Stem cell–ness: a "magic marker" for cancer

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Commentary

Transcriptional profiling of patient tumors is a much-heralded advancement in cancer therapy, as it provides the opportunity to identify patients who would benefit from more or less aggressive therapy and thus allows the development of individualized treatment. However, translation of this promise into patient benefit has proven challenging. In this issue of the *JCI*, Glinsky and colleagues used human and murine models to identify a potential stem cell mRNA signature, based on the hypothesis that tumors with stem cell–like characteristics are likely to have a poor prognosis. Remarkably, an 11-gene "expression signature" associated with "stem cell–ness" separated patients with different cancers into good- and poor-prognosis groups. Such a "magic marker" would, if validated, have a major impact on patient care. However, there remain challenges incumbent with creating and validating such signatures.

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Stem cell–ness: a "magic marker" for cancer

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Transcriptional profiling of patient tumors is a much-heralded advancement in cancer therapy, as it provides the opportunity to identify patients who would benefit from more or less aggressive therapy and thus allows the development of individualized treatment. However, translation of this promise into patient benefit has proven challenging. In this issue of the *JCI*, Glinsky and colleagues used human and murine models to identify a potential stem cell mRNA signature, based on the hypothesis that tumors with stem cell–like characteristics are likely to have a poor prognosis (see the related article beginning on page 1503). Remarkably, an 11-gene "expression signature" associated with "stem cell–ness" separated patients with different cancers into good- and poor-prognosis groups. Such a "magic marker" would, if validated, have a major impact on patient care. However, there remain challenges incumbent with creating and validating such signatures.

Prediction and cancer

The inception of high-throughput analyses using oligonucleotide microarrays has given biologists the ability to globally assess RNA levels in a patient's tumor sample. A typical microarray study generates data on the expression of the approximately 20,000 human genes and soon studies will be able to analyze the more than 150,000 splice variants of RNA that are likely to have functional roles. The inherent challenge is to convert this data into applicable knowledge. A potential strength of the technology lies in its ability to uncover complex gene interaction patterns and correlate those patterns with clinically relevant outcomes. This "holy grail" could ultimately predict not only the therapeutic response of the tumor present in each patient, but also the patient's survival, which would subsequently lead to the development of individualized therapy for each patient based both on the genetic aberrations in the tumor and on the patient's own genetic makeup. However, despite early enthusiasm, there have been considerable challenges in converting the promise of individualized molecular medicine into clinical practice.

In this issue of the *JCI*, Glinsky and colleagues outline a possible expression signature comprising 11 genes that has the ability, according to the authors' analysis, to segregate tumor samples from multiple tumor lineages into those that have good or poor prognoses (1). The authors have applied this gene set to multiple tissue types from disparate data sets and have repeatedly observed its predictive power. The application of their 11-gene signature to these independent sets addresses an analytical limitation that is often overlooked when "predictive" gene expression signatures are found in microarray experiments (2, 3). When thousands of measurements are taken on each patient, the number of ways to select some of those measurements as a pattern classifying tumors or predicting outcomes is enormous. When selecting multiple gene measurements, the probability of finding a combination with apparent clinical relevance just by chance is even higher.

This multiple-measurements problem can be addressed using a "training and test set" approach, wherein predictive models are validated on separate, independent

Table 1
Gene expression signature detected by Glinsky et al. (1) and reported to predict good or poor prognosis of patients with multiple types of cancer

Affymetrix MG-U74A probe set	Mouse UniGene no. (build 145)	Gene name (HUGO symbol)	Signature value (log$_{10}$)
94200_at	Mm.204730	*Gastrulation homeobox 2* (*GBX2*)	2.3
99457_at	Mm.4078	*Antigen identified by monoclonal antibody ki-67* (*KI67*)	2.9
160159_at	Mm.260114	*Cyclin B1* (*CCNB1*)	2.5
104097_at	Mm.2185	*Budding uninhibited by benzimidazoles 1* (*BUB1*)	2.1
93441_at	Mm.373656	*Kinetochore-associated protein 2* (*KNTC2*)	2.0
97960_at	Mm.30602	*Ubiquitin-specific protease 22* (*USP22*)	1.9
100901_at	Mm.248353	*Host cell factor C1* (*HCFC1*)	1.4
93164_at	Mm.31512	*Ring finger protein 2* (*RNF2*)	1.7
98477_s_at	Mm.235960	*Ankyrin 3* (*ANK3*, also known as *Ankyrin G*); *ankyrin 3* in mice	−0.5
93090_at	Mm.16340	*Fibroblast growth factor receptor 2* (*FGFR2*)	−0.6
101538_l_at	Mm.292803	*Carboxylesterase 1* (*CES1*); *carboxylesterase 3* (*CES3*) in mice	−2.7

data sets of adequate size. However, maintaining consistency of the thresholds and cutoffs used across data sets is fundamental to this paradigm. If a certain threshold is used to indicate a specific change on the training set, then it is important that the same threshold be used with subsequent test sets. While Glinsky et al. (1) do separate each data set into training and test sets, they require a separate training and test set and different cutoffs for each cell lineage and potentially for each RNA analysis platform. Since the authors use different cutoffs to identify prognosis in different data sets, one must remain cautious about whether their approach will be generally applicable to patient management. Furthermore, splitting a single data set into 2 smaller sets for training and test purposes also introduces statistical and analytical challenges, since the smaller data sets will have diminished power (4).

Stem cells and cancer progression

A key insight in the Glinsky et al. study (1) is the biological motivation driving their selection of the gene signature. The authors begin with the plausible hypothesis that transformed cells, in which self-renewal or stem cell–related pathways are activated, may contribute to the survival of cancer cells in tumors and promote tumor progression and poor prognosis for patients. They apply this idea across species, combining a study of stem cells in a *murine leukemia viral-1* (*Bmi-1*) knockout mouse model with a study of primary and metastatic tumors in a model of transgenic adenocarcinoma of the mouse prostate (TRAMP) in order to select genes that consistently display a stem cell self-renewal–like expression profile in

multiple models. This approach builds on the paradigm that cancer likely arises in a limited population of stem cells. These stem cells could potentially have a set of common characteristics, and thus gene expression patterns, across tumor lineages. Characterization of a common expression signature with supplemental tissue-specific gene changes could reflect the cell of origin of a cancer. This concept is compatible with the observation that most tumors are less differentiated than the putative precursor cell and that only a small number of normal cells have the potential for self-renewal (5).

Moreover, if this signature can delineate those patients whose tumors rely on stem cell–like gene expression, then targeting those genes within the tumors may result in a cell population with limited proliferative potential. There is strong support for the hypothesis that the clones that initiated the cancer are different from the majority of cells within the tumor (6). Different pathways may be activated within different clones, and thus therapeutic targeting of these initiating cells may lead to a better outcome. Traditional therapies are aimed at the rapidly dividing cells within the tumor; while these may reduce tumor mass, they may not lead to cures.

The significance of the *Bmi-1*–based 11-gene signature

Most statisticians (and many biologists) are leery of studies that claim to find "gene signatures" or "patterns of gene expression" that can be used to predict clinical outcomes across tumor lineages. Much of the uneasiness arises because many studies neglect to precisely define the notion of a signature. By contrast, Glinsky and

colleagues produce a concrete definition of a gene signature (1). Their signature is defined quantitatively as an 11-dimensional vector of expression fold-change values in the base-10 logarithm of 11 genes in the peripheral nervous system (PNS) neurospheres in the mouse *Bmi-1* knockout model. Deviations from the average expression of these 11 genes in individual tumor samples are correlated with this 11-dimensional vector. While this signature had classification and predictive value when assessed on human tumors, it is important to note that other potential "stem cell–ness" signatures, such as the 14-gene group discussed in the study, did not demonstrate predictive value.

The transcription factor *Bmi-1* appears to play a role in gene regulation in both cancerous and normal stem cell proliferation through epigenetic mechanisms — changes that affect gene expression without altering gene structure, such as methylation or acetylation of chromatin. As the authors note, *Bmi-1* has previously been shown to be required for maintenance of self-renewing HSCs (7) and for the self-renewal of leukemic stem cells (8). *Bmi-1* has been implicated in extending the replicative potential of human fibroblasts through the suppression of the senescence pathway dependent on *p16* (a cyclin-dependent kinase inhibitor) in a retinoblastoma protein–dependent manner (9). Further, a deficiency in the *p16*^{INK4a} gene partially reverses the self-renewal defect in *Bmi-1* dominant-negative neural stem cells (10). Additionally, a cooperative interaction between *Bmi-1* and the oncogene *c-myc* has been demonstrated, through enhancement of mouse embryonic fibroblast proliferation, as a result of

Table 2

Probe sets on the Affymetrix U95Av2 array representing the 11 genes

U95Av2 probe set	Gene
33688_at	GBX2
418_at	MKI67
419_at	MKI67
1945_at	CCNB1
34736_at	CCNB1
41081_at	BUB1
35699_at	BUB1
40041_at	KNTC2
39866_at	USP22
37910_at	HCFC1
33484_at	RNF2
36965_at	ANK3
36966_at	ANK3
36967_g_at	ANK3
1363_at	FGFR2
1970_s_at	FGFR2
34354_at	FGFR2
37203_at	CES1

inhibiting *c-myc*–induced apoptosis and $p19^{arf}$ (11). It is apparent that the Polycomb group gene *Bmi-1* is potentially involved in various phases of tumorigenesis; therefore, the 11-gene expression signature highlighted by Glinsky and colleagues (1), created from changes in gene expression induced by altering *Bmi-1* in different backgrounds and validated on data collected by different researchers at different times, must be taken seriously and subjected to extensive evaluation and validation in independent laboratories.

The 11-gene signature

At the very least, the 11-gene signature suggests the possibility of more accurately identifying patients with poor prognoses as candidates for more aggressive or investigational therapy. As the mode of treatment is different for each tumor lineage, it also suggests that the signature truly indicates prognosis rather than predicting response to therapy. However, it could include a general indication of sensitivity to cell death as a component of its prognostic load, although the individual components of the 11-gene signature have not been previously indicated as major regulators of cell survival.

The components of this 11-gene set vary in function and exist in different pathways (see Table 1 for gene names, signatures, and values). Both budding uninhibited by benzimidazoles 1 (BUB1) and kinetochore-associated 2 (KNTC2; also known as HEC) have been implicated as mitotic checkpoint

proteins, and as such, aberrations in their function could contribute to genomic instability and aneuploidy. Mutations in the *BUB1* gene lead to its inactivation and increase microsatellite instability in colon cancer as well as a predisposition to certain types of cancers (12, 13). Increased gastrulation homeobox 2 (GBX2) expression stimulates growth of human prostate cancer cells through upregulation of the gene coding for IL-6 (14). Overexpression of cyclin B1 (CCNB1) levels have been observed in high-grade large-cell and small-cell lung carcinoma (15) and have been shown to be downregulated as a result of p53 induction in non–small-cell lung cancer (16). Additionally, CCNB1 expression was highly correlated with the labeling index for antigen identified by mAb ki-67 (Ki-67, associated with increased tumor cell proliferation), which suggests a key role for CCNB1 in the regulation of neuroendocrine tumor cell proliferation (15). In breast cancer cell lines, overexpression of the *FGF receptor 2* (*FGFR2*) gene resulted in activation of the MAPK and PI3K pathways (17). Interestingly, restoration of this gene product into a malignant prostate epithelial cancer cell line, PC3, led to suppression of malignancy and restoration of nonmalignant traits. Thus, 3 of the 11 genes identified in the Glinsky et al. gene signature (1) are related to cell proliferation and 2 to transition through mitosis. While little is known regarding the specific biological functions of ubiquitin-specific protease 22 (USP22), ubiquitin-specific proteases have been implicated in control of regulatory molecules such as p53 and cyclins (18). The ring finger 2 (RNF2) protein is part of the Polycomb group of proteins, like Bmi-1, that play key roles in hematopoiesis and cell-cycle regulation (19). Ankyrins are transmembrane proteins shown to be involved in cellular functions relating to the influx and efflux of sodium and calcium (20, 21). Carboxylesterase (CES) enzymes are found in many animal species and play a role in the hydrolysis of drugs such as steroids and anticancer agents (22). Mutations in CES1 have been implicated pharmacogenomically in the activation status of cancer drugs and prodrugs (23). At this time, the role of host cell factor c1 (HCFC1) in cancer has yet to be evaluated; however, as the host cell factor family is implicated in immunomodulation, it is possible that HCFC1 plays a role in limiting the immune response to cancer or in the production of cytokines such as IL-6 or IL-8, which can contribute to neovascularization or tumor growth.

As demonstrated with the prostate cancer data set, the 11-gene set can be divided into 2 groups: those for which elevated expression levels are associated with stem cell–ness and a poor prognosis (*Ki67*, *CCNB1*, *GBX2*, *BUB1*, *KNTC2*, *USP22*, and *RNF2*, in descending order of strength of association) and those for which decreased expression levels are associated with stem cell–ness and a good prognosis (*CES1*, *FGFR2*, and *ankyrin 3* [*ANK3*]; see Table 1) (1). High levels of these stem cell–related genes indicate a potential for self-renewal within tumors and for increased tumor aggressiveness within patients. Intriguingly, those genes that are positively associated with tumor cell proliferation (*Ki67*, *CCNB1*, and *GBX2*) and with the mitotic spindle (*BUB1* and *KNTC2*) are also positively associated with the stem cell–ness signature. *FGFR2*, which has been shown to decrease the growth of prostate cancer cells, is negatively associated with the stem cell–ness signature. The association between this 11-gene signature and good and poor prognosis therefore makes sense, at least for those genes that have been characterized as associated with the behavior of cancer cells.

While the relevance to cancer progression and malignancy has been studied and even established in certain members of this 11-gene set, others have yet to be studied in detail. This underscores another strength of high-throughput analysis: the use of such a global approach may identify new and unex-
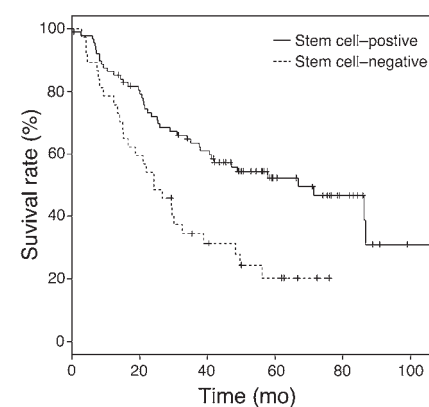

Figure 1

Stem cell–positive samples correlate with poor prognosis in lung adenocarcinoma patients. Cox proportional hazards model of lung cancer patients (*n* = 125) using the SPAI confirmed the correlation between patient samples with stem cell–like expression pattern and poor overall survival (*P* = 0.0179). Analysis based on patient data from the lung cancer study by Bhattacharjee et al. (26).

pected targets for study, some of which may prove to be potential therapeutic targets. The inclusion of genes involved in cancer cell proliferation as well as unexplored genes within this predictive group suggests that further analysis into their mechanisms and potential involvement in signaling pathways germane to cancer is warranted.

Can their analysis be replicated?
In many microarray studies, how the data are analyzed may be more important than how they were generated. It is therefore incumbent upon researchers to describe their analytical methods in enough detail to allow independent researchers to replicate their computations on the same data. Ideally, these details take the form of precise equations or algorithms or the actual code used to analyze the data (24–26). Although the Glinsky et al. study (1) reports many of the critical values needed to replicate their results, their reliance on purely verbal descriptions leaves room for some ambiguity.

We attempted to replicate part of their analysis and turned our attention to the same lung cancer study used by the authors (27), which included survival data on 125 patients and microarray data from Affymetrix U95Av2 GeneChips. Our estimate of the standard gene expression signature is listed in Table 1. Because of redundancy, a total of 18 probe sets represent these 11 genes on the U95Av2 array (Table 2). We used all 18 probe sets, which expanded the fixed gene signature vector from Table 1 into an 18-dimensional vector. For each of the 18 probe sets, we computed the average expression over all 125 samples. We divided the expression vectors in individual tumor samples by the average expression value and then transformed the ratios by computing the base-10 logarithm, producing 18-dimensional vectors. We then computed the Pearson correlation coefficient between the fixed gene expression signature vector and the individual vector of log ratios. This procedure yielded 1 number, x_i, for each tumor sample $i = 1, \ldots, 125$, which represented our best estimate of the stem cell–like phenotype association index (SPAI) described in the Glinsky et al. study (1).

We used SPAI as a continuous covariate in a Cox proportional hazards model to predict survival in the full data set and found that SPAI was significant (likelihood ratio test, $P = 0.0179$). Because the coefficient x_i in the model was positive (0.798), higher values of SPAI were associated with shorter survival. Using trial and error, we determined a threshold on the full data set that, when used to split the samples into 2 groups, yielded the best results in a survival analysis. The optimum threshold occurred at 0.32 and separated the data into 40 samples with a stem cell–like profile ($x_i > 0.32$) and 85 samples without a stem cell–like profile. To this extent, we were able to confirm the analysis reported by Glinsky et al. (Figure 1).

Based on this preliminary attempt to replicate the authors' analyses, we believe that their results should be greeted with cautious optimism. However, we were unable to validate their method when splitting the data multiple times into training and test sets. Our splits were more challenging than the ones used by the authors, since we did not use outcome information to balance the splits. It is also not clear whether we followed the same procedure for computing the SPAI that was used by Glinsky et al. (1).

Conclusions
Glinsky and colleagues (1) have produced a stimulating analysis of a collection of microarray data experiments. Using ideas that were well motivated by the underlying biology and combining data across species from 2 different microarray studies, they identified an 11-gene signature that might be related to tumor behavior, and thus patient survival, in cancer. In order to validate this signature, they tested it retrospectively in a broad spectrum of microarray studies of different kinds of cancer.

In our hands, unsupervised analyses of expression levels across some of the same data sets used by Glinsky and colleagues (1) did not reveal any clinically relevant or statistically significant correlations with outcome. By introducing a weighting coefficient into their predictor model, they are in fact adding an element of supervision into the analysis. Further, as these coefficients are re-derived on each subsequent data set, the statistical and analytical issues incumbent with high-throughput technologies are insufficiently addressed (2).

Glinsky et al. proposed several models, but only the 11-gene metastatic TRAMP tumor sample/PNS set consistently differentiated samples in a clinically relevant manner (1). An important aspect of this gene set and the corresponding coefficients used in determining the weighted survival predictor is that those genes that have been previously identified as potential indicators of poor prognosis are given the greatest coefficients. That is, negative weighting correlates with longer survival. For example, in the prostate cancer SPAI, the authors assign the largest coefficient value to Ki-67, which is associated with increased proliferation in cancer and could therefore lead to greater aggressiveness and poorer prognosis.

In the search for a powerfully predictive set of cancer genes, there have been various signatures proposed that contain anywhere from 10–100 genes. However, recent studies have cast doubt on the power of those sets that were validated within a single data set or even a single tumor type (2–4). As such, the need for validation of a proposed gene expression signature across independent data sets is warranted. To their credit, Glinsky and colleagues have gone to great lengths to validate their 11-gene signature, albeit in a supervised manner, across multiple tumor samples and multiple tissue types. As researchers begin to question the results reported from microarray studies (2–4, 28), the need for replicable analyses and independent corroboration grows more acute. Regardless of whether it was said by Niels Bohr or Yogi Berra, it is still the case that "Prediction is very difficult, especially about the future."

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Toward improved immunocompetence of adoptively transferred CD8+ T cells

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Adoptive transfer of autologous or allogenic T cells to patients is being used with increased frequency as a therapy for infectious diseases and cancer. However, many questions remain with regard to defining optimized procedures for preparation and selection of T cell populations for transfer. In a new study in this issue of the *JCI*, Gattinoni and colleagues used a TCR transgenic mouse model to examine in vitro–generated tumor antigen–specific CD8+ T cells at various stages of differentiation for their efficacy in adoptive immunotherapy against transplantable melanoma (see the related article beginning on page 1616). The results confirm that CD8+ T cells progressively lose immunocompetence with prolonged in vitro cultivation and suggest that effector CD8+ T cells alone may be considerably less potent at protecting hosts with advanced tumors than are less differentiated T cells.

In addition to the well-established donor lymphocyte infusion (DLI) approach to treating leukemia relapse after HSC transplantation, adoptive cell transfer therapy (ACT) is also being developed to treat EBV- and CMV-associated diseases, and more recent initiatives have focused on the use of ACT to treat solid human cancers, primarily melanoma (1–5). Unfortunately, in vitro–cultured antigen-specific T cells,

particularly T cell clones, often die only a few hours after adaptive transfer and generally do not survive more than a matter of days, which limits treatment efficacy (6, 7). By contrast, T cells adoptively transferred directly from donor to recipient show increased survival rates and are more likely to become immunoprotective; this has been confirmed by transfer experiments using T cells from TCR transgenic mice, which provide unprecedented amounts of naive antigen-specific donor T cells and thus circumvent the need for in vitro T cell cultivation (8). In humans, the most durable form of ACT is DLI, which usually involves direct peripheral wbc transfer from the allogeneic donor to the leukemia patient who has previously received HSCs

from the same donor. Most patients with solid tumors, however, have never undergone allogeneic stem cell transplantation and thus cannot receive donor cells, but instead depend on transfer of autologous cells. These cells must be selected and/or enriched in cell cultures in order to obtain large numbers of T cells with appropriate antigen specificity. One of the great challenges in ACT lies in the development of optimal procedures for lymphocyte selection and preparation.

T cell differentiation

The study by Gattinoni et al. (9) in this issue of the *JCI* addresses the question of whether progressive CD8+ T cell differentiation toward an effector T cell phenotype is associated with changes in the cells' capacity to protect the host from disease. The study provides detailed insight into the relationship among the duration of in vitro T cell culture, the functional and phenotypic characteristics of T cells at various stages of differentiation, and their immunocompetence upon adoptive transfer. The authors performed sequential rounds of in vitro stimulation in order to promote progressive CD8+ T cell differentiation. The longer the T cells were stimulated and cultured in vitro, the more they acquired the