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A PHASE II AND PHARMACODYNAMIC STUDY OF GEFITINIB IN PATIENTS WITH REFRACTORY OR RECURRENT EPITHELIAL OVARIAN CANCER

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Abstract

Purpose—The primary objective of this study was to evaluate the biochemical effects of gefitinib on its target signal transduction pathways in patients with relapsed epithelial ovarian cancer (EOC). The secondary aims included assessing clinical activity and toxicity, and determining the association between biochemical and clinical outcomes.

Methods—Twenty-four heavily pretreated EOC patients with good end organ function and performance status, and measurable disease were treated with gefitinib 500mg daily. Prospectively planned core tumor needle biopsies were obtained before treatment and after four weeks. Protein expression of total and phosphorylated (p) EGFR, AKT, and ERK was quantified in microdissected tumor cells using tissue lysate array proteomics.

Results—All tumor samples had detectable levels of EGFR and pEGFR. A decrease in the quantity of both EGFR and pEGFR was seen with gefitinib therapy in over half of patients. This was not associated with clinical benefit nor were responses observed. However, trends for increased gastrointestinal and skin toxicity were seen with greater phosphorylation or quantity of EGFR, ERK, and AKT in tumor samples ($p \leq 0.05$). Gefitinib had limited clinical activity as monotherapy, in spite of documented target inhibition.

Conclusion—We have demonstrated gefitinib inhibits phosphorylation of EGFR in EOC tumor cells, providing proof of target in a clinical setting. Combinatorial therapy with molecular therapeutics against complementary targets may prove successful.

Keywords

ovarian cancer; EGFR; gefitinib; proteomics; protein array

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INTRODUCTION

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancy among American women[1]. Debulking surgery followed by adjuvant platinum and paclitaxel chemotherapy is highly effective in remitting disease[2]. However, most women diagnosed with advanced stage disease eventually recur. A variety of cytotoxic agents are used with response rates in the range of 10-30%. In all, approximately 70% of all women diagnosed with EOC will die of disease. Therefore, there is a critical need for the development and assessment of new agents.

One potential target for anti-cancer therapy is the epidermal growth factor receptor (EGFR). EGFR, a regulator of cell growth and differentiation in normal tissues, plays a role in promoting cell proliferation, apoptosis inhibition, and angiogenesis in malignancy[3-5]. EGFR is overexpressed in 35-70% of epithelial ovarian cancers depending on the assessment technique used[6, 7]. Increased EGFR signaling has been shown to be associated with the development of an invasive phenotype in ovarian cancer cell lines[8, 9] and is detected more often in metastases than primary tumor samples[10]. The relationship between EGFR overexpression and clinical prognosis is less clear, with some reports suggesting a prognostic importance [5, 7], and others refuting that association[11]. EGFR homodimerizes or heterodimerizes with other members of the erbB family upon ligand binding[12, 13]. This leads to activation of the intrinsic tyrosine kinase domain, auto- and trans-phosphorylation, and initiation of downstream signaling cascades[5, 14].

EGFR is a promising target for molecular therapeutics because of its well-studied pro-survival role. Both monoclonal antibodies and small molecule tyrosine kinase inhibitors of EGFR have tested in the clinic[4, 15, 16]. Gefitinib (Iressa®) is a small molecule inhibitor of the tyrosine kinase of EGFR. It attaches to the ATP-binding domain of the receptor, preventing phosphorylation and activation of both EGFR and important downstream signaling molecules[17]. Gefitinib inhibits EGF-stimulated growth of ovarian cancer cells in vitro; this effect is cytostatic, with an increase in apoptosis seen at higher doses and when used in combination with conventional chemotherapy producing supra-additive growth arrest[18, 19]. Gefitinib has also been tested in xenograft models of ovarian cancer, resulting in inhibitory effects on cell growth and increased survival of mice in the treatment groups[16, 20].

Phase I trials of gefitinib in solid tumors including epithelial ovarian cancer demonstrated tolerable toxicity and promising clinical results[15, 21-24]. We hypothesized that gefitinib would inhibit activation of EGFR in ovarian cancer patients, effect downstream signal transduction events, and lead to regression or stabilization of disease. The primary objective of this study was to evaluate the biochemical effects of gefitinib on signal transduction pathways in tumor. The secondary aims included assessing the clinical activity and toxicity of gefitinib in epithelial ovarian cancer and determining the association between biochemical and clinical outcomes.

PATIENTS AND METHODS

Eligibility

This study was approved by the Institutional Review Board of the National Cancer Institute (Bethesda, MD). Written informed consent was obtained before enrollment. Eligible patients had histopathologically confirmed EOC. All patients had progressing disease and were at least four weeks from most recent therapeutic intervention. Other criteria included an ECOG performance status of 0-2, and leukocyte count $\geq 3000/mm^3$, platelets $\geq 100{,}000/mm^3$, serum creatinine ≤ 1.5 mg/dl, transaminases (ALT, AST) $\leq 2.5 \times$ the upper limit of normal, and bilirubin ≤ 1.5 mg/dl, measurable disease by CT, and a sentinel lesion amenable to percutaneous biopsy. Toxicity from prior therapies must have recovered to grade 1 or better. Patients with

evidence of CNS involvement, history of myocardial infarction or angina within the previous six months, history of another invasive malignancy within five years, ongoing or active infection, previous treatment with any EGFR inhibitor, and concurrent treatment with alternative or complementary medications were excluded from study.

Treatment Plan

Patients received oral gefitinib 500mg daily on 28 day cycles until progressive disease, unacceptable toxicity or withdrawal. They were seen every 4 weeks for history, physical and pelvic exams, and laboratory tests, including CA125. Response was assessed every eight weeks by imaging and scored according to RECIST criteria[25]. All patients were required to keep a diary to document compliance and adverse events. Toxicity was assessed using the NCI Common Toxicity Criteria (CTC) v2.0. Symptomatic management was provided to patients with gastrointestinal and dermatologic toxicities. Grade 3 or hematologic grade 4 toxicities required treatment interruption until resolution to grade 1. Patients were not eligible to resume therapy if the time to resolution was greater than two weeks, or four weeks if evidence of clinical benefit. Patients were dose reduced to 250mg daily in the setting of grade 3 or 4 toxicity or recurrent grade 2 dermatologic toxicity. Further dose reductions were not allowed.

The study prospectively planned collection of percutaneous 18g core needle tumor biopsies in order to evaluate target modulation. Biopsies were obtained under imaging guidance prior to initiation and after 4 weeks of gefitinib treatment and cryopreserved immediately in OCT compound (Sakura Finetek; Torrance, CA). Biopsy sections 6 μm were cut for pathology review and laser capture microdissection (LCM; Molecular Devices, Sunnyvale, CA). Samples with predominant necrosis or lymphocytic infiltration were not assayed. Tumor and stromal cells were collected using LCM as reported[26, 27] and analyzed independently.

Tissue lysate array (TLA) preparation and analysis

Captured cells were lysed and the proteins extracted as previously described[28] in a 1:1 preparation of Tris-glycine-SDS sample buffer:Tissue Protein Extraction Reagent (Pierce; Rockford, IL) plus 2.5% ß-mercaptoethanol for 30 mins at 75°C. An estimated 30,000 cells were obtained yielding 30 μl of lysate to print 30 replicate arrays. EGF-treated A431 cell lysate (BD Biosciences; San Jose, CA) was used as a positive control. Lysates were loaded into 384 well plates in a five point 1:1 dilution curve and printed in triplicate onto nitrocellulose-coated glass slides (Schleicher and Schuell Bioscience; Keene, NH) using a GMS 417 pin and ring arrayer (Affymetrix; Santa Clara, CA)[29] and stored dessicated at −20°C. Arrays were incubated with Reblot antibody stripping solution (Chemicon; Temecula, CA), rinsed in PBS and blocked in I-Block (Applied Biosystems; Foster City, CA). Each slide was probed with primary antibody (Supplemental Table) using an automated slide stainer (Dako; Carpinteria, CA) and detected with the Dako Catalyzed Signal Amplification system [30]. Primary antibody was omitted on one slide to serve as the nonspecific background control. One slide from each set was stained with Sypro ruby stain to quantitate total protein load (Molecular Probes; Eugene, OR) and visualized on a FluorChem imaging system (Alpha Innotech; San Leandro, CA). Protein intensity was quantified from stained arrays using ImageQuant v5.2 (Molecular Dynamics; Sunnyvale, CA). Expression signals were normalized to total protein content then standardized to a control lysate of A431 cells printed onto each slide.

Statistical Considerations

The primary endpoint was defined prospectively as the modulation of EGFR, AKT and ERK, after one month of gefitinib therapy. Secondary endpoints included clinical response and toxicity and the association of clinical and biochemical effects. A minimum of 15 paired tumor biopsies was estimated to be required to address the primary endpoint. This number would be adequate to detect a difference of one standard deviation of change with 80% power, assuming

a one-tailed t-test would be used at the 0.008 significance level based upon an assumption of 6 paired comparisons to be undertaken, in order to allow at least implicitly for a Bonferroni correction accounting for multiple comparisons.

The data themselves were standardized values:

$$\text{Normalized Intensity Value (NIV)} = \frac{\text{Intensity} - \text{Background Value}}{\text{Total Protein}}$$

Triplicate values were averaged to yield the single NIV value for a given TLA parameter at a given time (pre- or post- treatment) for either tumor or stroma. The relative difference used for analysis after determining that the actual difference was more dependent on pre-treatment than was the relative difference, and hence less suitable as an endpoint. It was defined as: (post treatment value − pre treatment value)/pretreatment value. A two-tailed Wilcoxon signed rank test was used to test whether the relative changes between post- and pre-treatment differ from zero. An exact Jonckheere-Terpstra trend test was used to test for an association between grade of GI toxicity or worst toxicity and each TLA parameter. Parameters in patients with or without diarrhea or skin toxicity were compared using an exact Wilcoxon rank sum test. In view of the large number of parameters ultimately evaluated in this exploratory study, although no formal correction for multiple comparisons was performed, a p-value <0.005 was considered necessary in order to interpret a result as being statistically significant, while $0.005 < p < 0.05$ would suggest a trend.

RESULTS

Patients

Twenty-four patients with recurrent EOC were enrolled between Nov. 2002 and Jan. 2005 in order to yield fifteen cases with matched biopsies. Patient demographics are summarized in Table 1. No patient had a kinase-activating EGFR mutation (data not shown).

Tumor response

Sixteen patients completed at least two cycles of therapy and thus were evaluable for clinical assessment. There were no complete or partial responses. Median time on treatment was 2 months (range 3d to 5 months). Nine patients (37%) had stable disease for longer than two months. CA125 values were consistent with radiographic changes with 15 patients having a steady rise during treatment, 8 had decreases at some point during treatment, of whom four were on study for 4+ months. One patient presented with a solid cervical node mass that became soft and fluctuant 3 months into therapy. CT showed development of cystic regions in the mass, histologically necrotic tumor tissue with few viable tumor cells in the floor of the cavity. She had progressive disease after 4 months of therapy despite this promising beginning.

Safety and Toxicity

Adverse events observed in this trial were generally mild. The most common adverse events attributed to gefitinib treatment are summarized in Table 2. Diarrhea was managed successfully with loperamide therapy in most patients. The acneiform rash was seen in 42% of patients and improved with topical clindamycin treatment. Five patients had asymptomatic elevations in hepatic transaminases and/or alkaline phosphatase. Grade 3 and 4 toxicities related to gefitinib administration were rare. Grade 4 hyponatremia was reported in a patient who had multiple baseline electrolyte abnormalities resulting from short bowel syndrome and underlying adrenal insufficiency; she experienced drug-related grade 3 liver enzyme elevations. A patient receiving enoxaparin for a synthetic aortic graft developed a grade 4 hemorrhage due to splenic

rupture. Its attribution to gefitinib treatment is unclear. Thirty percent of patients required dose modification to 250mg per day; one patient was reduced during cycle 1, three patients in cycle 2 and three patients at or after cycle 3. Seven patients required short treatment interruptions for recovery of diarrhea or fatigue (median 3.5, 1-10 days). Both patients with grade 4 toxicity had treatment discontinued.

Signal profiling

Pre-and post-treatment tumor specimens were obtained in 23 and 18 patients, respectively; sites are listed in Table 3. No second biopsy was obtained if there was no solid tumor in the first biopsy or if treatment was discontinued early. No complications occurred during sample acquisition. Several biopsies were considered unsuitable for microdissection due to the absence of tumor, excessive lymphocyte infiltration, or overwhelming necrosis. Matched data on protein expression in stroma are available for a limited number of patients because many biopsies had minimal dissectable stroma. Fifteen paired tumor biopsies were used for proteomic evaluation.

Demonstration of target modulation—Target quantity and phosphorylation could be measured in varied numbers of cases (Table 4). Figure 1 shows changes in the primary parameters of total and phosphorylated EGFR (pY-1148), AKT, and ERK with one month of gefitinib therapy. The ratio of NIV values are presented for expression of the protein in tumor obtained during gefitinib therapy as a function of baseline values. Reduction in total and p-EGFR was seen in several patients. However, no absolute or relative differences were considered suggestive of a significant change.

Clinical association with signaling events—Extent of adverse events observed was associated with changes in signaling parameters in several instances. Table 4 shows sample statistics for signaling parameter and clinical toxicity by grade and type of toxicity for those parameters for which there was at least a trend toward an association between relative change in the parameter and the type of toxicity noted. Increasing EGFR, AKT, p-ERK and p-EGFR moieties in tumor on treatment demonstrated at least a trend toward being associated with increasing overall toxicity ($p \leq 0.05$), gastrointestinal toxicity ($p < 0.05$), and skin toxicity ($p = 0.03$). No pretreatment biochemical parameters were predictive of toxicity. Several strong trends were observed in this study and need to be confirmed with a larger cohort due to the small number of subjects studied.

DISCUSSION

Demonstration of biochemical proof of action is important in the continued development of small molecule signal transduction inhibitors such as gefitinib. Newer semi-quantitative and quantitative techniques are being developed to facilitate this important endpoint[31]. This study shows that EGFR is present in epithelial ovarian cancers, and is activated in most patients despite lack of an activating mutation. We demonstrate gefitinib inhibits the phosphorylation of EGFR in patient ovarian cancer cells, providing proof of target in a clinical setting. Gefitinib has been shown to inhibit phosphorylation and activation of EGFR with corresponding inhibition of downstream signaling cascades and a decrease in cell proliferation and tumor cell growth in laboratory and animal models[16]. Prior preclinical and clinical studies have shown decreased EGFR and diminished activation in unproven surrogates[19, 32]. This is one of the first studies to provide tumor-specific evidence of tyrosine kinase inhibition by gefitinib in a clinical setting. We further demonstrate that inhibition of phosphorylated EGFR results in inhibition of downstream signaling molecules known to transmit the EGFR growth response. A decrease in phosphorylation of both AKT and ERK was observed with gefitinib therapy. These results in ovarian cancer are similar to recent data in breast cancer showing a down

regulation of pEGFR with gefitinib therapy, as measured by immunohistochemistry staining of sequential tumor biopsies[33]. Less downstream inhibition was observed in the breast cancer cohort with a decrease in pERK but not pAKT in tumor cells. A 500 mg dose of gefitinib daily is a biochemically effective dose in the tumor cells of ovarian cancer patients.

Gefitinib has limited activity as monotherapy in this population of heavily pretreated patients with recurrent or refractory ovarian cancer, despite some target inhibition in 82% of patients. No patient attained stabilization of disease of at least 6 months, the clinical target parameter. The gefitinib phase II trial for ovarian cancer patients coordinated by the Gynecologic Oncology Group (GOG 170C) had a primary endpoint of progression-free survival[34] and patients were limited to fewer prior treatment regimens. Similar to our trial, their median number of cycles received was two. Four patients achieved stable disease for longer than six months and one partial response was observed in a patient with an EGFR mutation. Activating mutations of EGFR have been shown to predict response to gefitinib in some patients. The majority of patients with NSCLC who respond to gefitinib, 77% in recently compiled data from multiple trials, have mutations of EGFR in or near the ATP-binding region of the kinase domain; these mutations are rarely seen in NSCLC patients who do not respond[35-38]. The somewhat more promising results in the GOG trial may be due to patient differences. Our more heavily pretreated patient population is a group known to have limited response to new agents[39].

The adverse events observed in this trial were generally mild and comparable to those observed in other phase II trials of gefitinib[33, 40-42]; diarrhea and rash are common adverse events of gefitinib. Greater quantity or phosphorylation of EGFR or its downstream signaling partners was associated with increased overall, gastrointestinal, and skin toxicities in our study. These toxicities and their associations imply effective levels of EGFR inhibition in the skin and GI tract. The lack of clinical activity of single agent gefitinib, despite some target effects in tumor tissue, indicates that the presence and extent of these toxicities cannot be used as surrogates of intratumoral effects.

The question presented by this trial is the lack of association between the biochemical and clinical effects of gefitinib. It appears that blocking the activity of the EGFR kinase is not sufficient to produce a clinical effect in ovarian cancer. There are three possible explanations: 1) the target is not important in the tumor biology; 2) the target is important but its modulation is not sufficient; 3) the target is important and sufficiently inhibited, but this effect can be overcome by paracrine or parallel signaling to the same downstream targets. The second explanation implies a possible incomplete inhibition of EGFR phosphorylation. The necessary threshold for EGFR inhibition and the specific key phosphorylation sites are unknown. Although a greater than 50% decrease is seen in the level of pEGFR expression, detectable levels of pEGFR are still present in all tumor biopsies and the proportion of EGFR activation is unchanged by treatment. This may be adequate to stimulate tumor growth and proliferation. There are other pro-malignant pathways that promote tumor development and survival in molecularly heterogeneous cancers such as ovarian cancer[43]. These pro-growth molecular signals may compensate for or overcome inhibition of the EGFR pathway. That EGFR phosphorylation has a limited clinical effect but biochemical activity provides additional rationale for the exploration of gefitinib in combination with signal transduction inhibitors that effect downstream targets. Inhibition of one pathway at multiple levels or several pathways in parallel may have an additive or even supra-additive effect.

This study also demonstrates that tissue lysate arrays may be a sensitive technique for detecting protein expression and activation in ovarian cancer. They provide a method for quantifying protein expression, something that cannot easily be achieved with immunohistochemistry[28, 29]. Tissue lysate arrays also allow the simultaneous study of a large number of protein

endpoints. As a pilot study, our proteomic evaluation was relatively limited; potentially up to 30 different proteins could be probed in the analysis of one small tumor biopsy. Although the use of tissue lysate arrays in the clinical setting is still exploratory, this study has demonstrated the feasibility and applicability of the technique to monitoring of biochemical response to molecularly targeted therapies.

Condensed Abstract

Gefitinib treatment of ovarian cancer patients inhibits EGFR activity in over half of patients. Modulation of EGFR activity correlates to gefitinib toxicity. EGFR inhibition is inadequate as a sole molecular therapeutic target in ovarian cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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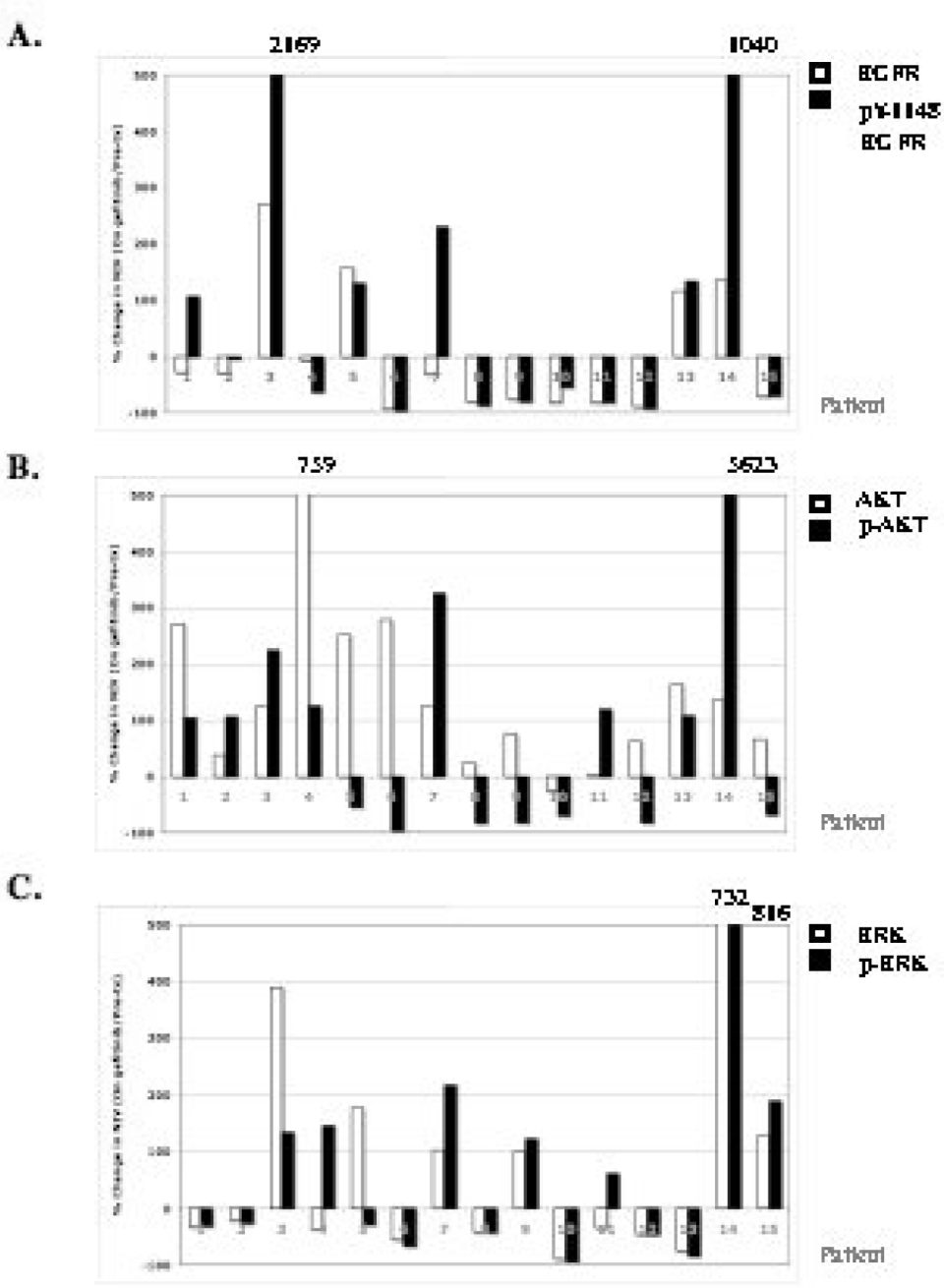

Figure 1.
Changes in parameters seen with one month of gefitinib therapy.

Table 1

Patient Characteristics. N = 24

Median Age (Range)	**53 (33-67)**
Histology	
Serous papillary	9
Endometriod	2
Clear cell	4
Undifferentiated adenocarcinoma	9
Tumor grade	
2	6
3	18
Sites of disease	
Abdomen	20
Liver	17
Pelvis	11
Lung	8
Lymph nodes	9
Spleen	4
Chest	4
Psoas muscle	1
Prior treatments	
< 5	1
5 - 8	8
> 9	15

Table 2

Common adverse events related to gefitinib administration seen in 2 or more patients.

Toxicity	Grade 2 N (%)	Grade 3 N (%)
Gastrointestinal symptoms		
Abdominal pain	0	2 (8)
Anorexia	2 (8)	0
Diarrhea	5 (21)	3 (12)
Nausea	3 (12)	0
Vomiting	3 (12)	0
Constitutional symptoms		
Fatigue	4 (17)	0
Weight loss	2 (8)	0
Skin changes		
Rash/desquamation	3 (12)	0
Laboratory abnormalities		
ALT elevation	1 (4)	1 (4)
Anemia	2 (8)	0
Hypoalbuminemia	4 (17)	1 (4)

Table 3

Sites of acquisition of core tumor biopsies for proteomic analysis.

Biopsy site	N
Liver parenchymal mass	8
Abdominal mass	8
Lymph nodes	5
Pelvic mass	3
Psoas muscle mass	1

Table 4A

Sample statistics for parameters with a trend toward an association with increasing toxicity.

Variable	Site	Group	Gr	N	$\bar{X}$	SEM	P_2
EGFR Post	Tumor	Worst tox =	0	2	13.7	0.8	0.006*
			1	1	6.9		
			2	4	12.4	1.4	
			3	6	51.5	12.6	
			4	2	35.3	12.9	
AKT Post	Tumor	Worst tox =	0	2	19.6	3.9	0.023
			1	1	10.4		
			2	4	12.2	1.1	
			3	6	67.3	17.2	
			4	2	62.6	39.8	
p-ERK Post	tumor	Worst tox =	0	2	10.6	0	0.053
			1	1	7.5		
			2	4	7.4	1.8	
			3	6	13.8	2.1	
			4	2	15.2	3.9	
pY^{1148}-EGFR Post	tumor	GI tox =	0	6	9.1	1.8	0.016
			1	2	24.4	18.1	
			2	3	32.0	21.0	
			3	4	46.9	16.6	
pY^{1173}-EGFR Post	tumor	GI tox =	0	3	5.6	1.5	0.005
			1	1	12.3		
			2	1	34.1		
			3	2	53.4	15.7	
pY^{992}-EGFR Post	tumor	GI tox =	0	5	3.5	0.7	0.026
			1	1	6.4		
			2	2	29.3		
			3	3	29.9	13.2	
pAKT Post	tumor	GI tox =	0	6	6.5	2.1	0.052
			1	2	14.5	7.3	
			2	3	22.4	10.6	
			3	4	26.2	14.2	
pERK Post	tumor	GI tox =	0	6	12.5	1.1	0.022
			1	2	11.6	1.6	
			2	3	17.7	4.7	
			3	4	23.6	2.4	

*Exact Jonckheere-Terpstra trend test

Table 4B

Sample statistics for parameters with a trend toward an association with increasing toxicity.

Variable	Site	Group	Y/N	N	$\bar{X}$	SEM	P_2
pAKT Post	tumor	Skin toxicity =	N	8	6.8	1.6	0.029*
			Y	7	26.5	8.2	
pY^{1148}-EGFR Post	tumor	diarrhea	N	6	9.1	1.8	0.05
			Y	9	37.0	10.1	
pY^{992}-EGFR Post	tumor	diarrhea	N	5	3.5	0.7	0.017
			Y	6	25.8	9.1	
pAKT Post	tumor	diarrhea	N	6	6.5	2.1	0.05
			Y	9	22.3	6.9	

*(Exact Wilcoxon rank sum test)