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Flow cytometric detection of altered signaling in myelodysplastic syndrome and cytopenia

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ABSTRACT

Multiparameter flow cytometric analysis allows for precise evaluation of growth factor stimulated intracellular signaling in distinct immunophenotype defined hematopoetic populations. Our analysis of intracellular phosphoprotein in response to major hematopoietic growth factors or cytokines showed several interesting findings. Although there was no characteristic signaling abnormality that was diagnostic for MDS, MDS cases were often associated with more signaling aberrancies involving more cellular populations. Higher than average response in the $CD34^+CD117^+$ progenitor cells to Flt3 ligand and stem cell factor stimulation was frequently associated with high risk features or disease progression in MDS. Although preliminary results hint an adverse prognostic role of dysregulated FLT3 pathway in MDS cases, whether this observation adds independent prognostic value to the existing prognostic system needs to be further explored in future prospective studies.

1. Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of bone marrow myeloid progenitor cells characterized by peripheral cytopenia, dysplasia and ineffective hematopoiesis. Current diagnosis of MDS relies on the clinical presentation, peripheral blood and bone marrow morphologic findings of dysplasia, as well as the presence of cytogenetic abnormalities [1]. However, the diagnosis of MDS can be challenging in clinical practice, especially when the morphologic findings are subtle. Multiparameter flow cytometric analysis of surface antigen maturation patterns of peripheral blood leukocytes has some diagnostic value, and is a recommended adjunctive tool for diagnosing MDS by the current WHO classification and the European Leukemia Net [1–3]. But the divergence from normal immunophenotypic maturation pattern is not specific to MDS [4]. Additionally, MDS is also associated with variable risks of transformation to acute myeloid leukemia. Multiple risk models have been developed to predict survival and leukemic evolution, including the recent World Health Organization classification based Prognostic Scoring System (WPSS) and the Revised International Prognostic Scoring System (IPSS-R) [5,6]. However, these models do not always accurately capture the kinetics of the disease course.

It is well known that normal hematopoiesis is precisely regulated by a complex network of signaling through cytokines and growth factors [7,8]. The binding of the ligands to receptors on the cell surface triggers a cascade of phosphorylation of intracellular proteins that provide signals for proliferation, maturation and differentiation amongst others. Previous work on the growth factor/cytokine critical for hematopoiesis shed light on this complex network and downstream signaling cascades. The receptors for Stem cell factor (SCF) and Flt3 ligand (FL) are predominantly expressed on early hematopoietic progenitor cells, indicating their critical role in early hematopoiesis. SCF regulates survival of hematopoietic stem cells through multiple pathways including the Ras–Raf–MAP kinase cascade and the JAK/STAT pathway [9,10], whereas FL more selectively activates the downstream signal transducer and activator Stat5a through a Janus kinase-independent mechanism [11,12]. On the other hand, role of G-CSF and GM-CSF are typically lineage specific to the precursors of granulocytes, and correlates well with activation of distinct JAK-Stat pathways [13,14]. Recent advances have considerably increased our understanding of signaling dysregulations in hematopoietic diseases like AML and myeloproliferative neoplasms (MPN). Studies indicate that aberrant cell signal transduction is present in virtually all AML and potentially representing the fundamental abnormalities in leukemogenesis [15,16]. Following the same lines, it is logical to

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believe that signaling abnormalities do contribute to MDS; however very limited published data exist in literature, on dissecting these abnormal signaling pathways in the MDS setting.

Recent advances in flow cytometric analysis provide a robust and reliable measurement of expression of intracellular phosphoproteins in immunophenotypically distinct populations. Ours as well as other published observations indicate that signaling patterns in distinct subpopulations of normal bone marrow cells in response to stimulation by exogenous cytokine/growth factors are highly predictable [17,18]. This observation lays a solid foundation to further explore the potential clinical utility of dissecting signaling transduction pathway abnormalities using multiparameter flow cytometric analysis. Spinelli et al. identified defective Stat5 and Erk1/2 activations in response to erythropoietin in a group of MDS patients [19]. Another study also reported MDS samples with increased myeloid frequency demonstrated increased Stat5 response to G-CSF stimulation [18]. The present study employs flow cytometry techniques to analyze signaling responses to the four major hematopoietic growth factors/cytokines (SCF, FL, G-CSF and GM-CSF) in immunophenotype distinct subpopulations from fresh bone marrow aspirate samples, thus limiting additional physical manipulations to isolate neoplastic cells, which may inevitably alter signal transduction responses as well as gene expression. Our study includes a cohort of newly diagnosed untreated MDS patients, as well as patients presenting for other causes of cytopenias or lymphoma staging during the same period of time.

2. Material and methods

2.1. Cases

Bone marrow (BM) aspirates from patients presented for initial diagnostic work up for cytopenia, at the Northwestern Memorial Hospital (NMH), were included in this study. Our cohort of patients in this present study included 15 cases of MDS or MDS/MPN (cases 1–15). These cases were classified as MDS NOS, MDS RARS, MDS RAEB-1, MDS RAEB-2 or MDS/MPN according to WHO 2008 criteria. In addition, there were 3 patients presenting with a presumptive diagnosis of MDS, but later reclassified as acute myeloid leukemia with myelodysplasia related changes (AML-MRC) based on the blast counts in the bone marrow aspirates, that were 32% (case 16), 22% (case 17) and 45% (case 18). Cytogenetic abnormalities were identified in 9 MDS cases and in 1 AML case. The clinical and pathologic features of these cases are summarized in Table 1. The final cohort of 10 cytopenias were most often as a result of chronic disease such as rheumatoid arthritis (cases 19, 21), idiopathic thrombocytopenia purpura (cases 22, 25), systemic lupus erythematosus (SLE) (case 27), serous atrophy (case 23) and unspecified etiology (cases 20, 24, 26). One additional patient (case 28) with newly diagnosed central nervous system diffuse large B cell lymphoma not involving the bone marrow was noted to have persistent mild anemia in the chart review; this patient was then re-classified into the cytopenia group. No cytogenetic abnormalities were present in any of the cytopenia cases. Ten patients with newly diagnosed B cell lymphoma with negative staging bone marrow and normal CBC were included in the study as normal controls (cases 29–38). Patients with prior history of hematologic disorders or history of chemotherapy for non-hematologic malignancy were excluded from the controls. Samples were acquired under Northwestern University—Institutional Review Board approved protocol.

2.2. Morphologic review and other ancillary studies

The complete morphologic work up included reviews of Wright–Giemsa stained peripheral blood and bone marrow aspirate smears, Prussian blue stained bone marrow aspirates and H&E stained trephine core biopsy sections. The morphologic diagnoses were made independently by two hematopathologists based on WHO classification diagnostic criteria. In cases of discrepancy, a consensus of diagnosis was made by a multiheaded microscopical review. Conventional cytogenetic analysis was performed following standard clinical laboratory protocol. Cytogenetic analysis was not performed for negative staging bone marrow.

2.3. FLT3 Internal Tandem Duplication (ITD) and D835 mutation analysis

FLT3 ITD and D835 mutation analysis were performed in all MDS and cytopenia cases. DNA was extracted from scraped unstained bone marrow aspirate smears on automated QIAsymphony SP instrument (Qiagen, Valencia, CA) following manufacture protocol. DNA was amplified using two sets of primers for both the ITD and D835 regions of the FLT3 following method previously Murphy et al. [24]. The D835 product was restriction enzyme digested and both run on the 3130xl DNA sequencer (Applied Biosystems, Foster City, CA).

2.4. Cytokine/growth factor stimulation

Flow cytometric analyses were performed on heparin anticoagulated bone marrow aspirates within 4 h of BM procedure. Cytokine/growth factor (GF) stimulation was performed as per previously published protocol [17]. Aliquots of BM samples were used for cytokine/GF stimulation or kinase inhibitor treatment at 3×10^6 cells/100 μL. Samples were incubated at 37 °C for 30 min before cytokine/GFs or inhibitor addition. Inhibition was carried out with a cocktail of 100 μM UO126 (EMD Chemicals, Gibbstown, NJ), 50 μM GDC0941 (Axon Medchem, Reston, VA) and 1 μM rapamycin (EMD Chemicals) for 30 min at 37 °C. Individual tubes were stimulated with either 10 ng/100 μL recombinant human (rh)-SCF (R&D Systems, Minneapolis, MN), 50 ng/100 μL rh-FL (R&D Systems), 10 ng/100 μL rh-GM-CSF (R&D Systems), or 10 ng/100 μLrh-G-CSF (ORF Genetics, Kopavogur, Iceland) and collected at 3.5 min and 15 min by immediate fixation with formaldehyde at 37 °C. One tube receiving no stimulant or inhibitor was also processed. After stimulation/inhibition, samples were fixed, permeabilized, and washed according to previously described protocol with Triton X-100 and ice cold 80% methanol permeabilization [17]. Because of negative effect on the antibody surface labeling staining quality by the Triton-X 100/methanol treatment, CD13-PC7 (Beckman Coulter, Brea CA), CD16-alexa 647 (BD, Billerica, MA), and CD64-A700 (Biolegend, San Diego, CA), CD16-A700 (BD), and CD64- PE (Beckman Coulter) were all stained prior to fixation and permeabilization.

2.5. Intracellular phosphoprotein staining

Following fixation and permeabilization, cell suspensions were washed twice with 4 ml of PBS + 2% BSA solution, centrifuged at 800× *g* for 6 min at 4 °C. The cells were then resuspended in 100 μls of blocking serum solution (25% Mouse + 25% Rabbit serum in PBS) and incubated for 5 min. Following this the cells were stained with appropriate concentrations (based on titration), of CD34-ECD (Beckman Coulter), CD117-PC5.5 (Beckman Coulter), CD45-APC-A750 (Beckman Coulter), pErk (Thr202/Tyr204)-A488 (cell Signaling Technology, Danvers, MA), and pStat5 (Tyr694)-PE or pStat5 (Tyr694)A647 (cell Signaling Technology) or CD117 PC7 (Beckman Coulter), CD45-Pacific Blue (Invitrogen, Carlsbad, CA). Suspensions were incubated in the staining solution for 45 min on ice, washed twice with ice-cold phosphate buffered saline (PBS)/bovine serum albumin (BSA), centrifuged at 800× *g* for 6 min