

Review

Gene Expression Profiling on Lung Cancer Outcome Prediction: Present Clinical Value and Future Premise

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Abstract

DNA microarray has been widely used in cancer research to better predict clinical outcomes and potentially improve patient management. The new approach provides accurate tumor classification and outcome predictions, such as tumor stage, metastatic status, and patient survival, and offers some hope for individualized medicine. However, growing evidence suggests that gene-based prediction is not stable and little is known about the prediction power of gene expression profiles compared with well-known clinical and pathologic predictors. This review summarized up-to-date publications in microarray-based lung cancer clinical outcome prediction and conducted secondary analyses for those with sufficient sample sizes and associated clinical information. Among the most commonly used analytic approaches, unsupervised clus-

tering mainly recaptures tumor histology and provides variable degrees of prediction for tumor stage, lymph node status, or survival. Overall, most studies lack an independent validation. Supervised learning and testing generally offer a better prediction. Noted is that when conventional predictors of age, gender, stage, cell type, and tumor grade are considered collectively, the predictive advantage of the gene expression profiles diminishes. We conclude that outcome prediction from gene expression signatures selected by current analytic approaches can be mostly explained by well-known conventional predictors, particularly histologic subtype and grade of differentiation. A strategy for establishing independent or more accurate signatures is commented. (Cancer Epidemiol Biomarkers Prev 2006;15(11):2063–8)

Introduction

Lung cancer, a disease of somatic gene mutations and regulation disturbance, causes dramatic gene expression changes in its tumor cells. These changes can be interrogated simultaneously by DNA microarray technology to distinguish the high from the low aggressive tumor natures, which, in turn, may lead to a more accurate clinical outcome prediction and a better treatment option. Published studies in this fast-growing field have provided some promising results. For example, gene expression profiling can help to identify a subtype of lung adenocarcinoma with poor prognosis (1), or a gene panel can reliably predict patient survival for lung adenocarcinoma (2). Emerging evidence also shows that the accuracy of expression-based outcome prediction varies greatly among studies (3) and the reliability of molecular signatures largely relies on the selection of patients (4). However, very few studies have compared the prediction performance of gene expression profiles with previously known (or conventional) predictors, including age, gender, tumor stage, and histologic features. Thus, converging questions have been raised from researchers and clinicians: Why does gene-based prediction vary? Can DNA expression profiles provide more accurate prediction than conventional predictors? Are gene panels or molecular signatures independent predictors or merely surrogates of conventional factors?

The usefulness of a novel factor or a genetic marker panel for clinical outcome prediction depends on whether it provides

additional and critical information compared with what is currently available in practice (5). In this review, we systematically examined the published results from microarray-based outcome studies in lung cancer. We first presented associations between gene expression profiles and tumor histology, stage, and patient survival. We then geared our focus on prediction performance comparison between molecular signatures and conventional predictors, with supplemental analysis using published data. Last, we provided our insights of future directions in gene expression profile-based outcome prediction studies. The main purpose was to use lung cancer as an example to illustrate the current advances and the issues in this promising research area.

Gene Expression Profiles and Histologic Features

Clustering Recapitulates Histologic Cell Type or Subtype. Hierarchical clustering (6) is one of the most commonly used approaches in microarray studies. Tumor clusters obtained from this approach closely reflect tumor histologic cell types. For example, adenocarcinoma, squamous cell carcinoma, small-cell carcinoma, or carcinoid is clearly distinguishable by forming their own clusters (1, 7–10). Similarly, a subtype of certain histologic cell types, such as adenocarcinoma, shows more diverse gene expression profiles (1, 7), reflecting the histologic complexity of the cell type at the tissue level where six subtypes and several sub-subtypes are defined according to the WHO classification of lung cancer (11). Although many studies did not provide sub-cell type information for adenocarcinoma, available data suggest that bronchioloalveolar carcinoma (BAC), a special subtype of adenocarcinoma with distinct clinical and pathologic features (12), has a very different gene expression profile from other subtypes of adenocarcinoma. In a study where the four distinct subclasses (C1–C4) of adenocarcinoma were identified by Bhattacharjee et al. (1), 10 of 15 tumors in C4 were BAC type. In Beer's (2)

Received 6/20/06; revised 8/8/06; accepted 8/17/06.

Grant support: NIH grant CA84354 (P. Yang) from the National Cancer Institute and Mayo Foundation Funds.

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doi:10.1158/1055-9965.EPI-06-0505

series with three distinct clusters, all BACs were in cluster 1 or 2 but none in cluster 3. The close association between the adenocarcinoma subtype and the sample cluster was statistically significant (Table 1).

The high correlation between histologic cell/sub-cell type and unsupervised sample cluster has three implications: First, genes related to cell type differentiation dominate differing gene expression patterns among tumors. Second, unsupervised clustering may be a useful tool to identify a subclass of a tumor with a distinct clinical behavior that is otherwise indistinguishable using current histologic tools. For instance, a subclass of adenocarcinoma of the lung with neuroendocrine features was found to have worse prognosis (1), and squamous cell carcinoma with different profiles gave rise to varied patient survivals (9, 10). More specifically, tumors with invasive growth pattern, active stromal reaction, and prominent keratinization had worse prognosis than those with a well-circumscribed border, minimal stromal reaction, and inconspicuous keratinization and nuclear pleomorphisms (10). However, these findings need to be further validated because the published results were obtained from a very limited number of samples. It is imperative to examine whether the molecular classification can be easily made by standard histologic examinations. Any new technique that does not significantly outperform less expensive and easily conducted approaches is less likely to be useful in clinical practice. The molecular classification by DNA microarray is better suited to the cases that challenge the conventional methods and tools. Third, studies lumping different histologic subtypes with proven distinct outcomes together for gene expression profiling may unnecessarily complicate result interpretations and exaggerate the prediction power. A typical example is the mix of BAC and other non-BAC adenocarcinoma. Because BAC is known for a favorable prognosis over non-BAC adenocarcinoma (12), when the two histologic subtypes are profiled together, it is very likely that the genes associated with clinical outcomes are surrogates for different subtypes. Those genes are not necessarily responsible for tumor-aggressive behaviors and when they are tested in a different set of patients with a different cell type composition from the original samples, they likely do not show independent or consistent predictive values.

Histologic Grade of Differentiation (Tumor Grade) Plays a Significant Role in a Gene Expression Profile. Tumor grade is a quantitative measurement of cell differentiation. It is separate but sometimes closely related to histologic cell type. For example, BAC is always well or moderately differentiated; whereas, large cell, sarcomatoid, or small cell by definition are always poorly differentiated or undifferentiated. This complicates the evaluation of the role of tumor grade in gene expression profiling when different cell types are investigated at the same time. Nonetheless, analyses conducted on the same histologic cell type suggest an important role of tumor grade in gene expression profiles: Tumors with similar grade tend to cluster together, as shown in Table 1 (2, 7). The effect of tumor grade on gene expression profiling is more evident in such tumors with obvious differentiation gradient as prostate (13) and renal cell carcinoma where heterogeneous cell types are less of a concern (14).

Gene Expression Profiles and Tumor Stages

Tumor-node-metastasis stage is the most important outcome predictor for lung cancer, and it often functions as a surrogate for survival. However, accurate staging could be challenging because micrometastasis are easily overlooked by conventional pathologic examinations or low sensitivity of clinical imaging. Because tumor cells go through various genetic changes from initiation to progression and metastasis, researchers have attempted, by analyzing gene expression profiles of a primary tumor, to conduct molecular tumor staging in achieving more accurate clinical outcome predictions and treatment options.

Molecular Tumor Staging. The correlation between unsupervised sample clusters and lung cancer stages was observed in several studies (2, 7). Beer et al. (2) found that more low-stage (stage I) tumors were clustered together; however, these tumors were mostly well-differentiated adenocarcinoma or BAC type. Meanwhile, more advanced-stage tumors (stage III) tended to form another cluster; likewise, they were more likely to be non-BAC type and/or of poor differentiation. The close relationship between tumor histologic features and gene expression patterns partly underlies the observed statistical association of tumor stage and cluster (2). This observation

Table 1. Association between sample cluster and clinical variables among three major studies

Study group	Chip type	Variable	Variable level	Cluster 1	Cluster 2	Cluster 3	Cluster 4	P		
Michigan (2)	Affymetrix HuGeneFL (7,129 features)	Stage	I	24	31	12		0.04		
			III	4	6	9				
		Cell type	BAC	11	8	0		<0.01		
			Other AD	17	29	21				
		Tumor grade	1	12	10	1		0.01		
			2	12	20	10				
Harvard (1)	Affymetrix U95Av2 (10,000 features)	Stage	3	4	7	10				
			I	3	4	12	14	<0.01		
			II	3	4	0	0			
		Cell type	III	2	1	2	0			
			AD	9	13	8	7	<0.01		
			ADSC	1	0	0	0			
			ADBAC	0	0	4	1			
		Tumor grade	BAC	0	0	3	7			
			1	0	0	4	6	<0.01		
			2	1	4	8	6			
		Stanford (7)	Two-channel cDNA microarray (24,000 features)	Stage	3	8	4	1	1	
					I	7	2	1		0.40
II	1				1	0				
Tumor grade	III			2	2	2				
	IV			6	1	5				
	1			1	0	0		0.07		
	2			11	3	2				
	3	4	3	7						

Abbreviations: BAC, bronchioloalveolar carcinoma; AD, adenocarcinoma; ADSC, adenosquamous carcinoma; ADBAC, adenocarcinoma with BAC feature.

suggests that the overall gene expression pattern of a tumor that is dominated by genes responsible for histologic phenotypes is less likely to accurately measure the stage of a tumor, and this resembles the scenario where purely histologic examination of a primary tumor is unable to reliably determine the presence or absence of a remote metastasis. However, noticeable gene expression profile differences have been postulated during tumor progression and metastasis, which may be better studied on specimens from the same individuals by comparing a metastatic tumor with its counterpart primary nodule. Some studies have shown that the gene expression profile of a metastatic tumor is much more similar to its counterpart primary tumor than to tumors (primary or metastatic) from other individuals (7). When 11 paired primary and metastatic tumors from the same individuals were compared using laser capture microdissection to achieve cell purity, the gene expression difference between the metastatic and primary tumors was very small, with 27 differentially expressed genes from over 20,000 features (15). If these genes are truly differentially expressed, they might be more predictive and provide useful information about the tumor metastatic mechanism, i.e., tumor metastasis needs additional mutations or abnormal gene expression regulations that are not present in the primary tumors. On the other hand, the minor difference in gene expression profiles may also suggest that tumor metastasis needs few further significant mutations and that a metastatic signature may be already embedded in a primary tumor. This latter hypothesis was supported by a study comparing gene expression profiles of adenocarcinoma metastases to unmatched primary adenocarcinomas. Ramaswamy et al. (16) found that some primary tumors shared the molecular signature with metastatic tumors, i.e., tumors with the signature were most likely to metastasize and advance to a higher stage. However, just based on the signature from this study, it is not possible to know whether a tumor has actually metastasized due to a lack of clinical follow-up information.

Lymph Node Status Prediction. Lymph node metastasis, including location and number of lymph nodes involved, is one of the most important determinants in separating an early-stage tumor from a late-stage tumor. Another focus in lung cancer molecular profiling is to compare gene expression profiles of tumors with lymph node metastasis and those without to find a signature that can predict lymph node status of a primary tumor. Using laser capture microdissection, Kikuchi et al. (8) analyzed 37 cases of non-small cell lung cancer and showed that unsupervised clustering was able to segregate 18 of 22 adenocarcinomas into two distinct clusters, one with and one without lymph node metastasis. In this study, no information concerning tumor histologic subtype or tumor grade was provided, which makes it difficult to evaluate whether the separation was affected by adenocarcinoma subtype and/or tumor grade. A similar study on 92 cases of bulk non-small cell lung cancer tumors (37 squamous cell carcinomas and 55 adenocarcinomas), using an optimized-feature-subset selection algorithm, achieved a very high accuracy of prediction (100% for squamous cell carcinoma using 23 genes and 94% for adenocarcinoma using 43 genes) for patients with or without lymph node metastasis (pN stage; ref. 17). However, these predictions were obtained on the same samples used for gene signature selection and no independent validation was conducted.

By applying prediction analysis of microarray, a software package using the nearest shrunken centroid methodology for classification (18), Xi et al. (19) selected 318 genes that were correlated with pathologic lymph node status in a patient series of 86 adenocarcinomas (2). When the panel was applied to a validation data set of 69 adenocarcinoma patients provided by a group from Harvard (1), the classification accuracy was 94% for the lymph node-positive cases (16 of 17), but the

accuracy for lymph node-negative cases (pN₀) was only 21% (11 of 52), highlighting the challenges in lymph node status prediction by gene expression profiling. This study may also imply the possibility of understaging in the original classification (19).

Gene Expression Profiles and Patient Survival

Published studies have applied two common approaches to assess the association between a gene expression profile and survival: (a) unsupervised clustering to identify a subclass of a tumor with a distinct clinical outcome and (b) supervised learning and testing to identify a predictive signature or a gene panel correlated with survival. The first approach does not take tissue annotations, such as cell type, into account, and tumor cluster formation is solely based on the similarity of gene expression patterns among samples under study. Using this approach, Bhattacharjee et al. (1) identified a subclass of adenocarcinoma showing worse survival than other types of adenocarcinoma and having neuroendocrine features. Carcinomas with different gene expression profiles as separated by clustering were correlated with varying prognoses. For example, in Garber et al.'s (7) adenocarcinoma series, three distinct clusters were identified, with one (group 3) having significantly worse prognosis than the other two (groups 1 and 2). Squamous cell carcinoma with different gene expression patterns also showed different survivals (9, 10). Unsupervised clustering was able to distinguish the tumors with recurrence from the tumors without recurrence regardless of their stages and histologic types (20), suggesting that tumors with a potential to recur may share similar molecular profiles. However, in their follow-up studies, testing on 11 highly distinctive genes on 92 independent non-small cell lung cancer samples did not show the expected prognostic values (21).

In a supervised learning and testing approach, researchers first identify a subset of genes based on predefined classes (short versus long survival; metastasis versus no metastasis) or time to an event (survival length) and then apply these genes to an independent set of patients for outcome prediction (22-24). Using survival analysis, Beer et al. (2) selected sets of genes that were highly correlated with survival. The top genes could accurately predict the survival of independent groups of patients from not only their own cohort but also another institution.

Comparable Outcome Predictions by Conventional Predictors

Comparing outcome prediction between gene expression profiles and conventional predictors, such as tumor stage, histologic type, or tumor grade, is difficult for most studies due to limited sample sizes and incomplete information. Evaluation on the studies from Beer et al. (2) and Bhattacharjee et al. (1), each with over 80 patient samples and clinical information, may provide hints on whether gene expression panels can achieve more accurate prediction than conventional predictors or are simply their surrogates. Table 2 lists the raw and adjusted risk ratios for five variables often reported in the literature for the two public data sets. The last column is the result from a cohort of 2,598 cases of adenocarcinoma at Mayo Clinic (25). The point estimates for these variables are very comparable between the two public data sets and between the public data and Mayo Clinic data. To compare the survival prediction by these clinical variables with the prediction by the gene signature originally selected from Beer et al. (2), we used more conservative and reliable predictive factors obtained from the Mayo Clinic patients to calculate the risk index for each case in the Harvard study, just as the prediction conducted by the authors using their 50 gene-based signature

Table 2. Raw and adjusted risk ratios for conventional predictors in survival of lung adenocarcinoma: two public data sets and a large cohort from a single institution

	Michigan, raw (<i>n</i> = 86)	Harvard, raw (<i>n</i> = 115)	Combined, adjusted (<i>n</i> = 201)	Mayo cohort, adjusted (<i>n</i> = 2,598)
Age (1-y increment)	1.03 (0.99-1.07)	1.02 (0.99-1.05)	1.04 (1.01-1.06)	1.02 (1.01-1.02)
Sex				
Female	1	1	1	1
Male	1.6 (0.7-3.5)	1.3 (0.8-2.1)	1.6 (1.0-2.6)	1.4 (1.2-1.5)
Stage				
I	1	1	1	1
II	NA	1.9 (1.1-3.5)	2.6 (1.4-4.9)	2.0 (1.6-2.5)
III	7.0 (3.0-16.7)	3.8 (1.9-7.6)	5.1 (3.0-8.9)	4.0 (3.4-4.7)
Cell type				
BAC & like	1	1	1	1
Other AD	1.8 (0.5-6.2)	2.2 (1.1-4.5)	2.1 (0.7-5.8)	1.6 (1.3-2.0)
Tumor grade				
1	1	1	1	1
2	2.2 (0.6-7.9)	2.1 (0.8-5.5)	1.0 (0.3-2.9)	1.1 (1.0-1.3)
3	3.0 (0.8-11.5)	3.5 (1.4-9.0)	1.6 (0.5-4.8)	1.5 (1.3-1.8)

NOTE: The analysis was conducted using a Cox proportional hazard model for each variable. The first level of each variable was compared as baseline, and proportional hazard ratios were presented with 95% confidence intervals in parentheses.

Abbreviations: NA, not applicable; BAC & like, bronchioloalveolar adenocarcinoma and adenocarcinoma with BAC feature.

panel (2). As shown in Fig. 1, the gene panel achieved very good prediction for patient survival (green color) but did not outperform the prediction (red color) by the combination of five conventional variables (age, gender, stage, cell type, and tumor grade). Notably, when using tumor pathology data (cell type and grade) alone, the prediction (blue color) provided only a slightly reduced power compared with using the gene panel. This suggests that most prediction from the gene panel is reflected by tumor pathology information; thus, the enhanced predictive accuracy from the 50-gene panel is limited. This observation was supported by another study when all these variables were matched in patient selection (23, 24). In that study, gene expression profiles were compared between two groups of patients with squamous cell carcinoma, one with survival >5 years and another with survival <2 years. The two groups were matched on stage, age at diagnosis, gender, cell type, tumor grade, and smoking status. The two contrasting groups were indistinguishable in unsupervised clustering and differentially expressed genes between the two groups were minimal, although promising gene expression differences may exist in a pathway-based gene subset analysis (24).

Like histologic examination, gene expression profiling is a snapshot of a tumor at certain point of its growth, only it is at the molecular level. If there are genes critical for metastasis, they are likely overwhelmed by highly expressed genes responsible for an obvious histologic phenotype, such as tumor cell type or grade, and are not easily detectable by common analytic approaches. These genes may be expressed at levels that are below the detection limit of current DNA microarray technology. These observations were supported by findings that little difference exists between paired primary and metastatic tumors from the same individuals (15) and significant difference between primary and metastatic tumors from different individuals (16). In contrast, metastatic genes may be part of a signature that was embedded in tumor differentiation because poorly differentiated tumors grow faster and metastasize earlier than well-differentiated carcinomas (25, 26). This may explain the overlap prediction between gene signatures and histologic features.

Current Status and Future Directions

As a high-throughput tool at the molecular level, DNA microarray has clear advantages over traditional histologic examinations. The simultaneous interrogation of thousands of genes offers a unique opportunity to measure a tumor from

multiple angles, which generally provides a more accurate measurement about biological behaviors than histologic variables alone. The molecular measurement is more objective and often detects the difference that routine pathology fails. More importantly, the DNA microarray provides a closer look at gene activities in tumors and creates an opportunity to find therapeutic targets. However, available data to date from most studies concerning clinical outcome prediction indicate that microarray-based studies on tumors are more challenging than previously expected, and its clinical applications are still questionable. Reasons include the following: (a) There is a significant overlap for clinical outcome prediction between gene expression profiles and pathologic features, and most

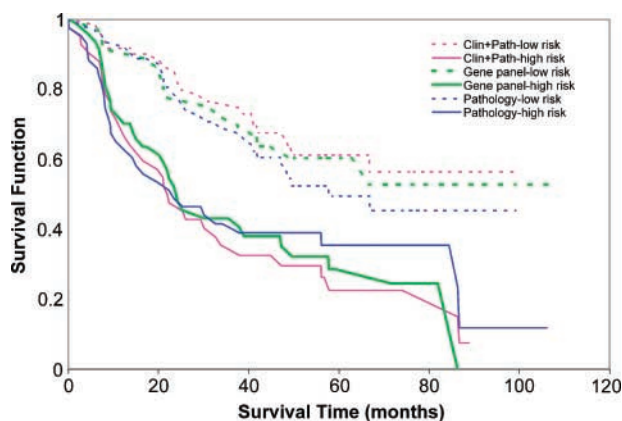


Figure 1. Comparison of survival predictions by a 50-gene signature and combination of clinical and pathologic variables. Survival prediction for adenocarcinoma of the Harvard data (1) was conducted by calculating the risk index for each case using a 50-gene signature set or clinical and pathologic variables. Patients were separated into low- and high-risk groups by the 60 percentile risk index. Survival curves were generated by stratifying the prediction risk group. The 50-gene signature set was selected by Beer et al. (2) on their 86 cases of adenocarcinoma with survival analysis and cross-validation. Clinical variable risk estimates were obtained from analyzing 2,598 adenocarcinomas from Mayo Clinic (Table 2). *Gene panel*, the 50-gene signature set. *Clin+Path*, five variables, including age, gender, stage, cell type, and tumor grade. *Pathology*, tumor cell type and grade only. The log-rank test *P* values for the gene signature set, the clinical and pathologic variables, and the histologic features were 0.003, <0.0001, and 0.009, respectively.

studies have not shown a superior performance using the new technology over conventional predictors, particularly when evaluated collectively. (b) Most studies had a limited number of cases and an independent validation was not adequately conducted. (c) Current analytic algorithms favor genes at high expression or genes highly differentially expressed, most of which are related to tumor differentiation and may not correlate with clinical outcomes; conversely, genes expressed at low levels or in a subtle difference are often overlooked, which may be quite relevant biologically to clinical questions. (d) There are still some unsolved technical issues about DNA microarray; for example, different microarray platforms (27) or studies from different laboratories using the same platform (28) often produce inconsistent results even when the same RNA samples were used for hybridization. (e) Results from different analytic approaches also differ (23). As an undesirable consequence, consistent or overlapped genes selected for predicting the same outcome from multiple studies are rare.

To overcome these drawbacks, future microarray studies or analyses should pay serious attention to the following six key points:

(a) To clearly define a study aim. Researchers should be fully knowledgeable about what the currently established clinical predictors are and seek answers for what microarray can offer beyond these predictors. The approach without taking previously accumulated knowledge into consideration is likely to be repetitive and not practically useful. For example, many published studies have applied unsupervised clustering with a hope that it provides a better tumor classification correlated with clinical outcomes. The problem is that if tumors included in the studies are easily separable under conventional histologic examinations, the microarray analysis merely provides the same information as the conventional methods. The main focus in microarray studies should explore the molecular explanations for varied clinical outcomes given a group of patients with similar clinical and pathologic characteristics.

(b) To layout and compare alternative study designs. As a histologic type may have a significant effect on gene expression profiles, special care should be taken before mixing different cell types or sub-cell types together. Independent validation of study findings should be an integral part of the analysis.

(c) To carefully select samples. This includes a sufficient size, good quality, and unambiguous clinical outcomes. Consideration of multiple testing problems in the design phase is also important.

(d) Be fully aware of the limitations of DNA microarray and of what you are expecting for a chosen platform. Particularly, DNA microarray is prone to various sources of variations, and genes at low expression are less reliably detected, if possible at all.

(e) To conduct a knowledge- or context-based analysis. As shown earlier, genes related to tumor differentiation often dominate most analyses, but they may not be an interest of a specific study aim. For example, a small subset of genes may be involved in tumor metastasis, but these genes may not have dramatic changes for easy detection. A pathway approach may provide a better solution. Developments in this area are emerging and are expected to evolve fast, such as the newly described gene set enrichment analysis (29). This approach applies prior biological knowledge to define gene sets, such as biochemical pathway or coexpression in previous experiments so that evaluation of a gene set is placed within a biological context. This approach, for an example, allows for identification of genes with subtle changes in human normal and diabetic muscles (30), and, in another example, several functional-related gene sets are correlated with poor outcome of lung adenocarcinoma and shared between two independent data sets (29).

(f) To provide clinical relevant interpretation from the study results and the value added in practice. For example, how much better are the newly identified markers compared with the conventional predictors? How can these markers be applied clinically for improved patient care?

Summary

Gene expression profiling has been applied in a wide range of lung cancer outcome studies and has provided varied accuracy of outcome prediction. Genes related to a tumor histologic phenotype, including cell subtype or tumor grade, generally dominate an expression profile of a tumor. These genes are responsible for maintenance of structural proteins and certain functions specific to that histologic differentiation. The high correlation between a gene expression profile and a tumor histologic phenotype explains, to a certain extent, the observed association of an expression profile with tumor stage or survival. A common problem in most published studies is lacking evaluation of the value-added utility of expression profiling results in the context of known prognostic factors. To date, even a thoroughly validated molecular signature does not outperform combined conventional clinical and pathologic variables in non-small cell lung cancer survival prediction, with prediction mostly explained by histologic differentiations. This may also explain the relatively low predictability of gene expression profiles for tumor stage or lymph node metastatic status because gene signatures only provide a modestly enhanced prediction over combined histologic features; in other words, histologic features cannot reliably predict tumor stage or lymph node status of tumor metastasis.

As a collective measure for a tumor from multiple angles, gene expression profiling is expected and proved to be able to provide a better prediction of clinical outcomes than more subjective histologic variables alone. Future microarray studies in search for prognostic markers should focus on resolving the issues that conventional histologic methods are challenged and on identifying those markers that can provide predictive values beyond conventional predictors. It would also be valuable to find those markers that can provide accurate and objective measures for a proven histologic variable, such as tumor grade, in assisting molecular diagnosis. A valid study design and a meticulous implementation are the keys to achieve these goals. Further improvement of microarray technology on sensitivity and reliability and development of feasible analytic approaches are also crucially needed.

Acknowledgments

We thank Susan Ernst for her technical assistance with the manuscript.

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