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# 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

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The tumor models are available to investigators through collaboration and/or Institutional Material Transfer Agreement.

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**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.

responses to treatment *in vivo* (4), and that those tumors that do grow *in vitro* represent a subset of patients with poorer clinical outcome (7, 8), other more clinically relevant models of NSCLC are required.

Primary tumor xenografts (PTXG) are formed by taking tumor fragments from patients and directly implanting them into another host who usually is immunocompromised to prevent rejection of the graft. They may be more relevant models of cancer, as they better represent the heterogeneity seen in the primary malignancy than cell lines and murine allografts (3, 5). However, there are also limitations to the model including loss of species–specific tumor cell–stromal interface and differences in pharmacokinetic and pharmacodynamic interactions, which potentially may limit their predictive value in drug development (9, 10). As a model, xenografts derived from cell lines and primary tumors are used frequently in preclinical studies such that nearly every clinically approved anticancer drug has shown some degree of activity in xenograft models (11).

Similar to *in vitro* cell line studies, however, not all primary tumors when implanted into immunocompromised mice will engraft and grow. Despite the increasing use of PTXGs for their potentially improved clinical relevance, only 3 studies have investigated the model in NSCLC by correlating clinicopathologic characteristics with engraftment rates (12–14) and *no* correlation was found. However in those studies, the number of NSCLC tumors included and the numbers that subsequently engrafted were small. We report here that similar to cell lines, primary early-stage NSCLC tumors that engraft and can be passaged serially *in vivo* are associated with distinct histologic, genetic, and clinical features. However, rates of engraftment are much higher and subtypes that engraft are more heterogeneous than those seen in cell lines. These observations may have important implications for the interpretation of preclinical studies that use PTXGs in the context of early-phase clinical trials.

## 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 $cm^3$, 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

**Table 1.** Clinical and pathologic features of 157 patients and their tumors

| All characteristics ($n = 157$) | No. of patients (%) XG ($n = 63$) | No-XG ($n = 94$) | $P$ value[a] |
|---|---|---|---|
| *Age*, median (range), y | 66 (44–84) | 67 (44–88) | ns |
| *Sex* | | | |
| Male | 38 (60) | 37 (39) | 0.014 |
| Female | 25 | 57 | |
| *Pathologic TNM stage* | | | |
| Median T size (range) | 4.5 cm (1.7–11.5) | 3.2 cm (0.6–13.5) | 0.003[b] |
| I | 35 (56) | 54 (57) | ns |
| II | 9 (14) | 23 (25) | |
| III/IV | 19 (30) | 17 (18) | |
| *Histologic type* | | | |
| Adenocarcinoma | 30 (48) | 67 (71) | <0.001 |
| Squamous cell | 29 (46) | 15 (16) | |
| Large cell | 3 (5) | 3 (3) | |
| Carcinoid | 0 | 5 | |
| Sarcomatoid | 1 | 0 | |
| Lymphoepithelial | 0 | 1 | |
| Adenosquamous | 0 | 1 | |
| Mixed small/large cell | 0 | 2 | |
| *Differentiation* | | | |
| Well | 2 (3) | 17 (19) | 0.003 |
| Moderate | 26 (41) | 41 (46) | |
| Poor | 35 (56) | 31 (35) | |

Abbreviation: ns, not significant.
[a]Fisher's exact test.
[b]Wilcoxon test.

*in vivo* (Table 1). 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 and clinical outcome

To determine the impact of engraftment on clinical outcome, we assessed DFS for patients with stage I–IIIA tumors with at least 12 months of follow-up if they had not progressed or died within this time. This included 109 patients with a median follow-up time of 1.9 years (range: 0.003–4.4 years). Twenty-nine patients had developed clinical recurrence and 3 patients had died without relapse. Patients whose tumors engrafted had significantly shorter DFS than those with no-XG tumors [hazard ratio (HR): 5.2, 95% CI: 2.38–11.21. $P < 0.001$; ref. Fig. 2A). Among patients with XGs, those with adenocarcinoma had shorter DFS than those with squamous cell cancers (Fig. 2B). However, patients with squamous cell carcinoma had better DFS than those with adenocarcinomas, regardless of engraftability. In multivariate analysis (Table 2) in which patient age, sex, histology, differentiation, and stage were included in the model, the ability to form a XG remained an independent predictor of shorter DFS (HR: 7.0, 95% CI: 3.1–15.81, $P < 0.000003$).

### 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

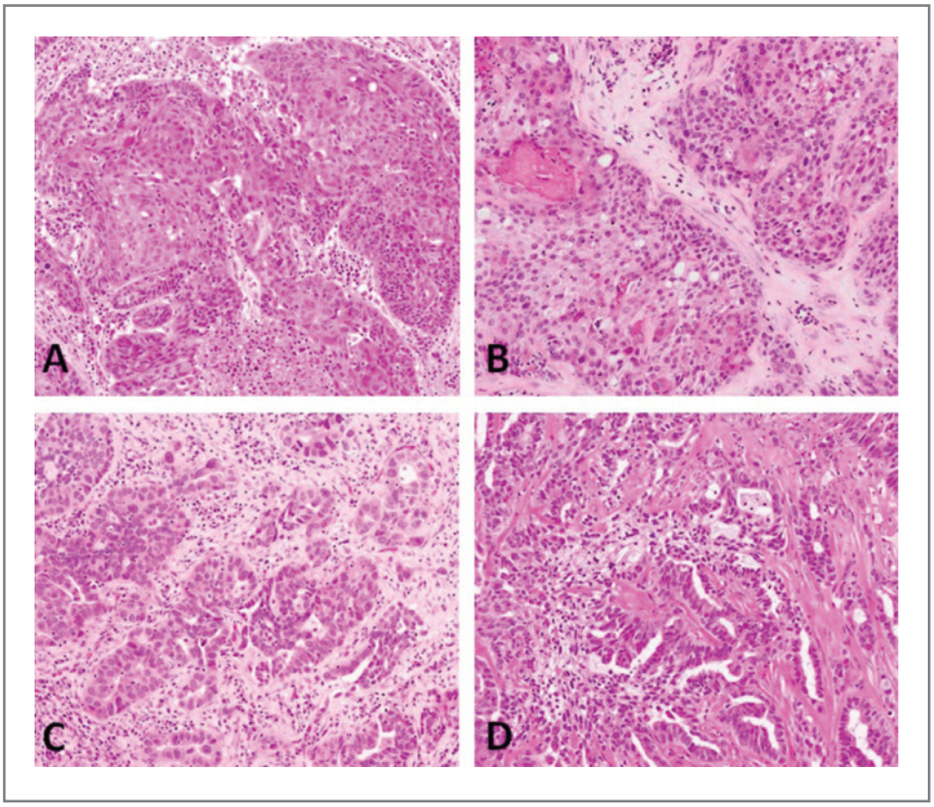


Figure 1. Histology of representative primary tumor xenografts and the primary tumor they were derived from: moderately differentiated squamous cell carcinoma patient primary tumor (A) and derived xenograft with the same histology (B). Moderately differentiated adenocarcinoma patient primary tumor (C) and derived xenograft with the same histology (D). Original magnifications × 100.

*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 rare single nucleotide polymorphisms (SNP; ref. 18), 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* mutants 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).

**Table 2.** Disease-free survival according to patient and tumor characteristics

| Factor | Univariate HR (95% CI) | Univariate *P* value | Multivariate HR (95% CI) | Multivariate *P* value |
|---|---|---|---|---|
| Age | 0.99 (0.96–1.03) | 0.63 | 1.01 (0.97–1.04) | 0.72 |
| Sex (M vs. F) | 0.93 (0.46–1.86) | 0.83 | 0.89 (0.43–1.83) | 0.75 |
| Histology (AD vs. other) | 1.31 (0.62–2.78) | 0.47 | 2.35 (1.05–5.25) | 0.038 |
| Stage (I vs. II/III) | 1.44 (0.71–2.92) | 0.31 | 1.42 (0.69–2.94) | 0.34 |
| Engraftment | 5.16 (2.38–11.21) | <0.00001 | 7.00 (3.1–15.81) | <0.000003 |

Abbreviations: M, male; F, female; AD, adenocarcinoma.

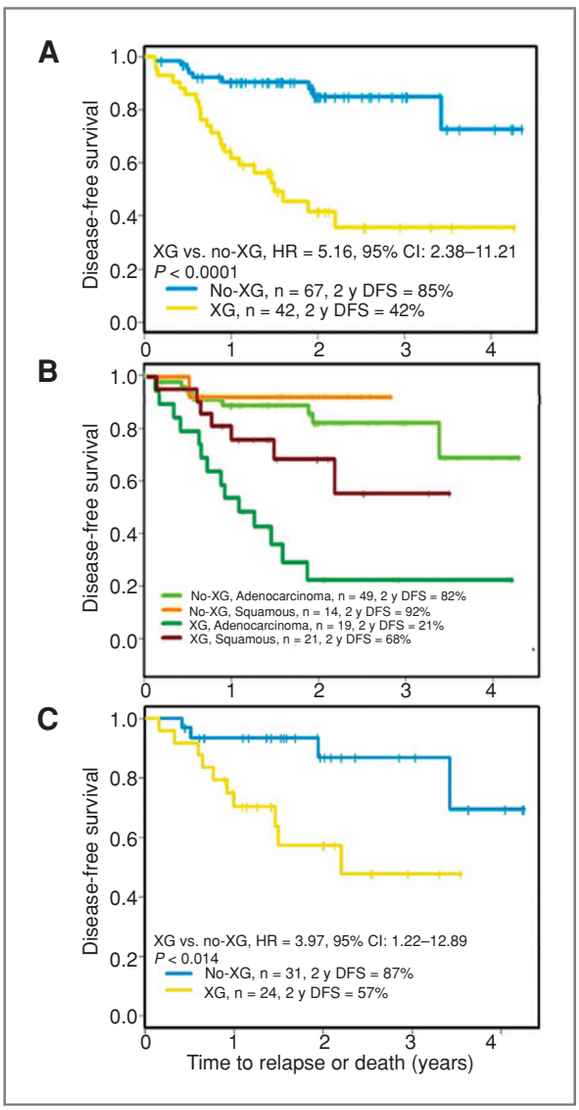


**Figure 2.** Correlation of ability to xenograft with patients' prognosis. (A) Kaplan–Meier survival curves for XG and no-XG patients showing a significant difference in DFS. (B) DFS according to histology showing significantly poorer survival in XG adenocarcinoma patients. (C) DFS in all wild-type (by OncoCarta panel profiling) patients showing significant difference based on engraftment.

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 engraftablilty 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

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

| Gene | No. of samples (%) | Mutation type | Number of samples |
|---|---|---|---|
| **Tumors that formed xenografts ($n = 63$)** | | | |
| *EGFR* | 2 (3) | E746_A750del | 1 |
| | | L858R | 1 |
| *K-ras* | 15 (24) | G12A | 1 |
| | | G12C | 7 |
| | | G12D | 4 |
| | | G12V | 2 |
| | | Q61H | 1 |
| *MET*[b] | 3 (5) | T992I | 3 |
| *PIK3CA* | 3 (5) | E542K | 2 |
| | | E545K | 1 |
| *BRAF* | 1 (1) | G469V | 1 |
| No mutation | 39 (62) | | |
| **Tumors that failed to engraft (n = 76)** | | | |
| *EGFR* | 14 (18) | E746_A750del | 4 |
| | | L858R | 7 |
| | | >1 mutation | 3[a] |
| *K-ras* | 15 (20) | G12C | 6 |
| | | G12D | 2 |
| | | G12V | 4 |
| | | G13D | 2 |
| | | G13V | 1 |
| *MET*[b] | 7 (9) | R970C | 2 |
| | | T992I | 5 |
| *PIK3CA* | 2 (3) | E545K | 1 |
| | | H1047R | 1 |
| No mutation | 38 (50) | | |

[a]Samples with >1 mutations: T790M/L858R; T790M/L747_T751del; S768I/G719C.
[b]Corresponding normal lung tissue DNA also showed the presence of these sequence variants. We have therefore considered them to be SNPs.

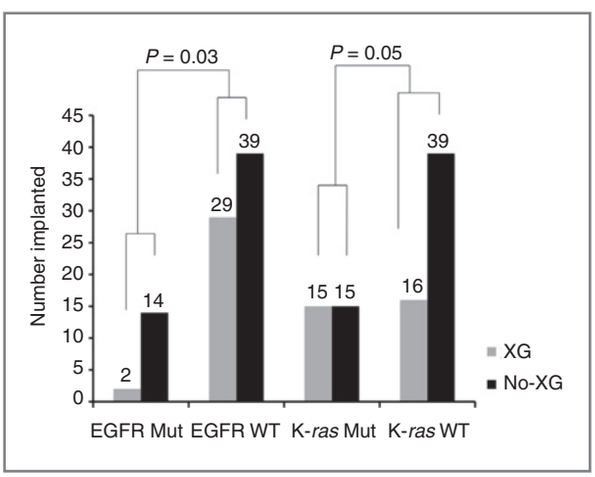


Figure 3. Differences in rate of engraftment by mutation status among adenocarcinomas. Mut, mutation; WT, wild type.

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

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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