Review

Developing gene expression signatures of pathway deregulation in tumors

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Abstract

Recent advances in our understanding of cancer biology have led to the development of therapies targeting specific signaling pathways. Molecular targeting promises to improve our ability to predict who will respond by assessing the state of these targeted pathways in patients. However, a single pathway can be deregulated by multiple mechanisms, and for some pathways it may be difficult to assess activation state by analyzing a single oncogene or tumor suppressor. Therefore, developing gene expression signatures of pathway activation status using model systems or human tumor samples may enable a more reliable measurement of pathway activity. This review discusses recent advances in the identification of gene expression–based signatures of pathway deregulation and how this information may lead to improved therapeutic response prediction. [Mol Cancer Ther 2006;5(10):2444–9]

Introduction

The identification of patient subpopulations most likely to respond to therapy is a central goal of modern molecular medicine. This notion is particularly important for cancer due to the large number of approved and experimental therapies (1), low response rates to many current treatments, and clinical importance of using the optimal therapy in the first treatment cycle (2). In addition, the narrow therapeutic index and severe toxicity profiles associated with currently marketed cytotoxics results in a pressing need for accurate response prediction. Although recent studies have identified gene expression signatures associated with response to cytotoxic chemotherapies (3–6), these examples (and others from the literature) remain unvalidated and have not yet had a major effect on clinical practice. In addition to technical issues, such as lack of a standard technology platform and difficulties surrounding the collection of clinical samples, the myriad cellular processes affected by cytotoxic chemotherapies may hinder the identification of practical and robust gene expression predictors of response to these agents. One exception may be the recent finding by microarray that low mRNA expression of the microtubule-associated protein Tau is predictive of improved response to paclitaxel (6).

To improve on the limitations of cytotoxic chemotherapies, current approaches to drug design in oncology are aimed at modulating specific cell signaling pathways important for tumor growth and survival (7–9). In cancer cells, these pathways become deregulated resulting in aberrant signaling, inhibition of apoptosis, increased metastasis, and increased cell proliferation (reviewed in ref. 10). Although normal cells integrate multiple signaling pathways for controlled growth and proliferation, tumors seem to be heavily reliant on activation of one or two pathways (''oncogene addiction''). In addition to the well-known dependence of chronic myelogenous leukemia on BCR-ABL, studies of the epidermal growth factor receptor and MYC pathways showed that inactivation of a single critical oncogene can induce cell death or differentiation into cells with a normal phenotype (11–16). The components of these aberrant signaling pathways represent attractive selective targets for new anticancer therapies. In addition, responder identification for targeted therapies may be more achievable than for cytotoxics, as it seems logical that patients with tumors that are ''driven'' by a particular pathway will respond to therapeutics targeting components of that pathway. Therefore, it is crucial that we develop methods to identify which pathways are active in which tumors and use this information to guide therapeutic decisions. One way to enable this is to identify gene expression profiles that are indicative of pathway activation status. This article will review recent examples from the literature that have used different methods to identify such signatures.

How to Measure Pathway Activation

Current methods for assessing pathway activation in tumors involve the measurement of drug targets, known oncogenes, or known tumor suppressors. However, one pathway can be activated at multiple points, so it is not

Received 6/8/06; revised 7/31/06; accepted 8/25/06.

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doi:10.1158/1535-7163.MCT-06-0340

always feasible to assess pathway activation by evaluating known cancer-associated genes (17). To illustrate this situation, consider signaling through phosphatidylinositol 3-kinase (PI3K; Fig. 1). This pathway is activated by multiple growth factors through receptor tyrosine kinases and has effects on multiple processes, including cell growth and survival, metastatic competence, and therapy resistance. PI3K signaling is often activated in human cancers, and many pharmaceutical companies are developing inhibitors of one or more pathway components (18). Therefore, accurate determination of PI3K pathway activation will be critical for the identification of potential responders to these emerging novel therapeutics.

However, the PI3K pathway can be activated by aberrations at multiple points, and assessing pathway activity may not be straightforward (19). For example, PI3K itself is frequently mutated in cancers. PI3K somatic missense mutations are common in HER2-amplified, hormone receptor–positive breast cancers, and PI3K mutation/amplification has been observed in ovarian cancer, gastric cancer, lung cancer, brain cancer, etc. (20–25). In addition, activating mutations in RAS occur in pancreatic and lung cancers (26), and a recent large-scale sequencing project in colorectal cancers recently identified novel infrequent mutations in PDK1 (27). Finally, AKT (activation, amplification) and PTEN (mutation, deletion, epigenetic inactivation) are also deregulated in many human cancers (28–34). Although PI3K pathway activation can be assessed by immunohistochemical analysis of PTEN or phosphorylated AKT levels in clinical samples (35), this may not be the optimal way to measure pathway activation. These assays are subject to the technical limitations of immunohistochemistry and are not quantitative. In addition, oncogenic pathways are complex (e.g., RAS signaling contributes to PI3K activation), so important pathway mediators may be missed by testing only a few well-characterized pathway components. The difficulty of measuring PI3K pathway activation by these means is reflected by inconsistent results reported in the literature when individual pathway components are analyzed in isolation (36, 37).

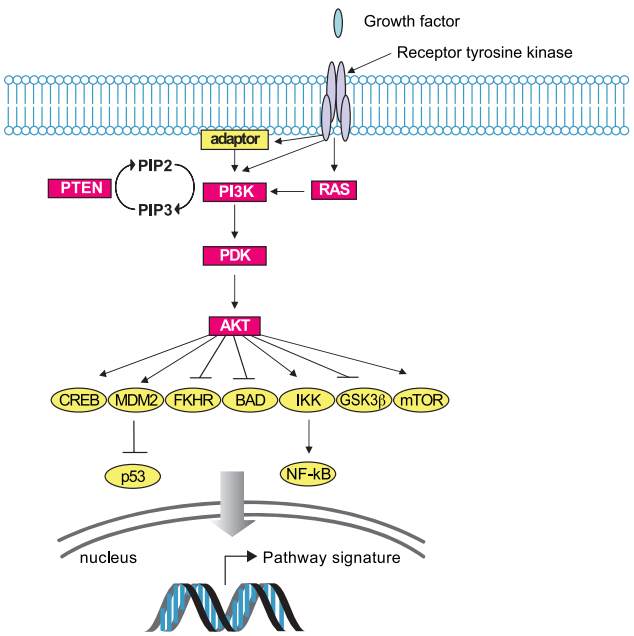

Figure 1. PI3K pathway activation and gene expression signatures. PI3K is activated by growth factors through receptor tyrosine kinases. In addition, PI3K can be activated by RAS, resulting in cross-talk with other signaling cascades (data not shown). On activation, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (*PIP2*) to phosphatidylinositol-3,4,5-trisphosphate (*PIP3*), a process that is reversed by PTEN. PIP3 signals activate the kinase PDK1, which in turn activates the kinase AKT. This signaling cascade affects multiple cellular processes and results in a gene expression ''signature'' of pathway activity. Activation of this pathway has been implicated in many cancers, and this activation can occur via aberrations in multiple pathway components (*red*). Because activation of various pathway components may lead to the same gene expression profile, a signature of pathway activation is likely to provide more accurate information than the assessment of a single known oncogene or tumor suppressor.

Examples like this suggest that a gene expression signature-based readout of pathway activation may be more appropriate than relying on a single indicator of pathway activity, as the same signature of gene expression may be elicited by activation of multiple components of the pathway. In addition, by integrating expression data from multiple genes, a quantitative assessment of pathway activity may be possible. Here, we review three recent articles that have addressed this problem in different ways, providing potential strategies for choosing the most effective therapy based on the gene expression signature of a tumor.

Identification of Pathway Signatures Using Animal Models

One method for elucidating pathway signatures is to engineer pathway activation into animal models and assess the gene expression profiles that arise as a consequence of this activation. Sweet-Cordero et al. (38) recently took this approach using a mouse lung cancer model in which a mutant *Kras2* allele is sporadically activated through spontaneous homologous recombination (Fig. 2A). Gene expression profiling was done on a set of lung tumors from this model, and these profiles were compared with normal lung tissue to identify genes showing significant differences between groups. The resultant signature was assessed in a human lung cancer gene expression data set (39) and shown to accurately classify tumor versus normal human samples. The authors then used a statistical technique known as Gene Set Enrichment Analysis to compare the mouse *Kras2* signature with human tumor data sets. Gene Set Enrichment Analysis is used to assess whether a gene set identified in one comparison (i.e., mouse *Kras2* lung tumor versus normal tissue) significantly overlaps the top or bottom of a ranked-ordered list of genes derived from a separate comparison (i.e., human tumor versus normal tissue). This technique was originally designed to detect modest but coordinate changes in groups of genes when these differences are subtle at the

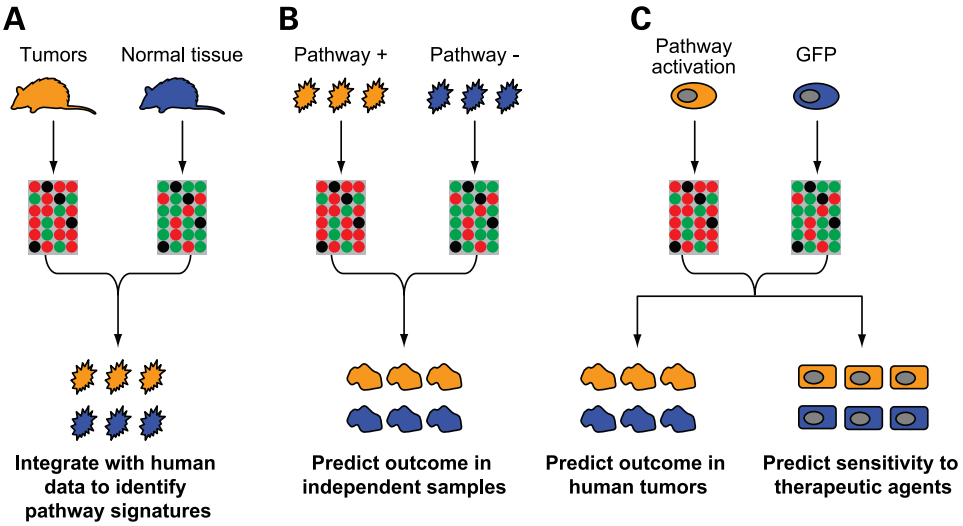

Figure 2. Multiple approaches to the identification of pathway signatures. **A,** using a *Kras2* mouse cancer model, Sweet-Cordero et al. (38) identified genes differentially regulated between tumor and normal tissue. These data were then integrated with human profiling data to identify a signature of *KRAS2* mutation in human lung cancers. **B,** using human tumor samples for signature discovery, Miller et al. (44) identified a signature of p53 pathway status. This signature was validated in independent human samples, and its ability to predict clinical outcome was shown to be superior to p53 mutation status alone. **C,** by engineering pathway deregulation into primary human cells *in vitro*, Bild et al. (52) identified signatures for five oncogenic pathways. These signatures were shown to be prognostic in human cancer cohorts and used to predict sensitivity to drugs targeting these pathways.

individual gene level (40) and has recently been used to identify conserved expression signatures between zebrafish and human liver tumors (41). When Gene Set Enrichment Analysis was used to compare the mouse *Kras2* signature with gene sets derived from tumor versus normal comparisons from a large panel of human tumors, significant enrichment was only detected in lung adenocarcinomas, a tumor type in which *RAS* is frequently mutated (42).

This result suggests that the mouse tumor signature is reflecting biology underlying lung adenocarcinomas. To identify a *KRAS2* signature using only human tumors, two lung adenocarcinoma gene expression data sets with known *KRAS2* mutation status (39, 43) were analyzed, and the number of genes differentially expressed between *KRAS2* versus wild-type groups was not larger than expected by chance. However, when the mouse *Kras2* signature was tested for enrichment in gene sets derived from these human comparisons using Gene Set Enrichment Analysis, genes up-regulated (but not down-regulated) in the mouse *Kras2* signature showed significant overlap with genes up-regulated in human mutant *KRAS2* adenocarcinomas. This suggests that a statistically significant signature can be identified when the mouse and human data are considered together and that *Kras2* has a stronger influence on up-regulation than down-regulation of gene expression. Integration of the mouse and human data resulted in a *KRAS2* signature that was shown to be relevant in pancreatic cancer, and the regulation of individual signature genes was shown when *KRAS2* was inhibited using RNA interference in human lung cancer cells.

Why was no significant signature detected when the human data were analyzed alone? One possible explanation is that assessing *KRAS2* mutations does not capture all information relevant to RAS pathway activation. For example, whereas some adenocarcinomas have *KRAS2* mutations, others may have mutations in other pathway components, such as epidermal growth factor receptor or *BRAF*. As these mutations are likely to result in a common mechanism of transformation, they may produce gene expression changes that are difficult to distinguish from *KRAS2* mutant tumors. As such, simply segregating human tumors based on *KRAS2* mutation status and assessing differences is not likely to capture alternative means of activating the same pathway. An alternative explanation involves the inherent difficulties in profiling heterogeneous clinical samples that may prevent the identification of more subtle gene expression signatures. Indeed, each individual gene in the final *KRAS2* signature identified in this study is only marginally differentially expressed in human tumors, and none meet statistical significance on their own. It is only when viewed as a collection of genes does the coordinate regulation of this signature reach statistical significance. It is important to note that these explanations are not mutually exclusive. In any case, this example underscores the use of *in vivo* mouse model systems for the discovery of pathway signatures. Discovery of signature genes may more easily be done in these models, as pathway activation can be engineered at a known point and the relatively homogeneous nature of samples results in reduced variability. It is not unreasonable to expect that other pathway signatures could be elucidated from an integrative genomics approach comparing human tumors with their representative mouse cancer models.

Identification of Pathway Signatures in Human Cancers

Does the above result suggest that it will not be possible to identify pathway signatures using only human samples? A recent study from Miller et al. (44) assessing p53 pathway status shows that signature discovery is feasible in human samples, especially for pathways with a large effect on gene expression (Fig. 2B). The p53 tumor suppressor protein is a potent transcription factor that is mutated in >50% of human cancers (45, 46), and p53 status has been reported to be predictive of chemotherapy response in multiple tumor types (47–49). However, multiple mechanisms of p53 functional loss have been reported, including mutations, deletions, MDM2 amplification, and p14/ARF deletion. This has led to inconsistent reports on the importance of p53 status when assessed using any one particular method (examples: refs. 50, 51). Because the determination of p53 status may be an important prognostic or predictive factor for many clinical programs, a more accurate way to measure p53 pathway status is needed.

In this study, 251 human breast cancer samples with information about p53 mutations (determined by cDNA sequencing) were used to identify gene expression profiles indicative of p53 pathway status. Supervised analysis revealed that >5,000 genes were significantly differentially expressed between tumors with p53 mutations versus p53 wild-type tumors. This is in stark contrast to attempts to identify a *KRAS2* mutation signature using only human tumors as described above, underscoring the extensive effect of p53 status on gene expression in human tumors. This p53 signature was then reduced to a set of 32 genes with optimal performance. However, this gene set misclassified 26 of 193 tumors with no mutations as p53 mutants and 12 of 58 tumors with p53 mutations as wild-type. This suggests that the gene expression classifier may be flawed or that cDNA sequencing and mutation analysis is not a comprehensive means by which to determine p53 functional status.

To address this question, Miller et al. assessed the transcript levels of p53 in addition to six known p53 target genes in this tumor panel. Indeed, p53 and its targets showed significantly reduced transcript levels in the 26 tumors with no p53 mutations that were misclassified as mutants when compared with the other wild-type tumors. In addition, p53 and its targets showed significantly increased expression in the 12 tumors with mutations that were misclassified as wild-type when compared with the other mutant tumors. Finally, when the mutations harbored by the 12 p53 mutant tumors misclassified as wild-type were assessed in more detail, a significant lack of ''severe'' mutations (insertions, deletions, nonsense mutations) relative to the other mutant tumors was observed. These results suggest that the gene expression classifier is a more accurate reflection of p53 functionality than mutation status. This assertion was supported by the finding that the p53 gene expression classifier had greater prognostic significance for disease-specific survival in this cohort when compared with p53 mutation status. In addition, this pathway signature was able to predict outcome in independent breast cancer cohorts, including patients treated with postoperative radiation and adjuvant tamoxifen monotherapy, patients receiving systemic doxorubicin or 5-fluorouracil plus mitomycin, and patients treated with radiotherapy alone. These findings show that p53 functional status is determined by the interplay among multiple factors, of which mutation status is only one. Therefore, just as assessing one oncogene in an activated pathway may not reveal the total status of pathway activation, no single molecular assessment of p53 activity is likely to provide complete information about p53 functional status. In addition, it is now possible to assign a quantitative score to p53 functional status rather than simply categorizing tumors as ''mutant'' or ''wild-type.'' Although the biological and clinical implications of intermediate tumor p53 activity are not clear, a more accurate determination of the state of p53 activity in tumors may help improve prognosis and therapy selection.

In vitro Discovery of Pathway Signatures

An alternative approach to the identification of pathway signatures is to engineer pathway activation into cells in culture and assess the resultant gene expression changes. Bild et al. (52) recently used this approach to identify signatures of activation of MYC, RAS, SRC, and β-catenin or loss of RB function (Fig. 2C). Recombinant adenoviruses were used to express these oncogenic activities in human primary mammary epithelial cells, resulting in the isolation of gene expression signatures caused by specific pathway deregulation in a homogenous cellular background. Supervised analysis was used to identify gene expression signatures indicative of pathway activity in each case, and these signatures were shown to accurately distinguish deregulation of each particular pathway from the others. In addition, combined analysis of the signature genes was used to assign a quantitative score reflecting the relative probability of deregulation of each pathway in cell lines or tumor samples.

These signatures were then validated by comparison with mouse cancer models in which the initiating oncogenic event is known. For example, mouse tumors induced by mouse mammary tumor virus–driven expression of MYC showed the highest prediction of MYC pathway deregulation on the gene expression level, whereas the highest prediction of RAS pathway deregulation was observed in mouse mammary tumor virus-RAS tumors. The RAS result was further validated in the Sweet-Cordero et al. data set described above (38), in which RAS is activated by homologous recombination in adult animals and in human lung cancers bearing RAS mutations. Although such validation shows that these signatures are not specific to human primary mammary epithelial cells, the most attractive aspect of this approach is the ability to assess patterns of pathway deregulation in human tumors.

To address this, the predicted degree of pathway deregulation was used to do hierarchical clustering of

human tumor samples, showing that this information can be used to cluster tumors into bins that show correlation with clinical outcome. One important finding from this analysis was that tumors showing coderegulation of multiple pathways (e.g., RAS, β-catenin, SRC, and MYC) were associated with a very poor survival, and individual pathway status was not as effective at patient stratification as patterns of multiple pathway deregulation. This result underscores the need for analyzing multiple pathways when developing rational combination therapies for complex cancers.

In addition to prognosis, the ability of pathway signatures to predict drug response is a critical issue for personalized medicine. To test the use of pathway signatures for response prediction, Bild et al. used gene expression and biochemical techniques to assess RAS and SRC pathway deregulation in a series of breast cancer cell lines, and these cells were treated with drugs targeting the RAS or SRC pathways. In all cases, the degree RAS or SRC pathway activity as assessed by gene expression profiling accurately predicted *in vitro* sensitivity to drugs targeting these pathways. Interestingly, although the RAS signature predicted response to RAS inhibitors, this signature did not correlate with levels of activated RAS in the cell as assessed biochemically. This is more evidence to support the assertion that similar patterns of gene expression can be achieved by upstream or downstream components of a pathway, and for RAS, biochemical measurement of this oncogene alone is not a comprehensive assessment of pathway activity.

Conclusion

It is becoming increasingly clear that the activation of oncogenes or inactivation of tumor suppressor genes often results in the deregulation of complex cell signaling pathways. Because accurate determination of pathway status will be critical for cancer prognosis and response prediction, there is a need to move away from measuring a single oncogene or tumor suppressor in isolation. Recent evidence suggests that gene expression profiles provide a more comprehensive means by which to assess the functional consequence of pathway deregulation and therefore may be an effective tool for personalized medicine. Although there are clear examples for which pathway activation and drug response can be predicted by a single oncogenic mutation [i.e., epidermal growth factor receptor mutation and small-molecule epidermal growth factor receptor inhibitors (11, 12), *BRAF* mutation, and mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors (53)], it seems likely that more thorough genomic analyses will be necessary to accurately predict response to other therapeutics. This may be particularly true for drugs targeting complex signaling pathways.

Transcriptional profiling is certainly not the only technology available for pathway analysis. Proteomics technologies, including lower-throughput analyses of phosphoproteins involved in signal transduction cascades, are rapidly evolving and it may soon be possible to do comprehensive transcriptional, proteomic, metabolic, and single-nucleotide polymorphism profiling analyses to evolve a systems biology approach to the dissection of pathways. However, given the current state of technology, gene expression microarrays offer an exciting opportunity to comprehensively measure pathway activation and provide a means by which to rationally choose which pathway-specific drug or combination of drugs is likely to be most effective. Future work is likely to address the potential difficulty in defining a ''pathway'' because of branching and cross-talk between signal transduction cascades. Undoubtedly, further developments in our understanding of complex signaling mechanisms and their interplay will enable a more complete assessment of pathway structure and activation. Considering the large number of pathway-targeted therapies in clinical development and the pressing need to target these therapies to the appropriate patients, the development of pathway signatures promises to improve cancer therapy in the foreseeable future.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2006;5:2444-2449.