Going with the Flow: JAK-STAT Signaling in JMML

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Knowledge of the distinctive cellular and genetic traits of a cancer aids in diagnosis, prognosis, and potentially treatment. In this issue of *Cancer Cell*, Kotecha et al. (2008) demonstrate using a sophisticated flow cytometry approach that signal transduction responses to exogenous stimulation can inform diagnosis and pathobiology of myeloproliferative neoplasms.

The disorderly order of tumorigenesis makes it a wonder that cancer diagnostics and prognostics have been utilized to date with any efficacy. Cancers can be identified by tissue, cellular, and molecular characteristics, and much work has been committed to correlating these characteristics with diagnostic and prognostic utility, with several notable successes. However, recent technological advances promise to improve our current strategies for diagnosis and treatment of cancer, as exemplified herein by Kotecha et al. (2008).

Juvenile myelomonocytic leukemia (JMML) is an invasive and clinically aggressive myeloproliferative disorder (MPD) or neoplasm that arises from primitive stem or myeloid progenitor cells (Lapidot et al., 1996; Emanuel, 2008). Accurate diagnosis is essential given the aggressive nature of JMML and limited therapeutic options (see Emanuel, 2008 for diagnostic criteria). Although current clinical criteria are adequate in most cases, they represent indirect manifestations of the underlying pathogenetic basis of the disease.

The molecular underpinnings of JMML are exceptionally well characterized and can largely be attributed to mutational activation of the RAS/MAP kinase (MAPK) pathway. Mutations in the *RAS* family members *KRAS* or *NRAS*, the nonreceptor tyrosine phosphatase SHP2 (*PTPN11*), or the RAS-GAP *NF1* are observed in 65%–85% of JMML patients (Schubbert et al., 2007; Emanuel, 2008). Furthermore, these mutations are largely mutually exclusive between patients with the same clinical phenotype, indicating at least partially redundant function. Engagement of RAS signaling results in activation of multiple effectors including the canonical MAPK pathway converging on ERK activation, the PI3K/AKT pathway, the RAL pathway, and activation of PKC and RAC (Schubbert et al., 2007). The balance of these signaling outputs regulates numerous cellular processes including survival and proliferation, and thus, dysregulated pathway activation might reasonably result in the observed JMML phenotype. Indeed, evidence for causality of these mutations comes from data obtained using mouse models that recapitulate many of the phenotypic attributes of JMML (Schubbert et al., 2007; Emanuel, 2008). Although the identification of mutations in *RAS*, *PTPN11*, or *NF1* is potentially useful in diagnostic assessment of JMML, exclusion of their mutations does not eliminate a diagnosis of JMML.

Elevated RAS/MAPK pathway activation presumably explains the hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) that was described prior to genetic insights into disease. Myelomonocytic progenitors from the bone marrow (BM) or peripheral blood (PB) of JMML patients display enhanced colony growth in vitro in the presence of low doses of GM-CSF that do not support the growth of healthy BM or PB progenitors (Emanuel et al., 1991). GM-CSF normally stimulates hematopoietic development, as well as inflammatory responses, acting through its cognate GM-CSF receptor, which is comprised of a ligand-binding α subunit and a common β_c signal-transducing subunit (Guthridge et al., 1998). JMML mutations apparently confer hypersensitivity to stimulation with GM-CSF and may sensitize cells to GM-CSF antagonists (Iversen et al., 1997; Bernard et al., 2002; Kim et al., 2007).

Aberrant signaling networks are thought to be critical drivers of cancer cell growth, proliferation, and survival, a notion that has led to development of effective therapies that target these signaling networks. These observations provide an impetus for a detailed understanding of these pathways in cancer to inform novel therapeutic strategies, as well as our understanding of the dependence of cancer cells on these pathways. Technological advances reported by Garry Nolan and others have informed this effort through use of multiparametric flow cytometry at the single-cell level to study signal transduction from primary patient material. In a seminal paper (Irish et al., 2004), the Nolan laboratory measured both extracellular and intracellular parameters from primary acute myelogenous leukemia (AML) samples stimulated with a number of hematopoietic agonists. By measuring changes in phosphoprotein levels as surrogates for activation of their respective pathways, the authors could cluster AML samples according to several signaling signatures. Importantly, these signaling signatures correlated well with various clinical parameters, including response to chemotherapy, receptor tyrosine kinase mutations (namely *FLT3*), and cytogenetic status (Irish et al., 2004). Of further interest, individual signaling profiles for each AML patient could be constructed based on flow cytometry data. This study paved the way for using signaling signatures not only for drug target discovery purposes but also for diagnostic and possible prognostic use.

Extending these concepts to JMML, Kotecha et al. (2008) sought to identify aberrant signaling responses to GM-CSF in primary JMML cell samples, using the schema shown in Figure 1. The obvious suspects would have been members of the RAS/MAPK pathway. Quite surprisingly, phospho-ERK (p-ERK

[Thr202/Tyr204]) and p-S6 (Ser235/236, a readout of both RAS/MAPK and mTOR activation) responses to GM-CSF were quite heterogeneous in JMML samples and had minimal utility in identifying a unique signaling signature in JMML samples compared to controls. However, the authors did identify a subpopulation of monocytic cells in the PB and BM of JMML patients with the surface immunophenotype $CD33^{+}CD14^{+}CD34^{-}CD38^{lo}$ that displayed elevated levels of p-STAT5 (Tyr694) when stimulated with low doses of GM-CSF. This p-STAT5 response to low levels of GM-CSF was not observed in other types of childhood MPDs or in healthy individuals. The response was readily detectable at diagnosis, disappeared when the patient was treated and in remission, but recrudesced in patients at time of relapse or transformation to AML. The aberrant signaling signature was also shared with patients having related leukemias, including chronic myelomonocytic leukemia (CML) and the M4/M5 subtype of AML. Importantly, this signature was not merely a surrogate assay for standard progenitor colony growth. Patients with Noonan syndrome, who harbor relatively weak germline *PTPN11* gain-of-function mutations and develop transient MPDs, often did not display the hyperactive p-STAT5 signature yet had progenitors that were hypersensitive to GM-CSF in vitro. This hyperactive p-STAT5 response was abolished in JMML patient cells through pretreatment with a JAK2 inhibitor, but not with a MEK inhibitor, implying that hyperactive JAK2 activity underlies this p-STAT5 response in JMML monocytic cells.

This elegant report exemplifies the value of studying signaling networks in primary patient material. In the context of JMML, this approach has identified novel signaling elements at play in JMML in the form of the JAK-STAT pathway, suggests the possibility of therapeutic targeting of the pathway, and provides potential for the development of new and more accurate diagnostics. It should be noted in this context that these investigators are arguably the best in this arena and that technical requirements for single-cell flow cytometry from primary patient material—especially for labile signal transduction intermediates—may be challenging in conventional clinical laboratories. The report also raises a number of interesting questions for further study. For example, it would be of considerable interest to assess the role of JAK-STAT pathway inhibition in biological readouts and to understand how gain-of-function mutations in *RAS* or SHP2 (*PTPN11*) result in JAK-STAT pathway activation. Another interesting point raised by this report is that the immunophenotype described does not match the $CD34^{+}CD38^{-}$ phenotype previously reported to contain JMML-initiating cell activity in a xenotransplant mouse model (Lapidot et al., 1996). Does the GM-CSF-hypersensitive population identified by Kotecha et al. (2008) contain cancer stem cell activity or contribute to disease pathogenesis, and how do the two immunophenotypes correspond biologically and from a signal transduction perspective? In addition, it would be of value to assess the validity of this approach in the various murine models of disease—given the rarity of JMML and the technical challenges in working with primary human samples, it would be useful to determine whether some of these questions could be addressed in model systems. However, it is clear that technological advances, such as those described here, are continuing to have a dramatic impact on our understanding of pathophysiology, diagnosis, and potentially treatment of cancer.

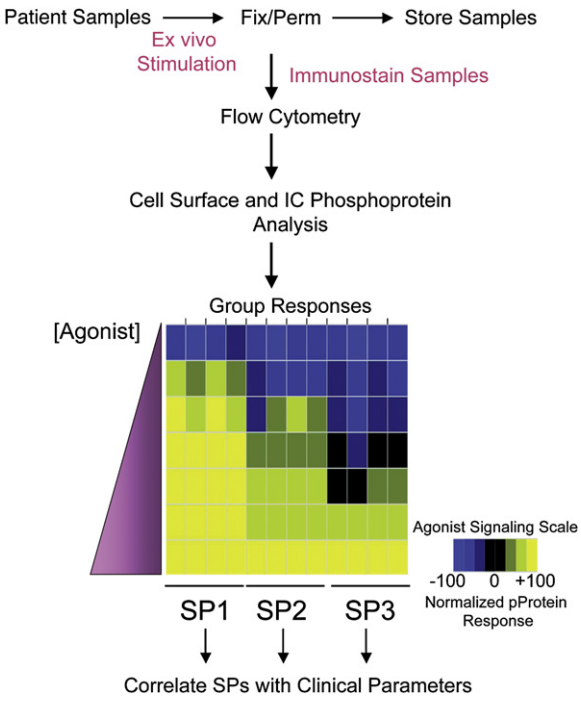

Figure 1. Identifying Unique Signaling Profiles in Primary Patient Samples

Shown is a flow chart essentially outlining the strategy used by Kotecha et al. (2008) to identify signaling abnormalities in primary juvenile myelomonocytic leukemia (JMML) samples. Cells from primary patient samples are stimulated with one or more agonists ex vivo and then undergo fixation and permeabilization. Samples can then be stored or stained for surface and/or intracellular (IC) antigens. The samples are then analyzed by flow cytometry, and signaling responses can be grouped (as shown in the hypothetical heat map representation) according to the strength of response to a given perturbation (in this example, increased concentration of agonist). Signaling profiles (SPs) can be deduced and potentially correlated with clinical parameters, such as type of disease, response to therapy, mutational status, etc. (as in Irish et al., 2004; Kotecha et al., 2008).

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