Differences in proliferative capacity of primary human acute myelogenous leukaemia cells are associated with altered gene expression profiles and can be used for subclassification of patients

H. Reikvam*,†,‡, A. M. Øyan§,¶, K. H. Kalland§,¶, R. Hovland**, K. J. Hatfield* and Ø. Bruserud*,†

*Division for Hematology, Institute of Clinical Science, University of Bergen, Bergen, Norway, †Division for Hematology, Department of Medicine, Haukeland University Hospital, Bergen, Norway, ‡Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway, §Department of Microbiology, Haukeland University Hospital, Bergen, Norway, ¶The Gade Institute, University of Bergen, Bergen, Norway, and **Centre for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway

Received 26 April 2013; revision accepted 12 June 2013

Abstract

Objectives: Proliferative capacity of acute myelogenous leukaemia (AML) blasts is important for leukaemogenesis, and we have investigated whether proliferative capacity of primary human AML cells could be used for subclassification of patients.

Materials and methods: *In vitro* proliferative capacity of AML cells derived from two independent groups was investigated. Cells were cultured under highly standardized conditions and proliferation assayed by ³H-thymidine incorporation after seven days culture. Patients were subclassified by clustering models, and gene expression profile was examined by microarray analyses.

Results: Based on proliferative capacity of the AML cells, three different patient clusters were identified: (i) autocrine proliferation that was increased by exogenous cytokines; (ii) detectable proliferation only in presence of exogenous cytokines; and (iii) low or undetectable proliferation even in presence of exogenous cytokines. Patients with highest proliferative capacity cells had no favourable prognostic impact by NPM-1 mutation. Analysis of gene expression profiles showed that the most proliferative cells generally had altered expression of genes involved in regulation of transcription/RNA functions, whereas patients with high proliferative capacity and internal tandem

duplications (ITDs) in the *FLT3* cytokine receptor gene had altered expression of several molecules involved in cytoplasmic signal transduction.

Conclusions: *In vitro* proliferative capacity of primary human AML cells was considerably variable between patients and could be used to identify biologically distinct patient subsets.

Introduction

Acute myelogenous leukaemia (AML) is a heterogeneous malignancy arising from transformed haematopoietic stem- and progenitor cells of the bone marrow (1,2). Bone marrow infiltration of leukaemic cells with suppression of normal haematopoiesis is a hallmark of the disease. AML cell proliferation can be stimulated by several haematopoietic growth factors as well as by other cytokines. *In vitro* studies have also demonstrated that for some patients, AML cells have spontaneous proliferation due to autocrine growth factor release by leukaemic blasts (3,4), whereas for other patients, leukaemic cells only proliferate in the presence of exogenous growth factors (3–6). Cytokines may in addition, induce AML blast differentiation (7). Abnormalities of cytokine- and growth factor-induced intracellular signalling are characteristics of myeloid malignancies (8), and gene expression profiles have emerged as powerful means to further classify AML patients (9,10). The aims of the present study were to investigate effects of various cytokines on AML blast proliferation, to use this proliferative responsiveness for subclassification of patients, and to compare global gene expression profiles for patient subsets with different proliferative capacity.

Correspondence: Ø. Bruserud, Medical Department, Haukeland University Hospital, N-5021 Bergen, Norway. Tel.: +55 97 50 00; Fax: +55 97 29 50; E-mail: oystein.bruserud@helse-bergen.no

Materials and methods

AML cells

This study was approved by the local Ethics Committee (Regional Ethics Committee III, University of Bergen, Norway) and samples were collected after written informed consent. We included patients with AML without signs of acute promyelocytic leukaemia (APL) based on clinical, morphological and genetic criteria. AML cells were isolated from peripheral blood of patients with high blood blast counts ($>7 \times 10^9/l$) by density gradient separation (11). Isolated cells included at least 95% leukaemia blasts (7,11,12). Two independent and consecutive patient groups were examined; they are referred to as group I and group II and their clinical and biological characteristics are summarized in Table 1. The two cohorts represent two different groups of consecutive patients that were analysed at two different time periods in two independent

Table 1. Clinical and biological characteristics of the included AML patients. The Table shows the clinical and biological characteristics for the two patient groups (absolute numbers). The classification of cytogenetic abnormalities as favourable, unfavourable or intermediate is based on the recent classification system (30).

Patient characteristics	Group I	Group II
Demographic data and disease history		
Gender (numbers)		
Male/female	20/20	27/26
Age (median, range)	64 (29–84)	61 (26–82)
History		
<i>De novo</i>	35	33
Secondary	2	13
Relapse	3	4
AML cell differentiation		
FAB classification		
M0-1	14	15
M2	7	12
M4-5	19	22
M6-M7	0	1
CD34 expression		
0–20%	13	20
20–50%	2	3
50–100%	22	18
Genetic abnormalities		
Cytogenetics		
Favourable	3	3
Unfavourable	2	6
Intermediate	6	6
Normal	22	17
<i>FLT3</i>		
ITD	22	19
TKD	2	4
Wt	16	26
<i>NPM-1</i>		
Mutated	13	9
Wt	26	31

sets of experiments, but patients were included according to the same criteria for both groups.

Proliferation assay

AML cells (5×10^4 cells in 150 μ l medium per well) were cultured in flat-bottomed 96-well plates (Nucleon™; Nunc, Roskilde, Denmark) in serum-free Stem Span SFEM™ medium with gentamicin 100 μ g/ml (Stem Cell Technologies, Vancouver, BC, Canada). Cells were cultured in medium alone and in presences of 20 ng/ml of either stem-cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, FLT3-ligand (FLT3-L), interleukin 3 (IL-3) or IL-1 β (all purchased from PeproTech Ltd, Rocky Hill, NJ, USA). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for seven days before proliferation was assayed by ³H-thymidine incorporation (13,14).

RNA preparation, labelling and microarray hybridization

Patient group I. Microarray experiments on AML blasts *in vitro* were performed using the Illumina iScan Reader, based on fluorescence detection of biotin-labelled cRNA. For each sample, 300 ng of total RNA was reversely transcribed, amplified and biotin-16-UTP-labelled by using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, USA). Amount and quality of biotin-labelled cRNAs were controlled both by Nano-Drop spectrophotometer and by Agilent 2100 Bioanalyzer. Thereafter, 750 ng of biotin-labelled cRNA was hybridized to HumanHT-12 V4 Expression BeadChip according to the manufacturer's instructions. HumanHT-12 V4 BeadChip targets 47 231 probes were derived primarily from genes of the NCBI RefSeq database (Release 38).

Patient group II. Agilent Human Whole Genome Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA, USA) was used to analyse 16 AML cell samples with FLT3-ITD mutations. RNA isolation, cRNA synthesis and hybridization were then performed as previously described (15). These oligonucleotide microarrays were scanned (Agilent Scanner G2505B) and features automatically extracted using Agilent Feature Extraction v.7.5.

Bioinformatic analyses of microarray data

Illumina iScan Reader data were investigated in GenomeStudio and J-Express 2012 for quality control measures (16). Before being compiled into an expression profile data matrix, all arrays within the experiment were

quantile normalized to be comparable. Analyses were performed using the J-Express 2012 analysis suite (MolMine AS, Bergen, Norway) (17). Unsupervised hierarchical clustering was performed with Euclidian correlation as distance measure and complete linkage. Agilent data were imported and analysed in J-Express software (Molmine, <http://www.molmine.com>). Mean spot signals were used as intensity measure and expression data normalized by using median over entire array. Analysis of variance (ANOVA) was used to identify differentially expressed genes in patients with *FLT3*-mutations.

Results

Cytokine-dependent AML cell proliferation showed wide variation between patients

Cytokine-dependent proliferation was investigated by using ^3H -thymidine incorporation assay for both patient groups, and amounts of DNA-incorporated ^3H -thymidine were then determined as counts per minute (cpm) (11). Overall results are summarized in Table 2. Only one subset of patients had spontaneous AML cell proliferation in medium alone, but all cytokines caused a statistically significant increase of AML cell proliferation ($P < 0.001$ for all cytokines, Wilcoxon's signed rank test) comparing overall results. However, for one subset of patients, leukaemia cells had no or only minimal proliferation even in the presence of exogenous cytokines.

Cytokine-dependent proliferation of primary human AML cells can be used to subclassify patients

Values for proliferation were log10 converted before unsupervised hierarchical clustering analysis was performed (Euclidean as distance metrics and complete linkage). On the basis of this analysis, we identified

Table 2. Cytokine-dependent AML cell proliferation. AML cells were cultured in the presence of 20 ng/ml of each cytokine (right column) for seven days before proliferation was assayed in the ^3H -thymidine incorporation assay. Results are given as median cpm $\pm$ standard error (SER) for each cytokine and for each of the two independent groups described in Table 1.

Cytokine added	Group I (n = 40)	Group II (n = 53)
FLT3-L	6992 ($\pm$ 2982)	2326 ($\pm$ 1964)
GM-CSF	6336 ($\pm$ 2941)	4550 ($\pm$ 3247)
SCF	5884 ($\pm$ 1288)	3754 ($\pm$ 3247)
IL-3	8433 ($\pm$ 2356)	5760 ($\pm$ 2880)
IL-1	3800 ($\pm$ 1057)	3079 ($\pm$ 2736)
G-CSF	6990 ($\pm$ 2571)	6483 ($\pm$ 3829)
M-CSF	5119 ($\pm$ 1869)	953 ($\pm$ 1487)
Medium alone	2767 ($\pm$ 1507)	430 ($\pm$ 726)

three distinct patient clusters that could be seen in both patient groups (Figs 1 and 2): (i) one cluster showed high proliferation both in medium alone and in presence of exogenous cytokines (blue, Figs 1 and 2); (ii) a second cluster showed high proliferation only in the presence of exogenous cytokines (green); and (iii) the final cluster had absent or low proliferation both with and without exogenous cytokines (brown). The cluster model also allowed us to identify different cytokine clusters. Even though cytokine clustering varied between the two patient groups, GM-CSF and IL-3 clustered close to each other and FLT-3 and SCF clustered together for both groups. Observations indicate that proliferative responsiveness showed similarities between cytokines that cluster together or close to each other.

Proliferative AML cell profile was associated with biological characteristics – no patients with high proliferative capacity had favourable prognostic impact of NPM-1 mutations

As proliferation patterns of AML blasts have been associated with prognosis for patients who receive intensive chemotherapy (3), we investigated whether any associations could be detected between proliferation and clinical or biological characteristics of the patients. No significant associations could be detected for FAB classification, cytogenetic abnormalities or *FLT3* mutations. However, favourable impact of *NPM-1* mutations could not be detected for any patients with both high spontaneous and cytokine-dependent proliferation (Figs 1 and 2, blue); these patients did not have *NPM-1* mutations (group II) or *NPM-1* mutations occurred either together with *FLT3*-internal tandem duplications (*FLT3-ITD*) or high-risk cytogenetic abnormalities (group I, 4 patients). Furthermore, generally higher CD34 expression corresponding to at least 50% positive cells, by standard flow cytometric analyses, was seen for patient cluster with high spontaneous as well as cytokine-dependent proliferation, but this difference reached statistical significance only for group II patients (Chi-square test, $P = 0.014$).

AML cells derived from patients with leukaemia relapse were heterogeneous with regard to proliferative capacity but early relapse seemed to be associated with high proliferative responses

Our study included 7 patients with AML relapse after intensive chemotherapy (Figs 1 and 2); duration of first remission varied between 4 and 42 months and only two of these patients had a duration of first remission longer than 12 months (16 and 37 months, respectively). The possibility of achieving a second haematological

Figure 1. Unsupervised hierarchical clustering analyses based on proliferation of different cytokines among 40 AML patients. Primary human AML cells derived from 40 consecutive patients (Group I, Table 1) were cultured for 7 days before proliferation was assayed using 3H-thymidine DNA incorporation. Values were log10 transformed and used in unsupervised hierarchical cluster modeling with Euclidian correlation distance measure with complete linkage. Patients could be divided into three distinct clusters: (i) high proliferation both in medium alone (autocrine proliferation) and in the presence of exogenous cytokines (indicated by a blue bar in the figure), (ii) high proliferation only in the presence of exogenous cytokines, but low/undetectable proliferation in medium alone (green bar), and (iii) low/undetectable proliferation both in medium alone and in the presence of exogenous cytokines (brown bar). Patient characteristics are indicated in the right column.

remission is higher if patients have a long duration of their first remission, and 1 year is often used as the cut-off point when this prognostic parameter is analysed in clinical studies (18,19). This was therefore used in further analysis of our relapsed patients. Both patients with long-lasting remission clustered in the subset showing low/absent spontaneous and cytokine-dependent AML cell proliferation (Figs 1 and 2; brown), whereas the other 5 patients with short first remission clustered among patients, showing high spontaneous and/or cytokine-dependent proliferation (blue or green). This difference in subset distribution is statistically significant (Chi-square test, $P = 0.0082$). Finally, one of the patients with 9 months duration first remission was tested both at first time diagnosis and at time of relapse; the first time, he clustered among patients with low proliferative capacity, whereas by time of relapse, he was included in patient subset showing high cytokine-dependent proliferation.

High cytokine-dependent proliferation was associated with distinct gene expression signature

To further explore differences between patients with high and low AML cell proliferation, we analysed

global gene expression profile for 33 patients in group I; 13 of these had high spontaneous and cytokine-dependent proliferation. Class comparison analysis using ANOVA between the 13 patients with high proliferation and 20 patients belonging to the other clusters (Fig. 1) showed that 140 genes differed significantly between these two groups with a P -value < 0.005 ; 65 of these genes were upregulated, whereas 75 were downregulated in high-proliferation patients (Table S1). We then performed new hierarchical clustering analysis based only on these 140 genes; all high-proliferation patients then formed a separate cluster without any overlap with other patients (Fig. 3). Thus, the AML patient subset having leukaemic cells with high *in vitro* proliferative responses could also be identified by analysis of expression profile of a limited number of genes.

Global gene expression profiles of primary AML cells with high and low proliferative capacity – two subsets showing major differences in expression of genes involved in regulation of transcription

Gene ontology (GO) mapping is used to analyse how differences in gene expression profiles affect molecular

Figure 2. Unsupervised hierarchical clustering analysis based on proliferation of different cytokines among 54 AML patients. Blasts derived from 54 consecutive AML patients (Group II, Table 1) were cultured for seven days before proliferation was assayed using the 3H-thymidine proliferation assay. Values were log₁₀ transformed and used in unsupervised hierarchical cluster modeling with Euclidian correlation distance measure with complete linkage. Patient could be divided into three distinct clusters: (i) high proliferation both in medium alone and in the presence of exogenous cytokines (indicated by a blue bar in the figure), (ii) high proliferation only in the presence of exogenous cytokines but undetectable/low proliferation in medium alone (green bar), (iii) low/undetectable proliferation both in medium alone and in the presence of exogenous cytokines (brown bar).

functions, cell compartments or biological processes. To explore differentially expressed GO-terms that are important for proliferative capacity of primary human AML cells, we selected those genes from the above study of Group I patients that were differentially expressed with a P -value <0.05 by ANOVA. We then identified 1694 genes that fulfilled these criteria; 849 were upregulated and 845 were downregulated for high-proliferative cluster compared to other patients. We next used these genes in an overrepresentation analysis to identify those GO-terms that had >30 genes in the annotation and, in addition, differed significantly between AML patients with high and low proliferative capacity. Fourteen different annotations were detected and these results are present in Table 3. A majority of the differentially expressed genes were downregulated in high-proliferation patients (Chi-square test, $p < 0.0001$), and this was true for all 14 annotations. It can be seen that (i) 6 of these GO terms describe functions of DNA/RNA, (ii) two additional terms describe macromolecular metabolic processes, whereas (iii) 5 terms describe cationic binding/function and in particular zinc ion binding. Zinc is important for

regulation of gene expression (20–23). Thus, difference in proliferative capacity between AML patients seems to be determined mainly by differences in regulation of gene expression, whereas differences in expression of growth factors/growth factor receptors/downstream intracellular mediators seem to be less important. The complete list of genes that belong to at least one of these 13 annotations in Table 3 is given in Table S2.

FLT3-ITD⁺ patients with high and low proliferative capacity of their AML cells differed in gene expression profiles and ITD length

For group II, global gene expression profiles were available for 16 consecutive patients with *FLT3-ITD* (10 with high and 6 with low proliferative capacity). *FLT3-ITD⁺* patients have a genetic abnormality in the same region of the gene and these mutations are associated with adverse prognosis and distinct gene expression profiles in leukaemic cells (1,9). However, despite these similarities, these patients are heterogeneous with regard to molecular structure of the ITD (24,25), prognosis (there are long-time

Figure 3. Hierarchical clustering based on differentially expressed genes. Gene expression data were obtained for 33 patients belonging to the group I cohort; 13 of the 33 patients belonged to the high-proliferation cluster (indicated by the blue bar in Fig. 1) and ANOVA analysis was used to compare these 13 patients with the other patients. A total of 140 genes were then found to differ significantly between the two groups (i.e. showing $P < 0.005$); 65 genes then showed higher and 75 genes showed lower expression in the high-proliferation patients. These 140 selected genes were used to perform a new hierarchical clustering analysis, Euclidean correlation distance measure with complete linkage. All high-proliferation patients clustered separately and are identified by the blue bar at the top of the figure and the other patients are indicated by the purple colour bar. Up-regulated genes are marked with red colour in the heat map and down-regulated genes with green colour.

Table 3. Gene annotations that were significantly altered in patients with high proliferative (autocrine- and cytokine-dependent) capacity compared with patients with low capacity patients (patient group I). The expression of 1694 genes differed significantly (ANOVA, $P < 0.05$) when comparing patients with high and low proliferative capacity, and an overrepresentation analysis was performed based on these genes. We then identified those GO-terms that differed significantly ($P < 0.05$) between the two groups and included at least 30 genes in the annotation; the 14 annotations that fulfilled these criteria are presented in the Table. A majority of the 1695 genes showed a lower expression in patients with high proliferative capacity compared with the low-capacity patients, and this difference was seen for all 14 annotations (Chi-squared test $P < 0.0001$, for all presented data).

Significantly differing GO term	Total number of genes included in the GO term	Number of downregulated genes	Number of upregulated genes
Nucleic acid binding	3364	142	60
Gene expression	2979	131	43
Regulation of RNA and metabolic processes	1542	102	42
DNA binding	2369	108	44
Regulation of RNA biosynthetic processes	1542	80	28
Transcription, DNA dependent	2256	115	38
Ion binding	4014	149	81
Cation binding	4005	148	81
Metal ion binding	1478	62	35
Transition metal ion binding	2250	103	36
Zinc ion binding	2014	95	31
Regulation of macromolecule metabolic processes	2262	101	49
Macromolecule metabolic processes	3020	134	43
Intracellular organelles	7979	286	140

survival also among these patients) (25,26) and proliferative capacity (Figs 1 and 2); *FLT3-ITD*s cause constitutive activation of this surface-expressed cytokine receptor (11). Comparison of global gene expression profiles for *FLT3-ITD*⁺ patients with high and low proliferative capacity showed that these two patient groups differed (especially in upregulated genes) in their expression of genes important for communication between cells and intracellular signal transduction. This is illustrated by genes presented in Table 4; it can be seen from their GO annotations that a major part of the differentially expressed genes with fold change >2 and ANOVA *P*-values <0.004 were important for cytoplasmic signal transduction (for example, the ubiquitin-proteasome pathway, JAK signalling or signalling downstream to surface receptors). Thus, the *FLT3-ITD*⁺ patients differ from other AML patients, in that variations in signal transduction seem more important than variation in gene expression regulation for proliferative capacity for them.

Association between length of *FLT3-ITD* and prognosis in AML has previously been described (25,27). We therefore investigated the association between size of the ITD and proliferation for group II patients (Fig. 2). Data regarding size of the ITD were available for 17 patients, five with high spontaneous proliferation (blue bars, Fig. 2) and 12 patients with low proliferation rates. *FLT3-ITD* patients with high spontaneous prolifer-

ation had significantly longer ITDs (mean 100 bp, range 66–153 bp) compared to ITD patients with low or undetectable spontaneous proliferation (mean 51 bp, range 24–90) (*P* = 0.0392, Mann–Whitney *U*-test). *FLT3-ITD*: *FLT3*-wt ratio did not differ between the two groups (data not shown).

Discussion

In vitro proliferative capacity of primary human AML cells has wide variation between patients (3,7). In our present study, we used a highly standardized experimental model (28,29) to analyse proliferative capacity of non-APL AML cells for two independent and unselected/consecutive patient groups. Based on ability of their AML cells to undergo spontaneous or autocrine proliferation and responsiveness of leukaemic cells to a limited number of haematopoietic growth factors, patients could be classified into three major subsets: (i) cells with both autocrine proliferation and responsiveness to a wide range of growth factors; (ii) no autocrine proliferation, but generally strong proliferative responses to exogenous growth factors; and (iii) no autocrine proliferation and no or only weak responsiveness to all or most growth factors.

Proliferative capacity of AML cells showed no (for example, cytogenetic abnormalities, *FLT3* mutations) or

Table 4. Differentially expressed genes when comparing *FLT3-ITD*⁺ patients (group II) with AML cells showing high (autocrine and cytokine-dependent) and low proliferative capacity. We identified 17 genes that were differently expressed (at least 2-fold altered and with *P* < 0.005); 10 GENES were upregulated in the high-proliferation group and seven were downregulated compared with the low-proliferation patients.

Upregulated genes in patients with high proliferative capacity			
Gene	Complete name	Fold change	GO-annotations
GPR27	G protein-coupled receptor 27	7.7	G-protein coupled receptor signalling pathway
SORT1	Sortilin 1	3.9	G-protein coupled receptor signalling pathway
HSPA1A	HSP 70 kDa protein 1A	3.4	Ubiquitin-proteasome pathway
RAB33A	Ras-related protein Rab-33A	3.4	Small GTPase-mediated signal transduction
EIF4E3	EIF4E3 eukaryotic translation initiation factor 4E	3.2	Cytokine-mediated signalling pathway
PHACTR1 by similarity	Phosphatase and actin regulator 1	2.8	Phosphatase and actin regulation
TTC39C	TTC39C tetratricopeptide repeat domain 39C	2.7	Ubiquitin-proteasome pathway
TOR4A	Torsin family 4, member A	2.2	Ubiquitin-proteasome pathway
CTNNB1	Beta-catenin	2.2	Transcription coactivator activity Wnt signalling
ENT1	Equilibrative nucleoside transporter 1	2.1	Nucleobase-containing compound metabolic process
Downregulated genes in patients with high proliferative capacity			
Gene	Full name	Fold change	GO-annotations
TSC22D1	TSC22 domain family, member 1	4.1	Regulation of apoptosis and cell proliferation
FLJ13197	Homo sapiens hypothetical protein FLJ13197	3.7	Not characterized
JAMIP2	Janus kinase and microtubule interacting protein 2	3.3	Jak and microtubule interacting protein
ADA	Adenosine deaminase	2.5	Negative regulation of migration
L1TD1 family	Member of the L1 transposable element family	2.4	ES cell-associated and transposase domain-containing protein
ZBED1 by similarity	Ac-like transposable element	2.3	Transposase activity
TJP3	Tight junction protein 3	2	Cell junction

only weak (CD34 expression) associations with other clinical or biological patient characteristics, the only exception being that favourable prognostic impact of *NPM-1* mutations (30) could not be detected in patients with highest proliferative capacity as *NPM-1* mutations then were either not present or combined with dominant adverse prognostic parameters. This may explain why previous clinical studies have shown association between adverse prognosis and autocrine *in vitro* proliferation of AML cells (3,4).

Our study included 7 patients with AML relapse, and AML cells at the time of relapse also differed in their proliferative capacity (Figs 1 and 2). The possibility of achieving second haematological remission is higher if patients have had long duration of their first remission, and 1 year is often used as a cut-off point, when this prognostic parameter is analysed in clinical studies (18,19). Here, patients with short (<1 year) duration of first remission, differed significantly from patients with long-lasting remission with regard to clustering, based on proliferative capacity; short duration was then associated with high proliferative capacity. Proliferative capacity is thus associated with prognosis also for patients with AML relapse. However, even though this difference reached statistical significance, data should be interpreted with great care as number of patients was small and verification in a larger study is definitely needed.

Patients with cells of different proliferative capacity also differed in their global gene expression profiles, and patient subset with high proliferative capacity could then be identified based on expression of a limited number of genes. This gene expression profile is more suitable for identification of this patient subset than analysis of *in vitro* proliferation, and such gene expression analyses can also be easily included in future clinical studies. Even though our subclassification is based on proliferative responsiveness to autocrine cytokine release or addition of exogenous cytokines, global gene expression analysis of unselected patients in patient group I did not show any major differences between patients from different clusters with regard to expression of cytokines, cytokine receptors or corresponding downstream mediators for cytoplasmic signal transduction. Rather, patients with high proliferative capacity differed with regard to expression of genes encoding regulators of DNA transcription/RNA synthesis. This is probably the explanation why patients either respond to a wide range of growth factors or show general low proliferative responsiveness; proliferative capacity is not determined by differences in expression of individual growth factor receptors or their downstream mediators, but rather by differences in common regulatory mechanisms that

integrate signalling from various cytokine-activated pathways at DNA/RNA level.

We performed a more detailed analysis of proliferative capacity of *FLT3*-ITD⁺ AML cells as (i) these patients have similar genetic abnormality of this cell surface receptor (11,30) although the prognostic impact of ITDs differs between patients and seems to depend on the ITD structure (25); and (ii) *FLT3*-L activates receptor signalling and functions as an AML growth factor, whereas *FLT3*-ITDs cause constitutive activation of the receptor (11). For these reasons, we analysed *FLT3*-ITD:*FLT3*-wt ratio, ITD length and global gene expression profiles for *FLT3*-ITD⁺ patients in one of our cohorts (group II, see Fig. 2). Thus, *FLT3*-ITDs are important for chemosensitivity and have adverse prognostic impact for patients receiving intensive chemotherapy (26,27). When comparing *FLT3*-ITD⁺ patients with high and low proliferative capacity, the two groups differed with regard to both ITD length and expression of molecules involved in intracellular signal transduction. Our hypothesis is that additional mechanisms at signal transduction levels (for example, the ubiquitin-proteasome pathway) are important for regulation of proliferative capacity in a minority of *FLT3*-ITD⁺ AML patients in addition to the general difference in regulation of DNA transcription/RNA synthesis (Table 3).

To conclude, AML patients can be subclassified based on proliferative capacity of their leukaemic cells. For most patients, this classification reflects differences in regulation of cell proliferation at the DNA/RNA level, but a minority of *FLT3*-ITD⁺ patients represents an exception where additional differences at the signal transduction level are also important (for example, proteasome function). Proliferation is important for disease progression, and our observations suggest that described differences between patients may translate into differences in susceptibility to new targeted therapies, such as proteasome inhibition, that may be most effective only for certain subsets of *FLT3*-ITD⁺ patients, whereas targeting gene expression through modulation of epigenetic regulation may be most effective in other patients.

Acknowledgments

The study received financial support from the Norwegian Cancer Society. Bioinformatic analysis was performed as cooperation project with the Norwegian Bioinformatics platform, funded by the FUGE programme, the Norwegian Research Council.

Conflicts of interest

The authors report no potential conflict of interest.

References

- Estey EH (2012) Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *Am. J. Hematol.* **87**, 89–99.
- Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC *et al.* (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell* **150**, 264–278.
- Lowenberg B, van Putten WL, Touw IP, Delwel R, Santini V (1993) Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. *N. Engl. J. Med.* **328**, 614–619.
- Hunter AE, Rogers SY, Roberts IA, Barrett AJ, Russell N (1993) Autonomous growth of blast cells is associated with reduced survival in acute myeloblastic leukemia. *Blood* **82**, 899–903.
- Bruserud O, Frostad S, Foss B (1999) In vitro culture of acute myelogenous leukemia blasts: a comparison of four different culture media. *J. Hematother.* **8**, 63–73.
- Bruserud O, Gjertsen BT, Foss B, Huang TS (2001) New strategies in the treatment of acute myelogenous leukemia (AML): in vitro culture of aml cells—the present use in experimental studies and the possible importance for future therapeutic approaches. *Stem Cells* **19**, 1–11.
- Bruserud O, Gjertsen BT (2000) New strategies for the treatment of acute myelogenous leukemia: differentiation induction—present use and future possibilities. *Stem Cells* **18**, 157–165.
- Van Etten RA (2007) Aberrant cytokine signaling in leukemia. *Oncogene* **26**, 6738–6749.
- Valk PJ, Verhaak RG, Beijnen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM *et al.* (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *N. Engl. J. Med.* **350**, 1617–1628.
- Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R *et al.* (2004) Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N. Engl. J. Med.* **350**, 1605–1616.
- Bruserud O, Hovland R, Wergeland L, Huang TS, Gjertsen BT (2003) Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of Flt3-ligand effects in AML cell populations with and without genetic Flt3 abnormalities. *Haematologica* **88**, 416–428.
- Bruserud O, Rynningen A, Wergeland L, Glenjen NI, Gjertsen BT (2004) Osteoblasts increase proliferation and release of pro-angiogenic interleukin 8 by native human acute myelogenous leukemia blasts. *Haematologica* **89**, 391–402.
- Stapnes C, Doskeland AP, Hatfield K, Ersvaer E, Rynningen A, Lorens JB *et al.* (2007) The proteasome inhibitors bortezomib and PR-171 have antiproliferative and proapoptotic effects on primary human acute myeloid leukaemia cells. *Br. J. Haematol.* **136**, 814–828.
- Reikvam H, Hatfield KJ, Ersvaer E, Hovland R, Skavland J, Gjertsen BT *et al.* (2012) Expression profile of heat shock proteins in acute myeloid leukaemia patients reveals a distinct signature strongly associated with FLT3 mutation status - consequences and potentials for pharmacological intervention. *Br. J. Haematol.* **156**, 468–480.
- Oyan AM, Anensen N, Bo TH, Stordrange L, Jonassen I, Bruserud O *et al.* (2009) Genes of cell-cell interactions, chemotherapy detoxification and apoptosis are induced during chemotherapy of acute myeloid leukemia. *BMC Cancer* **9**, 77.
- Stavrum AK, Petersen K, Jonassen I, Dysvik B (2008) Analysis of gene-expression data using J-Express. *Curr. Protoc. Bioinformatics* **7**, 7.3.
- Dysvik B, Jonassen I (2001) J-Express: exploring gene expression data using Java. *Bioinformatics* **17**, 369–370.
- Lazarus HM, Perez WS, Klein JP, Kollman C, Bate-Boyle B, Brede-son CN *et al.* (2006) Autotransplantation versus HLA-matched unre-lated donor transplantation for acute myeloid leukaemia: a retrospective analysis from the Center for International Blood and Marrow Transplant Research. *Br. J. Haematol.* **132**, 755–769.
- Ferrara F, Palmieri S, Mele G (2004) Prognostic factors and therapeutic options for relapsed or refractory acute myeloid leukemia. *Haematologica* **89**, 998–1008.
- Yang XJ, Ullah M (2007) MOZ and MORF, two large MYSTic HATs in normal and cancer stem cells. *Oncogene* **26**, 5408–5419.
- Morrison AA, Viney RL, Ladomery MR (2008) The post-transcriptional roles of WT1, a multifunctional zinc-finger protein. *Biochim. Biophys. Acta* **1785**, 55–62.
- Katsumoto T, Yoshida N, Kitabayashi I (2008) Roles of the histone acetyltransferase monocytic leukemia zinc finger protein in normal and malignant hematopoiesis. *Cancer Sci.* **99**, 1523–1527.
- Razin SV, Borunova VV, Maksimenko OG, Kantidze OL (2012) Cys2His2 zinc finger protein family: classification, functions, and major members. *Biochemistry* **77**, 217–226.
- Hatfield KJ, Hovland R, Oyan AM, Kalland KH, Rynningen A, Gjertsen BT *et al.* (2008) Release of angiopoietin-1 by primary human acute myelogenous leukemia cells is associated with mutations of nucleophosmin, increased by bone marrow stromal cells and possibly antagonized by high systemic angiopoietin-2 levels. *Leukemia* **22**, 287–293.
- Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, Du J *et al.* (2009) Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. *Blood* **114**, 2386–2392.
- Schlenk RF, Dohner K, Krauter J, Frohling S, Corbacioglu A, Bullinger L *et al.* (2008) Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N. Engl. J. Med.* **358**, 1909–1918.
- Stirewalt DL, Kopecky KJ, Meshinchi S, Engel JH, Pogosova-Agadjanyan EL, Linsley J *et al.* (2006) Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. *Blood* **107**, 3724–3726.
- Bruserud O, Gjertsen BT, von Volkman HL (2000) In vitro culture of human acute myelogenous leukemia (AML) cells in serum-free media: studies of native AML blasts and AML cell lines. *J. Hematother. Stem Cell Res.* **9**, 923–932.
- Bruserud O, Ulvestad E (1999) Effects of gamma-irradiation on acute myelogenous leukemia blasts: in vitro studies of proliferation, constitutive cytokine secretion, and accessory cell function during T cell activation. *J. Hematother. Stem Cell Res.* **8**, 431–441.
- Döhner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK *et al.* (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453–474.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes that are differentially expressed ($p < 0.005$) when comparing patients with high and low proliferative capacity (Group I patient cohort).

Table S2. List of genes that are differentially expressed ($p < 0.05$) when comparing patients with high and low proliferative capacity (Group I patient cohort) and each of these genes belonging to at least one of the G0-annotation listed in Table 3.