Immunophenotypic analysis of cell cycle status in acute myeloid leukaemia: relationship to cytogenetics, genotype and clinical outcome

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Running title: Cell cycle analysis in AML

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Summary

Cell cycle status may play an important role in directing patient therapy. We therefore determined the cell cycle status of leukaemic cells by immunophenotypic analysis of bone marrow trephine biopsies from 181 patients with acute myeloid leukaemia (AML) and correlated the results with biological features and clinical outcome. There was considerable heterogeneity between patients. The presenting white cell count significantly correlated with the proportion of non-quiescent cells (P<0.0001), of cycling cells beyond G_1 (P<0.0001), and the speed of cycling (P<0.0001). Profiles in acute promyelocytic leukaemia (APL) differed from non-APL and were consistent with more differentiated cells with reduced proliferative potential, but no significant differences were observed between non-APL cytogenetic risk groups. *NPM1* mutations but not *FLT3*^{ITD} were significantly associated with a higher proportion of cells beyond G_1 (P=0.002) and faster speed of cycling (P=0.003). Resistance to standard cytosine arabinoside and daunorubicin induction chemotherapy was significantly related to a slower speed of cycling (P=0.0002), as was a higher relapse rate (P=0.05), but not with the proportion of non-quiescent cells or actively cycling cells. These results show a link between the cycling speed of AML cells and the response to chemotherapy, and help to identify a group with a very poor prognosis.

Keywords: AML, cell cycle analysis, response to chemotherapy

Introduction

Knowledge of cell cycle status may play an important role in directing patient therapy. In a variety of solid tumors, it is indicative of which patients are likely to respond to cell cycle phase-specific agents, and may be predictive of the long-term outcome (Williams & Stoeber, 2007). However, in acute myeloid leukaemia (AML), although the main therapeutic agents are cytosine arabinoside (Ara-C), which is cell cycle S-phase specific, and topoisomerase II-inhibiting anthracyclines that have greater toxicity in rapidly proliferating cells, the results of studies addressing the relationship between the cell cycle status of leukaemic blast cells and clinical response have been inconsistent (Hiddemann *et al*, 1982a; Preisler *et al*, 1984; Raza *et al*, 1990, 1991; Vidriales *et al*, 1995; Braess *et al*, 2001). This may partly reflect the different techniques used but is most likely to represent the different types of samples used. It has been shown that fewer leukaemic cells in the blood are cycling compared to the bone marrow (Mauer & Fisher, 1962; Hiddemann *et al*, 1982b), and using direct immunophenotyping we demonstrated that nearly all the leukaemic blasts in the peripheral blood (PB) were arrested in G_1, with only 1% beyond G_1, compared to 24% in the bone marrow trephine (BMT) samples (Sellar *et al*, 2016). Furthermore, only 6% of cells were beyond G_1 in matched bone marrow aspirate (BMA) samples, considerably less than in the BMTs due to variable dilution with blood-derived blasts.

As the number of blasts in a BMA sample that are blood-derived is likely to depend on both the volume of the sample taken and the presenting white cell count (WCC), this suggests that in patients with a higher proliferative rate leading to a higher blast cell count in the PB, the degree of contamination of the BMA with PB is likely to be greater and, paradoxically, the cycling rate observed in the BMA sample would therefore be

spuriously lower. For accurate assessment of cell cycle status, it is therefore essential to analyse BMT samples.

Another factor that may influence cell cycle status is the molecular heterogeneity of AML that is now apparent (Khwaja *et al*, 2016). This is highly relevant to current therapy strategies as some molecular abnormalities such as internal tandem duplications in the *FLT3* gene (*FLT3*^{ITD}) and nucleophosmin gene mutations (*NPM1*^{MUT}) are highly predictive of outcome and used to risk-stratify patient management (Dohner *et al*, 2017; O'Donnell *et al*, 2017). Both are also associated with a higher presenting WCC (Dohner *et al*, 2005; Gale *et al*, 2008). The relationship between proliferative status and response to therapy has been reported to be dependent on the cytogenetic risk group (Braess *et al*, 2001) and, more recently, Behbehani and colleagues have reported that the proportion of AML early progenitor cells that are in cell cycle is lower in cases with a *FLT3*^{ITD} (Behbehani *et al*, 2015). However, BMAs were used for these studies.

In order to address these issues we have used immunohistochemistry of trephine biopsies from AML patients to assess cell cycle status and the proportion of cells undergoing apoptosis, and investigated the relationship between these parameters, the cytogenetic risk group, molecular subtype as defined by *FLT3* and *NPM1* mutational status, as well as clinical outcome. The data indicate that the proportion of cells beyond G_1 and the speed of cell cycling are predictive of outcome.

Materials and Methods

Patient cohort

All patients studied presented with newly diagnosed AML to UCLH between January 2003 and December 2011 and had a diagnostic biopsy performed prior to treatment. Local research ethics approval for the study was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research, and informed patient consent was obtained in accordance with the Declaration of Helsinki. Patient records were reviewed to define patient demographics, details of the therapy received, and response to treatment including induction death (ID), achievement of CR, cumulative incidence of relapse (CIR), time to relapse, and overall survival (OS). Response definitions are given in the Supplementary materials.

Molecular analysis

Molecular analysis was performed on DNA available from the UCL tissue bank or extracted from stored BMA smears using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK). *FLT3* and *NPM1* genotype were determined as previously described (Gale *et al*, 2008).

Immunohistochemistry (IHC)

Serial sections of formalin-fixed paraffin-embedded BMTs were processed and stained as previously described (Sellar *et al*, 2016) using antibodies to Mcm2 to assess non-quiescent cells regardless of whether or not they were actively cycling (positive in G_1, S, G_2 and M but not G_0.), MIB-1 (equivalent to Ki-67) for actively proliferating cells (positive in a proportion of cells in G_1, and cells in S, G_2 and M but not G_0) and geminin for cycling cells that have progressed beyond G_1 (positive in S, G_2 and M but not G_1 and G_0). In addition the sections were stained for cleaved caspase 3 (Cell Signaling Technology, Hitchin, UK) to assess the proportion of cells undergoing apoptosis (Suzuki

et al, 2012). The proportion of non-quiescent cells that are proliferating (MIB-1/Mcm2 ratio) is inversely related to the number of cells arrested in G_1, and the geminin/MIB-1 ratio is an indicator of the average speed of the cell cycle, with the time in S phase being relatively constant and the time in G_1 accounting for most of the variability of the cell cycle duration (Wharton *et al*, 2004). Tonsil tissue containing both glandular and epithelial components was processed at the same time as a positive control. The number of leukaemic cells positive for each of the antibodies was determined using the Ariol automated scanning microscope and image analysis system (Turashvili *et al*, 2009), counting at least 1000 cells per sample (see Supplementary materials). Results were expressed as the labelling index (LI), i.e. the number of positive cells as a proportion of the total cells after excluding most lymphoid and erythroid cells.

Statistical analysis

Full details of the statistical analysis are provided in the Supplementary materials.

Results

Patient characteristics and treatment

In total, 187 AML patients newly diagnosed between January 2003 and December 2011 and with available pre-treatment trephine biopsies were identified. Six were subsequently excluded: five had inadequate material for further immunohistochemistry and one had a coincident B cell malignancy. Median age of the remaining 181 patients was 55 years (range, 13-86); 55% were male (Table 1). Overall, 83% had *de novo* AML; the remaining patients had secondary disease due to a preceding haematological disorder (10%) or prior chemotherapy (7%) administered at least two years before the current presentation. Fifteen patients (8%) had acute promyelocytic leukaemia (APL),

and 166 had non-APL disease, of which 31% were *NPM1*^{MUT} and 22% *FLT*3^{ITD}. Of the 136 patients with known cytogenetics, 68% had intermediate-risk disease.

All but one APL patient received intensive chemotherapy plus a differentiating agent(s); the remaining patient received arsenic trioxide and all-trans retinoic acid without conventional chemotherapy. Of the non-APL patients, 140 (84%) received intensive induction therapy containing standard dose Ara-C plus an anthracycline as their first treatment; 13 (9%), 80 (57%) and 23 (16%) had favourable-, intermediate- and adverse-risk cytogenetics respectively; data were not available for 24 patients (17%). The frequency of *NPM1*^{MUT} was 34% and *FLT*3^{ITD} 22% in this cohort. A further three patients received an alternative intensive regimen and 17 received a non-intensive chemotherapy regimen, most commonly subcutaneous low-dose Ara-C. Twenty nine patients (16%) received an allogeneic transplant in first CR. Five patients received no treatment and one was lost to follow-up.

Outcome of Patients Receiving Intensive Induction Therapy

The CR rate after one cycle of induction therapy was 100% in the APL patients. In the non-APL patients treated with Ara-C and an anthracycline it was 66% (92%, 73% and 39% respectively in the favourable-, intermediate- and adverse-risk cytogenetic groups). Seventeen patients (12%) died during induction therapy and 31 (22%) were alive but not in CR. After further cycle(s) of induction therapy, the CR rate increased to 71%. OS at 3 years was 100% for APL and 41% for non-APL patients (P=0.0004). In the cytogenetic risk groups it was 76%, 41% and 26%, respectively (P=0.001). Patients aged <60 years had a significantly longer survival than those aged ≥60 years (median, 1478 vs 282 days respectively, P<0.0001).

Cell cycle analysis and cleaved caspase staining

Cell cycle profiling of all 181 patients indicated that the majority of leukaemic blasts in the BMTs were not quiescent (median Mcm2 LI, 81%), and approximately 40% of the non-quiescent cells were MIB-1 positive and therefore actively cycling (Table 2). The median geminin LI was 17%, and approximately half of the actively cycling cells had progressed beyond G_1 (median geminin/MIB-1 ratio, 0.51). There was, however, considerable heterogeneity within the cell cycle profile, with relatively wide interquartile and overall ranges (Table 2). Very few blasts were undergoing apoptosis (median cleaved caspase LI, 3%).

Relationship to biological risk factors

There was no correlation between any of the cell cycle parameters and patient age at presentation, but cleaved caspase staining was positively correlated with increasing age (r=0.3, *P*=0.0001). This relationship was maintained when either APL or secondary AML was excluded (r=0.3, *P*=0.0003 for both analyses).

The presenting WCC was significantly correlated with the proportion of non-quiescent cells (r=0.4, *P*<0.0001) but not the proportion of actively cycling cells (for MIB-1 LI, r=0.1, *P*=0.06). It did, however, correlate with the proportion of cycling cells beyond G_1 and the speed of cell cycle in cycling cells (for geminin LI, r=0.4, *P*<0.0001; geminin/MIB-1 ratio, r=0.3, *P*<0.0001). It was also inversely correlated with the proportion of apoptotic cells (for cleaved caspase LI, total cohort, r=-0.2, *P*=0.002; non-APL patients, r=-0.3, *P*<0.0001). These results suggest that a higher WCC typically occurs in those cases with faster cell cycle progression rates and lower levels of apoptosis. No

significant differences in cell cycle parameters or the proportion of apoptotic cells were observed between *de novo* and secondary AML patients (data not shown).

Relationship to FAB type

Patients with APL had significantly fewer non-quiescent cells, actively cycling cells and cells in $S/G_2/M$ compared to other FAB types, and a greater proportion of cells in a G_1-arrested state (Figure 1; Table 2). They also had a significantly lower median geminin/MIB-1 ratio, implying a longer average duration of G_1 in the cycling cells. There was no significant association between other FAB types and cell cycle markers (data not shown). The APL patients also had a significantly lower proportion of cleaved caspase labelled cells, with no differences observed between other FAB types.

Relationship to Cytogenetic Risk Groups

Differences in cell cycle parameters between the cytogenetic risk groups in the non-APL patients were not statistically significant (Table 2). However, the proportion of apoptotic cells was slightly, but significantly, higher in the poorer cytogenetic risk patients (respectively 1.9%, 2.8% and 3.4% in the favourable-, intermediate- and adverse-risk cytogenetic groups, P=0.03).

Relationship to molecular genotype in patients with intermediate-risk cytogenetics

As *NPM1* and *FLT*3^{ITD} mutations are predominantly associated with intermediate-risk cytogenetics, their association with cell cycle parameters was only considered in the 85 patients with intermediate-risk disease and available mutational data. The proportion of non-quiescent cells was marginally higher in the *NPM1*^{MUT} cases compared to those with wild-type *NPM1* (*NPM*1^{WT}) (median Mcm2 LI, 89% vs 85%, P=0.03). Although the

proportion of actively cycling cells was also higher, the difference was not significant (median MIB-1 LI, 40% vs 34%, *P*=0.4). However, the proportion of cycling cells that had progressed beyond G_1 was significantly higher in the *NPM1*^{MUT} cases (median geminin LI, 24% vs 15%, *P*=0.002), as was the geminin/MIB-1 ratio (0.68 vs 0.40, *P*=0.003) (Figure 2). These results indicate that although *NPM1*^{MUT} and *NPM1*^{WT} patients have a similar proportion of non-quiescent and actively cycling cells, in *NPM1*^{MUT} cases, those cells that are cycling spend a smaller proportion of time in G_1 and are thus cycling more quickly. Non-quiescent cells were slightly higher in *FLT3*^{ITD} than *FLT3*^{WT} patients (median Mcm2 LI, 92% vs 84%, *P*=0.03) but the proportion of actively cycling cells and of cells in $S/G_2/M$ was similar (median MIB-1 LI, 36% vs 36%, *P*=0.77; median geminin LI, 21% vs 19%; *P*=0.5). The geminin/MIB-1 ratio was also not significantly different (0.48 vs 0.52, *P*=0.8). When the *NPM1*^{MUT} and *NPM1*^{WT} populations were analysed separately, there was again no significant impact of the *FLT3*^{ITD} status (*P*=0.41 and 0.82 with regard to the geminin/MIB-1ratio in the *NPM1*^{MUT} and *NPM1*^{WT} populations respectively). Inspection of the geminin/MIB-1 ration in all non-APL cases (Figure 1E) and in the intermediate-risk disease category (Figure 2B) suggested that there were two distinct subpopulations. An exploratory analysis was therefore carried out comparing clinical, morphological, cytogenetic and limited genotype features between those patients with higher and lower geminin/MIB-1 ratios. No significant differences were found. The proportion of cells positive for cleaved caspase was not influenced by either the *NPM1* or *FLT3* genotype (*P*=0.2 and *P*=0.4 respectively).

Relationship between cell cycle status, cleaved caspase staining and response to therapy

For the 140 non-APL patients that received intensive induction therapy, 66% achieved CR after one cycle of chemotherapy, and neither the proportion of non-quiescent cells

nor of actively cycling cells differed between those who did or did not achieve CR (Mcm2 LI, 83% vs 75%, *P*=0.2; MIB-1 LI, 34% vs 37%, *P*=0.9). Nevertheless, patients who achieved an early remission did have a significantly higher proportion of cells beyond G_1 (geminin LI, 19% vs 13%, *P*=0.004) and geminin/MIB-1 ratio (0.54 vs 0.41, *P*=0.0002), and this relationship was still statistically significant when CR at any time was considered (71% of the cohort: geminin LI, 19% vs 12%, *P*=0.02; geminin/MIB-1 ratio, 0.52 vs 0.42, *P*=0.02). However, further inspection of the response data revealed that the CR rates were confounded by an excess of IDs in those patients with the higher geminin/MIB-1 ratios. In multivariate analysis of the total cohort considering biological, cytogenetic and molecular characteristics, the factors predicting for ID were WCC (*P*=0.01) and age (*P*=0.02), with the impact of the geminin/MIB-1 ratio not achieving significance (*P*=0.1). If IDs were excluded, the factors significantly predicting for attainment of CR after one cycle of therapy were the presence of an *NPM1* mutation (*P*=0.001) and the geminin/MIB-1 ratio (*P*=0.01).

When the patients were grouped according to quartiles of the geminin/MIB-1 ratio, it was apparent that there was an association between this ratio and the achievement of CR after one cycle of therapy, with the highest CR rate (74%) in the quartile with the highest geminin/MIB-1 ratio (Q1: median, 0.81) and the lowest CR rate (54%) in those with the lowest ratio (Q4: median, 0.30), although this difference did not reach significance (*P*=0.08 across all quartiles) (Table 3). IDs were, however, significantly higher in Q1 (26%) and lower in Q4 (6%; *P*=0.01 across all groups), which is probably due to the higher presenting WCC in Q1 patients, a known risk factor for ID and an independent adverse risk factor for ID in this study. In fact, of the nine IDs in Q1 patients, seven had a WCC above the median value of 56 $x10^9$/L for this quartile.

Furthermore, six of these seven patients were over the age of 60 years, and four of the nine patients with early deaths had secondary leukaemia. When the induction mortality as well as the CR rate was taken into account, there was a highly significant relationship between resistant disease and the geminin/MIB-1 ratio. In Q1, every patient who was alive after the first course of induction therapy was in CR compared to only 58% in Q4 (*P*=0.0002) (Table 3). Considering best response at any time, the values for resistant disease were 0% in Q1 and 24% in Q4 patients (*P*=0.02). It is notable that the incidence of *NPM1* mutations was much higher in Q1 than in Q4 and it is not possible to clearly separate the impact on chemo-resistance of the cell cycle profile *per se* and the *NPM1* mutational status. The number of patients who received a consolidation allograft in first remission was similar in each quartile (Table 3). After censoring patients at the time of transplant, the relapse rate was higher in Q4 (*P*=0.04). Furthermore, five out of six patients in Q4 who received and survived an allograft subsequently relapsed.

Relationship between geminin/MIB-1 ratio and long-term outcome

The CIR and OS at 3 years in the non-APL patients who received intensive induction therapy were 47% and 41% respectively. The CIR varied significantly between the different geminin/MIB-1 ratio quartiles; it was highest (73%) in Q4 patients with the lowest geminin/MIB-1 ratio (*P*=0.01) (Table 3) (Figure 3A). Although the difference in OS between the quartiles was not significant, this was again confounded by the higher rate of ID in the Q1 patients. In view of the high relapse rate in the Q4 patients, an exploratory landmark analysis was performed from the time at which patients received their second cycle of intensive chemotherapy, comparing Q4 (n=31) with Q1-3 patients (n=83). The difference in survival between these groups was borderline significant

(*P*=0.05) (Hazard ratio by logrank analysis, 1.65; 95% confidence intervals, 0.996 – 3.126). Median survival was 472 vs 1346 days in Q4 and Q1-3 respectively (Figure 3B).

Discussion

The results presented here on the cell cycle status of leukaemic cells and its relationship to outcome in a cohort of 181 AML patients are from a single centre, but the characteristics of the cohort with regard to age, cytogenetics, mutational status and response to treatment are similar to other reported series. An important feature of the study was that the cell cycle analysis of the leukaemic cells was determined from BMTs. This was essential in order to avoid the impact on the results of blood contamination of BMAs (Mauer & Fisher, 1962; Hiddemann *et al*, 1982b; Sellar *et al*, 2016).

The results showed that only a minority of bone marrow leukaemic cells were in G_0, which is similar to other malignancies where this parameter has been assessed (Dudderidge *et al*, 2005; Obermann *et al*, 2005; Kulkarni *et al*, 2007; Kayes *et al*, 2009; Loddo *et al*, 2009). There was also a relatively high proportion of actively cycling cells (approximately one-third of leukaemic cells), similarly comparable to other malignancies, although considerably less than in Burkitt's lymphoma where nearly all the cells are actively cycling (Obermann *et al*, 2005). A striking feature of the current findings, however, was that those AML cells that were actively cycling were doing so rapidly (geminin/MIB-1 ratio, 0.53), which is about twice the value observed in ovarian and penile cancer, and nearly three times that in breast cancer (Kulkarni *et al*, 2007; Kayes *et al*, 2009; Loddo *et al*, 2009). This speed of cycling may therefore contribute to the observed sensitivity of AML cells to the cell cycle phase-specific agents that have been the backbone of standard therapy for several decades.

Nevertheless, there was considerable heterogeneity in the cell cycle profile observed between patient samples and this highlighted several important factors. Firstly, a more proliferative profile with fewer quiescent cells, more cells in $S/G_2/M$, and a faster cycling rate was associated with a higher presenting WCC, as was a lower percentage of apoptotic cells. Secondly, APL patients formed a distinct subgroup, with more cells in G_0, fewer cells actively cycling and in $S/G_2/M$, and a greater proportion of cells likely to be in a G_1-arrested state than non-APL patients. They also had a lower geminin/MIB-1 ratio, implying a slower cell cycle time, and a lower cleaved caspase LI. This data is in accord with the fact that most of the cells in APL are differentiated to a relatively late maturational stage, by which time the proliferative potential is markedly reduced. No differences were observed between other FAB types. Thirdly, the cytogenetic risk group did not seem to impact on the cell cycle parameters, despite the fact that core binding factor leukaemias (good-risk cytogenetics) are known to be highly sensitive to high dose Ara-C. The presence of an *NPM1* mutation was, however, associated with more quickly cycling cells. This accords with the fact that *NPM1* mutations are associated with a higher presenting WCC (Dohner *et al*, 2005; Gale *et al*, 2008). Unexpectedly, a similar relationship was not observed for $FLT3^{ITD}$, which is counterintuitive in that an activating mutation in a growth factor receptor would be expected to induce more rapid proliferation. It is possible that the major impact of a $FLT3^{ITD}$ is on the more primitive leukaemic progenitor cells and not the bulk leukaemic cells, although recent studies have suggested that the more primitive AML progenitors in $FLT3^{ITD}$ AML have a lower proliferative fraction than the same phenotypic subpopulation in other forms of AML (Behbehani *et al*, 2015). It is possible that other mutations may have an impact on the

cell cycle profile of the leukaemic blast cells, but a much larger cohort of patients with trephine biopsies carried out at diagnosis will be required to ascertain this.

In spite of the general sensitivity of AML cells to cell cycle phase-specific agents, it was noteworthy that the proportion of actively cycling cells did not significantly correlate with the CR rate. Nevertheless, the speed of cell cycling as assessed by the geminin/MIB-1 ratio was inversely related to chemoresistance to Ara-C plus anthracycline combination chemotherapy, and although it was most significant after the initial cycle of intensive induction therapy, it was still apparent when best response at any time was considered. The impact on CR rate was much less apparent than on chemoresistance, and this was largely due to the higher treatment-related mortality in those patients with the most rapidly proliferative disease. The latter have, on average, a much higher presenting WCC, which is a known major risk factor for early death (Walter *et al*, 2011; Othus *et al*, 2014).

The rate of relapse was significantly higher in the quartile with the slowest cell cycling time than in the other quartiles and this was not obviously influenced by the use of consolidation with allogeneic transplantation in first CR. The higher rate of relapse in Q4 did not translate into a significantly lower OS, which was again likely to be due in part to the confounding effect of the increased treatment-related deaths in the cohort with the fastest cell cycling time. This finding has interesting biological implications. Although the attainment of CR is a reflection of the properties of the bulk of the leukaemic cells, relapse is thought to originate from the leukaemic stem cells (LSC), which have a more primitive phenotype and are thought to proliferate more slowly than the bulk of cells (Saito *et al*, 2010). Nonetheless, our data suggests that proliferation

kinetics in the bulk population may be a surrogate for the chemosensitivity of the LSCs, probably indicating that in those cases where the bulk cells are proliferating more quickly so too are the LSCs, albeit at a lower level than in the bulk population, and vice-versa. This may be testable by multi-parameter immunophenotypic analysis of AML progenitor cell populations using flow cytometry or mass cytometry on single cell suspensions made from BMTs, but the small size of BMT samples generally available for study after removal of the portion required for diagnostic purposes will make this challenging. Being able to predict the likelihood of chemo-resistance to standard therapy, and to ascertain which patients are most at risk of relapse, may be of clinical value by suggesting which patients might benefit from the early introduction of non-cell cycle specific agents and high dose therapy consolidation.

Acknowledgements

This work was supported by Cancer Research UK and Bloodwise (formerly Leukaemia and Lymphoma Research), and was undertaken at UCL, which receives a proportion of funding from the Department of Health's NIHR Biomedical Research Centre's funding scheme.

Author contributions

R.S.S., A.K., K.S., G.H.W. and D.C.L. designed the study; R.S.S. and M.L. performed experimental work; R.S.S., M.G. and D.C.L. analysed data; R.E.G. provided samples; R.S.S., A.K., R.E.G. and D.C.L. wrote the manuscript, which was reviewed by all authors.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Table 1. Characteristics of the total cohort of 181 patients

Parameter	
Median age (range), years	55 (13-86)
Sex	55% male; 45% female
Disease status	
De novo AML	150 (83%)
Secondary AML	31 (17%)
FAB type	
M0	12 (7%)
M1	40 (22%)
M2	64 (35%)
M3	15 (8%)
M4	17 (9%)
M5	25 (14%)
M6	2 (1%)
M7	1 (<1%)
Unknown	5 (3%)
Cytogenetic risk group	
Favourable	30 (17%)
Intermediate	92 (51%)
Adverse	29 (16%)
Unknown	30 (17%)
Genotype, all patients	
NPM1^{MUT}	49/159 (31%)
FLT3^{ITD}	35/161 (22%)
Genotype, intermediate-risk group	
NPM1^{MUT}	42/85 (49%)
FLT3^{ITD}	25/85 (29%)

Table 2. Immunohistochemistry results in the total cohort of 181 patients and specific subgroups

Antigen	Total cohort			APL and non-APL			Non-APL cytogenetic risk groups			
	Median LI	Interquartile range	Overall range	APL	Non-APL	*P*	Favour-able	Inter-mediate	Adverse	*P*
Mcm2	81%	69-92%	40.5-100%	68%	82%	0.03	80%	85%	78%	0.06
MIB-1	32%	23-49%	0.1-81.7%	16%	34%	0.0005	32%	34%	27%	0.1
Geminin	17%	10-24%	0-66.1%	6%	18%	<0.0001	18%	19%	17%	0.07
MIB-1/Mcm2 ratio	0.42	0.29-0.60	0-0.99	0.27	0.45	0.002	0.42	0.46	0.37	0.3
Geminin/MIB-1 ratio	0.53	0.35-0.79	0-0.97	0.33	0.52	0.005	0.54	0.5	0.53	0.4
Cleaved caspase	2.7%	1.1-5.2%	0-17.2%	0.9%	2.9%	0.003	1.9%	2.8%	3.4%	0.03

Table 3. Patient characteristics and outcome according to quartiles of the geminin/MIB-1 ratio in non-APL patients receiving intensive induction therapy

	Total cohort (n=140)	Q1 (n=35)	Q2 (n=35)	Q3 (n=35)	Q4 (n=35)	*P*
Geminin/MIB-1						
Mean		0.90	0.64	0.43	0.27	
Median		0.81	0.61	0.43	0.30	
Range		0.82-0.97	0.52-0.81	0.37-0.51	0.04-0.36	
Cell cycle parameters						
Geminin, median LI		26%	20%	18%	9%	0.0001
MIB-1, median LI		29%	34%	41%	40%	0.01
MIB-1/Mcm2 ratio		0.35	0.42	0.54	0.52	0.001
Cleaved caspase, median LI		2.3%	3.0%	2.7%	2.3%	1.0
Median age, yrs		54	57	47	50	0.2
Median WCC, $x10^9/L$		56	8	12	15	0.0004
Cytogenetics, n (%)						
Favourable	13	3 (23%)	4 (31%)	4 (31%)	2 (15%)	
Intermediate	80	26 (33%)	16 (20%)	20 (25%)	18 (23%)	
Adverse	23	2 (9%)	7 (30%)	7 (30%)	7 (30%)	
Unknown	24	4 (17%)	8 (33%)	4 (17%)	8 (33%)	
Genotype						
NPM1^{MUT}	43/128 (34%)	17/30 (57%)	8/30 (27%)	15/33 (45%)	3/35 (9%)	0.001
FLT3^{ITD}	29/129 (22%)	9/30 (30%)	7/31 (23%)	8/33 (24%)	5/35 (14%)	0.2
Response to therapy						
Induction deaths		26%	11%	6%	6%	0.01
CR post 1 cycle		74%	69%	66%	54%	0.1
CR at any time		74%	71%	71%	71%	1.0
Alive but not in CR						
Post 1 cycle		0%	23%	30%	42%	0.0002
At any time		0%	19%	24%	24%	0.02
Allograft in first CR, n		8	8	6	7	
Long-term outcome						
CIR at 3 yrs		48%	44%	29%	73%	0.01
Median time to relapse, (days)		304	452	319	265.5	0.4
OS at 3 years		32%	46%	52%	34%	0.4

Figure Legends

Figure 1. Differences in the cell cycle and apoptosis parameters between APL and non-APL patients. LIs for (A) Mcm2 (non-quiescent cells, positive in G_1, S, G_2 and M but not G0), (B) MIB-1 (actively proliferating cells, positive in a proportion of cells in G_1, and cells in S, G_2 and M but not G_0), (C) geminin (cycling cells that have progressed beyond G_1, positive in S, G_2 and M but not G_1 and G_0), (D) MIB-1/Mcm2 ratio (proportion of non-quiescent cells that are proliferating), (E) geminin/MIB-1 ratio (indicator of the average speed of the cell cycle), (F) cleaved caspase-3 (apoptotic cells).

Figure 2. Comparison between intermediate-risk *NPM1*^{WT} and *NPM1*^{MUT} patients according to (A) geminin expression and (B) geminin/MIB-1 ratio.

Figure 3. Outcome stratified according to the geminin/MIB-1 ratios. (A) CIR according to quartiles of the ratio, Q1 = highest, Q4 = lowest. (B) OS from the start of the second cycle of intensive chemotherapy, stratified according to the geminin/MIB-1 ratio at diagnosis and comparing the highest 3 quartiles (Q1-3) with the lowest quartile (Q4).

Figure 1

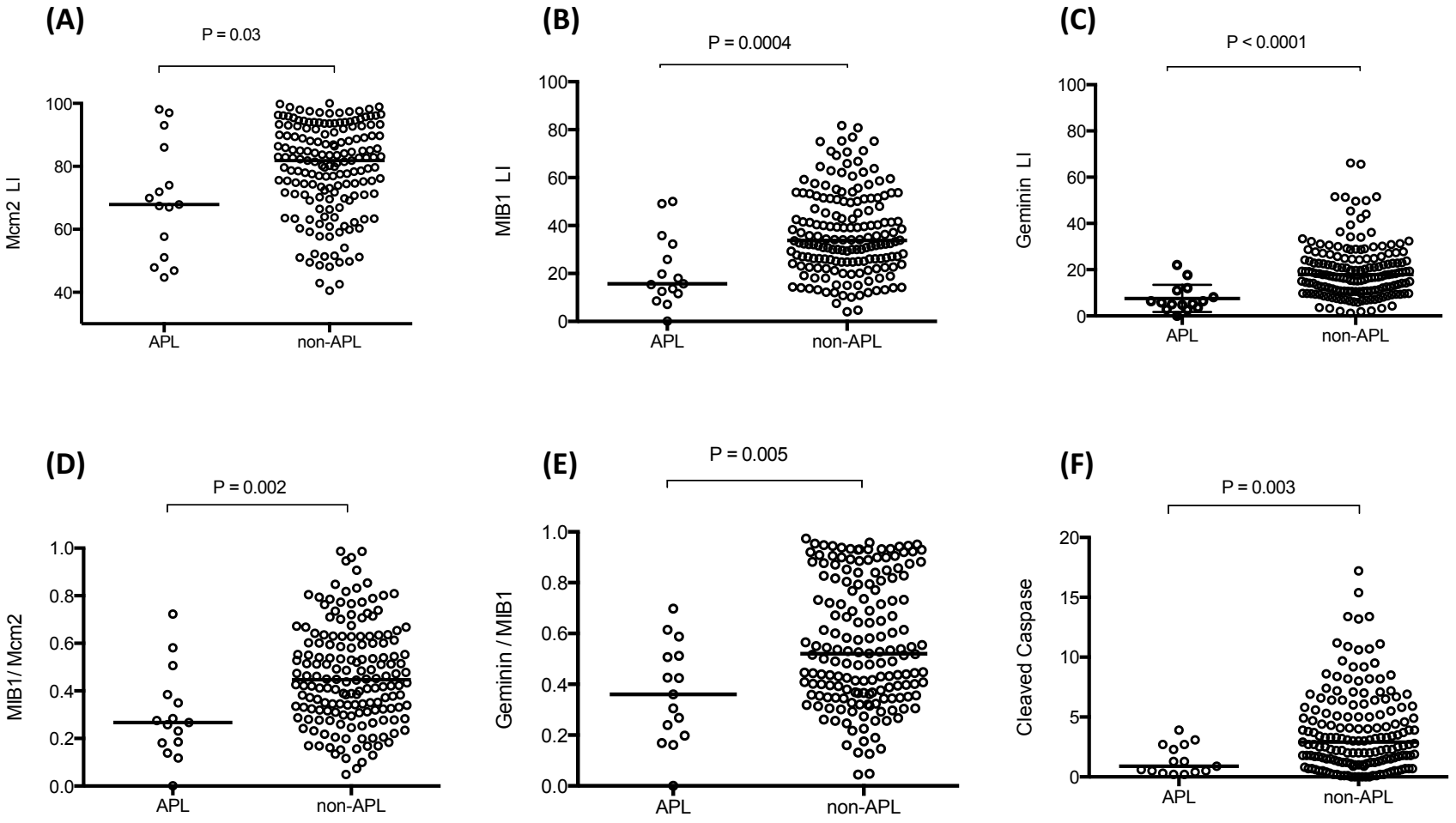

(A)
P = 0.03
Mcm2 LI
100
80
60
40
APL
non-APL
(B)
P = 0.0004
MIB1 LI
100
80
60
40
20
0
APL
non-APL
(C)
P < 0.0001
Geminin LI
100
80
60
40
20
0
APL
non-APL
(D)
P = 0.002
MIB1/ Mcm2
1.0
0.8
0.6
0.4
0.2
0.0
APL
non-APL
(E)
P = 0.005
Geminin / MIB1
1.0
0.8
0.6
0.4
0.2
0.0
APL
non-APL
(F)
P = 0.003
Cleaved Caspase
20
15
10
5
0
APL
non-APL

Figure 2.

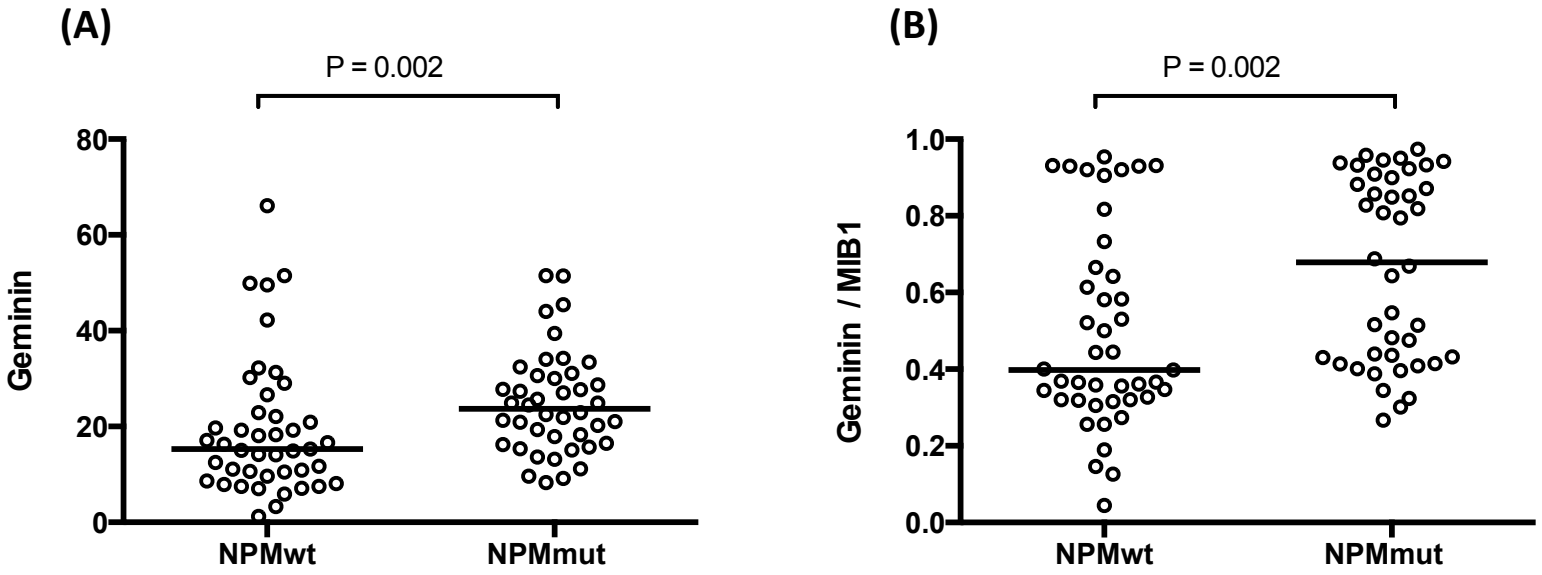
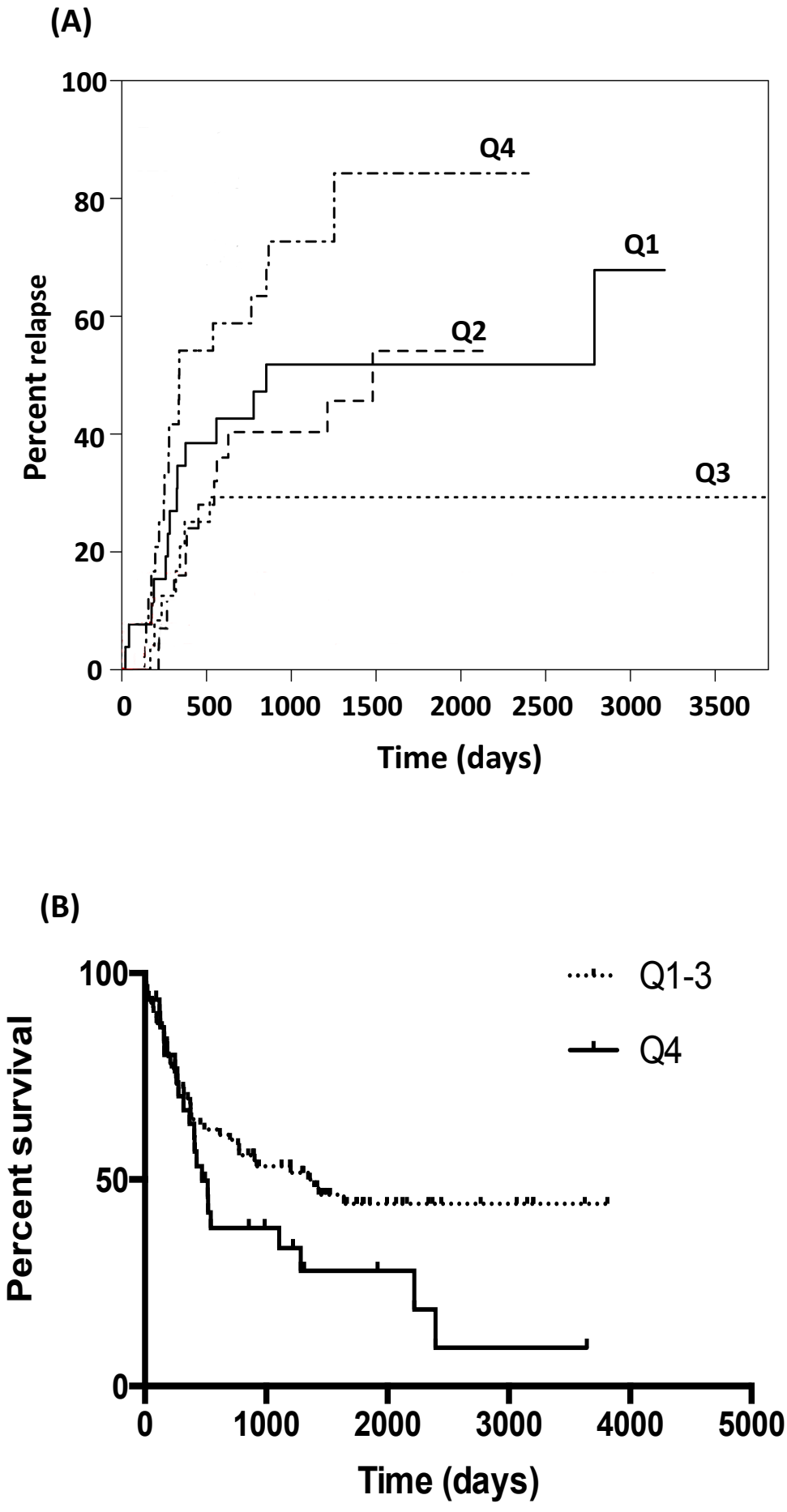

(A)
P = 0.002
Geminin
80
60
40
20
0
NPMwt
NPMmut
(B)
P = 0.002
Geminin / MIB1
1.0
0.8
0.6
0.4
0.2
0.0
NPMwt
NPMmut

Figure 3.


(A)
100
80
60
40
20
0
Percent relapse
Q4
Q1
Q2
Q3
0
500
1000
1500
2000
2500
3000
3500
Time (days)
(B)
100
50
0
Percent survival
Q1-3
Q4
0
1000
2000
3000
4000
5000
Time (days)

SUPPLEMENTARY MATERIALS

Automated Analysis of Cell Cycle status determined by immunocytochemistry

In order to validate an automated counting system, the proportion of blast cells positive for each of the primary antibodies, the LI, was first determined manually on biopsies from 30 patients. Digital files of all biopsy sections were created using a Leica SCN400 scanner. At least three separate representative areas from different sections of the biopsy were selected and JPEG files created and printed. The LI was then determined after counting a minimum of 1000 cells, with stromal cells, megakaryocytes, lymphocytes and late erythroid cells excluded by morphological criteria. The digital images for each primary antibody for these 30 biopsies were then evaluated using the Ariol® automated scanning microscope and image analysis system (Turashvili *et al*, 2009). This system had been trained to differentiate and enumerate positive and negative cells. It had also been trained to exclude stromal cells, megakaryocytes, lymphocytes, and late erythroid cells according to color, size and, shape criteria.

LIs determined by the manual method and automated system showed good correlation; for Mcm2, r= 0.97 (P<0.0001); for geminin, r = 0.98 (P<0.0001); for MIB-1, r = 0.99 (P<0.0001); for cleaved caspase 3, r=0.99 (P<0.0001). As r measures the strength of a relation between two variables, not the agreement between them, and two methods designed to measure the same variable are related, a Bland-Altman analysis was performed to assess agreement between the two methods. The results of the Bland-Altman analysis showed good agreements and minimal apparent bias (Supplementary Table S1). Based on these results, this automated system was then used to analyse the remaining biopsies.

Response Definitions

Complete Remission (CR) was defined as a normocellular BM containing < 5% blasts and showing evidence of normal maturation of other BM elements and with a PB neutrophil count of ≥1 x 10^9/L and a platelet count of ≥100 x 10^9/L. One patient achieved BM remission with incomplete count recovery but was counted as achieving CR for the purposes of analysis. Remission failures were classified as resistant disease (RD) or induction death (ID), i.e., related to treatment or hypoplasia. Where evaluation was not possible, deaths within 30 days of entry were classified as ID and deaths later than 30 days after entry as RD. Overall Survival (OS) was defined as the time from diagnosis to death. Cumulative Incidence of Relapse (CIR)

was defined as the probability of relapse at a given time from the point of achieving first remission. Data was censored at the point of three mutually exclusive events: alive in remission at last follow-up having never relapsed, the date of relapse, the date of death in remission from any cause other than disease relapse i.e. non-relapse mortality (NRM).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. Correlations were calculated using Spearman's rank correlation coefficient and the Bland Altman test. Differences between two cohorts were calculated using the Mann-Whitney-U test, and between more than two cohorts using the Kruskal-Wallis test. Survival data was calculated using Kaplan-Meier estimates. In addition, R version 3.2.1 was used for calculating CIR using a competing risks analysis with relapse and non-relapse mortality as competing events from the time of first remission (Scrucca *et al*, 2007). Nominal and binary logistic regression multivariate analysis was performed using SPSS version 22. All tests were two-tailed and a *P* value of <0.05 was considered significant.

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Supplementary Table S1. Overall results of the Bland-Altman analysis to assess agreement between the manual and automated counting systems.

Antigen	Bias	Standard Deviation of bias	95% limits of agreement
MCM2	-0.37	2.39	-5.05 – 4.30
MIB-1	0.27	2.14	-3.92 – 4.46
Geminin	-0.13	1.80	-3.66 – 3.40
Cleaved Caspase	-0.09	0.45	-0.98 – 0.80

A negative number shows bias towards a higher number in the automated LI, and a positive number shows bias towards a higher number in the manual LI.