Signal transduction in MDS bone marrow populations

DISTINCT SIGNAL TRANSDUCTION ABNORMALITIES AND ERYTHROPOIETIN RESPONSE IN BONE MARROW HEMATOPOIETIC CELL SUBPOPULATIONS OF MYELODYSPLASTIC SYNDROME PATIENTS

Elena Spinelli¹, Roberto Caporale², Francesca Buchi¹, Erico Masala¹, Antonella Gozzini¹, Alessandro Sanna¹, Francesca Sassolini¹, Ana Valencia¹, Alberto Bosì¹, and Valeria Santini¹.

²Department of Laboratory Medicine. Azienda Ospedaliero Universitaria (AOU) Careggi. Florence, Italy.

Running title: Signal transduction in MDS bone marrow populations.

Keywords: Hematologic, MDS, signal transduction, flow cytometry, cytokines.

Corresponding author:

Valeria Santini, MD. Functional Unit of Hematology, AOU Careggi, University of Florence.
Largo Brambilla 3 Firenze, Italy. Tel: 00390557947296; fax 390557947343; e-mail santini@unifi.it

Word count: 3941
Total number of figures: 4
Total number of tables: 2
Signal transduction in MDS bone marrow populations

**Statement of translational relevance**

This study provides one immediate translational application and two possible clinical developments. The accuracy and rapidity of the tests described in this report are therefore of major interest.

First, this study provides a possible means of improving the assessment and prediction of clinical sensitivity to erythropoietin using a rapid and relatively simple cytofluorimetric assay in MDS patients already indicated as possibly responsive on the basis of recognized predictive biological/clinical parameters. Moreover, from our experimental evidence, G-CSF stimulates CD34+ MDS cells at the same rate as normal progenitors. These data further support the safety of the use of G-CSF in MDS cases with severe neutropenia.

Finally, these findings demonstrate a means of determining the rate of apoptosis of MDS cells by evaluation of p38 phosphorylation and caspase-3 cleavage. These determinations could be indicative of the efficacy of anti-apoptotic therapy in hypoplastic, low-risk MDS cases.
Signal transduction in MDS bone marrow populations

Abstract

Purpose: Myelodysplastic syndromes (MDS) are heterogeneous clonal diseases characterized by cytopenias as a result of ineffective hematopoiesis. Little is known about alterations in signal transduction pathways in MDS.

Experimental design: Multiparameter flow cytometry was used to evaluate the proteolytic activation of caspase-3 and the phosphorylation of ERK1/2, p38 MAPK, and STAT5 specifically in defined CD34+, CD45+, or CD71+CD45- bone marrow (BM) cells from 60 MDS cases and normal controls, both at baseline and following stimulation with G-CSF and erythropoietin (EPO).

Results: In CD71+CD45- cells from a subpopulation of 36 MDS cases who were predicted to be responsive by clinical parameters (endogenous EPO levels, transfusion dependency, percentage of blasts in the BM), EPO failed to activate ERK1/2 or STAT5 in 23/36 cases, but it was effective in 13/36 cases, although to a significantly lower degree than in CD71+CD45- cells from healthy donor BM. The EPO response in vivo correlated with in vitro EPO-dependent STAT5 activation in 20/22 cases.

STAT5 was significantly activated at baseline in MDS cells compared to normal controls, while caspase-3 was activated in CD34+ and CD45+ MDS cells, and was activated more often in the RA and RAEB-1 MDS subtypes. G-CSF stimulation activated ERK1/2 and STAT5 equally in MDS and normal CD34+ cells.

Conclusions: Abnormalities in the response to growth factors are restricted to EPO stimulation in CD71+CD45- cells and correlate with the clinical response to EPO. Activation of baseline signal transduction for proliferative and apoptotic signals is altered in MDS, but with different patterns among the various BM subpopulations.
Introduction

Myelodysplastic syndromes (MDS) are a group of hematological neoplastic diseases heterogeneous for clinical presentation and genetic alterations. The characterization of biological properties of myelodysplastic cells in vitro is hampered by lack of animal models and by difficulties in isolating the cell populations responsible for the disease and/or its maintenance. Although high throughput techniques have recently identified many genetic lesions present in MDS, the majority of cases lack an easily detectable molecular or phenotypic marker. The burden of disease is routinely evaluated morphologically, by manually scoring dysplastic cells, and only recently have other methods been proposed, including flow cytometric analysis (1, 2) or molecular quantitation of oncogenes such as WT-1 (3, 4). In fact, at present, three diagnostic and prognostic evaluation systems are used to classify MDS, the French–American–British (FAB) classification (5), the International Prognostic Score System (IPSS) (6), and the World Health Classification (WHO) classification (7), along with the WHO classification-based prognostic scoring system, WPSS (8). Both prognostic systems are dependent on the morphological detection of immature cells and on the presence of cytogenetic abnormalities.

Cells from the BM of MDS patients have altered signal transduction pathways. In particular, the erythropoietin (EPO) receptor is expressed at a normal density on MDS cells, but STAT5 activation in response to EPO stimulation is defective (9). This observation was attributed to intracellular structural defects in the EPO receptors of MDS cells, but this was not specifically demonstrated (10, 11). Few studies have been conducted regarding the basal activation of proliferative signaling in MDS marrow progenitors (9, 12). Recently, altered p38 MAPK activation has been demonstrated in MDS (13). Moreover, little information is available to explain the progressive displacement of normal hematopoiesis by the MDS clone, for which the nature of the survival advantage has not been demonstrated (14).
Signal transduction in MDS bone marrow populations

On the other hand, one of the typical clinical manifestations of MDS, peripheral cytopenia with hyperproliferative BM and ineffective hemopoiesis, is caused by excessive premature apoptosis of hematopoietic precursors (15).

In this study, the signal transduction pathways controlling proliferation and apoptosis in distinct subpopulations of MDS BM cells were evaluated to identify a possible dysplastic signature. Differences in the basal activation of signaling pathways were evaluated among marrow cell subtypes and among MDS morphological subtypes, defined according to the WHO classification. Signal transduction was also evaluated in response to erythropoietic and granulopoietic stimulating agents, broadly used in clinics for the treatment of low-risk IPSS MDS patients. In particular, STAT5 phosphorylation in response to EPO was examined with respect to clinical outcome in the MDS cases who were treated with EPO based on clinical parameters (IPSS LOW/INT-1; endogenous EPO < 500U/l; no transfusion need), which indicated them as potentially responsive to erythropoiesis-stimulating agents (ESAs).

Materials and methods

Patients

BM blood samples were obtained from 60 patients diagnosed with MDS at the Hematology Unit, AOU Careggi, University of Florence, Italy. Patient characteristics are reported in Table 1. BM cells from six healthy donors were included in the study. Informed consent was obtained according to institutional guidelines. MDS diagnosis was made in accordance with the WHO criteria. Three CMML and two CMML-2 cases were diagnosed according to the morphologic and cytochemical criteria of the FAB classification and were included in the study. All analyses were performed at the time of diagnosis. BM cells were cultured and processed, and conventional cytogenetic analysis was performed on unstimulated BM cultures after 24 h by trypsin-Giemsa banding (GTG-banding). Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (16).
Signal transduction in MDS bone marrow populations

Endogenous EPO serum levels were routinely evaluated at diagnosis with a chemiluminescent immunometric assay (Immulite 2000 EPO; Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA).

Isolation of mononuclear cells from BM samples, cells, and culture conditions

For in vitro experiments, bone marrow mononuclear cells (BMMC) were purified by standard density gradient centrifugation (Lympholyte-H; Cedarlane Laboratories Ltd, Burlington, NC, USA) and were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Kasumi-1 cells, an AML1/ETO-positive cell line derived from a human AML (17), was also cultured with the same method.

Stimulation of BMMC

Kasumi-1 cells and BMMC from MDS patients and healthy donors were resuspended in RPMI 1640 medium supplemented with 5% FBS. Cells were stimulated with human recombinant G-CSF (100 ng/ml for 10 min), EPO (100 U/ml for 15 min) or 12-O-tetradecanoylphorbol-13-acetate (TPA; 400nM for 10 min) at 37°C (all from Sigma-Aldrich). Stimulations were chosen based on time-courses analyses (data not shown).

Cell lysis and Immunoblotting

Cells were washed once with ice-cold PBS and solubilized by incubating for 10 min at 95°C in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 0.005% blue bromophenol, and 2% SDS). Lysates were clarified by centrifugation (20000 g, 10 min, RT). The protein concentration of the supernatants was determined using the BCA assay (Pierce Chemical, Rockford, IL, USA) and 30 µg aliquots of each sample were boiled for 10 min in the presence of 100 mM of 2-mercaptoethanol. Proteins were separated by SDS-PAGE in a 9–15% polyacrylamide gel and...
Signal transduction in MDS bone marrow populations transferred onto PVDF membranes (Amersham Biosciences, Uppsala, Sweden) by electro-blotting. Membranes were blocked in PBS containing 0.1% Tween 20 and 1% BSA (T-PBS/1%BSA; 3 h, RT) and incubated in the same buffer with a rabbit anti-phospho-ERK1/2 primary antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) (6–18 h at 4°C). A horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St Louis, MO, USA) was added for an additional hour. Antibody-coated protein bands were visualized by ECL chemiluminescence detection (Amersham Biosciences). To confirm equal loading of samples, membranes were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol; 30 min, 50°C), extensively washed with T-PBS, and re-probed with a rabbit anti-p38 antibody (1:1000; Cell Signaling Technology).

**Intracellular phospho-specific flow cytometry**

Cells were fixed in BD Cytofix Buffer and permeabilized with BD Perm Buffer III (both from BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions, and were treated as described elsewhere (18). BMMC were incubated with the following monoclonal antibodies directed to surface proteins: allophycocyanin (APC)-conjugated CD34 (clone 581), phycoerythrin (PE)-conjugated CD71 (clone M-A712), and peridinin-chlorophyll (PerCP-)conjugated CD45 (clone 2D1) (all from BD Biosciences). At the same time, cells were incubated with an Alexa Fluor488-conjugated IgG1 isotype control (clone MOPC-21) or with Alexa Fluor488-conjugated specific antibodies directed to the following intracellular proteins: phospho-STAT5 (clone 47), phospho-ERK1/2 (clone 20a), or phospho-p38 MAPK (clone 36/p38) (all from BD Biosciences), or polyclonal cleaved caspase-3 (Cell Signaling Laboratories). For analysis, approximately 20,000 events were collected for each sample using a FACSCanto analyzer (BD Biosciences) and data were processed using the BD FacsDiva software.
Signal transduction in MDS bone marrow populations

**Statistical and cluster analysis**

Differences in basal phosphorylation were compared by calculating the specific MFI as the ratio of the mean fluorescence intensity (MFI) for each sample divided by the MFI of the isotype control. The MFI cutoff to consider activated phosphorylation (> 1.2) was established calculating the MFI median values +/- standard deviation of unstimulated normal controls.

A protein was considered strongly activated when the specific MFI was greater than 5.0, activated when the specific MFI was between 2.0 and 5.0, weakly activated when the specific MFI was between 1.2 and 2.0, and not activated when the specific MFI was less or equal to 1.2. Changes in the phosphorylation status of proteins following cytokine stimulation were determined by calculating the ratios of the specific MFIs of stimulated versus unstimulated cell populations.

The non-parametric Wilcoxon Kruskal-Wallis tests were used to evaluate statistical significance. \( P \leq 0.05 \) was considered statistically significant. The response to EPO treatment in vivo was evaluated according to IWG criteria as an increase of 2 g/dl in Hb levels after 8 weeks of EPO treatment or transfusion independence for > 8 weeks (19). The correlation between EPO responses in vivo and in vitro was calculated by the non-parametric Spearman’s test. Analyses were conducted using the StataCorp2003 software program. To assess the validity of the in vitro results with respect to in vivo response to EPO, we estimated the sensitivity, the specificity and the positive and negative predictive values with their 95% confidence intervals. Analyses of flow cytometry data were performed using unsupervised algorithms that allow for samples with similar patterns of protein basal activation or similar cytokine responses in vitro to be grouped together. Heat maps and dendrograms were drawn using the open-source versions of the Cluster and TreeView programs*.

Results

Spontaneous basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 in CD34+ cells

Basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 in CD34+ BMMC was evaluated in 60 MDS patients and six healthy donors. CD34+ cells were not detected in six MDS cases, leaving 54 evaluable samples (Supplementary Table 1). The pattern of activation is listed in Table 2.

STAT5 was phosphorylated in the majority of MDS cases, but particularly in 5/54 cases (1 RA, 1 RCMD, 2 RAEB-1, and 1 RAEB-2). Caspase-3 was also strongly activated in 5/54 cases (1 RA, 1 RARS, 1 RCMD, 1 RAEB-1, and 1 RAEB-2). Healthy donor cells did not exhibit basal activation of any of the proteins studied (Supplemental Figure 1).

Statistical analysis showed that STAT5 and caspase-3 were significantly activated in CD34+ MDS cells compared to normal donor cells (P = 0.001 and P = 0.002, respectively; Figure 1A), and that STAT5 was significantly phosphorylated in the RA and RAEB-1 WHO subgroups as compared to healthy donors (P < 0.001 and P < 0.001).

A heterogeneous pattern of basal activation was observed by cluster analysis, in that MDS cases belonging to the same WHO classification subtype did not cluster together (Figure 1B). Flow cytometric analysis of ERK1/2, STAT5, p38 MAPK, and caspase-3 basal activation in one representative case (#18) is shown in Supplementary Figure 2.

Spontaneous basal activation of ERK1/2, STAT5, p38 MAPK and caspase-3 in CD45+ cells

Basal activation of ERK1/2, p38 MAPK, STAT5 and caspase-3 was studied in CD45+ cells obtained from 45/60 MDS patients and six healthy donors (Supplemental Table 2). The pattern of activation is depicted in Table 2.

ERK1/2 and p38 MAPK phosphorylation was a rare event in these cells. The intensity of phosphorylation of STAT5 and caspase-3 was heterogenous but weak in the majority of cases (1.2
Signal transduction in MDS bone marrow populations

< MFI < 2). BM cells from the normal donors did not show any basal activation of the studied proteins (Supplemental Figure 3).

Statistical analysis showed that STAT5 and caspase-3 were significantly activated in CD45+ MDS cells compared with healthy donors controls ($P < 0.001$ and $P = 0.003$, respectively; Figure 1C) and that STAT5 was significantly phosphorylated in patients with the RA and RAEB-1 subtypes ($P < 0.001$ and $P = 0.001$, respectively) compared to normal cases.

Cluster analysis showed a heterogeneous pattern of activation, and the cases belonging to the same WHO subtypes did not cluster together, although normal cases did (Figure 1D).

**Spontaneous basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 in CD71+CD45- cells**

Basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 was studied in BM from 45/60 MDS patients and six normal donors. Two of the MDS cases analyzed did not contain CD71+CD45- cells. The activation of signaling pathways in this subpopulation was low overall and is shown in Table 2.

Healthy donor cells and MDS cases did not present basal activation of ERK1/2, p38 MAPK, or caspase-3 (Supplemental Figure 4). STAT5 was the only protein significantly activated in the CD71+CD45- subpopulation compared with normal cells ($P = 0.006$; Figure 1E). Cases of RA and RAEB-1 ($P = 0.001$ and $P = 0.001$, respectively) showed the highest levels of STAT5 activation in absence of any stimuli. Five RA cases with the highest STAT5 activation (MFI > 3) also grouped together by cluster analysis (Figure 1F).

**Cytokine response in vitro**

*Activation of signaling proteins in Kasumi-1 and healthy control cells.* To validate the method, Kasumi-1 cells (85% CD34+) were stimulated with TPA, a known activator of ERK1/2. Strong activation of ERK1/2 was observed by flow cytometry after TPA treatment. This result was qualitatively validated by immunolotting (Figure 2A-B).
Signal transduction in MDS bone marrow populations

In parallel, ERK1/2 and STAT5 activation was also studied in normal BMMC after EPO treatment for 5, 15, 30, and 60 min or TPA treatment for 10 min. After 15 min of EPO treatment, CD71+CD45- cells showed no ERK1/2 activation (Figure 2C, lower left), and STAT5 phosphorylation peaked at this time (data not shown). The activation of ERK1/2 was not visible by immunoblotting of total BMMC, which contains only 17.6% CD71+CD45- cells (Figure 2D).

Moreover, ERK1/2 activation following TPA stimulation was observed in CD45+ cells (79% of the total BMMC) and in CD34+ cells (1.4% of the total BMMC), but not in CD71+CD45- cells (Figure 2C, lower right). This result was qualitatively validated by immunoblotting analysis of total BMMC (Figure 2D).

**EPO stimulation.** The response to EPO stimulation was examined in 38/60 MDS cases and six healthy donors. Two MDS cases had no CD71+CD45- cells. EPO did not modify the activation of ERK1/2, p38 MAPK, or caspase-3 in CD34+, CD45+, or CD71+CD45- MDS cells, or the activation of STAT5 in CD34+ or CD45+ MDS cells. However, in CD71+CD45- cells, STAT5 phosphorylation was induced at a ratio of MFI 1.3–2.5-fold by EPO in 13/36 MDS patients belonging to various WHO subtypes and 2.1–2.9-fold in all healthy donors. The remaining MDS cases did not show phosphorylation of STAT5 after *in vitro* treatment with EPO (Supplementary Table 5). Flow cytometric analysis of STAT5 activation after EPO stimulation in one representative case (#34) is shown in Supplementary Figure 5A.

Statistical analysis indicated that STAT5 phosphorylation after EPO stimulation was significantly lower in CD71+CD45- MDS cells compared to CD71+CD45- cells from healthy donors (*P* < 0.001) (Figure 3A).

It is interesting to note that none of the MDS cases responsive to EPO *in vitro* showed basal activation of caspase-3 in CD71+CD45- cells, and conversely, none of the MDS cases with caspase-3 basal activation responded to EPO.
Signal transduction in MDS bone marrow populations

Three main groupings were visible upon cluster analysis. Cluster 1 included the six healthy donors, one RCMD case and one CMML-2 case. Cluster 2 contained 11 MDS responding to EPO and belonging to various WHO subtypes; STAT5 activation in these cases was lower than in healthy donors. Cluster 3 included MDS cases not responding to EPO (Figure 3B). Subsequently, the \textit{in vivo} response to EPO was evaluated in 22 non-transfused MDS patients. These patients were considered to have a high probability of response to EPO according to accepted clinical criteria, in that all 22 were transfusion-independent, with endogenous serum EPO levels < 500U/L, and 17/22 had an absence of blasts in the BM. Clinical response to EPO was defined according to IGW 2006 criteria (19) as an increase of 2 g/dl in Hb levels after 8 weeks of EPO treatment. EPO response \textit{in vitro} was defined as a greater than 1.2-fold MFI increase in STAT5 activation. Characteristics of patients treated with EPO are reported in Figure 3C. A strong correlation between the EPO response \textit{in vitro} and in the clinic was observed in 20 of the 22 cases (90.9%) (Spearman’s rho = 0.62 and $P$ = 0.002) (Figure 3D and Supplementary Table 6). The \textit{in vitro} response was highly predictive of \textit{in vivo} response to EPO, with a sensitivity of 71% (95% CI: 36.0 to 92.0), a specificity of 100% (80.0 to 100.0), a positive predictive value of 100% (56.6 to 100.0) and a negative predictive value of 88% (66.0 to 96.7).

The two MDS cases that did not show a correlation with EPO response \textit{in vivo} had high basal STAT5 phosphorylation ($2 \leq\text{MFI} < 5$). Moreover, in 8/22 cases the immunophenotype of CD34+ cells was evaluated with a panel of antibodies applied by Westers \textit{et al} (20). Consistent with these authors’ observations, in 5/8 MDS cases the aberrant immunophenotype of myeloid blasts correlated with EPO treatment failure, while 1/8 case did respond to EPO and presented a normal CD34+ phenotype. By contrast, the remaining 2/8 cases responded to EPO treatment \textit{in vivo}, despite an aberrant CD34+ phenotypic pattern.

\textit{G-CSF stimulation}. G-CSF stimulation was examined in 40/60 MDS cases and six healthy donors. This analysis could not be performed in six MDS cases in which CD34+ cells were absent. G-CSF
Signal transduction in MDS bone marrow populations did not modify the activation of p38 MAPK or caspase-3 in any cellular subpopulations. G-CSF stimulation had no effect on ERK1/2 or STAT5 phosphorylation in CD71+CD45- MDS cells, while it induced STAT5 activation in CD45+ cells in 9/40 cases (data not shown). CD34+ cells showed a 1.3–10-fold G-CSF-dependent activation of STAT5 in 32/34 MDS cases, while in normal cells, STAT5 was activated 1.9–2.8-fold in response to G-CSF (Supplementary Table 4). Statistical analysis indicated no significant difference in STAT5 activation in response to G-CSF in CD34+ cells from MDS cases as compared to normal controls ($P = 0.49$) (Figure 4A). Cluster analysis revealed that the pattern of STAT5 phosphorylation after G-CSF was heterogeneous (Figure 4B). Flow cytometric analysis of STAT5 activation after G-CSF stimulation in one case (#37) is represented in Supplementary Figure 5B. Moreover, CD34+ cells showed G-CSF-dependent activation of ERK1/2 in 6/8 MDS cases and in all normal cases with an MFI ratio of between 1.3 and 1.9 (Figure 4C).
Signal transduction in MDS bone marrow populations

Discussion

MDS are a complex group of diseases. Very few studies have focused on dissecting the signaling pathways in MDS cells (13, 21). This study evaluated the spontaneous basal activation of ERK1/2, p38 MAPK, STAT5, and caspase-3 in 60 primary human MDS cases, and the modulation of this activation by EPO and G-CSF using a flow cytometric method which allowed quantitation of phosphorylation in the distinct CD45+, CD34+, and CD71+CD45- BMMC subpopulations. The spontaneous activation of ERK1/2, p38 MAPK, STAT5, and caspase-3 differed markedly between normal and MDS cells and was highly heterogeneous among MDS cases and also among the different MDS cell subpopulations studied. This is due to the fact that, in MDS marrow, progenitors with different stages of differentiation and different genotypes are present. Our aim was not to study a selected subpopulation, as done in gene expression profiling studies, but to examine the difference in pathways among these subpopulations.

The intensity of activation of STAT5, caspase-3, and p38, and the number of cases in which activation was observed, was highest in CD34+ cells, suggesting that this is the most “active” subpopulation in MDS. This enhanced signaling could contribute to the progressive depletion of early hematopoietic cells seen in MDS patients due to defective self-renewal, differentiation and quiescence (22).

ERK1/2 is only marginally activated in basal conditions in CD34+ cells, and completely unphosphorylated in CD71+CD45- cells, both in normal donors and in MDS cases. The erythroid subpopulation appeared inert in normal BMMCs, while CD71+CD45- cells from myelodysplastic BMs showed basal phosphorylation of STAT5 in approximately half of the cases studied, which can be interpreted as a sign of ineffective erythropoiesis. There is a lack of correlation between the phosphorylation pattern and the WHO (or FAB) classification, reflecting possibly inadequacy of current MDS classification systems, which are based only on morphological features.
Signal transduction in MDS bone marrow populations

While few data for activated proliferative pathways are available, literature mainly concerns gene silencing or overexpression/mutation of oncogenes correlated with progression of MDS to AML (23-26).

In addition, STAT5 and ERK1/2 activation in response to EPO was defective in CD71+CD45- MDS marrow cells. A defect in STAT5 gene expression characterizes lenalidomide responsiveness irrespective of the presence of the 5q deletion and is an essential pre-requisite for lenalidomide sensitivity (27). As already shown by other authors, STAT5 activation in response to *in vitro* EPO was absent or significantly reduced in all MDS cases compared to normal controls. No significant differences in the presence of EPO-R, nor in the burden of the MDS clone, as indicated by abnormal metaphases (data not shown), were detected between responders and non-responders. This abnormality seems to allow a clear distinction between normal and MDS CD71+CD45- progenitors. These data are consistent with recent reports (21). This intrinsic defect in erythroid development is characteristic of the disease and mainly functionally affects CD71+CD45- cells (9, 27). Even when all metaphases scored are abnormal, the response to EPO may be present and thus attributed to the MDS clone (case #25; see results).

*In vitro* response to EPO, measured by cytofluorimetry in CD71+CD45- cells, correlated strictly with the *in vivo* response. This method could thus be used as a test that could accurately predict clinically responsive patients, even among those presenting with favorable biological/clinical parameters such as endogenous EPO levels < 500u/L, low marrow blasts, and transfusion independency. This analysis particularly focused on such MDS cases. These characteristics identify the group of MDS cases with the lowest Nordic score (28), and who therefore are predicted to have the highest probability of response to EPO (plus G-CSF) treatment, as confirmed in a recent meta-analysis (29). Within this subset of possibly responsive patients, the cytofluorimetric test described here strongly predicted the clinically non-responsive patients. Recently, an aberrant phenotype of CD34+ cells in MDS patients has been shown to be predictive of the response to EPO (20). The MDS cases with an aberrant CD34+ phenotype according to ELN guidelines (1) were also EPO-
Signal transduction in MDS bone marrow populations insensitive in the STAT5 phosphorylation test, confirming the correlation between EPO insensitivity and CD34 immunophenotype. An inconsistency of the two types of evaluation was observed only in one isolated case. Therefore, the test described in this report has a very high predictive power for the in vivo EPO response, particularly as it is independent of the presence of CD34+ cells. A correlation between the flow score of BM cells and the IPSS score has been shown in MDS (30). In that study, and in follow-up studies by the same group, the predictability of the response to treatments like HSCT and immunosuppressive drugs was evaluated (31, 32).

Attempts have been made to distinguish an EPO response signature in MDS and, in fact, differential expression of 37 genes could separate EPO-responsive and non-responsive cases with similar biological and clinical characteristics (33). None of the genes differentially expressed were directly linked to erythroid maturation. Similarly to what is observed for EPO, non-5q- MDS cases responding to lenalidomide did not vary significantly from non-responders with respect to clinical characteristics such as age, BM cell counts, or peripheral blood cell counts, but were identified based only on gene signature (34).

A correlation between activated ERK1/2 in BM cells and clinical response to EPO has been shown recently (21). We originally attributed our failure to observe a high level of phosphorylation of ERK1/2 in response to EPO to a methodology problem; however, TPA strongly activated ERK1/2 not only in MDS cells, but also in an AML cell line and in normal donor cells. It therefore seems that activation of the ERK1/2 pathway, although present, is not strong or frequent in MDS cells or normal BMMC.

Finally, G-CSF promotes STAT5 and ERK1/2 phosphorylation at a level that is comparable to that observed in normal CD34+ cells. The response to G-CSF is therefore not altered in CD34+ MDS cells, and thus does not identify a pattern typical of dysplastic hematopoietic stem cells. Most probably, earlier stem cell populations such as CD34+CD38- or CD133+ cells should be evaluated in this sense, as gene expression profiling has indicated significant differences compared to normal
Signal transduction in MDS bone marrow populations
counterparts (35). In addition, this data could in fact support a safe, and consequently broader, use of G-CSF in the treatment of MDS.

This study provides the first evidence that is possible to identify a dysplastic phosphorylation signature, opening novel possibilities for investigation of the heterogeneous pathophysiology of MDS. This data also demonstrates that the EPO clinical response may be predicted with a rapid cytofluorimetric test that is more accurate than previously applied biological/clinical parameters.

**Disclosure of Potential Conflicts of Interest**

The authors have no conflict of interest to declare.

**Acknowledgments**

The authors wish to thank Dr. V. Gattei and Dr. A. Zucchetto from Centro di Riferimento Oncologico, I.R.C.C.S., Aviano (PN), Italy, for helpful discussion and suggestions concerning the cluster analysis. We also thank Prof. Boddi from University of Florence, and Dr Gianni Ciccone, Centro di Riferimento per l'Epidemiologia e la Prevenzione Oncologica in Piemonte (CPO-Piemonte), Torino, for their support in the statistical analyses.

**Grant Support**

Research Fundings: Regione Toscana, Bando salute 2009; Ente Cassa di Risparmio di Firenze (ECR); Ministero per l’Istruzione, l’Università e la Ricerca (MIUR).

**References**

1. van de Loosdrecht AA, Alhan C, Bene MC, Della Porta MG, Drager AM, Feuillard J et al. Standardization of flow cytometry in myelodysplastic syndromes: report from the first European
Signal transduction in MDS bone marrow populations


11. Shimizu R, Komatsu N, and Miura Y. Dominant negative effect of a truncated erythropoietin receptor (EPOR-T) on erythropoietin-induced erythroid differentiation: possible involvement of
Signal transduction in MDS bone marrow populations


Signal transduction in MDS bone marrow populations


Signal transduction in MDS bone marrow populations


Figure and table legends

**Figure 1.** (A) ERK1/2, STAT5, p38 MAPK, and caspase-3 basal activation in CD34+ BMMCs from MDS patients (n=54) and normal controls (n=6) is indicated as the MFI of the sample divided by the MFI of the isotype control (**, P<0.01).
Signal transduction in MDS bone marrow populations

(B) Unsupervised hierarchical clustering of cytofluorimetric data for basal activation of CD34+ 54 MDS cases and six normal BMMC controls. Hierarchical clustering of MDS samples analyzed for the activation of four intracellular proteins and three surface markers. The hierarchical clustering algorithm is based on the complete-linkage method and uses Euclidean distances as measures of similar/dissimilar behavior. The heat map depicts the activation level values for each of individual sample; columns represent MDS samples and rows represent the proteins analyzed. The activation level from 0% (black) to 100% (intense red; pixel setting 5) was calculated as the MFI of staining with the specific antibody divided by the MFI of the isotype control. The MDS cases are labeled by their progressive patient number. (C) ERK1/2, STAT5, p38 MAPK, and caspase-3 basal activation in CD45+ MDS cells (n=45) and normal BMMC (n=6) is indicated as in Figure 1A. (D) Unsupervised hierarchical clustering of cytofluorimetric data regarding basal activation of CD45+ cells from 45 MDS cases and six normal BMMC samples. For details, see Figure 1B. (E) Basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 in CD71+CD45- MDS cells (n=45) and normal BMMC (n=6) is indicated as in Figure 1A. (F) Unsupervised hierarchical clustering of cytofluorimetric data regarding basal activation of CD71+CD45- MDS cells from 43 MDS cases and six normal controls. For details, see Figure 1B.

Figure 2. ERK1/2 activation in Kasumi-1 cells and normal BMMC.  (A) Flow cytometric analysis of ERK1/2 activation before (upper right) and after (lower right) TPA treatment in Kasumi-1 cells. (B) Immunoblot analysis of ERK1/2 activation before and after TPA treatment in Kasumi-1 cells. Full-length blots are presented in Supplementary Figure 6A. (C) Flow cytometric analysis of ERK1/2 activation before and after EPO and TPA treatment in BMMC obtained from healthy donors: isotypic control (upper left), P-ERK1/2 basal activation (upper right), P-ERK1/2 activation after EPO treatment (lower left), and P-ERK1/2 activation after TPA treatment (lower right). The CD45+ cells are indicated in orange, the CD71+CD45- cells are in violet, the CD34+ cells are shown in dark blue, and all activated cells are shown in light blue. (D) Immunoblot analysis of
Signal transduction in MDS bone marrow populations

ERK1/2 activation before and after TPA and EPO treatment of normal BMMC. Full-length blots are presented in Supplementary Figure 6B.

**Figure 3.** (A) STAT5 activation by EPO in CD71+CD45- cells. EPO-dependent activation of STAT5 in CD71+CD45- MDS cells (n=36) and healthy donor cells (n=6) is indicated as the ratio of the MFI from stimulated to unstimulated cells (**, \(P<0.01\)). (B) Unsupervised hierarchical clustering of cytofluorimetric data regarding STAT5 phosphorylation in CD71+CD45- cells from 36 MDS cases and six normal BMMC samples after EPO stimulation. The hierarchical clustering algorithm is based on the complete-linkage method and uses Euclidean distances as measures of similar/dissimilar behavior. The heat map depicts the activation level values for each of the individual sample. Columns represent MDS samples. Activation levels from 0% (black) to 100% (intense red; pixel setting 3) were calculated as the ratio of the STAT5-specific MFI in stimulated versus unstimulated CD71+CD45- cells.

(C) Characteristics of MDS patients treated with EPO. (D) Correlation between EPO response *in vitro* and *in vivo*. The X-axis indicates STAT5 activation following EPO stimulation while the Y-axis indicates the increase in hemoglobin levels after EPO treatment *in vivo*. The cases with positive or negative responses to EPO fall within quadrant I (n= 5) and III (n= 15), respectively (positive correlation between *in vitro* and *in vivo* responses). The cases with a contrasting response to EPO (negative correlation between *in vitro* and *in vivo* responses) fall within in quadrant II (n= 2) and IV (n= 0). The original graphic is presented in Supplementary Figure 6C.

**Figure 4.** (A) STAT5 activation by G-CSF in CD34+ cells. G-CSF-dependent activation of STAT5 in CD34+ MDS cells (n=34) and healthy donor cells (n=6) is calculated as the ratio of the MFI of stimulated cells to unstimulated cells. (B) Unsupervised hierarchical clustering of cytofluorimetric data of STAT5 phosphorylation in CD34+ cells from 34 MDS cases and six normal BMMC samples after G-CSF stimulation. The hierarchical clustering algorithm is based on the complete-linkage method and uses Euclidean distances as measures of similar/dissimilar behavior. The heat map depicts the activation level values for each individual sample. Columns represent MDS
Signal transduction in MDS bone marrow populations

samples. Activation level from 0% (black) to 100% (intense red; pixel setting 5) was calculated as the ratio of the STAT5-specific MFI in stimulated versus unstimulated CD34+ cells. (C) ERK1/2 activation by G-CSF in CD34+ cells. G-CSF-dependent activation of ERK1/2 in CD34+ MDS cells (n=8) and healthy donor cells (n=6) is indicated by the ratio of the MFI of stimulated versus unstimulated cells.

Table 1. Main patient characteristics. Abbreviations: RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB-1, RA with excess blasts-1; RAEB-2, RA with excess blasts-2; RCMD, refractory cytopenia with multilineage dysplasia; CMML-1, chronic myelomonocytic leukemia-1; CMML-2, chronic myelomonocytic leukemia-2; MPL/MDS, myeloproliferative disorders/myelodysplastic syndromes; N, normal controls.

Table 2. Pattern of basal protein activation. The ratio of MDS cases considered positive for spontaneous activation are reported with respect to the total number of cases analyzed.
Figure 1

**CD34+ cells**

**CD45+ cells**

**CD71+CD45- cells**

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 2

A

B

C

D

Figure 2

A

B

0 10 TPA (min)
P-ERK 1/2

p38

C

D

TPA EPO

10 15 0 (min)
P-ERK 1/2

p38
Figure 3

A STAT5 activation by EPO in CD71+CD45- cells

B Clustering analysis

C Characteristics of MDS patients treated with EPO

<table>
<thead>
<tr>
<th>IPSS LOW</th>
<th>IPSS INT-1</th>
<th>IPSS INT-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Median age</td>
<td>76</td>
<td>74.5</td>
</tr>
<tr>
<td>Clinical EPO response</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

WHO

| RA; RARS | 6; 1 | 4; 0 | 0; 0 |
| RCMD; 5q- | 3; 0 | 2; 0 | 0; 0 |
| RAEB-1; RAEB-2 | 0; 0 | 0; 0 | 3; 2 |
| MDS/MPL | 1 | 0 | 0 |

D Correlation between EPO response in vitro and in vivo
Figure 4

A  STAT5 activation by G-CSF in CD34+ cells

B  Clustering analysis

C  ERK1/2 activation by G-CSF in CD34+ cells
Table 1. Main characteristics of MDS patients.

<table>
<thead>
<tr>
<th></th>
<th>IPSS LOW</th>
<th>IPSS INT-1</th>
<th>IPSS INT-2</th>
<th>IPSS HIGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>31</td>
<td>17</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Median age</td>
<td>73</td>
<td>75</td>
<td>68.5</td>
<td>66.5</td>
</tr>
<tr>
<td>Gender M; F</td>
<td>16; 15</td>
<td>12; 5</td>
<td>7; 3</td>
<td>2; 0</td>
</tr>
<tr>
<td>WHO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA; RARS</td>
<td>20; 1</td>
<td>7; 1</td>
<td>0; 0</td>
<td>0; 0</td>
</tr>
<tr>
<td>RCMD; 5q-</td>
<td>6; 1</td>
<td>6; 0</td>
<td>0; 0</td>
<td>0; 0</td>
</tr>
<tr>
<td>RAEB-1; RAEB-2</td>
<td>0; 0</td>
<td>2; 0</td>
<td>5; 4</td>
<td>0; 1</td>
</tr>
<tr>
<td>MDS/MPL</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMML; CMML-2</td>
<td>2; 0</td>
<td>1; 0</td>
<td>0; 1</td>
<td>0; 1</td>
</tr>
<tr>
<td>KARYOTYPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>30</td>
<td>13</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>-7q; -5q</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>-5q</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-11q</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>t(9;21)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>COMPLEX</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2. Pattern of spontaneous pathway activation in MDS bone marrow cell subpopulations

<table>
<thead>
<tr>
<th>CD34+ cells</th>
<th>P- ERK1/2</th>
<th>P- STAT5</th>
<th>P- p38</th>
<th>Caspase 3 cleav.</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/54</td>
<td>45/54</td>
<td>11/54</td>
<td>43/54</td>
<td></td>
</tr>
<tr>
<td>CD45+ cells</td>
<td>3/45</td>
<td>34/45</td>
<td>6/45</td>
<td>34/45</td>
</tr>
<tr>
<td>1/43</td>
<td>28/43</td>
<td>4/43</td>
<td>12/43</td>
<td></td>
</tr>
<tr>
<td>CD71+CD45-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/43</td>
<td>28/43</td>
<td>4/43</td>
<td>12/43</td>
<td></td>
</tr>
</tbody>
</table>
distinct signal transduction abnormalities and erythropoietin response in bone marrow hematopoietic cell subpopulations of myelodysplastic syndrome patients

Elena Spinelli, Roberto Caporale, Francesca Buchi, et al.

Clin Cancer Res  Published OnlineFirst April 11, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-0686

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/05/29/1078-0432.CCR-11-0686.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.