

## Distinct Signal Transduction Abnormalities and Erythropoietin Response in Bone Marrow Hematopoietic Cell Subpopulations of Myelodysplastic Syndrome Patients

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### 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 extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), and STAT5 specifically in defined CD34<sup>+</sup>, CD45<sup>+</sup>, or CD71<sup>+</sup>CD45<sup>-</sup> bone marrow (BM) cells from 60 MDS cases and normal controls, both at baseline and following stimulation with granulocyte colony-stimulating factor (G-CSF) and erythropoietin.

**Results:** In CD71<sup>+</sup>CD45<sup>-</sup> cells from a subpopulation of 36 MDS cases who were predicted to be responsive by clinical parameters (endogenous erythropoietin levels, transfusion dependency, percentage of blasts in the BM), erythropoietin failed to activate ERK1/2 or STAT5 in 23 of 36 cases, but it was effective in 13 of 36 cases, although to a significantly lower degree than in CD71<sup>+</sup>CD45<sup>-</sup> cells from healthy donor BM. The erythropoietin response *in vivo* correlated with *in vitro* erythropoietin-dependent STAT5 activation in 20 of 22 cases. STAT5 was significantly activated at baseline in MDS cells compared with normal controls, whereas caspase-3 was activated in CD34<sup>+</sup> and CD45<sup>+</sup> 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<sup>+</sup> cells.

**Conclusions:** Abnormalities in the response to growth factors are restricted to erythropoietin stimulation in CD71<sup>+</sup>CD45<sup>-</sup> cells and correlate with the clinical response to erythropoietin. Activation of baseline signal transduction for proliferative and apoptotic signals is altered in MDS but with different patterns among the various BM subpopulations. *Clin Cancer Res*; 18(11); 3079–89. ©2012 AACR.

### Introduction

Myelodysplastic syndromes (MDS) are a group of hematologic neoplastic diseases heterogeneous for clinical presentation and genetic alterations. The characterization of biologic 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, 3 diagnostic and prognostic evaluation systems are used to classify MDS, the French-American-British (FAB) classification (5), the International Prognostic Score System (IPSS; ref. 6), and the World Health Organization (WHO) classification (7), along with the WHO classification-based prognostic scoring system, WPSS (8). Both prognostic systems are dependent on the morphologic detection of immature cells and on the presence of cytogenetic abnormalities.

Cells from the bone marrow (BM) of patients with MDS have altered signal transduction pathways. In particular, the erythropoietin receptor is expressed at a normal density on MDS cells, but STAT5 activation in response to erythropoietin stimulation is defective (9). This observation was attributed to intracellular structural defects in the erythropoietin receptors of MDS cells, but this was not specifically showed (10, 11). Few studies have been conducted

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-11-0686

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### 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 patients with MDS already indicated as possibly responsive on the basis of recognized predictive biologic/clinical parameters. Moreover, from our experimental evidence, granulocyte colony-stimulating factor (G-CSF) stimulates CD34<sup>+</sup> 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 show 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 antiapoptotic therapy in hypoplastic, low-risk MDS cases.

regarding the basal activation of proliferative signaling in MDS marrow progenitors (9, 12). Recently, altered p38 mitogen-activated protein kinase (MAPK) activation has been showed 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 showed (14).

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 morphologic 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 patients with MDS. In particular, STAT5 phosphorylation in response to erythropoietin was examined with respect to clinical outcome in the MDS cases who were treated with erythropoietin based on clinical parameters (IPSS low/int-1; endogenous erythropoietin < 500 U/L; no transfusion need), which indicated them as potentially responsive to erythropoiesis-stimulating agents (ESA).

## Materials and Methods

### Patients

BM blood samples were obtained from 60 patients diagnosed with MDS at the Hematology Unit, AOU Careggi,

University of Florence, Florence, Italy. Patient characteristics are reported in Table 1. BM cells from 6 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-1 and 2 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 conducted at the time of diagnosis. BM cells were cultured and processed, and conventional cytogenetic analysis was conducted on unstimulated BM cultures after 24 hours by Trypsin-Giemsa banding (GTG-banding). Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (16).

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

### 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.) and were maintained in RPMI-1640 supplemented with 10%

**Table 1.** Main characteristics of patients with MDS

	IPSS low	IPSS int-1	IPSS int-2	IPSS high
No. of patients	31	17	10	2
Median age	73	75	68.5	66.5
Gender, M/F	16/15	12/5	7/3	2/0
WHO				
RA; RARS	20; 1	7; 1	0; 0	0; 0
RCMD; 5q-	6; 1	6; 0	0; 0	0; 0
RAEB-1; RAEB-2	0; 0	2; 0	5; 4	0; 1
MDS/MPN	1	0	0	0
FAB				
CMML-1; CMML-2	2; 0	1; 0	0; 1	0; 1
Karyotype				
Normal	30	13	4	0
7q-; 5q-	0	0	1	0
5q-	1	0	0	0
11q-	0	0	1	0
t(9;21)	0	0	0	1
+8	0	3	1	0
Complex	0	1	3	1

Abbreviations: CMML-1, chronic myelomonocytic leukemia-1; CMML-2, chronic myelomonocytic leukemia-2; MDS/MPN, myelodysplastic/myeloproliferative neoplasms; 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.

FBS, glutamine and penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Kasumi-1 cells, an AML1/ETO-positive cell line derived from a human acute myelogenous leukemia (AML; ref. 17), was also cultured with the same method.

### Stimulation of BMMCs

Kasumi-1 cells and BMMCs from patients with MDS and healthy donors were resuspended in RPMI-1640 medium supplemented with 5% FBS. Cells were stimulated with human recombinant granulocyte colony-stimulating factor (G-CSF; 100 ng/mL for 10 minutes), erythropoietin (100 U/mL for 15 minutes) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 400 nmol/L for 10 minutes) 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 minutes at 95°C in Laemmli buffer (62.5 mmol/L Tris/HCl, pH 6.8, 10% glycerol, 0.005% blue bromophenol, and 2% SDS). Lysates were clarified by centrifugation (20,000 × *g*, 10 minutes, room temperature). The protein concentration of the supernatants was determined by the bicinchoninic acid (BCA) assay (Pierce Chemical) and 30 µg aliquots of each sample were boiled for 10 minutes in the presence of 100 mmol/L of 2-mercaptoethanol. Proteins were separated by SDS-PAGE in a 9% to 15% PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) by electroblotting. Membranes were blocked in PBS containing 0.1% Tween 20 and 1% bovine serum albumin (BSA; T-PBS/1% BSA; 3 hours, room temperature) and incubated in the same buffer with a rabbit anti-phospho-ERK1/2 primary antibody (1:1,000; Cell Signaling Technology; 6–18 hours at 4°C).

A horseradish peroxidase (HR)-conjugated secondary antibody (Sigma-Aldrich) was added for an additional hour. Antibody-coated protein bands were visualized by enhanced chemiluminescence detection (Amersham Biosciences). To confirm equal loading of samples, membranes were incubated in stripping buffer (62.5 mmol/L Tris-HCl, pH 6.7, 2% SDS, 100 mmol/L 2-mercaptoethanol; 30 minutes, 50°C), extensively washed with T-PBS, and reprobed with a rabbit anti-p38 antibody (1:1,000; Cell Signaling Technology).

### Intracellular phospho-specific flow cytometry

Cells were fixed in BD Cytotfix Buffer and permeabilized with BD Perm Buffer III (both from BD Biosciences) according to the manufacturer's instructions and were treated as described elsewhere (18). BMMCs 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 Fluor

488-conjugated IgG1 isotype control (clone MOPC-21) or with Alexa Fluor 488-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 with the BD FACS Diva software.

### Statistical and cluster analysis

Differences in basal phosphorylation were compared by calculating the specific mean fluorescence intensity (MFI) as the ratio of the MFI for each sample divided by the MFI of the isotype control.

The MFI cutoff value to consider activated phosphorylation (>1.2) was established by calculating the MFI median values ± SD 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 nonparametric Wilcoxon Kruskal-Wallis tests were used to evaluate statistical significance.  $P \leq 0.05$  was considered statistically significant. The response to erythropoietin treatment *in vivo* was evaluated according to IWG criteria as an increase of 2 g/dL in hemoglobin (Hb) levels after 8 weeks of erythropoietin treatment or transfusion independence for more than 8 weeks (19). The correlation between erythropoietin responses *in vivo* and *in vitro* was calculated by the nonparametric Spearman test. Analyses were conducted with the StataCorp2003 software program. To assess the validity of the *in vitro* results with respect to *in vivo* response to erythropoietin, we estimated the sensitivity, the specificity, and the positive and negative predictive values with their 95% confidence intervals (CI). Analyses of flow cytometric data were conducted 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 with the open-source versions of the Cluster and TreeView programs (<http://bonsai.ims.u-tokyo.ac.jp/~mdheoon/software/cluster/software.htm>).

## Results

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

Basal activation of extracellular signal-regulated kinase (ERK)1/2, STAT5, p38 MAPK, and caspase-3 in CD34<sup>+</sup> BMMCs was evaluated in 60 patients with MDS and 6 healthy donors. CD34<sup>+</sup> cells were not detected in 6 MDS

**Table 2.** Pattern of spontaneous pathway activation in MDS BM cell subpopulations: basal protein activation

	p-ERK1/2	p-STAT5	p-p38	Caspase-3 cleavage
CD34 <sup>+</sup> cells	7/54	45/54	11/54	43/54
CD45 <sup>+</sup> cells	3/45	34/45	6/45	34/45
CD71 <sup>+</sup> CD45 <sup>-</sup> cells	1/43	28/43	4/43	12/43

NOTE: The ratio of MDS cases considered positive for spontaneous activation are reported with respect to the total number of cases analyzed.

cases, leaving 54 evaluable samples (Supplementary Table S1). The pattern of activation is listed in Table 2.

STAT5 was phosphorylated in the majority of MDS cases but particularly in 5 of 54 cases (1 RA, 1 RCMD, 2 RAEB-1, and 1 RAEB-2). Caspase-3 was also strongly activated in 5 of 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 (Supplementary Fig. S1).

Statistical analysis showed that STAT5 and caspase-3 were significantly activated in CD34<sup>+</sup> MDS cells compared with normal donor cells ( $P = 0.001$  and  $P = 0.002$ , respectively; Fig. 1A) and that STAT5 was significantly phosphorylated in the RA and RAEB-1 WHO subgroups as compared with 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 (Fig. 1B). Flow cytometric analysis of ERK1/2, STAT5, p38 MAPK, and caspase-3 basal activation in one representative case (#18) is shown in Supplementary Fig. S2.

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

Basal activation of ERK1/2, p38 MAPK, STAT5, and caspase-3 was studied in CD45<sup>+</sup> cells obtained from 45 of 60 patients with MDS and 6 healthy donors (Supplementary Table S2). 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 heterogeneous but weak in the majority of cases ( $1.2 < \text{MFI} < 2$ ). BM cells from the normal donors did not show any basal activation of the studied proteins (Supplementary Fig. S3).

Statistical analysis showed that STAT5 and caspase-3 were significantly activated in CD45<sup>+</sup> MDS cells compared with healthy donors controls ( $P < 0.001$  and  $P = 0.003$ , respectively; Fig. 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 with 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 (Fig. 1D).

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

Basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 was studied in BM from 45 of 60 patients with MDS and 6 normal donors. Two of the MDS cases analyzed did not contain CD71<sup>+</sup>CD45<sup>-</sup> 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 (Supplementary Fig. S4). STAT5 was the only protein significantly activated in the CD71<sup>+</sup>CD45<sup>-</sup> subpopulation compared with normal cells ( $P = 0.006$ ; Fig. 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 (Fig. 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<sup>+</sup>) 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 (Fig. 2A and B).

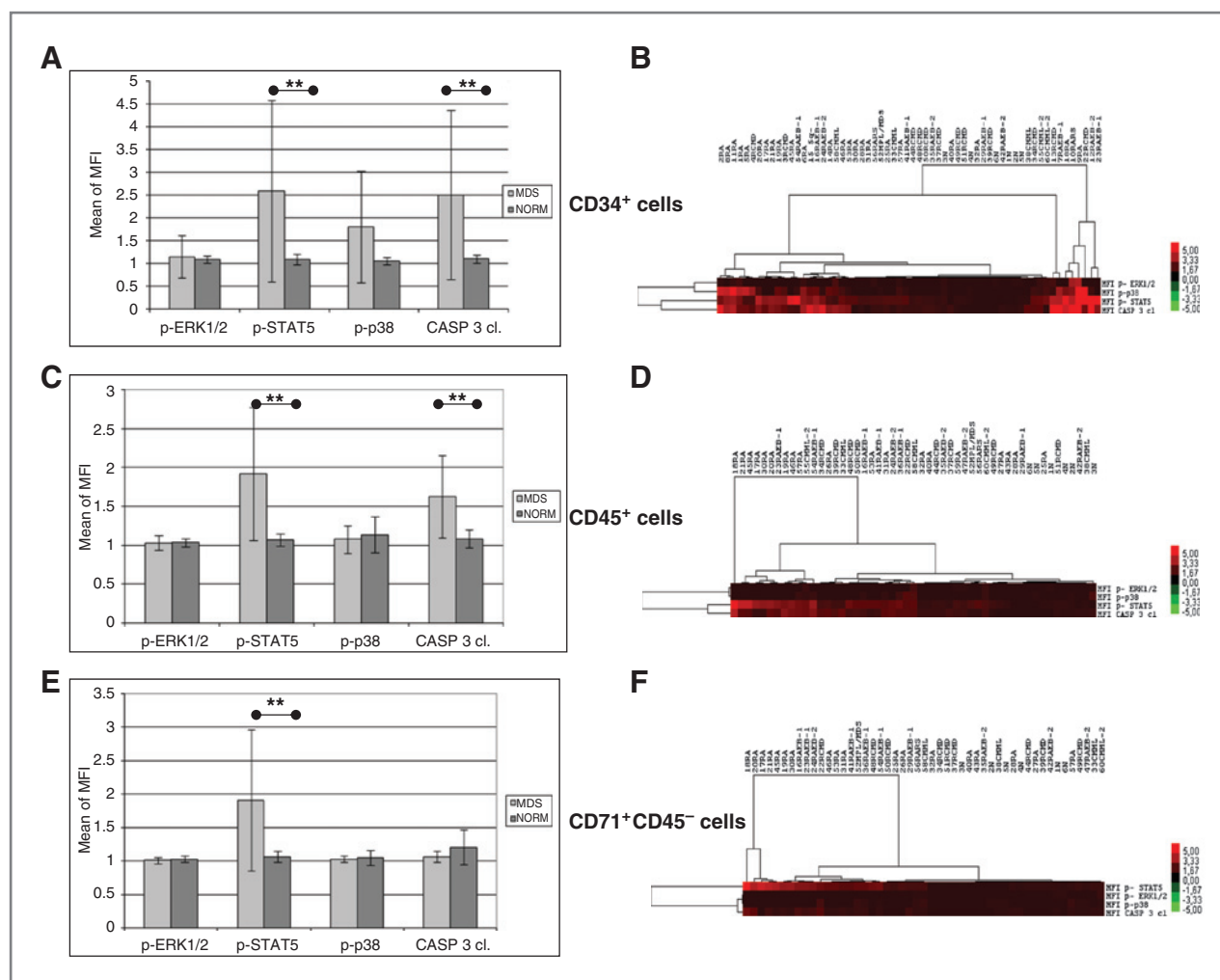
In parallel, ERK1/2 and STAT5 activation was also studied in normal BMMCs after erythropoietin treatment for 5, 15, 30, and 60 minutes or TPA treatment for 10 minutes.

After 15 minutes of erythropoietin treatment, CD71<sup>+</sup>CD45<sup>-</sup> cells showed no ERK1/2 activation (Fig. 2C, bottom left), and STAT5 phosphorylation peaked at this time (data not shown). The activation of ERK1/2 was not visible by immunoblotting of total BMMCs, which contains only 17.6% CD71<sup>+</sup>CD45<sup>-</sup> cells (Fig. 2D).

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

**Erythropoietin stimulation.** The response to erythropoietin stimulation was examined in 38 of 60 MDS cases and 6 healthy donors. Two MDS cases had no CD71<sup>+</sup>CD45<sup>-</sup> cells. Erythropoietin did not modify the activation of ERK1/2, p38 MAPK, or caspase-3 in CD34<sup>+</sup>, CD45<sup>+</sup>, or CD71<sup>+</sup>CD45<sup>-</sup> MDS cells or the activation of STAT5 in CD34<sup>+</sup> or CD45<sup>+</sup> MDS cells. However, in CD71<sup>+</sup>CD45<sup>-</sup> cells, STAT5 phosphorylation was induced at a ratio of MFI 1.3- to 2.5-fold by erythropoietin in 13 of 36 patients with MDS belonging to various WHO subtypes and 2.1- to 2.9-fold in all healthy donors. The remaining MDS cases did not





**Figure 1.** A, ERK1/2, STAT5, p38 MAPK, and caspase-3 basal activation in CD34<sup>+</sup> BMNCs from patients with MDS ( $n = 54$ ) and normal (NORM) controls ( $n = 6$ ) is indicated as the MFI of the sample divided by the MFI of the isotype control (\*,  $P < 0.01$ ). B, unsupervised hierarchical clustering of cytofluorimetric data for basal activation of CD34<sup>+</sup> 54 MDS cases and 6 normal BMNC controls. Hierarchical clustering of MDS samples analyzed for the activation of 4 intracellular proteins and 3 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<sup>+</sup> MDS cells ( $n = 45$ ) and normal BMNCs ( $n = 6$ ) is indicated as in (A). D, unsupervised hierarchical clustering of cytofluorimetric data about basal activation of CD45<sup>+</sup> cells from 45 MDS cases and 6 normal BMNC samples. For details, see (B). E, basal activation of ERK1/2, STAT5, p38 MAPK, and caspase-3 in CD71<sup>+</sup>CD45<sup>-</sup> MDS cells ( $n = 45$ ) and normal BMNCs ( $n = 6$ ) is indicated as in (A). F, unsupervised hierarchical clustering of cytofluorimetric data about basal activation of CD71<sup>+</sup>CD45<sup>-</sup> MDS cells from 43 MDS cases and 6 normal BMNCs. For details, see (B).

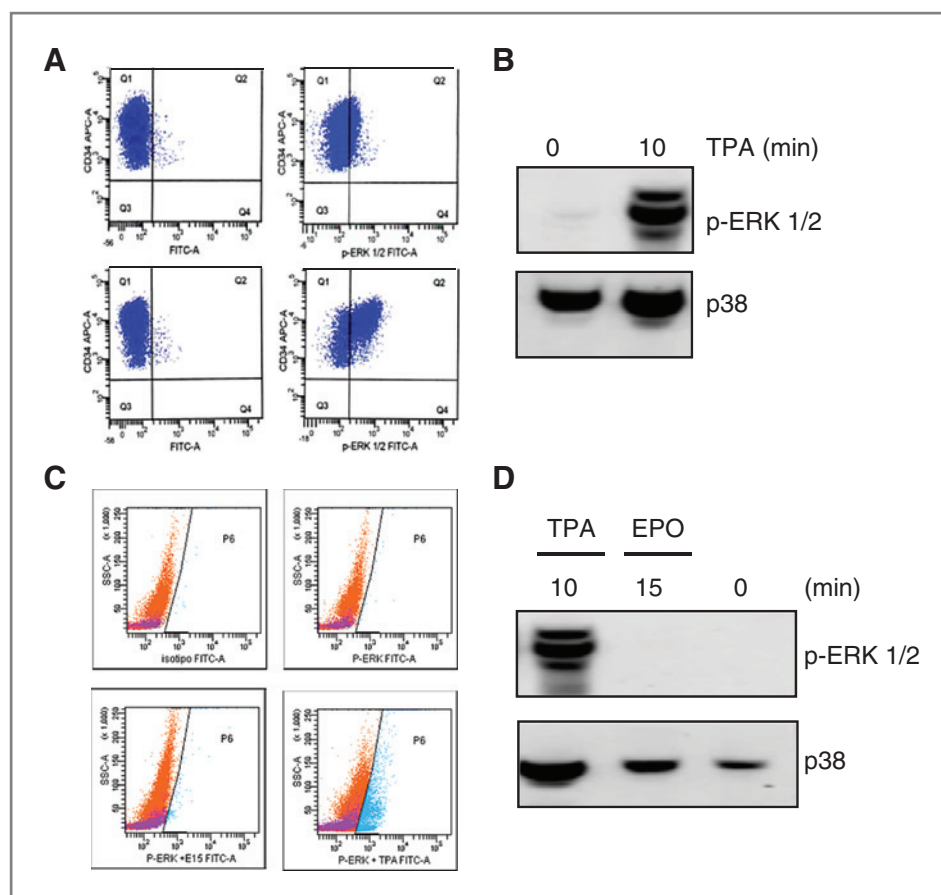
show phosphorylation of STAT5 after *in vitro* treatment with erythropoietin (Supplementary Table S5). Flow cytometric analysis of STAT5 activation after erythropoietin stimulation in one representative case (#34) is shown in Supplementary Fig. S5A.

Statistical analysis indicated that STAT5 phosphorylation after erythropoietin stimulation was significantly lower in CD71<sup>+</sup>CD45<sup>-</sup> MDS cells than in CD71<sup>+</sup>CD45<sup>-</sup> cells from healthy donors ( $P < 0.001$ ; Fig. 3A).

It is interesting to note that none of the MDS cases responsive to erythropoietin *in vitro* showed basal activation of caspase-3 in CD71<sup>+</sup>CD45<sup>-</sup> cells, and conversely, none of

the MDS cases with caspase-3 basal activation responded to erythropoietin.

Three main groupings were visible upon cluster analysis. Cluster 1 included the 6 healthy donors, one RCMD case and one CMML-2 case. Cluster 2 contained 11 MDS responding to erythropoietin 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 erythropoietin (Fig. 3B). Subsequently, the *in vivo* response to erythropoietin was evaluated in 22 nontransfused patients with MDS. These patients were considered to have a high probability of response to



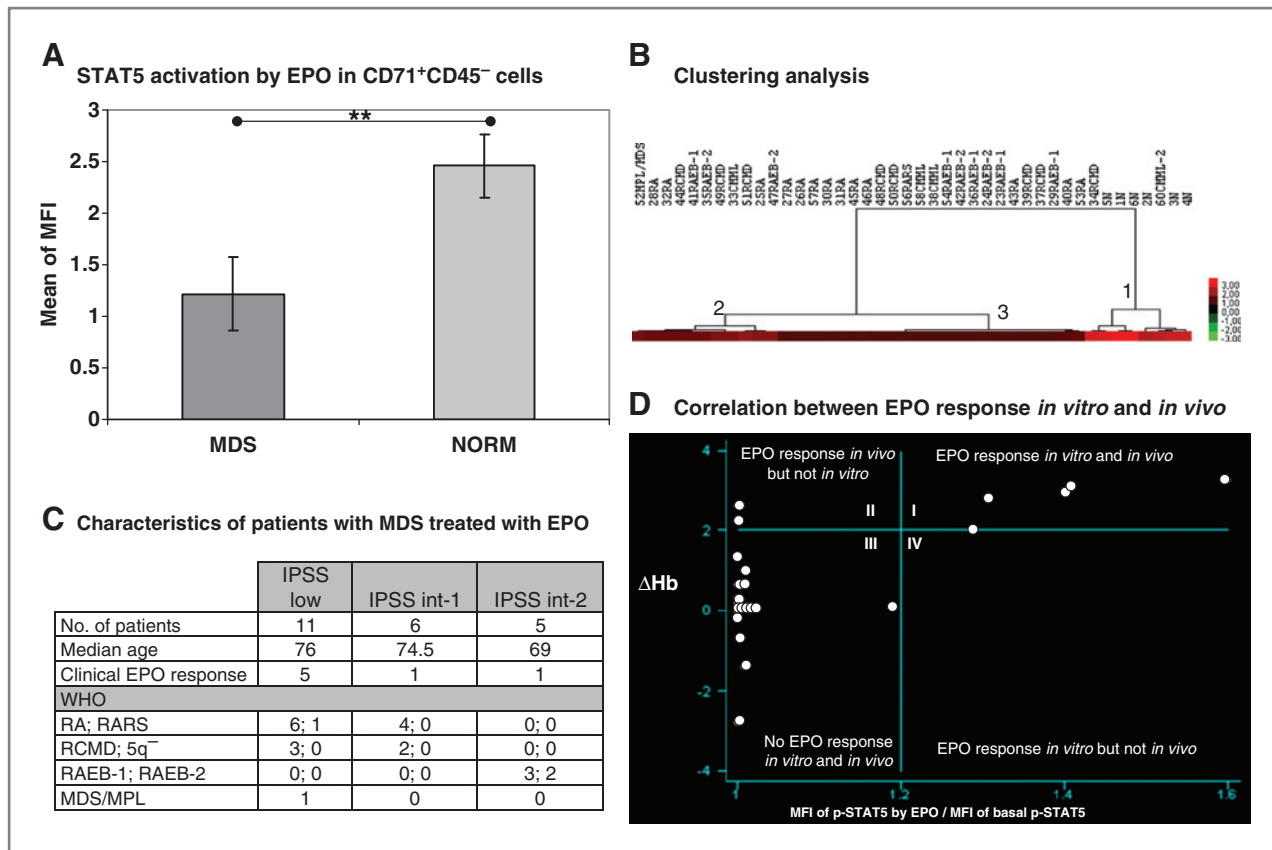
**Figure 2.** ERK1/2 activation in Kasumi-1 cells and normal BMMCs. A, flow cytometric analysis of ERK1/2 activation before (top right) and after (bottom right) TPA treatment in Kasumi-1 cells. B, immunoblot analysis of ERK1/2 activation before and after TPA treatment in Kasumi-1 cells. Full-length blots are presented in Supplementary Fig. S6A. C, flow cytometric analysis of ERK1/2 activation before and after erythropoietin (EPO) and TPA treatment in BMMCs obtained from healthy donors: isotypic control (top left), p-ERK1/2 basal activation (top right), p-ERK1/2 activation after erythropoietin treatment (bottom left), and p-ERK1/2 activation after TPA treatment (bottom right). The CD45<sup>+</sup> cells are indicated in orange, the CD71<sup>+</sup>CD45<sup>-</sup> cells are in violet, the CD34<sup>+</sup> cells are shown in dark blue, and all activated cells are shown in light blue. D, immunoblot analysis of ERK1/2 activation before and after TPA and erythropoietin treatment of normal BMMCs. Full-length blots are presented in Supplementary Fig. S6B.

erythropoietin according to accepted clinical criteria, in that all 22 were transfusion-independent, with endogenous serum erythropoietin levels <500 U/L, and 17 of 22 had an absence of blasts in the BM. Clinical response to erythropoietin was defined according to IGW 2006 criteria (19) as an increase of 2 g/dL in Hb levels after 8 weeks of erythropoietin treatment. Erythropoietin response *in vitro* was defined as a greater than 1.2-fold MFI increase in STAT5 activation. Characteristics of patients treated with erythropoietin are reported in Fig. 3C. A strong correlation between the erythropoietin response *in vitro* and in the clinic was observed in 20 of 22 cases (90.9%; the Spearman  $\rho = 0.62$  and  $P = 0.002$ ; Fig. 3D and Supplementary Table S6). The *in vitro* response was highly predictive of *in vivo* response to erythropoietin, with a sensitivity of 71% (95% CI, 36.0–92.0), a specificity of 100% (95% CI, 80.0–100.0), a positive predictive value of 100% (95% CI, 56.6–100.0), and a negative predictive value of 88% (95% CI, 66.0–96.7).

The 2 MDS cases that did not show a correlation with erythropoietin response *in vivo* had high basal STAT5 phosphorylation ( $2 \leq \text{MFI} < 5$ ). Moreover, in 8 of 22 cases the immunophenotype of CD34<sup>+</sup> cells was evaluated with a panel of antibodies applied by Westers and colleagues (20). Consistent with these authors' observations, in 5 of 8 MDS cases the aberrant immunophenotype of myeloid blasts correlated with erythropoietin

treatment failure, whereas 1 of 8 case did respond to erythropoietin and presented a normal CD34<sup>+</sup> phenotype. In contrast, the remaining 2 of 8 cases responded to erythropoietin treatment *in vivo*, despite an aberrant CD34<sup>+</sup> phenotypic pattern.

**G-CSF stimulation.** G-CSF stimulation was examined in 40 of 60 MDS cases and 6 healthy donors. This analysis could not be conducted in 6 MDS cases in which CD34<sup>+</sup> cells were absent. G-CSF 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<sup>+</sup>CD45<sup>-</sup> MDS cells, whereas it induced STAT5 activation in CD45<sup>+</sup> cells in 9 of 40 cases (data not shown). CD34<sup>+</sup> cells showed a 1.3- to 10-fold G-CSF-dependent activation of STAT5 in 32 of 34 MDS cases, whereas in normal cells, STAT5 was activated 1.9- to 2.8-fold in response to G-CSF (Supplementary Table S4). Statistical analysis indicated no significant difference in STAT5 activation in response to G-CSF in CD34<sup>+</sup> cells from MDS cases as compared with normal controls ( $P = 0.49$ ; Fig. 4A). Cluster analysis revealed that the pattern of STAT5 phosphorylation after G-CSF was heterogeneous (Fig. 4B). Flow cytometric analysis of STAT5 activation after G-CSF stimulation in one case (#37) is represented in Supplementary Fig. S5B. Moreover, CD34<sup>+</sup> cells showed G-CSF-dependent activation of ERK1/2 in 6



**Figure 3.** A, STAT5 activation by erythropoietin (EPO) in CD71<sup>+</sup>CD45<sup>-</sup> cells. erythropoietin-dependent activation of STAT5 in CD71<sup>+</sup>CD45<sup>-</sup> 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 about STAT5 phosphorylation in CD71<sup>+</sup>CD45<sup>-</sup> cells from 36 MDS cases and 6 normal BMMC samples after erythropoietin 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<sup>+</sup>CD45<sup>-</sup> cells. C, characteristics of patients with MDS treated with erythropoietin. D, correlation between erythropoietin response *in vitro* and *in vivo*. The x-axis indicates STAT5 activation following erythropoietin stimulation whereas the y-axis indicates the increase in hemoglobin levels after erythropoietin treatment *in vivo*. The cases with positive or negative responses to erythropoietin 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 erythropoietin (negative correlation between *in vitro* and *in vivo* responses) fall within quadrant II ( $n = 2$ ) and IV ( $n = 0$ ). The original graphic is presented in Supplementary Fig. S6C.

of 8 MDS cases and in all normal cases with an MFI ratio of between 1.3 and 1.9 (Fig. 4C).

## Discussion

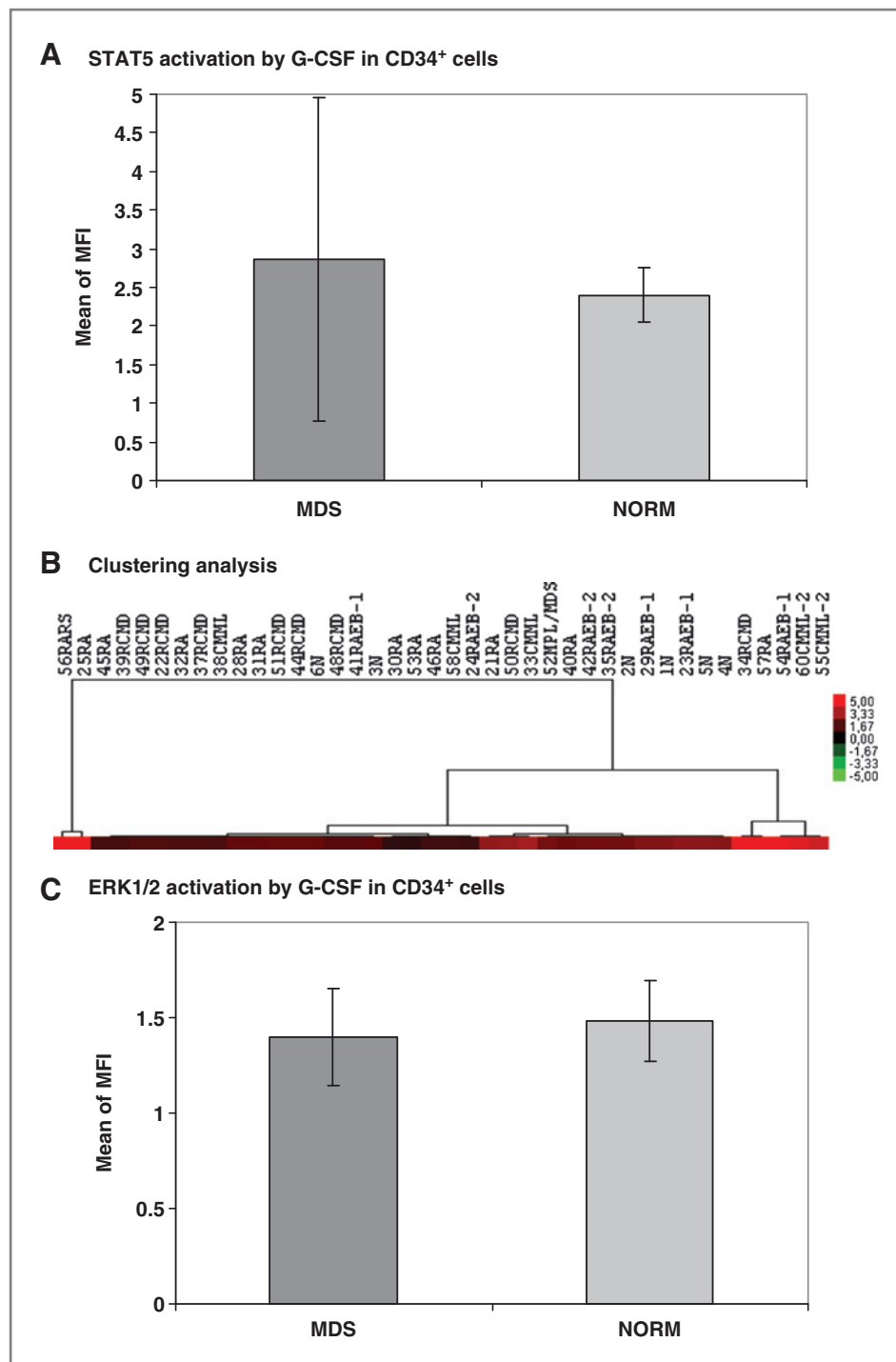
MDS are a complex group of diseases. Very few studies have focused on dissecting the signaling pathways in MDS cells (13, 21). This study evaluated the spontaneous basal activation of ERK1/2, p38 MAPK, STAT5, and caspase-3 in 60 primary human MDS cases, and the modulation of this activation by erythropoietin and G-CSF using a flow cytometric method which allowed quantitation of phosphorylation in the distinct CD45<sup>+</sup>, CD34<sup>+</sup>, and CD71<sup>+</sup>CD45<sup>-</sup> 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<sup>+</sup> 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 patients with MDS due to defective self-renewal, differentiation, and quiescence (22).

ERK1/2 is only marginally activated in basal conditions in CD34<sup>+</sup> cells, and completely unphosphorylated in



**Figure 4.** A, STAT5 activation by G-CSF in CD34<sup>+</sup> cells. G-CSF-dependent activation of STAT5 in CD34<sup>+</sup> 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<sup>+</sup> cells from 34 MDS cases and 6 normal BMNC 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 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<sup>+</sup> cells. C, ERK1/2 activation by G-CSF in CD34<sup>+</sup> cells. G-CSF-dependent activation of ERK1/2 in CD34<sup>+</sup> MDS cells ( $n = 8$ ) and healthy donor cells ( $n = 6$ ) is indicated by the ratio of the MFI of stimulated versus unstimulated cells.

CD71<sup>+</sup>CD45<sup>-</sup> cells, both in normal donors and in MDS cases. The erythroid subpopulation appeared inert in normal BMNCs, whereas CD71<sup>+</sup>CD45<sup>-</sup> 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 morphologic features.

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 erythropoietin was defective in CD71<sup>+</sup>CD45<sup>-</sup> MDS



marrow cells. A defect in STAT5 gene expression characterizes lenalidomide responsiveness irrespective of the presence of the 5q deletion and is an essential prerequisite for lenalidomide sensitivity (27). As already shown by other authors, STAT5 activation in response to *in vitro* erythropoietin was absent or significantly reduced in all MDS cases compared with normal controls. No significant differences in the presence of erythropoietin responsive, nor in the burden of the MDS clone, as indicated by abnormal metaphases (data not shown), were detected between responders and nonresponders. This abnormality seems to allow a clear distinction between normal and MDS CD71<sup>+</sup>CD45<sup>-</sup> 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<sup>+</sup>CD45<sup>-</sup> cells (9, 27). Even when all metaphases scored are abnormal, the response to erythropoietin may be present and thus attributed to the MDS clone (case #25; see Results).

*In vitro* response to erythropoietin, measured by cytofluorimetry in CD71<sup>+</sup>CD45<sup>-</sup> 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 biologic/clinical parameters such as endogenous erythropoietin levels <500 U/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 erythropoietin (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 nonresponsive patients. Recently, an aberrant phenotype of CD34<sup>+</sup> cells in patients with MDS has been shown to be predictive of the response to erythropoietin (20). The MDS cases with an aberrant CD34<sup>+</sup> phenotype according to ELN guidelines (1) were also erythropoietin insensitive in the STAT5 phosphorylation test, confirming the correlation between erythropoietin insensitivity and CD34 immunophenotype. An inconsistency of the 2 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* erythropoietin response, particularly as it is independent of the presence of CD34<sup>+</sup> 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 hematopoietic stem cell transplantation (HSCT) and immunosuppressive drugs was evaluated (31, 32).

Attempts have been made to distinguish an erythropoietin response signature in MDS and, in fact, differential expression of 37 genes could separate erythropoietin-responsive and nonresponsive cases with similar biologic and clinical characteristics (33). None of the genes dif-

ferentially expressed were directly linked to erythroid maturation. Similarly to what is observed for erythropoietin, non-5q<sup>-</sup> MDS cases responding to lenalidomide did not vary significantly from nonresponders 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 erythropoietin has been shown recently (21). We originally attributed our failure to observe a high level of phosphorylation of ERK1/2 in response to erythropoietin 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 BMMCs.

Finally, G-CSF promotes STAT5 and ERK1/2 phosphorylation at a level that is comparable with that observed in normal CD34<sup>+</sup> cells. The response to G-CSF is therefore not altered in CD34<sup>+</sup> MDS cells, and thus does not identify a pattern typical of dysplastic hematopoietic stem cells. Most probably, earlier stem cell populations such as CD34<sup>+</sup>CD38<sup>-</sup> or CD133<sup>+</sup> cells should be evaluated in this sense, as gene expression profiling has indicated significant differences compared with normal counterparts (35). In addition, these 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. These data also show that the erythropoietin clinical response may be predicted with a rapid cytofluorimetric test that is more accurate than previously applied biologic/clinical parameters.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

**Conception and design:** V. Santini

**Development of methodology:** E. Spinelli, R. Caporale

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** E. Spinelli, R. Caporale, E. Masala, A. Gozzini, A. Sanna, F. Sassolini, A. Valencia, V. Santini

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** E. Spinelli, R. Caporale, A. Sanna, V. Santini

**Writing, review, and/or revision of the manuscript:** E. Spinelli, R. Caporale, V. Santini

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** E. Spinelli, F. Buchi, A. Valencia

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#### Acknowledgments

The authors thank Drs. V. Gattei and A. Zucchetto from Centro di Riferimento Oncologico, I.R.C.C.S., Aviano (Pordenone), Italy, for helpful discussion and suggestions concerning the cluster analysis; 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

The study was supported by research fundings: Regione Toscana, Bando salute 2009; Ente Cassa di Risparmio di Firenze (ECR); and Ministero per l'Istruzione, l'Università e la Ricerca (MIUR).

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Received March 14, 2011; revised February 27, 2012; accepted March 24, 2012; published OnlineFirst April 11, 2012.

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# Clinical Cancer Research

## Distinct Signal Transduction Abnormalities and Erythropoietin Response in Bone Marrow Hematopoietic Cell Subpopulations of Myelodysplastic Syndrome Patients

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*Clin Cancer Res* 2012;18:3079-3089. Published OnlineFirst April 11, 2012.

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