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Sequential fluorescence *in situ* hybridization analyses for trisomy 12 in chronic leukemic B-cell disorders

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Background and Objectives. Trisomy 12 is one of the most common chromosomal abnormalities in B-cell chronic lymphocytic leukemia (CLL). The aberration is readily detected by fluorescence *in situ* hybridization (FISH). There are only a few reports in which FISH analyses have been used to study the expansion of the trisomy 12 clone over time.

Design and Methods. Repeat FISH analyses were performed in 77 patients with a chronic leukemic B-cell disorder. The aim was to study the development of the trisomy 12 clone throughout the course of the disease, to measure the effect of therapy on the proportion of trisomic cells, and to relate the findings to the response to therapy.

Results. Fifty-eight of the 60 patients with no trisomy 12 at the initial test were consistently disomic for chromosome 12, while 2 patients seemingly acquired trisomy 12 during follow-up. Seventeen patients showed trisomy 12 at the first test. Expansion of the trisomy 12 clone was seen in all patients with a progressive lymphocytosis. In contrast to poor responders, patients responding well to chemotherapy showed a significant decrease in the proportion of $CD19^+$ cells with trisomy 12. The effect of purine analogs in patients with trisomy 12 seemed inferior, both clinically and when studying the effect on the trisomic clone.

Interpretations and Conclusions. There is a strong association between expansion of the trisomy 12 clone and progressive disease, both in treated and untreated patients. Conversely, reduction of the trisomic B-cell clone was linked to clinical response to chemotherapy. Acquisition of trisomy 12 remains a rare event.
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Key words: chronic leukemic B-cell disorders, CLL, trisomy 12, APAAP-FISH, treatment.

Trisomy 12 is one of the most common chromosomal abnormalities in B-cell chronic lymphocytic leukemia (CLL),[1-4] but has also been found in a substantial proportion of cases with other chronic leukemic B-cell disorders such as lymphoplasmacytoid lymphomas.[5,6] It has been shown that the trisomy 12 aberration is restricted to the neoplastic B-cells, and is usually found only in a subset of them.[7–11] There are contradicting reports on the prognostic importance of trisomy.[12] In some reports, the abnormality has been associated with a poor prognosis, while in others no inferior survival was seen in patients with trisomy 12-positive CLL.[3-5,12-17] Trisomy 12 is predominantly found in CLL with atypical morphology, while it is a rare finding in typical CLL.[4,5,17,18] Despite the association between trisomy 12 and atypical CLL morphology, it has been shown by MGG/FISH studies of individual cells that the aberration is not restricted to lymphocytes with atypical morphology.[19] The karyotype in CLL is usually stable over time.[20,21] Repeat fluorescence *in situ* hybridization (FISH) in 15 CLL cases showed no case with acquisition of trisomy 12 during 13-73 months.[22] In three other FISH studies, trisomy 12 was acquired, overall, in 7 CLL patients with Richter's transformation.[23-25] Since the clonal evolution was associated with morphologic transformation, it was suggested that trisomy 12 was correlated with disease progression.[25]

In 12 CLL patients with trisomy 12 studied by repeat metaphase cytogenetic analyses, the trisomic clone resulted as being a constant finding.[20] Using FISH, a persistent mosaicism of the trisomy 12 clone was found in 4 cases, with the percentages of trisomic tumor cells remaining constant over a period of 15 to 81 months.[22] In another sequential FISH study no significant change in the percentage of trisomic cells was seen over a 4-year period, even in CLL patients with disease progression.[26] In contrast, a longitudinal FISH study of 30 CLL cases showed that all 14 patients with trisomy 12 had an increase of clonal cells during follow-up.[25] Little is known about the effect of therapy on the trisomy 12 clone in CLL. In some cases the number of trisomic cells increased following treatment with alkylating agents, while in others a reduction was observed in patients who achieved remission after combination regimens.[25,27] The aim of the present study was to perform sequential

FISH analyses to detect trisomy 12 in patients with chronic leukemic B-cell disorders and to study the development of the trisomy 12 clone during the course of the disease. We also wanted to measure the impact of chemotherapy on the proportion of cells with trisomy 12, and relate the findings to the response to therapy.

Design and Methods

Patients

Seventy-seven patients with a chronic leukemic B-cell disorder were followed prospectively at the Hematology clinic, Danderyd Hospital from diagnosis until April 2000. The FISH analyses were performed between September 1994 and April 2000. Seventeen patients (22%) showed trisomy 12 cells in the first FISH analysis, while 60 (78%) lacked the aberration. FISH analyses of peripheral blood were scheduled to be performed regularly at the time of clinical check-ups. The CLL diagnosis was made according to the FAB criteria and the REAL classification.[28,29] Leukemic disease was defined as presence of >5×10^9/L clonal, mature-appearing B-lymphocytes in the peripheral blood as shown by immunoglobulin (Ig) light chain restriction. The morphology and the immunophenotype of the trisomic cases are described in detail in Table 1. Only one of these patients had a typical CLL. Of the non-trisomic cases, 34 had morphologically typical CLL, while 23 had atypical CLL (aCLL) including 20 atypical CLL of mixed type (aCLL$_{mix}$) and 3 aCLL with lymphoplasmacytoid features (aCLL$_{LP}$), 1 CLL/PL, 1 PLL and 1 SLVL.

Table 1. Description of morphologic and immunophenotypic features of 17 patients with trisomy 12 (1st test).

Pt No.	*MNC with trisomy 12*	*Morphology (%)*	*CD19+/ CD5+*	*CD19+/ CD23+*	*Ig light chain*
8	6.8	aCLL$_{mix}$	pos	nd	dim
25	36.2	aCLL$_{mix}$	pos	pos	moderate
33	9.6	aCLL$_{mix}$	pos	pos	dim
34	2.6	aCLL$_{LP}$	pos	neg	moderate
44	12.8	aCLL$_{mix}$	pos	nd	dim
88	2.4	aCLL$_{mix}$	pos	pos	dim
97	12.8	aCLL$_{mix}$	pos	nd	dim
126	15.2	aCLL$_{mix}$	pos	nd	dim
130	8.6	aCLL$_{mix}$	pos	nd	dim
154	12.0	aCLL$_{mix}$	pos	pos	moderate
157	43.6	aCLL$_{mix}$	pos	pos	moderate
207	25.0	aCLL$_{mix}$	pos	pos	dim
236	27.4	aCLL$_{LP}$	pos	pos	dim
237	20.2	aCLL$_{mix}$	pos	pos	dim
241	13.8	aCLL$_{LP}$	pos	pos	moderate
256	9.8	aCLL$_{mix}$	pos	pos	dim
369	41.2	CLL	pos	nd	dim

CLL = chronic lymphocytic leukemia of B-cell type, aCLL$_{mix}$ = atypical CLL of mixed type and aCLL$_{LP}$ = aCLL with lymphoplasmocytoid features. nd = not done.

Twelve patients with trisomy 12 required therapy during follow-up. Altogether the proportion of cells with trisomy 12 before and after therapy was evaluable for 23 treatment episodes, including 8 episodes with purine analogs (CdA and fludarabine), 13 with oral alkylators (mainly chlorambucil) and 6 with different combination regimens (CHOP, MIME, DexaBEAM). All drugs were given according to standard protocols, which did not change during the study period. A major clinical response was defined as >50% tumor reduction and disappearance of all symptoms, a minor clinical response as 25-50% tumor reduction and regression of symptoms, and no response as <25% tumor reduction or progression. The study was approved by the Ethics Committee at Karolinska Hospital.

Fluorescence in situ hybridization (FISH)

Gradient-separated peripheral blood mononuclear cells (MNC) were obtained from all patients using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). A 175 bp large a-satellite probe specifying the centromeric region of chromosome 12 (Oncor, Gaithersburg, MD, USA) was used for the detection of trisomy 12, as previously described.[5] Briefly, the MNC fraction was resuspended in phosphate-buffered saline to a concentration of approximately 5×10^9 MNC/L. One or two drops of this cell suspension were dropped on slides and allowed to air dry. The films were frozen wrapped in foil paper and stored at –20°C until their use. After thawing, the slides were hybridized according to the recommendations from the manufacturer and analyzed in a fluorescence microscope (Zeiss EPI-Fluorescence Axioskop 20). In each case 500 well spread cells were scored. Cells with three distinct fluorescent signals were counted as trisomic. A sample was considered to be positive for trisomy 12 if >1.3% (mean of normal controls ± 3SD) of the cells showed three fluorescent signals. This cut-off limit was chosen after analyzing 10 peripheral blood samples from healthy individuals. The proportion of MNC with 3 signals in normal subjects was 0.2±0.3% (mean±SD), range 0-1.0%.

Immunocytochemistry

To detect the proportion of B-cells in a sample, immunocytochemistry staining with a monoclonal antibody (Dakopatts, Copenhagen, Denmark) against CD19 was performed using the alkaline phosphatase method (APAAP) as earlier described.[30] Five hundred cells were scored in each case. For combined immunocytochemical and FISH analysis (APAAP/FISH), the slides were fixed in 4% NBF for 5 min and blocked with 1% bovine serum albumin for 10 min before being incubated for 1 hour with a primary anti-CD19 mouse antibody (Dakopatts, Copenhagen) diluted 1:50. This step was followed by incubation with biotinylated rabbit anti-mouse antibody diluted 1:200 for 30 min, and alkaline-phosphatase conjugated avidin for 30 min. Tris-buffered saline was used to dilute the antibodies and in all washing steps. Naphthol AS-MX phosphate (Sigma, St. Louis, MO, USA) was

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Table 2. Clinical features of 77 patients with chronic leukemic B-cell disorders included in this study.

	Patients without trisomy 12[a)] n=60		*Patients with trisomy 12 n=17*		
Median (range) no. of FISH tests	3	(2-8)	6	(3-18)	
Median (range) time between FISH tests (months) [b)]	31	(1-67)	33	(7-65)	
No. of patients with progressive disease (%)	41	(68)	12	(71)	
Median (range) time to treatment (months)	37	(0-190)	21	(0-88)	p =0.28
No. of patients deceased (%)	20	(33)	7	(41)	
Median (range) survival time (months)	149	(9-286)	99	(28-147)	p =0.57

a) two patients acquired trisomy 12 during follow-up; b) if >2 FISH tests were done, the time between the first and the last sample was used.

used as enzyme substrate according to standard procedures. The immunostained slides were then refixed in 4% NBF and FISH for chromosome 12 was performed as described above. The percentage of CD19+ cells with trisomy 12 was determined in two ways. With the APAAP/FISH method, FISH and APAAP were performed simultaneously on the same slide which allowed identification of trisomy 12 in CD19+ cells. Alternatively, FISH and APAAP were done on separate slides of the same sample. The percentage of cells with trisomy 12 was then divided by the percentage of CD19+ cells. Comparison between the results obtained by the two methods showed that they were quite comparable (r=0.876, p <0.001). For the purposes of this study, we therefore chose to express the percentage of CD19+ cells with trisomy 12 using the less complicated method with separate FISH and APAAP analyses.

Statistical analysis

Descriptive statistics and graphical methods were used to characterize the data. Regression analysis was used to correlate the FISH and APAAP results in individual patients with combined APAAP/FISH. Student's paired t-test was used to compare changes in the trisomy 12 clone before and after therapy, and in the case of a statistically significant result, the probability value (p value) was given. In this statistical analysis, only the first treatment episode was included (n=23). The actuarial times to treatment and survival were calculated using Kaplan-Meier survival plots.

Results

Patients without trisomy 12

In the first FISH analysis 60 (78%) of 77 patients showed disomy for chromosome12, i.e. exhibited ≤1.3% MNC with 3 fluorescent signals. The number of repeat FISH tests and the interval between the first and the last test is shown in Table 2. Forty-one cases (68%) showed progressive disease during follow-up and the median time to treatment was 37 months (range: 0–190) (Table 2).

Fifty-eight (97%) of the 60 patients without trisomy 12 had a consistent FISH pattern with disomy for chromosome 12 in all tests. Two (3%) patients, #12 and 173, acquired an extra chromosome 12 during the course of their disease. Patient #12 with a typical CLL had asymptomatic disease for almost 12 years. During this period two FISH analyses showed a low percentage of trisomic cells, well below the cut-off level. When he developed a large tumor bulk in the abdomen, trisomy 12 was found in 2.2% of his peripheral blood MNC. No biopsy of the abdominal lymph nodes was performed, but the patient responded well to chlorambucil treatment and remains in a good clinical remission more than one year off therapy. Patient #173 with an atypical CLL had a good partial remission after 8 CHOP courses, and has not required further therapy during a follow-up period of more than 7 years. FISH studies were not performed before treatment, but at 24, 29 and 74 months after the CHOP therapy. The first two tests were negative, but the last one showed trisomy 12 in 40.4% of the MNC.

Patients with trisomy 12

Seventeen (22%) patients exhibited trisomy 12 in the first FISH test in 2.4-43.6% (median 12.8) of the MNC (Table 1), and in between 4.1 and 50.5% (median 18.9) of the CD19+ cells. The number of FISH tests performed in each patient and the time between the first and the last test are shown in Table 2. Twelve cases (71%) had progressive disease and required therapy during follow-up (Table 2).

Eight untreated patients showed an increasing lymphocytosis during follow-up; 4 had progressive disease and were given chemotherapy, while 4 had an otherwise clinically stable course and did not require therapy. In all these 8 cases the rise in lymphocyte count was associated with an increase in the proportion of CD19+ cells with trisomy 12 (Figures 1a-b).

Impact of therapy on the trisomy 12 clone

The proportion of CD19+ cells with trisomy 12 was studied before and after chemotherapy and related to the lymphocyte counts. Results from 23 treatment episodes in the 12 patients are summarized in Table 3. After the first evaluable treatment episode the median lymphocyte count of the 12 patients dropped from 20.6 to 4.5×10^9/L (p =0.0052), while the median percentage of CD19+ cells with trisomy 12 decreased from 33.0 to 17.6% (p =0.0009). When the three treatment groups

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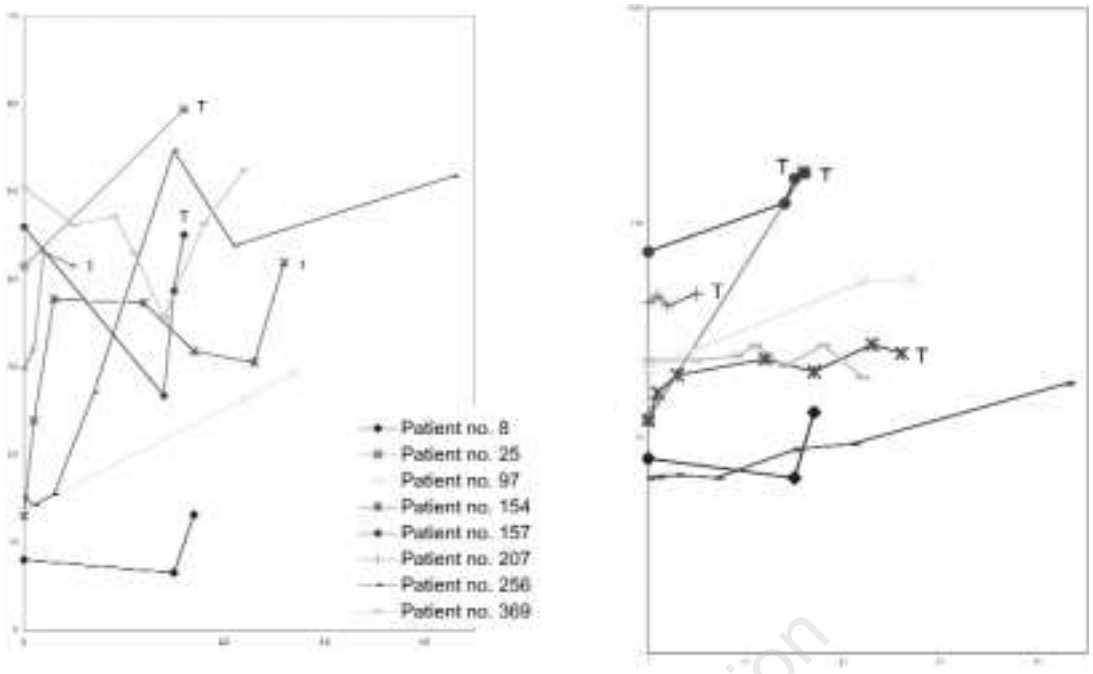

Figure 1. Diagrams showing (a) the increase in the percentage of CD19⁺ cells with trisomy 12 over time in 8 untreated patients with chronic leukemic B-cell disorders (T = start of therapy), and (b) changes in total lymphocyte counts during the same period.

were studied separately, it appeared that single alkylator and combination therapy both resulted in a significant decrease in the percentage of CD19⁺ cells with trisomy 12, while treatment with purine analogs did not have a significant impact on the proportion of trisomic cells (Table 3). However, in 5 of the 8 patients receiving purine analogs the relative number of CD19⁺ cells with trisomy 12 increased to ≥1% after therapy, whereas this occurred in only 1 of 15 patients in the other treatment groups (Table 4). The impact of treatment on the trisomy 12 clone was also analyzed in relation to the clinical outcome. Compared to patients with minor or no responses, patients with major responses showed a much more pronounced decrease in lymphocyte counts and percentage of CD19⁺ cells with trisomy 12 (Table 3).

Discussion

Trisomy 12 is the most common numerical chromosomal aberration in B-CLL and several studies have shown that the aberration is associated with features of a more aggressive disease, i.e. atypical morphology, higher stage, shorter time to treatment and impaired survival.[3-5,12-14] Trisomy 12 has been extensively studied in B-CLL and other chronic leukemic B-cell disorders, but there are only a few reports on the development of the trisomy 12 clone during the course of the disease. Juliusson *et al.*[20] reported that, using metaphase cytogenetics, the trisomy 12 clone in CLL appeared constant over time. Their results were later supported by some studies using sequential FISH,[22,26] but not by others.[24,25] In the present study, 77 patients with chronic leukemic B-cells disorders from a single center were followed prospectively with sequential FISH analyses. Between 2 and 8 FISH tests were performed in 60 cases that showed disomy for chromosome 12 initially, and between 3 and 18 tests in the 17 patients with trisomy 12. Even though the predetermined test schedule proved difficult to keep, sufficient numbers of FISH tests were performed to show clearly that the percentage of cells with trisomy 12 increased during the follow-up period in patients with signs of progressive disease requiring therapy. Our findings thus agree with those in the report by Garcia-Marco *et al.*,[25] who found an expansion of the trisomy 12 clone over a 4-year period in 14 CLL patients who had progressive disease requiring therapy. This is further supported by the present finding of an expansion of the trisomy 12 clone also in all the patients with progressive lymphocytosis not requiring therapy. There may be several explanations as to why not all sequential FISH studies in CLL have been able to show an association between progressive disease and expansion of the trisomic clone. In the study by Roghoebier *et al.*,[22] only 4 cases were investigated, and none of them required therapy during the study. Auer *et al.*[26] studied 14 patients on two occasions, 4 years apart. The percentage of cells with trisomy 12 in this study was ≤5% in half of the patients. Studies of such small clones, particularly in cases with low white blood cell counts and few leukemic cells, require special considerations since contaminating T-cells may dilute the tumor cells and

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Table 3. Effect of type of chemotherapy and clinical response on the median percentage of CD19+ cells with trisomy 12 and median lymphocyte counts.

	Lymphocyte counts (x10⁹/L)			*CD19+ cells with trisomy 12 (%)*		
No. of pts. (n)	*before*	*after*	*p*	*before*	*after*	*p*
Type of chemotherapy [a]						
PA (8)	6.3	4.0	0.11	21.4	29.0	0.47
SA (10)	22.9	5.2	0.01	41.4	21.8	0.02
Comb (5)	4.6	2.2	0.16	24.4	6.3	0.03
Response to chemotherapy [b]						
Major (11)	24.7	4.6	0.006	41.9	24.4	0.004
Minor/NR (12)	4.6	3.3	0.04	19.9	11.8	0.23

a) PA: purine analogs, SA: single alkylator, comb: combination regimens; NR: no response.

give rise to falsely low percentages of trisomy 12+ cells. To evaluate the size of the trisomic clone correctly in such cases, it is necessary to make corrections based on the number of T-cells in the sample,[22,31] or express the percentage of cells with trisomy 12 relative to the number of CD19+ cells (present study). Moreover, several of Auer's patients had been treated between the two tests.[26] This makes it more difficult to measure an expansion of the trisomy 12 clone, since the relative size of the trisomic clone decreases significantly after successful chemotherapy, as shown in the present study and two earlier ones.[25,27] Most data thus suggest that in CLL patients with trisomy 12, progressive disease is associated with an expansion of the trisomy 12 clone.

Acquisition of an extra chromosome 12 in CLL has so far been reported only in patients with signs of transformation to Richter's syndrome.[23-25] We now report 2 patients who seemingly acquired the aberration during follow-up without signs of blastic transformation. However, it is not possible to state for certain that the trisomy 12 clone was acquired in either case. In one of the patients, in whom the trisomic clone was first detected when the disease was progressing in abdominal nodes, no biopsy of the abdominal masses was performed. It is not, therefore, possible to rule out a Richter's syndrome, but the prompt response to chlorambucil treatment and the sustained remission after therapy speaks against a transformation. Furthermore, this case showed a small number of cells with 3 fluorescent signals in two earlier FISH tests, both below the cut-off level for trisomy 12. It is, however, feasible that such small numbers of trisomic cells might signify an early appearance of the trisomy 12 clone. In the second case with the trisomy 12 detected after treatment with CHOP, no FISH test had been performed prior to the therapy. In this patient, we cannot exclude the possibility that the chemotherapy had reduced the trisomic clone below the detection level of the FISH method. A more frequent use of repeat FISH in lymphoma patients without trisomy 12 is required to show whether true acquisition of trisomy 12 occurs, or whether the progression is associated with expansion of an already existing small trisomic clone.

The choice of therapy varied, mainly depending on the severity of clinical symptoms, with purine analogs and CHOP being given to those with more advanced disease. Moreover, to some extent different study protocols were used during the follow-up period. For the purpose of this analysis we divided the patients into three chemotherapy groups: purine analogs, single alkylator and combination therapy. When we analyzed the outcome in 23 treatment episodes, we found that both the percentage of MNC and CD19+ cells with trisomy 12 decreased significantly after single alkylator and combination therapy, but not after purine analogs. The small number of patients in each cohort may explain the differences, but it is also possible that the trisomic clone reacts differently to treatment than the rest of the leukemic cells do. Cuneo *et al.*[24] reported that the percentage of trisomic cells increased after treatment with alkylating agents in 2 CLL cases with trisomy 12, while Escudier *et al.*[27] showed comparable overall responses to

Table 4. Trisomy 12 in MNC measured by FISH and in CD19+ cells measured by FISH and APAAP before and after therapy. Analysis of 12 patients and 23 treatment episodes.

Patient No.	*MNC with trisomy 12 (%)*		*CD19+ cells with trisomy 12 (%)*		*Lymphocyte count (x10⁹/L)*		*Clinical response*
	before	*after*	*before*	*after*	*before*	*after*	
Purine analog							
25	53.6	30.6	56.5	38.2	173.0	9.9	major
33	9.6	0.6	11.8	1.3	4.5	1.2	major
34	2.6	4.4	4.1	12.1	1.8	2.6	NR
44	17.4	22.8	21.7	33.0	26.6	4.3	minor
126	21.4	4.6	24.9	7.3	4.6	3.6	minor
130	16.6	18.6	18.6	33.5	3.1	1.8	NR
207	37.8	45.8	43.1	48.1	41.2	21.0	NR
241	17.4	23.9	21.1	25.0	7.9	6.8	NR
Single alkylator							
25	53.4	38.8	58.4	40.8	192.0	57.9	major
	59.6	*57.0*	*68.5*	*57.8*	*109.0*	*98.0*	*minor*
33	40.2	42.0	40.8	53.0	165.0	9.2	maj
34	6.4	1.8	15.2	5.8	1.2	0.9	NR
	1.8	*1.2*	*5.8*	*5.3*	*0.9*	*4.6*	*minor*
126	23.0	21.4	27.0	24.9	42.4	4.6	major
154	39.6	16.2	41.9	19.1	24.7	10.2	major
157	43.8	40.0	45.1	42.1	171.0	15.1	major
207	45.8	20.2	48.1	24.4	21.0	2.2	major
236	27.4	2.8	30.3	5.5	8.4	0.7	major
237	37.4	3.4	63.2	7.8	16.5	1.9	major
	32.0	*33.2*	*43.0*	*34.7*	*6.2*	*0.5*	*NR*
241	13.6	14.0	15.5	16.1	10.7	5.7	minor
Combination regimens							
34	1.2	1.2	5.3	5.7	4.6	1.8	minor
130	16.2	8.8	35.7	10.8	5.2	0.8	major
207	20.2	4.8	24.4	6.3	2.2	2.2	minor
	16.2	*15.8*	*20.2*	*18.8*	*6.8*	*2.9*	*minor*
237	33.2	1.8	34.7	3.0	0.5	3.0	NR
241	14.0	7.4	16.1	11.4	5.7	3.5	NR

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fludarabine between CLL cases with and without trisomy 12. In the present study, compared to the disomic cases, patients with trisomy 12 seemed to respond well to alkylators, and more poorly to purine analogs. It is possible that differences in chemotherapy response may reflect the earlier reported differences in proliferative potential[31] between leukemic cells with and without trisomy 12.

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VH, RH and EK contributed equally to the drafting of the manuscript. The authors would like to express their sincere gratitude to Mrs. Ingrid Arvidsson, for skilfull FISH and APAAP analyses.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Potential implications for clinical practice

The results suggest that alkylating agents, rather than purine analogs, should be used as first-line therapy in cases with trisomy 12.

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