Going from genes to proteins in myelodysplastic syndromes

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The myelodysplastic syndromes (MDS) are a heterogeneous group of malignant clonal diseases of bone marrow stem and progenitor cells ranging clinically from mild asymptomatic anemia or other cytopenias to rapid transformation to acute leukemia. The early stages of MDS are characterized by dysplasia and increased apoptosis of bone marrow cells resulting in the characteristic pattern of bone marrow hypercellularity with peripheral blood cytopenias (1, 2). Immune mechanisms driven by autoreactive T cell clones and associated cytokines have been implicated in the pathogenesis of ineffective hematopoiesis in early MDS (3), and the degree of anemia correlates well with the serum concentration of TNF- α (4). During disease progression, increasingly complex genetic alterations within the marrow cells lead to profound differentiation defects with accumulation of blast cells in the marrow and ultimately development of frank acute leukemia in 30–40% of patients. MDS can therefore be regarded as a preleukemic disease state, which can be monitored to elucidate the successive genetic and cellular events important for leukemogenesis.

What is the value of the paper by Aivado *et al.* (5), published in this issue of PNAS, for our understanding of MDS? By generating serum proteome profiles in a large group of MDS patients and controls, Aivado *et al.* for the first time demonstrate that concentrations of the chemokines CXCL4 and CXCL7, but not other peptides, are selectively decreased in serum from MDS patients. Although this decrease was more pronounced in patients with advanced disease stage, it was also detectable in early stages of MDS. These observations were corroborated by CXCL4-specific immunoassays on both platelet-free plasma and platelets from MDS and non-MDS cytopenia patients. Deficiency of both CXCL4 and CXCL7 may be one of several causes for the increased rate of infections in MDS patients. Additional causes include decreased neutrophil numbers and absence of secondary neutrophil granulation.

MDS are classified mainly according to morphological changes in the bone marrow and in the blood (6). Classification schemes have been refined recently to better reflect the biological behavior of the disease including distinct cytogenetic

subentities (7). MDS risk scores defined by blast count, number of cell lines involved and cytogenetic abnormalities have been developed (8), resulting in risk strata with different prognoses. Most MDS patients are above age 60 and have transfusion-dependent anemia with white cell and platelet counts below normal but high enough to be compatible with prolonged survival. Nonetheless, most patients ultimately succumb to infections, bleeding complications, iron overload, or leukemic transformation. Treatment options are limited and mainly consist of supportive measures. High-risk MDS in young patients is treated with bone

Chemokines CXCL4 and CXCL7 are selectively decreased in serum from myelodysplastic syndromes patients.

marrow transplantation, and trials of aggressive chemotherapy are ongoing. Immunomodulatory therapy aimed at the reversal of suppression of hematopoiesis has been used successfully in early stage disease (9, 10).

Despite the advancements in our knowledge about etiology, pathogenesis, and treatment modalities in MDS, many questions remain. Furthermore, there continue to be uncertainties in the correct diagnosis of early stage MDS. Diagnosis is especially troublesome in MDS cases presenting with only slight morphological alterations, with no significant blast-cell population, and with normal karyotype. These MDS cases are difficult to distinguish from reactive changes of the bone marrow due to chronic inflammatory processes, which use the same inhibitory pathways (mainly IFN- and TNF-associated) that are also found in early MDS. In addition, it is difficult to foresee the clinical course of individual patients and to select the best therapy upon impending progression to a more advanced stage.

Gene Expression Pattern Is Controlled by Cytogenic Aberrations

Karyotypic abnormalities are found in $\approx 50\%$ of patients with MDS, resulting mainly in loss of genetic material. Deletions of chromosomes 5, 7, and 20 are most frequently found, whereas translocations are rarely observed. Although proteome analyses have not yet been reported in MDS, several studies have analyzed differential gene expression in the immature CD34⁺ blast cell population. These studies compared findings in MDS with those in normal controls or patients with acute myeloid leukemia but not with those in other patients with acquired cytopenias. *DLK1*, which encodes a transmembrane protein belonging to the epidermal growth factor-like superfamily, was the first gene found to have an increased expression in blast cells from MDS patients but not in blast cells from acute myeloid leukemia patients (11, 12). Distinctive expression profiles were reported for patients with monosomy 7, who have a high rate of progression to acute leukemia, and for those with trisomy 8, characterized by a rather benign disease course. In monosomy 7, CD34⁺ cells showed up-regulation of genes inducing transformation to acute leukemia and apoptosis and down-regulation of genes controlling cell growth and differentiation (13). In trisomy 8, genes primarily involved in immune and inflammatory responses were up-regulated, whereas genes inhibiting apoptosis were down-regulated (13). An independent gene expression analysis of CD34⁺ bone marrow cells from a large group of MDS patients including many with del(5q) showed an up-regulation of IFN-stimulated genes in the majority of patients (14). Peripheral cytopenias might be caused by up-regulation of IFN-stimulated genes or the recently described reduced expression of the human myeloid nuclear differentiation antigen (*MNDA*) (14, 15).

More closely related to the present work by Aivado *et al.* (5), several studies

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investigating MDS reported down-regulation of genes regulating megakaryocyte proliferation and platelet activity, i.e., *PF4* (platelet factor 4, also called *CXC* chemokine ligand 4, *CXCL4*) and *CD41b* (platelet glycoprotein IIb) (12–14). Expression of genes involved in actin cytoskeleton maintenance and, intriguingly, megakaryocyte/platelet-associated genes were up-regulated in patients with del(5q), with *PF4V1* being the most differentially expressed gene (14). This is most interesting because MDS patients usually present with either normal or more often low platelet counts, whereas platelet counts are regularly increased with abnormal megakaryocyte morphology in the 5q-syndrome.

CXCL4 and CXCL7 Distinguish Myelodysplastic Syndromes from Nonmalignant Cytopenias

In contrast to earlier gene expression studies that mainly focused on the molecular basis for disease progression (11–14), Aivado *et al.* (5) not only evaluated MDS patients and healthy controls but extended their study to include patients with other types of chronic cytopenias or marrow dysfunction. The present study by Aivado *et al.* demonstrates CXCL4 and CXCL7 to be strong candidates as potential biomarkers not only to distinguish MDS from non-MDS anemias but also to differentiate among the different MDS subtypes.

Previous gene expression analyses have predicted decreased levels of CXCL4, based on decreased RNA expression. However, why were differential protein concentrations only found for CXCL4 and CXCL7 and not for other gene products despite the differential expression of many other genes? This may be due to physiological roles of these two proteins, which are normally abundant in the α -granules of platelets and released into circulation upon platelet activation to recruit neutrophils at inflammation sites (16). A comparable association can be drawn between

increased expression of the *TNF α* gene in MDS (13) and enhanced TNF serum levels in correlation with the degree of anemia (4). However, this latter observation is not specific for MDS but can also be seen in other types of anemia in chronic disease. Moreover, most of the other differentially expressed and translated gene products described in previous studies mainly either act intracellularly or are released into tissues, and are therefore difficult to measure in the serum. Alternatively, lack of detection could also reflect technological limitations of the methods applied to screening of the sera of this patient cohort (17).

Initially, Aivado *et al.* (5) performed MS by SELDI (surface enhanced laser desorption ionization time of flight) to screen serum samples collected from patients and controls. Two peaks of differentially expressed proteins at molecular masses of 7,786 and 9,319 Da, respectively, were identified as CXCL4 and CXCL7. CXCL4 and CXCL7 were both found at lower levels in platelets of patients with MDS compared with the controls, and this was also reflected by the data obtained in serum of the patients. The group applied additional proteomic approaches, including Western blots and immunoassays to ascertain their previous findings.

The authors used SELDI for the detection of marker molecules. SELDI relies on elective adsorption of proteins to different active surfaces, thus reducing the complexity of biological samples without a separation before mass spectrometry (18), providing a low-resolution mass “fingerprint.” Advantages of SELDI include its easy use and high-throughput capacity. Disadvantages are the low mass resolution and loss of potentially interesting peptides due to the selection of molecules via different absorption properties before identification. To date, several options for high separation resolution exist. The highest resolution in peptide separation can be accomplished either by combining two

liquid chromatography-based technologies (2DE-LC; HPLC) or in a single step using capillary zone electrophoresis (CE) (19). With the introduction of the electrospray ionization mass spectrometry (ESI-MS), online LC- or CE-MS coupling became possible. Methods allowing high-resolution separation and high-resolution analyses of differentially expressed or secreted peptides/proteins hold great promise for identification of clinically relevant markers. Limitations of LC-MS include difficulties with comparative analysis, in part due to the variability in multidimensional separations, and the substantial time required for the analysis of a single sample. Nevertheless, 2DE- and/or HPLC-MS are currently the methods of choice for analysis of high-molecular-mass proteins or biomarkers (20).

CE-MS has been successfully applied to diagnosis of several clinical conditions, including detection of graft versus host disease by proteomics pattern established in urine collected from patients after allogeneic HSCT (18, 21). Limitations of CE-MS include the difficulty in separating very-high-molecular-mass molecules and the small sample volume that can be applied to the system. Nonetheless, CE-MS has developed into an important alternative to the currently used methods for the detection and analyses of low-molecular-mass peptides.

The use of proteomics in clinical studies will gain importance in the future because the technique holds the promise of allowing evaluation of many peptides and proteins simultaneously. Thus, changes occurring because of modification of proteins or peptides or by the differential up- or down-regulation of molecules can be evaluated in a single assessment. Improved proteomic techniques will lead to changes in the disease diagnosis, improve follow-up and response to therapy evaluations, and may allow more complete clarification of the causative events of carcinogenesis.

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