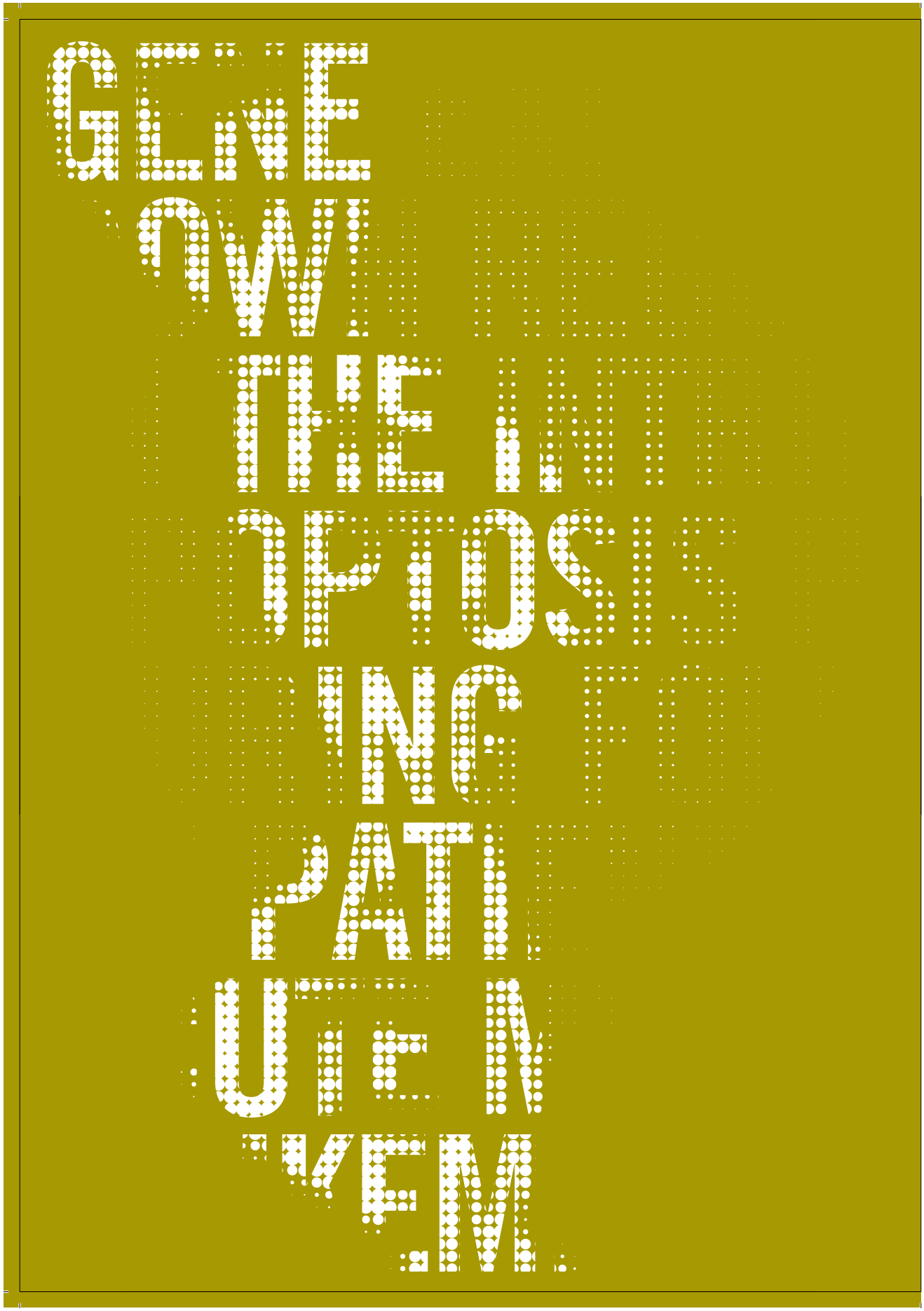
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GENE EXPRESSION DOWN REGULATION IN THE INTRINSIC APOPTOSIS PATHWAY DURING FOLLOW-UP OF PATIENTS WITH ACUTE MYELOID LEUKEMIA

C.J. Hess[1], J. Berkhof[2], F. Denkers[1], N. Feller[1], A. Kelder[1] G.J. Ossenkoppele[1], Q. Waisfisz[1], G.J. Schuurhuis[1]

[1] Department of Hematology, VU Medical Center, Amsterdam, The Netherlands
[2] Department of Clinical Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Purpose
By expression profiling of 31 apoptosis related genes in acute myeloid leukemia (AML) during the course of the disease we determined whether relapse is caused by emergence of apoptosis resistant subpopulations at diagnosis.

Experimental design
Samples were obtained from patients diagnosed with AML; 40 at diagnosis, 35 during follow-up and 20 at relapse. Blasts were isolated by FACS-sorting based on leukemia associated phenotypes. Reverse Transcriptase-Multiplex Ligation dependent Probe Amplification was applied for simultaneous quantification of transcripts. Transcript levels were compared between the different subgroups at all time points. Sequential correlations were estimated by mixed effects modelling.

Results
Pro- and anti-apoptotic expression at diagnosis was higher than controls, both in refractory ($p=.004$, $p=.028$) and non-refractory patients ($p=.001$, $p=.07$).
Non-refractory patients showed a decline in expression in response to chemotherapy for pro-apoptotic ($p=.028$), but not for anti-apoptotic genes ($p=.51$).
Refractory patients showed no change in gene expression in response to chemotherapy, levels remained higher compared to the controls ($p=.001$, $p=.014$).
At relapse, pro-apoptotic gene expression was higher ($p=.019$), while anti-apoptotic gene expression remained in the ranges of controls.

Conclusions
Activation of the apoptosis pathway, expressed by high expression of both pro- and anti-apoptotic genes, is affected by chemotherapy and post-therapy changes.

INTRODUCTION

Treatment resistance, reflected by high frequencies of minimal residual disease (MRD) after induction and consolidation chemotherapy, is strongly associated with poor prognosis and impending relapse in acute myeloid leukemia (AML)[1-4].
Characterizing leukemic blasts that survive chemotherapy (MRD cells) could provide useful information on the biology of AML and its responsiveness to treatment either during or after therapy.
Most cytotoxic drugs ultimately result in the induction of apoptosis in their target cells. Defects in the ability to undergo apoptosis has been suggested to contribute to resistance to a variety of chemotherapeutic agents[5-7].
In theory, this would imply that chemotherapy surviving MRD cells would have a more apoptosis resistant profile. However, by sequential characterization of AML blasts we unexpectedly found MRD cells in patients that achieved complete remission to gain a more apoptosis sensitive protein expression profile[8]. This was concluded from the observation that mean expression of the anti-apoptotic proteins Bcl-2, Mcl-1 and Bcl-xl was lower during remission, compared to diagnosis and relapse.
To further substantiate and refine this observation, parallel characterization of a larger number of parameters, including a broader range of pro-apoptotic parameters, was demanded. Assessment of multiple parameters has, up to now, been impossible due to the low availability of MRD cells.
Recently, we have validated the application of a novel one tube PCR method for the simultaneous measurement, without prior RNA amplification, of transcript levels of 31 apoptosis related genes in samples with very few cells, as they appear in MRD follow up bone marrow[9].
Parallel to reports on the independent prognostic value of the anti-apoptotic Bcl-2 protein[10,11], apoptosis related transcript levels at diagnosis, as examined with the novel RT-MLPA method, allowed risk stratification with respect to achievement of complete remission (CR), overall survival (OS) and relapse free survival (RFS)[12].
It was observed that, contrary to expectation, high expression of both anti- and pro-apoptotic genes predicted poor OS, thereby suggesting that apoptosis resistance is represented by a pathway wide activation of the apoptosis pathway.

The present study was designed to gain support for our previous preliminary observations of an apoptosis sensitive protein expression profile in MRD, which was based almost exclusively on the analysis of three anti-apoptotic proteins[8].
While using expression of multiple genes of the intrinsic apoptosis pathway, we attempted to establish whether the observations included other anti-apoptotic genes or even included the whole apoptosis pathway, i.e. both anti- and pro-apoptotic genes[12].
To determine whether putative changes seen between diagnosis and remission would be reversible at relapse, expression at diagnosis and at remission eveluation were compared with relapse.
Lastly, to confirm that the gene expression changes are a characteristic of the malignant origin of the blasts, all observations were compared with the expression profiles of normal bone marrow CD34 positive counterpart.
The changes observed are discussed in the context of the hypothesis that changes in the bone marrow microenvironment, be it cellular and/or soluble, related to AML blast cells[13-15] or to accompanying bone marrow stromal cells[16,17], underlie the changes found during therapy and thereafter.

MATERIALS AND METHODS

Patients and controls

Forty patients (median age 51, range 19-72 years) diagnosed with acute myeloid leukemia (AML) at the Hematology department of the VU Medical Center (Amsterdam, the Netherlands) were enrolled in this study.

Numbers according to the FAB classification were: M0 (n=2), M1 (n=4), M2 (n=10), M4 (n=9), M5 (n=7), M6 (n=2), RAEB-t (n=3) and missing (n=3). The median white blood cell (WBC) count in the samples at diagnosis was $34.45 \star 10^9$ cells/l (range 1.00 – $322 \star 10^9$ cells/l).

Patients <60 years of age were treated according to HOVON-29 (during 1998-2000) and HOVON-42 (during 2001-2006) protocols. Patients ≥60 years were either treated according to the HOVON-32[18] (during 1996-1999) or the HOVON-43 protocol (during 2000-2006). All protocols are outlined in detail at www.hovon.nl and in references[19,20].

Complete remission or resistance to induction treatment was assessed by bone marrow evaluation at the day of hematological recovery according to standard criteria. Remission was defined as complete in bone marrow with normal hematopoiesis of all cell types, less than 5% blast cells and a peripheral blood count of at least 1,500/μL ($1.5 \star 10^9$/l) neutrophils and 100,000/μL ($100 \star 10^9$/l) platelets.

Refractoriness was defined as failure to induce CR after 2 cycles of induction chemotherapy. Relapse was defined as re-infiltration of the bone marrow by ≥5% leukemic blasts or a proven infiltration of leukemic blasts at any other site.

Bone marrow (BM) aspirates were obtained at diagnosis (n=40), at the time of hematological recovery; after first cycle of induction chemotherapy (n=15), after second cycle of chemotherapy (n=16), or after consolidation therapy (n=4), and at relapse (n=20). Out of the 35 BM follow up samples after 1st or 2nd cycle of induction chemotherapy or consolidation chemotherapy, 16 were scored as CR whereas 19 were scored as non-CR at the time of sampling. Of the latter 19 samples, 11 were obtained from patients that remained refractory throughout the courses of chemotherapy, whereas 8 samples were obtained from patients that achieved complete remission after 2 cycles of induction chemotherapy, though not at the time of sampling but at a later point in time.

Eight control BM samples were received from healthy individuals or patients that underwent cardiac surgery. In the control samples the $CD34^+$ subpopulation was considered to be the normal counterpart of the leukemic blasts.

Antibodies and Reagents

Ficoll Paque was obtained from Pharmacia Biotech (Uppsala, Sweden), RPMI 1640, Iscove's modified Dulbecco's medium and fetal calf serum (FCS) were from Life Technologies (Paisley, UK). 7-AAD (Via Probe, Pharmingen, San Diego) was used in a dilution of 1:20.

FITC-conjugated monoclonal antibodies (Moab's) anti-CD13 (Dako, Glostrup, Denmark) was diluted 1:10, anti-CD7 (1:10), anti-CD15 (1:100), anti-CD33 (1:10), anti-CD34 (1:10), were all obtained from BD.

All Moab's conjugated to PE were from BD and used in the following dilutions: anti-CD11b (1:10), anti-CD33 (1:10), anti-CD117 (1:10), anti-CD22 (1:10), anti-CD56 (1:10), anti-CD7 (1:10), anti-CD13 (1:10), anti-CD34 (1:10), and anti-CD4 (1:10). Applied APC-conjugated Moab's were; anti-CD34 (1:50), anti-CD117 (1:10), anti-CD45 (1:50), all acquired from BD.

Detection of Minimal Residual Disease
Leukemia associated phenotypes (LAPs) were established at diagnosis as previously described[4,21]. In normal bone marrow these LAPs are per definition absent or present in very low frequencies[22].
Since the penetrance of LAP positivity on leukemic blasts at diagnosis is usually lower than 100%, the actual LAP frequency at diagnosis has to be taken into account when the frequency of leukemic blasts is calculated on the basis of LAP positive leukemic blasts in follow-up samples.
In the present cohort every patient displayed one or more LAPs, thus enabling MRD detection during follow-up.

Sample preparation
Patient bone marrow samples were Ficoll-purified, and cryo-preserved[9]. For analysis cells were rapidly thawed[9], occasionally fresh samples were supplemented. Former studies did not reveal effects of freeze-thawing procedures on gene expression levels[9] of viable leukemic cells or on LAP expression[21].
Immunophenotypic staining procedure with FITC-, PE- and APC-conjugated monoclonal antibodies (Moab's) was performed as previously described[19]. In the studied patient cohort no phenotypic shifts took place during the course of the disease.
The leukemic subpopulations of interest were isolated by means of FACS-sorting on bases of LAPs, using a FACS Aria flow cytometer (Becton and Dickinson, Alphen aan den Rijn, the Netherlands).
Since cryo-preserved samples were used, additional staining with 7-AAD was necessary. By combining 7-AAD in the FL3 channel with the required combinations of surface markers we were able to optimally isolate the viable blast population on bases of their aberrant phenotype under MRD conditions.
In case of samples with low MRD frequencies and as a consequence, more time consuming FACS-sorts, target populations were sorted directly into RNABee solution, in order to prevent experimentally induced apoptosis.
The $CD34^+$ normal counterpart subpopulation obtained from the controls was also isolated by means of FACS-sorting, using 7-AAD and APC labeled CD34.

RNA isolation
RNA was isolated using RNABee solution (Tel-test Inc., Friendswood, TX), according to the manufacturer's recommendations. Total RNA was stored at -80°C.

RT-MLPA
Reverse Transcriptase-Multiplex Ligation dependent Probe Amplification (RT-MLPA) was performed as described previously[9] to analyze the expression levels of 31 genes in the mitochondrial apoptosis pathway (for detailed description of probe design: www.mrc-holland.com).

Statistics
The RT-MLPA expression data were log-transformed yielding unskewed variables. Expression data were optimally adjusted for possible effects of unequal amounts of mRNA (diagnosis and relapse samples being more cell rich compared to MRD samples) using GUS-B (ß-Glucoronidase) as internal control. Cleaned expression data were transformed into z-scores, indicating expression levels to be higher or lower compared to the mean value.

For identification of differences between classes/patient subgroups (e.g. refractory, non-refractory) differences in normalized transcript levels were calculated between groups of patients at different time points during the course of the disease. In case more than one sample was obtained during a point of time from one patient mean values were calculated.
A mixed effects model was used to underpin the intra-patient structure of the repeated measurements data.
For comparison with normal bone marrow the median z-score of the reference samples was used.
The significance level was set at .05 and all reported p-values were two-sided.
For analysis of individual gene expression levels a Bonferroni correction was used. Calculations were performed using SPSS 11.0.1 (SPSS, Chicago, IL).

RESULTS

Apoptosis transcript levels of 19 pro- and 12 anti-apoptotic genes in the mitochondrial mediated apoptosis pathway were determined by RT-MLPA, in samples obtained from patients diagnosed with AML: at the time of diagnosis, remission evaluation and at relapse.
Mean expression of all pro-apoptotic genes, all anti-apoptotic genes, as well as expression levels of individual genes were thereby determined in refractory patients and non-refractory patients.
Gene expression levels were compared between these different subgroups at particular defined time points. Conversely, changes in time within each patient group were determined.
Since RT-MLPA is a semi-quantitative method, all expression levels were denoted as fold-differences. Expression levels in normal BM $CD34^+$ cells were used as a reference.
For all data presented it should be realized that the MLPA panel contained 19 genes designated as pro-apoptotic and 12 genes as anti-apoptotic.
Figure 1 shows a summary of gene expression data for all pro-apoptotic genes (Figure 1A) and anti-apoptotic genes (Figure 1B) at the three different disease conditions (diagnosis, follow up and relapse) for the two different groups of patients (refractory, and non-refractory).
In the following paragraphs, mean gene expression for a particular patient group at a particular time point is in all cases designated as fold-differences with other groups of interest. In addition, a similar approach is followed for expression of individual genes.

Apoptosis profile at diagnosis

A. Comparison with normal BM
At diagnosis the mean expression of all 19 pro- as well as the mean expression of all 12 anti-apoptotic genes was higher compared to $CD34^+$ control samples, both in the refractory and non-refractory patients.
For the refractory patients the mean expression of pro- and anti-apoptotic gene compared to $CD34^+$ controls was 1.46-fold (95%CI 1.15-1.86, p=.004) and 1.33-fold (95%CI 1.04-1.70, p=.028) higher, while for the non-refractory patients the mean expression was 1.30-fold (95%CI 1.12-1.50, p=.001) and 1.17-fold (95%CI 1.01-1.39, p=.07) higher (Figure 1).
The differences in gene expression between diagnosis and $CD34^+$ controls was significant (p<.0002) for 7 out of 31 individual genes, these included the pro-apoptotic genes PMAIP1, MCL1(s), BAX(s), BAX(l), APAF1(1), APAF1(3) and the anti-apoptotic gene MCL1(l).

B. Comparison between refractory and non-refractory patients
Although mean expression of pro- and anti-apoptotic genes was higher in refractory patients compared to non-refractory patients, the differences were non-significant (p=.21 and p=.23).

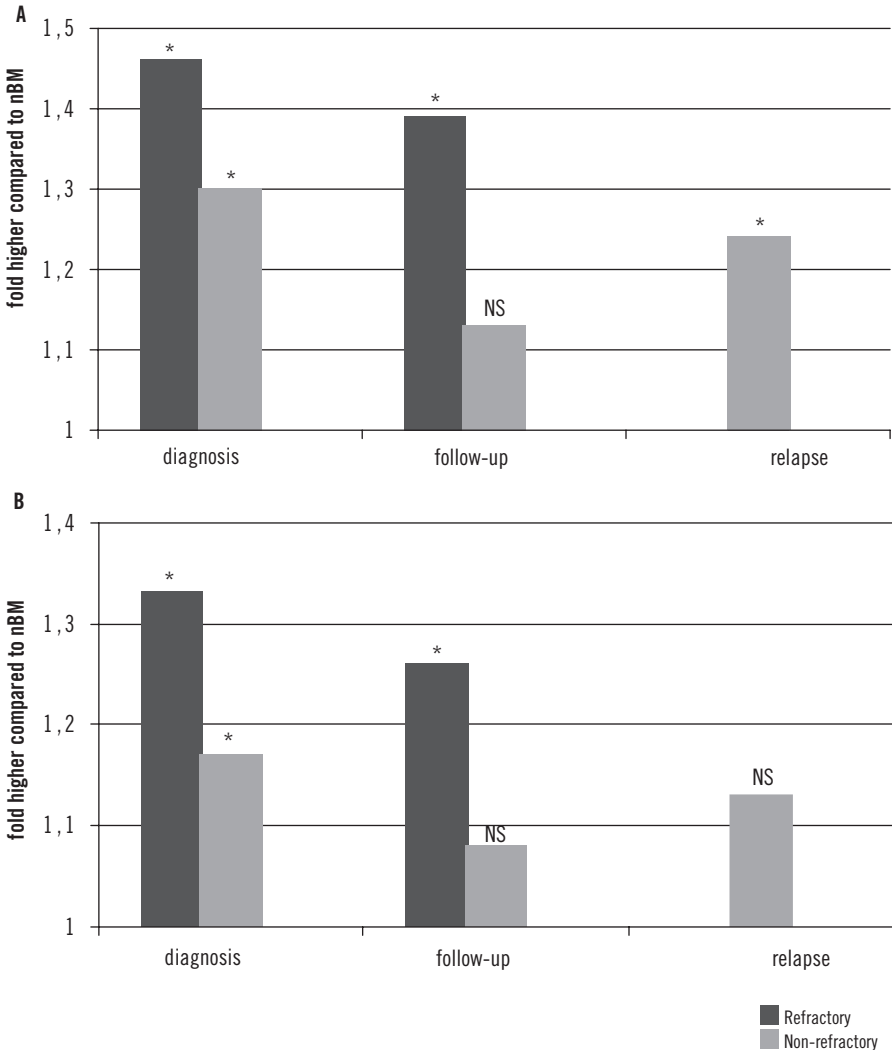

Figure 1. Expression levels, relative to control bone marrow CD34+ cells, at different stages of disease in patient groups differing in clinical status.
All levels of gene expression refer to mean expression of all pro-apoptotic genes (Figure 1A) or all anti-apoptotic genes (Figure 1B).
Y-axis values indicate fold difference with normal bone marrow.
At diagnosis, the black box indicates patients who later on turned out to be refractory to therapy.
At diagnosis the grey boxes represent all patients who later on turned out to enter into remission.
Relapses originate from patients in the grey box at remission.
Significant differences are depicted with an asterisk. NS, indicates non significance.

Apoptosis profile at remission evaluation

A. Comparison with normal BM
The mean expression levels of both the pro- and anti-apoptotic genes in the follow-up of the refractory patients were 1.39-fold and 1.26-fold higher compared to $CD34^+$ controls, respectively (95%CI 1.62-1.19, p=.001 and 1.52-1.06, p=.014, respectively) (Figure 1).
On individual bases 7 out of 31 genes were significantly higher (p<0.002) in refractory patients compared to $CD34^+$ controls, these included the pro-apoptotic genes PMAIP1, MCL1(s), BAX(l) and the anti-apoptotic genes BCL2L2, BCL2A1, MCL1(l), and BIRC1.
In the non-refractory patients the mean pro- and anti-apoptotic gene expression was 1.13- and 1.08-fold higher, respectively than $CD34^+$ cells (95%CI 1.28-0.99, p=.06 and 1.25-0.93, p=.31, respectively) (Figure 1).
Except for MCL1(l), which was expressed significantly (p<.002) higher in the non-refractory patients during follow-up than in the controls, all other individual genes were in the range of the controls.

B. Comparison between refractory and non-refractory patients
At remission evaluation, mean pro- and mean anti-apoptotic gene expression in refractory patients was non-significantly higher compared to the non-refractory patients (95%CI 1.41-0.99, p=.07 and 1.36-0.92, p=.25, respectively).

C. Comparison with diagnosis
Figure 1 suggests decrease of mean gene expression from diagnosis to remission for both patient groups and both types of genes.
To determine the effect of chemotherapy on the apoptosis related gene expression profile we compared expression profiles of both refractory and non-refractory patients at follow-up with those at diagnosis.
For the refractory patients the decrease of mean pro- and anti-apoptotic gene expression (factor 1.05 and 1.06, respectively) was not significant.
In the non-refractory patients the 1.15-fold decrease was significant for the mean pro-apoptotic gene expression (95%CI 1.18-1.01, p=.03), while the 1.08-fold decrease for mean anti-apoptotic expression was not.

6. APOPTOSIS PROFILE AT RELAPSE

A. Comparison with normal BM
Mean pro-apoptotic and mean anti-apoptotic gene expression in relapse samples was 1.24- and 1.13-fold higher, respectively, than in $CD34^+$ control samples (95%CI 1.04-1.49, p=.019 and 0.94-1.37, p=.19, respectively).
On individual bases 5 genes, the pro-apoptotic genes PMAIP1, MCL1(s), BAX(s), BAX(l) and the anti-apoptotic gene MCL1(l), were significantly higher (p<0.002) at relapse compared to the control samples, whereas expression of *BIRC5* was significantly lower (p<0.002) compared to the $CD34^+$ controls.

B. Comparison with follow-up
Since patients that experience relapse have, per definition, achieved complete remission, we have compared gene expression levels in relapse samples with gene expression at the time of their remission evaluation.

Although mean expression of pro- and anti-apoptotic genes in the non-refractory patients was higher in relapse compared to follow-up (factor 1.10 and 1.05, respectively), this difference was non-significant.

DISCUSSION

In this paper we present an expression analysis of 31 apoptosis related genes in leukemic blasts obtained at diagnosis, at relapse and the different scenario's at follow-up: 1) refractoriness i.e. no remission achieved after different courses of chemotherapy; 2) remission, at the follow-up time point or at a later follow-up time point.
Also, all situations were compared with gene expression in the normal $CD34^+$ counterparts.
The results show down-regulation of both pro-apoptotic genes (significant) and anti-apoptotic genes (not significant) in the non-refractory patients, who entered remission at some time point during therapy, down to levels of normal bone marrow.
In the refractory patients no significant effects on pro- or anti- apoptotic gene expression were seen in response to chemotherapy.
Comparisons with normal bone marrow $CD34^+$ cells (nBM) confirmed the overall picture: refractory patients show large differences with these controls both at diagnosis and at follow up.
The differences with nBM seen at diagnosis for the patients who later entered remission, disappeared at follow up and reappeared at relapse. These changes were most pronounced for pro-apoptotic genes (Figure 1).
For a number of individual genes, including the MCL1 and BAX splice variants and PMAIP1 significant changes were observed, which is consistent with the general observation that both pro- and anti- apoptotic genes change in the same direction.
Although factors change are not that large, such changes may have profound functional effects.
In two earlier papers we discussed two unexpected phenomena that should shed light on the above observations: Firstly, in contrast to what might be expected from chemotherapy, cells surviving chemotherapy had lower expression of the anti-apoptotic proteins Bcl-2, MCL-1 and Bcl-Xl, compared to diagnosis[12].
We hypothesized that apoptosis resistance is in part sustained by soluble pro-survival factors excreted mainly by leukemic blasts. Chemotherapy-induced blast reduction in that concept would thus result in concentration decreases for such factors and thus lead to more apoptosis sensitive profiles, largely resembling now the normal bone marrow blast cells[12].
Secondly, although the well known adverse prognostic impact of anti-apoptosis proteins was seen also in expression of corresponding genes as well as in expression of other anti-apoptotic[16,17] genes, quite unexpectedly, adverse prognosis correlated strongest with high expression of pro-apoptotic genes. This pathway-wide activation was discussed in the context of a concept referred to as oncogenic addiction[12].

The results presented in this study thus show that, in line with the decrease of expression of the few anti-apoptotic proteins studied earlier, it is gene expression of the whole intrinsic pathway that is down-regulated after chemotherapy. Further, in agreement with the important prognostic role for the pro-apoptotic gene expression at diagnosis, it is the expression of the pro-apoptotic genes that is affected most by therapy.
Whether lower gene expression always translates to lower protein expression needs to be verified: in the present paper we saw down-regulation of expression of all pro-apoptotic genes during therapy, while in our previous report we did not observe this for Bax protein expression[8], although this was the only pro-apoptotic protein studied.

The results of the present paper and our previous paper suggests a large contribution from soluble factors to apoptosis characteristics of the leukemic cells.
Direct evidence for the role of soluble factors was found by incubating apoptosis- "sensitive" MRD cells with supernatants produced by their corresponding more apoptosis resistant diagnosis blasts (submitted).
It can not be excluded, however, that part of cellular resistance originates from inherent resistance characteristic not that sensitive to external influences. Although it remains speculative how to translate the current findings to therapeutic options, the results presented here suggest that a) targeting the soluble apoptosis pathway activating microenvironment at diagnosis would be a promising therapeutic approach and b) cells surviving chemotherapy might still be vulnerable to apoptosis inducing therapies.

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