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Chromosome anomalies detected by interphase fluorescence *in situ* hybridization: correlation with significant biological features of B-cell chronic lymphocytic leukaemia

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Summary. Fluorescence *in situ* hybridization (FISH) was used to detect 6q–, 11q–, +12, 13q–, 17p– and translocations involving 14q32 in interphase nuclei from blood and/or bone marrow from 113 patients with B-cell chronic lymphocytic leukaemia (B-CLL). A total of 87 patients (77%) had a FISH anomaly: 13q– × 1 was most frequent (64%) followed by 13q– × 2 (28%), +12 (25%), 11q– (15%), 17p– (8%) and 6q– (0%). FISH results for blood and bone marrow cells in 38 patients were similar. Purified $CD5^+/CD19^+$ cells from blood were studied in eight patients and results indicate that in some patients not all B cells have FISH anomalies. We used a defined set of hierarchical FISH risk categories to compare FISH results by stable versus progressive disease, age, sex, Rai stage, $CD38^+$ expression and IgV_H mutational status. Significant differences in FISH risk distributions were associated with Rai stage, disease status and $CD38^+$, but not by age, sex or IgV_H mutational status. To look for baseline factors associated with high-risk disease, multivariate analysis of age, sex, Rai stage, $CD38^+$ and disease status versus FISH risk category was performed. Importantly, only $CD38^+$ was significantly associated with high-risk FISH categories (+12, 11q– and 17p–) after adjustment for the effects of other variables.

Keywords: B-CLL, Rai stage, FISH, CD38 expression, IgV_H mutations.

The prognosis and clinical course of patients with B-cell chronic lymphocytic leukaemia (B-CLL) are highly variable. Some patients have stable disease while others develop progressive disease that requires therapy. Studies with conventional cytogenetics, fluorescence *in situ* hybridization (FISH) with chromosome-specific DNA probes, immunophenotyping and mutational analysis of the immunoglobulin heavy chain variable regions (IgV_H) have all been used to study B-CLL. However, the oncogenic events that lead to the origin and progression of B-CLL remain unknown (Han *et al*, 1984; Juliusson *et al*, 1990; Brito-Babapulle *et al*, 1997; Fais *et al*, 1998; Damle *et al*, 1999; Dohner *et al*, 2000).

Studies of the variable region of Ig genes indicate that approximately 50% of patients with B-CLL have mutated IgV_H clones (Fais *et al*, 1998). These patients purportedly have a better prognosis than patients with non-mutated IgV_H clones (Damle *et al*, 1999). Other investigations indicated that expression of surface membrane CD38 in B-CLL cells might be an important indicator of poor prognosis in B-CLL (Hamblin *et al*, 1999; Hamblin *et al*, 2000; Ibrahim *et al*, 2001; Jelinek *et al*, 2001).

The most common cytogenetic anomalies in B-CLL involve chromosomes 6, 11, 12, 13, 14 and 17 (Han *et al*, 1984; Finn *et al*, 1998; Larramendy *et al*, 1998; Buhmann *et al*, 2002). These anomalies have been associated with differing prognoses (Han *et al*, 1984; Juliusson *et al*, 1990; El Rouby *et al*, 1993; Neilson *et al*, 1997; Zhang *et al*, 1997). New FISH methods detect these chromosome anomalies in non-dividing neoplastic cells (interphase nuclei) (Dohner *et al*, 2000; unpublished observations). In B-CLL, these anomalies have also been associated with different clinical outcomes and may be associated with IgV_H mutation status and CD38 expression (Dohner *et al*, 2000; Krober *et al*, 2002).

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We used FISH to detect anomalies of chromosomes 6, 11, 12, 13, 14 and 17 in interphase nuclei from two groups of patients: those with stable B-CLL who did not require treatment and those with progressive B-CLL who did require treatment. For statistical analysis of data, we followed a similar hierarchical risk model of FISH anomalies in B-CLL to that defined by Dohner *et al* (2000) with 17p– being the most aggressive → 11q– → 6q– → +12 → (normal) → 13q– × 2 → 13q– × 1 (least aggressive). The FISH results were studied for associations with stable or progressive disease, Rai stage, percentage of $CD5^+/CD19^+$ B cells, percentage of abnormal nuclei in blood versus bone marrow, CD38 expression and IgV_H mutation status.

MATERIALS AND METHODS

Patient acquisition. The immunophenotypic criteria for diagnosis of CLL required monoclonal surface or cytoplasmic immunoglobulin (dim), dual CD5/CD20 with dim CD20 expression and dual CD5/CD23 staining. A total of 113 patients were accrued between September 1999 and February 2002. Patients were classified into two clinical subsets, stable or progressive. Stable disease was defined as any Rai stage without evidence of progressive disease for at least 6 months. Progressive disease was defined as any Rai stage and clinical criteria for progressive disease requiring therapy (Cheson *et al*, 1996).

Fifty-six (49·6%) of the 113 patients had stable disease: 38 consecutive patients seen at the Mayo Clinic between September 1999 and February 2001, and 18 patients enrolled in a North Central Cancer Treatment Group (NCCTG) clinical trial (988151) for theophylline. Both blood and bone marrow specimens were collected prior to treatment from NCCTG patients. The progressive disease group consisted of 57 (50·4%) patients enrolled in any one of three NCCTG clinical trials for B-CLL. Blood specimens from these patients were obtained immediately prior to treatment. Of these 57 patients, 29 were enrolled in NCCTG trial 978151 for alternating cycles of fludarabine and cyclophosphamide, 17 were enrolled in NCCTG trial 988152 for gemcitabine, and 11 were enrolled in NCCTG trial N9986 for thalidomide. The Rai stage for each patient was recorded at the time of specimen collection for this study.

Flow cytometric immunophenotyping. Flow cytometric immunophenotyping of citrate-anticoagulated peripheral blood was performed in each patient to establish the diagnosis of B-CLL. A standard, whole blood assay with erythrocyte cell lysis was used for preparing all specimens. Determination of monoclonality was based on a combination of flow cytometric histogram evaluation and correlation with the percentage of cellular positivity for CD45-peridinin chlorophyll (PerCP)/CD19-phycoerythrin (PE)/kappa-fluorescein isothiocyanate (FITC) versus CD45-PerCP/CD19-PE/lambda-FITC antibody combinations. The primary monoclonal antibody combinations used included: CD19-PE/CD10-FITC, CD5-PE/CD20-FITC, CD23-PE/CD20-FITC, CD11c-PE/CD22-FITC and CD19-PE/CD103-FITC (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Two-colour and three-colour 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 either a lymphoid subpopulation based on cell blank; size or as back gating on CD19-positive B-cell staining events.

CD38 expression. CD38 expression levels were determined by anti-CD38-PE reactivity on $CD19^+$ (anti-CD19-FITC) cells. CD38 expression of the B-CLL population was performed by first incubating cells with FITC-conjugated CD5 and PE-conjugated CD19 to verify the B-CLL population, and then incubating cells with FITC-conjugated CD19 and PE-conjugated CD38 (Jelinek *et al*, 2001). $CD38^+$ was defined as ≥ 30% B cells with CD38 expression and $CD38^-$ was defined as < 30% B cells with CD38 expression (Jelinek *et al*, 2001).

IgV_H gene mutation analysis. DNA sequencing of the variable region of Ig genes was performed on 62 patients (41 with stable disease and 21 with progressive B-CLL) using a previously published method (Jelinek *et al*, 2001). Nucleotide sequences were aligned with those in the V BASE sequence directory using DNAPLOT software. Non-mutated IgV_H clones were defined as < 2% DNA sequence deviation from the most similar IgV_H gene in the V BASE sequence directory. Mutated IgV_H clones were defined as ≥ 2% differences in DNA sequence from the V BASE sequence directory.

Purification of CLL B-cells. Human peripheral blood mononuclear cells (PBMC) were separated from CLL blood samples by passage through a standard histopaque gradient. Highly purified $CD19^+$ B cells (> 95%) were then obtained from PBMC by a standard negative selection process using a cocktail of subset-specific antibodies conjugated with magnetic beads (Miltenyi Biotech, Auburn, CA, USA).

FISH studies. Blood (7 ml) was collected in sodium heparin from each patient at initial presentation. For 38 of the 56 stable patients, bone marrow (1 ml) was also collected within 24 h of the blood specimen. Each blood and bone marrow specimen was processed with hypotonic solution (0·075 mol/l potassium chloride) and fixed with three changes of 2 : 1 methanol and glacial acetic acid (Dewald *et al*, 1982). Blood specimens from 18 patients with stable disease and 57 patients with progressive disease were first processed by Ficoll density gradient centrifugation prior to cell preparation and FISH study.

Fluorescent-labelled DNA probes were used to detect anomalies of chromosomes 6, 11, 12, 13, 14 and 17 in interphase nuclei (Table I). FISH for each probe was performed in a similar fashion to *BCR* and *ABL*, as previously reported (Dewald, 2002). Two-hundred consecutive qualifying interphase nuclei were scored from blood and/or bone marrow for each probe. Representative signal patterns for normal and abnormal nuclei are demonstrated in Fig 1. Results were considered clonal when the percentage of cells with any given chromosome abnormality exceeded the normal cut-off value.

Statistical methods. 'Exact' Wilcoxon and chi-squared tests were used to compare distributions of ordered and unordered, respectively, categorical variables among subsets of patients defined by FISH anomalies and baseline patient and disease characteristics. The Kruskal–Wallis test

Table I. Summary of panel FISH test: DNA probes, fluorophor colours, hybridization locus and normal cut-off values.

Most common anomaly	Probes (chromosome locus)		Upper limit of normal*			
	SpectrumOrange™	SpectrumGreen™	Deletion	Trisomy	Monosomy	Translocation
6q–	c-MYB (6q23)	D6Z1 (6 centromere)	6·5%	1·5%	4·0%	NA
11q–	ATM (11q23)	D11Z1 (11 centromere)	5·0%	1·5%	4·5%	NA
+12	D12Z3 (12 centromere)	MDM2 (12q15)	7·5%	1·5%	5·0%	NA
13q– × 1	D13S319 (13q14)	D13S327 (13qter)	7·0%	5·0%	6·5%	NA
13q– × 2	D13S319 (13q14)	D13S327 (13qter)	1·5%	5·0%	6·5%	NA
17p–	P53 (17p13.1)	D17Z1 (17 centromere)	8·5%	1·5%	6·5%	NA
t(11;14)	CCND1 (11q13)	IgH (14q32)	NA	NA	NA	2·5%

*Based on blood sample analysis from 20 normal individuals and a one-sided 95% CI for observing the maximum number of nuclei for each false-positive signal pattern seen in 200 scoreable nuclei using the binomial distribution.

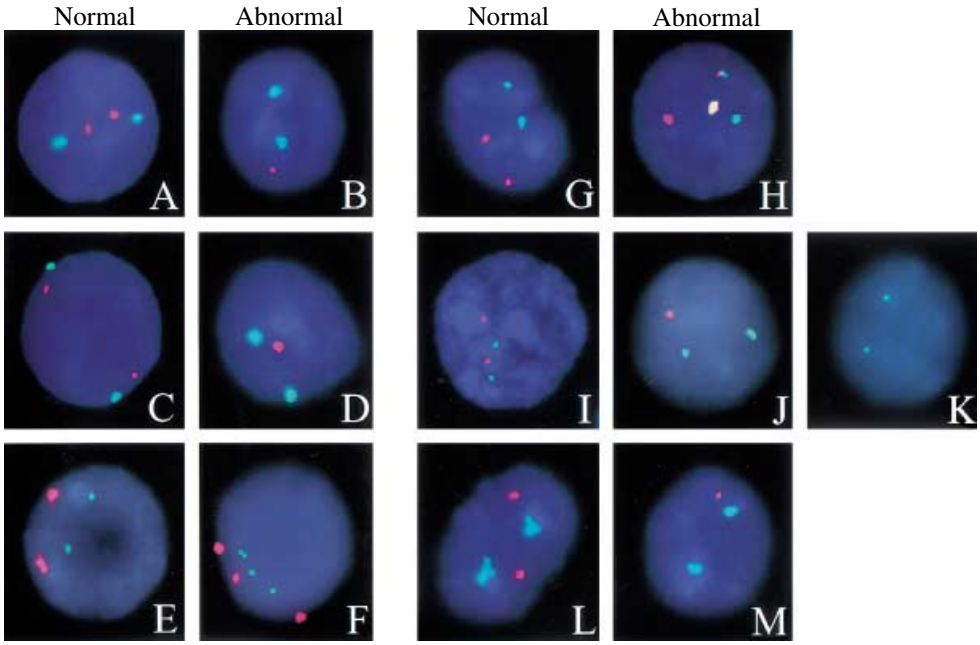

Fig 1. Representative nuclei showing normal and abnormal signal patterns for D6Z1 and c-MYB, D11Z1 and ATM, D12Z3 and MDM2, CCND1 and IgH, D13S319 and D13S327, and D17Z1 and P53. For more information about each probe see Table I. Fluors that produced a red or orange signal are denoted as R, green signals as G, and yellow or adjacent R and G signals as F to indicate an R/G signal fusion. The normal signal pattern was 2R2G for chromosomes 6 (A), 11 (C and G), 12 (E), 13 (I), 14 (G) and 17 (L). The abnormal signal pattern was: 1R2G for 6q– (B), 11q– (D), 13q– × 1 (J) and 17p– (M); 0R2G for 13q– × 2 (K); 3R3G for +12 (F); and 1R1G2F for t(11;14) (H).

was used to compare the equality of ordered variables among more than two subsets of patients. The Spearman's correlation coefficient was used to assess the degree of linear association between pairs of variables. The multivariate logistic model was used to look for baseline factors that might be associated with high-risk disease.

RESULTS

Patient characteristics

Table II summarizes clinical characteristics for 113 patients. The 56 patients with stable disease included 23 women and 33 men; mean age was 61 years (median 60 years, range 39–82 years). The 57 patients with progressive disease included 11 women and 46 men; mean age was 64 years (median 64 years, range 38–83 years).

Fish anomalies at clinical presentation

Chromosome anomalies. Table III summarizes the results of FISH studies for blood samples from 113 patients. Eighty-seven patients (77%) were abnormal, including 48 (55%) with a single anomaly, 31 (36%) with two anomalies and eight (9%) with ≥3 anomalies. The mean percentage of abnormal nuclei for 87 patients was 65·4 (range 6–100)

Table II. Characteristics of 113 patients with B-CLL, classified by clinical disease status.

	All patients		Stable		Progressive		
Patient characteristics	Number	%	Number	%	Number	%	*P*-value
Age (years)							0·18*
≤60	56	49·5	29	52	21	37	
>60	56	49·5	27	48	35	61	
Not available	1	1	–	–	1	2	
Sex							0·0142*
Male	79	70	33	59	46	81	
Female	34	30	23	41	11	19	
Rai Stage							0·0001†
0	41	36	38	68	3	5	
1	16	14	9	16	7	12	
2	15	13	4	7	11	19	
3	15	13	1	2	14	25	
4	25	22	4	7	21	37	
Not available	1	1	–	–	1	2	
CD38							0·0042*
Positive	28	25	7	12	21	37	
Negative	77	68	44	79	33	58	
Not available	8	7	5	9	3	5	
IgVH status							0·0063*
Non-mutated	28	25	13	23	15	26	
Mutated	34	30	28	50	6	11	
Not available	51	45	15	27	36	63	

*'Exact' chi-squared test comparing the distributions of the baseline characteristic between the stable and the progressive subsets.

†'Exact' Wilcoxon test comparing the distributions of the baseline characteristic between the stable and the progressive subsets.

among patients with different FISH anomalies. Among 87 patients with any FISH anomaly, 13q– × 1 was most frequent (64%) followed by 13q– × 2 (28%), +12 (25%), 11q– (15%) and 17p– (8%). Anomalies of 6q– were not observed.

Except for 13q– × 2, no association between any two or more different chromosome anomalies was observed (Table III). We used multiple probes including RB1, D13S319 and D13S327 to study nine patients with 13q– × 2 as the sole anomaly (i.e. bi-allelic deletions in chromosome 13q). The RB1 probe hybridizes to 13q14 adjacent to D13S319 on the centromeric side. In four patients, all abnormal nuclei lacked hybridization sites of D13S319 loci but retained one copy of RB1 and two copies of the control target D13S327. Of the five remaining patients, four lacked hybridization sites for both D13S319 loci, but retained two copies of RB1 and two copies of the control target D13S327. The final patient lacked hybridization sites for RB1 and D13S319 loci, but retained two copies of the control target D13S327.

$CD5^+/CD19^+$ B-cells. For eight patients, $CD5^+/CD19^+$ B cells were isolated from whole blood using magnetic beads and then studied by FISH. The difference between the percentage of $CD5^+/CD19^+$ B cells and that of abnormal nuclei by FISH was calculated (delta) for each patient. The delta was <15% for three patients: each of these patients had 13q– × 1. The delta was >28% in three patients including two with only 13q– × 1 and one with +12. In these patients, it is probable that only a subset of B cells had FISH anomalies. The remaining two patients each had two FISH anomalies. The delta for one patient was 39% and 27% for each anomaly, suggesting that a subset of neoplastic B cells had both +12 and 17p–. The delta for 13q– × 1 and 11q– for the other patient was 28% and 6%, respectively, suggesting that 13q– × 1 originated earlier in the disease than 11q–.

Blood versus bone marrow. We studied blood and bone marrow collected on the same day for each of 38 patients with stable B-CLL. FISH results were normal in both blood and bone marrow for 13 (34%) patients. The same chromosome anomalies were found in both the blood and bone marrow samples from each of the other 25 patients. The percentages of abnormal nuclei for blood and bone marrow were similar for each patient and were significantly ($P < 0·0001$) correlated with each other, with Spearman's correlation coefficients of 0·97 and 0·90 when patients with no FISH anomalies were included and excluded respectively.

Correlation of fish results with clinical and other laboratory prognostic factors

Stable versus progressive disease. Table III summarizes FISH anomalies for all 113 patients classified by disease status.

Table III. FISH anomalies in B-CLL patients classified by disease status, Rai stage, IgV_H status and CD38 expression.

	All B-CLL patients ($n = 113$)		Disease status ($n = 113$)				Rai stage ($n = 112$)										IgV_H status ($n = 62$)				CD38 expression ($n = 105$)			
			Stable		Progressive		0		1		2		3		4		Mutated		Non-mutated		Positive		Negative	
FISH results	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Normal	26	23	19	33·9	7	12·3	13	32	5	31	2	13	1	7	5	20	12	35	7	25	2	7·1	21	27·3
Abnormalities	87	77	37	66·1	50	87·7	28	68	11	69	13	87	14	93	20	80	22	65	21	75	26	92·9	56	72·7
13q– × 1	31	35·6	20	54·1	11	22·0	16	57	3	27	3	23	1	7	8	40	11	50·0	5	23·8	5	18·0	25	44·6
+12	13	14·9	4	10·8	9	18·0	2	7	2	18	3	23	5	36	1	5	0	0·0	5	23·8	6	23	7	12·5
13q– × 2	9	10·3	1	2·7	8	16·0	1	4			1	8	2	14	5	25	2	9·1	1	4·8	2	7·7	7	12·5
13q– × 1/ 13q– × 2	8	9·2	3	8·1	5	10·0	4	14	1	9	1	8			2	10	2	9·1	2	9·5	0	0·0	6	10·7
11q–/13q– × 1	6	6·9	2	5·4	4	8·0			2	18	1	8	2	14	1	5	1	4·5	2	9·5	4	15·4	2	3·4
13q– × 1/+12	4	4·6	3	8·1	1	2·0	1	4	1	9			1	7	1	5	1	4·5	2	9·5	2	7·1	2	3·6
17p–/+12	2	2·3	1	2·7	1	4·0	2	7									1	4·5	1	4·8	2	7·7	0	0·0
11q–	3	3·4	1	2·7	2	4·0			1	9	2	15					1	4·5	0	0·0	2	7·7	1	1·8
13q– × 1/17p–	2	2·3	1	2·7	1	2·0	1	4			1	8					1	4·5	0	0·0	0	0·0	2	3·6
11q–/13q– × 1/ 13q– × 2	2	2·3	0	0·0	2	4·0							1	7			0	0·0	2	9·5	2	7·1	0	0·0
13q– × 1/ 13q– × 2/+12	2	2·3	0	0·0	2	4·0					1	8	1	7			1	4·5	0	0·0	0	0·0	2	3·6
11q dup	1	1·1	1	2·7	0	0·0	1	4									0	0·0	0	0·0	0	0·0	0	0·0
11q–/13q– × 2	1	1·1	0	0·0	1	2·0			1	9							0	0·0	0	0·0	1	3·8	0	0·0
13q– × 2/17p–	1	1·1	0	0·0	1	2·0									1	5	0	0·0	0	0·0	0	0·0	1	1·8
13q– × 1/ 13q– × 2/ 17p–/–17	1	1·1	0	0·0	1	2·0								1	5	1	4·5	0	0·0	0	0·0	1	1·8	
Hyperdiploid all probes	1	1·1	0	0·0	1	2·0							1	7			0	0·0	1	4·8	0	0·0	0	0·0
Number of FISH anomalies																								
0	26	23·0	19	33·9	7	12·3	13	32	5	31	2	13	1	7	5	20	12	35·3	7	25·0	2	7·1	21	27·3
1	48	42·5	26	46·5	22	38·6	19	46	6	38	8	53	6	40	9	36	12	35·3	10	35·7	13	46·4	33	42·9
2	31	27·4	11	19·6	20	35·1	9	22	4	25	4	27	5	33	9	36	8	23·5	8	28·6	10	35·7	19	24·6
3	6	5·3	0	0·0	6	10·5	0	0·0	1	6	1	7	2	13	1	4	1	2·9	2	7·1	3	10·7	3	3·9
4	1	0·9	0	0·0	1	1·8	0	0·0	0	0·0	0	0·0	0	0·0	1	4	1	2·9	1	3·6	0	0·0	1	1·3
6	1	0·9	0	0·0	1	1·8	0	0·0	0	0·0	0	0·0	1	7	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
'Exact' Wilcoxon *P*-value =			0·0001				0·055										0·3225				0·0256			

Table IV. Characteristics of 110 patients with B-CLL, classified by FISH risk categories.

Patient characteristics	FISH risk categories												
	1 = 13q− × 1		2 = 13q− × 2		3 = normal		4 = +12		5 = 11q−		6 = 17p−		
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	Kruskal–Wallis *P*-value
Age (years)													0·77
≤ 60	17	34	3	6	15	30	6	12	7	14	2	4	
> 60	14	23	14	23	11	18	13	22	4	7	4	7	
Sex													0·93
Male	22	28	13	17	15	19	14	18	12	15	2	3	
Female	9	27	4	12	11	33	5	15	0	0	4	12	
Rai stage													0·007
0	16	40	5	12	13	32	3	8	0	0	3	8	
1	3	19	1	6	5	31	3	19	4	25	0	0	
2	3	20	2	13	2	13	4	27	3	20	1	7	
3	1	7	2	14	1	7	7	50	3	21	0	0	
4	8	32	7	28	5	20	2	8	1	4	2	8	
CD38													0·0008
Negative	25	32	13	17	21	27	11	14	3	4	4	5	
Positive	5	18	2	7	2	7	8	29	9	32	2	7	
IgVH status													0·12
Non-mutated	5	19	3	11	7	26	7	26	4	15	1	4	
Mutated	11	32	4	12	12	35	2	6	2	6	3	9	
Disease status													0·0478
Stable	20	36	4	7	19	35	7	13	3	5	2	4	
Progressive	11	20	13	23	7	13	12	21	9	16	4	7	

FISH was abnormal for 37 [66%; 95% confidence interval (CI) 52% to 78%] of 56 patients with stable B-CLL and 50 (88%; 95% CI 76% to 95%) of 57 patients with progressive disease. The numbers of FISH anomalies were significantly different in the two clinical disease groups (Wilcoxon $P = 0{\cdot}0001$). Thus, patients with progressive disease tended to have more FISH anomalies. The mean percentage of abnormal nuclei was 55 ± 24 (median 58, range 9–92) for 37 patients with stable disease and 73 ± 26 (median 85, range 6–100) for 50 patients with progressive disease (details not shown). No apparent differences in distribution were noted for anomalies of 6q–, 11q–, +12, 13q– or 17p– among patients with stable and progressive disease.

Rai stage. FISH results were analysed for correlation with Rai stage for 112 patients (Table III). The percentages of patients that were abnormal by FISH were 68%, 69%, 87%, 93% and 80% for Rai stages 0, 1, 2, 3 and 4 respectively. The percentages of abnormal patients with ≥ 2 anomalies were 32%, 45%, 38%, 57% and 55% for Rai stages 0, 1, 2, 3 and 4 respectively. No consistent pattern in the distributions of either the FISH type of anomalies or the number of FISH anomalies among various Rai stages was apparent.

IgV_H mutational status. We compared FISH results in 62 patients classified by IgV_H mutational status (Table III). Among 28 patients with a non-mutated IgV_H clone, 21 (75%; CI 55% to 89%) were abnormal by FISH. Among 34 patients with mutated IgV_H clone, 22 (65%; CI 46% to 80%) were abnormal by FISH. Each of the five patients with +12 alone had a non-mutated IgV_H clone. Among six patients that had +12 and other anomalies, three had a non-mutated IgV_H clone and three had a mutated IgV_H clone. Otherwise, no apparent differences in FISH anomalies were observed among patients with non-mutated versus mutated IgV_H clones.

$CD19^+/CD38^+$ cells. We compared FISH results in 105 patients classified by CD38 expression (Table III). The numbers of FISH anomalies were significantly different in the two groups ('exact' Wilcoxon $P = 0{\cdot}0256$). Among 77 $CD38^-$ patients, 56 (73%; CI 61% to 82%) were abnormal by FISH, whereas 26 (93%; CI 76% to 99%) of 28 $CD38^+$ patients were abnormal by FISH. Different FISH anomalies were also observed between $CD38^+$ patients versus $CD38^-$ patients. Among 26 $CD38^+$ patients, 18 (69%; CI 48% to 86%) had an 11q–, +12 or 17p–, which purportedly are associated with poor prognosis in B-CLL (Dohner *et al*, 1997a, 2000). By comparison, among 56 $CD38^-$ patients, only 19 (34%; CI 22% to 48%) had 11q–, +12 or 17p–. As a sole anomaly, 13q– × 1 or 13q– × 2 were observed in 38 (56%) of 56 $CD38^-$ patients and seven (27%) of 56 $CD38^+$ patients. Multiple FISH anomalies were observed in 13 (50%) of 26 $CD38^+$ patients and 23 (41%) of 56 $CD38^-$ patients.

Defining CLL risk categories using FISH. Table IV summarizes FISH risk distributions for the subsets of study patients classified by age, sex, disease status (stable versus progressive disease), Rai stage, IgV_H mutation status and $CD38^+$ expression. Significant ($P < 0{\cdot}05$) differences in FISH risk distributions were found in the groups defined by Rai stage, disease status and $CD38^+$ but not by age, sex or IgV_H mutation status. In particular, larger proportions of patients with progressive disease or higher Rai stage, and those who were $CD38^+$, were classified as high risk.

Multivariate analysis was performed using the logistic model to look for an association of baseline variables with high-risk FISH anomalies (+12, 11q– and 17p–). The dependent variable was high-risk disease (= 1 for risk categories 4–6, = 0 for risk categories 1–3) and the following five variables were independent baseline variables: age (continuous, in years), male (1 = yes, 0 = no), Rai stage (0, 1, 2, 3, 4), $CD38^+$ (1 = yes, 0 = no) and stable disease (1 = yes, 0 = no). Only $CD38^+$ was significantly associated with high-risk disease defined by FISH risk categories after adjustment for the effects of the other variables in the model.

DISCUSSION

We observed significant ($P < 0{\cdot}05$) differences in FISH risk distributions in the groups defined by Rai stage, disease status and $CD38^+$ (Table IV). However, the results of multivariate analysis of age, sex, Rai stage, $CD38^+$ and disease status versus FISH risk category indicated that only $CD38^+$ was significantly associated with high-risk disease as defined by FISH risk categories after adjustment for the effects of other variables in the model. We also found that $CD38^+$ patients were more likely to have multiple FISH anomalies. As our results indicated that favourable (13q–) and unfavourable (+12, 11q–, 17p–) FISH anomalies can occur in stable and progressive disease, FISH results together with CD38 expression may provide valuable prognostic information about patients with B-CLL.

Two previous investigations suggest that 11q– and 17p– might be associated with non-mutated IgV_H clones (Rai *et al,* 2001; Krober *et al,* 2002;). Our results are different, as 11q– or 17p– was observed in five (15%) of 34 patients with mutated IgV_H clones and five (19%) of 27 patients with non-mutated IgV_H clones (Table IV). Interestingly we observed +12 as a sole anomaly in five of 28 (18%) patients with non-mutated IgV_H clones but in none of the 34 patients with mutated IgV_H clones (Table III). This could be significant, but we also observed +12 together with other anomalies in three patients with non-mutated IgV_H clones and with three patients with mutated IgV_H clones. Our patient sample size precludes any accurate conclusion.

In this study, we used our standard cut-off values, ≥ 30% B cells with CD38 expression and ≥ 2% with IgV_H differences in DNA sequence, to define $CD38^+$ and mutated IgV_H clones respectively. Other investigators have used different cut-off values (Ibrahim *et al,* 2001; Krober *et al,* 2002). Thus, we re-analysed our FISH results, using ≥ 7% and ≥ 20% B cells with CD38 expression and ≥ 3% with IgV_H differences in DNA sequence, to define $CD38^+$ and mutated IgV_H clones. The results of statistical studies using the alternate cut-offs were similar to our previous findings, although the *P*-values were somewhat larger, suggesting somewhat weaker associations between FISH risk categories and the redefined $CD38^+$ and IgV_H mutation status variables.

The hierarchy of FISH risk categories used in this investigation is arbitrary, but is supported by investigations of survival and progression in other studies of B-CLL (Dohner *et al,* 2000). Our study results confirmed that +12, 11q– and 17p– are strongly correlated with $CD38^+$ (Table IV). Other investigators have shown that CD38 expression is associated with poor prognosis in B-CLL (Hamblin *et al*, 1999; Hamblin *et al,* 2000; Ibrahim *et al,* 2001; Jelinek *et al,* 2001). To validate any hierarchy of FISH risk categories, investigations of much larger cohorts of patients from a very early stage of their disease must be studied over a long period of time.

The results of our study are consistent with the hypothesis that chromosome anomalies detected by our FISH assay are associated with chromosome evolution and disease progression. For example, we detected an abnormal clone in 66% of patients with stable disease and 88% of patients with progressive disease. Also patients with progressive disease tended to have more FISH anomalies (Table III). In addition, experiments with purified $CD5^+$/$CD19^+$ cells from patients with stable disease revealed various percentages of nuclei with FISH anomalies among B-CLL patients.

The most frequent chromosome anomaly in this study was 13q–. This same observation has been reported by other investigators using either cytogenetic or FISH studies (Han *et al*, 1984; Juliusson *et al*, 1990; Dohner *et al*, 1997b, 1999, 2000; Bullrich *et al,* 2001). If 13q– were a true initiating oncogenic event, we would expect to find it in all purified $CD5^+$/$CD19^+$ cells among patients with 13q–. Indeed, our results indicate that all purified $CD5^+$/$CD19^+$ cells in four of six patients had a 13q–. However, in two patients 13q– was observed in only a subset of purified $CD5^+$/$CD19^+$ cells. Thus, 13q– appears to be a product of chromosome evolution within neoplastic clones.

Twenty-four patients in our series had deletions involving the expected maternal and paternal chromosome 13 targets for D13S319, but retained both of the expected parental control targets for D13S327. Herein, we refer to homozygous deletion of D13S319 as 13q– × 2 and hemizygous deletion of D13S319 as 13q– × 1. Other investigators have reported homozygous or hemizygous loss at 13q14 in more than 50% of patients with B-CLL by FISH (Bullrich *et al,* 2001). We used multiple fluorescent-labelled DNA probes for different loci on chromosome 13 to study nine patients with 13q– × 2 as the sole anomaly: four of these patients had different 13q– deletions. These results suggest 13q– × 2 results from independent deletions on the maternal and paternal chromosomes 13. Consequently, 13q– × 2 may result from chromosome evolution and represent a more aggressive FISH anomaly than 13q– × 1. Thus, we arbitrarily classified 13q– × 2 in our hierarchical scheme for FISH risk categories as more aggressive than 13q– × 1.

Prior FISH-based investigations of B-CLL indicate that 17p– and 11q– are associated with a relatively poor survival and increased probability for disease progression (Dohner *et al*, 1997a, 2000). We do not have sufficient follow-up information to confirm this observation for our cohort of patients. However, we did not observe any difference in the distribution of 6q–, 11q–, +12, 13q– or 17p– for patients with stable disease versus progressive disease. We are currently conducting sequential studies on this CLL cohort to learn whether patients with stable disease and unfavourable FISH anomalies will develop disease progression.

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