Mesenchymal Stromal Cell Density Is Increased in Higher Grade Myelodysplastic Syndromes and Independently Predicts Survival

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ABSTRACT

Objectives: We retrospectively tested the prognostic and diagnostic significance of CD271+ mesenchymal stromal cell (MSC) density in cytopenic patients who underwent bone marrow biopsy to evaluate for myelodysplastic syndromes (MDS).

Methods: CD271+ MSC density was quantitated by automated image analysis of tissue microarray cores in 125 cytopenic patients: 40 lower grade MDS (<5% marrow blasts), 24 higher grade MDS, and 61 benign.

Results: CD271+ MSC density was increased in higher grade MDS compared with benign (P = .006) and lower grade MDS (P = .02). CD271+ MSC density was predictive of survival among patients with MDS independent of Revised International Prognostic Scoring System (IPSS-R), history of transfusion, therapy-related MDS, and fibrosis (hazard ratio, 3.4; P < .001). Among low or intermediate IPSS-R patients, median survival was significantly shorter in the high CD271+ MSC density group (47 vs 18 months, P < .02).

Conclusions: High CD271+ MSC density is characteristic of higher grade MDS and is associated with poor risk independent of known prognostic factors.

Persons with myelodysplastic syndromes (MDS) experience symptomatic cytopenias, and many die of complications of bone marrow failure or transformation to acute myeloid leukemia (AML). Diagnosis, prognostication, and therapeutic decision making for MDS are complicated by significant cytogenetic, clinical, and morphologic heterogeneity. Mesenchymal stromal cells (MSCs) are components of the normal bone marrow microenvironment capable of supporting self-renewing proliferation of CD34+ hematopoietic progenitor/stem cells. Perturbation of MSC populations has been shown to be sufficient to induce myelodysplasia in a mouse model. MSCs from patients with MDS are cytogenetically and functionally abnormal and demonstrable cytogenetic abnormalities in isolated MSCs correlate with worse clinical outcomes. The heterogeneity of MDS may be a manifestation of the reciprocal relationship of dysfunctional hematopoietic stem/progenitor cells with their also dysfunctional MSC partners.
Low-affinity nerve growth factor receptor/CD271 is expressed on an MSC population that is multipotent in vitro,\textsuperscript{14} merit the term mesenchymal stem cell.\textsuperscript{15} We have previously found that CD271 immunostaining in intact bone marrow selectively highlights a delicate arborizing MSC population that is in direct contact with CD34+ hematopoietic progenitor/stem cells.\textsuperscript{16} We also used image analysis to quantitate CD271+ MSC area in intact bone marrow core biopsy specimens and noted an increase in the area of CD271+ MSCs in MDS vs benign bone marrow biopsy specimens in a small cohort.\textsuperscript{16} We therefore hypothesized that CD271+ MSC density could be useful as an additional diagnostic tool to discriminate MDS from benign cytopenias. Given the biological differences between MSCs derived from higher risk compared with lower risk patients with MDS,\textsuperscript{17,18} we also hypothesized that MSC density as determined by CD271 immunohistochemistry on routine diagnostic bone marrow core biopsy specimens could be of utility as a novel prognostic factor in MDS. Herein we have systematically assessed the density of the CD271+ MSC network in intact diagnostic bone marrow core biopsy specimens of 125 cytopenic patients with or without MDS using both automated quantitation by image analysis and subjective scoring of bone marrow core biopsy tissue microarrays. We have compared CD271+ MSC density in benign cytopenias, lower grade MDS, and higher grade MDS and explored the prognostic significance of CD271+ MSC density in a Cox proportional hazards model incorporating known clinically relevant prognostic variables, including Revised International Prognostic Scoring System (IPSS-R),\textsuperscript{19} transfusion,\textsuperscript{20} history of chemotherapy/radiation,\textsuperscript{21} and fibrosis.\textsuperscript{22,23}

Materials and Methods

Research was approved by the institutional review board at Stanford School of Medicine and the R&D committee at the Veterans Affairs (VA) Palo Alto Health Care System. Requirement for individual patient consent was waived by the institutional review board. Bone marrow biopsies to rule out MDS, performed within 4 months of the initial diagnosis and prior to any disease-altering MDS therapy, were included. Exclusion criteria were (1) age younger than 18 years, (2) non-MDS malignancy morphologically involving the bone marrow at baseline, and (3) current nonmyeloid malignancy, with the exception of nonmelanoma skin cancer and small lymphocytic lymphoma. Ninety-eight patients were from the VA and 27 from the Stanford School of Medicine, with biopsy specimens obtained between 1995 and 2009. All diagnostic materials were reviewed by one author (D.G.), a board-certified hematopathologist, and classified according to World Health Organization (WHO) 2008 criteria\textsuperscript{24} plus the Refractory Anemia with Excess Blasts in Transformation category as recommended by National Comprehensive Cancer Network guidelines.\textsuperscript{25} Patients with MDS whose clinical record mentioned a history of chemotherapy or radiation as defined by WHO 2008 criteria were classified as having therapy-related disease. Cases that did not meet WHO 2008 criteria for a diagnosis of MDS were classified as benign. Bone marrow fibrosis was graded based on European consensus guidelines.\textsuperscript{26}

Bone marrow biopsy specimens were fixed in Bouin’s solution and decalified before routine processing and embedding in paraffin. Tissue microarrays were constructed using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD).\textsuperscript{16} Each bone marrow core biopsy was sampled in duplicate; in 80% of cases, both cores were suitable for evaluation, and in 20% of cases, one core was evaluable due to technical factors (partial or complete tissue loss). Immunostaining was performed using rabbit polyclonal antibody against CD271 at 1:500 (HPA004765; Sigma, St Louis, MO).\textsuperscript{16} Reticulin and trichrome staining of the tissue microarray was performed on the Dako Artisan autostainer (Dako, Carpinteria, CA). For CD271+ MSC quantitation using CellProfiler 2.0 image analysis software (Broad Institute, Cambridge, MA),\textsuperscript{27} tiff files were obtained on an Olympus BX45 upright microscope (Olympus, Center Valley, PA) with a UPlan FL \( \times 40/0.75 \) dry lens and SpotFlex Model 15.2 camera (Diagnostic Instruments, Sterling Heights, MI). The red AEC (3-amino-9-ethylcarbazole) chromogen (labeling CD271+ MSCs) and the blue hematoxylin counterstain (labeling the total cellular area) were separated using the “unmix colors” module; primary objects (CD271+ MSCs) were identified and their area measured in the red channel image, and the cellular area was measured as the thresholded area of the blue channel image (for entire pipeline, see Supplementary Figure 1; all supplemental materials can be found at http://www.ascp.org/docs/default-source/pdf/press/johnsondec14.pdf). The CD271+ MSC density of duplicate cores was averaged. Qualitative CD271 scoring was performed in a blinded manner on a BX45 upright microscope (Olympus) with a UPlan FL \( \times 20/0.5 \) dry lens and WH15X/14 oculars using a low/medium/high scale. The maximum score of the duplicate cores was used in subsequent statistical analysis.

Statistics were performed using STATA (STATA/IC 11.2; StataCorp, College Station, TX). Correlation coefficients were calculated using the nonparametric Spearman \( \rho \) test. Variables were compared using the nonparametric Kruskal-Wallis test adjusted for tied ranks. Survival analysis was performed for variables that passed a test for proportionality of hazards using Schoenfeld residuals and showed no evidence of time dependence. Survival curves were plotted using the Kaplan-Meier method and compared
using log rank analysis. Putative prognostic variables were assessed by Cox regression analysis; multivariate analysis was performed using a Cox proportional hazards model with robust standard error estimation. Estimated power for Cox proportional hazards regression was performed using a Wald test. Intraobserver and interobserver agreement was calculated using the \( \kappa \) statistic.

**Results**

**Patient Characteristics**

The study cohort included 125 patients with diagnostic bone marrow core biopsies performed for evaluation of persistent cytopenias and to assess for MDS. Sixty-one patients had no evidence of hematolymphoid malignancy (benign group), whereas 64 were diagnosed with MDS. The 125 patients had a median follow-up of 23.4 months (range, 0.33–160 months) with 60 deaths and 14 cases of AML recorded. The MDS and benign patient groups were similar in baseline demographic characteristics, with an average age in the upper 60s and a male predominance Table 1.  

Depths of cytopenia were similar between the two MDS and benign groups for hemoglobin and platelets, but absolute neutrophil count was significantly lower (\( P = .0001 \)) in the MDS group. Chronic renal failure and/or cirrhosis were mentioned more frequently on the requisition sheets in the MDS group compared with the benign group (\( P = .001 \)), suggesting a higher prevalence of marrow-extrinsic causes of cytopenia in cases that did not meet WHO 2008 criteria for MDS. Patients were divided into categories of benign (n = 61), lower grade MDS (<5% marrow blasts, n = 40), and higher-grade MDS (≥5% marrow blasts and/or therapy related, n = 24). Log rank analysis showed no difference in overall survival (OS) between the benign cytopenia and lower grade MDS groups (\( P = .7 \)) but significantly shorter survival in the higher grade MDS group (lower grade vs higher grade MDS, \( P = .02 \)). AML-free survival, by contrast, differed significantly between the benign and lower grade MDS groups (\( P = .02 \)) and the lower grade vs higher grade MDS groups (\( P = .002 \)).

**Increase in CD271+ MSC Density for Higher Grade MDS vs Benign Cytopenia and Lower Grade MDS**

CD271+ MSC density was calculated as the percentage of the hematoxylin-stained cellular area covered by CD271+ MSCs using automated image analysis with object identification Image 1. An alternate quantitative measurement of CD271+ MSC density using total area covered by CD271 immunostain without object identification produced very similar data (Spearman \( \rho \), 0.998; \( P < .0001 \)), confirming the replicability of the automated CD271+ MSC density measurement. CD271+ MSC density was found to differ significantly across categories of benign cytopenia, lower grade MDS, and higher grade MDS (\( P = .02 \)) Figure 1. CD271+ MSC density differed significantly between the higher grade MDS and lower grade and benign groups (\( P = .02 \) vs lower grade MDS, \( P = .006 \) vs benign), but there was no significant difference in CD271+ MSC density between the lower grade MDS and benign cytopenia categories (\( P = .8 \)). CD271+ MSC density was also weakly correlated with poorer risk as assessed by the IPSS-R (Spearman \( \rho \), 0.28; \( P = .032 \)). CD271+ MSC density was independent of age at diagnosis (\( P = .8 \)) and fibrosis score (\( P = .8 \)).
High CD271+ MSC Density Is Independently Associated With Shorter OS in MDS

Median follow-up of OS for patients with MDS was 22 months (range, 0.5-160 months), with 34 deaths among 64 patients, yielding 53% power to identify a doubling of the hazard ratio (HR) for death with \( P < .05 \). Increasing CD271+ MSC density showed a significant association with poorer OS on univariate analysis (\( \chi^2 = 10.1, P = .0015 \)). CD271+ MSC density was split into thirds (low, medium, and high density); there was no difference in OS between the low and medium CD271+ MSC density groups (Supplementary Figure 2), and these were therefore combined for further analysis. OS was significantly shorter in the high compared with the medium/low CD271+ MSC density category (18 vs 71 months; \( P = .01 \)) \( \text{Figure 2A} \). CD271+ MSC density split IPSS-R low- and intermediate-risk patients into distinct survival categories (median survival, 18 vs 57 months; \( P = .02 \)) \( \text{Figure 2B} \). Multivariate analysis for OS among patients with MDS confirmed the independent prognostic significance of CD271+ MSC density from known prognostic factors, including IPSS-R,\(^19\) history of RBC transfusion,\(^28\) fibrosis,\(^22,23\) and history of chemotherapy/radiation prior to the diagnosis of MDS\(^24\) \( \text{Table 2} \). Patients with high CD271+ MSC density were at more than three times the risk of death (HR, 3.4; 95% CI, 1.7-6.9; \( P = .001 \)) compared with those with low/medium CD271+ MSC density. Transformation to AML was documented in 13 patients with MDS; there was no significant difference in AML-free survival between the high and medium/low CD271+ MSC categories (\( P = .5 \)). The power of this study to detect a twofold increase in risk of leukemic transformation was estimated at 22%, and thus the sample size is insufficient to exclude an association of CD271+ MSC density with leukemic transformation.
Among the 61 patients who did not meet diagnostic criteria for MDS, median follow-up was 30 months (range, 0.3-130 months), with 26 deaths. On univariate analysis, increasing CD271+ MSC density did not reach statistical significance with respect to overall survival ($\chi^2 = 2.9, P = .09$). Only one patient of 61 in the benign cohort developed AML, so no analysis was performed for AML-free survival. The single patient who developed AML had a history of radiation therapy for a solid tumor, so the subsequent AML may represent therapy-related disease.

**Manual Scoring of CD271+ MSC Density**

A three-part semiquantitative CD271+ MSC density scoring schema was modeled on the myelofibrosis scoring system as follows: low, no or scattered CD271+ MSCs; medium, loose or partial CD271+ MSC network; and high, prominent uniform CD271+ MSC network involving more than 75% of the tissue microarray core. The subjective CD271+ MSC scoring scheme positively correlated with the results of automated image analysis (Spearman $\rho$, .68, $P < .0001$). This three-part subjective scoring system was internally reproducible (84% agreement, unweighted intraobserver $\kappa$ value of 0.72, $P < .0001$) and moderately externally reproducible (scorer 2, 73% agreement, unweighted interobserver $\kappa$ value of 0.52, $P < .0001$; scorer 3, 65% agreement, $\kappa$ value of 0.43, $P < .0001$). Qualitative scoring of CD271+ MSC density provided results concordant with those provided by CD271+ MSC density quantitation: for example, CD271+ MSC scores varied significantly across benign, lower grade MDS and higher grade MDS diagnostic categories ($P = .005$) and also weakly correlated with poorer risk as assessed by the IPSS-R (Spearman $\rho$, 0.32, $P = .019$). Increasing CD271+
MSC density score was significantly associated with poorer OS (P = .005) on univariate Cox regression analysis; as with quantitative CD271+ MSC density, poorer OS was restricted to the high CD271+ MSC score category, and there was no difference in survival between the medium and low CD271+ MSC score groups (Supplementary Figure 3). High CD271+ MSC density score could identify a poor-risk category among patients with low and intermediate IPSS-R (median survival, 18 vs 40.5 months; P = .02) and was independent of IPSS-R, transfusion, history of chemotherapy/radiation, and fibrosis score (HR, 1.5; 95% CI, 1.01-2.1; P = .044).

### Discussion

On the basis of our prior studies of the MSC compartment in benign and MDS bone marrow, we hypothesized that assessment of CD271+ MSC density of diagnostic bone marrow core biopsy specimens could aid in distinguishing benign causes of cytopenias from MDS and that CD271+ MSC density could serve as a novel prognostic marker in MDS. To rigorously address our hypotheses, we assembled a cohort of 125 cytopenic patients who underwent diagnostic bone marrow biopsy to rule out MDS. Those patients who did not meet criteria for MDS provided an age-matched cohort with similar levels of anemia and thrombocytopenia as the patients ultimately diagnosed with MDS. Cytopenias in the non-MDS or “benign” group may be due in part to non–marrow-related underlying conditions such as chronic renal failure or liver disease. Other patients not meeting diagnostic criteria for MDS could nevertheless be harboring a clonal myeloid neoplasm with ineffective hematopoiesis that is not detectable by morphology, blast count, and standard cytogenetics; these patients could be classified as having idiopathic cytopenia of uncertain significance. Limitations of this study include its retrospective nature; sampling in tissue microarray format, which does not reflect clinical practice but does allow efficient and quantitative analysis; and relatively small sample size limiting the power of the analysis.

We assessed CD271+ MSC density—the percentage of the hematopoietic area of the biopsy specimen encompassed by CD271+ MSCs—using an automated image analysis procedure by implementing an object-based quantification method using CellProfiler software. CD271+ MSC density was significantly increased in the higher grade MDS group compared with either lower grade MDS or cytopenic patients without MDS. We note that the higher grade MDS group (≥5% marrow blasts and/or therapy related) is an amalgam of the categories of therapy-related myeloid neoplasia and refractory anemia with excess blasts, and the lower grade MDS group (<5% marrow blasts and not therapy related) is an amalgam of refractory cytopenia with unilineage dysplasia, refractory cytopenia with multilineage dysplasia, and MDS with isolated deletion 5q. This study is not large enough to examine individual WHO diagnostic categories. In clinical practice, the distinction between benign cytopenia and higher grade MDS is generally not problematic based on blast count and often cytogenetic abnormalities. CD271+ MSC density did not appear to be useful in separating benign cytopenias from lower grade MDS, an area of significant diagnostic difficulty. The IPSS-R is a powerful composite measure of prognostic risk in MDS based on cytopenias, cytogenetic studies, and the bone marrow blast count; there was a weak but statistically significant positive correlation between CD271+ MSC density and IPSS-R risk category. Interestingly, biological differences have been demonstrated in the adhesion and immunoregulatory properties of MSC derived from high-risk compared with low-risk patients with MDS. The association of high CD271+ MSC density with higher grade and poor-risk MDS could reflect an abnormal positive feedback loop between the myeloid clone and its dysfunctional mesenchymal niche.

We next assessed our hypothesis that CD271+ MSC density in routine diagnostic bone marrow core biopsy specimens could be of utility as a novel prognostic factor in MDS. Indeed, high CD271+ MSC density was associated with shorter OS among patients with MDS independent of IPSS-R, history of transfusion (a stand-in for the known risk factor of transfusion dependence), myelofibrosis, and history of chemotherapy or radiation. Of clinical interest is the finding that patients with low and intermediate scores in the IPSS-R schema could be further subdivided on the basis of high CD271+ MSC density (median survival, 18 vs 57 months); if validated, this finding could lead to more highly personalized treatment algorithms for these patients. Quantitative CD271+ MSC density analysis is unlikely to come into routine clinical practice; we therefore also tested our hypotheses using an independent qualitative scoring method similar to those in current clinical use. Qualitative assessment of the density of a stromal cell population presents logistical difficulties similar to those encountered in the grading of marrow fibrosis; we developed

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<th>Characteristic</th>
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<th>P Value</th>
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<td>CD271+ MSC density top third</td>
<td>3.4 (1.7-6.9)</td>
<td>3.5</td>
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<tr>
<td>IPSS-R</td>
<td>1.7 (1.3-2.4)</td>
<td>3.7</td>
<td>.000</td>
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<td>3.4</td>
<td>.001</td>
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<td>.1</td>
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<tr>
<td>Fibrosis</td>
<td>0.9 (0.5-1.6)</td>
<td>-0.4</td>
<td>.7</td>
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IPSS-R, Revised International Prognostic Scoring System; MDS, myelodysplastic syndrome; MSC, mesenchymal stromal cell.

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a semiquantitative CD271+ MSC density scoring schema based conceptually on a broadly accepted consensus bone marrow fibrosis grading schema.\textsuperscript{26} Interobserver reproducibility was moderate and similar to published interobserver $k$ values for morphologic MDS diagnostic criteria such as bone marrow blast percentage ($k$, 0.42) and 2008 WHO subtype ($k$, 0.43).\textsuperscript{30} Optimal cut points for manual scoring remain to be determined and may well differ in whole core biopsy sections in contrast to 1-mm tissue microarray cores. Nevertheless, the manual scoring data mirror the quantitative analysis data, confirming higher CD271+ MSC density among patient with higher grade MDS as well as the independent prognostic significance of high CD271+ MSC density for overall survival. A bone marrow core biopsy is a standard component of the diagnostic evaluation of cytopenic patients for MDS; CD271 immunohistochemistry has the potential to provide additional independently prognostic information based on the abnormal MDS bone marrow microenvironment. Like other components of existing prognostic systems, CD271 immunohistochemistry will need to be used in combination with multiple parameters to assess prognosis. As shown here, CD271+ MSC density indeed represents an independent prognostic factor based on a separate bone marrow compartment from those used currently and is independent of IPSS-R, transfusion history, history of chemotherapy/radiation, and fibrosis in the Cox proportional hazards model for overall survival among patients with MDS. Last, we also note the potential for therapeutic targeting of the abnormal bone marrow MSC compartment in higher grade MDS, analogous to the targeting of the MSC compartment in AML to potentiate the effectiveness of standard chemotherapeutic agents.\textsuperscript{31,32}

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