Leukemia (2013) **27**, 2149–2156

www.nature.com/leu

ORIGINAL ARTICLE

Newly diagnosed adult AML and MPAL patients frequently show clonal residual hematopoiesis

C Fernandez[1], MC Santos-Silva[2], A López[1], S Matarraz[1], M Jara-Acevedo[1], J Ciudad[1], ML Gutierrez[1], ML Sánchez[1], C Salvador-Osuna[3], MJ Berruezo[4], JÁ Díaz-Arias[5], AM Palomo-Hernández[6], E Colado[7], N González[8], D Gallardo[9], A Asensio[10], R García-Sánchez[11], R Saldaña[12], C Cerveró[13], A Carboné-Bañeres[14], O Gutierrez[15] and A Orfao[1]

Adult acute myeloid leukemia (AML) is a highly heterogeneous stem cell malignancy characterized by the clonal expansion of immature myeloid precursors. AML may emerge *de novo*, following other hematopoietic malignancies or after cytotoxic therapy for other disorders. Here, we investigated the clonal vs reactive nature of residual maturing bone marrow cells in 59 newly diagnosed adult AML and mixed phenotype acute leukemia (MPAL) patients as assessed by interphase fluorescence *in situ* hybridization analysis of AML and myelodysplastic syndrome-associated cytogenetic alterations and/or the pattern of chromosome X inactivation, in females. In addition, we investigated the potential association between the degree of molecular/genetic involvement of hematopoiesis and coexistence of altered immunophenotypes by flow cytometry. Our results indicate that residual maturing neutrophils, monocytes and nucleated red cell precursors from the great majority of newly diagnosed AML and MPAL cases show a clonal pattern of involvement of residual maturing hematopoietic cells, in association with a greater number of altered immunophenotypes. These findings are consistent with the replacement of normal/reactive hematopoiesis by clonal myelopoiesis and/or erythropoiesis in most newly diagnosed AML and MPAL cases, supporting the notion that in most adults presenting with *de novo* AML, accumulation of blast cells could occur over a pre-existing clonal hematopoiesis.

Leukemia (2013) **27**, 2149–2156; doi:10.1038/leu.2013.109

Keywords: *de novo* AML; clonal hematopoiesis; immunophenotype

INTRODUCTION

The current World Health Organization (WHO) classification of myeloid neoplasms identifies four major subgroups of heterogeneous diseases —acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN) and mixed myelodysplastic/myeloproliferative neoplasms— with a significant degree of overlap among them.[1] Within AML, disease heterogeneity translates into six major subgroups, each of which still contains several specific diagnostic entities. Except for a small proportion of AML cases, which carry specific recurrent cytogenetic alterations, diagnostic criteria for AML requires >20% of myeloid lineage blast cells in the bone marrow (BM), with or without previous cytotoxic therapies or MDS-associated features.[1] Among AML patients, both cases occurring *de novo* and secondary to cytotoxic therapies or a prior myeloid neoplasia (for example, MDS or MPN) are included, their subclassification being of utmost clinical relevance because of their distinct prognosis.[2–4] In addition, among cases classified as *de novo* AML, a significant percentage of patients show AML with myelodysplasia-related changes, this also represents a unique poor-prognosis WHO category of the disease, independently of the lineage and cytogenetic alterations of myeloid blast cells.[5] Overall, these criteria confirm the clinical relevance of the presence of MDS-associated features in AML, for unequivocal differential diagnosis among *de novo* AML, secondary AML and AML with myelodysplasia-related changes, an intermediate subgroup of AML with dysplastic BM features potentially reflecting the presence of an underlying clonal disorder of residual hematopoiesis.

At present, it is well known that neither cytomorphology nor cytogenetics alone is sensitive enough to assess the potential clonal nature of residual hematopoietic cells, in every *de novo* AML patient.[2,3,6,7] To the best of our knowledge, no study has been reported so far in which the clonal vs reactive nature of residual mature/maturing BM cells other than blast cells, has been systematically analyzed in patients presenting with AML, as currently defined by the WHO 2008 classification. In the present study, we investigated the clonal vs reactive nature of residual maturing BM cells in newly diagnosed adult AML and mixed phenotype acute leukemia (MPAL) patients as assessed by interphase fluorescence *in situ* hybridization (iFISH) analysis of those chromosomal alterations that are frequently observed in

[1]Servicio General de Citometría and Departamento de Medicina, Centro de Investigación del Cáncer (Instituto de Biología Molecular y Celular del Cáncer and IBSAL; CSIC-USAL), Universidad de Salamanca, Salamanca, Spain; [2]Laboratório de Oncologia Experimental e Hemopatias do Departamento de Análises Clínicas da Universidade Federal de Santa Catarina, Florianopolis, Brazil; [3]Servicio de Hematologia, Hospital Miguel Servet, Zaragoza, Spain; [4]Hospital Punta de Europa, Algeciras, Spain; [5]Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain; [6]Hospital Xeral Calde, Lugo, Spain; [7]Hospital Universitario Central de Asturias, Oviedo, Spain; [8]Hospital Obispo Polanco, Teruel, Spain; [9]Servicio de Hematología Institut Català d'Oncologia, Hospital Josep Trueta, Girona, Spain; [10]Hospital San Jorge-Huesca, Huesca, Spain; [11]Hospital Virgen de la Victoria de Málaga, Málaga, Spain; [12]Hospital de Jerez de la Frontera, Jerez de la Frontera, Spain; [13]Servicio de Hematología, Hospital General Virgen de la Luz, Cuenca, Spain; [14]Unidad de Oncopediatría, Hospital Universitario Miguel Servet, Zaragoza, Spain and [15]Servicio de Hematología, Hospital Rio Hortega, Valladolid, Spain. Correspondence: Professor A Orfao, Servicio General de Citometría and Departamento de Medicina, Centro de Investigación del Cáncer (Instituto de Biología Molecular y Celular del Cáncer and IBSAL; CSIC-USAL), Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.
E-mail: orfao@usal.es
Received 5 March 2013; revised 2 April 2013; accepted 5 April 2013; accepted article preview 12 April 2013; advance online publication, 10 May 2013

AML and in MDS[1] and/or the pattern of inactivation of the X chromosome in females.[8] In addition, the potential association between the molecular findings and both dysplastic features by cytomorphology and altered immunophenotypes by multiparameter flow cytometry (FCM) was also investigated. Overall, our results indicate that residual maturing neutrophils, monocytes and nucleated red blood cells (NRBC) from the great majority of the newly diagnosed AML and MPAL cases (mostly including *de novo* AML) show a clonal pattern of involvement, in association with a greater number of altered immunophenotypes. These findings are consistent with the replacement of normal/reactive hematopoiesis by clonal myelopoiesis and/or erythropoiesis in most adults with newly diagnosed AML and support the notion that in the great majority of these patients, accumulation of blast cells could occur over a pre-existing clonal (dysplastic or not) hematopoiesis.

MATERIALS AND METHODS

Patients and samples

A total of 59 adult patients (23 males and 36 females; median age of 61 years) newly diagnosed with either AML ($n=53$) or MPAL ($n=6$) were included in this study. In all cases, diagnosis was established according to the WHO 2008 criteria[1] based on blood counts, clinical findings, morphological, immunophenotypical and cytogenetic/molecular data (Supplementary Table 1).

In every case, EDTA-anticoagulated BM samples were collected at diagnosis, after informed consent was given by each individual; the study was approved by the local Ethics Committee (Institutional Review Board) of the University Hospital of Salamanca (Salamanca, Spain).

Immunophenotypic studies

Erythrocyte-lysed, freshly obtained BM samples were analyzed by a panel of four-color combinations of monoclonal antibodies (MAb) directed against cell surface membrane markers alone or cell surface membrane markers in combination with cytoplasmic (Cy) and nuclear (n) antigens, according to previously described techniques.[9–12] The following pairs of fluorochrome-conjugated MAb—fluorescein isothiocyanate, phycoerythrin— were used in combination with CD45-peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5) and CD34-allophycocyanin (APC), (1) nTdT, CyMPO; (2) CD19, CyCD79a; (3) CyCD3, CD7; (4) CD2, CD56; (5) HLA-DR, CD117; (6) HLA-DR, CD123; (7) CD11b, CD13; (8) CD15, CD16; (9) CD65, 7.1/NG2; (10) CD36, CD64 and CD14-APC; (11) CD71, CD235a (or CD105); (12) CD38, CD203c; (13) CD61, CD25; (14) CD22, CD33 and; (15) CD300e (IREM-2), CD14. All MAb reagents were purchased from Becton/Dickinson Biosciences (BD, San Jose, CA, USA), except CD65, 7.1/NG2, CD11b, CD36, CD64, CD16, CD235a and CD203c, which were obtained from Immunotech (Marseille, France), CD38, which was purchased from Cytognos SL (Salamanca, Spain), and CD300e (IREM-2) that was obtained from Immunostep SL (Salamanca, Spain). Immediately after sample preparation, data acquisition was performed for $\geqslant 5 \times 10^4$ cells per sample aliquot using a FACSCanto II flow cytometer (BD) equipped with the FACSDiva software program (BD). For data analysis, the INFINICYT software (Cytognos SL) was used.

Cell purification

Purification of specific BM cell populations was performed using a FACSAria flow cytometer (BD) equipped with the FACSDiva software. Before sorting, cells were stained with $\geqslant$4-color combinations of fluorochrome-conjugated MAb aimed at specific and simultaneous identification of the different cell populations of interest present in the sample (for example, blast cells, maturing neutrophils, monocytic cells, nucleated erythroid precursors and mature lymphocytes). The purity achieved for the FACS-sorted cell populations was systematically $>97\%$.

iFISH studies

iFISH studies aimed at detection of t(9;22), t(8;21), inv(16), 11q23 abnormalities, -5/del(5q), -7/del(7q), del(20q), trisomy 8 and -Y, were performed on interphase nuclei from the different FACS-purified cell populations, after they were fixed in 3/1 (v/v) methanol/acetic. For this purpose, the following panel of Spectrum Orange (SO) and Spectrum Green (SO) DNA probes (Vysis Inc, Downers Grove, IL, USA) was used: LSI *BCR/ABL*, LSI *AML1/ETO*, LSI *CBFB* dual-color breakapart, LSI *MLL* dual-color breakapart, LSI *EGR1*/D5S23, D5S721, LSI D7S486/CEP7, LSI D20S108, CEP8 and CEPY (satellite III) probes. Hybridization with fluorochrome-labeled FISH probes was performed according to the recommendations of the manufacturer with slight modifications, as described elsewhere.[13] Fluorescence signals were evaluated as previously described[13] using a BX60 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a $\times 100$ oil objective.

HUMARA assay

Assessment of clonality was performed on FACS-purified cell populations from female patients ($n=36$) using the human androgen receptor X-chromosome inactivation (HUMARA) assay. For that purpose, genomic DNA was extracted from FACS-purified blast cells, maturing neutrophils, monocytic cells, NRBC and lymphocytes using the QIAamp mini and micro DNA extraction kits (Qiagen, Valencia, CA, USA), according to the instructions of the manufacturer. Extracted DNA (20 ng) was digested overnight at 37 °C, in the presence vs absence of the HAPII (10 U/μl) methylation-sensitive restriction endonuclease (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a final volume of 50 μl. Afterward, restriction enzymes were inactivated by heating at 95 °C for 10 min. Both digested and non-digested DNA was then amplified by PCR using two sets of primers flanking the trimeric CAG repeat specific for exon 1 of the HUMARA gene: round-one primer pairs (forward: 5′-TGTGGGGCCTCTA CGATG- 3′; reverse: 5′-TCCAACACCTACCGA-3′) were used for 28 cycles (1 min at 95 °C, 1 min at 55 °C and 45 s at 72 °C, with an initial denaturation step at 95 °C for 10 min) in a iCycler (Bio-Rad, Hercules, CA, USA) and round-two primer pairs (forward: 5′-CCGAGGAGCTTTCGAGAATC-3′; reverse-FAM5′-TACGATGGGCTTGGGGAGAA-3′) were used for 20 reaction cycles (1 min at 95 °C, 1 min at 58 °C and 45 s at 72 °C, with an initial denaturation at 95 °C for 10 min). Following the second round of amplification, PCR products were checked to confirm the amplification of the HUMARA target using 0.8% agarose gel electrophoresis stained with ethidium bromide (0.5 μg/ml). For fragment analyses, 1 μl of the PCR product was placed in 12.25 μl of Hi-DiTM formamide (Applied Biosystems) plus 0.5 μL of GENESCAN 400HD (ROX) size standard (Applied Biosystem). The mixture was sequentially denatured at 95 °C for 5 min and cooled in ice. The PCR products were examined by fragment analysis in an automated ABI PRISM 3100 genetic analyzer (Applied Biosystems). Data was analyzed using the GeneScan software (version 3.1; Applied Biosystems). Clonality was defined when the corrected ratio (CR) showed an excess of representation of one of the parental alleles over the other of $\geqslant 50\%$ (CR $\leqslant 0.33$ or $\geqslant 3$).[8,14–16]

KIT mutation studies

Presence of *KIT* mutation was assessed in DNA samples from FACS-purified cell fractions, using a previously described PCR-clamping technique.[17,18]

Statistical methods

To determine the statistical significance of differences observed between groups, the Chi-square and the Mann–Whitney U tests were used for categorical and continuous variables, respectively (SPSS software package, SPSS, Chicago, IL). *P*-values <0.05 were considered to be associated with statistical significance.

RESULTS

Cytogenetic and molecular markers of blast cell clonality

Overall, the clonal nature of blast cells was demonstrated in all (59/59; 100%) newly diagnosed AML and MPAL patients analyzed (Table 1). In detail, a clonal pattern of inactivation of chromosome X was detected in 31/31 (heterozygous) females investigated, one or more chromosomal alterations were found in 32/48 (69%) cases analyzed by iFISH and the presence of *KIT* mutation was detected in three patients. In six female AML patients, clonality was confirmed by both the HUMARA test and iFISH, either in the absence ($n=5$) or in the presence of the *KIT* mutation ($n=1$). The specific chromosomal alterations detected by iFISH in leukemia blast cells included: isolated trisomy 8, 5 cases; del (5q31), 3 cases; del (7q31), 4 cases; monosomy 7, 1 case; nulisomy Y, 2 cases; del (20q12), 2 cases; t(9;22), 3 cases; t(8;21), 1 case; inv (16), 2 cases; del

Table 1. Frequency of clonality found for different hematopoietic cell populations from patients with newly diagnosed AML and MPAL as detected by HUMARA, *KIT* mutation and/or iFISH

Percentage of AML and MPAL cases	*Clonal by HUMARA test*[a]	*Positive KIT mutation*	*Cytogenetic alterations by iFISH*	*Total*
Percentage of cases with clonal markers on blast cells	31/31 (100%)	3/3 (100%)	32/48 (69%)	59/59(100%)
Percentage of cases with clonal markers on maturing granulomonocytic and/or NRBC	25/31 (81%)	3/3 (100%)	28/48 (58%)	49/59 (83%)

Abbreviations: AML, acute myeloid leukemia; HUMARA, human androgen receptor assay; iFISH, interphase fluorescence *in situ* hybridization; MPAL, mixed phenotype acute leukemia; NRBC, nucleated red blood cells. [a]Only heterozygous female cases were analyzed by the HUMARA test.

(7q31) in association with del (20q12), 1 case; del (7q31) plus nulisomy Y, 1 case; del (5q31) associated with trisomy 8, 2 cases; del (5q31) plus del (20q12), 1 case; del (5q31) associated with both trisomy 8 and del (7q31), 3 patients and del (5q31), del (20q12) and trisomy 8, 1 patient (Table 2).

Clonal involvement of residual mature/maturing BM cells

Overall, the clonal nature of residual BM compartments of mature/maturing neutrophils and monocytic cells and/or NRBC was confirmed for at least one of these three cell compartments in most newly diagnosed AML and MPAL cases (49/59; 83%) (Figure 1, Tables 1 and 2). In the majority of cases showing clonal residual mature/maturing myeloid and/or erythroid cells (clonal cases), chromosomal alterations by iFISH and/or the *KIT* mutation (31/49; 68%) were detected in their blast cells, while cases carrying these cytogenetic/molecular alterations represented a lower fraction (4/10, 40%; $P = 0.17$) among newly diagnosed AML and MPAL patients in whom the presence of residual clonal maturing neutrophils, monocytes and/or NRBC could not be demonstrated (Table 2). Of note, all the later four altered cases, were males: two showed isolated trisomy 8, one had isolated del(7q31), and one simultaneously carried del(5q31) and del(20q12) in their blast cells with apparently normal maturing myeloid and erythroid precursors (Table 2). Conversely, all cases carrying *KIT* mutation, t(8;21), t(9;22) and inv(16) displayed clonal involvement of residual neutrophils, monocytes and/or NRBC. However, differences in the frequency of clonal cases in these genetic subgroups vs other AML and MPAL cases did not reach statistical significance ($P > 0.05$) due to the reduced number of the former AML cases (Table 2).

Frequency of cases with clonal residual hematopoiesis in distinct WHO 2008 subtypes of AML

Overall, no statistically significant differences were observed as regards the distribution of cases with clonal vs non-clonal residual hematopoiesis among the different diagnostic subgroups of newly diagnosed AML (Table 3). Despite this, it should be noted that all therapy-related AML patients (secondary AML; 3/3 cases) and CBF AML cases (5/5 cases), as well as the majority of *de novo* AML with myelodysplasia-related changes (AML–MRC; 24/28 cases, 86%) and MPAL (5/6 patients, 83%) patients had an underlying clonal residual hematopoiesis. Conversely, the frequency of clonal cases appeared to be slightly ($P > 0.05$) lower among *de novo* AML not otherwise specified (NOS; 11/15 cases, 73%) (Table 3 and Supplementary Table 1).

Immunophenotypic profile of residual BM mature/maturing granulomonocytic and NRBC

The immunophenotypic profile of residual BM mature/maturing neutrophils, monocytic cells and NRBC could be analyzed in all but one AML case (58/59 cases). Overall, immunophenotypic alterations similar to those recurrently described for MDS[9,10,19–22] were found in 52/58 cases (90%). When present, such alterations typically involved any combination of the three granulomonocytic/erythroid cell compartments analyzed. Accordingly, immunophenotypically altered neutrophils, monocytes and NRBC were found in 51/58 (88%), 38/52 (73%) and 27/45 (60%) patients, respectively (Table 4). The majority of cases showed altered

Table 2. Specific markers of clonality detected in blast cells from patients with newly diagnosed AML and MPAL classified according to the clonal nature of residual mature/maturing granulomonocytic cells and/or nucleated red blood cells

Molecular/genetic marker of clonality	*Newly diagnosed AML and MPAL*	
	Cases with non-clonal RH, n = *10*	*Cases with clonal RH* n = *49*
HUMARA[a] (*n* = 31)	6/31	25/31[b]
KIT mutation (*n* = 3)	0/3	3/3[c]
Cytogenetic alterations by iFISH (n = 32)	4/32	28/32[b]
nuc ish 8q22(RUNX1T1x3), 21q22(RUNX1x3) (RUNX1 with RUNX1T1)	0	1
nuc ish (ABL1x3), (BCRx3), (ABL1 con BCRx2)	0	3
nuc ish (CBFBx2), (5′CBFB sep3′CBFBx1)	0	2
nuc ish 5q31(*EGR1*x1)	0	3
nuc ish 7q31(D72486x1)	0	4
nuc ish 8cen (D8Z2x3)	2	3
nuc ish DYZ1-	0	2
nuc ish 20q12(*ZNF217*x1)	0	2
nuc ish 5q31(*EGR1x1*), nuc ish 8cen (D8Z2x3)	0	2
nuc ish 5q31(*EGR1x1*), nuc ish 20q12(*ZNF217*x1)	1	0
nuc ish 7q31(D72486x1), nuc ish 7p11.1(D7Z1x1)	1	0
nuc ish 7q31(D72486x1), nuc ish 20q12(EGR1x1)	0	1
nuc ish 7q31(D72486x1), nuc ish DYZ1-	0	1
nuc ish 5q31(*EGR1x1*), nuc ish 7q31(D72486x1), nuc ish 8cen (D8Z2x3)	0	3
nuc ish 5q31(*EGR1x1*), nuc ish 20q12(EGR1x1), nuc ish 8cen (D8Z2x3)	0	1
Total (*n* = 59)	10/59 (17%)	49/59 (85%)

Abbreviations: AML, acute myeloid leukemia; HUMARA, human androgen receptor assay; iFISH, interphase fluorescence *in situ* hybridization; MPAL, mixed phenotype acute leukemia; RH, residual hematopoiesis. [a]Only heterozygous female cases were analyzed by the HUMARA test. [b]In five cases, clonality was simultaneously detected by iFISH and the HUMARA test. [c]In two cases, clonality was detected by the *KIT* mutation and the HUMARA test and/or iFISH.

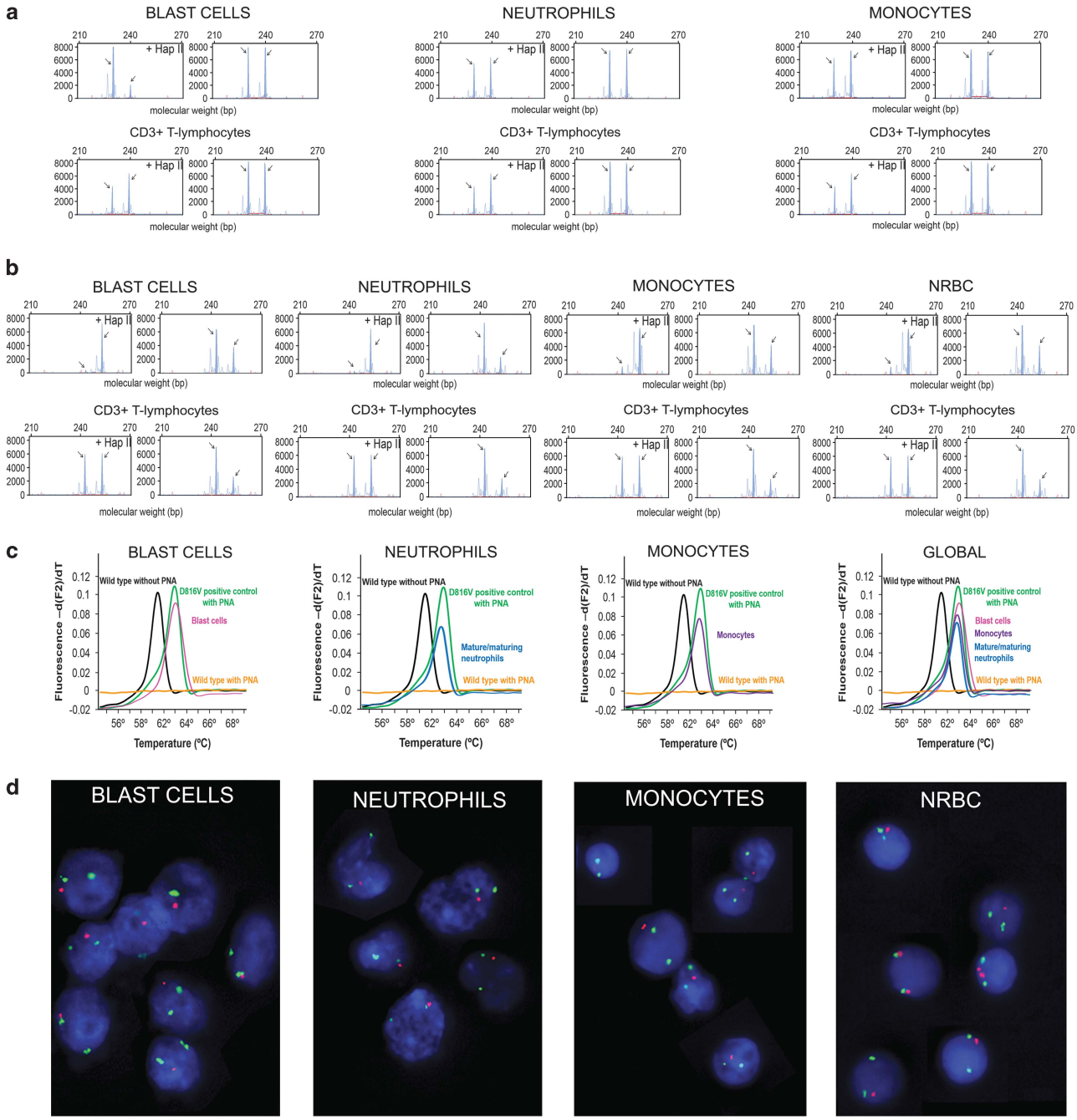

Figure 1. Assessment of clonal residual hematopoiesis based on the HUMARA test, interphase FISH and/or the *KIT* mutation assays in purified blast cells as well as purified mature/maturing neutrophils, monocytic and erythroid BM cells. The HUMARA assay results in panels **a** and **b** show two different cases analyzed with the HUMARA test. An AML with (poly)clonal residual hematopoiesis is shown in panel **a**, where the blast cell population shows one allele to be markedly reduced in intensity after HpaII digestion compared with the level of this allele found in normal/polyclonal CD3+ T-lymphocytes, mature/maturing granulomonocytic and erythroid precursors. An AML with clonal residual hematopoiesis is shown in panel **b**, where one allele is markedly reduced in intensity after HpaII digestion in each of the purified myeloid populations (for example, blast cells, neutrophils, monocytes and NRBC) analyzed vs that of CD3+ T-lymphocytes. In panel **c**, melting curves positive for the D816V *KIT* mutation in all purified BM cell populations (blast cells, neutrophils and monocytes) vs the wild-type sequence with a melting curve at 59 °C is shown. In panel **d**, iFISH patterns found with the D7S486/CEP7 dual-color probe, in a patient with AML and clonal residual hematopoiesis is shown. Please note that all populations from this patient, which are displayed in panel **d**, show the same aberrant pattern consistent with del(7q) —loss of one red signal in the presence of two green signals is interpreted as 7q- deletion— with a higher frequency of del(7q) in blast cells, neutrophils and monocytes vs erythroid nucleated precursors.

immunophenotypic profiles in two (16/58 cases; 27%) or three (29/58; 50%) cell populations, while phenotypic alterations were restricted to a single maturation–associated cell compartment in 13/58 cases (22%) (Table 4). Once the frequency of phenotypic alterations in distinct cell populations was compared between cases with 'clonal' vs 'non-clonal' residual hematopoiesis, the former showed a significantly higher frequency of aberrant phenotypes among maturing neutrophils (98% vs 50%;

Table 3. Presence of molecular/genetic markers of clonality in different bone marrow compartments of mature/maturing neutrophils, monocytic and nucleated red blood cells from newly diagnosed AML and MPAL patients grouped according to the WHO 2008 classification

WHO 2008 subtype of AML	*Cases with non-clonal RH*	*Cases with clonal RH*	P-*value*
Therapy-related myeloid neoplasms ($n=3$)	0 (0%)	3 (100%)	0.42
AML–MRC[a] ($n=28$)	4 (14%)	24 (86%)	0.60
AML with recurrent genetic abnormalities ($n=5$)	0 (0%)	5 (100%)	0.29
AML NOS ($n=15$)	4 (27%)	11 (73%)	0.18
MPAL[b] ($n=6$)	1 (17%)	5 (83%)	0.94
Other[c] ($n=2$)	1 (50%)	1 (50%)	0.44

Abbreviations: AML, acute myeloid leukemia; AML–MRC, acute myeloid leukemia with myelodysplasia-related changes; AML NOS, acute myeloid leukemia not otherwise specified; MPAL, mixed phenotype acute leukemia; RH, residual hematopoiesis. [a]Defined by multilineage dysplasia by morphology and/or MDS-associated cytogenetic alterations. [b]Three cases were further classified as mixed phenotype B/myeloid acute leukemia NOS and the other three as mixed phenotype T/myeloid acute leukemia NOS. [c]Two cases of myeloid proliferation related to Down syndrome.

Table 4. Frequency of immunophenotypic alterations on different bone marrow hematopoietic cell populations from patients with newly diagnosed AML and MPAL grouped according to the clonal nature of residual mature/maturing neutrophils, monocytes and NRBC

Cell subpopulation	*Cases with non-clonal RH*		*Cases with clonal RH*	
	No. of altered cases (%)	*No. of phenotypic alterations*	*No. of altered cases (%)*	*No. of phenotypic alterations*
Monocytes	3/10 (30%)	1.0 ± 1.2	35/42 (83%)*	2.5 ± 1.3**
Maturing neutrophils	5/10 (50%)	2.0 ± 2.2	47/48 (98%)**	4.2 ± 1.6**
NRBC	1/8 (13%)	0.9 ± 1.2	26/37 (71%)***	1.7 ± 0.6**
Total	5/10 (50%)	3.7 ± 4.2	47/48[a] (98%)**	6.9 ± 3.0**

Abbreviations: AML, acute myeloid leukemia; MPAL, mixed phenotype acute leukemia; NRBC, nucleated red blood cells; RH, residual hematopoiesis.*$P=0.003$, **$P<0.001$ and ***$P=0.01$ vs non-clonal cases. [a]One case could not be properly evaluated due to inadequate sample conditions for flow cytometry immunophenotypic assessment of residual BM cell populations.

$P<0.001$), monocytic cells (83% vs 30%; $P=0.003$) and NRBC (71% vs13%; $P=0.01$) (Table 4). In addition, for each of these three cell populations, cases with clonal residual hematopoiesis also showed a higher mean number of phenotypic alterations/case (6.9 ± 3.0 vs 3.7 ± 4.2, $P<0.001$) as well as greater number of altered cell populations/case (2.4 ± 0.8 vs 1 ± 1.2, $P<0.03$), than the other cases (Tables 4 and 5).

DISCUSSION

AML is a poor-prognosis but still highly heterogeneous disease in adults, which significantly reduces overall patient survival rates.[23,24] For decades, it is well established that AML may emerge *de novo* (*de novo* AML) or following other hematopoietic malignancies as well as cytotoxic therapy for other disorders (secondary AML), the latter two groups being associated with a particularly unfavorable patient outcome.[2–4,25] Because of this, the distinction between primary *de novo* and secondary AML represents a major goal in the classification of the disease.[26–29] Differential diagnosis criteria for primary vs secondary AML have been traditionally based on the existence of another previously well-documented myeloid stem cell disorder (for example, MDS or MPN) and/or concurrence of prior administration of leukemogenic cytotoxic therapies (associated or not with specific genetic alterations).[4,30–36] More recently, the WHO defined a new subgroup of AML cases, which have specific myelodysplasia-related cytogenetic alterations and/or exhibit morphological dysplasia in ⩾50% of the cells from ⩾2 myeloid lineages.[1] Such AML with MRC cases, also show a worse prognosis vs other *de novo* AML cases.[5,23,32,37–39] Even though, within those *de novo* AML patients who do not meet the criteria for AML–MRC, still a relatively significant proportion of cases show dysplastic features that are highly suspicious of an underlying clonal hematopoiesis.[40] Despite this, to the best of our knowledge, no study has been reported so far in which the clonal vs reactive nature of residual mature/maturing BM hematopoietic cells has been systematically investigated in AML.

Table 5. Frequency of immunophenotypic alterations detected by multiparameter flow cytometry on different populations of hematopoietic cells from adult patients with newly diagnosed AML and MPAL classified according to the clonal vs non-clonal nature of residual mature/maturing granulomonocytic and/or NRBC

No. of cell populations with an altered phenotype	*Newly diagnosed AML and MPAL*		P-*value*
	Cases with non-clonal RH n = *10*	*Cases with clonal RH* n = *48*[a]	
0	5/10 (50%)	1/48 (2%)	<0.001
1	2/10 (20%)	5/48 (10%)	0.60
⩾2	3/10 (30%)	42/48 (88%)	0.02
Mean (± one s.d.)	1 ± 1.2	2.4 ± 0.8	0.03

Abbreviations: AML, acute myeloid leukemia; MPAL, mixed phenotype acute leukemia; RH, residual hematopoiesis. [a]One case could not be properly evaluated due to inadequate sample conditions for flow cytometry immunophenotyping.

Here, we investigated the presence of markers of clonality in residual mature/maturing hematopoietic BM cells from a series of 59 newly diagnosed adult AML and MPAL cases. Overall, our results suggest that the great majority of newly diagnosed AML cases display an underlying clonal hematopoiesis as defined by the presence of specific cytogenetic alterations and/or a clonal pattern of inactivation of chromosome X in residual BM mature/maturing neutrophils, monocytes and/or NRBC. Interestingly, the frequency of AML cases with clonal residual hematopoiesis was invariably high among the different WHO diagnostic subtypes of the disease. Although a relatively limited number of cases were included within each specific WHO diagnostic category of AML, overall our results showed that the great majority of the *de novo* AML cases, including *de novo* AML with MRC and AML with CBF-associated recurrent genetic alterations, carry an underlying clonal hematopoiesis. Similarly, the majority of cases with AML NOS also showed blast cells coexisting with clonal residual mature/maturing hematopoietic cells in the absence of myelodysplasia-related changes, suggesting that by adding more AML NOS cases, significant differences will potentially be detected. Of note, the frequency of AML cases showing underlying clonal hematopoiesis within these specific subgroups of *de novo* AML was also similar to that of therapy-related AML and MPAL cases.

Altogether, these results suggest that except for a limited number of patients, most newly diagnosed adult AML and MPAL cases develop over a pre-existing clonal hematopoiesis. The demonstration that all AML and MPAL cases carrying recurrent genetic abnormalities in their blasts —for example, t(8;21),[41–43] inv(16),[44–46] *BCR/ABL* gene rearrangements,[47–49] *KIT* mutation[50]— shared these same alterations in their residual mature/maturing myeloid hematopoietic cell compartments, suggests that such alterations potentially occur in hematopoietic (myeloid) stem cells capable of multilineage differentiation at least among the cases here analyzed, in line with previous observations in some AML and MPAL,[23,25,32,51–53] and also in systemic mastocytosis patients who progress to AML.[53] At the same time, these observations also indicate that such alterations would not be sufficient for transformation to acute leukemia, other genetic hits being potentially required in this process. In line with this hypothesis, previous studies have shown that most genetic alterations here investigated are also found in maturing myeloid cells from MDS and MPN patients.[18,23,25,30,32,52,54–60] Of note, we investigated the presence of *KIT* mutations in all sorted bone marrow cell compartments of three patients who were suspected of having a coexisting systemic mastocytosis; for this purpose, we used a DNA-based, low-sensitive technique that allowed us to rule out contamination by other *KIT*-mutated cell populations. Usage of similar approaches in future studies for the evaluation of other AML-associated mutations (for example, *FLT3* and *NPM1)* may further contribute to increase the frequency of clonal markers in AML blasts, particularly among males with no other markers of clonality.

Despite all the above, it should be noted that the presence of an underlying clonal hematopoiesis in adults with newly diagnosed AML and MPAL, appears to be independent of the specific diagnostic subtype of the disease or the presence of myelodysplasia-related changes. However, we should be cautious in deriving such a conclusion, as the specific markers for clonality used here might have not been informative in specific cases. As an example, all female cases diagnosed with AML with MRC showed an underlying clonal hematopoiesis, whereas this was not detected in 3/14 male patients. These results may indicate that only the cytogenetic alterations present in the blast cells of the three male patients with AML with MRC might not represent a primary but a secondary cytogenetic event, restricted to the blast cell population, while the primary cytogenetic/molecular event might have gone undetected with the set of probes used. In line with this latter hypothesis, all three male AML with MRC patients who showed no clonal residual hematopoiesis on cytogenetic grounds carried multilineage morphological dysplasia, which is highly suggestive of an underlying clonal hematopoiesis.

Based on all the above, new sensitive markers for clonality directed either at the detection of primary genetic events or their consequences on the phenotypic and/or morphological appearance of the cells are needed. In this regard, multiparameter flow cytometry immunophenotyping has emerged as an attractive approach, as the presence of multiple phenotypic alterations in different compartments of BM cells have been recurrently reported in MDS.[9,10,19–22,40] Interestingly, our results also showed that newly diagnosed AML and MPAL patients who showed clonal hematopoiesis on molecular/cytogenetic grounds, more frequently displayed aberrant patterns of protein expression by FCM together with a greater number of aberrant phenotypes on the different populations of residual mature/maturing BM hematopoietic cells analyzed vs all other cases. However, aberrant phenotypes were also detected at lower frequencies among the latter cases. These results may suggest that in such cases, the markers used to assess clonality could be not sensitive enough (for example, assessment of clonality by the HUMARA test requires that clonally related cells represent $\geqslant$70% of the cells investigated[8]); at the same time, they point out the potential utility of FCM immunophenotyping, in addition to conventional cytomorphology, for the identification of MRC in the BM of newly diagnosed AML and MPAL cases presenting as *de novo* cases.[9,20] Of note, three of our AML cases –two males and one female- in whom clonal hematopoiesis could not be demonstrated by FISH and/or HUMARA, showed aberrant phenotypes in all residual hematopoietic lineages investigated (neutrophils, monocytes and NRBC); interestingly, all these three cases were classified as AML–MRC based on cytomorphology. Taken together, these results would support the notion that FCM could contribute to increase the sensitivity of conventional cytomorphology in those cases where mild or no dysplastic features are seen in residual mature/maturing BM cells, or where they are present at relatively low frequencies among a major population of AML blasts, hampering adequate morphological assessment. Further prospective studies are required in this regard to demonstrate the combined utility of FCM and cytomorphology for the identification of AML with MRC.

In summary, our results indicate that the vast majority of adults with newly diagnosed AML and MPAL displays an underlying clonal hematopoiesis, residual mature/maturing granulomonocytic and/or erythroid cells displaying chromosomal alterations, which are frequently shared by the blast cells, in addition to multiple aberrant phenotypes; noteworthy, this appears to involve most WHO 2008 diagnostic subtypes of AML and also MPAL. Whether the presence vs absence of clonal residual hematopoiesis contributes to a better prognostic stratification of newly diagnosed adult AML and MPAL patients deserves further investigations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fondo de Investigaciones Sanitarias (FIS) of the Ministerio de Economía y Competitividad, Madrid, Spain (RETICS RD12/0036/0048-FEDER); Junta de Castilla y León (Ayuda al Grupo GR37 de Excelencia de Castilla y León). CFG was supported by a grant from the Fondo de Investigaciones Sanitarias (FIS) of the Ministerio de Economía y Competitividad, Madrid, Spain (PI08/90881). MCS-S was supported by a grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior -CAPES (1386/06-9) do Ministério de Educação do Brasil.

REFERENCES

1 Arber DA, Brunning RD, Vardiman JW, Le Beau MM, Orazi A, Bain BJ *et al.* Acute myeloid leukaemia and related precursor neoplasms. In: Swerdlow SH *et al.* (eds) *WHO Classification of Tumours and Haematopoietic and Lymphoid Tissues*. IARC: Lyon, 2008, pp 106–145.
2 Mrozek K, Bloomfield CD. Chromosome aberrations, gene mutations and expression changes, and prognosis in adult acute myeloid leukemia. *ASH Education Book* 2006; **2006**: 167–177.
3 Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? *Blood* 2007; **109**: 431–448.
4 Larson RA. Is secondary leukemia an independent poor prognostic factor in acute myeloid leukemia? *Best Pract Res Clin Haematol* 2007; **20**: 29–37.
5 Arber DA, Stein AS, Carter NH, Ikle D, Forman SJ, Slovak ML. Prognostic impact of acute myeloid leukemia classification. Importance of detection of recurring cytogenetic abnormalities and multilineage dysplasia on survival. *Am J Clin Pathol* 2003; **119**: 672–680.
6 Mrozek K, Dohner H, Bloomfield CD. Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. *Curr Opin Hematol* 2007; **14**: 106–114.
7 Radmacher MD, Marcucci G, Ruppert AS, Mrozek K, Whitman SP, Vardiman JW *et al.* Independent confirmation of a prognostic gene-expression signature in adult acute myeloid leukemia with a normal karyotype: a Cancer and Leukemia Group B study. *Blood* 2006; **108**: 1677–1683.
8 Kopp P, Jaggi R, Tobler A, Borisch B, Oestreicher M, Sabacan L *et al.* Clonal X-inactivation analysis of human tumours using the human androgen receptor gene (HUMARA) polymorphism: a non-radioactive and semiquantitative strategy applicable to fresh and archival tissue. *Mol Cell Probes* 1997; **11**: 217–228.
9 Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores J *et al.* The immunophenotype of different immature, myeloid and B-cell lineage-committed

CD34+ hematopoietic cells allows discrimination between normal/reactive and myelodysplastic syndrome precursors. *Leukemia* 2008; **22**: 1175–1183.
10 Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores-Montero J *et al.* Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: a pilot study on a series of 56 patients. *Cytometry B Clin Cytom* 2010; **78**: 154–168.
11 Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Bottcher S, Ritgen M *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012; **26**: 1986–2010.
12 van Dongen JJ, Lhermitte L, Bottcher S, Almeida J, van der Velden VH, Flores-Montero J *et al.* EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia* 2012; **26**: 1908–1975.
13 Rasillo A, Tabernero MD, Sanchez ML, Perez de Andres M, Martin Ayuso M, Hernandez J *et al.* Fluorescence in situ hybridization analysis of aneuploidization patterns in monoclonal gammopathy of undetermined significance versus multiple myeloma and plasma cell leukemia. *Cancer* 2003; **97**: 601–609.
14 Gale RE, Mein CA, Linch DC. Quantification of X-chromosome inactivation patterns in haematological samples using the DNA PCR-based HUMARA assay. *Leukemia* 1996; **10**: 362–367.
15 Nakahara Y, Suzuki H, Ohashi H, Hatano S, Tomita A, Kinoshita T *et al.* Clonality analysis of granulocytes and T lymphocytes in healthy females by the PCR-based HUMARA method. *Int J Hematol* 1999; **69**: 237–243.
16 Uchida T, Ohashi H, Aoki E, Nakahara Y, Hotta T, Murate T *et al.* Clonality analysis by methylation-specific PCR for the human androgen-receptor gene (HUMARA-MSP). *Leukemia* 2000; **14**: 207–212.
17 Sotlar K, Escribano L, Landt O, Mohrle S, Herrero S, Torrelo A *et al.* One-step detection of c-kit point mutations using peptide nucleic acid-mediated polymerase chain reaction clamping and hybridization probes. *Am J Pathol* 2003; **162**: 737–746.
18 Garcia-Montero AC, Jara-Acevedo M, Teodosio C, Sanchez ML, Nunez R, Prados A *et al.* KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: a prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. *Blood* 2006; **108**: 2366–2372.
19 Westers TM, Ireland R, Kern W, Alhan C, Balleisen JS, Bettelheim P *et al.* Standardization of flow cytometry in myelodysplastic syndromes: a report from an international consortium and the European LeukemiaNet Working Group. *Leukemia* 2012; **26**: 1730–1741.
20 Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 2008; **111**: 3941–3967.
21 Della Porta MG, Picone C, Pascutto C, Malcovati L, Tamura H, Handa H *et al.* Multicenter validation of a reproducible flow cytometric score for the diagnosis of low-grade myelodysplastic syndromes: results of a European LeukemiaNET study. *Haematologica* 2012; **97**: 1209–1217.
22 van de Loosdrecht AA, Ireland R, Kern W, Della Porta MG, Alhan C, Balleisen JS *et al.* Rationale for the clinical application of flow cytometry in patients with myelodysplastic syndromes: position paper of an International Consortium and the European LeukemiaNet Working Group. *Leuk Lymphoma* 2012; **54**: 472–475.
23 Gahn B, Haase D, Unterhalt M, Drescher M, Schoch C, Fonatsch C *et al.* De novo AML with dysplastic hematopoiesis: cytogenetic and prognostic significance. *Leukemia* 1996; **10**: 946–951.
24 Lowenberg B. Diagnosis and prognosis in acute myeloid leukemia--the art of distinction. *N Engl J Med* 2008; **358**: 1960–1962.
25 Baldus CD, Mrozek K, Marcucci G, Bloomfield CD. Clinical outcome of de novo acute myeloid leukaemia patients with normal cytogenetics is affected by molecular genetic alterations: a concise review. *Br J Haematol* 2007; **137**: 387–400.
26 Ohyashiki JH, Ohyashiki K, Kawakubo K, Fujimura T, Shimamoto T, Nakazawa S *et al.* Comparison between immunogenotypic findings in de novo AML and AML post MDS. *Leukemia* 1993; **7**: 1747–1751.
27 Pagano L, Mele L, Fianchi L, Rutella S, Piscitelli R, Leone G *et al.* Immunophenotypic analysis in 119 patients with acute myeloid leukemia following a previous malignancy: a comparison with the immunophenotype of 231 de novo AML. *Haematologica* 2003; **88**: 225–227.
28 Ostgard LS, Kjeldsen E, Holm MS, Brown Pde N, Pedersen BB, Bendix K *et al.* Reasons for treating secondary AML as de novo AML. *Eur J Haematol* 2010; **85**: 217–226.
29 Preiss BS, Bergmann OJ, Friis LS, Sorensen AG, Frederiksen M, Gadeberg OV *et al.* Cytogenetic findings in adult secondary acute myeloid leukemia (AML): frequency of favorable and adverse chromosomal aberrations do not differ from adult de novo AML. *Cancer Genet Cytogenet* 2010; **202**: 108–122.
30 Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med* 1999; **341**: 1051–1062.
31 Tefferi A, Thiele J, Vardiman JW. The 2008 World Health Organization classification system for myeloproliferative neoplasms: order out of chaos. *Cancer* 2009; **115**: 3842–3847.
32 Milosevic JD, Puda A, Malcovati L, Berg T, Hofbauer M, Stukalov A *et al.* Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *Am J Hematol* 2012; **87**: 1010–1016.
33 Estey E, Dohner H. Acute myeloid leukaemia. *Lancet* 2006; **368**: 1894–1907.
34 Arber DA, Slovak ML, Popplewell L, Bedell V, Ikle D, Rowley JD. Therapy-related acute myeloid leukemia/myelodysplasia with balanced 21q22 translocations. *Am J Clin Pathol* 2002; **117**: 306–313.
35 Mauritzson N, Albin M, Rylander L, Billstrom R, Ahlgren T, Mikoczy Z *et al.* Pooled analysis of clinical and cytogenetic features in treatment-related and de novo adult acute myeloid leukemia and myelodysplastic syndromes based on a consecutive series of 761 patients analyzed 1976-1993 and on 5098 unselected cases reported in the literature 1974-2001. *Leukemia* 2002; **16**: 2366–2378.
36 Leone G, Mele L, Pulsoni A, Equitani F, Pagano L. The incidence of secondary leukemias. *Haematologica* 1999; **84**: 937–945.
37 Leith CP, Kopecky KJ, Godwin J, McConnell T, Slovak ML, Chen IM *et al.* Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood* 1997; **89**: 3323–3329.
38 Miyazaki Y, Kuriyama K, Miyawaki S, Ohtake S, Sakamaki H, Matsuo T *et al.* Cytogenetic heterogeneity of acute myeloid leukaemia (AML) with trilineage dysplasia: Japan Adult Leukaemia Study Group-AML 92 study. *Br J Haematol* 2003; **120**: 56–62.
39 Yanada M, Suzuki M, Kawashima K, Kiyoi H, Kinoshita T, Emi N *et al.* Long-term outcomes for unselected patients with acute myeloid leukemia categorized according to the World Health Organization classification: a single-center experience. *Eur J Haematol* 2005; **74**: 418–423.
40 Miesner M, Haferlach C, Bacher U, Weiss T, Macijewski K, Kohlmann A *et al.* Multilineage dysplasia (MLD) in acute myeloid leukemia (AML) correlates with MDS-related cytogenetic abnormalities and a prior history of MDS or MDS/MPN but has no independent prognostic relevance: a comparison of 408 cases classified as "AML not otherwise specified" (AML-NOS) or "AML with myelodysplasia-related changes" (AML-MRC). *Blood* 2010; **116**: 2742–2751.
41 Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G *et al.* The importance of diagnostic cytogenetics on outcome in AML: analysis of 1612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood* 1998; **92**: 2322–2333.
42 Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC *et al.* Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood* 2002; **100**: 4325–4336.
43 Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH *et al.* Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood* 2010; **116**: 354–365.
44 Marlton P, Keating M, Kantarjian H, Pierce S, O'Brien S, Freireich EJ *et al.* Cytogenetic and clinical correlates in AML patients with abnormalities of chromosome 16. *Leukemia* 1995; **9**: 965–971.
45 Claxton DF, Marlton P, Siciliano MJ. Molecular genetics of inversion 16 leukemia: implications for leukemogenesis. *Cancer Treat Res* 1996; **84**: 1–18.
46 Speck NA, Gilliland DG. Core-binding factors in haematopoiesis and leukaemia. *Nat Rev Cancer* 2002; **2**: 502–513.
47 Chen Z, Morgan R, Berger CS, Pearce-Birge L, Stone JF, Sandberg AA. Identification of masked and variant Ph (complex type) translocations in CML and classic Ph in AML and ALL by fluorescence in situ hybridization with the use of bcr/abl cosmid probes. *Cancer Genet Cytogenet* 1993; **70**: 103–107.
48 Hamaguchi H, Suzukawa K, Nagata K, Yamamoto K, Yagasaki F, Morishita K. Establishment of a novel human myeloid leukaemia cell line (HNT-34) with t(3;3)(q21;q26), t(9;22)(q34;q11) and the expression of EVI1 gene, P210 and P190 BCR/ABL chimaeric transcripts from a patient with AML after MDS with 3q21q26 syndrome. *Br J Haematol* 1997; **98**: 399–407.
49 Tefferi A, Vardiman JW. The diagnostic interface between histology and molecular tests in myeloproliferative disorders. *Curr Opin Hematol* 2007; **14**: 115–122.
50 Yang Y, Huang Q, Lu Y, Li X, Huang S. Reactivating PP2A by FTY720 as a novel therapy for AML with C-KIT tyrosine kinase domain mutation. *J Cell Biochem* 2012; **113**: 1314–1322.
51 Appelbaum FR, Kopecky KJ, Tallman MS, Slovak ML, Gundacker HM, Kim HT *et al.* The clinical spectrum of adult acute myeloid leukaemia associated with core binding factor translocations. *Br J Haematol* 2006; **135**: 165–173.

52 Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K *et al.* Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med* 2012; **366**: 1090–1098.
53 Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev* 2004; **18**: 115–136.
54 Greenberg P, Cox C, LeBeau MM, Fenaux P, Morel P, Sanz G *et al.* International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood* 1997; **89**: 2079–2088.
55 Bernell P, Arvidsson I, Hast R, Jacobsson B, Stenke L. Differences in cell lineage involvement between MDS-AML and de novo AML studied by fluorescence in situ hybridization in combination with morphology. *Eur J Haematol* 1997; **58**: 241–245.
56 Preisler HD. Evolution of secondary hematologic disorders: preMDS-->MDS-->sAML. *Cancer Treat Res* 2001; **108**: 185–230.
57 Kelly L, Clark J, Gilliland DG. Comprehensive genotypic analysis of leukemia: clinical and therapeutic implications. *Curr Opin Oncol* 2002; **14**: 10–18.
58 Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet* 2002; **3**: 179–198.
59 Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A *et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 2009; **114**: 937–951.
60 Dicker F, Haferlach C, Sundermann J, Wendland N, Weiss T, Kern W *et al.* Mutation analysis for RUNX1, MLL-PTD, FLT3-ITD, NPM1 and NRAS in 269 patients with MDS or secondary AML. *Leukemia* 2010; **24**: 1528–1532.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)