The Ability to Form Primary Tumor Xenografts Is Predictive of Increased Risk of Disease Recurrence in Early-Stage Non–Small Cell Lung Cancer

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Abstract

**Purpose:** Primary tumor xenografts (PTXG) established directly from patients’ primary tumors in immunosuppressed animals might represent the spectrum of histologic complexity of lung cancers better than xenografts derived from established cell lines. These models are important in the study of aberrant biological pathways in cancers and as preclinical models for testing new therapeutic agents. However, not all primary tumors engraft when implanted into immunosuppressed mice. We have investigated factors that may influence the ability of primary non–small cell lung cancer (NSCLC) to form xenografts and their association with clinical outcome.

**Experimental Design:** Tumor fragments from patients undergoing curative surgery were implanted into NOD-SCID (nonobese diabetic–severely combined immunodeficient) mice within 24 hours of surgery. Patient characteristics for tumors that engrafted (XG) and did not engraft (no-XG) were compared. Patient tumor DNA was profiled for the presence of 238 known mutations in 19 cancer-associated genes by using the MassARRAY platform.

**Results:** Xenografts were established and passaged successfully from 63 of 157 (40%) implanted NSCLCs. Tumor factors associated with engraftment included squamous histology, poor differentiation, and larger tumor size. Significantly fewer EGFR (epidermal growth factor receptor)-mutated tumors engrafted ($P = 0.03$); conversely, more K-RAS–mutated tumors engrafted ($P = 0.05$). In multivariate analysis including age, sex, stage, and mutation, patients with XG tumors had significantly shorter disease-free survival compared with no-XG patients (hazard ratio: 7.0, 95% CI: 3.1–15.81; $P < 0.000003$).

**Conclusion:** PTXGs closely mirror the histology and molecular profiles of primary tumors and therefore may serve as important preclinical models. Tumors that engraft are biologically more aggressive and may be more representative of cancers with a higher propensity to relapse after surgery. *Clin Cancer Res; 17(1): 134–41. ©2010 AACR.*

Introduction

Reproducing the complexity of tumor growth in preclinical models is important for mechanistic and functional studies of cancer biology. In non–small cell lung cancer (NSCLC), identifying biological targets for novel therapies and predictive markers for treatment selection will be of increasing importance in the future for individualized patient care. Its importance is further highlighted by the fact that NSCLC has the highest cancer mortality in the United States for both sexes with over 159,000 people dying from the disease in 2009 (1). In patients, lung cancer biology is difficult to study longitudinally as invasive procedures are required to obtain tissue samples. Historically therefore, such biological studies have been conducted mainly on cancer cell lines grown in vitro or implanted into immunocompromised mice to form xenografts. Although cell lines are practical, reproducible, and easy to manipulate, correlation of treatment results in these models with clinical results in patients has been suboptimal. Although some studies have shown some degree of correlation between in vitro and in vivo responses to treatment (2, 3), others have reported considerable differences (4, 5). Considering that only a minority of primary tumors may form cell lines (6, 7), that growth as a monolayer in vitro does not mirror the human tumor microenvironment or reproduce similar
Translational Significance

On the basis of high failure rate of newly developed novel targeted drugs in clinical trials, there is increasing doubt concerning the suitability of traditional cell line models in preclinical studies. This work is currently topical and will become increasingly important as more research groups are employing primary tumor xenograft (PTXG) models in preclinical investigations of novel targeted agents. Although the establishment of PTXGs has been reported, none of the previous studies have looked into the clinical relevance of their establishment. Importantly, there are no studies that have investigated mutational status in this context. As such, this is the first report in primary non–small-cell lung cancer xenografts to document that the ability to form a xenograft is a poor prognostic marker. We believe that these results are highly translational and of significant interest to Clinical Cancer Research readers.

Patients and Methods

The University Health Network Human Research Ethics and Animal Care Committee approved the protocol, which included harvesting tumor samples from surgically resected early-stage NSCLCs for establishment of PTXGs. The fresh samples were cut into 5-mm sections by using a razor blade and mixed with 10% matrigel at 4°C (BD Biosciences). These fragments were implanted immediately into the subcutaneous tissues of 3 NOD-SCID (nonobese diabetic severely combined immunodeficient) mice. All mice were bred in our facility, housed under sterile conditions, and given autoclaved food and water ad libitum. They were monitored twice weekly for evidence of tumor growth. Once tumors reached 1.5 cm³, which was considered a humane endpoint by the Institutional Animal Care Committee, the mice were sacrificed and the tumors reimplanted into 3 further mice for up to 5 passages. Tumors were considered engrafted (XG) if they could be passaged at least once in vivo or not engrafted (no-XG) if growth was not detected by 8 months postimplantation.

Mutational profiling

Formalin-fixed and paraffin-embedded tumor blocks of the primary tumors were reviewed histologically and multiple 1.5-mm cores were taken from areas of high tumor cellularity, using a tissue microarray corer (Beecher Instruments Inc.). Among 157 samples, 139 were available and suitable for DNA isolation. The cores were deparaffinized by serial passages in xylene and ethanol solution, DNA was isolated as previously described (15) and subjected to mass spectrometric profiling for mutational sequence variants using the Sequenom (San Diego, CA) OncoCarta™ panel v1.0 (16). All mutations identified were verified in both the primary and xenograft tumors by using standard PCR-sequencing methods. For MET mutations, we also investigated surrounding normal lung tissue to determine whether mutations detected in the primary tumor were present in normal tissues as well.

Statistical analysis

Engraftment rate was correlated with clinicopathologic features, mutation status, and disease-free survival (DFS). Fisher’s exact test was used to determine the association of individual features with engraftment. DFS was calculated for patients with documented follow-up of at least 12 months and was defined as time between surgery and relapse or death. DFS percentages were calculated using the Kaplan–Meier method (17) and the survival curves were compared with a log-rank test. Variables of interest were tested in the presence of other clinical factors using a Cox proportional hazards model.

Results

Engraftability of primary resected NSCLC

Between April 2005 and June 2009, 157 primary NSCLC tumors were implanted into NOD-SCID mice. Among these, 63 (40%) engrafted and were passaged serially.
Histologic comparison between the corresponding xenograft and primary tumors revealed a high degree of similarity within the 5 passages in vivo (Fig. 1).

Squamous cell carcinomas had a significantly greater rate of engraftment (29/45, 64%; \(P < 0.001\)) than adenocarcinoma (30/96, 31%) or other histologic types (4/16, 25%). None of the 5 implanted carcinoid tumors engrafted. Two tumors with small cell components in the primary failed to engraft; however, on histologic review of the implanted tissue, no small cell component was seen. Moderately and poorly differentiated tumors (39% and 53%, respectively) were significantly more likely to engraft than well-differentiated tumors (11%, \(P = 0.003\)). Tumors that formed xenografts had a greater median tumor (T) size (4.5 vs. 3.5 cm, Wilcoxon test, \(P = 0.003\)). There was no significant difference in clinical stage between the XG and no-XG group (Table 1). In multivariate analysis poor differentiation, squamous cell histology, and larger T size were independently associated with increased rates of engraftment.

**Correlation of engraftability to the presence of mutations**

MassARRAY analysis detected mutations in 61 of 139 (43%) patient samples (Table 3). These included K-RAS mutations in 30 tumors, 29 of which were adenocarcinoma and 1 combined small and large cell carcinoma. The combined small and large cell tumor also harbored a
MET mutation. Twenty-six of the K-RAS mutations were in codon 12, 3 in codon 13, and 1 in 61, with the latter in a combined small and large cell tumor. EGFR (epidermal growth factor receptor) mutations were detected in 16 adenocarcinomas; 5 were exon 19 15-nucleotide deletions, and 8 were exon 21 L858R mutations. Two samples that did not engraft harbored T790M mutations concomitant with other EGFR mutations (1 L858R point mutation and 1 L747_T751 deletion). PIK3CA mutations were detected in 3 squamous cell carcinomas and 1 adenosquamous carcinoma. Two different MET mutations were found in 10 samples (T992I in 8 samples and R970C in 2). On the basis of a recent report suggesting these MET sequence variants were SNPs, we sequenced the DNA of corresponding normal lung tissue from these patients for the same mutation. The results confirmed the presence of identical sequence variants, indicating that these “mutations” were SNPs. Three tumors harbored more than 1 mutation. A significantly higher proportion of EGFR mutants were in the no-XG adenocarcinomas ($P = 0.027$) and more K-RAS mutations formed XG adenocarcinomas ($P = 0.054$; Fig. 3). Other mutations were present infrequently, but equally between the 2 groups. The derived xenografts, including samples with more than 1 mutation present in the primary tumor, harbored the same mutations.

To determine whether the impact of engraftment on DFS was driven by mutational status, all patients whose tumors harbored mutations were excluded. As the MET sequence variants were SNPs, these samples were also counted as wild-type. In univariate analysis, a significant difference in DFS persisted in wild-type (as profiled by the OncoCarta panel) patients between the XG and no-XG group ($HR: 3.97, CI 1.22–12.89, P = 0.014$), confirming that the association of engraftability with poorer outcome occurred irrespective of mutational status (Fig. 2C).

**Discussion**

In this study, we have shown that early-stage NSCLCs that form xenografts in NOD-SCID mice reproduce with significant fidelity, the histologic appearances of the patients’ primary tumors. The xenograft take rates were higher for squamous cell carcinoma (65.2%) than adenocarcinoma (30.5%). The ability to engraft was also correlated with larger size of the primary tumors, poorer differentiation grade, and K-RAS mutations among adenocarcinomas. In contrast, adenocarcinomas that harbored sensitizing EGFR tyrosine kinase domain mutations showed poor engraftability. Most important, patients whose tumors could form xenografts had significantly poorer DFS than patients whose tumors failed to engraft. All these results indicate that tumor engraftability is correlated with more aggressive biological and clinical behavior. Therefore, these primary xenografts are suitable models for mechanistic studies of NSCLC biology and as models to evaluate the efficacy of novel therapies that are being developed against NSCLC (19).
The 31.5% engraftment rate for adenocarcinomas is similar or only slightly higher than the success rate of establishing cell lines in culture (7, 8). In contrast, the ability to establish xenografts in 64.4% of squamous cell carcinomas is significantly greater than the reported ability to culture and establish cell lines for this tumor type [3/18, 16% reported by Stevenson et al. (8); and 1/25, 4% reported by Anderson et al. (13)]. This difference in the ability to establish squamous cell xenografts was also observed by some (12, 20) but not all (13) researchers using a similar protocol to our own; however, the total number of squamous cell xenografts was only 16 in these studies combined. Furthermore, the differences we have described in patient outcome with engraftability were not seen in other PTXG studies (12–14), which may also be reflective of the small numbers of patients included. Our observations suggest, though, that PTXGs may provide an excellent resource to study tumors from patients at high risk of relapse but also squamous cell tumors, a subgroup that has received limited attention in recent molecular studies.

The negative correlation of engraftability with EGFR TK domain mutations and positive correlation with K-RAS mutations are in keeping with the observed prognostic value of these mutations in NSCLC patients (21, 22). EGFR mutations are associated with an improved prognosis and K-RAS mutations with poorer outcome (21). Nevertheless, the negative prognostic significance of engraftment remained among patients whose tumors did not harbor an identifiable mutation. The association of mutation status and engraftment has not been reported previously, although other molecular studies have shown that jun, N-ras, and cyclin D protein upregulation is associated with increased engraftability (14). Recent studies in NSCLC have shown that many adenocarcinomas harbor mutations in important genes such as P53, K-RAS, STK11, and EGFR (23). As the OncoCarta panel is not specific to lung cancer and has limitations in the number of exons that can be interrogated in the 1 assay, several important genes are not reportable including p53. Given that p53 mutations have not been shown to be prognostic or predictive (21, 24), we would expect to see mutants in both the XG and no-XG groups. A recent study found p53 mutations in 13 of 25 PTXG samples (12), which is similar to the rate seen in clinical studies (23, 24). The
low frequency of other mutations seen in our study did not permit correlation with outcome. However, given that engraftability remained an independent predictor of disease relapse in multivariate analysis, further molecular characterization of these tumors promises to identify clinically relevant prognostic markers.

The success of targeted therapies in personalizing treatment has underscored the importance of performing mechanistic and functional investigations in NSCLC. As PTXGs parallel the original tumor more closely, they may result in more clinically translatable models. Our data show that similar to cell lines, PTXGs are more representative of biologically aggressive tumors, irrespective of their clinical stage at diagnosis and their mutation status. Therefore, the engraftable models bear a closer resemblance to patients who experience rapid postoperative disease progression rather than patients with more indolent disease. Although we specifically chose patients undergoing curative surgery and have not conducted similar studies by...

**Table 3. Mutations found in primary tumors that engrafted or failed to engraft**

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of samples (%)</th>
<th>Mutation type</th>
<th>Number of samples</th>
</tr>
</thead>
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<tr>
<td><strong>Tumors that formed xenografts (n = 63)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>2 (3) E746_A750del</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 L858R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-ras</td>
<td>15 (24)</td>
<td>G12A</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>G12C</td>
<td>7</td>
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<td></td>
<td></td>
<td>G12D</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>G12V</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G61H</td>
<td>1</td>
</tr>
<tr>
<td>MET&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3 (5) T992I</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>PIK3CA</td>
<td>3 (5)</td>
<td>E542K</td>
<td>2</td>
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<td></td>
<td></td>
<td>E545K</td>
<td>1</td>
</tr>
<tr>
<td>BRAF</td>
<td>1 (1) G469V</td>
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<td></td>
</tr>
<tr>
<td>No mutation</td>
<td>39 (62)</td>
<td></td>
<td></td>
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<tr>
<td><strong>Tumors that failed to engraft (n = 76)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>EGFR</td>
<td>14 (18) E746_A750del</td>
<td>4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>L858R</td>
<td>7</td>
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<tr>
<td></td>
<td></td>
<td>&gt;1 mutation</td>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>K-ras</td>
<td>15 (20)</td>
<td>G12C</td>
<td>6</td>
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<td></td>
<td></td>
<td>G12D</td>
<td>2</td>
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<td>G12V</td>
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<td></td>
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<td>G13D</td>
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<td></td>
<td></td>
<td>G13V</td>
<td>1</td>
</tr>
<tr>
<td>MET&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 (9) R970C</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T992I</td>
<td>5</td>
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<tr>
<td>PIK3CA</td>
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<td></td>
<td></td>
<td>H1047R</td>
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</tr>
<tr>
<td>No mutation</td>
<td>38 (50)</td>
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</table>

<sup>a</sup>Samples with >1 mutations: T790M/L858R; T790M/L747_T751del; S768I/G719C.

<sup>b</sup>Corresponding normal lung tissue DNA also showed the presence of these sequence variants. We have therefore considered them to be SNPs.
using biopsies from metastases or advanced cancer patients, our results suggest that the ability of tumors to engraft from the latter group of patients could potentially be much greater. Although of the 36 stage III or IV patients in this data set, we were able to form a xenograft in 19 (52%), this was not significantly greater than other stages.

We believe PTXGs may represent better models than cell lines or cell line–derived xenografts, although there remain potential limitations to the model that need to be investigated further. Neither in vitro nor in vivo models can account adequately for the complex pharmacokinetic or pharmacodynamic interactions or can they reproduce the role of immunoregulation in tumor response and tumor biology (25–27). As experimental models, they are more costly to maintain and to perform experiments on and their genomic stability with increasing passages remains to be defined. Nevertheless, some of these limitations also apply to cell lines, yet there are many advantages to using PTXGs. Unlike cell lines, the histologic representation of the original tumor remains intact. Others have shown that in terms of chemosensitivity, morphology, and gene expression, PTXGs closely correlate with the primary NSCLC (12, 28, 29). PTXGs representing the spectrum of NSCLC can be formed including well and moderately differentiated tumors, squamous cell carcinomas, and EGFR mutant tumors, whereas relatively few squamous cell carcinoma cell lines have been established. Subsequently, most cell lines are highly passaged poorly differentiated carcinomas with no resemblance to the initial tumor. Finally, from the perspective of testing therapies and determining biomarkers, treating PTXGs in vivo via the intravenous, oral, or intraperitoneal route, although imperfect, more closely parallels the clinical situation than adding therapies at different concentrations to cell cultures. For these reasons, PTXGs are becoming more widely adopted for preclinical studies, especially for therapeutic testing (30). To date, however, there are few reports that correlate the therapeutic activity of PTXGs to patient response data. The Pediatric Preclinical Testing Program, which uses PTXGs, was implemented by the National Cancer Institute to improve drug development in pediatric malignancies and has shown that PTXGs closely parallel responses observed clinically (31, 32). This approach may also be promising in NSCLC as PTXGs have much higher engraftment rates than cell line establishment and therefore a wider biological spectrum of the disease is available for testing.

As engraftable tumors represent a subgroup of patients with poorer biological features and shorter DFS independent of current known prognostic factors, they may be a superior model for preclinical studies in drug development because they reflect the subset of patients most in need of newer and better therapies. Such an approach potentially may improve the success rate of early-phase trials of novel targeted agents. Evaluation of specific therapies for specific targets in PTXG models may improve the paradigm for personalization of care.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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