Optimizing the development of targeted agents in pancreatic cancer: tumor fine-needle aspiration biopsy as a platform for novel prospective *ex vivo* drug sensitivity assays

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Abstract

At the present time, the optimal development of molecularly targeted anticancer agents is limited by the lack of clinically applicable tools to predict drug effects. This study aimed to develop methods that might be useful in predicting the efficacy of targeted agents in a novel model system of human pancreatic cancer. A series of xenografts were established in nude mice by implanting human pancreatic cancer tissue surgically resected from cancer patients. Animals were treated with the epidermal growth factor receptor inhibitor erlotinib, the mammalian target of rapamycin inhibitor temsirolimus, or vehicle. Tumor cells were sampled by fine-needle aspiration biopsy (FNAB) before (baseline, day 0) and at the completion of 28 days of treatment. Cells obtained at baseline were exposed to erlotinib or temsirolimus in short-term cell culture conditions (*ex vivo*). Western blot analysis was done to determine the degree of inhibition in the phosphorylation of extracellular signal-regulated kinase 1/2 and S6-ribosomal protein (downstream effectors of epidermal growth factor receptor and mammalian target of rapamycin, respectively) *ex vivo* and *in vivo*. Five of six xenografted tumors responded to temsirolimus, whereas only one tumor responded to erlotinib. The results of the *ex vivo* studies correctly predicted the pharmacodynamic effect of the agents *in vivo* as well as their gross antitumor effects. Finally, we showed the clinical feasibility of this approach, performing *ex vivo* assessment of drug-target response in FNAB samples from three patients with pancreatic cancer. Cancer cells obtained by FNAB, an established minimally invasive diagnostic procedure, can be used to test *ex vivo* the effects of targeted anticancer agents. These effects correlate with antitumor activity *in vivo* and may therefore provide an important tool applicable to clinical trials. Ultimately, an approach of this nature may facilitate the further refinement of patient selection in favor of individuals with molecular profiles, predicting a greater likelihood of therapeutic benefit. [Mol Cancer Ther 2007;6(2):515–23]

Introduction

Accumulating clinical experience suggests that molecularly targeted therapeutics may hold the promise of revolutionizing the treatment landscape available to cancer patients. Nevertheless, it is becoming increasingly evident that traditional drug development paradigms may not be ideally suited to realize the full clinical potential of these new agents. One logical organizing principle is that targeted therapeutics will be effective against tumors in which the target is biologically important and is adequately blocked by the drug (1–3).

The development of imatinib mesylate offers one example of a situation where conventional taxonomic schemes corresponded to a critical and, in this case, effectively treatable molecular target in a preponderance of cases (4, 5). Somewhat in contrast, more recent experience has revealed the striking differential efficacy of epidermal growth factor receptor (EGFR)–targeted agents among subgroups of solid tumor patients with distinct molecular profiles (6–12). This suggests the potential of tools for rational patient selection that may define these biologically discrete subpopulations and better realize the potential of these agents for larger numbers of patients (13–15). One principal limitation in this area is the lack of sophisticated preclinical models permitting the development of tissue acquisition protocols and candidate biomarkers predictive of drug actions.

Despite substantial progress made in recent years, there are currently no clinically validated tests to predict the efficacy of a given agent for an individual patient. Although there is consensus supporting the need to develop and integrate the evaluation of predictive biomarkers in clinical trials, the practical application of such an approach is still lagging behind (16). Three main issues define the obstacles in the way of realizing this conceptual goal. As a start, robust and well-validated assays that

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faithfully predict treatment outcomes are needed. Next, such assays must be applicable to readily available clinical materials. Finally, there is a need to develop practical, minimally morbid means of collecting reliable yields of tumor material for correlation of biomarker readout with clinical response.

In this context, we have recently shown that tumor fine-needle aspiration biopsy (FNAB) samples provide enriched cancer cells to assess the efficacy of targeted therapies *in vivo* (17). In that work, we showed that protein-based markers of target pathway inhibition *in vivo* correlated with antitumor response to EGFR and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase/ERK blockers. To build on that conceptual foundation, we have developed a novel prospective pharmacodynamic system for drug testing in a pancreatic cancer model using patients' tumor tissue obtained at the time of cancer resection. Tumors were heterotransplanted in athymic mice, expanded to numbers suitable for the evaluation of multiple treatments and then tested with different targeted drugs. Our prior studies showed that these tumors maintain the main features of the originating cancer (18); hence, we believe that their use in preclinical studies reproduces more accurately the clinical scenario compared with studies done with cell lines. Using this model system, in addition to validating the correlation of target pathway inhibition *in vivo* with antitumor response shown in our earlier work, the present study aimed to develop and evaluate a simple and reliable short-term biological (*ex vivo*) assay to predict the efficacy of targeted drugs before systemic treatment.

Materials and Methods

Drugs

Erlotinib (OSI-774, Tarceva, OSI Pharmaceuticals, Melville, NY) was dissolved in DMSO as a stock solution and then further diluted to desired concentrations for *ex vivo* experiments, or prepared for i.p. administration as described (17, 18). Temsirolimus (CCI-779, Wyeth Research, Colleville, PA) was prepared in ethanol and was further diluted for *ex vivo* experiments or for i.p. injection in xenograft mice as described (19, 20). Drug doses and treatment schedules were based on previous studies (19, 20).

Tumor Xenograft Development and Assessment

Four-week-old female athymic (nu+/nu+) mice were purchased from Harlan Laboratories (Washington, DC). The research protocol was approved by the Johns Hopkins University Animal Care and Use Committee, and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. The tissue acquisition protocol was reviewed and approved by the Johns Hopkins Institutional Review Board. Briefly, cancer tissue was obtained from surgical specimens of patients undergoing pancreatic resection for adenocarcinoma at the Johns Hopkins Hospital and established as s.c. xenografts in nude mice (F1 generation). The tumor xenografts from this initial passage were harvested and reimplanted s.c. in groups of five mice for each patient, with two small pieces per mouse (F2 generation). Tumors were allowed to grow to a size of 1.5 cm, at which point they were harvested, divided into small $\sim 3 \times 3 \times 3$ mm pieces, and transplanted to another 18-22 mice, with two tumors per mouse (F3 generation). Tumors from this second mouse-to-mouse passage were allowed to grow until reaching ~ 200 mm^3, at which time mice were randomized in the following three treatment groups, with six mice in each group per index patient tumor sample: control (vehicle), erlotinib (50 mg/kg/d i.p.), and temsirolimus (20 mg/kg days 1–5 i.p.). Mice were monitored daily for signs of toxicity and were weighed three times per week. Tumor size was evaluated three times per week by caliper measurements using the following formula: tumor volume = (length $\times$ width^2) / 2 as previously reported (21). Tumor growth inhibition was calculated by tumor growth differential of treated mice divided by tumor growth of control mice: $(C - T/C) \times 100$. With this design, xenografts of each index primary human pancreatic adenocarcinoma served as an internal control for drug response across the panel of six models. Experiments were terminated on day 28. Tumor response was defined as sensitive when tumor growth inhibition was >50% on day 28.

Ex vivo Studies

Tumor cells were collected by FNAB from the xenograft animals before the start of treatment using a sterile 25G short needle, as described in Fig. 1. All samples were confirmed to be enriched for cancer cells by microscopic assessment of a staff cytopathologist at the Johns Hopkins Hospital (S.A.). Tumor samples were immediately transferred into 10-mL sterile prewarmed complete RPMI 1640 culture medium containing 10% fetal bovine serum, penicillin (200 μg/mL), and streptomycin (200 μg/mL). Cells were incubated with 0.04% trypan blue (Sigma, St. Louis, MO) dissolved in PBS [9.1 mmol/L Na_2HPO_4, 1.7 mmol/L NaH_2PO_4, and 150 mmol/L NaCl (pH 7.4)] to assess viability. The viable (membrane intact) and dead cells were then counted, and the total viable cell count was used to calculate final working volumes. Approximately 25,000 viable tumor cells were seeded into each well of a six-well polypropylene microplate. Cells were treated in duplicates with vehicle (control), erlotinib (5 μmol/L), or temsirolimus (1 μmol/L) in a humidified 5% CO_2 incubator

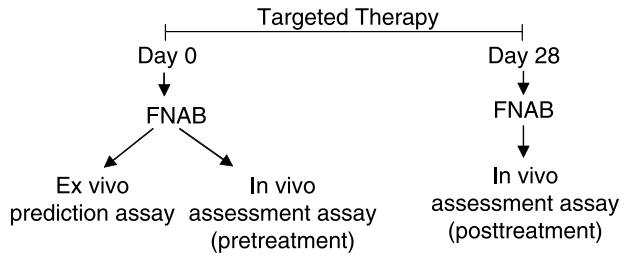

Figure 1. Schematic of experimental design. FNAB samples were collected before (day 0) and after (day 28) the initiation of therapy. *Ex vivo* and *in vivo* sensitivity assays were done to predict and assess the efficacy of therapy in xenograft models of human pancreatic carcinoma.

at 37°C for 6 h. No fibroblast or endothelial cell growth was observed. Following treatment, nonadherent and adherent cells (only a few, collected by scraping) were pooled together and centrifuged at 500 × *g* for 5 min at 4°C. After washing with PBS, cells were lysed in 100 μL of ice-cold lysis buffer [50 mmol/L Tris-HCl, 0.25 mol/L NaCl, 0.1% (v/v) Triton X-100, 1 mmol/L EDTA, 50 mmol/L NaF, and 0.1 mmol/L Na_3VO_4 (pH 7.4)] containing protease and phosphatase inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) and analyzed by Western blot.

In vivo FNAB Studies

In parallel with the FNAB samples obtained for the *ex vivo* studies, additional FNAB samples were collected from each animal before (day 0) and at the end (day 28) of treatment (Fig. 1), as previously described (17). The aspirated material was smeared onto clear glass slides, and all smears were allowed to air-dry and then stained with Diff-Quick stain (Baxter Healthcare, Miami, FL). Five air-dried and Diff-Quik (AD/DQ)–stained cytologic smears were prepared from each tumor sample. The cellular composition of each aspirate was assessed by a certified staff cytopathologist (S.A.) at the Johns Hopkins Hospital under the microscope before protein extraction. To prepare whole-cell lysates, the cells were collected from AD/DQ slides by scraping into ice-cold buffer with protease and phosphatase inhibitors (Roche Molecular Biochemicals). Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4°C, and the supernatants were used in Western blot experiments.

Tissue Preparation and Immunohistochemical Analysis

At the completion of the treatment course, xenografted tissues were harvested and fixed in formalin for 24 h. The fixed tissues were paraffin embedded and cut in 0.5-μm sections onto positively charged glass slides for immunohistochemical labeling. Pharmacodynamic effects of the drug in the targeted pathway were analyzed by immunohistochemistry. For immunohistochemical staining, slides were deparaffinized and rehydrated in graded concentrations of alcohol by standard techniques before antigen retrieval in citrate buffer pH 6 for 20 min. Next, the slides were cooled for 20 min before washing in 1× TBST (DAKO Corp., Carpinteria, CA). All staining was done using a DAKO Autostainer at room temperature. Slides were incubated in 3% H_2O_2 for 10 min followed by the appropriate dilution of primary antibody for 60 min. Tris-HCl (0.2 mol/L, pH 7.5; Quality Biological, Inc., Gaithersburg, MD) was used as the antibody diluent solution. Slides were incubated in 3% H_2O_2 for 10 min followed by the appropriate dilution of primary antibody for 60 min. Dilutions of antibodies used were as follows: total ERK1/2 (Cell Signaling Technology, Beverly, MA), 1:25; phosphorylated ERK1/2 (Thr^{202}/Tyr^{204}; Cell Signaling Technology), 1:50; p70S6K (Santa Cruz Biotechnology, Santa Cruz, CA), 1:50; and phosphorylated p70S6K (Cell Signaling Technology), 1:50. Negative controls were incubated for 60 min with the antibody diluent solution [0.2 mol/L Tris-HCl (pH 7.5) from Quality Biological].

Western Blot Analysis

Protein concentrations obtained from FNAB samples were quantified before each experiment. Protein extracts (15 μg) were electrophoresed on a 10% (w/v) SDS-PAGE. After electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA), membranes were blocked at room temperature using SuperBlock (Pierce, Rockford, IL) for 1 h. The primary antibodies were diluted at 1:1,000 in 1:10 dilution of SuperBlock solution in TBS, and the membranes were incubated with primary antibodies overnight at 4°C. The antibodies tested were phosphorylated ERK1/2 (Cell Signaling Technology), phosphorylated S6-ribosomal protein (S6-RP; Cell Signaling Technology), total ERK1/2 (Cell Signaling Technology), and total S6-RP (Cell Signaling Technology). The next day, the membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies, rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology), or mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) at a final dilution of 1:3,000. Antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

Results

Experimental Model

To determine whether cancer cells obtained by FNAB before systemic treatment can be used in an *ex vivo* assay to predict tumor response to targeted therapeutics *in vivo*, we used primary pancreatic cancer xenograft mouse models bearing tumors surgically resected from pancreatic cancer patients. Cancer cells were harvested by FNAB on day 0 (Fig. 1) and treated with temsirolimus or erlotinib for 6 h, after which signal pathway inhibition was analyzed by Western blot. We found the pharmacodynamic effects at 6 h of treatment to be representative of changes seen at longer incubation times (data not shown). Under these cell culture conditions, no fibroblast and endothelial cell growth was detected (data not shown).

Correlation of *Ex vivo* Assay with Sensitivity to Temsirolimus

As shown in Fig. 2, treatment with temsirolimus *ex vivo* inhibited phosphorylation of S6-RP, an important regulatory kinase of the mammalian target of rapamycin pathway, in cells collected from tumor A198, sensitive to therapy, but not in cells from tumor A194, and resistant to the antitumor effect of temsirolimus. *Ex vivo* treatment of cells did not affect the total levels of S6-RP protein (Fig. 2). The reproducibility of these findings was evaluated in a panel of six primary pancreatic cancer xenografts. Figure 3A (*top*) summarizes the correlation of inhibition of S6-RP phosphorylation in the *ex vivo* pharmacodynamic assays done with cells obtained by FNAB before systemic treatment and drug-mediated tumor growth inhibition *in vivo*. As shown in Fig. 3A, in xenograft animals treated with temsirolimus *in vivo*, the tumor growth inhibition ranged from 18% to 80%. Except for tumor A194, all other tumors were sensitive to therapy, with >50% tumor growth

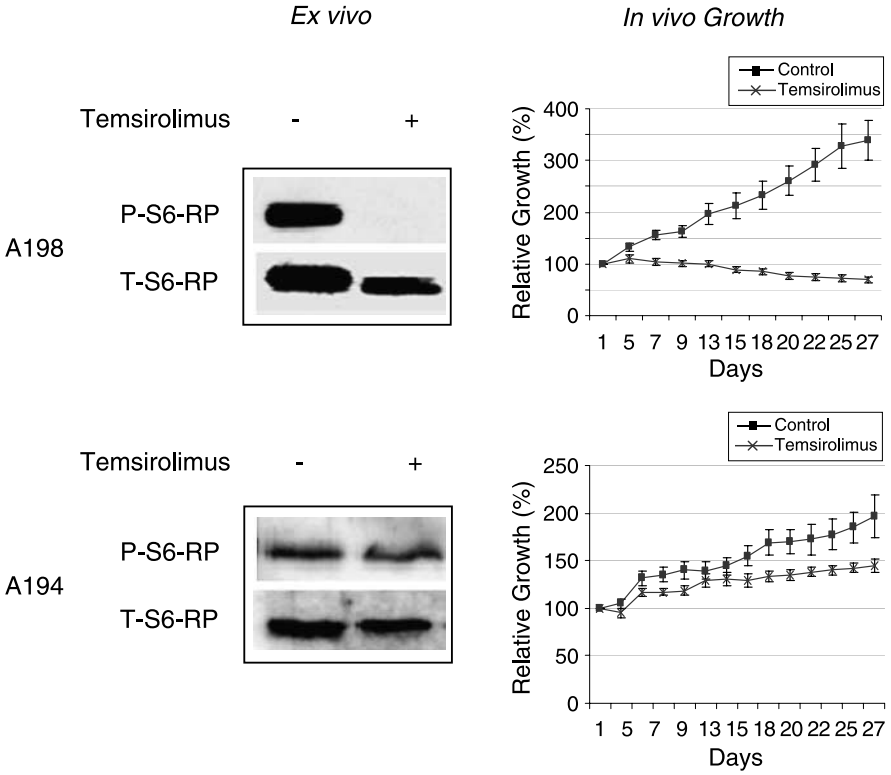

Figure 2. Representative tumor growth (*right*) and *ex vivo* assay data (*left*) from a temsirolimus-susceptible (A198) and temsirolimus-resistant (A194) pancreatic cancer xenograft. Cancer cells were obtained by FNAB before systemic treatment. Exposure to 1 μmol/L temsirolimus *ex vivo* inhibited phosphorylation (*P-S6-RP*) of S6-RP in the tumor cells from the susceptible tumor (A198), but not from the resistant tumor (A194). No change was observed in total (*T-S6-RP*) level of S6-RP in cells treated *ex vivo*.

inhibition (in bold). As illustrated in Fig. 3A, temsirolimus treatment blocked S6-RP phosphorylation *ex vivo* only in cancer cells obtained from tumors sensitive to therapy *in vivo*.

Correlation of *Ex vivo* Assay with Resistance to Erlotinib

In contrast, erlotinib was less active against the panel of primary pancreatic cancers. Figure 4 illustrates that *ex vivo* erlotinib treatment of cells from tumor A198, sensitive to erlotinib, resulted in a dramatic inhibition of phosphorylation of ERK1/2, downstream effectors of EGFR. However, *ex vivo* erlotinib treatment failed to inhibit ERK1/2 phosphorylation in cells obtained from the resistant tumor A265. No changes were observed in the expression levels of total ERK1/2 in treated animals (Fig. 4). As summarized in Fig. 5A, except for A198 (in bold), all the remaining tumors had <50% tumor growth inhibition and were therefore defined as resistant to erlotinib. Consistent with the above described results for the *ex vivo* analyses of temsirolimus response (Fig. 3A), erlotinib therapy failed to inhibit ERK1/2 activation *ex vivo* in all resistant tumors (Fig. 5A). These data show that the *ex vivo* assays can predict tumor response to temsirolimus and erlotinib in pancreatic tumors before *in vivo* treatment.

Correlation of *In vivo* Assay to *Ex vivo* Assay and *In vivo* Tumor Growth Inhibition

To further analyze the pharmacodynamic efficacy of temsirolimus and erlotinib *in vivo*, AD/DQ–stained smears were prepared from FNAB samples obtained from tumor tissue before initiation (day 0) and at the end (day 28) of treatment (Fig. 1), as previously described (17). Morphologic assessment of the cytologic smears showed that, on average, 90% of the cells were neoplastic with some RBC and negligible amount of connective tissue fragments in the background. No significant apoptosis or necrosis was detected in tumors of control- or drug-treated animals (data not shown), indicating that these agents had a cytostatic rather than cytotoxic effect on tumor cells. Following morphologic evaluation, whole-cell extracts were prepared from AD/DQ–stained tumor FNAB samples, and the expression levels of total and phosphorylated S6-RP and ERK1/2 proteins were determined on Western blot analysis. Overall, across the panel of xenografted primary pancreatic tumors, the pharmacodynamic effect of each drug *ex vivo* (*top*) was concordant with *in vivo* target effect (*middle*) as well as with changes in tumor volume (Figs. 3A and 5A). The phosphorylation of S6-RP and ERK1/2 was inhibited *in vivo* only in tumors sensitive to temsirolimus (Fig. 3A) or erlotinib (Fig. 5A), respectively.

Immunohistochemical Analysis

To corroborate the changes observed by FNAB analysis, we did immunohistochemical staining of tumor tissue resected from vehicle- and drug-treated animals (Figs. 3B and 5B) and compared results with the Western blot data obtained from *in vivo* FNAB samples (Figs. 3A and 5A). As illustrated in Fig. 3B, in tumor A198, which was sensitive to treatment, temsirolimus strongly decreased staining for the phosphorylated form of p70S6K (pS6K), a kinase in the mammalian target of rapamycin pathway that regulates the activity of S6-RP. However, no

effect was seen in tumor A194, which did not respond to temsirolimus *in vivo*. No significant changes were observed in total pS6K staining in treated tumors. These immunohistochemical results correlate with findings observed in Western blot analysis of FNAB specimens from the *in vivo* treated tumors (Fig. 3A). With erlotinib, however, the immunohistochemical results were rather inconclusive, partly due to the low intensity and focal staining pattern of phosphorylated ERK protein in both vehicle- and erlotinib-treated tumor samples (A198 and A265 xenografts depicted in Fig. 5B). This observation is likely due to the low sensitivity of the immunohistochemical assays to detect phosphorylated ERK1/2 proteins in selected cases, rather than problems associated with the antibody used in these assays, because the same antibody was able to detect ERK1/2 expression in immunohistochemical assays done with other pancreatic tumor samples (data not shown). These results show that the FNAB-based approach is a viable alternative to conventional immunohistochemistry to evaluate morphologic and molecular features of tumor cells in small tumor samples.

Feasibility of *Ex vivo* Assay in Routine Diagnostic FNAB Material

Finally, to determine whether routine clinical FNAB specimens provide adequately cellular tumor samples to perform *ex vivo* prediction assays, adenocarcinoma cells were collected by ultrasound-guided FNAB technique from pancreatic cancer patients during routine diagnostic procedures. Tumor cells were isolated by centrifugation from the needle rinse suspensions and treated with vehicle (control), erlotinib, or temsirolimus *ex vivo* for 6 h. As illustrated in Fig. 6, adenocarcinoma cells with similar cytomorphologic features showed variation in their responses to targeted therapeutics *ex vivo*. In cancer cells collected from patient 1, erlotinib dramatically blocked ERK1/2 phosphorylation, whereas temsirolimus only partially decreased S6-RP phosphorylation *ex vivo*. In tumor cells of patients 2 and 3, however, erlotinib did not effectively block ERK1/2 activity, whereas temsirolimus inhibited S6-RP phosphorylation. No inhibition was observed in the expression of total ERK1/2 and S6-RP proteins in drug-treated cells (Fig. 6). Although these patients were not subsequently treated with the same agents to correlate *ex vivo* drug effects with clinical outcome, these results support the conjecture that the *ex vivo* drug sensitivity assay employed in our preclinical model can be applied to clinical studies for evaluation as a predictor of patient response to targeted therapeutics before the initiation of treatment.

Discussion

The advent of targeted therapy offers the potential of new paradigms in the treatment of human cancer. In spite of

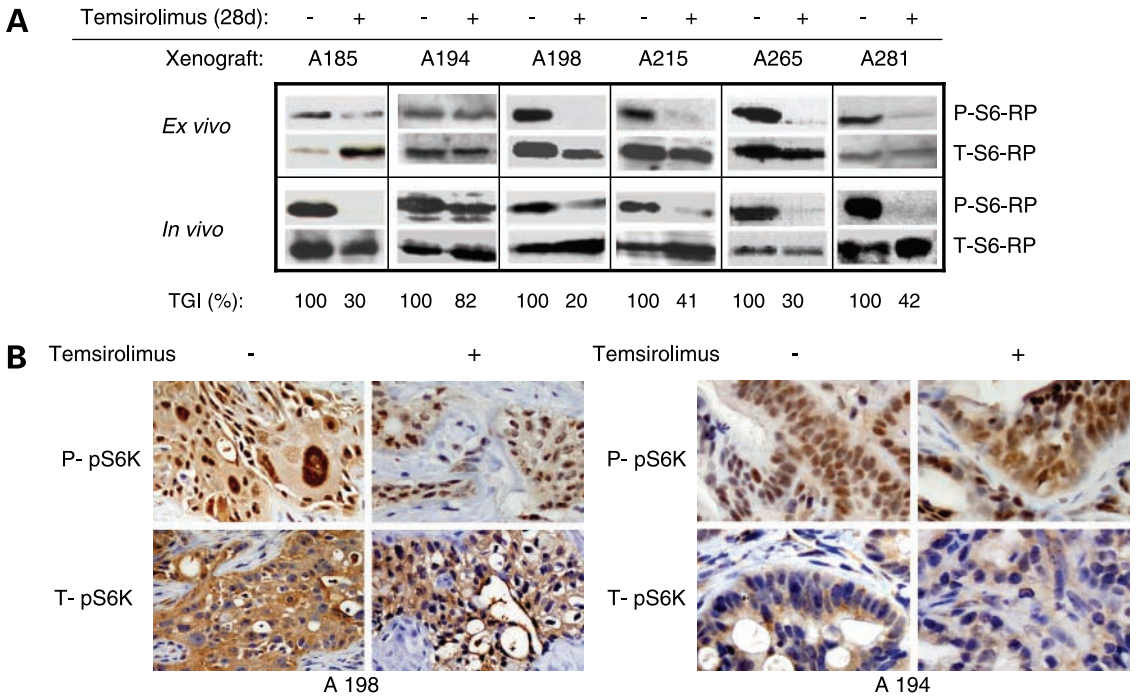

Figure 3. **A,** *ex vivo* (*top*) and *in vivo* (*bottom*) studies with temsirolimus in six different xenograft mice bearing primary human pancreatic carcinoma tumors. *Ex vivo* studies were done with tumor cells collected by FNAB before initiation of systemic treatment (day 0). For the *in vivo* assessment analysis, tumor FNAB samples were collected from the same animals before (untreated, day 0) and 28 d after treatment with temsirolimus. Cell lysates were prepared from AD/DQ – stained FNAB slides to determine protein phosphorylation (P-S6-RP) and total expression (T-S6-RP) levels of S6-RP protein on Western blot analysis. Results were correlated with tumor growth inhibition (*TGI*). **B,** representative immunohistochemical staining of phosphorylated p70S6k (*P-pS6K*) and total p70S6K (*T-pS6K*) in two representative tumors (A198 and A194) treated with vehicle or temsirolimus.

encouraging early results in select settings, contemporary experience has begun to illuminate the relatively substantial challenges in the way of realizing the full promise of this new field. To the extent that we accept the relatively straightforward concept that a given agent will be most effective against those tumors where its target is biologically critical, the obvious next step is developing clinically useful means of recognizing that scenario. The identification and validation of clinically relevant biomarkers of treatment efficacy could provide tools applicable to the enrichment of clinical trials and perhaps, ultimately, individualized tailoring of therapy. Nevertheless, at present, we are limited by the dearth of reliable tools to rationalize treatment selection and monitor efficacy of a given regimen. In this context, we sought to evaluate a novel prospective pharmacodynamic assay in xenograft mouse models of human pancreatic cancer, with source tissue obtained from primary clinical material. Our prior studies showed that these xenograft tumors maintain the features of the index tumor and are representative of the genetic heterogeneity of pancreatic cancer (18).

The results of this study show that relatively small samples of tumor cells obtained by a well-established, minimally invasive diagnostic technique (FNAB) can be used for reproducible assays to predict how tumors will respond to targeted anticancer agents before initiation of therapy. In our panel of xenografts, we found a strong correlation between the pharmacodynamic effects of the drugs on activation of downstream targets in *ex vivo* conditions and tumor response to therapy with temsirolimus and erlotinib *in vivo*. These findings build on our earlier work showing the potential utility of pharmacodynamic markers of drug sensitivity (17) with the added value of predicting response before systemic treatment. An additional strength of our study lies in the fact that the source material for the xenograft models was obtained from primary clinical specimens. We believe that such models add value to translational research in oncology, to the extent that they more closely recapitulate the biology of clinical cancers, without the potential confounding selection pressures inherent to the development of cell lines.

We found no meaningful correlation between tumor responsiveness to erlotinib and EGFR expression level (data not shown). Previously published sequencing of the *EGFR* gene in this panel of tumor xenografts revealed no mutations in the *EGFR* gene (18). The resistance to erlotinib observed in the majority of our xenograft panel may be due, at least in part, to the high prevalence of activating mutations of K-*ras*, in pancreatic cancer (22), also seen in this panel of tumor xenografts (18). In fact, studies in lung cancer have shown associations of K-*ras* mutation with resistance to EGFR-targeted interventions (9, 23). Remarkably, however, primary human pancreatic adenocarcinomas evaluated in this study were highly sensitive to temsirolimus, supporting the importance of mammalian target of rapamycin signaling to proliferation in pancreatic cancer (24). These findings illustrate the importance of validating candidate target markers, a prerequisite for pharmacodynamic-driven drug development.

The impediments to further development in this area may be organized under several broad themes. These relate to the selection and validation of end points or criteria of drug efficacy, the development of assays to evaluate those criteria, and tissue collection and sampling. Prospective determination of antibiotic sensitivity and resistance has been the standard of care in infectious diseases for many years. In contrast, due in part to the lack of reproducible predictive assays, treatment protocols for cancer patients have been empirical and driven by the taxonomy of tumor

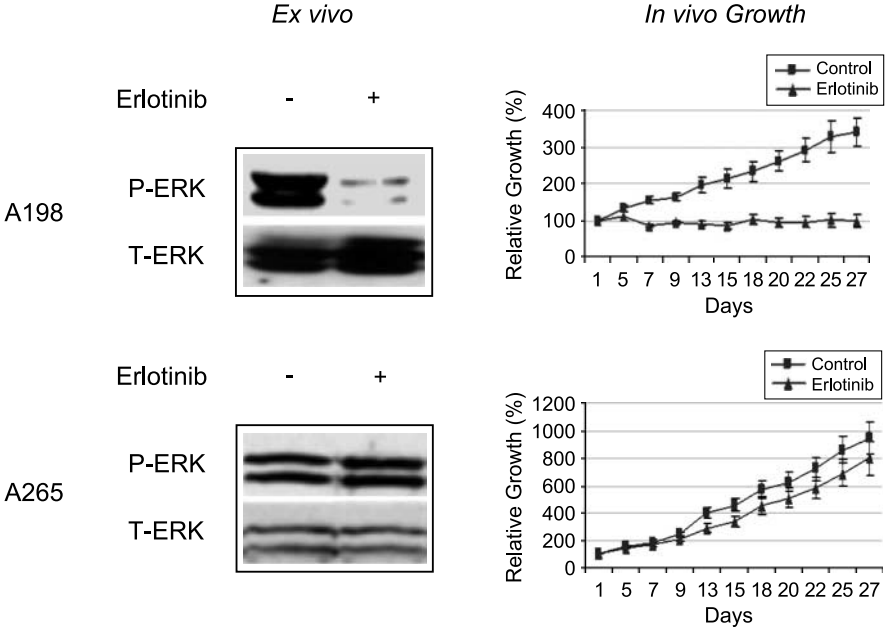

Figure 4. Representative tumor growth (*right*) and *ex vivo* assay data (*left*) from an erlotinib-susceptible (A198) and an erlotinib-resistant (A265) pancreatic cancer xenograft. Tumor cells were obtained by FNAB before systemic treatment. Treatment with 5 μmol/L erlotinib *ex vivo* inhibited phosphorylated (*P-ERK*) ERK1/2 in the tumor cells obtained by FNAB from the susceptible tumor (A198), but not from the resistant tumor (A265). No changes were observed in total (*T-ERK*) levels of ERK1/2 in cells treated *ex vivo*.

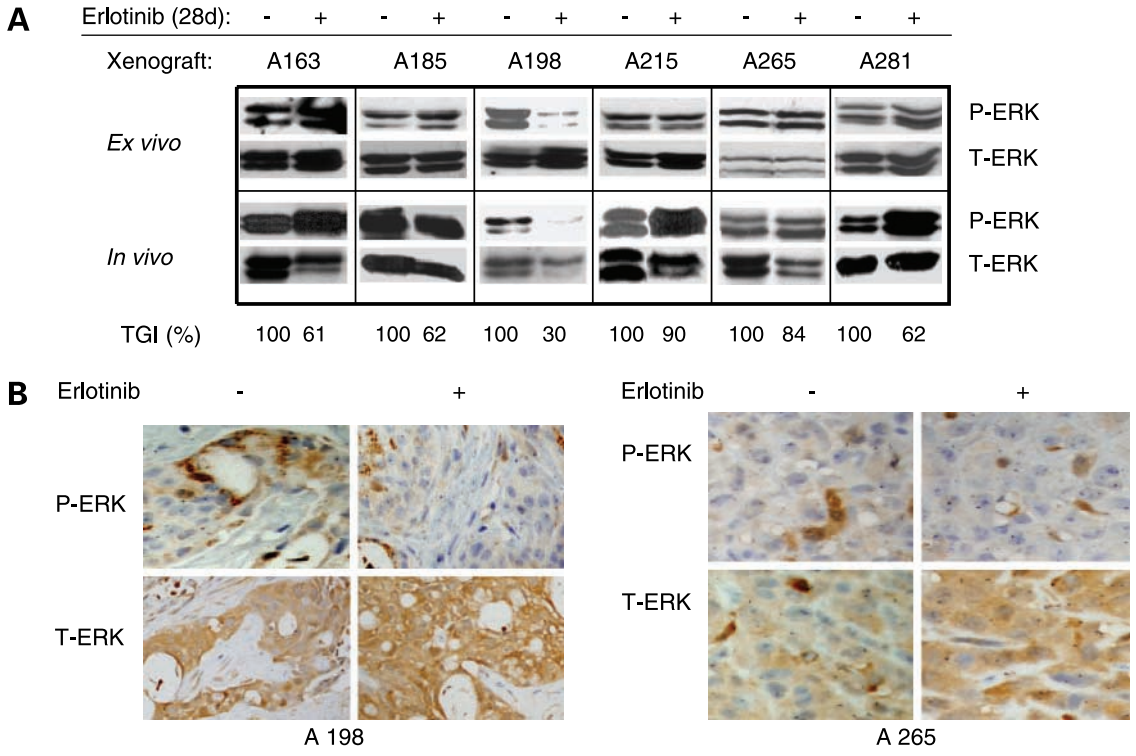

Figure 5. **A,** *ex vivo* (*top*) and *in vivo* (*bottom*) studies with erlotinib in six different xenograft models of human pancreatic cancer. Tumor cells were collected by FNAB before initiation of *in vivo* treatment and were exposed to erlotinib *ex vivo*. For the assessment of drug efficacy *in vivo*, tumor FNAB samples were collected from the same animals before (untreated, day 0) and after treatment (28 d) with erlotinib. Cell lysates were prepared from AD/DQ–stained smear slides, and phosphorylation and total expression levels of ERK1/2 proteins were analyzed on Western blot. Results were correlated with tumor growth inhibition. **B,** representative immunohistochemical staining of phosphorylated and total ERK1/2 in two representative tumors (A198 and A265) treated with vehicle or erlotinib.

histology rather than a tumor's sensitivity to a given chemotherapeutic agent. Growth inhibition or cell death has been used in previous iterations of assays of sensitivity to conventional chemotherapeutic agents (25–31). However, due to poor tumor growth under assay conditions, labor-intensive and time-consuming methods, and the use of uncertain criteria for defining ''sensitivity'' or ''resistance,'' these assays have not gained wide clinical acceptance. In this context, we present a strategy for rational sensitivity testing predicated upon pharmacodynamic markers specific to the molecular pathways targeted by individual agents.

The majority of available studies attempting to correlate candidate biomarkers and response to targeted agents have been retrospective in nature and focused on static measurements of drug target expression and molecular evidence of dysregulation or activation in tissues (6–12). There are several limitations with this approach. First, the detection of target protein expression in archived pretreatment samples may be inadequate to predict the activity of drugs because the anticancer effect of a given agent may, in actuality, depend upon alterations in signaling both upstream and downstream of the target protein (9, 12). This biological complexity provides a point of departure to begin to understand the range of responses to targeted therapies among individual patients with apparently identical target protein expression levels (32, 33). Furthermore, the conventional approach does not account for potential changes in the biological status of targets over the natural history of an individual case. This is underscored by recent demonstrations of spatial and temporal variation in EGFR expression following chemotherapy as well as in paired primary and metastatic colorectal cancers (34, 35).

We propose that the pharmacodynamic ability of a drug to inhibit the target pathway is more important as a predictor of efficacy than the expression or activation of the target per se. Taking this view, the challenge lies in identifying and characterizing the features of signaling nodes corresponding to biologically important pathway effectors. A distinct advantage of targeted agents is the potential to develop assays specific to the molecular actions of the drug. For this purpose, we used S6-RP and ERK1/2 phosphorylation as two well-validated and frequently used pharmacodynamic markers of mammalian target of rapamycin and EGFR pathway blockade, respectively (36, 37). Because the positive predictive value of pharmacodynamic assays of target inhibition may be tempered by cross-talking pathways downstream of the marker of interest and by factors such as tumor vasculature, metabolism, and

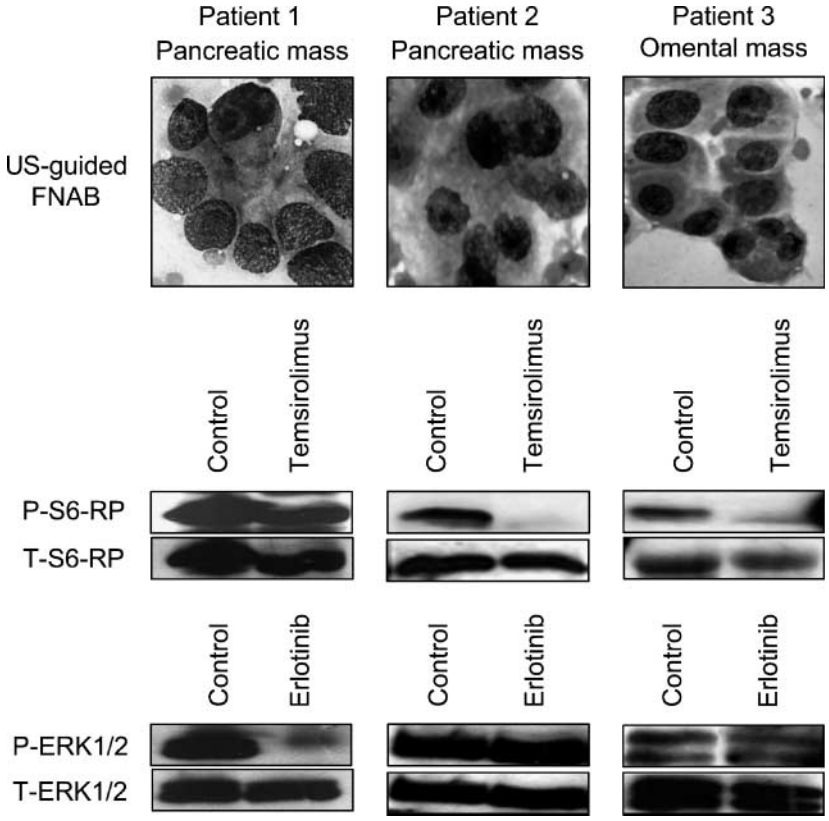

Figure 6. Tumor FNAB samples were collected from three pancreatic cancer patients, and cancer cells were treated *ex vivo* with vehicle (control), temsirolimus, or erlotinib for 6 h. Whole-cell extracts were prepared, and total expression and phosphorylation levels of ERK1/2 or S6-RP were analyzed on Western blot.

drug distribution to the tumor tissue, we believe that the potential utility of assays such as those described herein may be greatest as a tool with high negative predictive value.

The development of assays to predict tumor response to treatment is also hindered in the past by problems of tissue acquisition. Previously explored chemosensitivity assays required relatively large tumor specimens (i.e., surgical biopsies) that necessitated general anesthesia for safe and reliable acquisition (38). FNAB is a minimally invasive, established diagnostic procedure that allows acquisition of enriched tumor cell populations to perform analytic molecular tests (39–45). Our results show that cytologically confirmed tumor FNAB samples can yield viable tumor cells and sufficient protein quantities to analyze the efficacy of targeted drugs before (*ex vivo*) and during (*in vivo*) systemic treatment. Given its safety, minimal morbidity, and relative technical ease, FNAB is also suitable for serial sampling over the course of treatment to monitor therapy effect *in vivo*.

The performance of the FNAB studies seems quite feasible in xenograft tumors that, at the size sampled here, contain viable tumor cells with minimal necrotic contamination. An obvious question is whether similar materials can be obtained from patients' tumors. To address this concern, we evaluated the feasibility of *ex vivo* assays in FNAB materials from diagnostic biopsy materials. The results presented herein suggest that similar results as seen in the animal studies can be obtained from standard clinical materials. Future studies will determine the degree to which the results of such assays correspond to clinically observed treatment effects in humans.

In summary, in a novel *in vivo* model system for drug development and biomarker discovery in pancreatic cancer, FNAB-guided *ex vivo* drug assays seem to be a promising candidate tool to aid in the clinical development of targeted agents. Implementation of approaches such as those outlined herein in clinical studies may result in improved patient selection to maximize potential benefit while sparing patients unlikely to respond to a given agent. Furthermore, this approach theoretically provides a platform for the incorporation of multiple dynamic molecular analytic methods as well as the evaluation of more than one agent simultaneously. In the immediate term, this approach may offer a means of enriching clinical trials to better identify effective candidate regimens for patients with given tumor types. Ultimately, if validated in clinical trials, tools such as these may afford a means of tailoring the most efficient therapeutic regimen for individual patients.

References

1. Arteaga CL, Baselga J. Tyrosine kinase inhibitors: why does the current process of clinical development not apply to them? Cancer Cell 2004;5:525–31.

2. Baselga J, Arribas J. Treating cancer's kinase 'addiction'. Nat Med 2004;10:786–7.

3. Sawyers C. Targeted cancer therapy. Nature 2004;432:294–7.

4. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med 2002;346:645 – 52.

5. O'Brien SG, Guilhot F, Larson RA, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 2003;348:994 – 1004.

6. Bell DW, Lynch TJ, Haserlat SM, et al. Epidermal growth factor receptor mutations and gene amplification in non-small-cell lung cancer: molecular analysis of the IDEAL/INTACT gefitinib trials. J Clin Oncol 2005;23:8081 – 92.

7. Hirsch FR, Varella-Garcia M, McCoy J, et al. Increased epidermal growth factor receptor gene copy number detected by fluorescence *in situ* hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a Southwest Oncology Group Study. J Clin Oncol 2005;23:6838 – 45.

8. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643 – 55.

9. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005;2:e17.

10. Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012 – 4.

11. Ogino S, Meyerhardt JA, Cantor M, et al. Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. Clin Cancer Res 2005;11:6650 – 6.

12. Haas-Kogan DA, Prados MD, Tihan T, et al. Epidermal growth factor receptor, protein kinase B/Akt, and glioma response to erlotinib. J Natl Cancer Inst 2005;97:880 – 7.

13. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003;21:4342 – 9.

14. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304: 1497 – 500.

15. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129 – 39.

16. Twombley R. Identity crisis: finding, defining and integrating biomarkers still a challenge. J Natl Cancer Inst 2006;98:11 – 2.

17. Hidalgo M, Amador ML, Jimeno A, et al. Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration. Mol Cancer Ther 2006;5:1895 – 903.

18. Rubio-Viqueira B, Jimeno A, Cusatis G, et al. An *in vivo* platform for translational drug development in pancreatic cancer. Clin Cancer Res 2006;12:4652 – 61.

19. Jimeno A, Rubio-Viqueira B, Amador ML, et al. Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. Cancer Res 2005;65:3003 – 10.

20. deGraffenried LA, Friedrichs WE, Russell DH, et al. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. Clin Cancer Res 2004;10:8059 – 67.

21. Grunwald V, DeGraffenried L, Russel D, Friedrichs WE, Ray RB, Hidalgo M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. Cancer Res 2002;62:6141 – 5.

22. Adjei AA, Hidalgo M. Intracellular signal transduction pathway proteins as targets for cancer therapy. J Clin Oncol 2005;23:5386 – 403.

23. Magne N, Fischel JL, Dubreuil A, et al. Influence of epidermal growth factor receptor (EGFR), p53 and intrinsic MAP kinase pathway status of tumour cells on the antiproliferative effect of ZD1839 (''Iressa''). Br J Cancer 2002;86:1518 – 23.

24. Agbunag C, Bar-Sagi D. Oncogenic K-*ras* drives cell cycle progression and phenotypic conversion of primary pancreatic duct epithelial cells. Cancer Res 2004;64:5659 – 63.

25. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science 1977;197:461 – 3.

26. Gerhardt RT, Perras JP, Sevin BU, et al. Characterization of *in vitro* chemosensitivity of perioperative human ovarian malignancies by adenosine triphosphate chemosensitivity assay. Am J Obstet Gynecol 1991;165:245 – 55.

27. Kern DH, Weisenthal LM. Highly specific prediction of antineoplastic drug resistance with an *in vitro* assay using suprapharmacologic drug exposures. J Natl Cancer Inst 1990;82:582 – 8.

28. Meitner PA. The fluorescent cytoprint assay: a new approach to *in vitro* chemosensitivity testing. Oncology (Huntingt) 1991;5:75 – 81; discussion 81 – 72, 85, 88.

29. Andreotti PE, Cree IA, Kurbacher CM, et al. Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. Cancer Res 1995;55:5276 – 82.

30. Hirano Y, Ushiyama T, Suzuki K, Fujita K. Clinical application of an *in vitro* chemosensitivity test, the Histoculture Drug Response Assay, to urological cancers: wide distribution of inhibition rates in bladder cancer and renal cell cancer. Urol Res 1999;27:483 – 8.

31. Sharma S, Neale MH, Di Nicolantonio F, et al. Outcome of ATP-based tumor chemosensitivity assay directed chemotherapy in heavily pre-treated recurrent ovarian carcinoma. BMC Cancer 2003;3:19.

32. Campiglio M, Locatelli A, Olgiati C, et al. Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. J Cell Physiol 2004;198:259 – 68.

33. Bishop PC, Myers T, Robey R, et al. Differential sensitivity of cancer cells to inhibitors of the epidermal growth factor receptor family. Oncogene 2002;21:119 – 27.

34. Scartozzi M, Bearzi I, Berardi R, Mandolosi A, Fabris G, Cascinu S. Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: implications for treatment with EGFR-targeted monoclonal antibodies. J Clin Oncol 2004;22:4772 – 8.

35. De Pas T, Pelosi G, de Braud F, et al. Modulation of epidermal growth factor receptor (EGFR) status by chemotherapy in patients with locally advanced non small cell is rare. J Clin Oncol 2004;22: 4966 – 70.

36. Peralba JM, DeGraffenried L, Friedrichs W, et al. Pharmacodynamic evaluation of CCI-779, an inhibitor of mTOR, in cancer patients. Clin Cancer Res 2003;9:2887 – 92.

37. Baselga J, Albanell J, Ruiz A, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. J Clin Oncol 2005;23:5323 – 33.

38. Schrag D, Garewal HS, Burstein HJ, Samson DJ, Von Hoff DD, Somerfield MR. American Society of Clinical Oncology Technology Assessment: chemotherapy sensitivity and resistance assays. J Clin Oncol 2004;22:3631 – 8.

39. Pelosi G, Bresaola E, Rodella S, et al. Expression of proliferating cell nuclear antigen, Ki-67 antigen, estrogen receptor protein, and tumor suppressor p53 gene in cytologic samples of breast cancer: an immunochemical study with clinical, pathobiological, and histologic correlations. Diagn Cytopathol 1994;11:131 – 40.

40. Makris A, Allred DC, Powles TJ, et al. Cytological evaluation of biological prognostic markers from primary breast carcinomas. Breast Cancer Res Treat 1997;44:65 – 74.

41. Rao JY, Apple SK, Hemstreet GP, Jin Y, Nieberg RK. Single cell multiple biomarker analysis in archival breast fine-needle aspiration specimens: quantitative fluorescence image analysis of DNA content, p53, and G-actin as breast cancer biomarkers. Cancer Epidemiol Biomarkers Rev 1998;7:1027 – 33.

42. Nizzoli R, Bozzetti C, Naldi N, et al. Comparison of the results of immunocytochemical assays for biologic variables on preoperative fine-needle aspirates and on surgical specimens of primary breast carcinomas. Cancer 2000;90:61 – 6.

43. Assersohn L, Gangi L, Zhao Y, et al. The feasibility of using fine needle aspiration from primary breast cancers for cDNA microarray analyses. Clin Cancer Res 2002;8:794 – 801.

44. Kuner R, Pollow K, Lehnert A, et al. [Needle biopsy vs. conventional surgical biopsy: biochemical analysis of various prognostic factors]. Zentralbl Gynakol 2000;122:160 – 4.

45. Pusztai L, Ayers M, Stec J, et al. Gene expression profiles obtained from fine-needle aspirations of breast cancer reliably identify routine prognostic markers and reveal large-scale molecular differences between estrogen-negative and estrogen-positive tumors. Clin Cancer Res 2003;9: 2406 – 15.