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Investigation of clonal involvement of myeloid cells in Philadelphia-positive and high hyperdiploid acute lymphoblastic leukemia

A Kasprzyk, CJ Harrison and LM Secker-Walker

Cytogenetics Laboratory, The Royal Free and University College School of Medicine, Pond Street, London NW3 2QG, UK

Acute lymphoblastic leukemia (ALL) with a high hyperdiploid clone has a good prognosis for both childhood and adult patients while patients with Philadelphia-positive (Ph) ALL do badly at all age groups. It has been suggested that different responses to treatment might be related to the cell of origin of the leukemia with 'stem cell' cases responding less well than those arising in a lymphoid committed progenitor cell. The clonal involvement of different cell lineages in 12 patients with acute lymphoblastic leukemia has been examined by applying fluorescence *in situ* hybridization (FISH) to detect chromosomal abnormalities in bone marrow cells previously identified by morphology and/or immunology. The karyotype of the malignant clone was either high hyperdiploid or Philadelphia translocation (Ph) positive with a breakpoint in the minor breakpoint cluster region of the *BCR* gene (m-*BCR*) or in the major breakpoint cluster region of the *BCR* gene (M-*BCR*). The high hyperdiploid clone, in each case, was found in cells of the B-lymphoid ($CD19^+$) lineage but not in T cells ($CD3^+$) or in cells of the myeloid ($CD13^+$) or erythroid (glycophorin A^+) lineages, indicative of a lymphoid committed progenitor cell. Heterogeneity of lineage involvement was found in Ph^+ ALL: the m-*BCR* Ph^+ clone was found in lymphoid/blast cells but not in neutrophils or eosinophils. In contrast both M-*BCR* Ph^+ clones were detected in myeloid as well as lymphoid lineages, indicative of a stem cell origin.

Keywords: acute lymphoblastic leukemia; FISH; high hyperdiploidy; lineage; Philadelphia translocation

Introduction

Acute lymphoblastic leukemia (ALL) with a high hyperdiploid clone is associated with a good prognosis while clones with the Philadelphia (Ph) translocation t(9;22)(q34;q11) signify a poor prognosis in all age groups.[1–6] It has been suggested that the chemosensitivity of different leukemias may be related to the proliferative properties of the cell in which neoplastic transformation has occurred.[7,8] Leukemias originating in a pluripotent stem cell would be chemoresistant, while those originating in a committed progenitor cell would be relatively chemosensitive. The identification of the leukemic progenitor cell may therefore have important therapeutic implications. Acute lymphoblastic leukemia is characterized by the proliferation and accumulation of clones of genetically altered immature cells of the lymphoid series. Thus ALL was originally presumed, in all cases, to arise in a lymphoid committed progenitor cell but this has been proven not to be so for Ph-positive ALL. In some cases the Ph translocation has been found in myeloid and lymphoid cells suggestive of a stem cell origin.[7,8]

We have investigated lineage involvement in cases of ALL with a high hyperdiploid clone (nine cases) or with the Philadelphia translocation (three cases) in order to discover the probable level of commitment of the target cell in these prognostically distinct subgroups of ALL. This involved first, the detection of different cell types by immunophenotyping and/or by morphological examination followed by the application of fluorescence *in situ* hybridization (FISH) to individual cells using probes to detect the clonal, chromosomal abnormalities.[9–12]

Correspondence: C Harrison; Fax: 0171 794 1417
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Materials and methods

Patients

Patients were diagnosed as having ALL by standard clinical, hematological and immunological investigations. Cytogenetic analysis of the bone marrow had identified high hyperdiploidy (nine cases), or the Philadelphia translocation (three cases). Peripheral blood samples from three healthy males and three healthy females were used as controls.

Methods

Cells remaining from the cytogenetic investigation and stored in Carnoy's fixative at −20°C were used for the metaphase FISH investigations. Investigations of lineage involvement and interphase FISH were performed on cytospins which had been prepared from the mononuclear layer or high density fraction of cells from the patients' bone marrow.

Mononuclear (MNC) and high density (HD) cell isolation

Samples were diluted 50:50 with phosphate-buffered saline (PBS). Ten ml of diluted bone marrow/peripheral blood was added to 10 ml of lymphoprep (Nycomed; Pharmacia, Oslo, Norway) and centrifuged at 800 *g* for 20 min. Red cell lysis buffer (15 ml) was added to both the mononuclear cell (MNC) layer and to the high density (HD) fraction, left at room temperature (5 min) and spun at 400 *g* (5 min). The supernatant was removed and the cells were washed in 20 ml of phosphate-buffered saline (PBS) and centrifuged at 400 *g* (5 min). The separated cells were resuspended in PBS and cytospins were prepared, wrapped in aluminium foil and stored at −20°C.

Investigation of the clonal involvement of different cell lineages

Cells, belonging to particular cell lineages, were first identified on cytospins of bone marrow by visualizing either their morphology using May–Grünwald-Giemsa (MGG) stain or their immunophenotype using the alkaline-phosphatase, anti-alkaline-phosphatase (APAAP) technique. Subsequently, the same preparations were used for FISH investigation. Following the

MGG staining, cytospins were scanned and the location of cells of interest was recorded using low (×20) magnification. Cell morphology was recorded using high (×100) magnification, a CCD camera coupled to a Zeiss Axioskop microscope (Carl Zeiss, Germany) and an Apple Macintosh Computer equipped with the Smart Capture Software (Vysis, UK). When recording was complete, slides were destained in 100% methanol for 1 min, air dried and prepared for FISH.

APAAP technique: To determine cell immunophenotype, monoclonal antibodies from DAKO were used as follows: T-lymphoid (CD3), B-lymphoid (CD19), myeloid (CD13) and erythroid (glycophorin A). These were applied and visualized as directed by the manufacturers. Following APAAP staining, the coverslips were removed by soaking in deionized water, air dried and prepared for FISH.

Fluorescence in situ hybridization (FISH): Chromosome-specific, alpha-satellite probes (Oncor, Gaithersburg, MD, USA) were obtained for chromosomes X, 6 and 7. Probes to indentify t(9;22) from the fusion of *ABL* with the minor (m) or the major (M) *BCR* were obtained from Oncor. The probes were used in accordance with the manufacturer's instructions.

Five hundred cells were scored on each control sample. Control values for all probes were calculated as the mean plus twice the standard deviation.

Results

Patients

The clinical data and karyotypes of the patients are shown in Table 1.

The control values for cells with an extra signal to chromosomes 6 or X, a missing signal to chromosome 7 or with *BCR/ABL* fusion are shown in Table 2.

Results of APAAP/FISH and MGG/FISH investigation

High hyperdiploidy: Nine patients with high hyperdiploidy at diagnosis (cases 1–7) or in relapse (cases 8 and 9) were investigated with APAAP/FISH and MGG/FISH using an alpha-satellite chromosome-specific probe to one of the chromosomes gained (trisomies). The FISH investigation confirmed the presence of the relevant trisomy on metaphase spreads in cases where such material was available (cases 7–9). In the remaining cases the presence of the trisomy was confirmed on interphase nuclei on cytospins.

The results of the APAAP/FISH and MGG/FISH studies and the probe used for interphase detection of the chromosomal abnormality are shown in Table 3. Significant numbers of cells with one additional signal to chromosome X (+X) (cases 1, 2, 4, 6–9), two additional signals to chromosome X (+X,+X) (case 5) or one extra signal to chromosome 6 (case 3) were observed as follows: Using APAAP/FISH the clone was detected in B-lineage ($CD19^+$) cells but not in T cells ($CD3^+$) nor in cells of the myeloid ($CD13^+$) or erthroid (glycophorin A^+) lineages. An example of APAAP/FISH is shown in Figure 1A–D. In some cases the MGG/FISH investigation yielded rather few myeloid cells for analysis but again significant numbers of clonal cells were seen only in immature lymphoid blasts. Myeloid cells (neutrophils and eosinophils) and erythroid cells had no extra signals in 8/8 and 3/3 evaluable cases, respectively. An example of MGG/FISH is shown in Figures 1E and F.

Philadelphia positive cases: Metaphase spreads from three patients with t(9;22)(q34;q11) (Table 1, cases 10–12) were first examined with the M-*BCR-ABL* and m-*BCR-ABL* probes to confirm the translocation breakpoint. Case 10 had m-*BCR/ABL* fusion (examined in refractory disease) (Figure 2C) and cases 11 and 12 had an M-*BCR/ABL* fusion (Figure 2A). The detection of cell lineage in Philadelphia-positive cells was limited to MGG-FISH (Table 3, Figures 2B and D). Both patients with M-*BCR* (cases 11 and 12) had elevated numbers of cells with M-*BCR-ABL* fusion in myeloid cells (neutrophils) as well as in lymphoid blasts (Figure 2B). Bone marrow from case 10 was available for MGG staining only in refractory disease when both homologues of chromosomes 9 and 22 were translocated t(9;22)(q34;q11),t(9;22)(q34;q11). Two m-*BCR-ABL* fusion signals were observed in blast cells but fusion signals in myeloid cells were within control values (Figure 2D). APAAP proved to be technically impossible with *BCR/ABL* probes due to the relatively low intensity of the fluorescent signals in the brightly fluorescing CD^+ cells but clonality of the diagnostic sample from case 10 (karyotype 44,XY,–7,t(9;22)(q34;q11),-22) was examined by MGG and APAAP/FISH using a chromosome 7 centromeric probe (Figures 2E and F). Both techiques confirmed the findings in relapse, namely that the clone was present only in lymphoid blast or $CD19^+$ cells and was not in neutrophils, $CD13^+$, glycophorin A^+, or $CD3^+$ cells.

Discussion

Patients with ALL and a high hyperdiploid clone were investigated using FISH combined with both immunphenotyping and with morphology in order to discover the clonal involvement of different cell lineages. Each of nine patients with a high hyperdiploid clone showed the chromosomal abnormality only in cells of the lymphoid lineage, strongly suggesting that the clone had arisen in a lymphoid committed progenitor cell. We are aware of only one other similar study. Larrermendy *et al*[13] reported finding clonal cells restricted to the lymphoid lineage in 5/6 cases with high hyperdiploid ALL. In the remaining patient, the leukemic cells also expressed myeloid-associated antigens and the high hyperdiploid clone was found also in the erythroid cells.

Others have shown lymphoid restriction to be a feature of ALL with other karyotypic abnormalities. Dow *et al*[14] demonstrated restriction of clonality (identified by the presence of a single iso-enzyme type A or B) to the lymphoid lineage in 19 patients with ALL who were heterozygous for the X chromosome-linked enzyme, glucose-6-phosphate dehydrogenase (G6PD). Clonal karyotypes in these cases were low hyperdiploid or pseudodiploid. Knuutila *et al*[15] used the morphology, antibody, chromosome (MAC) technique to show that chromosomal abnormalities t(5;14) and +X were restricted to cells of lymphoid morphology in a male patient with ALL and that low hyperdiploid clones with trisomy 7 and trisomy 8 respectively were restricted to the lymphoid lineage.[16] Therefore, with only one exception, clones with trisomies in patients with ALL have been found only in cells of the lymphoid lineage. While these findings suggest that cases of ALL with trisomies originate in a lymphoid committed progenitor

Table 1 Clinical data and karyotypes of patients with ALL investigated for lineage involvement

Case No.	*Age (years)/ Sex*	*Immunophenotype*	*Stage*	*Karyotype*	*REL (months)*	*BMT Y/N (months)*	*Survival (months)*
1	3/F	common	diag	53,XX,+X,+4,+6,del(6)(q),+17,+21,+22,inc	–	No	48+
2	4/M	NK	diag	53,XY,+X,+14,+18,+21,+21,+2mar	33	No	46+
3	4/M	common	diag	54,XY,+Y,+4,+6,+10,+14,+17,+21,+21	–	12	38+
4	5/F	common	diag	52,XX,+X,+4,+18,+21,inc	–	No	52+
5	11/F	common[a]	diag	65–72,XX,+X,+X,+1,+1,+2,+5,+6,+6,+8,+9,del(9)(p22),+10,+11,+12,+16,–17,+18, +19,+20,+20,+21,+21,+22,+2mar	21	25	25
6	19/M	NK	diag	54–55,XY,+X,+4,+11,+21,inc	NK	NK	NK
7	23/M	common	diag	79–87,XX,XY,–4,–8,–8,–11,–11,–12,–15,–15,+22,+2mar,inc	–	No	53+
8	4/M	Pre-B	rel	63–66,XY,+X,der(1)t(1;?)(q21;?),der(1)t(1;?)(q21;?),+2,+4,+5,+6,+7,+8,+10,+11,+12, +16,+17,+18,+19,+20,+21,+22	36	43	82+
9	4/M	common	rel	65,XY,+X,+1,+4,+5,+6,+6,+8,+9,+10,+10,+11,+1,+2,+14,+add(17)(p),+18,+21, +21,+21,+22	31	41	46
10	38/F	common	diag	44,XX,–7,t(9;22)(q34;q11),–22	–	No	10
			refractory	54,XX,+X,t(1;7)(p11;q34),+5,+8,+8,t(9;22)(q34;q11),t(9;22)(q34;q11),+11, del(12)(p),+12,+17,+19			
11	18/M	common	refractory	46,XY,t(9;22)(q34;q11)	–	3	5
12	61/M	NK	diag	46,XY,t(9;22)(q34;q11)	NK	NK	NK

[a]with myeloid marker expression
NK, not known

Table 2 Cells with chromosomal gain, loss or gene fusion in control blood samples from three females and three males

Chromosomal abnormality or gene fusion probed	*Hybridization signals*	*Percentage of abnormal cells*		
		Mean (M)	*Standard deviation (s.d.)*	*Control values (M + 2 × s.d.)*
+X (males)	2 signals	1.2	0.4	2
+X (females)	3 signals	1.1	0.4	1.9
+X,+X (females)	4 signals	0.8	0.4	1.6
+6	3 signals	0.9	0.3	1.5
–7	1 signal	3.2	0.3	3.8
M-*BCR-ABL*	M-BCR-ABL fusion	5.1	0.4	5.9
m-*BCR-ABL*	m-BCR-ABL fusion	5.9	0.6	7.1

Table 3 The results of morphology/FISH and immunology/FISH investigation

Case No.	*Chromosomal abnormality or gene fusion probed*	*Morphology*				*Immunology*			
		Neu	*Eo*	*Er*	*Lymphoid/Blast*	*CD13+*	*Glyco A+*	*CD3+*	*CD19+*
		No. abnormal/total cells (%)				*No. abnormal/total cells (%)*			
1	+X	0/46 (0)	0/3 (0)	NCD	164/168 (97.6)	0/81 (0)	0/32 (0)	0/37 (0)	234/247 (94.7)
2	+X	0/53 (0)	0/16 (0)	0/35 (0)	191/198 (96.5)	1/71 (1.4)	0/55 (0)	1/155 (0.6)	203/207 (98.1)
3	+6	0/32 (0)	0/3 (0)	0/28 (0)	110/119 (92.4)	0/50 (0)	0/38 (0)	0/34 (0)	124/132 (93.9)
4	+X	0/9 (0)	NCD	NCD	162/167 (97.0)	0/32 (0)	0/31 (0)	0/40 (0)	131/139 (94.2)
5	+X,+X	0/20 (0)	0/35 (0)	0/12 (0)	251/256 (98.0)	0/50 (0)	0/41 (0)	0/56 (0)	301/310 (97.1)
6	+X	0/11 (0)	0/1 (0)	0/7 (0)	159/163 (97.5)	0/32 (0)	0/40 (0)	0/30 (0)	119/125 (95.2)
7	+X	0/9 (0)	0/3 (0)	NCD	135/137 (98.5)	0/34 (0)	0/37 (0)	0/30 (0)	100/103 (97.1)
8	+X	0/61 (0)	0/21 (0)	NCD	154/163 (94.5)	1/90 (1.1)	0/50 (0)	0/45 (0)	201/203 (99.0)
9	+X	0/17 (0)	0/1 (0)	NCD	166/168 (98.8)	0/42 (0)	0/33 (0)	0/38 (0)	357/361 (98.9)
10	–7	2/100 (2)	NCD	NCD	165/189 (87.3)	2/127 (1.6)	1/30 (3.3)	2/56 (3.6)	87/100 (87.0)
	m-*BCR-ABL*	2/34 (5.9)	1/22 (4.5)	NCD	95/100 (95.0)	NI	NI	NI	NI
11	M-*BCR-ABL*	41/45 (91.1)	0/4 (0)	NCD	121/132 (91.6)	NI	NI	NI	NI
12	M-*BCR-ABL*	42/52 (80.8)	0/2 (0)	NCD	111/117 (94.9)	NI	NI	NI	NI

NI, not investigated; NCD, no cell detected; Neu, neutrophilic lineage; Eo, eosinophilic lineage; Er, erythroid lineage; Glyco A, glycophorin A.

cell the possibility of a pluripotent stem cell origin with subsequent restriction to lymphoid development cannot be ruled out. Quijano *et al*[17] demonstrated cytogenetic abnormalities in CD34+/lineage-negative cells in 5/19 patients with ALL. These comprised three of the 12 patients with a high hyperdiploid (50–65 chromosomes) clone and two of the seven patients with a low hyperdiploid (47–49 chromosomes) clone. This suggests that high hyperdiploid clones in ALL may arise in a lineage uncommitted stem cell but whether such cells are capable of forming myeloid cells is, at present, unknown.[17]

In contrast, Philadelphia-positive clones showed heterogeneity in terms of lineage involvement. Both our cases with Ph+ M-*BCR* showed the clone in lymphoid and myeloid cells indicative of a pluripotent stem cell origin. These cases presented as Ph+ ALL but, as in many such cases, the possibility of CML with a previously undiagnosed chronic phase could not be ruled out. In contrast, the m-*BCR/ABL* Ph+ clone was seen in lymphoid cells only. Heterogeneity of the cell of origin in Ph+ ALL has been shown in a number of other studies using various techniques. Cytogenetic studies of hemopoietic colonies derived from a total of 19 patients with Ph+ ALL have shown that Ph+ clones can be lymphoid restricted (11 cases) or may occur both in the lymphoid and in the myeloid compartment (eight cases).[18–20] A distinction can now be made between the M-*BCR* (CML-like) and m-*BCR* (ALL-like) variant of Ph+ ALL. The use of molecular techniques on six of these 19 cases enabled the demonstration that two m-*BCR* and one M-*BCR* cases were lymphoid restricted while one m-*BCR* and two M-*BCR* cases were stem cell cases.[19] Bone marrow from six further cases, separated by density gradient centrifugation, was investigated by Southern blotting for M-*BCR* or m-*BCR* rearrangements in the different cell components. Of four cases with M-*BCR*, two were lymphoid restricted and two also showed myeloid involvement. Both cases with m-*BCR* were restricted to the lymphoid lineage.[21,22] Two more recent studies, combining FISH with morphology, have together demonstrated lymphoid restriction in four cases with m-*BCR* and multilineage involvement in eight cases with M-*BCR* and five cases with m-*BCR*.[23,24] No correlation has been found between breakpoint location and patient survival. However, patients in whom the clone occurs in both lymphoid and myeloid cells have been shown to survive significantly longer than those with lymphoid involvement only.[22,23] This finding is at odds with the predicted features of stem cell leukemia

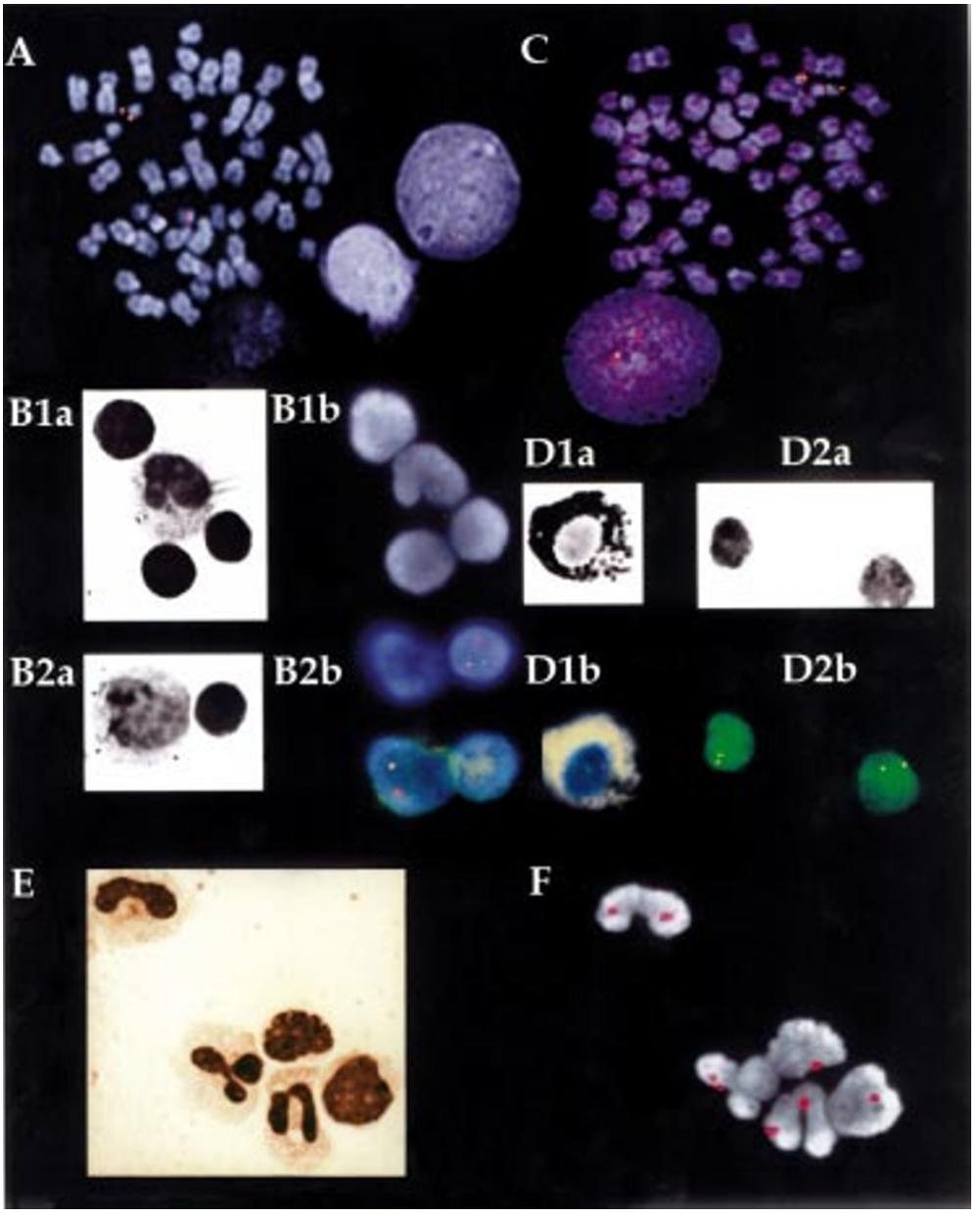

Figure 1 (A–D) The results of APAAP/FISH investigation in case 5 (female). Cells positive with a given antibody are stained red. Green dots represent hybridization signals with alpha-satellite chromosome X specific probe. (A) Note two signals in $CD13^+$ cell and four signals in the remaining cells. (B) Note two signals in glycophorin A^+ cell and four signals in the remaining cells. (C) Note four signals in $CD19^+$ cells. (D) Note two signals in $CD3^+$ cell and four signals the remaining cells. (E and F) The results of MGG/FISH investigation in case 1 (female). Red dots represent the hybridization signals with alpha-satellite chromosome X specific probe. (E) Cells stained with MGG to identify the morphology. (F) The same cells after hybridization with the X chromosome probe. Note two signals in the eosinophilic and neutrophilic cells and three signals in the blast cells.

by which stem cell involvement should equate with lack of chemosensitivity and the involvement of a committed progenitor cell with relative chemosensitivity.[7,8]

Our case with m-*BCR/ABL* Ph^+ restricted to the lymphoid lineage was of particular interest. The Ph^+ clone at diagnosis was hypodiploid with monosomy 7, which is characteristic of chronic myeloid leukemia in lymphoid blast crisis. However, lineage restriction was characteristic of ALL. The high hyperdiploid clone seen in relapse would, in Ph-negative patients, be associated with a good prognosis but lymphoid restriction of the clone in a Ph^+ patient indicates a particularly poor prognosis. In relapse two copies of t(9;22) involving both homologues of 9 and 22 were present. This has rarely been reported. Since both translocations resulted in m-*BCR/ABL* fusion it is impossible to say whether these represent a duplication of both translocation partners with loss of the normal homologues or whether the translocation has occurred separately in each pair of homologues.

The present study confirms the heterogeneous nature of the target cell for Ph+ ALL. More importantly it demonstrates that high hyperdiploid clones in ALL are found only in the lymphoid cell population, indicative of the origin of this subtype of

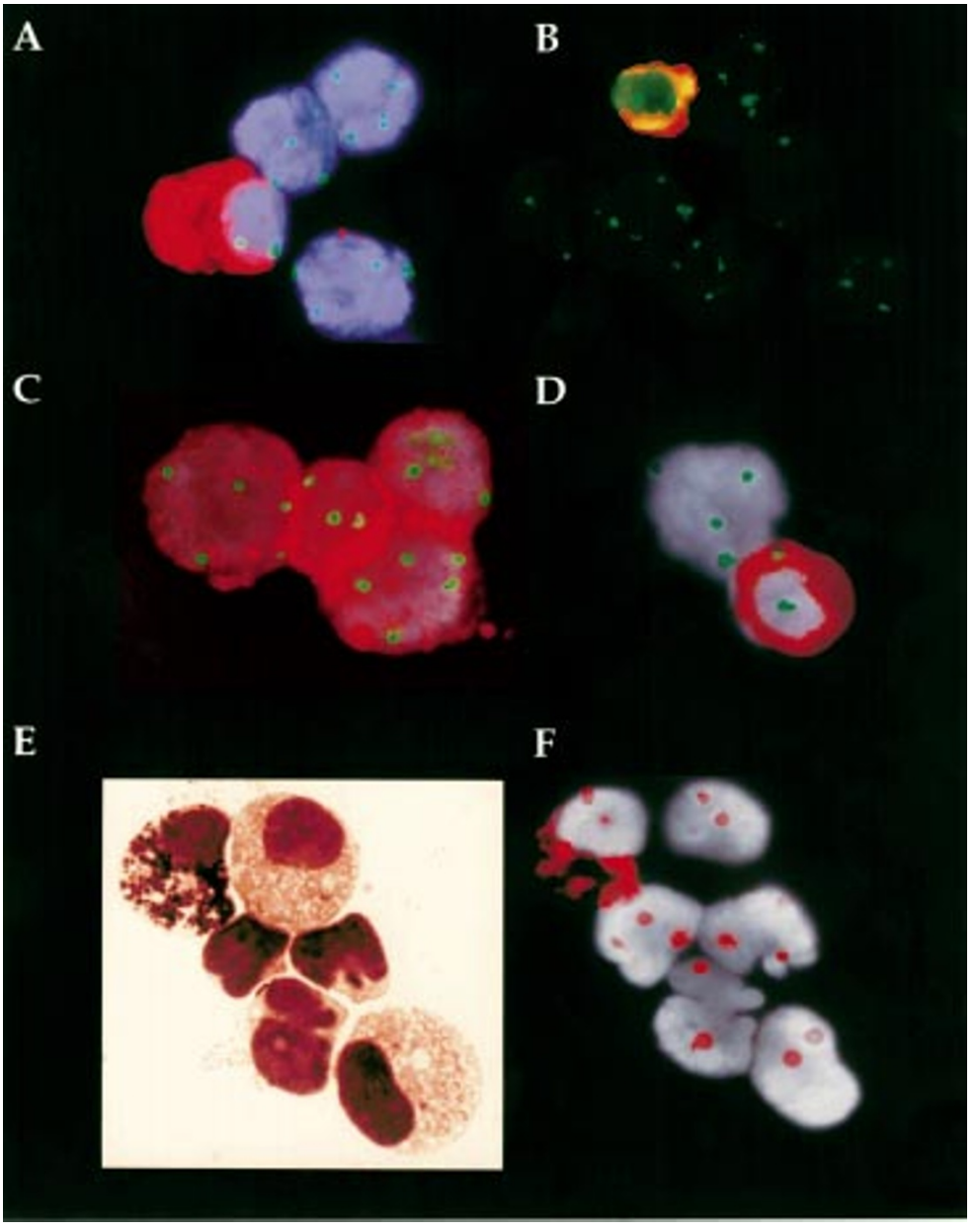

Figure 2 (A and B) The results of MGG/FISH investigation in case 11. Red dots represent hybridization signals with *BCR* and green with *ABL* probes, respectively. Yellow signals represent *BCR-ABL* fusion. (A) Metaphase spread and interphase cells hybridized with M-*BCR-ABL* probe. Note yellow signals representing M-*BCR-ABL* fusion. (B1a) MGG stained bone marrow showing three lymphoid cells and one myeloid cells. (B1b) The same cells hybridized with M-*BCR-ABL* probe. Note yellow signals representing M-*BCR-ABL* fusion in a representative myeloid cells. (B2a) MGG stained bone marrow showing one lymphoid cell and a myeloid cell. (B2b) The same cells hybridized with M-*BCR-ABL* probe, focused on the lymphoid cell (top) and on the myeloid cell (bottom). Note yellow signals representing M-BCR-ABL fusion in myeloid and lymphoid cells. (C and D) The results of MGG/FISH investigation in case 10 in relapse with t(9,22), t(9;22). Red dots represent hybridization signals with *BCR* and green with *ABL* probes, respectively. Yellow signals represent *BCR-ABL* fusion. (C) Metaphase spread and interphase cell hybridized with m-*BCR-ABL* probe. Note two yellow signals representing m-*BCR-ABL* fusion. (D1a) Eosinophil stained with MGG. (D1b) The same cell hybridized with m-*BCR-ABL* probe. Note the lack of yellow signal. (D2a) Blasts stained with MGG. (D2b) The same cells hybridized with m-*BCR-ABL* probe. Note two yellow signal in each cell. (E and F) Case 10 at diagnosis with monosomy 7. (E) Bone marrow stained with MGG to show the three cells of the myeloid lineage and two blasts. (F) The same cells hybridized with chromosome 7 probe. Note one signal in blast cells and two signals in myeloid cells.

leukemia in a lymphoid committed progenitor cell. This study requires confirmation on a larger sample of patients. Meanwhile, if treatment is to be directed against the leukemic cell of origin studies of this kind will have to be carried out on individual patients.

Acknowledgements

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