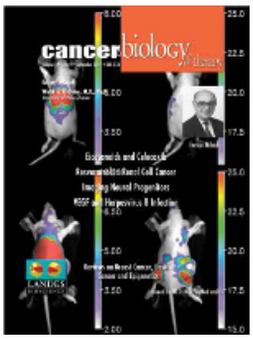

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Research Paper

Comparison of Genetic Changes Between Interphase and Metaphase Nuclei in Monitoring CML and APL Treatment Using DC-FISH Technique

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KEY WORDS

leukemia, FISH, interphase, metaphase, monitoring

ABSTRACT

In leukemias, the monitoring techniques on the response after the treatment have clinical importance for evaluating new therapeutic approaches and identifying the risk of relapse. In this study, genetic changes before and after chemotherapy in interphase and metaphase nuclei of bone morrow of adults with provisional diagnosis of leukemia were compared to understand the molecular characterization and pathogenesis of the leukemia for the classification of diagnosis and prognosis. We examined bone morrow cells of 47 chronic myeloid leukemia (CML) cases (29 of 47 at the time of diagnosis, 31 of 47 after chemotherapy) with the bcr/abl translocation probes and of 10 acute promyelocytic leukemia (APL) cases (7 of 10 at the time of diagnosis, 4 of 10 after chemotherapy) with the PML/RARα translocation probes by using dual color-flourescence in situ hybridization (DC-FISH). For each case, 400 interphase nuclei and 11 to 25 metaphases nuclei were analysed. The ratios of translocations before and after chemotherapy were compared between interphase and metaphase nuclei. After chemotherapy, though, translocations were detected in interphase nuclei of 29 of the 31 CML and 4 of the 4 APL cases, these translocations were determined in metaphase nuclei of only 14 of the 31 CML and 1 of the 4 APL cases with very low ratios ($p < 0.01$). The results showed that the rates of translocation positive interphase nuclei were higher than the rates of translocation positive metaphase nuclei ($p < 0.01$) after chemotherapy, so there may be some factors effecting proliferative activity of metaphase formation in leukemias.

INTRODUCTION

The molecular characterization of leukemias by using karyotyping, fluorescence in situ hybridization (FISH), southern analysis or polymerase chain reaction has provided insight into the pathogenesis of these neoplasms, aided in diagnosis, classification and prognosis, and improved recognition of minimal residual disease.[1-3] These techniques rely on the fact that virtually all leukemias are clonal and demonstrate alterations of DNA.[3] Chronic myeloid leukemia (CML) is a clonal stem cell disease characterized by the Philadelphia (Ph^1) chromosome [t(9;22)(q34;q11)] which is the genetic marker of CML and occurs in approximately 95 % of patients.[4,5] The patients carrying the Ph^1 chromosome have a dismal prognosis and need specific therapy, thus the ratio of Ph^1 positive cells after treatment are important to evaluate the dynamics of the disease for the therapeutic management.[6,7] This translocation constitutes bcr/abl fusion gene, the product of which has tyrosine kinase activity and is assumed to be the major cause of the chronic phase of CML.[4] Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) which has a special clinical and pathological hematological disorder characterized by a reciprocal balanced translocation between chromosome 15 and 17. This translocation constitutes PML/RARα chimerical gene, the transcript product of which could contribute the leukemic phenotype. This translocation [t(15;17)(q22;q12-21)] is diagnostic for APL. The detection and monitoring of this translocation in neoplastic APL cells is very vital in the diagnosis, therapy, prognosis and choosing the dose of treatment.[8-11] For patients with CML and APL, techniques for monitoring response to treatment are clinically important to identify, as early as possible, the patients who are at the a high risk of relapse, since some therapeutic approaches such as allogenic bone marrow transplantation may result in long-term disease-free survival.[4,12-15] The determination and quantification of residual leukemic cells in patients with CML and APL in remission exposes those who might be at risk of relapse and could require close clinical monitoring. Prognostically valuable structural and numerical chromosomal aberrations may sometimes go undetected by using conventional GTG-banding. Cytogenetic analysis of CML and APL is often handicapped by poor chromosome morphology and few malignant metaphases, and sometimes only metaphases

of normal cell clones may be detected after cell culture. In addition, the conventional cytogenetic techniques are time-consuming and their sensitivity is low because of obtaining less metaphases. Recently, the dual color fluorescence in situ hybridization (DC-FISH) has been shown to be useful in the direct visualizing of rearrangements such as bcr/abl, PML/RARα in both of the metaphase and the interphase nuclei. Thus, DC-FISH is used to monitor the response to therapy in various hematological malignancies.[8,16-19]

In this study, the rates of specific genetic changes were compared between interphase and metaphase cells of the translocation positive CML and APL patients at time of the diagnosis and after chemotherapy, by using bcr/abl and PML/RARα dual color locus specific probes with DC-FISH technique. Therefore, the value of translocation rates in interphase and metaphase nuclei in monitoring leukemia were examined at the time of diagnosis and after treatment.

MATERIAL AND METHODS

Patients and Preparing Specimens. Between March 2001 and June 2003, the heparinized bone marrow samples aspirated from cases of provisional diagnosis of CML and APL were referred to cytogenetic analysis. Interphase and metaphase nuclei of CML and APL patients were prepared successfully to be studied at the time of diagnosis and after chemotherapy. DC-FISH was applied to the 48 h cultured bone marrow samples by using the bcr/abl and PML/RARα locus specific probes. The translocation rates were compared between the interphase and metaphase cells of 47 CML cases who were bcr/ abl translocation positive and 10 APL patients who were PML/RARα translocation positive. Among the 47 CML patients, 29 cases (Cases 1–29) were examined at the time of diagnosis and 31 cases (Cases 1–13 and 30–47) were examined after chemotherapy. Similarly, 7 of the 10 APL patients (Cases 1–7) and 4 of the 10 APL cases were examined at the time of diagnosis (Cases 1 and 8–10) and after chemotherapy, respectively. The ages of the patients ranged from 19 to 71 years. The mean age was 50.5 ± 12.2 (± SD) years for CML and 37.2–10.85 years for APL patients. Peripheral blood samples of ten healthy persons were studied as the control group to determine the cut-off values of FISH signals. Bone marrow samples were cultured for 48 h in RPMI 1640 medium 1X including 15% fetal bovine serum. The cells were treated with colcemid (0.1 μg/ml) before harvesting by established cytogenetic technique which included hypotonic treatment (0.075 mol/L KCL) with fixation in acetic acid:methanol (1:3) at least three times. The cell suspension was stored in a freezer at -20°C until use. The metaphase spreads were also studied from the same preparations which were also used for the interphases belong to translocation positive cases.

FISH Analysis. FISH was performed using fixed cells including the metaphase and interphase nuclei on the cytogenetic preparations of bone marrow slides. The dual color probes specific to bcr/abl and PML/RARα fusion gene locuses which were available commercially (Vysis, Downers Grove, IL, USA) were used. All available metaphase nuclei as well as the interphase nuclei which show FISH signals were evaluated for translocation rates. The probes were denatured for 5 minutes at 72°C. At the same time, slides were placed into 40 μl pepsin (100 mg/ml) in 100 ml HCL (0.01 N) solution at 37°C for ten minutes, washed in 2xSSC and PBS solution, denatured in 70% formamide/2x SSC at 70°C for 5–6 minutes and dehydrated through an ascending alcohol series (70, 85 and 100%) and air-dried. The denatured probes were applied on the slides, overlaid with a cover slip and sealed with rubber cement. Hybridization was delayed overnight at 37°C, a post hybridization wash was performed twice in 50% formamide at 42°C for 3 minutes and then twice in 2xSSC at room temperature for 3 minutes. Slides were counterstained with DAPI (4,6 diamino-2-phenyl-indole) in mounting solution. The overlapping nuclei, disrupted nuclei, indistinguishable cells, and nuclei with diffuse and indistinguishable signals were eliminated. In specimens of both the control group and the patients, the mean percentage values of clearly identifiable signals of each probe and each nucleus were calculated separately. Therefore 11 to 25 metaphase nuclei and 400 interphase nuclei were analyzed for each specimen on a Quips Imaging System (Applied, UK) equipped with Nikon E 600 (Japan) standard conventional epifluorescence microscope and a filter set (triple;dapi/red/green, dual colour;red/green, single red and single green, Vysis, USA). The color photomicrographs were taken with a Cool Snap camera (Photometriks) equipped with a computerized system. "Wilcoxon Signed Ranks" test was used for the comparison of statistical differences between translocation rates in the interphase and metaphase nuclei at the time of diagnosis and after chemotherapy.

RESULTS

The interphase and metaphase nuclei of peripheral blood samples of control group (n = 10) were examined to determine the sensitivity and specificity of the dual-color FISH technique. The mean percentages of nuclei showing two normal signals for locus specific bcr/abl and PML/RARα dual-color probes were 98% and 97%, respectively. The distributions of the bcr/abl and PML/RARα translocation frequencies in interphase and metaphase nuclei at the time of diagnosis and after chemotherapy are listed in the Table1A and B and Table 2A and B respectively. At the time of diagnosis, the bcr/abl translocations were determined in interphase nuclei (Fig. 1A) of all 29 CML cases (Cases 1–29) with a ratio ranging from 71% to 100%. The bcr/abl translocations were also determined in metaphase nuclei of all 29 CML cases with a ratio ranging from 35.2% to 100% which were less than the ratio of the interphase cells (Table 1A). After chemotherapy, bcr/abl translocation was not determined in interphase nuclei of 2 of 31 (6.4%) CML cases (Cases 8 and 37), however these were determined in the interphase nuclei of the remaining 29 cases with a ratio ranging from 3% to 72% (Table 1B). After chemotherapy, though the bcr/abl translocations were determined in the metaphase nuclei (Fig. 1B) of only 14 of 31 CML cases (Cases 3, 5, 10, 11, 12, 31, 33, 38–40 and 42–46), no translocation was determined in metaphase nuclei of remaining 17 of 31 (54.8%) CML cases (Cases 1, 2, 4, 6–9, 13, 30, 32, 34–37, 41, 44, 47) (Table 1B). At the time of diagnosis, the PML/RARα translocations were determined in the interphase nuclei (Fig. 2A) of 7 all APL cases (Cases 1–7) with a ratio ranging from 47% to %100 and in the metaphase nuclei of 6 of 7 APL cases (Case; 1,3-7) with a ratio ranging from 21% to 90% which were less than the ratio of the interphase nuclei. So, at the time of diagnosis no translocation was determined in one APL case (Case 2) (Table 2A). After chemotherapy, though 4 APL cases (Cases; 1 and 8–10) had PML/RARα translocations in the interphase nuclei with a ratio ranging from 6 to 10.5% (Table 2B), PML/RARα translocation was found in metaphase nuclei of only 1 of these 4 (25%) APL cases (Case 1) (Fig. 2B). Translocation rates of both the interphase and metaphase nuclei were calculated and differences between the values at the time of diagnosis and after chemotherapy were compared by using "Wilcoxon Signed Ranks Test".

Wilcoxon Signed Ranks Test Results. At the time of diagnosis, among the all cases, the mean translocation percentage in the metaphase nuclei showed a small reduction of 19.76% (-19.76) compared to the interphase nuclei. Furthermore, after chemotherapy, the translocation percentages of the metaphase nuclei showed a reduction of 75.14% (-75.14) compared with the interphase nuclei. After chemotherapy, the ratio of reduction in the translocation rates of the metaphase nuclei was significantly higher than the reduction rate of translocations in interphase nuclei, ($p < 0.01$). On the other hand, no prominent statistical significance was found at the PML/RARα translocation rates of the metaphase nuclei after chemotherapy in comparison to the interphase nuclei ($p > 0.05$). At the time of diagnosis, while 35 of 36 cases had translocation in metaphase nuclei (29 of 29 CML and 6 of 7 APL cases), after chemotherapy, only 15 of 35 cases had translocation in metaphase nuclei (14 of 31 CML and 1 of 4 APL cases). So, statistically, the number of patients having translocations in their metaphases after chemotherapy was quite significantly less than those in metaphases at the time of diagnosis ($p < 0.01$).

Table 1 **The Distributions of the bcr/abl Translocation Frequencies in the Interphase and Metaphase Nuclei of 29 CML Patients at the Time of Diagnosis and of 31 CML Patients After Chemotherapy Respectively**

(A) The number and percent of the cells with bcr/abl translocation at the time of diagnosis				(B) The number and percent of the cells with bcr/abl translocation after chemotherapy			
Case No	**Age/ Sex**	**For interphase nuclei**	**For metaphase nuclei**	**Case No**	**Age/ Sex**	**For interphase nuclei**	**For metaphase nuclei**
1	66/M	368/400 (92%)	22/25 (88%)	1	66/M	56/400 (14%)	0/20 (0%)
2	53/F	392/400 (98%)	19/20 (95%)	2	53/F	152/400 (38%)	0/19 (0%)
3	52/F	376/400 (94%)	16/20 (80%)	3	52/F	72/400 (18%)	1/15 (6.6%)
4	38/M	352/400 (88%)	10/16 (62.5%)	4	38/M	140/400 (35%)	0/20 (0%)
5	58/F	376/400 (94%)	19/20 (95%)	5	58/F	112/400 (28%)	1/17 (5.8%)
6	34/F	376/400 (94%)	12/15 (80%)	6	34/F	128/400 (32%)	0/18 (0%)
7	28/F	312/400 (78%)	12/19 (63.1%)	7	28/F	32/400 (8%)	0/20 (0%)
8	55/M	368/400 (92%)	18/20 (90%)	8	55/M	0/400 (0%)	0/17 (0%)
9	38/M	288/400 (72%)	14/22 (59%)	9	38/M	12/400 (3%)	0/18 (0%)
10	50/M	372/400 (93%)	7/17 (41.1%)	10	50/M	192/400 (48%)	4/13 (30.7%)
11	67/F	364/400 (91%)	17/20 (85%)	11	67/F	248/400 (62.5%)	5/20 (25%)
12	38/F	296/400 (74%)	9/20 (45%)	12	38/F	272/400 (68%)	3/16 (18.7%)
13	39/M	272/400 (68%)	9/22 (40.9%)	13	39/M	232/400 (58%)	1/20 (5%)
14	48/M	344/400 (86%)	13/17 (76.4%)	30	71/M	20/400 (5.5%)	0/20 (0%)
15	50/M	384/400 (96%)	15/18 (83.3%)	31	61/F	34/400 (8.5%)	1/16 (6.2%)
16	28/M	400/400 (100%)	15/15 (100%)	32	62M	24/400 (6%)	0/20 (0%)
17	70/F	392/400 (98%)	19/20 (95%)	33	54/M	88/400 (22%)	3/25 (12%)
18	50/M	304/400 (76%)	6/17 (35.2%)	34	70/M	24/400 (6%)	0/22 (0%)
19	31/M	380/400 (95%)	14/14 (100%)	35	58/F	40/400 (10%)	0/20 (0%)
20	47/F	324/400 (81%)	6/11 (54.5%)	36	53/M	36/400 (9%)	0/19 (0%)
21	63/F	284/400 (71%)	8/17 (47%)	37	70/M	0/400 (0%)	0/17 (0%)
22	29/F	328/400 (82%)	11/19 (57.8%)	38	48/F	232/400 (58%)	0/20 (0%)
23	50/M	376/400 (94%)	17/17 (100%)	39	45/M	192/400 (48%)	4/18 (22.2%)
24	42/F	376/400 (94%)	20/24 (83.3%)	40	36/F	272/400 (68%)	4/14 (28.5%)
25	46/F	368/400 (92%)	15/20 (75%)	41	42/F	12/400 (3%)	0/16 (0%)
26	70/F	344/400 (86%)	13/16 (81.2%)	42	44/F	288/400 (72%)	7/20 (35%)
27	46/M	392/400 (98%)	19/20 (95%)	43	68/M	72/400 (18%)	1/20 (5%)
28	53/F	384/400 (96%)	12/16 (75%)	44	38/M	16/400 (4%)	0/17 (0%)
29	57/F	364/400 (91%)	12/15 (80%)	45	57/F	176/400 (44%)	6/20 (3%)
				46	56/F	128/400 (32%)	3/21 (14.2%)
				47	45/M	32/400 (8%)	0/16 (0%)

DISCUSSION

Rapid detection and true assessment of genetic changes are very effective in cancer genetics, because certain chromosomal abnormalities have been shown to be significant in terms of diagnosis and prognosis of the disease.[4,14,20,21] The identification of certain translocations or chromosomal abnormalities in these cases shows an important advantage of interphase analysis over conventional chromosome analysis, because the interphase method does not depend on the proliferative activity or the presence of metaphase spreads.[1,3,22] A further advantage of DC-FISH analysis of interphase nuclei is indicated by the finding that some preallograft and post-allograft specimens in clinical remission where no translocations was detected cytogenetically exhibited some nuclei with evidence of the translocation as bcr/abl.[14,21] So, it may be suggested that the 9;22 translocation may be present as a minor clone at the diagnosis even though not detected cytogenetically. The detection of the rearrangement in remission, which was considered to be cytogenetically abnormal, indicates that interphase DC-FISH may be able to detect a low frequency of abnormality in interphase nuclei where the number of fusion signals is higher than that found in control cases.[13,17] The determination of the bcr/abl translocation before treatment is also quite important for differential diagnosis of atypical CML from typical CML, for diagnostic purpose, for evaluating hematological and cytogenetic stages, and for prediction of the prognosis. Similarly, the determination of the PML/RARα translocation in APL is also quite important for diagnosis, post-treatment follow-up, and prognosis. The evaluation of genetical changes for both CML and APL is also important for the progression of leukemic clone, especially after chemotherapy. This is further supported with

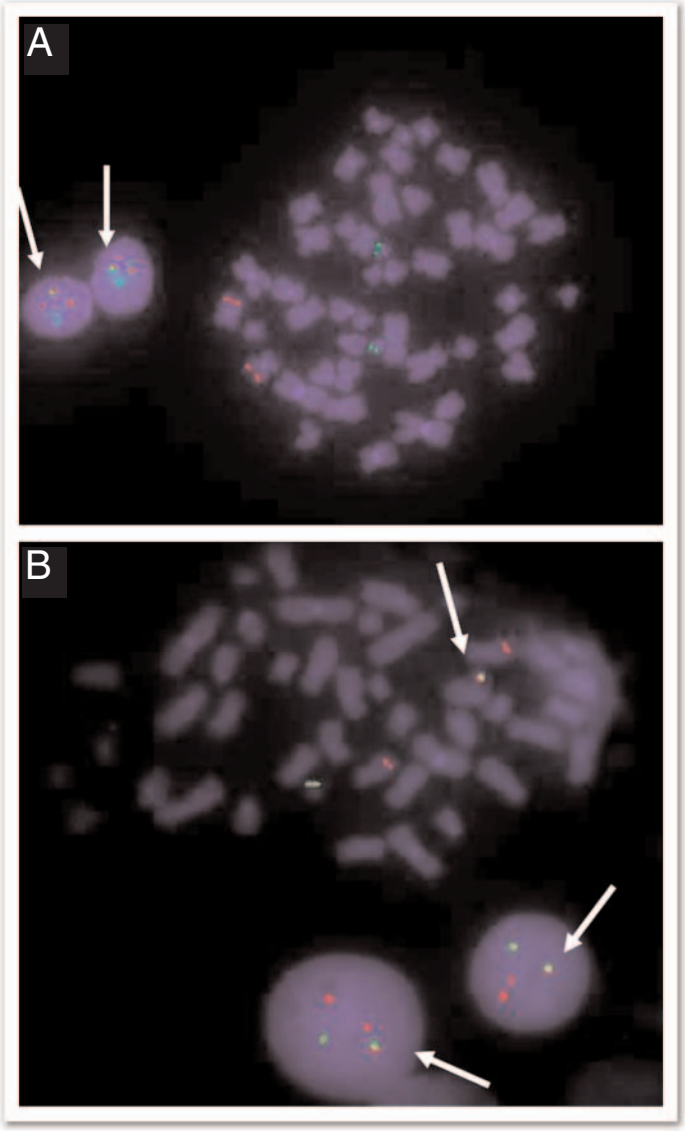

Figure 1. (A) Two arrows show bcr/abl translocation in interphase nuclei, there are no translocation in metaphase nuclei. (B) Three arrows show bcr/abl translocation in interphase and metaphase nuclei. Green signals show bcr region on chromosome 22 and red signals show abl region on chromosome 9, overlapped green/red or yellow signals belong to bcr/abl fusion gene. Third red signals belong to remain signal on translocated chromosome 9 (extra signal).

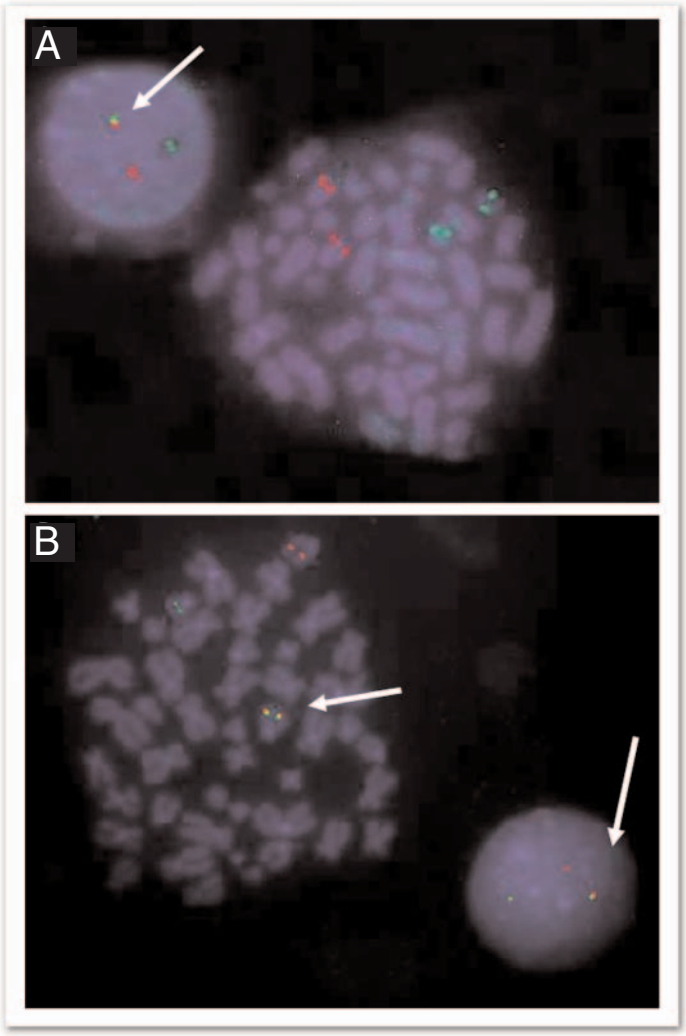

Figure 2. (A) Arrow shows PML/RARα translocation on interphase nucleus, there are no translocation on metaphase nucleus. (B) Two arrows show PML/RARα translocation in interphase and metaphase nuclei. Green signals show RARα region on chromosome 17 and red signals show PML region on chromosome 15, overlapped green/red or yellow signals belong to PML/RARα fusion gene.

some studies showing that cytogenetic evaluation has some disadvantages due to the low quality of metaphases and cultural problems when compared to the FISH and PCR methods.[23-27] With DC-FISH method, optimal results can be obtained on the standard cytogenetic pellets or bone marrow smears even if stored for years. Our findings which were correlated with other previous studies were considered as quiet useful for the diagnosis and monitoring of both CML and APL.[2,6-8,18,23,26,27] In the literature, some studies revealed that it is not always possible to detect genetic changes with conventional cytogenetic methods in metaphase nuclei of leukemic cells.[5,9,16,17,20,26] In our study, the ratios of translocations detected in the interphase and metaphase nuclei of the cultured bone marrow cells were compared by using DC-FISH technique. The aim of this was to investigate the reason of the failure for detecting genetic changes with real value in metaphases by conventional techniques and to compare efficiency of the DC-FISH and cytogenetic techniques for the follow-up of residual disease in monitoring CML and APL treatments. In the first group, 29 of 47 CML cases were analyzed at the time of diagnosis for the bcr/abl translocation in both of the interphase and metaphase nuclei. The results showed that the ratio of changes in the interphase and in the metaphase nuclei at the time of diagnosis did not differ significantly (Table 1A). In addition, 7 of 10 APL cases were analyzed at the time of diagnosis for the PML/RARα translocation both in the interphase and metaphase nuclei and the results similarly showed no statistically significant differences in the ratio changes, though no translocation was detected in the metaphase nuclei in one of these 7 APL cases (Table 2A and Case 2). In the second group, 31 CML cases were analyzed after chemotherapy for the bcr/abl translocation in both of the interphase and metaphase nuclei. The results showed a highly significant difference between these two groups. Although, bcr/abl translocation was detected in the interphase nuclei of 29 of 31 CML cases, this translocation was only detected in the metaphase nuclei of 14 of 31 CML cases after chemotherapy (Table 1B). Similarly, although the

Table 2 **The Distributions of the PML/RARα Translocation Frequencies in the Interphase and Metaphase nuclei of 7 APL Patients at the Time of Diagnosis and of 4 APL Patients After Chemotherapy Respectively**

(A) The number and percent of the cells with PML/RARα translocation at the time of diagnosis				(B) The number and percent of the cells with PML/RARα translocation after chemotherapy			
Case No	**Age/ Sex**	**For interphase nuclei**	**For metaphase nuclei**	**Case No**	**Age/ Sex**	**For interphase nuclei**	**For metaphase nuclei**
1	32/F	288/400 (72%)	7/15 (46.6%)	1	32/F	42/400 (10.5%)	1/20 (5%)
2	55/M	188/400 (47%)	0/20 (0%)	8	39/M	34/400 (8.5%)	0/17 (0%)
3	19/M	288/400 (72%)	13/20 (63.6%)	9	33/F	24/400 (6%)	0/20 (0%)
4	38/F	376/400 (94%)	12/19 (63.1%)	10	49/M	24/400 (6%)	0/18 (0%)
5	44/F	288/400 (72%)	4/19 (21%)				
6	24/M	376/400 (94%)	13/25 (52%)				
7	39/M	400/400 (100%)	18/20 (90%)				

PML/RARα translocation was detected in the interphase nuclei of all 4 APL cases, the translocation was detected only in one of 4 CML cases in the metaphase nuclei after chemotherapy (Table 2A). No significance was found in the differences of the interphase and metaphase nuclei percentages of APL cases after chemotherapy; the insufficient number of cases may be a reason for this unsignificance, since only one case had PML/RARα translocation in the metaphase nuclei (Table 2B and Case 1). The results show that the cytogenetic outputs may not be always confidential in the monitorization of the leukemia treatment, so there may be a meaningful failure in the determination of the translocations in the metaphase nuclei rather than in the interphase ones, especially after chemotherapy ($p < 0.01$). The determination of low translocation rates in the metaphase nuclei after treatment by using DC-FISH in a few cases (14 of 31 CML and 1 of 4 APL cases) may suggest that the chemotherapeutic drugs could have an effect on the leukemic clone cells, especially on the cell division cycle. Chemotherapeutics probably had an effect on the metaphase phase of the cell division cycle and for this reason the count of the metaphase nuclei in the leukemic clone could be less than nonleukemic ones. To our knowledge, there are only a couple of studies indicating the effects of the chemotherapeutics on the cell cycle in leukemias.[28,29] So, further studies examining the effects of chemotherapeutics on all stages of the cell division are required. The use of DC-FISH and RT-PCR methods in the clinical progress of the minimal residual disease will be both qualitatively and quantitatively more beneficial than the cytogenetic methods. It is suggested that, false-negative results may be detected in the cytogenetic evaluation especially after chemotherapy in monitoring of leukemia with less genetic changes.

In this study, the comparison of the DC-FISH results of the interphase and metaphase nuclei for the translocatios mentioned above indicates that the interphase FISH is more sensitive in detecting low rate rearrangements in leukemias than the conventional cytogenetic analysis. This method is also quite effective in the follow-up of patients especially with the genetic changes even at low quantity after treatment. The quite reduced translocation rates or absence of translocation after chemotherapeutic treatment may give an idea of full remission or exact cytogenetic or hematological cure.

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