Mining our ABCs: Pharmacogenomic approach for evaluating transporter function in cancer drug resistance

The association of transporter proteins and cancer drug resistance has been known for approximately 25 years, with recent discoveries pointing to an ever-increasing number of ATP binding cassette (ABC) transporter proteins involved with the response of cancer cells to pharmacotherapy. As reported in this issue of *Cancer Cell*, Szakács et al. couple quantitative, real-time PCR assays for all 48 human ABC transporters with chemosensitivity information mined from the NCI-60 cancer cell line database. Predictions of transporter involvement in drug effect were validated in selected cases, and furthermore produced novel leads relating ABC transporter expression and chemoresistance or chemosensitivity.

Genes encoding transporter proteins comprise approximately 25% of the human genome, which attests to the importance of these molecules in normal cellular function. Not surprisingly, cancer cells utilize transporters, notably members of the ATP binding cassette (ABC) superfamily, to enhance their survival and chemoresistance. Comprised of seven families designated A through G, certain ABC transporters couple the hydrolysis of ATP to move drugs and xenobiotics unidirectionally out of cells, thereby effecting drug resistance. The ability of ABC transporters to efflux diverse drugs provided an explanation for the clinical phenomenon of multidrug resistance, where resistance to multiple agents accompanies recurrent cancer following initial successful treatment.

The first ABC transporter described in association with multidrug resistance to chemotherapeutic agents was ABCB1 (P-glycoprotein, the product of the MDR1 gene), responsible for pleiotropic resistance to multiple classes of anticancer agents with very different chemical structures and mechanisms of action (e.g. anthracyclines, Vinca alkaloids, taxanes). The expression of ABCB1 in human tumors, particularly in acute myelogenous leukemia (AML), and the correlation of ABCB1 expression with poor outcome in this disease (Leith et al., 1999), led to trials employing ABCB1 inhibitors in combination with cytotoxic agents effluxed by that transporter. These trials, overall, proved disappointing, suggesting that resistance mechanisms in addition to or other than ABCB1 overexpression may be involved in clinical multidrug resistance. Hence, the value of ABCB1 inhibition as a therapeutic strategy is currently unsettled.

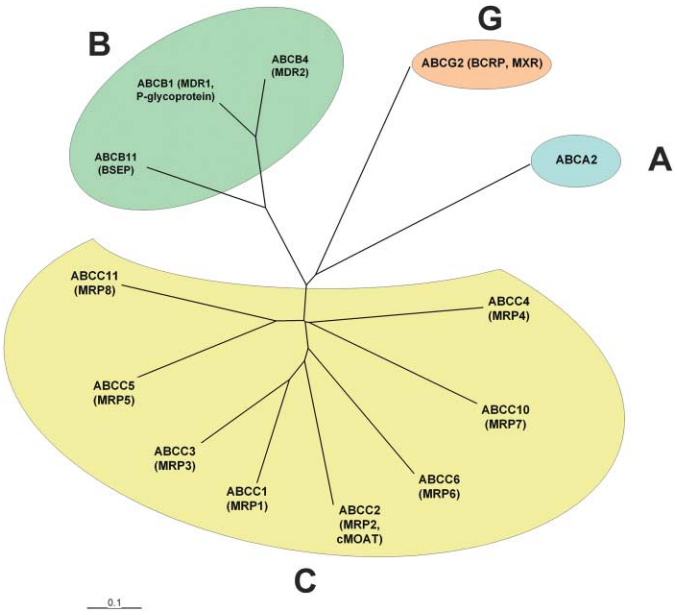

Figure 1. ABC transporters currently known or suspected to cause resistance to cancer chemotherapeutic drugs

ABC transporters are depicted as a radial, unrooted phylogenetic tree. To produce the figure, the protein sequences were aligned and assembled into a phylogenetic tree using ClustalX 1.8 (Thompson et al., 1997), then displayed and printed using TreeView (Win32) 1.6.6 (Page, 1996). The horizontal bar in the figure represents the relative phylogenetic distance for pairwise alignments. Representative antineoplastic drugs known or suspected to be subject to attenuation by these transporters are as follows: ABCA2, estramustine; ABCB1 (P-glycoprotein or MDR1), anthracyclines, etoposide, imatinib, taxanes, vinca alkaloids; ABCB4 (MDR2), paclitaxel, vinblastine; ABCB11 (BSEP), paclitaxel; ABCC1 (MRP1), anthracyclines, etoposide, methotrexate, but not taxanes (a point of distinction from ABCB1 drug resistance spectrum); ABCC2 (MRP2, cMOAT), cisplatin, doxorubicin, etoposide, methotrexate, mitoxantrone, vinca alkaloids; ABCC3 (MRP3), cisplatin, doxorubicin, etoposide, methotrexate, vinca alkaloids; ABCC4 (MRP4), methotrexate, thiopurines; ABCC5 (MRP5), 6-mercaptopurine, 6-thioguanine; ABCC6 (MRP6), anthracyclines, etoposide, teniposide; ABCC10 (MRP7), docetaxel, paclitaxel, vinca alkaloids; ABCC11 (MRP8), purine and pyrimidine nucleotide analogs, NSC 671136 (reported in this issue of *Cancer Cell* by Szakács et al., 2004); ABCG2 (BCRP, MXR), mitoxantrone, methotrexate, topotecan, SN-38, imatinib, flavopiridol, anthracyclines (if mutation present at codon 482).

The clinical trials with ABCB1 reversal agents and the recognition that not all drug-resistant cancer cells with an ATP-dependent, drug efflux phenotype express ABCB1 initiated a search for additional drug transporters. The first of these to be discovered was the multidrug resistance protein 1 (MRP1 or ABCC1; Cole et al., 1992), which quickly led to a search for homologs in the human genome, and the discovery of other MRP family members within the ABCC subfamily (Borst et al., 2000). The MRP family is represented by nine distinct members, of which the first eight are currently associated with cancer drug resistance in laboratory models. MRPs overlap, in part, in their chemotherapeutic substrate specificities with ABCB1, but broaden the classes of agents exported to include topoisomerase I inhibitors, cisplatin, methotrexate, nucleosides, nucleotides, and fluoropyrimidines. The importance of MRP transporters in conferring clinical drug resistance is still undetermined.

Other ABC transporters have been shown recently to confer multidrug resistance through transport of clinically important antineoplastic agents. ABCG2, or breast cancer resistance protein (BCRP) (Doyle et al., 1998) transports mitoxantrone, methotrexate, topoisomerases I inhibitors, flavopiridol, CI-1033, and, shown most recently, imatinib mesylate (Burger et al., 2004). The bile

salt transporter, ABCB11 (BSEP), is expressed at high levels in liver tissue, and confers resistance to paclitaxel (Childs et al., 1998). ABCA2, a transporter expressed intracellularly in endosomal/lysosomal vesicles and involved in steroid transport, confers resistance to estramustine, a nitrogen mustard derivative of estradiol (Vulevic et al., 2001). Figure 1 displays a phylogenetic tree of the ABC family members currently implicated in resistance to cancer chemotherapeutic agents.

Of the 48 human ABC transporters, only approximately a dozen (Figure 1) are associated with cancer drug resistance, and a relatively small number of known resistance-associated transporters are extensively characterized for substrate specificity. Szakács and colleagues (Szakács et al., 2004), in this issue of *Cancer Cell*, have taken a pharmacogenomic, hypothesis-generating approach to expand knowledge of the specificity of known multidrug resistance ABC transporters for candidate anticancer agents, and also to discover whether any other ABC transporter, hitherto unknown to cause drug resistance, can transport anticancer drugs. By using real-time RT-PCR methods to quantify the mRNA of all 48 human ABC transporters, they were able to obtain precise correlations of databased drug sensitivity in a subset of 1,429 potential anticancer drugs tested on the NCI-60 cell line panel with mRNA levels of ABC transporters for these cells. For a given drug-transporter pair, a negative Pearson correlation coefficient for cell sensitivity to drug versus transporter expression across the 60 cell lines suggests the transporter may cause drug resistance; conversely, a positive correlation coefficient would indicate the transporter enhances cellular sensitivity to the drug.

As expected, the methodology found good agreement (negative correlations) for known substrates of ABCB1 (P-glycoprotein) versus ABCB1 expression, and also predicted 18 compounds, previously unrecognized as such, to be substrates for ABCB1. Surprisingly, the cytotoxicity of a number of compounds displayed a positive correlation with ABCB1 expression, suggesting that P-glycoprotein expression may, in some cases, make drug therapy more effective. For one of the compounds with a positive correlation coefficent (NSC 73306), this phenomenon was validated extensively. One possible explanation for this phenomenon might be that the expression of ABCB1 alters the growth or differentiation of the cells in a way that makes them more sensitive to the cytotoxic effects of some drugs. For example, in *Dictyostelium* cells, an ABC transporter activity defined by rhodamine efflux blocks differentiation and maintains cells in an undifferentiated state by expelling a differentiation-inducing factor, DIF-1, from the interior of prespore cells (Good and Kuspa, 2000).

When the expression of all 48 transporters was correlated with the cytotoxicity data for 1,429 compounds across the 60 cell lines, the study identified 131 negatively correlated transporter-drug pairs, which included some ABC transporters known to cause anticancer drug resistance (e.g., in order of pair frequency, ABCB1, C2, C3, B11, and C1) and some previously unknown in this regard (e.g., in order of frequency, ABCD1, C7, A3, and B5). Two transporters included within these pairs (ABCC2 and ABCC11) were selected for further study, and the predictions were found to be valid using transfected cell lines, illustrating that the method can identify novel ABC transporters capable of causing drug resistance. However, two ABC transporters for which considerable evidence supports a role in multidrug resistance—ABCC1 (MRP1) and ABCG2 (BCRP)—displayed only weak correlation for a small number of compounds that were not known substrates for these transporters. This ambiguity may represent a limitation of the new methodology for ABC transporter screening. Perhaps for these ABC transporters, there is a poor correlation between mRNA transcript and/or protein expression or transporter function.

Because precise quantitative measurements of transcripts are required to correlate ABC transporter expression with databased drug sensitivity, the real-time RT-PCR method described by Szakács et al. (2004) offers a distinct advantage over array-based transcriptional profiling methods, which are not sufficiently sensitive to detect low levels of mRNA expression. Such low levels of expression for a gene, undetectable by microarray, could nevertheless be associated with biologically significant activity of that gene. Hence, the high sensitivity of the real-time PCR approach allows precise correlations of specific gene function and expression, as demonstrated by Szakács et al. A limitation of the real-time RT-PCR approach is that the scope of the studies must be narrowed to a relatively small number of genes of interest. Array technology, on the other hand, offers the ability to detect alterations in expression of vastly more genes than is possible or practical with real-time PCR methods; however, the array results may lead to more generalized conclusions. For example, oligonucleotide microarray analysis of the NCI-60 panel revealed surprisingly good prediction of cell line chemosensitivity based on overall gene expression profiles alone (Staunton et al., 2001), however, no specific molecular mechanism(s) of drug resistance emerged from this study. These global expression profiles may ultimately allow prediction of resistance to a given drug, yet considerable effort is required to sift through the many alterations observed to identify those which are involved in the resistance process.

The work by Szakács et al. provides evidence that this pharmacogenomic approach is generally valid, and the number of leads selected for validation was reasonable. Furthermore, the method provides an unbiased means to discover novel substrates and transporters involved with multidrug resistance compared to traditional methods, where generally a limited number of compounds are tested against a small number of cell lines that overexpress a given ABC transporter as the result of drug selection or transfection. Indeed, other leads obtained from the current work should be pursued further, and perhaps studies expanded to more drugs for which sensitivity data are available in the NCI-60 database (over 100,000 proven and/or candidate anticancer drugs have been tested in these cell lines). Furthermore, if combined with in vitro cytotoxicity testing, the methodology may ultimately lend itself to studies of clinical samples of tumor cells, and may provide insights as to the extent, globally, to which ABC transporters are upregulated and contribute to resistance to antineoplastic therapy.

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Toward a functional taxonomy of cancer

Interrogating the genomes of tumor cells with genomic and proteomic methods is becoming a mainstay of modern cancer classification efforts. This notion is brought to a new level by a paper in the July 23 issue of *Cell*, in which the dynamic responses of leukemia cells to perturbation are cataloged by flow cytometry, and the leukemias classified in terms of their functional responses. This study paves the way for more systematic attempts to bring functional genomics to the study of human cancer.

The beginning of the 21st century has brought the hope and expectation that an emerging understanding of the human genome will bring about a transformation of the practice of medicine. In the field of cancer, it now becomes likely that over the next decade or two, it will become possible to classify all cancers on the basis of their underlying genetics and physiology. These goals are being largely addressed through the use of DNA microarrays for monitoring the RNA profiles of tumor specimens. Significant progress has been made in many areas including breast cancer (van de Vijver et al., 2002), lymphoma (Rosenwald et al., 2002), and most recently, acute myeloid leukemia (AML) (Bullinger et al., 2004; Valk et al., 2004), among others. A molecular taxonomy of cancer thus appears feasible.

Yet, RNA profiles of tumor biopsies or resected specimens cannot possibly capture all of the relevant molecular detail of a given cancer. For one, much of cellular behavior is governed by translational and posttranslational control mechanisms that are not reflected in RNA profiles. This has led some to argue that a definitive molecular classification of cancer would require proteomic analysis. While this may be correct in principle, the ability to perform high-throughput, detailed proteomic analysis of tumors is at least several years off. As such, RNA profiling still represents the most tractable, high information content, high-throughput classification platform.

Far more important than the RNA versus protein debate is the fact that molecular analysis of tumors creates a snapshot of the biological state of the tissue at the time of biopsy. Differences in dynamic response to environmental conditions (e.g., growth factor stimulation, microenvironmental effects) are not exposed. That is, the resting profiles of two tumors could be the same, yet their response to provocation entirely different—and highly relevant to understanding the clinical behavior of human cancers.

This notion of classifying cancers according to their dynamic response to perturbation is explored for the first time in an important paper by Garry Nolan and colleagues in the July 23 issue of *Cell* (Irish et al., 2004). In this work, the authors use flow cytometry of leukemic cells to assess the phosphorylation state of 6 signaling proteins (Stat1, Stat3, Stat5, Stat6, p38, and Erk1/2) in response to 5 cytokine perturbations (FLT3 ligand, GM-CSF, G-CSF, IL-3, and interferon γ). The experiments were first conducted in leukemic cell lines, and then extended to primary blasts from patients with AML. The studies show quite convincingly that the phosphorylation status of signaling proteins at baseline is not predictive of their response to cytokine stimulation. For example, Stat5, known to be a downstream effector of the receptor tyrosine kinase FLT3, exhibited equivalent phosphorylation in FLT3 wild-type versus mutant (resulting in constitutive FLT3 activity) AMLs. However, Stat5 phosphorylation in response to cytokine stimulation differed significantly between FLT3 wild-type and mutant leukemias. In fact, FLT3 status could be predicted based on Stat5 (and other Stat proteins) response to cytokine treatment. Along those same lines, clustering of these dynamic responses led to successful prediction of response to chemotherapy.

This paper is noteworthy for several reasons. First and foremost, it demonstrates the feasibility of classifying tumors (or in fact any cell) on the basis of their response to cellular perturbation, thereby exposing a new dimension of cellular activity not otherwise accessible. Second, the study demonstrates the power of phospho-proteomic analysis, where single cell phosphorylation status is examined. Standard flow cytometry is reinvented as “single cell profiling” in