. 21, arrow:fusion chromosome).

Table 5: Mann-Whitney U test P values showing the association between the expression levels of hsa-miR-15a gene and 13q14.3 deletions.

	(hsa-miR-15a)			
	2G/2R	2G/1R	1G/1R	2G
P value	0.913	0.477	0.701	0.542

Table 6: Mann-Whitney U test P values showing association between expression levels of hsa-miR-16-1a gene and 13q14.3 deletions.

	(hsa-miR-16-1)			
	2G/2R	2G/1R	1G/1R	2G
P value	0.017	0.008	0.585	0.007

correlated with deletions of 13q14 [Table 5] but decreased miRNA-16-1 expressions were found to be correlated [Table 6].

Discussion

CLL is a blood and bone marrow disease developing slowly over time resulting with over production of lymphocytes by bone marrow mostly affecting older adults. Many genetic and epigenetic factors contribute to the formation of the disease. [14-16] Therefore, detecting molecular and cytogenetic abnormalities has vital importance in diagnosis and treatment of the disease. [17,18] However, it is extremely difficult to diagnose the abnormalities using only conventional karyotyping techniques due to the low mitotic index of lymphocytes. Stimulating the CLL cells using various agents for cytogenetic analysis has major importance in diagnosis. One of these effective immunostimulatory agents is DSP30. Recent studies have shown the efficacy of DSP30 on CLL cells either alone or with another agent (IL-2). [17-22]

In this study, we used conventional karyotyping, iFISH and qRT-PCR techniques in 30 CLL patients and 15 healthy controls to detect the abnormalities developed in CLL. As karyotyping is considered as a low efficient method due to low proliferation of CLL cells, iFISH technique and DSP30 have found a wider area of application to stimulate CLL lymphocytes in order to detect chromosomal abnormalities.

Regarding the chromosome banding, we found that clonal chromosomal abnormalities were present in 16/23 (69.5%) patient samples induced with DSP30+IL-2 using conventional karyotyping method. The occurrence of del(13q), del(11q), +12, and del(17p) was established in 8.6% (2/23), 4.3% (1/23), 8.6% (2/23) and 4.3% (1/23), respectively. These values are comparable to the data published so far.[18,23,24] Other chromosomal abnormalities we determined are shown in Table 1 and common abnormalities diagnosed in CLL causing bad prognosis such as t(11;14)(q13;q32), [25-27] dup(12)(q13q22), t(14;19)(q32;q13.1) accompanying with +12 andadd(14)(q32) [26-28] were also present in our study.

Trisomy 12 mechanism and target genes are not clear in CLL and associate with t(14;19)(q32;q13). So; previous studies and our results support that these three abnormalities can be clonal changes in leukemiagenesis of CLL and investigating this mechanism can be beneficial in order to understand the pathogenesis of CLL and develop new treatment options. We also determined t(1;13)(q32;q34), inv(2)(p25q21),t(3;17)(q21;p11.2),del(13)(q22q32), t(14;19)(q24;q13), dup(17)(q21q23), rob(21;21)(p13;p13) which have not been reported previously. To our knowledge, this is the first study reporting t(14;19)(q24;q13) in CLL. Previous studies have reported t(14;19)(q32;q13.1) in CLL. However, our new translocation spans a larger area on chromosome starting from q24 region. This large area may be associated with CLL. Balanced rearrangements are rare in CLL and we detected balanced translocation rob(21;21)(p13;p13).

In non-induced samples; we determined successful karyotyping in 6/30 (20%) of the patients and detected clonal chromosomal abnormalities in 2/6 (33%) of the patients. However, no chromosomal abnormalities were detected in 4 induced samples. Also we determined that DSP30+IL-2 caused an increase in mitotic index by 2.5-folds. So our results showed that using DSP30+IL-2 in CLL patients is very effective to increase mitotic index which allows us to detect sufficient number and quality metaphases and detect chromosomal abnormalities by inducing division of leukemic cells as reported in other studies. [17-20]

We investigated 13q14.3 deletion with both conventional karyotyping and iFISH techniques. del(13q) was detected only in 8% of patients with karyotyping while iFISH revealed it in 65.3%. Previous studies have reported rates varying between 1%-56%. [18,23,29,30] Similar to previous studies, our results indicate that iFISH technique is a very sensitive and specific method for routine diagnosis of CLL. In order to detect the abnormalities seen in chromosomes, conventional karyotyping is the most common method. On the other hand, it is obligatory to know the abnormality in advance to apply iFISH method which uses specially designed probes. Use of both methods at the same time can yield better results. In conclusion, DSP30+IL-2 should be preferred for routine conventional karyotyping of CLL and both techniques should be complementarily applied.

Our study showed decreased expression of miR-16-1 which correlates with monoallelic and biallelic deletion. Recent studies reported decreased expression of miR-15a and miR-16-1 in CLL cases. Calin et al. reported decreased miR expression in their study. [16] Smonskey et al. showed that the decrease in miR-15a expression correlated with deletions but no significant association between miR-16-1 expression and deletions was found in their study. [9]

Conclusion

In conclusion, we didn't find any correlation between miR-15a expression levels and deletions but some patients with del(13q) had increased miRNA expression levels or some patients without del(13q) decreased expression levels. miR-16-1 expression was found to be decreased but it was not related to deletions. So these findings can suppose that expression of both miRNAs not only regulates chromosomal loss but also other mechanisms such as epigenetic changes. Also, increased expression levels of both miRs in del(13q) positive patients may be due to the presence of homologous gene loci miR-15b/miR-16-2 in the 3q25-26.1. Our results indicate that miR16-1 plays an effective role in CLL pathogenesis when compared with miR-15a.

According to our results, FISH method is more specific to detect

deletion of 13q14.3 than conventional karyotyping methods. We also suggest that using DSP30+IL-2 combination can be very useful for karyotyping methods of CLL. Also miR-16-1 expression levels can be used to investigate in CLL patients with del(13q).

Competing Interests

The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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