p-ERK1/2 is a predictive factor of response to erythropoiesis-stimulating agents in low/int-1 myelodysplastic syndromes

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ABSTRACT

Serum erythropoietin level less than 100U/L and a transfusion requirement of less than 2 units *per* month are the best predictive factors for response to treatment by erythropoiesis-stimulating agents in low/int-1 myelodysplastic syndromes. To investigate the factors influencing the response to erythropoiesis-stimulating agents, we enrolled 127 low/int-1 myelodysplastic syndrome patients at diagnosis in a biological study of erythropoiesis. The 54 non-responders had a significantly lower number of burst-forming unit-erythroid and colony-forming unit-erythroid than responders. Erythropoietin-dependent proliferation and survival, and phospho (p)-ERK1/2 expression in steady state and after erythropoietin stimulation were defective in cultured erythroblasts. By flow cytometry, p-ERK1/2 was significantly lower in bone marrow $CD45^-/CD71^+/GPA^-$ cells from non-responders compared to responders or controls. Receiver Operator Characteristic curve analysis showed that this flow cytometry test was a sensitive biomarker for predicting the response to erythropoiesis-stimulating agents.

Key words: myelodysplastic syndromes, red cells, MAPkinase, erythropoiesis-stimulating agents.

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Introduction

Treatments in low/int-1 risk myelodysplastic syndromes (MDS) aim at improving cytopenias by red blood cell (RBC) transfusions and/or erythropoiesis-stimulating agents (ESA) like epoietin α/β, darbepoietin, alone or in combination with granulocyte colony-stimulating factor (G-CSF).[1-3] Recent studies demonstrated that ESA treatments may have a favorable impact on overall survival.[4,5] A randomized phase III study comparing best supportive care to ESA did not confirm the favorable impact of ESA treatment on overall survival, but demonstrated that responders had significantly better survival than non-responders.[6] Serum erythropoietin (EPO) levels less than 100U/L, and transfusion requirement of less than 2 units of red blood cell (RBC) *per* month have been associated with the best response rates hardly reaching 39% to 62%.[6-9] A decision model has been proposed and validated for anemic MDS patients.[10] However, the limited response rate suggests that other factors may influence the response.[3] The aim of our study was to identify these factors.

Design and Methods

Patients

One hundred and twenty-seven unselected patients were included in a biological study between June 2004 and December 2009 at Cochin Hospital. Inclusion criteria were a diagnosis of MDS according to the WHO classification, a low or int-1 IPSS, a serum EPO level less than 500U/L, hemoglobin (Hb) level less than 11 g/dL for more than two months or a transfusion requirement for more than one RBC *per* eight weeks for four months or more. ESA treatment was epoietin α or β at doses of 60,000 UI per week, or darbepoietin α at

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a dose of 300 μg per week ± G-CSF. Response to treatment was evaluated at week 12 according to the IWG 2006 response criteria.[11] Dyserythropoiesis was evaluated as absent, mild or major. A flow chart is shown in the *Online Supplementary Figure S1*. Control bone marrows (BM) were obtained from 34 volunteers. This study was approved by the local ethics committee and informed consent was obtained from patients and controls.

Cell cultures

Semi-solid cultures in methylcellulose (StemCell Technologies, Vancouver, Canada) containing IL-3, IL-6, EPO, GM-CSF, and SCF were used to quantify erythroid or granulo-monocytic progenitors. *In vitro* erythroid progenitor expansion was obtained in liquid culture conditions from BM CD34$^+$ cells as described.[12] Cells were harvested for immunofluorescence or flow cytometry studies.

Immunofluorescence (IF)

Cultured erythroblasts were cytospinned, fixed with 3.7% paraformaldehyde for 15 min at room temperature, and permeabilized with an ice-cold methanol/acetone mixture (1:1) for 10 min. After saturation in 10% horse serum, 1% BSA for one hour in room temperature, cells were incubated with p-Thr202/Tyr204-ERK1/2 rabbit polyclonal antibody (Cell Signaling Technology, Vancouver, Canada) at 1:100 in 1% BSA 0.2% Triton X-100 for one hour at 37°C and with chicken anti-rabbit Alexa Fluor 594-conjugated antibody or goat anti-rabbit Chromeo 642-conjugated antibody (Invitrogen, Carlsbad, CA). Slides were visualized with a Leica DMI 6000 and photographed with a 63X lens.

Immunohistochemistry (IHC)

BM biopsies were collected at diagnosis before ESA treatment, fixed in formalin and included in paraffin. Before labeling, 4μ-thick sections of BM tissue were deparaffined in xylem, dehydrated in 100% ethanol and washed in TRIS-HCl 0.05M, NaCl 0.15 M pH7.6 before performing antigen retrieval in sodium citrate 0.1 M, citric acid 0.1 M pH6.6 for 20 min at 95°C. Incubation with the p-Thr202/Tyr204-ERK1/2 or ERK1/2 (Cell Signaling), and then with a biotinylated secondary antibody (Dako, Glostrup, Denmark) in TBS containing 0.3% BSA was followed by the addition of horseradish peroxidase coupled to streptavidin. Diaminobenzidine was used as peroxidase substrate. The slides were countercolored with hematoxylin and fixed in ethanol/xylen. Microphotographs were taken using a Leica DXC 950P.

Flow cytometry (FC)

For phospho (p)-ERK1/2 labeling, total BM cells were incubated with CD45-phycoerythrin cyanine (PC)7, CD71-fluorescein isothiocyanate (FITC) and glycophorin A (GPA)-phycoerythrin (PE; Beckman Coulter, Miami, FL) and fixed with 4% paraformaldehyde (Sigma Aldrich, Saint-Louis, MO, USA) for 10 min. Cells were permeabilized using Triton X-100 0.1% PBS for 30 min at 37°C, and for 10 min at 4°C in methanol:PBS (v:v) and then labeled with the monoclonal antibody to p-Thr202/Tyr204 ERK1/2 or isotypic control coupled to Alexa-647 (BD Biosciences, San Jose, CA, USA).[13] All analyses were performed using a Cytomics FC500 flow cytometer (Beckman Coulter) equipped with CXP software.

Statistical analysis

Values are expressed as median and interquartile range (IQR) for continuous variables and were compared using the Wilcoxon's test or as mean and standard deviation (SD) compared using Student's t-test. Categorical variables are reported as count and percentage and were compared using Fisher's exact test. Sensitivity and specificity of p-ERK1/2 assay and other parameters were evaluated with the Receiver Operating Characteristic (ROC) curve. The areas under the curve (AUC) were computed and compared across the parameters by the method of Hanley and McNeil.[14] The Kaplan-Meier estimator was used to evaluate the probability of failure to ESA treatment over time, according to p-ERK1/2 level. Comparisons between the corresponding estimates were carried out with the log rank test. All statistical analyses were two-sided and P values <0.05 were considered statistically significant. Analyses were performed using the SAS package version 9.01 (SAS Institute, Cary, NC, USA).

Results and Discussion

Response to ESA treatment

This biological study enrolled 127 patients at diagnosis referred as refractory anemia or cytopenia (RA or RC) and 5q- syndrome (n=37), RA with ringed-sideroblasts (RARS, n=26), RC with multilineage dysplasia (RCMD, n=18), RCMD with ringed-sideroblasts (RCMD-RS, n=10) and RA with an excess of blasts below 10% (RAEB-1, n=36), according to the WHO classification. IPSS was low in 67 patients and int-1 in 50 patients. Median Hb level was 9.6 g/dL and median serum EPO level was 50 U/L. Among 115 informative patients, 53 received RBC transfusion (Table 1). All patients received ESA treatment. After 12 weeks, 73 patients met IWG 2006 criteria for erythroid improvement and were referred to as the responder group and 54 were non-responders to ESA. There was no difference between responders and non-responders in terms of age, gender, WHO, Hb level, median percentage of blasts or erythroblasts, dyserythropoiesis, karyotype, IPSS, type of ESA treatment, and additional G-CSF treatment. As expected, the number of patients with serum EPO level 100U/L or over or receiving 2 units or more RBC *per* month was significantly higher in the non-responders compared to the responders, and the median delay before treatment was equivalent in the two groups (Table 1).

Impaired erythropoiesis in the non-responsive patients

Hematopoiesis was investigated by the quantification of BFU-E and CFU-E erythroid progenitors and CFU-GM granulo-monocytic progenitors in methylcellulose assays. BFU-E and CFU-E were significantly decreased in non-responders as compared to responders and to controls (P<0.001; Figure 1 and *data not shown*), while CFU-GM were equivalent in the three groups (*data not shown*). Erythroblasts were amplified *in vitro* from BM CD34$^+$ cells. The mean cumulated number of cultured erythroblasts and the mean number of CD34$^+$-deriving BFU-E were approximately 3-fold lower for non-responders than for responders or normal controls (Student's t-test; P<0.05; *Online Supplementary Figure S2A and B*). The failure of *in vitro* BFU-E/CFU-E growth in the presence of EPO is a major characteristic of non-responders to ESA. It has been demonstrated that low circulating BFU-E correlated with the non-response and was a good prognostic marker for the response to treatment.[15] In addition, we observed here that erythroid precursor apoptosis was significantly increased in non-responders compared to responders (*Online Supplementary Figure S2C*; Wilcoxon's test; P=0.035). Non-responders exhibited a defect of EPO-

dependent proliferation and survival. EPO-R mRNA level was normal (*data not shown*). Neither mutations in the *EPOR* gene nor abnormal expression of EPO binding sites at the cell surface have been reported so far, supporting the hypothesis of EPO-R signaling abnormality.

Defective p-ERK1/2 expression in the non-responders

The ERK1/2 pathway is required for cell proliferation and, depending on the intensity of activation, may control cell differentiation of primary normal human or avian erythroid cells.[16,17] To investigate the EPO-R signaling pathway, we analyzed the activation of ERK1/2 in cultured erythroblasts by indirect IF using anti-p-ERK1/2 antibodies. Cells were harvested in steady-state conditions or after cytokine starvation and subsequent stimulation with 10 UI/mL EPO for 10 min. Both control (n=1) and MDS erythroblasts from responders (n=3) expressed p-ERK1/2 in steady state and after stimulation with EPO. Erythroblasts deriving from non-responders (n=3) lacked p-ERK1/2 labeling whatever the conditions (Figure 2A). By contrast with that observed in AML and high risk MDS,[13,18,19] p-ERK1/2 was never detected after cytokine starvation of cultured erythroblasts showing that the ERK1/2 pathway was not constitutively activated in low/int-1 MDS patients whatever their responder or non-responder status to ESA treatment.

We then assessed p-ERK1/2 levels on BM biopsies by IHC. In all 4 responders, we detected a strong cytoplasmic p-ERK1/2 staining in immature cells recognized upon the morphological appearance of their outlying nuclei, which displayed one or two prominent nucleoli. By contrast, p-ERK1/2 was detectable only in a few scattered cells of granulocytic lineage in all the 5 non-responders (Figure 2B).

To analyze p-ERK1/2 expression on fresh BM samples at diagnosis, we developed a four-color FC assay. p-ERK1/2 expression was quantified in the $CD45^-/CD71^+/GPA^-$ population in 55 consecutive patients (30 responders and 25 non-responders) and 10 controls as a ratio of median fluorescence intensity (RFI) between specific to isotypic control antibodies (Figure 2C). The UT-7 cell line was used as

Table 1. Clinical and biological parameters of MDS patients.

	All	Responders	Non-responders	*P* value
n 127	73	54		
Age median [IQR]	74 [69-81]	76 [71-81]	74 [69-81]	0.325
Gender M/F ratio	1.2	1.4	1.1	0.472
WHO				
RA/RC/5q-	37	21 (29%)	16 (30%)	
RCMD	18	12 (16%)	6 (11%)	
RAEB-1	36	20 (27%)	16 (30%)	0.721
RARS	26	14 (20%)	12 (22%)	
RCMD-RS	10	6 (8%)	4 (7%)	
Hb (g/dL) median [IQR]	9.6 [8.7-10.4]	9.9 [9.3-10.4]	9.2 [8.3-10.4]	0.184
Bone Marrow				
Blasts median % [IQR]	4 [2-5]	4 [2-5]	4 [3-6]	0.227
Erythroblasts median % [IQR]	30 [22-42]	30 [22-42]	30 [23-41]	0.829
Dyserythropoiesis n (%)	82 (69%)	48 (70%)	34 (68%)	0.851
Karyotype				
Good	101	62	39	
Intermediate	14	5	9	0.273
Poor	2	2	0	
missing	10	4	6	
IPSS				
Low	67	40 (58%)	27 (56%)	1.000
Int-1	50	29 (42%)	21 (44%)	
missing	10	4	6	
Serum EPO (U/L)				
median [IQR]	50 [22-162]	35 [17-98]	122 [45-234]	0.005
<100 U/L (n)	58	42	16	
≥100 U/L (n)	33	14	19	0.006
Transfusions				
n	53/115	23/66 (35%)	29/49 (59%)	
< 2 units/month (n)	8	8	0	
≥2 units/month (n)	45	16	29	<0.001
Time before treatment				
Median duration in months [IQR]	1.5 [0.7-11.2]	1.3 [0.4-7.8]	3.4 [1.0-19.8]	0.146
Treatment (n)				
Epoietin α or β	79	48 (66%)	31 (57%)	
Darbepoietin	48	25 (34%)	23 (42%)	0.360
G-CSF	42	20 (27%)	22 (41%)	0.101

Indicated Hb level was the value at diagnosis. Dyserythropoiesis was evaluated as absent, mild or major (if both nuclear and cytoplasmic abnormalities were present). Values were expressed as median (IQR: interquartile range) for continuous variables and were compared using the two-tailed Wilcoxon's tests. Categorical variables were reported as count and percentage and were compared using Fisher's exact tests. P values <0.05 were considered as significant.

control to test the inter-assay variability. Median RFI for p-ERK1/2 was 1.56 [IQR: 1.23-3.32] and 1.36 [IQR: 1.16-1.84] in controls and responders, respectively, and there was no significant difference between the two groups (Wilcoxon's test; P=0.193). By contrast, there was a significant difference in median RFI between non-responders (1.12 [IQR: 1.02-1.17]) and responders (P<0.001; Figure 2C). Thus, altogether, these results show that ERK activation is greatly decreased in patients non-responsive to ESA treatment compared to responders. These low p-ERK1/2 levels in non-responders may reflect the early defect of progenitor growth capability.

p-ERK1/2 in $CD45^-/CD71^+/GPA^-$ cells, as well as the EPO serum level, the number of transfusions *per* month and the number of BFU-E, represented significant variables between responders compared to non-responders (Table 1 and Figure 1). To evaluate their power to predict the response to ESA, ROCs were constructed for each parameter and the AUCs were calculated. ROC analysis of the FC test demonstrated a 0.84 area under the curve [95%CI: 0.73-0.94]. For a calculated cut-off value at 1.30, the sensitivity was 92% [95%CI: 74-99] and the specificity was 67% [95%CI: 47-83] indicating that p-ERK1/2 was a good predictive factor for the response to ESA (Figure 2D). The AUCs for the serum EPO level, the number of transfusions *per* month and the number of BFU-E were 0.72 [95% CI: 0.61-0.83], 0.65 [95% CI: 0.54-0.75] and 0.75 [95% CI: 0.65-0.85], respectively. Comparison of AUCs demonstrated that p-ERK1/2 was equivalent to BFU-E number (P=0.261) and serum EPO level (P=0.134), and was superior to the number of transfusions per month (P=0.011) for predicting the response to ESA. A Kaplan-Meier curve showed that the probability of failure of response to ESA treatment significantly increased in patients with low p-ERK1/2 expression compared to the patients with p-ERK1/2 level over the threshold (Figure 2E; log rank test; P=0.015). Finally, we identified p-ERK1/2 level as a sensitive biomarker predictive of the response to ESA which could be available in all MDS irrespective of the size of BM samples.

FC analysis of phosphoproteins has been used to evaluate the prognosis of acute leukemias.[20,21] An FC score based on evidence of aberrant markers on blasts was recently proposed as a predictor of the response to ESA in MDS.[22]

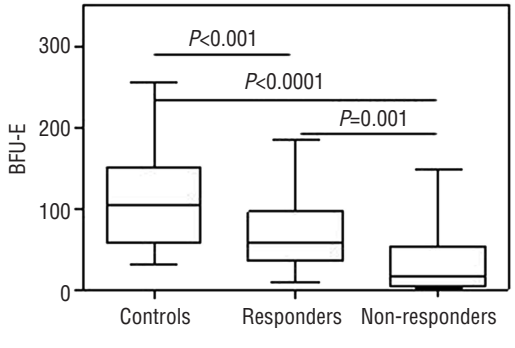

Figure 1. Quantification of erythroid progenitors in responders, non-responders and controls. BFU-E type derived-colonies were quantified in 73 responders, 54 non-responders and 34 controls in methylcellulose assays. Results are expressed as colony number *per* 105 BM mononuclear cells. Median value is indicated as a horizontal bar on the graphs (Wilcoxon's test for *P* values).

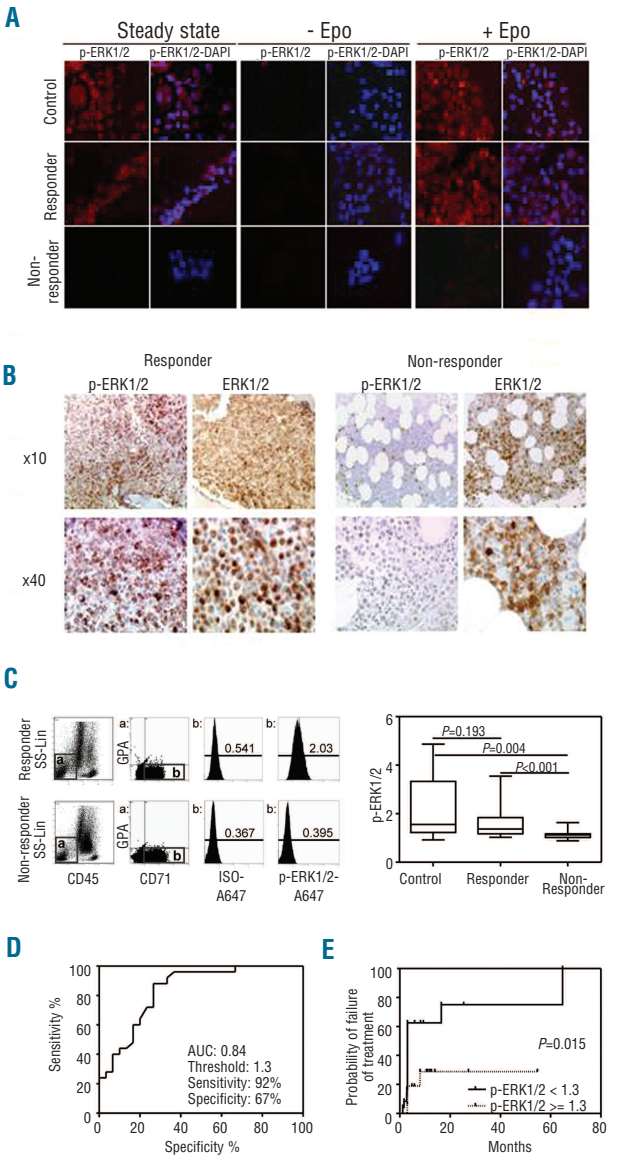

Figure 2. Defective expression of p-ERK1/2 in non-responders. (A) Immunofluorescence to p-ERK1/2 in cultured erythroblasts: cells were harvested from liquid cultures either in steady state condition, or after EPO starvation for 4 h, or after starvation and re-stimulation with 10 UI/mL EPO for 10 min, and then labeled with rabbit anti-p-ERK1/2 (red). DAPI staining was used to visualize the nucleus. Slides were examined under fluorescence microscope (magnification x63) and representative images of 3 responders, 3 non-responders and one control are presented. (B) Immunohistochemistry to p-ERK1/2 on BM biopsies: sections stained with the anti-p-ERK1/2 or anti-ERK1/2 antibodies, and then with a biotinylated secondary antibody. DAB was used as substrate for horseradish peroxidase coupled to streptavidin. The slides were countercolored in hematoxylin. Microphotographs at x10 and x40 magnification are representative of 4 responders and 5 non-responders. (C, D and E) Flow cytometry assay for p-ERK1/2 quantification in total BM aspirates. (C) Total BM cells were permeabilized and labeled with anti-p-ERK1/2-Alexa647, CD45-PC7, CD71-FITC and anti-GPA-PE antibodies. RFI of p-ERK1/2 to isotypic control in the $CD45^-/CD71^+/GPA^-$ population was determined. (Left) Representative histograms of one responder and one non-responder are shown. (Right) Boxplots comparing RFI between responders (n=30), non-responders (n=25), and controls (n=10). Median values are indicated as horizontal bars. Wilcoxon's test for *P* values. (D) Receiver Operating Characteristic (ROC) curve of p-ERK1/2 FC test. AUC: area under curve. (E) Kaplan-Meier analysis. Probability of failure of response to ESA treatment according to the threshold value of RFI (log rank test for *P* value).

In the present study, among patients with less than 100U/L serum EPO and receiving less than 2 units RBC *per* month, those with high p-ERK1/2 level were all responsive to ESA, while only 20% of patients with low p-ERK1/2 level achieved an erythroid improvement. Although this should be confirmed in a larger cohort of patients, our data suggest that the combination of p-ERK1/2 to serum EPO level and transfusion requirement could help predict the response to ESA.

Authorship and Disclosures

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