Abnormal Hedgehog pathway in myelodysplastic syndrome and its impact on patients' outcome

Myelodysplastic syndromes (MDS) are clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis and by cytopenias. Approximately 30% of patients with MDS progress to acute myeloid leukemia.[1] Thus, it is important to identify risk factors for AML progression and to guide treatment decisions that can have a positive impact on patient mortality. The Hedgehog (Hh) pathway is an important mediator of early hematopoietic development[2] and, for the last two decades, a number of studies have linked an abnormal Hh signaling to distinct human malignancies.[3] Previous evidence has suggested that Hh signaling plays a role in hematopoietic malignancies.[4] Kobune *et al*. showed activation of Hh signaling in primary CD34$^+$ blasts from AML[5] and, recently, these same authors reported the expression of Indian Hh (IHH) and Smoothed (SMO) in AML- and MDS-derived CD34$^+$ cells.[6] Here, we characterize expression of central components of the Hh pathway in the bone marrow (BM) of MDS patients. Moreover, we investigated the impact of the mRNA expression of these key elements on MDS outcome and survival.

Patients with diagnosis of *de novo* MDS (n=69), untreated at the time of sample collection, were included in the study. Among these 69 patients, 63 had their BM collected for mRNA, 49 had biopsies collected for immunohistochemistry (IHQ) of whom 40 had both mRNA and IHQ. Patients' characteristics are described in Table 1. Nineteen healthy donors and 7 patients with megaloblastic anemia (MA) were analyzed as control; these had their BM collected for mRNA and protein expression analysis, respectively. All subjects provided written informed consent and the study was approved by the local Ethics Committee. Analysis of comparison between groups was performed by the Mann-Whitney method and co-variance (ANCOVA) controlling for age, followed by *post hoc* comparisons using the Tukey test, when applied. Univariate and multivariate Cox regression were used to estimate overall survival (OS), event-free survival,[7] and AML evolution for MDS patients. OS was defined as the time (in months) between the date of sampling and the date of death (for deceased patients) or last follow up (for censored patients). Event-free survival (EFS) was defined as the time (in months) between the sampling and the date of the first event (death or MDS progression to a higher risk MDS category by WHO or to AML with myelodysplastic-related changes) or last follow up (for censored patients). AML evolution was defined as the time (in months) from the date of sampling to diagnosis of AML. Kaplan-Meier analysis, based on the gene expression categorized as median, and log rank test were used to construct survival curves. All tests were two-tailed and $P<0.05$ was considered statistically significant.

For protein expression analysis, the expression of Hh ligands, Dessert Hh (DHH), Sonic Hh (SHH) and Indian Hh (IHH) were compared in BM biopsies from MDS and from megaloblastic anemia (MA) patients by immunohisto-

Table 1. Patients' characteristics.

Characteristics	Value
Age (yr) at time of sampling; median (range)	65 (16-91)
Gender, n (%)	
Male/female	42 (61) / 27 (39)
WHO 2008, n	
RCUD/RCMD/RARS	12/29/4
RAEB-1/RAEB-2	14/10
IPSS risk group, n	
Low/ Int-1	23/31
Int-2/High	8/3
Not available	4

WHO: World Health Organization; RCUD: refractory cytopenia with unilineage dysplasia; RCMD: refractory cytopenia with multilineage dysplasia; RARS: refractory anemia with ringed sideroblasts; RAEB-1: refractory cytopenia with excess blasts – 1; RAEB-2: refractory cytopenia with excess blasts – 2; IPSS: International Prognostic Scoring System; Int: intermediate.

Table 2. Univariate analyses of survival outcomes.

Factor	Event-free survival		Overall survival		AML evolution	
	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*	Hazard Ratio (95% CI)	*P*
Gender						
Male *vs.* female	1.70 (0.80-3.60)	0.16	1.60 (0.75-3.41)	0.21	2.18 (0.71-6.66)	0.17
WHO 2008 classification*						
RAEB-1/RAEB-2 *vs.* RCUD/RCMD/RARS	5.07 (2.43-10.59)	<0.0001	5.85 (2.73-12.5)	<0.0001	7.75 (2.33-25.7)	0.001
IPSS risk group*						
Int-2 / High *vs.*						
Int-1 /Low	5.53 (2.42-12.6)	<0.0001	6.56 (2.85-15.0)	<0.0001	4.63 (1.33-16.1)	0.01
Gene expression; continuous variable&						
Age at sampling	1.00 (0.98-1.03)	0.51	1.00 (0.98-1.03)	0.63	0.99 (0.96-1.03)	0.81
SMO expression#	1.009 (1.00-1.01)	0.02	1.00 (1.00-1.01)	0.04	1.01 (1.00-1.02)	0.00 3
PTCH1 expression	1.02 (0.97-1.07)	0.44	1.00 (0.94-1.06)	0.95	1.05 (0.98-1.12)	0.13
SUFU expression	1.04 (0.90-1.19)	0.58	1.00 (0.86-1.17)	0.94	1.09 (0.91-1.31)	0.32
GLI1 expression	1.09 (0.31-3.75)	0.89	0.18 (0.006-5.90)	0.33	0.41 (0.01-13.7)	0.62
GLI2 expression	2.46 (0.25-23.7)	0.43	3.40 (0.34-33.7)	0.29	2.00 (0.03-143.5)	0.75
GLI3 expression	1.005 (0.99-1.02)	0.47	1.00 (0.99-1.02)	0.38	1.004 (0.98-1.02)	0.75

*MDS: myelodysplastic syndromes; AML: acute myeloid leukemia; WHO: World Health Organization; RCUD: refractory cytopenia with unilineage dysplasia; RCMD: refractory cytopenia with multilineage dysplasia; RARS: refractory anemia with ringed sideroblasts; RAEB-1: refractory cytopenia with excess blasts – 1; RAEB-2: refractory cytopenia with excess blasts – 2; IPSS: International Prognostic Scoring System; Int: intermediate. *Hazard ratio >1 indicates that the first feature has the poorest outcome. #Higher SMO expression predicts poorest outcome. &The number of patients tested for each gene is indicated: SMO=62, PTCH1= 62, SUFU=57, GLI1=62, GLI2=52, GLI3=56.*

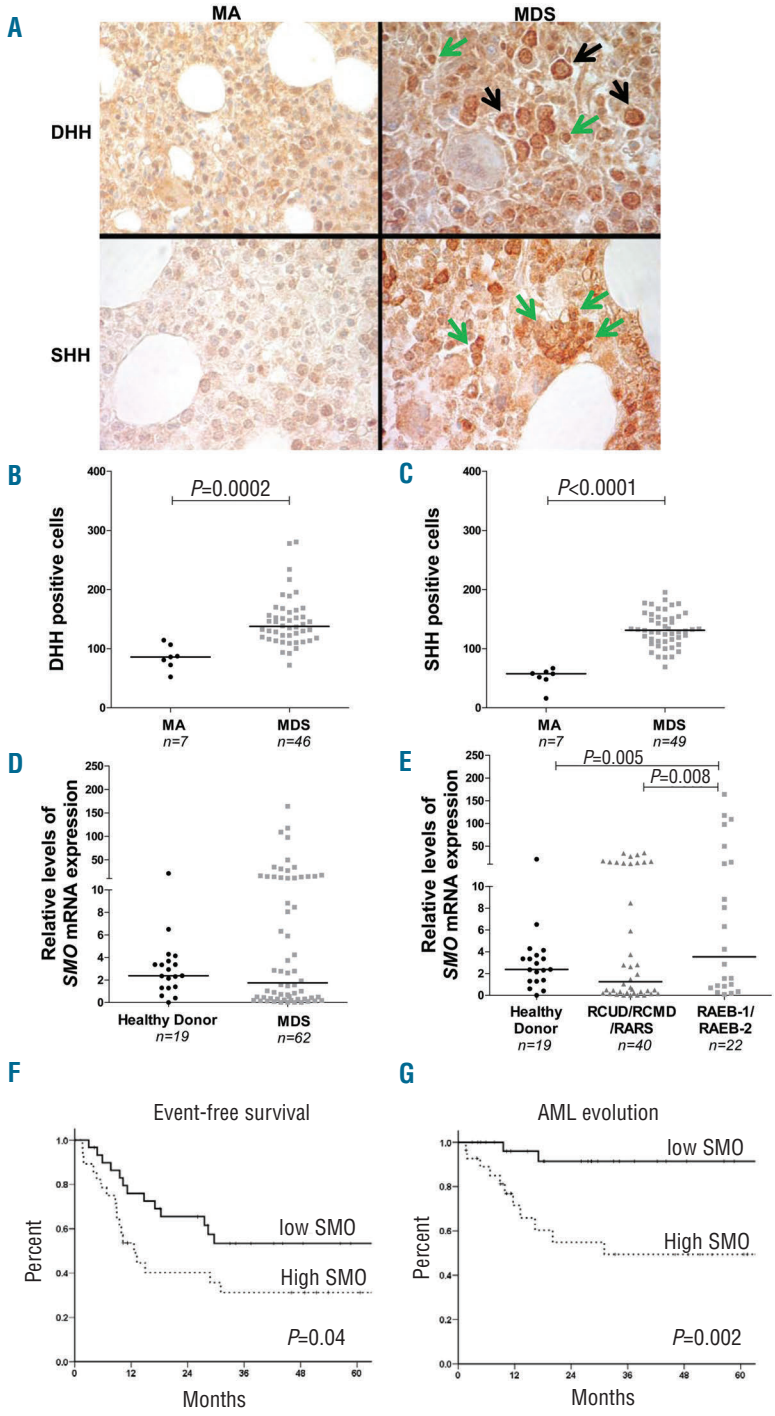

Figure 1. Hedgehog pathway is deregulated in MDS total bone marrow. (**A**) Immunohistochemical expression of DHH (upper panel) and SHH (lower panel) in bone marrow biopsies from MDS, and from megaloblastic anemia (MA) patients. Both antibodies were purchased from Santa Cruz and diluted at 1:50 in citrate buffer. Green and black arrows indicate likely erythroblasts and myeloid precursors, respectively. Graph shows quantitative analysis of IHQ, where four random high-powered fields from stained slides were captured at 20x objective magnification and visualized for manual scoring for positive cells, using ImageJ (*http://imagej.nih.gov/ij/*). Horizontal lines indicate medians of positive cells for DHH (**B**) and SHH (**C**). Quantitative PCR analysis of *SMO* mRNA expression in (**D**) total bone marrow cells from healthy donors and from patients with MDS and (**E**) stratified according to the WHO 2008 classification. Expression of *HPRT* transcripts was used as an endogenous control. The relative gene expression was calculated using the equation $2^{-\Delta\Delta CT}$ [11]. Horizontal lines indicate medians. Mann-Whitney test was used for comparisons between two groups (control *vs.* MDS). ANCOVA followed by *post hoc* Tukey was used for comparisons among control, RAEB-1/RAEB-2 and RCUD/RCMD/RARS groups. The numbers of individuals and *P* values are indicated in the graphs. Event-free survival (**F**) and freedom from AML evolution (**G**) of MDS patients categorized as median of *SMO* expression levels (Kaplan-Meier curves). Patients were subgrouped by high *SMO* expression (above median; 1.74) and low *SMO* expression (below median). *P* values are indicated in the graph (log rank test).

chemistry. We chose MA as a control, as ineffective hematopoiesis is a marker of both diseases although the causes are different.[8] Based on morphological aspects, DHH was likely expressed in both erythroid and myeloid precursors and SHH predominated in erythroid precursors, as positive cells were arranged in a nest (Figure 1A). DHH and SHH expressions were significantly higher in the MDS group (P=0.0002 and P<0.0001, respectively) (Figure 1B and C). There was no statistical difference between the MDS and the MA groups for IHH expression (*Online Supplementary Figure S1A*). Hh ligand protein expression did not predict either EFS nor OS (all P>0.05). There was no correlation of percentage of blasts with Hh ligand expression level (*data not shown*). Few studies have shown the deregulation of Hh signaling in the BM of hematologic malignancies by immunohistochemistry; high SHH protein levels, for example, have been described in the BM samples of patients with acute promyelocytic leukemia, AML and multiple myeloma.[9,10] This is the first time, to our knowledge, that an increase in Hh ligands has been described in the BM of MDS.

Subsequently, we used quantitative polymerase chain reaction (qPCR) to analyze the mRNA expression of molecules belonging to the Hh pathway in total BM cells from healthy donors and MDS patients. There was no statistical difference in *SMO* expression levels between control and

the MDS group (Figure 1D). Nevertheless, when MDS samples were classified, according to WHO 2008, we observed a significant increase of age-adjusted expression of *SMO* in the group of patients with RAEB-1 and RAEB-2 when compared to the control and to 5% or less BM blast MDS patients (RCUD, RCMD and RARS) (*P*=0.005 and *P*=0.008, respectively) (Figure 1E). There was no significant difference in age-adjusted expression of *Patched1 (PTCH1), Suppressor of fused protein (SUFU), GLI1, GL2* and *GLI3* transcripts between MDS patients and healthy donors (*Online Supplementary Figure S1B-F*). Consistent with these findings, high expression of SMO has been reported in human colonic tumors.[12] Recently, experimental data have shown that SMO was significantly increased in a mouse model for familial adenomatous polyposis. The authors also mentioned that SMO expression was increased in 9 of 20 cancer samples compared with the normal epithelium of the same patients. Unexpectedly, in that study, reduced expression of SMO did not suppress GLI-dependent Hh signaling in intestinal tumor cells, suggesting that SMO could contribute to the proliferation of those cells by a mechanism independent of GLI-mediated transcription.[13]

In our study, Kaplan-Meier analysis indicated a 5-year EFS of 31% *versus* 53% for patients with higher *SMO* expression (above median) *versus* lower *SMO* expression, respectively (*P*=0.04) (Figure 1F). The same was observed for 5-year AML evolution (49%>1.74 *SMO vs.* 91% <1.74 *SMO*; *P*=0.002) (Figure 1G). Univariate analysis showed that WHO diagnosis of RAEB-1 and RAEB-2, IPSS intermediate-2 and high risk, together with increased *SMO* expression (continuous variable) predicted worse EFS, OS and AML evolution (Table 2). As expected, the usual prognostic factors, including WHO 2008 classification and IPSS, remained independent predictors for EFS, OS, AML evolution, and for EFS and OS, respectively (*Online Supplementary Table S1*). Our findings corroborate other studies that have identified SMO overexpression as being significantly associated with poor prognosis of hepatoblastoma,[14] malignant pleural mesothelioma,[15] and also as an independent prognostic factor for post-operative liver metastasis-free survival in colon cancer.[16] Recently, SMO expression was reported to induce fatal AML in a mouse model of MDS, resulting in a reduced survival and widespread expansion of immature myeloid cells.[17] Taken together, it seems reasonable to suggest that alterations in the Hh pathway might be involved in MDS progression; however, whether this mechanism is dependent or not on the canonical Hh pathway requires further investigation.

Hedgehog signaling has gained interest as a prognostic marker and as a potential therapeutic strategy in a range of cancers.[4] Here, we describe an abnormal Hh signaling pathway in total BM cell from MDS patients and the possible involvement of SMO in MDS progression.

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