Resistance in the land of molecular cancer therapeutics

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The fusion tyrosine kinase Bcr-Abl plays a fundamental role in the pathogenesis of chronic myeloid leukemia (CML). Imatinib, a potent inhibitor of Bcr-Abl, has shown impressive clinical activity in CML patients. However, primary and acquired resistance occurs in many patients and is associated with reactivation of Bcr-Abl in primary leukemia cells. Studies reported over the past year have begun to elucidate the molecular basis of imatinib resistance, which may involve amplification of *BCR-ABL* or, more commonly, mutations that introduce amino acid substitutions into the Bcr-Abl kinase. Biochemical analysis and molecular modeling indicate that these mutant proteins retain kinase activity but are less sensitive to inhibition due to structural changes that perturb drug binding. These studies establish a paradigm for elucidating resistance to targeted therapeutics.

Forty years ago, the Philadelphia chromosome was identified in hematopoietic cells from patients with chronic myeloid leukemia (CML). This discovery heralded an extraordinary series of experiments that have illuminated many fundamental principles of cancer biology (reviewed in Sawyers, 1999). The basic facts are now ingrained in the canon of modern medicine. The Philadelphia chromosome results from a reciprocal translocation that joins the *BCR* gene on chromosome 22 with the *ABL* gene on chromosome 9. This fusion gene encodes Bcr-Abl, a chimeric protein with aberrant tyrosine kinase activity. The clinical course of CML is characterized initially by overproduction of differentiated myeloid lineage cells (chronic phase) and can be controlled for months to years by treatment with antiproliferative agents such as hydroxyurea. Despite normalization of peripheral blood counts, the bone marrow cells of CML patients treated in this manner invariably retain the Philadelphia chromosome. Chronic-phase CML ultimately evolves to blast crisis, with expansion of immature elements (blasts), effacement of normal hematopoiesis, and rapid demise. Blast crisis CML is almost invariably refractory to conventional chemotherapy; the few remissions that occur are short-lived. In addition to being present in >95% of patients with CML, *BCR-ABL* fusions are found in ~25% of adults and in 2%–5% of children with lymphoblastic leukemia (ALL). Importantly, *BCR-ABL* portends a poor clinical prognosis in both CML and ALL, with hematopoieitic stem cell transplantation (HSCT) representing the only known curative treatment. Unfortunately, HSCT requires an HLA-matched donor and is frequently associated with substantial acute and chronic toxicities, particularly in older individuals.

A major milestone in CML research was the recent development of imatinib, a small molecule inhibitor of the Bcr-Abl kinase that has been renamed a number of times (it is also known as CGP 571148B, STI-571, and Gleevec) (reviewed in Druker, 2002). Imatinib demonstrated remarkable activity in an elegant series of trials performed by Brian Druker, Charles Sawyers, and their colleagues that carefully assessed in vivo pharmacodynamic endpoints in primary target cells and correlated these data with clinical responses. In a landmark paper that appeared last year, the Sawyers group opened yet another new chapter when they identified *BCR-ABL* point mutations in CML patients that conferred resistance to imatinib and reactivated the kinase (Gorre et al., 2001). In this issue of *Cancer Cell,* Shah and associates (Shah et al., 2002) describe a comprehensive molecular analysis of a larger series of cases in which they conclusively establish the presence of imatinib-resistant Bcr-Abl species in a high proportion of resistant and relapsed CML patients, and they make a number of new insights. These findings raise general questions regarding the nature of cancer genomes and how the inherited and somatic mutations that underlie the malignant phenotype modulate both the primary response to targeted therapeutics and the subsequent development of resistance.

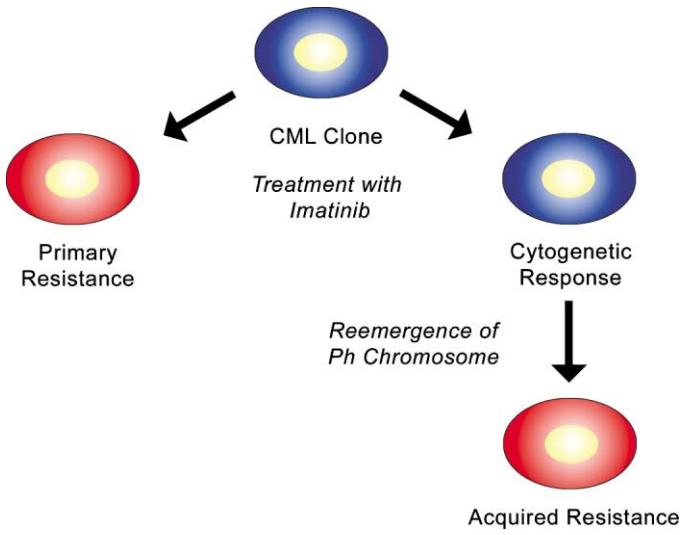

Figure 1. Patterns of imatinib resistance in CML

Patients treated with imatinib either clear the Philadelphia chromosome from the bone marrow (complete cytogenetic response) or demonstrate primary molecular resistance to the drug. Many patients with molecular resistance have a sustained hematologic response and benefit from continuing treatment. Among patients with cytogenetic responses, some will acquire resistance to imatinib and will relapse with reemergence of the Philadelphia chromosome in the bone marrow.

Two general types of molecular responses can be distinguished in CML patients who are treated with imatinib (Figure 1). In the first, there is both normalization of the peripheral blood counts and disappearance of the

Philadelphia chromosome from unstimulated bone marrow cells (major cytogenetic response). This occurs in a majority of chronic-phase patients that receive imatinib as a single agent (Kantarjian et al., 2002). It is not known what proportion of patients that achieve a complete cytogenetic response will ultimately relapse with reemergence of the Philadelphia chromosome. The second type of response is hematologic; that is, patients show normalization of their blood counts but a significant percentage of the bone marrow cells retain the Philadelphia chromosome. Although these individuals certainly benefit clinically from imatinib, they demonstrate primary resistance at the molecular level because treatment does not fully suppress the mutant clone and permit regrowth of normal hematopoietic elements.

With this background in mind, how does the study of Shah et al. advance the field, and what new questions do these studies raise? The authors focused on two cohorts of CML patients—the first included patients who relapsed after a hematologic response, while the second was comprised of chronic-phase patients with sustained hematologic responses but primary molecular resistance (i.e., lack of a durable cytogenetic response). The first group demonstrated substantial clinical heterogeneity at the time they were treated (16 myeloid blast crises, 5 lymphoid blast crises, and 11 with chronic disease). The first major finding was the high overall incidence of *BCR-ABL* mutations ($\sim$90%) that was similar in patients who were treated in chronic phase and blast crisis. There was a brief controversy last year when some laboratories initially failed to detect *BCR-ABL* mutations in CML patients that had been treated with imatinib (Hochhaus et al., 2001). Subsequent studies confirmed the observations of the Sawyers group, and the new work definitively establishes somatic *BCR-ABL* point mutations as pervasive in CML. The very high frequency of mutations identified by Shah et al. can be attributed to their decision to clone and sequence multiple independent cDNA clones from each case in order to identify mutant transcripts that comprised a minority of the *BCR-ABL* transcripts in a given sample. The authors make a further point is this regard, which is relevant to the issue of primary versus acquired imatinib resistance, when they note that mutant transcripts are especially likely to be in the minority in patients who experience a hematologic, but not a cytogenetic, response. This, in turn, infers that clones in these individuals that express "wild-type" *BCR-ABL* are intrinsically resistant to imatinib by alternative mechanisms because they are not selected against by drug treatment.

What might these mechanisms be? Rapid drug efflux from hematopoietic cells and inactivation of imatinib by binding to serum α 1 acid glycoprotein have been proposed (Gambacorti-Passerini et al., 2000; Mahon et al., 2000), but supporting in vivo data are lacking (Jorgensen et al., 2002). Are there other scenarios under which CML clones that express wild-type *BCR-ABL* and are exposed to therapeutic levels of the drug might nevertheless demonstrate primary resistance? An obvious idea is that *BCR-ABL* is not required to maintain the proliferative advantage of some CML cells. However, there is no direct evidence in support of this proposal, and a formidable body of experimental data argues against it. First, introducing *BCR-ABL* into mouse bone marrow causes leukemia (Daley et al., 1990; Elefanty et al., 1990; Kelliher et al., 1990), and recent experiments using a tetracyclene-regulated allele have shown that *BCR-ABL* expression is tightly correlated with the leukemic phenotype in vivo (Huettner et al., 2000). Second, studies in another mouse model support the general idea that oncogenes that initiate lymphoma are required for tumor maintenance (Felsher and Bishop, 1999). Finally, reactivation of the Bcr-Abl kinase as a consequence of somatic *BCR-ABL* mutations that confer resistance to imatinib in CML patients who relapse argues strongly for an essential role of the fusion protein in maintaining the disease.

The paper of Shah et al. extends our knowledge considerably in this area. A compelling aspect of this work is the elegant molecular modeling of the *BCR-ABL* mutations detected in CML samples performed by John Kuriyan and his colleagues that is based on the crystal structure of the c-Abl protein. One cluster of mutations detected in CML patient samples changed each of the invariant "X" positions in the Gly-X-Gly-X-X-Gly-X-Val sequence of P loop, which contains a number of contact points for binding ATP. These substitutions are likely to disrupt the distorted conformation that the P loop adopts to permit binding of imatinib. Another group of mutations change amino acids in Bcr-Abl that contact imatinib and are thought to alter its interaction with the drug independent of ATP binding. Shah et al. assessed the functional significance of individual *BCR-ABL* mutations by transfecting Ba/F3 cells. As expected, these mutant alleles retained kinase activity and transforming potential and demonstrated variable resistance to imatinib. Therapeutic doses of imatinib efficiently inhibit the c-Kit kinase, which regulates the growth and differentiation of discrete populations of hematopoietic cells. The authors sequenced the kinase domain of c-Kit in five patients with *BCR-ABL* mutations, but found no mutations. Together, these studies provide strong support for the idea that imatinib-resistant *BCR-ABL* mutations represent nonrandom genetic lesions that are selected for in vivo because they confer a proliferative advantage. This hypothesis predicts that clones that express mutant *BCR-ABL* transcripts would dominate in the clinical setting in which imatinib resistance develops in those patients that initially achieved complete cytogenetic responses. This data of Shah et al. are not definitive on this point because only five of their patients experienced major cytogenetic responses; however, relapse samples from a number of these cases demonstrated 100% mutant transcripts.

If *BCR-ABL* is required to maintain the proliferative advantage of CML cells in vivo, what might account for primary drug resistance in clones that do not express mutant kinases? Inasmuch as a substantial proportion of chronic-phase CML patients treated with imatinib do not achieve complete cytogenetic responses, this question is of fundamental biologic and clinical importance. One such mechanism—genomic amplification of *BCR-ABL*—has been described in imatinib-treated patients (Gorre et al., 2001). A logical starting point for investigations of primary imatinib resistance involves assessing bone marrow cells for evidence of Bcr-Abl kinase activity (this is usually done by measuring the phosphorylation levels of target molecules such as c-Cbl and Dok). If the Bcr-Abl kinase is active, it is possible that the drug either is not reaching its intracellular target or does not accumulate because of rapid exclusion from target cells, or there is intrinsic resistance within certain hematopoietic compartment or during certain stages of the cell cycle. Each of these potential mechanisms merits investigation. One important issue involves defining the relevant population(s) of cells to focus on. Chronic-phase CML is a disease that originates in hematopoietic stem cells, which produce differentiated progeny that populate the lymphoid, myeloid, erythroid, and megakaryocytic lineages. Studies of leukemia-initiating cells in

NOD/SCID mouse models support the idea that malignant hematopoiesis involves cells at multiple stages of maturation and that leukemia-initiating stem cells comprise a small fraction of the bone marrow (Bonnet and Dick, 1997). Similarly, cells that are capable of initiating and maintaining CML are likely to represent <0.1% of bone marrow mononuclear cells. From the perspective of understanding imatinib resistance, studying their vastly more numerous differentiated progeny may or may not be relevant to the behavior of CML-initiating cells. Ascertaining the percentage of early hematopoietic precursors that show *BCR-ABL* expression in different CML patients, and the sensitivity of cells at various levels of differentiation to imatinib, may uncover novel mechanisms of resistance and might identify patients that are likely to enter cytogenetic remission after treatment. Expression microarray analysis of pediatric ALL samples correlated discrete molecular signatures with specific chromosomal translocations and identified expression patterns that predicted a higher rate of relapse in some of these groups (Ferrando et al., 2002; Yeoh et al., 2002). These data suggest that stem cell populations that initiate leukemic growth specify distinct molecular programs that can be distinguished in their progeny. Similarly, prospective expression profiling of chronic-phase CML bone marrow cells (or of subpopulations enriched for leukemia-initiating cells) might identify pathways that modulate responsiveness to imatinib.

The data of Shah et al. also raise general questions regarding the role of genetic instability in tumorigenesis and in the development of resistance to targeted therapeutics. By definition, chronic-phase CML is a genetically unstable disorder, since patients invariably progress to blast crisis over time. Blast crisis cells frequently demonstrate secondary cytogenetic abnormalities, including acquisition of a second Philadelphia chromosome. While the degree of genetic instability is difficult to quantify in different cancers, it is perhaps useful to compare what prevails in CML with some other malignancies. In particular, certain inherited cancer predispositions such as hereditary nonpolyposis colon cancer, Fanconi anemia, and Bloom syndrome are associated with somatic mutation rates that are increased thousands of times. Many epithelial cancers and therapy-induced leukemias show widespread chromosomal aberrations that have been termed "genomic chaos" by some commentators. By contrast, chronic-phase CML and many other de novo hematopoietic malignancies demonstrate chromosomal translocations without other numerical or structural aberrations. This has given rise to the notion that these cancers have relatively stable genomes. The data of Shah et al. provide some support for this idea in that they did not detect mutations in the c-Kit kinase domain or in a 700 bp segment of *BCR-ABL* immediately upstream of the kinase domain. Rather, the genetic instability in CML cells is distinctly nonrandom and is intimately related to achieving a growth advantage. This aspect of chronic-phase CML bears an uncanny (and somewhat unsettling) resemblance to human immunodeficiency virus, which mutates rapidly to overcome antiviral agents. However, it is important that Shah et al. found *BCR-ABL* mutations in some patients before they received imatinib; this indicates that the development of these mutations does not simply represent genetic adaptation to selective pressure but is an intrinsic characteristic of CML cells. In summary, the prevalence of mutant Bcr-Abl kinases in relapsed CML likely results from multiple interacting factors including the essential contribution of *BCR-ABL* to the growth advantage of CML clones, some degree of genetic instability, a high rate of proliferation in the bone marrow, and the strong selective pressure of imatinib in facilitating outgrowth of mutant clones. As molecularly targeted small molecule inhibitors are used to treat other cancers, it will be interesting to ascertain if the paradigm of acquired resistance identified in CML is broadly applicable. In particular, the widespread genomic changes and occurrence of multiple oncogene and tumor suppressor gene mutations in many epithelial cancers may modulate their ability to become resistant to targeted agents.

What are the implications of these data for treating CML? A particularly interesting aspect of the work of Shah et al. is the study that chronic-phase patients had sustained hematologic responses to imatinib but did not achieve major cytogenetic responses. They found *BCR-ABL* mutations in four patients, three of whom relapsed within 18 months. By contrast, a single patient without a mutation relapsed over the same period (a *BCR-ABL* gene amplification was identified). Besides reinforcing the need for additional research to understand the mechanisms of primary resistance in chronic-phase CML patients without identifiable mutations, these preliminary data hint that *BCR-ABL* mutational status may predict clinical outcome. If this proves true in larger prospective trials, patients at high risk of relapsing might be considered for combination therapies or, in some instances, might be treated with higher doses of imatinib to suppress mutations that confer an intermediate level of sensitivity. To address this issue, it will be important to exploit methodologies for identifying mutations in a minority of cells that are more sensitive and less cumbersome than sequencing multiple independent clones. Single-strand conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC) analysis are robust screening methodologies that can detect mutations in ~5% of a sample. Reanalyzing the specimens included in the current study by SSCP and/or DHPLC would be a simple way of comparing the specificity and sensitivity of these strategies to the approach of sequencing multiple independent clones. Regardless of how samples are screened, prospective studies of the incidence and impact of preexisting and acquired *BCR-ABL* mutations on the responses of chronic-phase CML patients to imatinib should go forward.

The presence of imatinib-resistant BCR-ABL mutations in a high proportion of relapsed CML patients is an obvious concern for clinicians, patients, and their families. However, many of the patients reported by Shah et al. had advanced disease (blast crisis) before treatment and may have therefore had both a greater degree of genomic instability and more time to develop resistant clones. While the actual incidence of imatinib-resistant *BCR-ABL* species will need to be monitored closely in CML patients who are treated early in the chronic phase, it may be substantially lower. Indeed, Shah et al. found only four mutations in their group of chronic-phase patients without cytogenetic responses to imatinib.

Two historical precedents provide some additional perspective. In pediatric ALL, single-agent treatment with aminopterin or corticosteroids induced transient remissions in many patients in the 1950s and 1960s. It was not until these agents were deployed in combination with vincristine, methotrexate, and mercaptopurine that a substantial percentage of patients were cured (reviewed in Pui, 1995). Progress has continued in pediatric ALL with ~80% of children cured with modern regimens. A more recent example is that of acute promyelocytic leukemia (APL), which is associated with a t(15;17) that fuses the *PML*

and *RARA* genes. Treatment with all *trans* retinoic acid induces differentiation of the leukemic clone but is not curative. However, when this agent is administered with moderate doses of conventional chemotherapeutic agents, ~90% of patients can be cured (reviewed in Warrell, 1996). With respect to CML and imatinib, it is extraordinary that a majority of patients with a lethal hematologic malignancy enter cytogenetic remission following treatment with an oral agent that is well tolerated. However, the experience in pediatric ALL, APL, and other cancers suggest that combining drugs with distinct mechanisms of action may be required to achieve high rates of cure. Administering imatinib with cytotoxic agents such as cytosine arabinoside or with small molecules that target hyperactive Ras (reviewed in Le and Shannon, 2002) might be synergistic in CML and in *BCR-ABL*-positive ALL. The recent progress in CML research has been remarkable, and the work of Shah et al. raises a new set of biologic and clinical questions. Most of all, these data remind us that cancer is a tough and resilient adversary that can become resistant to new therapeutics through genetic mechanisms that result in highly specific modifications of relevant biochemical targets.

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