A set of commercially available fluorescent in-situ hybridization probes efficiently detects cytogenetic abnormalities in patients with chronic lymphocytic leukemia

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Purpose: To investigate a simplified panel of fluorescent in-situ hybridization (FISH) probes for evaluation of patients with chronic lymphocytic leukemia (CLL) and to correlate results from this technique with known prognostic factors. **Methods:** We retrospectively reviewed the FISH and conventional cytogenetic results, and clinical and laboratory data of 44 patients with CLL. **Results:** FISH was more sensitive than conventional cytogenetics in detecting genomic aberrations (75% vs. 16%, $P < 0.0001$). Trisomy 12 was significantly correlated with the cell surface marker of CD38 expression ($P = 0.0017$). **Conclusion:** This FISH panel reliably detects prognostically important genomic abnormalities in CLL and is suitable for widespread use. *Genet Med* 2004;6(1):48–53.

Key Words: FISH, molecular cytogenetics, B-CLL, CD38 expression, hematological malignancy

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Western world. The disease is characterized by the accumulation of mature-appearing lymphocytes in the blood, bone marrow, lymph nodes, and spleen. CLL has a highly variable clinical course, and some patients die from the disease within a few months of the time of diagnosis, whereas others live for 20 years or more. The commonly used clinical staging systems developed by Rai et al.¹ and Binet et al.² have been effective in classifying patients into broad prognostic groups that appear to correlate with the gross tumor burden and its effect on the function of the bone marrow. However, these staging systems do not accurately predict the clinical course of the disease in individual patients, especially those patients who have a low tumor burden at the time of diagnosis.³ Therefore, there is considerable interest in characterizing molecular markers that could identify patients with more rapidly progressive forms of the leukemia for whom the “watch and wait” approach may not be appropriate.

Molecular markers that have been shown to be of prognostic value in CLL include somatic hypermutation of the immunoglobulin heavy-chain gene variable-region (IgV_H) in the leukemic cells, surface CD38 expression, and chromosomal ab-

normalities identified by cytogenetic analysis.^{4–6} As B cells traverse through the germinal center during differentiation and maturation, somatic hypermutation of the IgV_H occurs. CLL cells can be divided into two groups, those that appear to have traversed the germinal center and have undergone somatic hypermutation, and those that have not encountered the germinal center and have unmutated IgV_H gene regions.^{4,7} Patients with unmutated IgV_H genes have much more aggressive disease, with a predicted median survival of 95 months, whereas those with mutated IgV_H genes have a relatively benign course with a predicted median survival of 293 months.⁴ However, establishing the mutation status is technically challenging, time-consuming, and not readily available to most clinicians. High CD38 expression, originally described as a surrogate for unmutated IgV_H genes, has been correlated with aggressive disease, but expression levels can change over time during the course of the disease, a fact that affects the predictive value of this marker.⁸ Finally, conventional karyotyping has been hampered by the lack of growth of the CLL cells in cell cultures, and chromosomal abnormalities are often missed by this technique.

A novel technique, fluorescent in-situ hybridization (FISH), allows detection of chromosomal aberrations in both actively dividing cells and interphase nuclei. Recently, this technique has been used to detect the most common genomic abnormalities in CLL, including trisomy 12, rearrangements involving 14q32, and deletions of 13q14, 6q21, 17p, and 11q22–23.⁹ Genomic aberrations have been detected in 82% of a population of 325 CLL patients using this technique.⁹ Some of these abnormalities have been found to be significant predictors of disease progression and patient survival.⁹ Unfortunately, the FISH analysis reported by Dohner et al.⁹ used thirteen probes and is too cumbersome for routine clinical use in most labora-

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tories. Our group has therefore compiled a simplified "CLL FISH Panel" designed to detect the genomic aberrations with proven clinical significance. This panel utilizes just 5 commercially available probes and reliably detects the prognostically important genomic abnormalities, thus allowing clinicians to assess the biological risk of disease progression in individual patients with CLL.

MATERIALS AND METHODS

Patients

The cytogenetics laboratory of the University of Utah identified 79 patients who had cytogenetics and FISH analysis performed on their blood or bone marrow samples for evaluation of lymphocytosis from January 1, 2001 to December 1, 2002. IRB approval was obtained to retrospectively review the clinical data of these patients. Out of the initial group, 44 patients were confirmed to have CLL. Clinical diagnosis of CLL in each patient was based on a lymphocytosis of $> 5 \times 10^9$ cells/L with $> 50\%$ mature appearing lymphocytes, and the characteristic immunophenotype of CD5, CD19, CD20, and CD23 expression, monoclonal κ or λ expression, and dim surface immunoglobulin expression.¹⁰ Clinical features at diagnosis were assessed, and a modified Rai stage was assigned for 42 patients.

Flow cytometric immunophenotyping

Flow cytometric immunophenotyping of anticoagulated peripheral blood was performed on each patient to help establish the diagnosis of B-CLL. Erythrocytes were lysed before antibody staining with ammonium chloride. Determination of B-cell monoclonality was based on finding a κ/λ ratio of $> 8:1$ or $< 0.2:1$ using κ/λ /CD19 and/or κ/λ /CD20 antibody combinations. Other antibody combinations used were FMC-7-FITC/CD38-PE/CD19-ECD/CD5-PC5 and CD79b-FITC/CD23-PE/CD19-ECD/CD5-PC5 (Becton Dickinson Immunocytometry Systems, San Jose, CA). Three-color and four-color flow cytometric analyses were utilized. Both forward/side light scatter and CD45/side light scatter were used in each case as the gating methodologies. Further gating was done as necessary on CD19-positive B-cell staining events. CD38 expression levels were determined by anti-CD38-PE reactivity on CD19⁺CD5⁺ cells.

Chromosome analysis

Karyotypic analysis was performed on all patients. Peripheral blood or bone marrow lymphocytes were cultured for 72 to 96 hours at 37°C in RPMI1640 medium supplemented with 15% of fetal calf serum. One culture was left unstimulated, and two were stimulated with pokeweed mitogen and phytohemagglutinin/Interleukin-2, respectively.¹¹ Chromosomes were characterized by a trypsin G-banding method. Karyotypes were described using the 1995 International System for Human Cytogenetic Nomenclature.

CLL FISH panel analysis

All the peripheral blood or bone marrow specimens were also analyzed by FISH using a set of commercially available FISH probes specific for chromosome 12 centromere, RB-1 (13q14), D13S25 (13q14.3), and p53 (17p13.1) loci (Vysis, Downers Grove, IL). For 27 patients in our sample, FISH was also performed using a probe for the ATM (11q22.3-q23.1) locus (Vysis). These five probes constituted the "CLL FISH panel." All five probes were set up separately on different slides for each patient. Hybridization and detection of hybridization signals were performed according to the manufacturer's protocols. At least two technologists scored the same case. Damaged and overlapping nuclei as well as areas of the slide where hybridization was absent or suboptimal were avoided for scoring. For each probe, two hundred nuclei were evaluated. Peripheral blood samples from twenty individuals without hematological diseases and with normal karyotypes were used as controls. Means and standard deviations (SD) of the percentages of nuclei with 1, 2, and 3 hybridization signals were calculated. Results were considered abnormal if the percent of nuclei with the abnormal hybridization signal was > 3 SD from the mean. Therefore, the following cut-off values were used: 4.5% for trisomy 12, 6.5% for the ATM deletion, 9.6% for the RB-1 deletion, 10.2% for the deletion of D13S25, and 10.1% for the deletion of p53.

Statistical analysis

Statistical analysis was performed using Statistica software (Releases 5.1 and 6.0, Statsoft, Inc.) and StatXact (Release 5, Cytel Software, Inc.). All reported significance levels are two-sided. Differences between the proportion of abnormalities detected by FISH and karyotypic analysis were tested using McNemar's test for correlated proportions. Fisher's exact test was used to test whether there was significant association between Rai stage, FISH characteristics, or CD38 status. Spearman rank correlation coefficients and associated 95% exact confidence interval were also calculated.

RESULTS

Patient characteristics

The median age of our patients was 64.5 years. There were 37 men and 7 women in our series, and 7/44 patients had previous treatment for their disease. The median white blood cell count at diagnosis was 19,000/uL (range = 7,000/uL to 370,000/uL); the median hemoglobin was 15.0 g/L (range = 4.5 to 17.5 g/L); the median platelet count was 209,000/uL (range = 51,000/uL to 400,000/uL). Using the modified Rai staging system, 19 patients had low risk disease, 18 had intermediate risk, and 5 patients had high-risk disease.¹²

Cytogenetics and FISH analysis

Karyotypic analysis detected chromosomal abnormalities in 7/44 (16%) of patients. Among them, 3 patients had trisomy 12 alone. One patient had trisomy 12, trisomy 14, and trisomy 19;

one patient had trisomy 12, deletion 6q, and deletion 20q; one patient had trisomy 12 and trisomy 22; and another patient had a deletion 6q alone.

In contrast, the CLL FISH panel detected genomic abnormalities in 33/44 (75%) patients. Therefore, our CLL FISH panel was significantly more sensitive than conventional karyotypic analysis in detecting chromosomal abnormalities in leukemic cells ($P < 0.0001$). Among the abnormalities identified, trisomy 12 was detected in 10/44 (23%) of the patients. Of these, 8/44 (18%) patients had only trisomy 12. Also, 1/44 (2.3%) patients had both trisomy 12 and the deletion of D13S25, and in 1/44 (2.3%) trisomy 12 was identified in conjunction with the ATM deletion. Further, 10/44 (23%) patients had the deletion of D13S25 alone detected. Only 2/44 (4.5%) patients had the RB-1 deletion alone, and 10/44 (23%) patients had codeletion of the RB-1 and D13S25 loci. In addition, 1/44 (2.3%) patients had a p53 deletion identified in conjunction with the codeletion of the D13S25 and RB-1 loci. We were able to test for the ATM deletion on the specimens of 27 patients. Of these patients, 1/27 (3.7%) had the ATM deletion alone and 1/27 (3.7%) patients had both the ATM deletion and trisomy 12. The percentage of cells positive for each probe was in the range of 33.5% to 87.5% for the ATM deletion, 32% to 87% for trisomy 12, 14.5% to 80% and 17.5% to 92%, respectively, for codeletion of RB-1 and D13S25, 30% to 85% for only the D13S25 deletion, 39.5% to 44% for only the RB-1 deletion and 18.5% for the p53 deletion. FISH identified trisomy 12 in all patients who had this abnormality detected by conventional karyotyping (see Table 1). The clinical features of the patients with abnormal FISH and/or conventional cytogenetic results are illustrated in Table 2.

Table 1

The number of patients^a with chromosomal abnormalities as detected by FISH versus conventional cytogenetic analysis

Chromosomal abnormality	Conventional cytogenetics	FISH
ATM deletion	0	2
Trisomy 12	6	10
RB-1 deletion	0	12
D13S25 deletion	0	21
Co-deletion of D13S25 and RB-1	0	10
P53 deletion	0	1
Other abnormalities (deletion 6q, 20q, trisomy 14, 19, 22)	4	0
Normal karyotype	37	11
Total number of patients with abnormalities detected	7	33 ($P < 0.0001$)

^a $n = 44$ patients for all karyotypic and FISH analyses except for the ATM deletion ($n = 27$).

Correlation between FISH abnormalities and previously described prognostic markers

In the 28 patients who had CD38 expression evaluated, 7 (25%) had CD38 expression $\geq 30\%$, and 8 (29%) had CD38 expression $\geq 20\%$. CD38 expression and trisomy 12 were positively correlated ($r = 0.71$, $P = 0.0017$) if a cutoff level for CD38 expression was $\geq 30\%$. The correlation was stronger ($r = 0.81$, $P = 0.0002$) if the cutoff level for CD38 expression was $\geq 20\%$. No significant correlation was found between CD38 expression and deletions of the ATM, p53, RB-1 loci, or the normal FISH category. A slightly negative correlation ($r = -0.45$) was found between high CD38 expression and the D13S25 deletion ($P = 0.032$).

No correlation was found between the FISH findings or CD38 expression and the modified Rai stage at the time of diagnosis.

DISCUSSION

By using a simplified CLL FISH panel, we were able to detect chromosomal abnormalities in 75% of CLL patients compared to 16% by conventional cytogenetics. Previous studies involving the FISH technique have demonstrated a similar rate of detection of 77% to 82%.^{9,13–15} With the exception of the ATM gene deletion, the frequencies of the individual genomic abnormalities detected with this panel in our study population are similar to the frequencies observed in prior studies. A comparison of genomic abnormalities as detected by FISH in various studies is listed in Table 3. The difference in the incidence of the ATM gene deletion between our investigation and the other studies can most likely be accounted by the fact that only a subset of our study population was analyzed with the ATM gene probe.

It is worth mentioning that the IgH locus-specific FISH probe was not included in our CLL FISH Panel for evaluation. As we know, somatic mutation of the IgV_H region has been shown to be of prognostic value in CLL.⁴ However, prognostically significant IgV_H mutations can be detected only by molecular methods, but not by FISH analysis. 14q32/IgH related rearrangements as detected by traditional cytogenetics or FISH have not been established as a significant prognostic marker in CLL.⁹ Our CLL FISH Panel was generated primarily for prognostic purpose. Even though FISH detection of 14q32/IgH rearrangements does have some diagnostic value in confirming the clonality of a disease, the fact that in general most CLL can be accurately diagnosed based on cell morphology and flow cytometric immunophenotyping analyses further supports the appropriateness of selecting probes in the Panel.

In concordance with reported data, monoallelic deletion of D13S25 was more frequent than the monosomy of RB-1 in our study.⁹ The coexistence of trisomy 12 and 13q14 deletions as well as trisomy 12 and the ATM deletion has been observed in previous FISH studies and in our own series.^{9,15} One patient (Case 65) had a similar percentage of cells with trisomy 12 (32%) or the D13S25 deletion (30%), and another patient

Table 2

The clinical features and results of the conventional cytogenetics and FISH analysis (listed in percentage of nuclei for each probe) for each patient with either abnormal cytogenetic and/or FISH results

Case	Age ^a	Sex ^b	Rai stage ^c	Conventional cytogenetics ^d	ATM probe ^e	Tri-12 probe	RB-1 probe	D13S25 probe	p53 probe
1	66	M	LOW	NL	0	0	4	78.5	4.5
2	65	M	INT	NL	2.5	0	5.5	32.5	3.5
3	67	M	NA	NL	2	0	3.5	78.5	4.5
4	72	F	INT	NL	0.5	0	71.5	84	6.5
5	69	M	LOW	NL	1	1	2.5	69	4.5
7	67	M	HIGH	NL	1	1	14.5	18.5	5
14	51	M	LOW	NL	ND	0	19	21	7
15	65	M	INT	Tri 12, Tri 14, Tri 19	ND	64	6	4.5	6
18	63	M	LOW	NL	1.5	87	2.5	0	1
19	75	F	LOW	Tri 12	3	57	0.5	1.5	0
20	74	M	INT	NL	ND	77	2	4	6
21	58	M	HIGH	Del 6q	ND	0	67	68	2
22	46	M	INT	NL	ND	0	21	17.5	6
24	63	M	INT	NL	1	82.5	3	2.5	2
25	41	M	INT	NL	2	0	55.5	78.5	0
31	49	M	INT	NL	ND	0	44	67	0
34	67	M	NA	NL	ND	0	44	0	3
38	46	F	INT	Tri 12	0	73.5	0	0	2
40	65	M	LOW	NL	ND	0	22.5	62.5	2
41	64	M	LOW	Tri 12	ND	61	2.5	2	4.5
43	63	M	LOW	NL	1	1.5	9	82	4
49	74	M	INT	NL	ND	0	3	80	2
56	54	M	LOW	NL	ND	0	39.5	1	4.5
58	71	M	LOW	NL	4	0	8.5	84	0
59	62	F	INT	Tri 12, Del 6q, Del 20q	33.5	73.5	2	1	0.5
60	69	F	INT	NL	1	56	0	0	5
61	37	M	INT	NL	87.5	3.5	0.5	4	2
65	69	M	INT	Tri 12, Tri 22	3	32	7.5	30	7
66	59	M	LOW	NL	0	2	80	92	2
67	51	M	LOW	NL	ND	1	2	75	2.5
68	53	M	LOW	NL	1	2	8.5	58	0
69	73	M	HIGH	NL	0.5	0	17	56	18.5
71	55	M	LOW	NL	4	0	6	85	3

^aAge in years old.

^bM = male; F = female.

^cModified Rai Stage at the time of diagnosis; INT = intermediate, NA = not available.

^dNL = normal, Tri = trisomy, Del = deletion.

^eND = not done.

(Case 59) had 73.5% of cells with trisomy 12 and 33.5% of cells with the deletion of ATM. In the latter case, it appears that trisomy 12 was the prevalent clone, suggesting that the deletion

of ATM was a secondary event, occurring as a result of clonal evolution. Another case (Case 69) had 17% of cells with the RB-1 deletion, 56% with the D13S25 deletion, and 18.5% with

Table 3

The incidence of genomic abnormalities in our series as compared with four other CLL studies

FISH result	Our study (n = 44)	Dohner et al ⁹ (n = 325)	Bullinger et al ^{a13} (n = 258)	Stilgenbauer et al ^{b14} (n = 139)	Dewald et al ¹⁵ (n = 113)
13q deletions	52%	55%	62%	51%	64%
Trisomy 12	23%	16%	10%	12%	25%
ATM deletion	7.4% (n = 27)	18%	11%	25%	15%
p53 deletion	2.3%	7%	6%	3%	8%
Normal	25%	18%	22%	19%	23%
Total abnormalities	75%	82%	78%	81%	77%

^aCLL1 trial of the German CLL Study Group (GCLLSG) for Binet A patients.^bCLL3 trial of the GCLLSG evaluating high dose therapy followed by autologous stem cell transplantation among Binet B and C patients.

the p53 deletion, suggesting that the D13S25 deletion was the predominant clone and the p53 and RB-1 deletions were minor clones involved in the pathogenesis of the disease. This observation warrants further study.

It is noteworthy that conventional cytogenetics did identify abnormalities involving chromosomes 6, 14, 19, 20, and 22 that could not be detected by the FISH technique in our series. Although it has been shown that patients with complex karyotypes have poorer survival, the specific prognostic significance of each of these chromosomal aberrations is not known.⁶ On the other hand, the prognostic significance of the individual chromosomal abnormalities that were identified by the FISH technique has been established, and five major prognostic groups have been identified including those with a median survival times of 32 months (the p53 deletion), 79 months (the ATM deletion), 111 months (normal FISH), 114 months (trisomy 12), and 133 months (the 13q14 deletions).⁹ Our study was not able to confirm the above prognostic findings due to the short follow-up of the study patients. However, the lack of well-established prognostic significance of some chromosomal abnormalities as detected by traditional cytogenetics, coupled with the high degree of sensitivity and reproducibility of the FISH technique, makes conventional karyotyping analysis less appealing as a standard clinical test for patients with CLL.

CD38 has been considered to be a surrogate marker for IgV_H mutational status but this correlation has been questioned.^{16,17} Nevertheless, several studies have shown that positive CD38 expression is a significant marker of disease progression and shorter patient survival.^{5,18,19} There is controversy regarding the appropriate cutoff level for CD38 expression. One study identified 20% expression as the cut-off level; other studies have used 30% expression as the cut-off level, and a recent study has even characterized the pattern of CD38 expression to risk stratify patients.^{5,17,19,20} It is important to note that CD38 expression analysis may be more complex than previously described.

To date, two other studies have reported on the relationship between CD38 expression and the chromosomal abnormalities detected by FISH. One study showed a correlation between abnormalities at the ATM locus and positive CD38 expression.²¹ Another study found a correlation between high-risk

FISH categories (trisomy 12, ATM, and p53 deletions) and positive CD38 expression.¹⁵ We did not observe the correlation between CD38 expression and the p53 or ATM deletions, possibly because only a subset of our patients had both CD38 expression and the ATM abnormality investigated and because the overall incidence of the p53 deletion was low in our study population. However, we did observe a positive correlation between trisomy 12 and CD38 expression in the present study. Both of these markers have been correlated with more aggressive disease progression and poorer survival. In addition, we observed a negative correlation between CD38 expression and the D13S25 deletion. This observation is consistent with previous studies that have shown that patients with the D13S25 deletion have generally better outcomes than those with positive CD38 expression.^{5,9}

In conclusion, our investigation reveals the higher sensitivity of a panel of commercially available FISH probes as compared with conventional cytogenetic analysis in identifying prognostically significant genomic aberrations in CLL. The frequencies of genomic abnormalities observed with our CLL FISH panel are similar to previously reported data. Our investigation also provides additional data regarding the relationship between CD38 expression and genomic aberrations in CLL. We believe that this study has demonstrated the ease and reliability of this FISH technique and argues for its suitability for widespread use by clinicians who are caring for patients with CLL.

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