



Role of cytogenetic abnormalities detected by fluorescence *in situ* hybridization as a prognostic marker: Pathogenesis & clinical course in patients with B-chronic lymphocytic leukaemia

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Background & objectives: B-cell chronic lymphocytic leukaemia (B-CLL) is one of the most common forms of adult leukaemia, with a highly variable clinical course. Specific chromosomal and genetic aberrations are used clinically to predict prognosis, independent from conventional clinical markers. Molecular cytogenetic methods such as fluorescence *in situ* hybridization (FISH) detect aberrations in up to 80 per cent B-CLL patients. This study was conducted to score the frequencies of recurrent aberrations, *i.e.*, del(13q14), trisomy 12, del(11q22), del(17p13), del(6q21) and *IgH* (immunoglobulin heavy chain) translocations and to understand their role in prognostication and risk stratification.

Methods: FISH studies were performed on bone marrow aspirate or peripheral blood of 280 patients using commercially available disease-specific probe set. The data were correlated with clinical and haematological parameters such as low haemoglobin, splenomegaly and lymphadenopathy.

Results: Chromosomal aberrations were detected in 79 per cent of patients, with del(13q14) (57%) as the most common cytogenetic aberration, followed by trisomy 12 (27%), del(11q22) (22%), t(14q32) (19%), del(17p13) (18%) and del(6q21) (9%). Single or in coexistence with other aberration del(13q14) had a favourable outcome in comparison to del(11q22), t(14q32), del(17p13) and del(6q21) which were associated with advanced stages of the disease. Trisomy 12 had a variable clinical course.

Interpretation & conclusions: FISH was found to be a sensitive and efficient technique in detecting the prevalence of recurrent cytogenetic abnormalities. Each of these aberrations is an important independent predictor of disease progression and survival which aids in designing risk-adapted treatment strategies for better disease management.

Key words B-cell chronic lymphocytic leukaemia - chromosomal aberration - fluorescence *in situ* hybridization - lactate dehydrogenase - prognosis - time-to-first treatment - treatment-free survival

B-chronic lymphocytic leukaemia (B-CLL) is highly heterogeneous in terms of its clinical course and prognosis which impacts the clinical

approaches, treatment strategies and survival times from diagnosis¹. The most prevalent symptoms in B-CLL are lymphadenopathy, organomegaly,

fever, fatigue, weight loss and night sweats, though 25 per cent of patients are asymptomatic². Rai and Binet clinical staging systems are the most useful methods for predicting survival of B-CLL patients, but these cannot be used to predict the individual risk of disease progression and survival during the early stages of disease detection³. Thus, determination of new prognostic markers is essential for providing appropriate counselling and designing better treatment strategies. Specific chromosomal and genetic aberrations are currently used clinically to predict prognosis independent of conventional clinical markers for a risk-adapted management of B-CLL patients. Conventional karyotyping can detect chromosome abnormalities in up to 40-50 per cent of patients with B-CLL⁴. Due to low mitotic index of B-CLL leukaemic cells *in vitro* and poor response to mitogens, analysis by conventional karyotyping is often hampered as there are only a few metaphases wherein smaller cryptic aberrations, and recurrent deletion cannot be detected⁵. In contrast, technique like fluorescence *in situ* hybridization (FISH) allows the detection of chromosomal aberrations not only in dividing cells but also in interphase nuclei. FISH is used for identifying abnormalities important to overall prognosis, disease progression and response to treatment. FISH also identifies abnormalities that are too small to be detected by study of metaphase chromosomes. It detects chromosome abnormalities in up to 80 per cent of B-CLL patients⁶. Thus, FISH has increased sensitivity and detection rate in comparison to conventional karyotyping. The most frequent recurrent cytogenetic abnormalities in B-CLL are 13q14 deletion (40-60%), trisomy 12 (20-40%), 11q22 (*ATM*) deletion (10-25%), 14q32 (*IgH*) translocation (4-21%), 17p13 (*TP53*) deletion (4-15%) and 6q21 (*MYB*) deletion (5-9%)⁷⁻¹².

The objective of this study was to score and evaluate the frequencies of the recurrent aberrations, *i.e.*, del(13q14), trisomy 12, del(11q22), del(17p13), del(6q21) and *IgH* translocation by FISH in B-CLL patients, and to understand the role of recurrent aberrations in prognostication, risk stratification of B-CLL patients in terms of treatment-free survival (TFS), time-to-first treatment (TTFT) based upon the correlation of each aberration with clinical and haematological parameters. Another objective was to characterize *IgH* translocation positive cases into partner chromosome and their clinical assessment.

Material & Methods

Newly diagnosed consecutive B-CLL patients referred to the Cancer Cytogenetics department, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India, were enrolled in this study from January 2016 to December 2017. Follow up cases were excluded from the study. The diagnosis of B-CLL was based on the WHO 2016 guidelines¹³. The baseline workup included clinical and haematological parameters such as persistent lymphocytosis (peripheral lymphocytes $\geq 5 \times 10^9/l$ and $\geq 40\%$ lymphocytes in bone marrow), haemoglobin (Hb) level ≤ 10 g/dl, lactate dehydrogenase (LDH) levels (normal range 110-210 U/l), Rai stages (0, I, II, III or IV), presence/absence of splenomegaly and lymphadenopathy. All the patients included in the study were positive for B-cell immunophenotype markers such as CD5, CD19 and CD23. The clonality of the circulating B lymphocytes as CLL was confirmed by flow cytometry and thus lymphoma cases were excluded. The study was approved by the Institutional Ethics Committee-III and informed consent was obtained from all participants.

FISH studies were performed on heparinized bone marrow aspirate or peripheral blood of 280 B-CLL patients using commercially available disease specific deletion probes LSI D13S319 (13q14)/LSI 13q34, LSI *TP53* (17p13)/CEP17 (Zytovision, Germany); LSI *ATM* (11q22)/CEP11, LSI 6q21/SE6 (Kreatech Diagnostics, Netherlands); centromere CEP 12 and LSI break apart *IgH* translocation probe (Vysis Abbott Molecular, Germany) according to the manufacturer's protocol. Patients showing splitting of one fluorescence signal with the break apart *IgH* probe were analyzed for partner chromosome: t(8;14), t(11;14), t(14;18) and t(14;19) using dual colour, dual fusion probes LSI *IgH/MYC*:t(8;14) [Zytovision, Germany], *IgH/CCND1*:t(11;14), *IgH/BCL2*:t(14;18) [Vysis Abbott Molecular, Germany] and *IgH/BCL3*:t(14;19) (Kreatech Diagnostics, Netherlands), respectively. The limit of detection was five per cent for deletion probes, six per cent for break apart probe and two per cent for centromeric and dual-colour, dual-fusion probes. The limit of detection was standardized by in-house validation and it varied from laboratory to laboratory according to the FISH probes used in the assay¹⁴.

In brief, peripheral blood or bone marrow sample was cultured using 5 ml complete culture medium consisting of Ham's-F12 + Glutamax

medium (Gibco, USA) supplemented with 10 per cent foetal bovine serum (Gibco, USA) and one per cent antibiotic (Gibco, USA) for 24 h. The cultured samples were incubated at 37°C without any mitogens and cells were harvested by treatment with hypotonic solution (KCl) and preserved in their swollen state with Carnoy's fixative (3:1 ratio of methanol:glacial acetic acid)¹⁵. Cells were dropped on to slides and after addition of the probe, initial co-denaturation was done at 73°C, followed by hybridization at 37°C by overnight incubation. Excess probe was washed off and after addition of a counterstain 4',6-diamidino-2-phenylindole, the slides were examined with an Olympus BX61 fluorescence microscope (Olympus Corp., Japan) equipped with appropriate filters. In every specimen, 200 interphase cells were analyzed for each probe by two observers and images were captured using the Genesis ASI System (Applied Spectral Imaging Ltd, Israel). The identified abnormalities were described according to the International System for Human Cytogenomic Nomenclature (ISCN 2016)¹⁶.

Statistical analysis: To evaluate clinicopathological significance of cytogenetic aberrations (TFS and TTFT) based on their correlation with clinical and haematological parameters, FISH-positive cases were divided into three groups. (i) Group 1: patients with single aberration were studied, to assess the importance of a specific aberration without the influence of additional abnormalities; (ii) Group 2: patients with coexistence of two aberrations, *i.e.*, those with coexistence of del(13q14) and other aberrations were compared with those with coexistence of two aberrations other than del(13q14); and (iii) Group 3: disease progression in patients with coexistence of >2 aberrations was studied by comparing them with cases with either single aberration or coexistence of two aberrations.

Data were analyzed using IBM SPSS version 21 (IBM Corp. NY, USA). A Chi-square test for independence was used to test if there was any association between the clinical parameters and the cytogenetic aberrations. One of the endpoints was to analyze the TFS and TTFT among the cytogenetic aberrations which was done using the Kaplan–Meier (log-rank) analysis.

Results

The number of males and females included in the study was 215 and 65, respectively (n=280) with an

age range of 30–86 yr (median: 60 yr). Among the 280 patients, 220 (79%) had genetic aberrations, of whom 137 (62%) had one aberration, 60 (27%) had two aberrations and 23 (10%) patients revealed coexistence of >2 aberrations. The most common cytogenetic aberration was del(13q14) (57%), followed by trisomy 12 (27%), del(11q22) (22%), t(14q32) (19%), del(17p13) (18%) and del(6q21) (9%) (Fig. 1 and Table I). Of the 126 patients with 13q14 deletions, 13 (10%) had biallelic (0R2G) or coexistence of monoallelic (1R2G) and biallelic deletions, while the remaining had only monoallelic deletion. *IgH* aberrations were identified 19 per cent (43/220) patients, of whom 49 per cent (21/43) had translocation (1Y1R1G), 47 per cent (20/43) had deletion in variable region of *IgH* (1Y1R/1Y1RdimG) and five per cent (2/43) had deletion in 3' *IgH* region (1Y1G) (Fig. 2). Of the 43 patients with translocation t(14q32), three had t(11;14)(q13;q32) (*IgH/CCND1*), seven had t(14;18)(q32;q21) (*IgH/BCL2*), four had t(14;19)(q32;q13) (*IgH/BCL3*) and the rest 26 patients had t(14q32) with an unidentified partner chromosome (Fig. 3). *IgH* characterization for three cases was not possible due to insufficient sample.

Correlation with clinical and haematological parameters: In group 1, of the 137 patients with single cytogenetic aberration, two prognostic categories were identified. The first category was patients with del(13q14) a sole abnormality which was associated with good prognostic markers having less number of patients with low Hb ($P>0.06$), high LDH ($P<0.001$), lymphadenopathy ($P<0.05$), splenomegaly ($P<0.02$), Rai stage 3 or 4 ($P<0.01$) and more number of patients having TFS ($P<0.001$). The second prognostic category was patients positive for either del(11q22) or del(17p13) in which the disease progression was more rapid due to their association with more aggressive clinical and haematological parameters, *i.e.*, low Hb ($P>1.00$, $P<0.02$), high LDH ($P>0.23$, $P>0.13$), lymphadenopathy ($P<0.03$, $P>0.26$), splenomegaly ($P<0.04$, $P<0.00$), Rai stage 3 or 4 ($P<0.01$, $P<0.02$) and lower number of patients with TFS ($P<0.03$, $P>0.25$) (Table II and Fig. 4). The median TTFT for del(13q14) was 380 days, and for del(11q22) and del(17p13), it was 16 and 11 days, respectively. Although insignificant, disease progression for patients with trisomy 12 was variable, and for del(6q21), it was aggressive. Patients with trisomy 12 were associated with both high-risk parameters such as high LDH ($P<0.001$), lymphocytosis ($P>1.00$), lymphadenopathy ($P>0.44$) and less TFS ($P>0.57$) and intermediate-risk

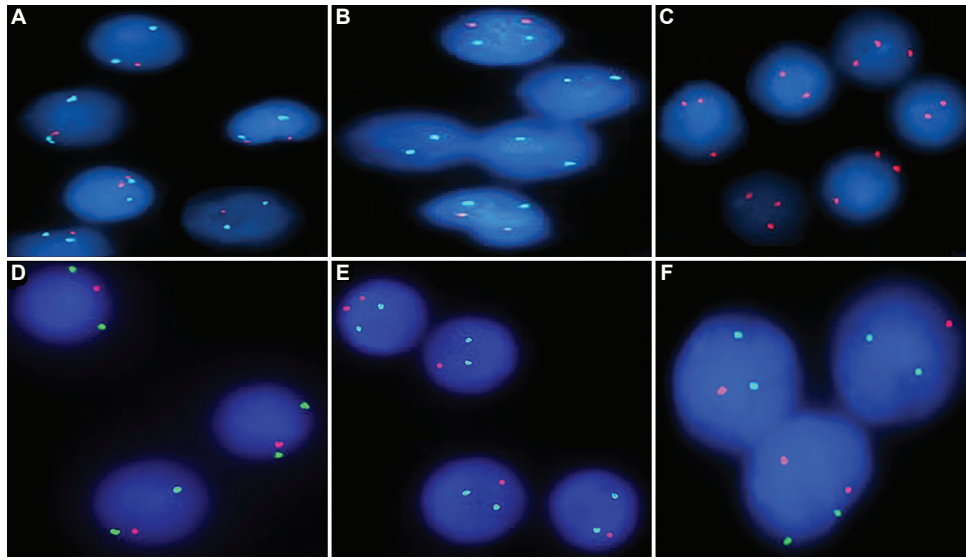


Fig. 1. (A) LSI 13S319 (13q14.3) probe shows monoallelic deletion of 13q14.3 locus (one red and two green signals); (B) LSI probe 13S319 (13q14.3) shows biallelic deletion of 13q14.3 locus (zero red and two green signals); (C) CEP 12 probe shows trisomy 12 (three red signals); (D) LSI ATM (11q22.3)/CEP11 probe shows monoallelic deletion of ATM (one red and two green signals); (E) LSI 6q21/SE6 probe shows monoallelic deletion of 6q21 (one red and two green signals); (F) LSI TP53 (17p13.1)/CEP17 probe shows monoallelic deletion of TP53 (one red and two green signals).

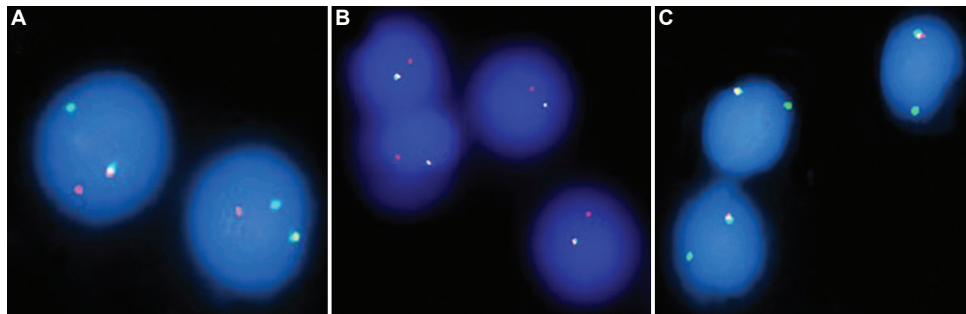


Fig. 2. (A) Dual-colour *IgH* break apart probe on interphase cells shows normal *IgH* allele (one yellow signal) and residual *IgH* (one red and one green signal); (B) Dual-colour *IgH* break apart probe on interphase cells shows normal *IgH* allele (one yellow signal), Residual 3' *IgH* (one red signal); (C) Dual-colour *IgH* break apart probe on interphase cells shows normal *IgH* allele (one yellow signal), deletion in 3' *IgH* region (one green signal).

Table I. Frequency of cytogenetic aberrations in B-chronic lymphocytic leukaemia patients (n=220)

Cytogenetic aberration	Single aberration, n (%)	Double aberration, n (%)	>2 aberration, n (%)	Total, n (%)	Incidence in literature (%) ^{7-12,17}
del(13q14)	67 (30)	41 (19)	18 (8)	126 (57)	40-60
Trisomy 12	26 (12)	21 (10)	10 (5)	57 (27)	20-40
del(11q22)	17 (8)	19 (9)	10 (5)	46 (22)	10-25
t(14q32)	14 (6)	17 (8)	12 (5)	43 (19)	4-21
del(17p13)	9 (4)	18 (8)	14 (6)	41 (18)	4-15
del(6q21)	4 (2)	4 (2)	11 (5)	19 (9)	5-9

parameters such as less patients with splenomegaly ($P < 0.01$), low Hb ($P > 0.59$), Rai stage 3 or 4 ($P > 0.10$) and median TTFT of 89 days (Fig. 4). Deletion

6q21-positive patients had low Hb ($P > 0.14$), high LDH ($P > 0.57$) and Rai stage 3 or 4 ($P > 0.59$); all the patients had lymphocytosis ($P > 1.00$), splenomegaly

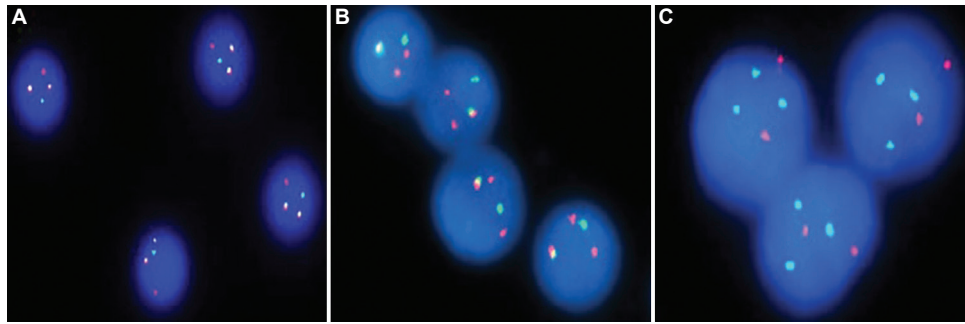


Fig. 3. (A) *IgH/CCND1* dual-fusion probe shows *IgH-CCND1* fusion (two yellow signals), 1 normal *IgH* allele and 1 normal *CCND1* allele (one green and one red signal, respectively); (B) *IgH/BCL3* dual-fusion probe shows *IgH-BCL3* fusion (one yellow signal), one *IgH* allele and two normal *BCL3* allele (one green and two red signals); (C) *IGH/MYC* dual-fusion probe shows *t(14;?)(q32;?)* with an unidentified partner, 3' *IgH* allele and 2 normal *MYC* allele (3 green and 2 red signals).

Table II. Correlation of cytogenetic aberrations with clinical and haematopathological parameters in a cohort of 137 B-chronic lymphocytic leukaemia patients (Group 1)

Parameters	del(13q14)	Trisomy 12	del(11q22)	t(14q32)	del(17p13)	del(6q21)
Number of patients	67	26	17	14	9	4
Haemoglobin (<10 g/dl) (%)	17	24	23	25	63	67
<i>P</i>	0.06	0.59	1.00	1.00	0.02	0.14
Lymphocytes ($\geq 5 \times 10^9/l$) (%)	100	96	93	92	88	100
<i>P</i>	0.12	1.00	0.39	0.34	0.24	1.00
Lactate dehydrogenase (>210 U/l) (%)	40	80	77	45	88	33
<i>P</i>	0.001	0.001	0.23	0.28	0.13	0.57
Splenomegaly (%)	42	29	79	73	100	100
<i>P</i>	0.02	0.01	0.04	0.20	0.00	0.24
Lymphadenopathy (%)	60	64	93	58	88	100
<i>P</i>	0.05	0.44	0.03	0.52	0.26	0.55
Rai stage (%)						
0, 1, 2	65	68	17	45	13	33
3, 4	35	32	83	55	87	67
<i>P</i>	0.01	0.10	0.01	0.38	0.02	0.59
Treatment free survival (%)	51	36	8	25	13	0
<i>P</i>	0.001	0.57	0.03	0.53	0.25	0.29

($P > 0.24$) and lymphadenopathy ($P > 0.55$) and none of the patients had TFS ($P > 0.29$) and median TTFT of 18 days (Fig. 4).

Overall disease progression in patients with coexistence of two aberrations is more rapid, but when 41 patients positive for del(13q14) and any other aberration were compared with 19 patients with combination of any two cytogenetic aberrations other than del(13q14), the number of patients with TFS was higher due to the association with low-risk clinical and haematological parameters (Table III). Median TTFT

for cases with del(13q14) was 256 days and for cases without del(13q14), it was 25 days (Fig. 5).

Patients with coexistence of >2 aberrations (23 cases) had a more advanced disease. High risk prognostic features *i.e.* low Hb levels, lymphocytosis, high LDH, lymphadenopathy, Rai stage 3 or 4 and splenomegaly were found in 75-85 per cent of patients and 5 per cent of patients had TFS. TTFT was of 10 days in comparison to those having single or double aberration, for which the TTFT was 130 days.

Table III. Comparison of clinical and laboratory data among 60 patients with del(13q14) and other aberration versus coexistence of other two aberrations (Group 2)

Parameters	Coexistence of two aberrations with del(13q14)	Coexistence of two aberrations without del(13q14)
Haemoglobin (<10 g/dl) (%)	18	53**
Lymphocytes ($\geq 5 \times 10^9/l$) (%)	98	100
Lactate dehydrogenase (>210 U/l) (%)	45	73
Splenomegaly (%)	43	75*
Lymphadenopathy (%)	34	73**
Rai stage (%)		
0, 1, 2	63	33*
3, 4	37	67
Treatment-free survival (TFS) (%)	47	13*

* $P < 0.05$, ** $P < 0.01$ as compared to coexistence of two aberrations with del(13q14)

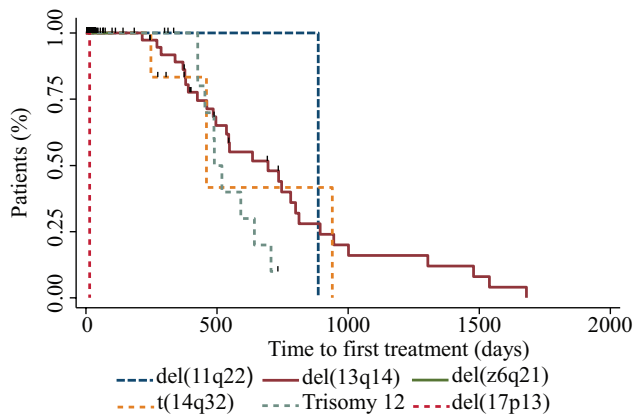


Fig. 4. Probability of disease progression, as indicated by the treatment-free survival and time-to-first treatment in 137 patients with single cytogenetic aberrations.

The clinical significance of *IgH* translocation-positive patients characterized into partner chromosomes was also assessed. Patients positive for t(14;18)(q32;q21) had an intermediate disease progression with none of patients having low Hb. Lymphocytosis was present in all the patients, while high LDH lymphadenopathy, Rai stage 3 or 4 and splenomegaly were present in 20- 60 per cent patients. The median TTFT was 499 days and 40 per cent of the patients had TFS. The clinical correlation of other *IgH* partner chromosomes was not significant, but they represented an unfavourable disease outcome. Patients positive for t(11;14)(q13;q32), t(14;19)(q32;q13) and t(14;?)(q32;?) had low Hb count (67, 25 and 59%), high LDH (100, 100 and 65%), lymphocytosis (66, 100 and 95%), lymphadenopathy (100, 75 and 64%), splenomegaly (100, 50 and 70%),

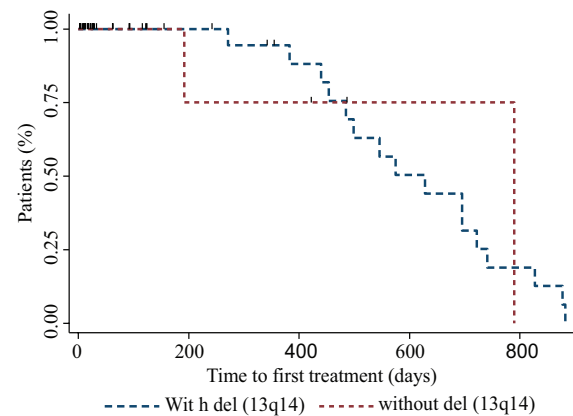


Fig. 5. Probability of disease progression, as indicated by the treatment-free survival and time-to-first treatment in 60 patients with or without coexistence of del(13q14).

Rai stage 3 or 4 (100, 50 and 64%) and TFS (0, 25 and 18%). The median TTFT for each partner chromosome was 216, 8 and 16 days, respectively.

Discussion

In the present study, chromosomal abnormalities were detected in 79 per cent of B-CLL patients by FISH which was in accordance to reported studies (50-80%)⁶. The incidence of del(13q14) (57%), trisomy 12 (27%), del(11q22) (22%), t(14q32) (19%) and del(6q21) (9%) was not deviated from that of previously reported western and Indian studies, *i.e.*, del(13q14) (40-60%), trisomy 12 (20-40%), del(11q22) (10-25%), t(14q32) (4-21%) and del(6q21) (5-9%)⁷⁻¹². In comparison to western literature (4-15%), the incidence of del(17p13) (18%) was higher in our study and previously reported studies which may be responsible for a different

biology¹⁷. The study showed the efficiency of FISH to detect various types of chromosomal abnormalities such as deletion, translocation/partial deletion and aneusomy.

The most frequent abnormality associated with favourable prognosis was a deletion involving chromosome band 13q14, which occurred in 57 per cent of cases, a finding similar to literature^{5,6}. The significance of large 13q14 deletions is emphasized by involvement of multiple gene loci such as *miR15a/16-1 micro RNA*, *RBI* and *DLEU7* genes involved in key biological pathways like cell cycle control, NF-κB signalling which is responsible for tumour suppressor activity and different clinical courses of the disease^{18,19}. Trisomy 12 was the second most frequent recurrent chromosomal aberration (27%). The prognostic effect of trisomy 12 has been controversial and our study was also consistent with the previous study¹². Consistent clonal del(13q14) and trisomy 12 appeared to be relatively specific drivers of B-CLL or B-cell malignancies^{11,20}.

Apart from the reported independent prognostic factor for poor prognosis, *i.e.*, del(17p13) reported in 18 per cent cases in the present study, two other adverse prognostic markers were identified – del(11q22) and del(6q21) with a frequency of 22 and 9 per cent, respectively. The higher frequency of TP53 deletion may reflect population difference. Inactivation of *TP53* gene which has a role in cell death and cell cycle, is involved in del(17p13)²¹. Genes having an important role in pathogenesis of the del(11q22) disease are *ATM* located within the minimal region of loss at 11q23 and *BIRC3* which is located near *ATM* gene²¹⁻²⁴. According to Cuneo *et al*²⁵, B-CLL with del(6q21) showed distinct haematological features and intermediate prognosis, but in our study, del(6q21) was identified as high-risk cytogenetic prognostic marker²⁶. Edelmann *et al*²⁷ defined a region of 2.5Mb at 6q21 that was affected in 80 per cent of del(6q21) patients; however, no specific gene has been identified as responsible for the 6q pathogenesis.

Presence of specific *IgH* translocations is not a characteristic of B-CLL; however, it has been described that 2-26 per cent of patients carry a wide range of translocations²⁸ which in our study was 19 per cent. *IgH* translocations result in the deregulated expression of genes involved in several pathways having a role in control of cell proliferation, apoptosis, *etc.* In a majority of the cases, the

translocated partner gene becomes transcriptionally deregulated as a consequence of its transposition into the immunoglobulin (IG) locus. Put *et al*²⁹ revealed that t(14;18)(q32;q21) in B-CLL was not associated with an unfavourable clinical outcome in a large patient cohort with a median TFS time of 48 months. Davids *et al*³⁰ demonstrated that the TTFT was significantly shorter among patients harbouring t(14q32) translocations without t(14;18)(q32;q21), compared with those having t(14;18)(q32;q21) which was consistent with our study. Patients with t(11;14)(q13;q32) and t(14;19)(q32;q13) were associated with poor prognosis^{31,32}.

Frequencies of combinations of different chromosomal changes were in accordance to other studies⁸⁻¹². The clinical outcome for the patients with combined chromosomal aberrations was adverse except for patients with coexistence of two aberrations having del(13q14) as one of the aberrations, the disease progression was slow³³. Thus, for patients with coexistence of aberrations, the clinical course will depend on the combination of aberrations present.

In conclusion, our study showed FISH to be a sensitive and efficient technique in detecting the prevalence of various recurrent cytogenetic abnormalities. These recurrent aberrations are implemented as strong, independent prognostic and diagnostic markers for predicting the individual risk of the disease progression and, survival during early stages of disease detection. Continued understanding of the relationship between specific FISH abnormalities and clinical outcome will lead to improved risk stratification and better disease management. The molecular basis for clinical differences in the outcome may provide important insights into disease biology and should be subjected to further investigations.

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Conflicts of Interest: None.

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