The role of gene expression profiling in early-stage non-small cell lung cancer

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ABSTRACT

For patients with identical clinical-pathological characteristics or the same stage of lung cancer, great uncertainties remain regarding how some patients will be cured while other patients will have cancer recurrence, metastasis, or death after surgical resection. Identification of patients at high risk of recurrence, those who are unlikely to respond to specific chemotherapeutic agents, is the rationale for measuring specific biochemical markers. Thus, main investigational studies nowadays are focused in identifying molecular markers of recurrence, beyond pathologic stage, after surgical treatment and factors that can predict a benefit from adjuvant chemotherapy in poor prognosis subgroups, to individualize treatments. Advances in genomics and proteomics have generated many candidate markers with potential clinical value. Gene expression profiling (GEP) by microarray or real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) can be useful in the classification or prognosis of various types of cancer, including lung cancer. A number of prognostic gene expression signatures have been reported to predict survival in non-small cell lung cancer (NSCLC). In this review, we focus on the role of GEP in early-stage NSCLC as predictive and prognostic biomarker and its potential use for a ‘personalized’ medicine in the years to come.

Key Words: non-small cell lung cancer; prognostic biomarker; gene expression profiling


Introduction

The incidence of lung cancer in 2007 is estimated to be 213,380 with 160,390 deaths in the U.S. It will contribute to 31% of male and 26% of female cancer-related deaths and is the largest cause of cancer-related mortality in both men and women. Non-small-cell lung cancer (NSCLC) accounts for approximately 75% of all cases of lung cancer, which is one of the most common tumors affecting humans in the world (1). The current standard of treatment for patients with stage I NSCLC is surgical resection (2-4), despite the observation that nearly 30 to 35 percent will relapse after the initial surgery and thus have a poor prognosis (5,6), similarly, as many as 66% of stage II and 75% of stage IIIA patients will develop recurrence and die as a result of their disease within 5 years of resection (3,4), indicating that a subgroup of these patients might benefit from adjuvant chemotherapy. Thus, the ability to identify subgroups of patients more accurately may improve health outcomes across the spectrum of disease.

Although other clinical and pathologic markers have prognostic significance (7-10), the clinic-pathologic staging system has been the standard for determining NSCLC prognosis (8). But this classification scheme is probably an imprecise predictor of the prognosis of an individual patient. For patients with identical clinical-pathological characteristics or the same stage of lung cancer, great uncertainties remain regarding how some patients will be cured while other patients will have cancer recurrence, metastasis, or death after surgical resection. Identification of patients at high risk of recurrence, those who are unlikely to respond to specific chemotherapeutic agents, is the rationale for measuring specific biochemical markers. Thus, main investigational studies nowadays are focused in identifying molecular markers of recurrence, beyond pathologic stage, after surgical treatment and factors that can predict a benefit from adjuvant chemotherapy in poor prognosis subgroups, to individualize treatments. This ability to identify subgroups of patients more accurately...
may improve health outcomes across the spectrum of disease. The study of molecular factors that influence drug responsiveness is also a potentially promising approach to decrease treatment toxicity and costs by avoiding the administration of ineffective therapy to patients destined not to benefit (11).

Advances in genomics and proteomics have generated many candidate markers with potential clinical value (12). Gene expression profiling (GEP) by microarray or quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) can be useful in the classification or prognosis of various types of cancer, including lung cancer (13-15). A number of prognostic gene expression signatures have been reported to predict survival in NSCLC. In this review, we will focus on the role of GEP in NSCLC as predictive and prognostic biomarker and its potential use for a ‘personalized’ medicine in the years to come.

**Techniques for gene expression profiling**

GEP (i.e., the systematic identification and characterization of those genes activated or expressed in a cell) can be conducted on different levels depending on the specific research objectives. This could involve analysis of DNA, mRNA, and/or protein as a measure of gene expression. A variety of techniques that have been used to profile the genome and to assess gene activity at the mRNA or protein level (Table 1).

**Microarray-based molecular profiles**

Both genetic changes within a precancerous cell and epigenetic changes in the tumor microenvironment are thought to promote tumorigenesis (16-18). In particular, it is now well accepted that alterations in the expression levels of certain genes strongly correlate with and are considered causative for cancer (19). These changes in gene expression are reflected by quantitative changes in mRNA levels. Detecting these changes was traditionally done by identifying single genes of interest and assaying mRNA expression by techniques such as PCR and Northern blotting. In 1995, Schena et al described a GEP technique adapted from Southern blotting that used strands of cDNA spotted onto a piece of glass to examine multiple mRNA expression levels at once (20). Known as a microarray, this technology was quickly developed into a tool that could be used to take a genome-wide snapshot of mRNA transcription levels within a tissue of interest in a single experiment (21).

**qRT-PCR–based molecular signatures**

In response to the concern that microarray-based profiles are difficult to translate into a clinical setting, several recent efforts have focused on developing qRT-PCR...
–based molecular signatures. It is unlikely that every gene in the molecular profiles obtained by microarray analysis has equal relevance with respect to prognosis. Ideally, a handful of genes could be isolated that convey close to the same prognostic information as microarray-based gene signatures. The disadvantage would be that only a small number of such genes could be tested by the current gold standard assay for gene expression, qRT-PCR. However, qRT-PCR has significant advantages over microarray-based assays, including widespread availability, cost, simplicity, reproducibility, and ability to use stored paraffin-embedded versus snap-frozen tissues (22-24). In addition, the limited number of genes in qRT-PCR–based signatures allows these signatures to be validated with protein expression by immunohistochemistry (25).

**Lung developmental pathways in lung cancer**

Current paradigms suggest that lung carcinomas arise from pluripotent stem and progenitor cells capable of differentiation into one or several histologic cell types. These paradigms suggest that lung tumor cell ontology is determined by the consequences of gene transcriptional activation and/or repression events that recapitulate embryonic lung development (26,27). The hypothesis that lung cancer arises from aberrant expression of genes involved in lung development is supported by gene expression studies demonstrating similarities between signatures obtained from human lung tumors and signatures characteristic of normal lung development. In an analysis of 32 NSCLC specimens and 7 normal specimens, unsupervised hierarchical analysis segregated tumors on the basis of histologic type and differentiation (28). Supervised clustering analysis of tumors identified numerous genes with known important function in embryonic lung development. Comparison of human lung tumor histology classifiers with genes temporally activated during mouse lung development reveals that genes expressed by large cell carcinoma (LCC) are similarly expressed during the early pseudoglandular and canalicular stages of lung development, while those expressed by adenocarcinoma mirror those expressed during the later terminal sac and alveolar stages. In addition to highlighting the expression of proliferation-associated genes by LCC and of differentiation-associated genes by adenocarcinoma, these results suggest a recapitulation of developmentally regulated pathways in lung tumors. In addition, Glinsky and colleagues reported that a gene signature of ‘stemness’ derived from BMI-1–regulated genes in normal stem cells is associated with metastasis and survival in several tumor types, including NSCLC (29). Taken together, these observations suggest that poor differentiation is linked to molecular parameters of early development representing lung stem and progenitor cell programs, and that gene signatures of these phenotypes are important for lung cancer differentiation, progression, and clinical outcome.

**Predicting response to treatment by gene expression profiling**

GEP has been used to predict response to treatment. The first clinical study of microarray as a predictor of benefit from chemotherapy in NSCLC used tissues from 133 patients enrolled in the JBR.10 study. JBR.10 is a North American phase III Intergroup trial led by the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG), in which 482 patients with completely resected stages IB and II—excluding T3N0-NSCLC were randomly assigned to receive four cycles of adjuvant cisplatin plus vinorelbine or observation alone (30). Chemotherapy-treated patients enjoyed a significant survival advantage (HR, 0.70; P=0.03), although a significant interaction with stage was seen, with benefit limited to stage II patients. By use of a supervised analysis, a 15-gene signature that correlated with survival, and was independent of stage, histology, age, and sex was derived from patients in the observation group (HR 15.02, 95% CI 5.12–44.04; p<0.0001). In the high-risk group, treatment with vinorelbine plus cisplatin conferred significant survival benefit compared with observation alone (0.33, 0.17–0.63; p=0.0005), whereas in the low-risk group, patients who received this chemotherapy regimen had shorter survival compared with observation alone (3.67, 1.22–11.06; p=0.0133). This interaction was highly significant (p=0.0001) (31). If the 15-gene signature is validated by further testing, it may improve the current method for deciding which patients should receive adjuvant chemotherapy.

Staunton et al. used DNA microarrays to measure gene expression in the NCI-60 panel (a collection of 60 human cancer cell lines) (32-34). By combining the untreated gene expression profile of each cell line together with information about each cell lines’ chemosensitivity profile, they were able to predict drug sensitivity in an independent test set of cell lines. A subsequent study by Potti et al. (35) repeated and built upon Staunton’s work. Potti and colleagues used molecular profiles from cell lines to establish sensitivity to chemotherapy. The signature that predicted response to individual agents was then further validated in cell lines, but also in clinical samples from patients with other tumor types (35). The usefulness of this approach is that one tumor sample can be interrogated for response to many agents on the basis of cell-line derived signatures. For example, a relationship between
docetaxel resistance and deregulation of the PI3-kinase pathway was observed. Using a panel of 17 NSCLC cell lines a significant association was found between docetaxel resistance and sensitivity to a PI3-kinase inhibitor (LY-294002), suggesting its use as a second-line therapy (36).

For many years, we have been discovering that expression of certain genes or the presence of certain gene mutations has implications in the prognosis of NSCLC or response to specific therapy. The improved responses seen with the use of tyrosine kinase inhibitors (TKIs; eg, erlotinib or gefitinib) in patients carrying mutations in the epidermal growth factor receptor (EGFR) gene are a good example of an attempt to stratify tumors that are more sensitive to these agents (37). We know that no more than 10% of the general population will have a response to these agents; however, when only selected patients who carry gene mutations are treated, the response rate to these agents can be as high as 70% (38,39), and now we are still trying to define which are the best techniques (gene mutation analysis, FISH, or others) to detect these mutations and moving these discoveries into our clinical practice. Similar efforts to identify predictive markers for the EGFR inhibition have been undertaken in the area of proteomics (40,41).

Recently, Altorki et al. examined safety and efficacy of short-term, preoperative pazopanib (an oral angiogenesis inhibitor targeting VEGFR, platelet-derived growth factor receptor, and c-kit; 800 mg/d for 2 to 6 weeks) monotherapy in 35 patients with operable stage I/II NSCLC, and gene-expression profiling was performed on 77 pre- and post-treatment lung samples from 34 patients. They found that several target genes were dysregulated after pazopanib treatment, validating target-specific response and indicating a persistent pazopanib effect on lung cancer tissue (42). In further study, they conducted a broad profiling of cytokine and angiogenic factors (CAF) to investigate the relationship between baseline CAF levels, CAF changes during treatment, and tumor shrinkage. Plasma samples were collected before treatment and on the last day of therapy from 33 patients with early-stage NSCLC. Levels of 31 CAFs were measured by suspension bead multiplex assays or ELISA and correlated with change in tumor volume. Pazopanib therapy was associated with significant changes of eight CAFs; sVEGFR2 showed the largest decrease, whereas placental growth factor underwent the largest increase. Increases were also observed in stromal cell-derived factor-1alpha, IP-10, cutaneous T-cell-attracting chemokine, monokine induced by IFN-gamma, tumor necrosis factor-related apoptosis-inducing ligand, and IFN-alpha. Posttreatment changes in plasma sVEGFR2 and interleukin (IL)-4 significantly correlated with tumor shrinkage. Baseline levels of 11 CAFs significantly correlated with tumor shrinkage, with IL-12 showing the strongest association. Using multivariate classification, a baseline CAF signature consisting of hepatocyte growth factor and IL-12 was associated with tumor response to pazopanib and identified responding patients with 81% accuracy. These data suggest that CAF profiling may be useful for identifying patients likely to benefit from pazopanib, and merit further investigation in clinical trials (43).

**Predicting survival and recurrence by gene expression profiling**

GEP has been used to predict response to treatment and patients’ outcome (13,31,44-68). Beer et al. analyzed the genetic profile in 86 patients with primary lung adenocarcinoma, and found that the genes most associated with survival were identified to create a risk index based on the top 50 genes that separated patients into high-risk and low-risk groups. When applying this risk predictor to a test data set of 62 stage I patients from another study, they were able to predict survival with statistical significance difference (P=0.006) (51). This study also identified certain patients with stage I along with stage III disease with poor prognosis based on gene profile. This demonstrated the ability for GEP to identify a patient with poor prognosis that is independent of the stage at the time of diagnosis.

Guo et al. devised a computational model system that predicted the clinical outcome of individual patients based on their GEP. A 37-gene signature was created, and the authors studied a cohort of 86 patients diagnosed with lung adenocarcinoma. The gene signature was then applied to predict the survival of the other 84 patients with adenocarcinoma. The predictive accuracy of the gene signature was 96%. The cluster analysis, using the 37-gene signature, aggregated the test patient samples into 3 groups with good (mean survival, 66.9 months), moderate (mean survival, 27.6 months), and poor (mean survival, 22.4 months) prognoses (Kaplan-Meier analysis; P < .0005; log-rank test) (Fig 1). Notably, when the results were reviewed, all patients who had grouped together in cluster 1 (good prognosis) had stage I disease, with N0 lymph node status (no metastasis) and smaller tumor size (T1 or T2) (63).

Landmark studies such as the one conducted by Potti et al. from Duke University have identified GEP, which predicted the risk of recurrence following surgery from a cohort of patients with early-stage NSCLC (52). The accuracy was > 70%. The investigators were also able to identify a subgroup of patients with stage IA disease who were at high risk for recurrence, with a very poor survival, and who might be suitable for adjuvant chemotherapy. This
is clinically relevant when the current standard of care for patients with stage IA disease is just clinical observation (no adjuvant chemotherapy is offered) because of a 70% chance of 5-year survival. This genetic strategy was then validated in two separate cohorts from multicenter cooperative group trials: 25 patients from the American College of Surgeons Oncology Group Z0030 study and 84 from the prospective CALGB 9761 trial, this genomic strategy had an overall predictive accuracy of 72 and 79%, respectively. This gene expression profile also was applied to 68 patients with stage IA disease, who are not usually candidates for adjuvant chemotherapy. Kaplan-Meier survival curves were generated for the group as a whole and for the subgroups predicted to be at high or low risk for recurrence by the lung metagene model. Although the survival rate for the group was approximately 70% at 4 years, the survival rate for those predicted to be at low risk was 90% and less than 10% for those predicted to be at high risk, thus identifying the subgroup of patients with stage IA NSCLC at high risk of recurrence, who might benefit from adjuvant chemotherapy (Fig 2).

In another important study from Taiwan University (13), authors examined the expression of multiple genes associated with invasive activity in frozen specimens of lung-cancer tissue from 125 randomly selected patients who underwent surgical resection of NSCLC and not received adjuvant chemotherapy, to identify a gene signature that is correlated with clinical outcome.

Sixteen genes were initially identified by analyzing microarray data and then confirmed by RT-PCR. From these, the authors further identified five genes that were significantly associated with survival. The levels of expression of these five genes were used to construct a decision tree to classify patients as having a high-risk gene signature or a low-risk gene signature. The five selected genes were: dual-specificity phosphatase 6 (DUSP6), monocyte-to-macrophage differentiation-associated protein (MMD), signal transducer and activator of transcription 1 (STAT1), v-erb b2 avian erythroblastic leukemia viral oncogene homolog 3 (ERBB3), and lymphocyte-specific protein tyrosine kinase (LCK).

The authors identified 59 patients with high-risk gene signatures and 42 with low-risk gene signatures, according to gene expression as measured with RT-PCR and decision-tree analysis. The five-gene signature was strongly associated with OS (sensitivity 98%; specificity 93%; positive predictive value 95%; negative predictive value 98%; and overall accuracy 96%). The presence of a high-risk five-gene signature in the NSCLC tumors was associated with an increased risk of recurrence and decreased OS. With a median follow-up of 20 months, the patients with a high-risk gene signature had a shorter median OS than the patients with a low-risk gene signature (20 months versus 40 months, P<0.001). The high-risk gene signature was associated with a median RFS of 13 months, whereas the low-risk gene signature was associated with a
the use of a set of published microarray data from 86 patients from a Western population with NSCLC.

The identification of five genes that are closely associated with the outcomes in patients with NSCLC has clinical implications. Patients who have tumors with a high-risk gene signature could benefit from a cisplatin-based adjuvant chemotherapy, whereas those with a low-risk gene signature could be spared what may be unnecessary treatment. Prospective, large scale, multicenter studies are necessary to test this idea. These five genes that can predict the clinical outcome in patients with NSCLC may also reveal targets for the development of therapy for lung cancer. STAT1 causes arrested growth and apoptosis in many types of cancer cells by inducing the expression of p21WAF1 and caspase (53,54). MMD is preferentially expressed in mature macrophages (55). Some studies have shown that macrophage activation promotes cancer metastasis (56), although the function of the MMD protein is unknown. DUSP6 inactivates extracellular signal-regulated kinase 2 (also known as mitogen-activated protein kinase 1), resulting in tumor suppression and apoptosis (57). ERBB3, a member of the epidermal growth receptor kinase 1 (P=0.04); fibronectin (P=0.002); insulin-like growth factor receptor (IGF1R), (P=0.05); EGF (P=0.002); EGFR (P=0.05); KIAA0974 (P=0.02); ANLN (P=0.02); carbonic anhydrase IX (CA IX), which is regulated by hypoxia and plays a role in chemoresistance (P=0.007); VEGFC (P=0.03); neurotrophic tyrosine receptor kinase 1 (P=0.04); fibronectin (P=0.002); insulin receptor (P=0.03). In the multivariate analysis of survival, CSF1, EGFR and CA IX, and tumor size emerged as significant variables (P=0.005, 0.02, <=0.0001, 0.02, respectively).

Shao et al. examined the expression pattern of 29 genes selected by cDNA studies to test their clinical prognostic value in early-stage squamous cell carcinoma (SCC) of the lung (49). From 2000 to 2004, freshly frozen primary tumor specimens were obtained at the time of the surgery from 66 SCC patients and gene expression of the 29 genes was assessed by quantitative RT-PCR using low-density arrays. Expression values were dichotomized using the median value as the cutoff. The univariate analysis showed 10 genes with prognosis value: PH4 (P=0.01); macrophage-colony stimulating factor (CSF1), which attracts macrophages and induce them to express EGF (P=0.002); EGFR (P=0.05); KIAA0974 (P=0.02); ANLN (P=0.02); carbonic anhydrase IX (CA IX), which is regulated by hypoxia and plays a role in chemoresistance (P=0.007); VEGFC (P=0.03); neurotrophic tyrosine receptor kinase 1 (P=0.04); fibronectin (P=0.002); insulin receptor (P=0.03). In the multivariate analysis of survival, CSF1, EGFR and CA IX, and tumor size emerged as significant variables (P=0.005, 0.02, <0.0001, 0.02, respectively).

Roepman et al. aimed to develop a gene expression profile for stage I and stage II NSCLC, allowing identification of patients with a high risk of disease recurrence within 2 to 3 years after initial diagnosis.
Fig 3. A, Kaplan-Meier curves comparing OS among patients with low- and high-risk (all stages) using the four-gene prognostic model (all stages); B, comparing disease-free survival (DFS) among patients with low- and high-risk scores (all stages); C, comparing OS among stage I patients with low- and high-risk scores using the four-gene prognostic model; D, comparing DFS among stage I patients with low- and high-risk scores; E, comparing OS among stage I patients with low- and high-risk scores using the 5-gene model published by Chen and colleagues (ref 13).
Whole-genome gene expression microarrays were used to analyze frozen tumor samples from 172 NSCLC patients (pT1-2, N0-1, M0) from five European institutions, who had undergone complete surgical resection. A 72-gene expression prognostic NSCLC classifier was developed. Based on the classifier score, patients were classified as either high or low risk of disease recurrence. Patients classified as low risk showed a significantly better recurrence-free survival both in the training set (P < 0.001; n = 103) and in the independent validation set (P < 0.05; n = 69). It was found that the 72-gene signature was closely associated with recurrence-free and overall survival in early-stage NSCLC patients and may become a tool for patient selection for adjuvant therapy (62).

Reed et al. proposed a qRT-PCR-based 2-gene signature for adenocarcinoma (48). Pooling microarray analysis of NSCLC cell lines in conjunction with correlation mapping of genes highly expressed in other tumors produced 14 candidate genes. These genes were tested by qRT-PCR on 20 adenocarcinoma samples yielding a 2-gene signature (CK19 and EpCAM2). This 2-gene signature revealed survival differences in high- and low-risk patients in their training cohort (HR, 6.2) and in a separate validation cohort (HR, 4.5) by Kaplan-Meier analysis. Raz et al. proposed a qRT-PCR-based 4-gene signature for adenocarcinoma (50). Seventy-six cancer-related candidate genes were selected from 217 genes demonstrated to have prognostic significance in previously published studies by content experts and literature review. Sixty-one of these genes for which reliable qRT-PCR data could be produced were assayed using qRT-PCR in a cohort of 120 adenocarcinoma samples. Cross-validation using Cox proportional hazards regression supported a 4-gene signature (WNT3A, RND3, LCK, and ERBB3). When applied to a cross-validated cohort of 70 patients with stage I adenocarcinoma, statistically significant differences in OS (87% vs. 38%) and disease-free survival (77% vs. 35%) were shown for high- and low-risk patients. This compared favorably with the 5-gene signature of Chen et al. (13) (Fig 3). When applied to the Raz et al cross-validated cohort, the signature developed by Chen et al demonstrated 5-year OS of 80% and 47%, respectively, for high- and low-risk patients. Notably, 2 of the genes (ERBB3 and LCK) overlapped between the Raz et al and Chen et al signatures.

Identified prognostic classifiers for early-stage NSCLC indicated large differences in sample numbers, microarray platform, and classifier design. Although a great variety of statistical models have been used, the performance of the different classifiers is similar with overall accuracies between 70% and 80% and a hazard ratio of 3 to 4. The overlap in profile genes, however, is limited to only 5 of a total of 327 genes (Fig. 4) even though it includes two studies (63,64) that reanalyzed existing data (51) but showed respectively no and three genes in overlap (62). Ein-Dor and coworkers (65) demonstrated that biological heterogeneity leads to thousands of samples being required to identify robust and reproducible subsets for most tumor types. These conclusions are supported by the finding that thousands of genes display intratumor heterogeneity, likely caused by the diversity of tumor microenvironments and cell populations (66,67). However, Boutros and coworkers hypothesized that differing statistical methodologies contribute to this lack of overlap (68). To test the hypothesis, they analyzed our previously published quantitative RT-PCR dataset with a semisupervised method. A 6-gene signature was identified and validated in 4 independent public microarray datasets that represent a range of tumor histologies and stages. The result demonstrated that at least 2 prognostic signatures can be derived from this single dataset. They further estimated the total number of prognostic signatures in this dataset with a 10-millionsignature permutation study. Their 6-gene signature was among the top 0.02% of signatures with maximum verifiability, reaffirming its efficacy. Importantly, the analysis identified 1,789 unique signatures, implying that their dataset contains >500,000 verifiable prognostic signatures for NSCLC. The result appears to rationalize the observed lack of overlap among reported NSCLC prognostic signatures (68).

Conclusions

GEP has demonstrated a tremendous potential to drive
personalized medicine in the near future when its use could be applied as a diagnostic tool, molecular staging classification, and more importantly, as prognostic and predictive biomarkers. Even patients with early-stage lung cancer demonstrate significant recurrence rates and lower-than-expected survival rates after surgical resection. The development of genomic prognostic models holds significant promise in our ability to differentiate among those patients who might benefit from additional therapy or lesser surgical procedures. However, we need to improve this technology in order to get results that are reproducible in most instances. Also, the technology must be available and be less cumbersome so it can be easily applied into common clinical practice.

References

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