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# Increased circulating normal and *BCR-ABL*+Ve progenitor numbers in Philadelphia chromosome-positive acute myeloid leukaemia

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## Abstract

We recorded elevated numbers of circulating myeloid and erythroid colony-forming cells in 15 adult patients with acute myeloid leukaemia (AML) who presented with high blood white cell counts. Since leukaemic blasts from three of these patients were Philadelphia chromosome-positive (Ph+), we were able to determine if blood progenitors from these particular patients arose from the leukaemic clone or from residual normal progenitors. Blasts and colonies were intensively investigated using a combination of cell surface marker analysis by flow cytometry, RT-PCR and interphase fluorescence in situ hybridization (FISH). FISH detected rearrangements within the major breakpoint *BCR* (*M-BCR*) region in blasts and in some myeloid and erythroid colonies from patients 1 and 2. The minor breakpoint (*m-BCR*) region was detected in blasts and in some myeloid and erythroid colonies from patient 3. RT-PCR detected long b2a2 *BCR-ABL* transcripts in blasts from patients 1 and 2, although misspliced short e1a2 transcripts were also seen in patient 1. Only e1a2 transcripts were found in blasts from patient 3. Flow sorting demonstrated the B-cell marker CD19 on blasts and on a proportion of myeloid and erythroid progenitors from patients 1 and 3. RT-PCR also detected IgH rearrangements, further evidence of B-cell differentiation, in blasts from these two patients. We conclude that both normal and clonal circulating progenitor numbers can be raised in both *M-BCR* and *m-BCR* Ph+ AML. The underlying cause, perhaps efflux from a congested marrow, may be common to AML patients with a high blood white cell count.

## 1. Introduction

In adult acute myeloid leukaemia (AML), myeloid and erythroid colonies can develop from normal polyclonal progenitors or from progenitors derived from the leukaemic stem cell [1–5]. The distinction is important, since malignancy in primitive stem cells makes adult AML hard to eradicate [6].

High blood colony-forming cell numbers have been reported in occasional AML patients [7–11] and a recent study

of 15 cases of AML in our laboratory demonstrated that considerable numbers of myeloid and erythroid progenitors circulate in those AML patients who present with high blood white cell counts.

Blasts from three of these 15 AML patients showed a translocation; all exhibited the Philadelphia chromosome-positive (Ph+) t(9;22)(q34;q11) karyotype by clinical G banding. The *BCR-ABL* translocation in this disease provides a convenient marker which can be detected either by using suitable primers for reverse transcriptase polymerase chain reaction (RT-PCR) from mRNA, or by using DNA probes for interphase fluorescence in situ hybridization (FISH). We have used these procedures to resolve whether the colonies in these three patients derive from clonal or normal progenitors.

Breakpoints in both chronic myeloid leukaemia (CML) [12] and AML typically involve the major-*BCR* (*M-BCR*) region on chromosome 22, giving rise to b2a2 or b3a2

*Abbreviations:* AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; GMCFC, granulocyte–monocyte colony-forming cells; FISH, interphase fluorescence in situ hybridization; MNC, mononuclear cells; Ph+, Philadelphia chromosome-positive; RT-PCR, reverse transcriptase polymerase chain reaction

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*BCR-ABL* transcripts and a constitutive, cytoplasmic p210 tyrosine kinase [13]. A p190 kinase with augmented activity can be generated from shorter e1a2 transcripts when the breakpoint involves the minor-*BCR* (*m-BCR*) region, as in Ph+ acute lymphatic leukaemia [14–18]. Consistent with its higher kinase activity, p190 tends to generate aggressive B lymphoid neoplasms in transgenic mice [19–23]. Nevertheless, it is also quite clear that the *m-BCR* rearranged gene too can transform multipotential target cells [15]. Short transcripts have also been detected in some cases of Ph+ AML [24,25] and CML [26–29] where they have been associated with higher blood counts [30] and more aggressive terminal disease [31]. Furthermore, short and long transcripts can co-exist as a consequence of alternative splicing from an underlying *M-BCR* rearrangement [30].

We have combined flow cytometric, RT-PCR and FISH analyses to characterize surface marker expression, *BCR-ABL* transcript types and underlying DNA rearrangements within blasts and colonies from our three Ph+ patients to see whether the accumulation of blood progenitors in AML is restricted to the *BCR-ABL*+ compartment.

## 2. Materials and methods

### 2.1. Patient samples

Blood from patients with counts exceeding  $20 \times 10^6$ /ml was collected into preservative-free heparin (10 U/ml) and mononuclear cells (MNC) prepared by centrifugation over lymphocyte separation medium (043-03010E, Gibco). MNC were washed once in Hepes-buffered minimum essential medium (MEM; 22370-027, Gibco) and resuspended in MEM supplemented with 40% fetal bovine serum (Gibco or Imperial) and 10% dimethyl sulphoxide (Sigma) before placing in a Cryo 1 freezing container (Nalgene) at room temperature and allowing to cool to  $-70^\circ\text{C}$ . Cryovials were thawed quickly and diluted dropwise, slowly, using MEM +10% FCS containing 10 U/ml preservative-free heparin, and resuspended in MEM for dilution into culture. MNC were discarded if contaminated with more than 15% non-blast cells and immunophenotyping confirmed a dominant leukemic phenotype. Viable cells were assessed in 0.2% trypan blue and were seeded at  $10^6$ /ml in liquid culture and between  $5 \times 10^4$  and  $5 \times 10^5$ /ml for semi-solid culture. Cell counts after culture also refer to viable cells excluding trypan blue.

Normal patients were visitors to the eye clinic from whom blood was removed with informed consent and the project had the approval of the South Glamorgan Ethics Committee.

### 2.2. Semi-solid colony assays

Serum-free medium was an adaptation of that first described by Drouet et al. [32]. Iscoves modified Dulbeccos medium (Gibco, 42200-014) was made to 450 mOsm

supplemented with extra glycine (75  $\mu\text{g}/\text{ml}$ , Analar, Phillip Harris), 100 U penicillin and 100  $\mu\text{g}$  streptomycin per ml (15070-822, Gibco), 0.24% sodium bicarbonate (25080-021, Gibco), 2% deionised bovine serum albumin (Fraction V, A4503, Sigma), 60  $\mu\text{M}$  thioglycerol (M1753, Sigma), 15  $\mu\text{g}/\text{ml}$  cholesterol (C3045, Sigma), 10  $\mu\text{g}/\text{ml}$  insulin (I5500, Sigma) and 545  $\mu\text{g}/\text{ml}$  human transferrin (652202, Boehringer Mannheim). This mix was diluted with a one-third volume of 1% agar (Difco, Noble) for semi-solid cultures. Colonies were scored after 14 days of semi-solid culture in 24 well Nunc multi-well plates (1-43982A). All cultures were kept at  $37^\circ\text{C}$  in a fully-humidified incubator in 5%  $\text{CO}_2$  in air. Colonies of more than 50 cells were enumerated and plucked from culture between 10 and 20 days incubation.

### 2.3. Purified, recombinant cytokines

Stem cell factor (Amgen, Thousand Oaks, California) was used as a source of c-kit ligand at 20 ng/ml final concentration. Interleukin-3 (Sandoz, Camberley, UK), granulocyte-macrophage colony-stimulating factor (Schering-Plough) and granulocyte colony-stimulating factor (G-CSF, Sandoz) were all used at 5 ng/ml. Recombinant erythropoietin (Boehringer Mannheim) was used at 2 U/ml of culture.

### 2.4. Colony cell numbers

Colonies were transferred, using a narrowed pasteur pipette, into 100  $\mu\text{l}$  of MEM followed by brief vortexing to suspend cells. Cells were diluted into an equal volume of 0.4% trypan blue and counted on a haemocytometer.

### 2.5. Surface markers on blasts, progenitors and cells from colonies

The following monoclonal antibodies obtained from commercial sources were used in double or triple combinations: CD13PE (BD, 347837), CD15FITC (BD, 347423), CD19FITC (Dako, F0768), CD34PE (BD, 348057), CD235a/Glycophorin-AFITC (Dako, F0870). Negative mouse controls included IgG1PE (BD, 349043), IgG1FITC (Dako, X0927) and IgMFITC (Dako, X0934). Cells were washed with phosphate buffered saline with 1% bovine serum albumin and 0.2% sodium azide (PB-SAA buffer), resuspended in residual buffer and stained with one–three antibodies (5–10  $\mu\text{l}$ ) for 30 min at  $4^\circ\text{C}$ . Cells were then either sorted (FACS 440, Becton Dickinson) or washed twice with PBSAA and resuspended in 300  $\mu\text{l}$  of 2% paraformaldehyde before data acquisition on a Becton Dickinson FACScan (within 2 days), set up using standardised beads (Dako Fluorospheres, K0110) with identical settings each time. Win MDI software was used to analyse data, setting light scatter gates around viable cells.

Table 1  
RT-PCR primers

| Primer                 | Sequence                               | Concentration |
|------------------------|--|---------------|
| ABL-ABL                |  |               |
| ABL-S                  | 5' CAGTAGCATCTGACTTTGAGCCTC            | 1 $\mu$ M     |
| ABL-A                  | 5' CCCATTGTGATTATAGCCTAAGAC            | 1 $\mu$ M     |
| b3a2/b2a2 first round  |  |               |
| BCR-1                  | 5' AGCATGGCCTTCAGGGTGCACAGTTGTAACGGCAA | 1 $\mu$ M     |
| ABL-1                  | 5' TCACTGGGTCCAGCGAGAAGGTTTTCCTTGAGTT  | 1 $\mu$ M     |
| b3a2/b2a2 second round |  |               |
| BCR-2                  | 5' GTTCCTGATCTCCTCTGACTATGAGCGTGCA     | 0.5 $\mu$ M   |
| ABL-2                  | 5' CTCAGACCCTGAGGCTCAAAGTCAGATGCT      | 0.5 $\mu$ M   |
| e1a2 first round       |  |               |
| ABL-3                  | 5' TGATTATAGCCTAAGACCCGGA              | 1 $\mu$ M     |
| BCR-3                  | 5' ACCATCGTGGGCGTCCGCAAGA              | 1 $\mu$ M     |
| e1a2 second round      |  |               |
| ABL-4                  | 5' ATCTCCAGTGGCCAGAAAATCATACA          | 1 $\mu$ M     |
| BCR-4                  | 5' AGATCTGGCCCAACGATGGCGAGGGC          | 1 $\mu$ M     |
| IgH                    |  |               |
| NV-2                   | 5' GGACACSGCYGYGTATTACTG               | 1 $\mu$ M     |
| NJ-2                   | 5' CTYACCTGCAGAGACRGTGACC <sup>a</sup> | 1 $\mu$ M     |

<sup>a</sup> Fluorescently labeled.

## 2.6. RT-PCR

Total RNA was isolated from either flow-sorted samples or individual colonies using RNAzol B (Biogenesis Ltd., Poole, England, UK) and resuspended in 15  $\mu$ l H<sub>2</sub>O following the manufacturer's instructions. An amount of 5  $\mu$ l of RNA was used for reverse transcription (GeneAmp RNA PCR kit, Applied Biosystems, Warrington, England, UK) with slight modifications to manufacturers instructions; 25 °C for 10 min, 42 °C for 30 min and 95 °C for 5 min.

All first round PCRs were carried out with the reagent concentrations specified by Perkin-Elmer using 5  $\mu$ l of cDNA in a volume of 25  $\mu$ l, with the exception of the IgH amplification. Primer sequences and final concentrations can be found in Table 1. An ABL-ABL control PCR was run using the ABL-S/ABL-A primer set with denaturation at 94 °C for 3 min followed by 1 min at 94, 58 and 72 °C for 38 cycles and a final extension at 72 °C for 10 min, producing a product of 197 bp from cDNA and 600 bp if genomic DNA contamination was present.

The IgH reactions, using primer sets NV2 and NJ2, were initiated in a reaction volume of 25  $\mu$ l containing 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP, 0.025 U/ $\mu$ l Taq and 5  $\mu$ l cDNA. Cycling parameters were initial denaturation at 94 °C for 4 min, then 1 min at 94, 60 and 72 °C for 30 cycles, followed by a 72 °C, 10 min final extension. IgH samples were analysed using an ABI Prism 310 Genetic Analyser (Applied Biosystems, Warrington, England, UK).

Reagent concentrations for the first round of b3a2/b2a2 and e1a2 amplifications followed the protocol of the GeneAmp RNA PCR Kit (Perkin-Elmer) but the total volume was reduced to 25  $\mu$ l. In the second round, 1  $\mu$ l of the first

round product was transferred into a 24  $\mu$ l volume containing 1.25 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP and 0.025 U/ $\mu$ l Taq. Both rounds of the b3a2/b2a2 amplification comprised an initial denaturation at 94 °C for 4 min, then 94 °C for 1 min, 72 °C for 1 min 30 s for 35 cycles. The b3a2 product runs at 300 bp and the b2a2 at 225 bp. The e1a2 conditions were initial denaturation at 94 °C for 4 min, then 1 min at 94, 60, 72 °C for 30 cycles and final extension at 72 °C for 10 min. The e1a2 product is 197 bp.

All of the PCRs with the exception of the IgH were analysed by running 8  $\mu$ l of the final product on a 2% agarose gel. Both positive and negative controls were run for the reverse transcription and all PCR amplifications.

## 2.7. Interphase fluorescence in situ hybridization

Individual colonies were transferred, in 1–2  $\mu$ l, from semi-solid cultures into 100  $\mu$ l MEM, dispersed by vortexing, treated with 0.075 M KCl for 10 min, centrifuged for 10 min, fixed in 3:1 methanol:acetic acid fixative and stored at –20 °C. Fixed cells (5  $\mu$ l) were placed onto untreated, dry slides, allowed to dry and denatured for 5 min in 70% formamide (Vysis protocol) at 73 °C followed by 1 min transfers through 70, 80 and 100% ethanol. BCR locus Spectrum Green/ABL locus Spectrum Orange probe (Vysis, Cat no. 32-190022) was prepared and denatured according to the manufacturer's instructions and 10  $\mu$ l was applied per slide. Slides were incubated overnight at 37 °C in a humidified chamber and were subsequently immersed in 0.4  $\times$  SSC/0.3% NP-40 at 73 °C for 2 min followed by immersion in 2  $\times$  SSC/0.1% NP-40 for 30 s at room temperature. Slides were then air-dried and nuclei counterstained

with 15  $\mu$ l DAPI at 125 ng/ml (Vysis, Cat no. 30-804931). Fluorescence was assessed on a Zeiss Axiophot fluorescence microscope using Digital Scientific SmartCapture software.

### 3. Results

#### 3.1. Blood colony numbers and transcripts in patients

The ranges for circulating clonogenic cells in seven haematologically normal donors and 15 AML patients with high blood counts, are summarised in Table 2 alongside the individual blood colony numbers for the three Ph+ patients and the *BCR-ABL* transcript types in their blasts. Blood erythroid progenitors in patient 2 amounted to a striking 22% of all blood MNC!

#### 3.2. Immunophenotype of blasts

Blasts from our three Ph+ patients commonly expressed myeloid markers along with the primitive marker CD34 (Table 3). CD19 was present on the blasts of patients 1 and 3. Also of note was the absence of glycophorin on blasts from patient 2.

#### 3.3. Colony maturation

Colonies from all three patients appeared to mature normally—at least to the extent that CD34 and CD19 were lost and CD15 or glycophorin gained (Figs. 1 and 2).

#### 3.4. Progenitors in blast fractions

Granulocyte–monocyte colony-forming cells (GMCF) were recovered in both the CD34+/CD19<sup>high</sup> and CD34+/

CD19<sup>low</sup> fractions in patients 1 and 3 (Fig. 3). Although most erythroid progenitors in patient 1 were found in the CD34+/CD19<sup>low</sup> fraction and, so possessed a normal phenotype, more CD34+/CD19<sup>high</sup> erythroid progenitors were detected in patient 3. Erythroid progenitors in patient 2 were restricted to the CD34+/CD13– fraction.

#### 3.5. *BCR-ABL* transcripts in blast fractions and IgH rearrangements

The distributions of b2a2 and e1a2 transcripts in blasts sorted from patients 1–3 on the basis of CD34 and CD19 or CD13 expression are given in Fig. 3. There was no clear distinction between CD19+ and CD19– blasts in patients 1 and 3, so the gates set were probably subjective since these are single populations with a gradation in CD19 levels. In patient 1, b2a2 was common to all fractions with e1a2 mRNA absent from the double negative fraction. In patient 2, b2a2 transcripts only were detected and were confined to the CD34+/CD13– population. In patient 3, where only e1a2 was detected, these transcripts were common to all fractions (Fig. 3). Representative gels are shown in Fig. 4.

Two clonal IgH rearrangements were detected in blasts from patient 1 (Fig. 5) and one in patient 3. In addition, the minor CD34–/CD19+ populations seen in both patients 1 and 3 (Fig. 3) were polyclonal (Fig. 5) and, so were presumably residual normal B-cells. IgH from patient 2 blasts was polyclonal.

#### 3.6. RT-PCR on individual colonies

When processing colonies for RT-PCR, there is a danger of false positives originating from surviving, non-proliferating blasts seeded into culture or from inward migration of cells from adjacent *BCR-ABL*+ colonies, so we

Table 2  
Colony-forming cells/ml of blood and *BCR-ABL* transcript types in leukaemic blasts

| Blood donor                  | CFC per ml blood |            | <i>BCR-ABL</i> transcripts in blood blasts |
|------------------------------|------------------|------------|--|
|                              | GMCF             | ECFC       |  |
| 7 Normals <sup>a</sup>       | 198–537          | 651–1513   |  |
| 15 AML patients <sup>a</sup> | 3029–47780       | 3290–71580 |  |
| Patient 1                    | 28500            | 58000      | b2a2 + e1a2                                |
| Patient 2                    | 146000           | 5425000    | b2a2                                       |
| Patient 3                    | 4185             | 2934       | e1a2                                       |

<sup>a</sup> Ranges from log-transformed data (patient 2 is an outlier).

Table 3  
Immunophenotype of blood leukaemic blasts

| Pt | Percentage of cells + for surface CD type |   |    |    |     |    |    |    |    |    |    |    |    |    |    |    | IgM | TdT | MPO | GPA |     |
|----|---|---|----|----|-----|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|
|    | 2   | 3 | 7  | 10 | 11b | 13 | 15 | 19 | 20 | 22 | 25 | 33 | 34 | 45 | 56 | DR |     |     |     |     | 117 |
| 1  | 2   | 3 | 3  | 21 | –   | 82 | –  | 66 | 12 | –  | –  | 62 | 69 | 94 | –  | –  | 21  | 2   | 45  | 1   | –   |
| 2  | 2   | – | 25 | 0  | 45  | 78 | 0  | 1  | –  | –  | 9  | 60 | 90 | 97 | 7  | 90 | –   | –   | 0   | 0   | 2   |
| 3  | 6   | – | 4  | 6  | –   | 84 | 0  | 90 | 7  | 7  | –  | 3  | 87 | 99 | –  | 97 | –   | 6   | 87  | 48  | –   |

Pt: patient; MPO: myeloperoxidase; GPA: glycophorin A; –: not measured.

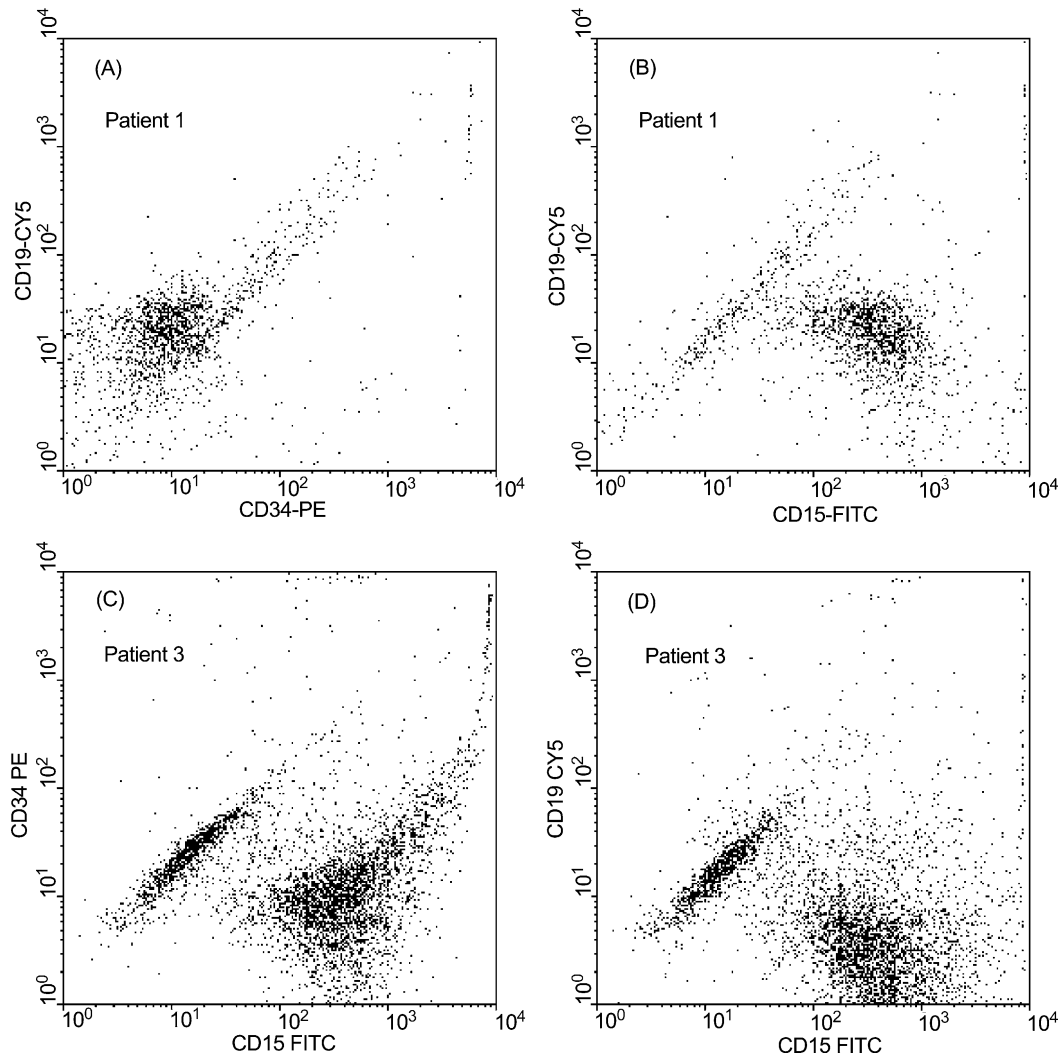


Fig. 1. Surface CD markers on maturing cells from granulocyte–monocyte colonies from patients 1 and 3.

performed RT-PCR on control agar blocks of equivalent volume (1–2  $\mu$ l) taken from between colonies. Positive RT-PCR from control background agar can be seen in lines 4 and 5 of Table 4 and the results from colonies from these cultures need to be interpreted with caution. Nevertheless, these RT-PCR results are largely supported by the FISH data below.

GM colony-forming cells from patient 1 were primarily b2a2+ (Table 4). Unlike the bulk blast population, e1a2 transcripts were not detected in this patient’s progenitors. Most GM progenitors from patient 3 expressed e1a2 transcripts, whereas, only half the erythroid colony-forming cells did (Table 4 and Fig. 6). In contrast, myeloid and erythroid

Table 4  
BCR-ABL transcript types in single colonies and background agar (Bx)

| Patient   | Culture type | Number of colonies plucked | Transcripts in colonies |      | Number of Bx plucked | Transcripts in Bx agar |      |
|-----------|--------------|----------------------------|-------------------------|------|----------------------|------------------------|------|
|           |              |                            | b2a2                    | e1a2 |                      | b2a2                   | e1a2 |
| Patient 1 | Myeloid      | 5                          | 4                       | 0    | 5                    | 0                      | 0    |
|           | Erythroid    | 4                          | 0                       | 0    | 4                    | 0                      | 0    |
| Patient 2 | Myeloid      | 5                          | 4                       | 0    | 5                    | 0                      | 0    |
|           | Erythroid    | 6                          | 6                       | 0    | 6                    | 1                      | 0    |
| Patient 3 | Myeloid      | 5                          | 0                       | 4    | 5                    | 0                      | 1    |
|           | Erythroid    | 4                          | 0                       | 2    | 4                    | 0                      | 0    |

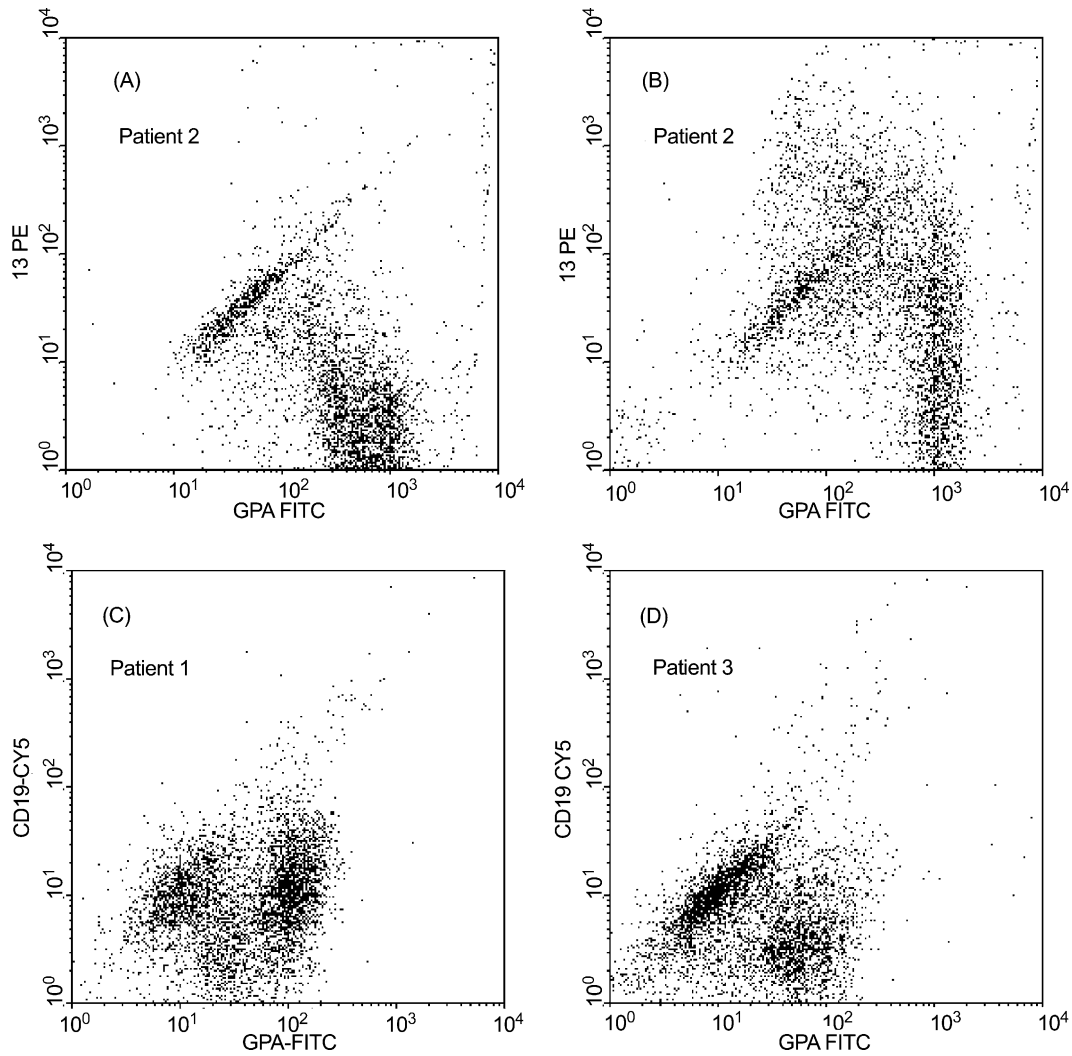


Fig. 2. Surface CD markers on cells from erythroid colonies from all three patients (GPA = glycoporin A).

progenitors from patient 2 were always b2a2 positive (Table 4 and Fig. 6).

### 3.7. Interphase *in situ* hybridization on blasts and individual colonies

FISH was not performed on the same individual colonies as RT-PCR. Unfractionated blasts from all three patients

showed a single *BCR-ABL* translocation pattern with a single, colocalised yellow signal together with a single red and, usually a single green signal. An additional green signal (Fig. 7) was frequently visible in blasts and some colonies from patient 3 suggesting a rearrangement in the *m-BCR* region on chromosome 22 [15]. *BCR-ABL* positive myeloid and erythroid colonies were common in patients 1 and 3 and predominant in patient 2 (Table 5).

Table 5  
Interphase FISH on colonies

| Patient | Culture type | Number of colonies plucked | <i>BCR-ABL</i> +/ <i>BCR-ABL</i> - colonies |
|---------|--------------|----------------------------|---|
| 1       | Myeloid      | 10                         | 4/6   |
|         | Erythroid    | 10                         | 5/5   |
| 2       | Myeloid      | 10                         | 10/0  |
|         | Erythroid    | 10                         | 10/0  |
| 3       | Myeloid      | 10                         | 5/5   |
|         | Erythroid    | 10                         | 3/7   |

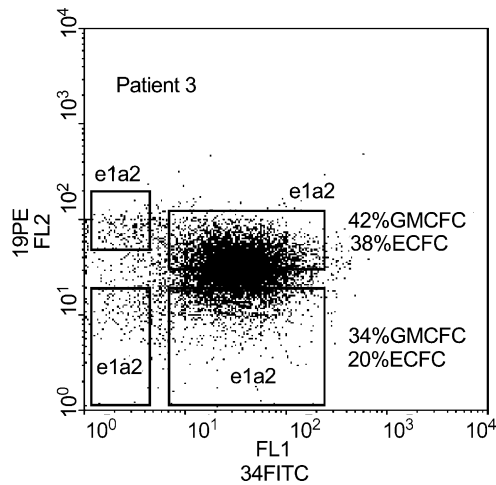
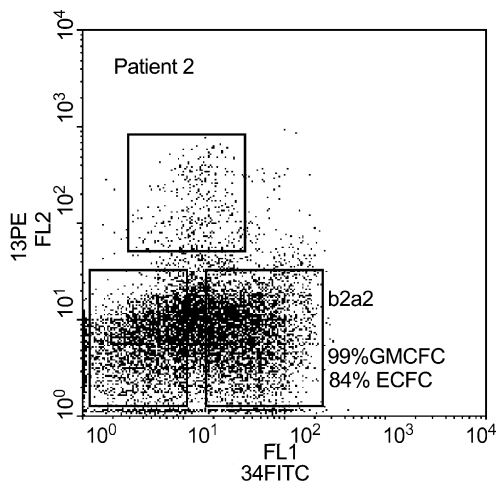
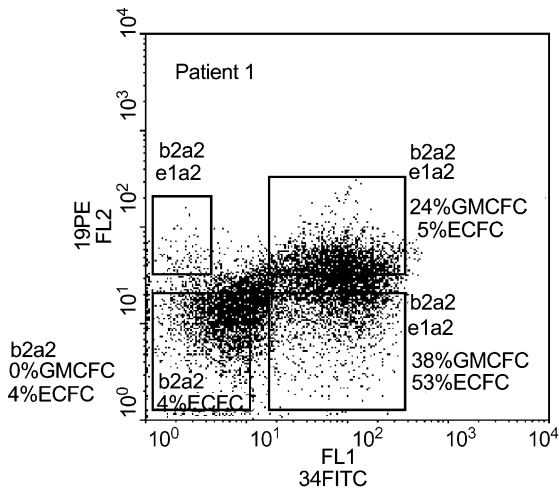


Fig. 3. Flow cytometric analysis of blasts from patients 1–3 showing surface phenotype of progenitors and distribution of *BCR-ABL* transcripts in the various fractions.

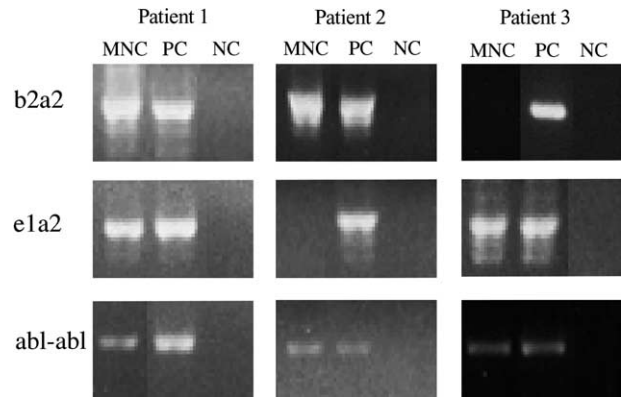


Fig. 4. *BCR-ABL* transcript types in unfractionated blasts (MNC) from patients 1–3 (PC = positive control; NC = negative control).

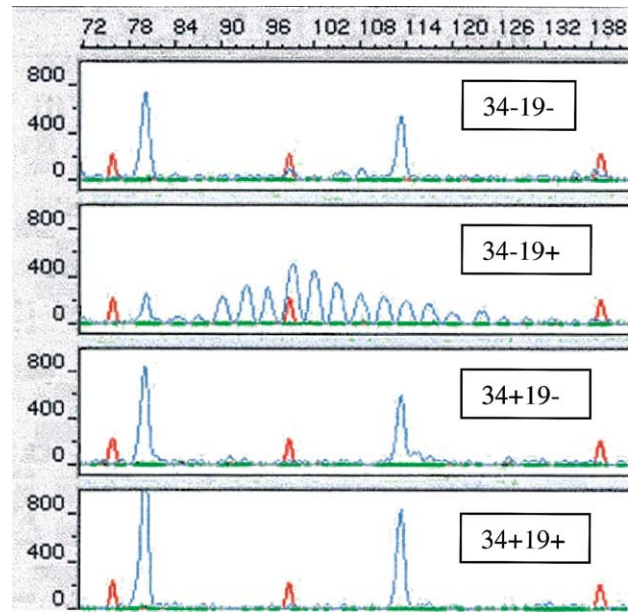


Fig. 5. IgH rearrangements (in blue) in blast fractions sorted from patient 1 on the basis of CD34 and CD19 expression: red peaks represent fluorescent DNA markers.

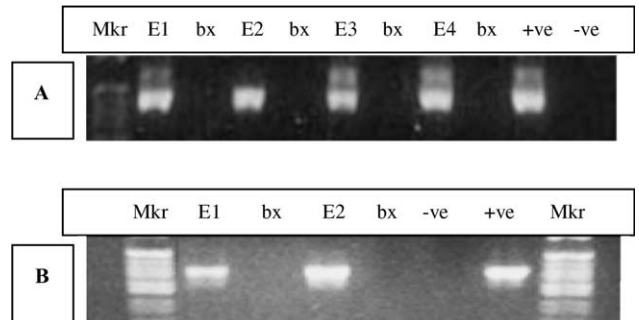


Fig. 6. Transcript types in erythroid colonies from patient 2 (b2a2 upper panel A) and patient 3 (e1a2 lower panel B): E1 etc = individual erythroid colonies; bx = background agar culture control; Mkr = marker lane; +/- = PCR+ and - controls.



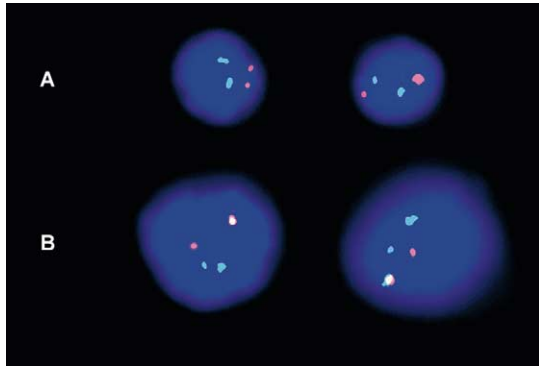


Fig. 7. Fluorescence in situ hybridization using Vysis Spectrum Green *BCR*/Spectrum Orange *ABL* probes on *BCR-ABL*<sup>-</sup> cells from a patient 3 granulocyte-monocyte colony (upper panel A) and on cells from a *BCR-ABL*<sup>+</sup> erythroid colony (lower panel B): yellow fusion signals are seen in the lower panel alongside two green signals in the erythroid cells suggestive of an *m-BCR* translocation.

#### 4. Discussion

The purpose of the current study was to determine if circulating colony-forming cells are clonal in Ph<sup>+</sup> AML, since this would imply that *BCR-ABL* plays a role in the expansion of blood progenitor numbers in the disease. The demonstration, by flow sorting, of aberrant CD19 on a proportion of myeloid and erythroid colony-forming cells together with the detection of *BCR-ABL* translocations and transcripts by FISH and RT-PCR in cells from some of these colonies, shows that many blood myeloid and erythroid colonies do indeed derive from clonal progenitors. Nevertheless, *BCR-ABL* was not detected in a substantial proportion of blood colonies so their accumulation is not dependent on the *BCR-ABL* translocation.

As would be expected from the published data on CML [12], the presence of b2a2 transcripts did not impair colony growth and maturation in vitro as was readily apparent from the development of large, fully haemoglobinised and glycophorin-positive colonies from the blood of patient 2. Likewise, e1a2-positive erythroid colonies from patient 3 also showed no obvious size or maturation defects compared to *BCR-ABL*<sup>-</sup> colonies within the same dish. Myeloid colony growth and maturation were similarly unimpaired by the various transcripts present in differentiating GMCFC from these patients, with acquisition of monocytic/granulocytic markers paralleled by a loss of CD19, where present.

The expression of CD19 suggests partial B-cell differentiation and, indeed, RT-PCR detected clonal IgH rearrangements within blasts from our two CD19<sup>+</sup> patients. This could mean that these leukaemias derive from a myeloid/B-lymphoid progenitor cell, the evidence for which has been reviewed recently [33]. Since we detected two clonal IgH rearrangements in the blast population from patient 1, separate IgH rearrangements must have occurred during the development of this clone towards frank leukaemia.

Although blasts from both patients 1 and 3 express CD19 and both have IgH rearrangements, the underlying *BCR-ABL* translocations differ. The detection of b2a2 transcripts in blasts and colonies from patient 1, indicates an underlying translocation within the *M-BCR* breakpoint region of chromosome 22, whereas a *m-BCR* breakpoint is indicated by the sole presence of e1a2 transcripts in patient 3. Our FISH data support these conclusions, since Schenk et al. [15] have proposed that enough of the *BCR* is translocated to chromosome 9, in *m-BCR-ABL* translocations, to be detected with FISH probes to the region and this can generate an extra green signal using probes like ours. This was apparent in interphase spreads of blasts and colonies from patient 3 but not from patient 1. These data have subsequently been confirmed using another (ES) probe which gives two fusion signals in the presence of *m-BCR-ABL*. Consequently, it seems likely that the short e1a2 transcripts detected in blasts from patient 1, are misspliced from *M-BCR-ABL* [30,34].

Despite being clearly involved in some way, it appears that *BCR-ABL*<sup>+</sup> colony-forming cells may not be directly related to the leukaemic process. This is highlighted in patient 2 where leukaemic blasts showed myeloid surface markers yet it was the erythroid progenitor compartment that had expanded. Again, in patients 1 and 3, colony maturation from CD19<sup>+</sup> progenitors appears to be unaffected, whereas, blasts from the leukaemic population are arrested at the CD19<sup>+</sup> stage of maturation. Although, the cytokines added to culture may force progenitors to complete their maturation in vitro, it seems more likely that leukaemic blasts have undergone further genetic change, as happens in CML blast crisis. It follows that *BCR-ABL*<sup>+</sup> colony-forming cells may derive either from a chronic phase of the disease, in which the final leukaemic step has yet to occur in one or more of their progeny, or alternatively, such progenitors may represent a benign offshoot of a more primitive preleukaemic stem cell and may not be directly involved in the leukaemic transformation at all.

In conclusion, it seems that neither *M-BCR-ABL* nor *m-BCR-ABL* translocations are directly responsible for the accumulation of blood progenitors in Ph<sup>+</sup> AML. A more general mechanism is probably involved such as efflux from a congested marrow which presumably applies to the other AMLs studied here. Our data also suggest that clonal colony-forming cells in AML are probably not fully malignant.

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