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PILOT STUDY OF GEFITINIB AND FULVESTRANT IN THE TREATMENT OF POST-MENOPAUSAL WOMEN WITH ADVANCED NON-SMALL CELL LUNG CANCER

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SUMMARY

Introduction—Estrogen receptor beta (ER β) has been detected in NSCLC cell lines and tumor specimens. The ER down-regulator, fulvestrant, blocked estradiol-stimulation of tumor growth and gene transcription in NSCLC preclinical models and showed additive effects with the EGFR inhibitor gefitinib. The safety and tolerability of combination therapy with the EGFR inhibitor, gefitinib, and fulvestrant was explored.

Methods—Post-menopausal women with advanced NSCLC received gefitinib 250 mg po daily and fulvestrant 250 mg IM monthly.

Results—Twenty-two patients were enrolled. Eight patients had adenocarcinoma, 6 NSCLC-NOS, 4 squamous cell, and 4 BAC. Seven patients were never smokers. Eight patients received ≥ 2 lines of prior chemotherapy, 6 received one prior chemotherapy, and 8 were treatment naive. One patient experienced grade 4 dyspnea possibly related to treatment; all other grade 3/4 toxicities were unrelated to treatment. Twenty patients were evaluable for response: 3 PRs were confirmed (response rate of 15%, 95% CI: 5% – 36%). The median PFS, OS, and estimated 1 yr OS were 12 wks (3–112 wks), 38.5 weeks (7–135 wks), and 41% (95% CI 20–62%), respectively.

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Conflict of Interest

No author of this article had any financial or personal relationships with other people or organizations that could inappropriately influence or bias this study.

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Survival outcomes did not differ by prior lines of therapy. A subset analysis revealed that OS in the 8 patients whose tumors exhibited at least 60% ER β nuclear IHC staining measured 65.5 weeks, while that of the 5 patients with ER β staining of less than 60% was 21 weeks. One patient with BAC and a PR had an EGFR L858R mutation in exon 21. There was no correlation between ER β IHC expression and histology or smoking history.

Conclusions—Combination therapy with gefitinib and fulvestrant in this population was well-tolerated and demonstrated disease activity.

Keywords

epidermal growth factor receptor; hormonal treatment; non-small cell lung cancer; sex; age; estrogen receptor

1.0 Introduction

Lung cancer is the leading cause of cancer-related mortality in women in the US, comprising 25% of all cancer-related deaths (1). Certain clinical characteristics distinguish lung cancer in women: a higher percentage of women, compared to men, are diagnosed under the age of 50, non-smokers diagnosed with lung cancer are more likely to be women, women are more likely than men to be diagnosed with adenocarcinoma or bronchioalveolar histologies, and survival is superior for women for all stages of disease, even after controlling for treatment (2–5).

Epidemiologic studies in smokers have yielded conflicting results as to whether sex impacts the risk of developing lung cancer (6–9). Multiple mechanisms may account for any excess risk of developing lung cancer in women. For example, female smokers have been shown to exhibit expression of gastrin-releasing peptide receptor (GRPR) mRNA at a lower mean pack-year tobacco exposure than male smokers. Gastrin-releasing peptide receptor is the receptor for gastrin-releasing peptide, a bombesin-like peptide that stimulates cell proliferation and acts as an autocrine growth factor in lung cancer (10). The gene for GRPR is located on the X chromosome near a cluster of genes that escape X-inactivation.

Further, patterns of polymorphisms of phase I enzymes may influence the risk of disease in women. In that regard, *CYP1A1*, an enzyme active in the conversion of carcinogens to DNA-reactive metabolites, is also involved in the metabolism of estradiol. Retrospective analyses have detected a higher frequency of DNA adducts per smoking dose and higher levels of *CYP1A1* gene expression in females, compared to males, in both lung tumor specimens and in normal lung tissue adjacent to tumor tissue (11). Patterns of polymorphisms of *CYP1A1* have been associated with an increased risk of developing lung cancer in women (12). Studies in normal human bronchoepithelial cells demonstrate that the estrogen receptor (ER) α enhances the expression of *CYP1B1* and *CYP1A1*, key bioactivating enzymes in the carcinogen metabolism pathway, and this may serve as a mechanism for enhanced female sex-related susceptibility to tobacco-mediated DNA mutations (13).

Lastly, hormonal mechanisms may potentially contribute to distinctions in disease risk and in the clinical characteristics of lung cancer between women and men. Hormone replacement therapy was found to be associated with shorter overall survival in women diagnosed with lung cancer, suggesting estrogen signaling might lead to lung cancer progression (14). In contrast, hormone replacement therapy before lung cancer diagnosis appears to protect against development of an ER-expressing lung tumor (15). These conflicting results suggest there is a shift in the balance between tumor-protective effects of estrogen, such as inducing differentiation and stimulating the immune system, and the

tumor-promoting effects of estrogen, such as stimulation of growth-promoting gene expression in normal tissues compared to neoplastic tissues.

Two wild-type ER forms, ER α and ER β , have been identified that share considerable amino acid homology in the DNA and ligand binding regions, but differ considerably in their tissue distribution (16,17). ER α is found in the alveoli, where it plays a role in estrogen-induced alveolar regeneration and is expressed by an immortalized human bronchiolar epithelial cell line (18,19). ER β is highly expressed in airway epithelial tissue, in areas of atypical adenomatous hyperplasia, and in most types of lung cancer in both men and women, while lung tumors express little ER α (20). ER β was found to modulate the expression of platelet derived growth factor A and granulocyte-macrophage colony stimulating factor, key regulators of alveolar formation and surfactant homeostasis, respectively, in mouse models of alveolar development and function (21). Levels of phase I and II carcinogen-activating enzymes in lung tissue were also shown to correlate with ER β expression (22). Estrogen receptor β has induced transcriptional responses from both an estrogen response element and an activator protein (AP-1) element in lung cancer cells; administration of the ER down-regulator, fulvestrant, blocked these effects both *in vitro* and *in vivo* (23). These results have been confirmed using a colony stimulating assay (20). Fulvestrant, also known as ICI 182,870, is a pure ER antagonist that blocks activity of both ER α and ER β (24,25). Estrogen also stimulated expression of E-cadherin, cyclin D1 and ID5, genes associated with tumor progression and growth (26). Retrospective reviews of resected non-small cell lung cancer (NSCLC) specimens from both male and female patients have demonstrated ER β staining per IHC in at least 45% of tumors. Multivariate analyses have shown that the presence of ER β is a positive prognostic variable, although in 2 of these 4 series, this favorable association was restricted to male patients (27–30).

Inhibition of the epidermal growth factor receptor (EGFR) and its downstream effectors represents an important therapeutic target in the treatment of NSCLC and other epithelial malignancies. Monotherapy with erlotinib, a small molecule inhibitor of the EGFR tyrosine kinase activity, yielded prolonged survival and improved symptom control in patients with advanced NSCLC, compared to supportive care, resulting in FDA approval for this indication (31). Prospective and retrospective studies have attempted to identify predictors of clinical benefit from EGFR inhibitors; examples of these have included absence of smoking history, Asian ethnicity, adenocarcinoma histology, EGFR protein expression, the presence of somatic EGFR mutations, amplification of gene copy number, and the severity of treatment-related rash (31–36). Although early reports with these agents identified female sex as being associated with radiographic response to treatment, sex did not fall out as a predictor of survival in the only placebo-controlled monotherapy experience with an EGFR blocking agent (31,37). Investigation of this class of agents remains robust in the treatment of patients with NSCLC, including means to optimize their combination with cytotoxics, other targeted agents, and as radiosensitizers (38–40).

The objective of our therapeutic combination was to exploit the potential interactions between the estrogen and EGFR pathways in patients with progressive lung cancer. Bidirectional signaling between the ER and EGFR pathways has been documented in breast and ovarian cells, and has recently come under investigation in lung cancer models (23,41–44). In addition to nuclear ER activation, a nonnuclear ER pool has been proposed that can exert its effect via rapid signaling through various kinase cascades, including the EGFR pathway and its downstream effectors, such as MAPK (23,41). Stabile et al. demonstrated that EGFR protein expression was up-regulated in response to anti-estrogens *in vitro*, and that ER β expression was decreased in response to EGF and was increased in response to the EGFR tyrosine kinase inhibitor (TKI), gefitinib (23). This work suggests that the EGFR pathway is more activated when estrogen is depleted in lung cancer cells, establishing a

rationale for this combined therapy. Additionally, Stabile and others have shown *in vitro* and *in vivo* that the combination of fulvestrant and an EGF TKI in lung cancer models can maximally inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways (23,41). Therefore, we conducted a pilot study of gefitinib, an EGFR TKI, in combination with the anti-estrogen, fulvestrant, in post-menopausal women with progressive NSCLC, in order to assess the safety and tolerability of this novel combination and to explore molecular predictors of response and toxicity.

2.0 Patients and Methods

2.1 Patient Selection

Patients with pathologically confirmed advanced (stage IIIB with pleural or pericardial effusion, stage IV, or recurrent) NSCLC who gave informed consent according to institutional and Food and Drug Administration guidelines were eligible for this study provided that the following criteria were met: ECOG performance status (PS) of 0, 1, or 2; brain metastases, if present, must have been clinically stable after treatment with surgery and/or radiotherapy; subjects must have been female and postmenopausal, defined as a woman fulfilling any 1 of the following criteria: age ≥ 60 years, age ≥ 45 years with amenorrhea ≥ 12 months with an intact uterus, having undergone a bilateral oophorectomy, or FSH levels in post menopausal range (utilizing ranges from the testing laboratory facility); adequate bone marrow, liver and renal function; PT/INR and PTT within the upper limit of institutional normal (low dose warfarin prophylaxis was permissible); no prior therapy with gefitinib, fulvestrant, or an aromatase inhibitor; life expectancy of at least 3 months; no use of estrogen replacement therapy within 4 weeks of registration and must have agreed to remain off for the duration of the study; and no clinical diagnosis of active interstitial lung disease. Patients who previously received chemotherapy or radiotherapy treatment for their NSCLC were eligible to participate.

2.2 Treatment Plan

Gefitinib was administered at 250 mg orally once daily. Treatment with gefitinib and fulvestrant commenced on day 1 of a 28 day cycle. Toxicities were evaluated per the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Enrollment was restricted to post-menopausal women; safety parameters of fulvestrant in pre-menopausal women and in men were not documented prior to study activation.

For the first 18 subjects enrolled, fulvestrant was administered on day 1 of each cycle at a dose of 250 mg IM. The final four subjects received fulvestrant at a loading dose of 500 mg IM on day 1, followed by 250 mg IM day 15 and day 29, then administered every 28 days (± 3 days) thereafter. Treatment on day 1 consisted of fulvestrant, 500 mg administered slowly as two 5.0 mL injection (50 mg/mL) into the buttock, or four 2.5 mL injections. Treatment on days 15 and 29, and every 28 days thereafter, consisted of fulvestrant, 250 mg administered slowly as one 5.0 mL injection (50 mg/mL) into the buttock, or two 2.5 mL injections into each buttock, for a total of 250 mg (fulvestrant was provided at a concentration of 50 mg/mL in a volume of 5.0 mL).

This addition of a loading dose of fulvestrant was amended into the protocol as an increasing amount of clinical data showed that use of such a loading dose could speed the time to steady-state plasma levels (45). It can take 3 to 6 months for fulvestrant to reach steady-state concentrations using the 250 mg/monthly dose. Through development of this protocol and patient enrollment, the sponsor devised a loading dose schedule for fulvestrant, given at a dose of 500 mg IM on day 1, followed by 250 mg IM day 15 and day 29, then administered every 28 days thereafter. Steady-state drug levels were obtained within 1 month using this

dosing schedule (45). As such, we incorporated it into this trial for the final 4 patients. Although the direct relationships between fulvestrant plasma levels, ER down-regulation, and clinical efficacy are not defined, an experience in preoperative breast cancer reveals that ER down-regulation increases with higher dose of fulvestrant and corresponding plasma concentrations (46).

Patients were examined and evaluated before each 28 day treatment cycle. Chest CT scans were obtained to assess tumor response at the completion of cycle 1. Thereafter, CT scans and chest X-rays were alternatively obtained every cycle, and tumor evaluation measurements were recorded, per RECIST criteria, after every 2 cycles of treatment, using CT scans. Patients with complete response (CR), partial response (PR) or stable disease (SD) continued on treatment until progressive disease (PD) or unacceptable toxicities developed. Patients with PD, unacceptable toxicities at any time, treatment delay exceeding 14 days, or patients requesting treatment discontinuation were removed from protocol treatment.

Dose reductions for either gefitinib or fulvestrant were not permitted. Protocol treatment was withheld for up to 14 days for the following adverse events: new onset of symptoms suggestive of interstitial lung disease, or any grade 3 toxicities possibly, probably, or likely related to either study agents. Treatment was resumed when toxicities resolved to grade 1 or less. The appearance of any grade 4 toxicity, of any attribution, resulted in protocol treatment termination.

2.3 Correlative Assays

Paraffin embedded tumor samples were analyzed for IHC and EGFR mutation status and amplification. Immunohistochemistry was performed for ER α , ER β , EGFR, P-MAPK, and P-AKT. Staining was compared with breast tumor slides as positive controls and slides untreated with primary antibodies served as negative controls. Antibodies used for immunostaining included ER α (clone ID5, Biogenix, 1:20 dilution), ER β (1:50 dilution, Invitrogen Corporation, Carlsbad, CA), EGFR (clone H11, 1:500 dilution, DakoCytomation California, Inc., Carpinteria, CA), P-MAPK (Thr202/Tyr204, 1:100 dilution, Cell Signaling Technology, Beverly MA), P-AKT (Ser473 IHC specific, 1:10 dilution, Cell Signaling Technology, Beverly MA). The secondary antibody was a biotinylated IgG specific for the primary antibody. Paraffin was removed from the slides with xylenes, and slides were stained according to standard procedures. Immunostaining results are expressed as total amount of overall staining on a score of 0–4: 0=no detectable staining; detectable = 1%–10% cells stained; 1=11%–25%; 2=26%–50%; 3=51%–75%; 4=76%–100%. For ER α and ER β , the percentage of positive cells in the nucleus was recorded.

For EGFR mutation and amplification analyses genomic DNA was isolated from specimens using the PUREGENE[®] DNA Purification Kit (Gentra Systems, Minneapolis, MN). EGFR mutation status in exons 18–21 of the EGFR tyrosine kinase domain was examined by polymerase chain reaction (PCR) and sequencing. Exon 18 primers: (F) 5'-TCCAAATGAGCTGGCAAGTG-3'; (R) 5'-TCCCAAACACTCAGTGAAACAAA-3'; Exon 19 primers: (F) 5'-GTGCATCGCTGGTAACATCC-3'; (R) 5'-TGTGGAGATGAGCAGGGTCT-3'; Exon 20 primers: (F) 5'-ATCGCATTCATGCGTCTTCA-3' (R) 5'-ATCCCCATGGCAAACCTCTTG-3'; Exon 21 primers: (F) 5'-GCTCAGAGCCTGGCATGAA-3'; (R) 5'-CATCCTCCCCTGCATGTGT-3'. PCR products were resolved by agarose gel electrophoresis. DNA was purified using the QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA). Sequencing fragments were analyzed via capillary electrophoresis using an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA) and Vector NTI software (Invitrogen Corporation, Carlsbad,

CA) followed by manual review. Candidate mutation samples in both directions were reamplified and resequenced as described above to confirm the existing mutations.

Single strand EGFR conformation polymorphism analyses were conducted as per methods previously described (47).

Fluorescent in situ hybridization (FISH) analysis of EGFR amplification was performed using the dual-color EGFR SpectrumOrange/CEP7 SpectrumGreen probe (Vysis, Inc., Downer's Grove, IL) and paraffin pre-treatment reagent kit (Vysis, Inc.). In brief, paraffin sections were de-paraffinized, dehydrated in ethanol and air-dried. Sections were digested with proteinase K (0.5mg/ml) at 37°C for 28 minutes. The slides were denatured for 5 minutes at 75°C prior to hybridization. Slides were hybridized overnight at 37°C and washed in 2XSSC/0.3% NP40 at 72°C for 2 minutes. Nuclei were counterstained with DAPI/antifade 1 (Vysis, Inc.) Each FISH assay included normal lung tissue sections as a negative control, and sections of lung non-small cell carcinoma previously identified as carrying EGFR gene amplification as a positive control. Analyses were performed using a fluorescence microscope (Nikon Optishot-2 and Quips Genetic Workstation) equipped with Chroma Technology 83000 filter set with single band excitors for Texas Red/Rhodamine, FITC, and DAPI (UV 360nm). The histological areas previously selected on the hematoxylin and eosin stained sections were identified on the FISH-treated slides. Only individual and well-delineated cells were scored. Overlapping cells were excluded from the analysis. At least 60 cells were scored for each case and control.

Each tumor was assessed by average number of copies of *EGFR* gene per cell, average ratio of EGFR to chromosome 7 copy numbers (CEP7), and ploidy. Amplification was defined as a ratio of EGFR signals to chromosome 7 centromere signals of ≥ 2.0 .

A single blood sample was obtained in a DNA Paxgene (Qiagen, Germantown, MD) tube at baseline for analysis of polymorphisms. All samples were evaluable for ABCB1 C3435T genotype; one sample did not amplify for the ABCB1 G2677T genotype and two samples did not amplify for ABCB1 C1236T and CYP3A4*1B. DNA was extracted as recommended by the manufacturer (Qiagen, Germantown, MD).

Specific oligonucleotide primers for the human ABCB1 and CYP3A4 gene fragments from genomic DNA were derived from known sequences using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR amplification for pyrosequencing was performed in 40 μ l reactions containing 20 μ l PCR Master Mix (Promega, Madison, WI), 10 pmol forward and reverse primer (IDT Technology, Coraville, IA), 14 μ l of nuclease free water and 10 ng of genomic DNA. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 5 minutes, 50 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension step at 72°C for 5 minutes.

The pyrosequencing primers were designed using SNP Primer Design Software Version 1.01 (<http://www.pyrosequencing.com>). Pyrosequencing was performed as follows. First, 35 μ l of biotinylated PCR product was immobilized on streptavidin-coated Sepharose beads (Amersham Biosciences, Piscataway, NJ) with binding buffer (10 mmol/L Tris-HCl, 2 mol/L NaCl, 1 mmol/L EDTA, and 0.1% Tween 20, pH 7.6). After room temperature incubation with constant agitation for 10 minutes, the strands were separated and treated with 70% ethanol, denaturation solution (0.2 mol/L NaOH) and washing buffer (10 mmol/L Tris-Acetate, pH 7.6). The beads, containing the biotinylated template, were released into wells with a 40 μ l mixture of annealing buffer (20 mmol/L Tris-Acetate, 2 mmol/L Magnesium Acetate Tetrahydrate, pH 7.6) and 21 pmol of sequencing primer (IDT Technology, Coraville, IA). Incubation was carried out at 80°C for 2 minutes.

Genotyping was subsequently performed using a PSQ 96 SNP Reagent Kit and PSQ 96MA system (Biotage AB, Uppsala, Sweden). Genotypes were resolved on the basis of peak height measurements using PSQ96 SNP Software, version 1.2 AQ. This software automatically performs genotyping and quality assessment of the raw data utilizing an algorithm. Genotype is scored automatically based on pattern recognition. Quality value is assigned based on several parameters such as the difference in match between the best and next best choice genotypes, agreement between expected and obtained sequence around the SNP, signal-to-noise ratios, variance in peak heights around the SNP, and peak width.

2.4 Statistical Considerations

The primary objective of this pilot study was to describe the safety and tolerability of gefitinib, combined with fulvestrant, in post-menopausal women with advanced NSCLC, per the NCI CTCAE (version 3.0). Secondary objectives included an estimation of the anti-tumor response rate per RECIST criteria, progression free survival (PFS), and overall survival (OS) using the Kaplan-Meier method. All patients were included for analyses of toxicity. Survival was analyzed on an intent-to-treat basis. Patients had to complete one cycle of therapy to be evaluable for antitumor response. Exploratory objectives included descriptive assessment of possible correlations between response to treatment, toxicities, and results from laboratory correlates.

The sample size chosen was sufficient to provide accuracy in estimating the toxicity rates. Specifically, with 20 evaluable participants, toxicity rates were estimated with a standard error of less than 12%. Moreover, there is a 65% chance of observing at least one incidence of a serious toxicity (grade 3 or higher) in 20 patients if the true toxicity rate is as low as 5% and an 88% chance of observing at least one incidence of a severe toxicity in 20 patients if the true complication rate is 10%.

The University of Wisconsin Paul P. Carbone Comprehensive Cancer Center (UWCCC) Clinical Trials Monitoring Committee was responsible for monitoring data quality and patient safety according to UWCCC guidelines. In addition, each patient's treatment was reviewed weekly by the UWCCC Lung Cancer Research Disease Oriented Working Group. Criteria for early study termination were in place based upon marginal rates of excessive grade 3 toxicities (defined as present in 7 of 20 patients), grade 4 toxicities (defined as present in 5 of 20 patients), and in either grade 3 or 4 toxicities (defined as present in 9 of 20 patients).

Categorical variables were summarized using frequencies and percentages while all continuous variables were summarized and reported in terms of medians and ranges. Progression-free survival and overall survival were analyzed using the Kaplan-Meier methodology. The log-rank test was used to compare survival curves between subgroups, e.g., ER β staining $\geq 60\%$ vs. $< 60\%$. All statistical tests were two-sided, and $P < 0.05$ was used to indicate statistical significance. Statistical analyses were performed using SAS version 9.1.

3.0 Results

3.1 Patient Characteristics

A total of 22 patients were enrolled in the study between February 2004 and February 2005. Twenty patients were evaluable for response. One patient was not evaluable due to the development of grade 2 blepharoconjunctivitis probably related to gefitinib within two weeks of initiating therapy; the patient self-discontinued treatment acutely and declined further protocol participation. A second patient was deemed not evaluable for efficacy due to developing grade 3 erythema multiforme related to an allergic reaction to phenytoin

within cycle 1 of therapy. All patients were evaluable for toxicity and survival assessments. The median follow-up is 38 weeks.

Table 1 shows clinical characteristics of all 22 patients. Median age was 66 years (range 56 – 81 years). Eight patients had adenocarcinoma (NSCLC-A), 6 had NSCLC-NOS, 4 had squamous cell (NSCLC-S), and 4 had bronchioalveolar carcinoma (BAC). Ten patients had PS of 0, 9 had PS of 1, and 3 had PS of 2. Seven patients were never-smokers, including 3 with NSCLC-A. Thirteen patients were former smokers and 2 were actively smoking at the start of treatment. Eight patients had ≥ 2 lines of prior chemotherapy, 6 patients had 1 prior line of chemotherapy, and 8 were chemotherapy naïve.

3.2 Treatment

Treatment compliance with gefitinib was good; only two patients reported missing one dose each of this daily medication. There were no missed doses of fulvestrant. One patient was inadvertently over-dosed with fulvestrant, receiving 500 mg IM on day 15. She experienced transient grade 2 nausea, vomiting, and diarrhea following this medication error. These symptoms were likely treatment-related, and resolved spontaneously. The median number of cycles administered was 2.5 (range 1 to 21).

3.3 Toxicities

One patient died on study: she had stable disease, experienced no prior toxicity, and died from an acute coronary event during cycle 4 of treatment. Her history was notable for hypertension and rheumatoid arthritis, 2 known risk factors for ischemic heart disease. Reviews of the literature and Investigational Drug Brochures for both study agents did not identify ischemic heart disease as a risk factor for either drug. As such, this event was deemed not related to study treatment.

Two patients experienced grade 4 dyspnea: 1 patient came off study with dyspnea related to progressive disease from an enlarging pleural effusion. The second patient experienced grade 4 dyspnea, possibly related to either or both study medications, during her first cycle of treatment. She inadvertently remained on study for an additional 2 weeks, as indicators of progressive disease (hepatic metastases) were evaluated. She came off study half-way through cycle 2.

Table 2 lists grade 3 toxicities, the majority of which were related to progressive disease and coincided with the patient coming off study. No grade 3 toxicities were possibly, probably, or definitely attributed to either study drug. One patient with grade 3 confusion demonstrated increased vasogenic edema on cranial imaging, attributed to prior irradiation. One patient with grade 3 dizziness was found to have experienced an acute embolic stroke while on study, while one patient with grade 3 erythema multiforme was believed to have developed this condition secondary to an acute allergic reaction to phenytoin. The attribution of this patient's rash to her phenytoin was confirmed after a skin biopsy and in-patient dermatologic consultation. One patient experienced grade 3 pneumonia on study. Lastly, one patient developed grade 3 rectal bleeding while on treatment; she was subsequently diagnosed with rectal adenocarcinoma and was taken off study with stable lung cancer. Two patients died within 30 days following completion of protocol treatment, both due to progressive disease.

Two patients experienced grade 2 treatment-related toxicity: one patient with grade 2 blepharoconjunctivitis probably related to gefitinib and one patient who was inadvertently overdosed with fulvestrant. She experienced transient grade 2 nausea, vomiting, and diarrhea likely related to fulvestrant that resolved spontaneously. No additional patients experienced grade 1 or 3 treatment-related toxicities.

3.4 Treatment Efficacy

Table 3 summarizes outcome and survival data. No patient experienced a CR. There were 3 patients with confirmed PRs, all of whom were never smokers. Two of these 3 patients were treatment-naïve. The third patient had achieved SD with chemoradiation for stage IIIB disease approximately 2 months prior to study entry. The median duration of response measured 76 weeks. The overall anti-tumor response rate was 15% (95% CI: 5% – 36%) for the 20 evaluable patients. Over half of patients (11 patients) had SD as the best response to treatment, with a median duration of 9.5 weeks. Median PFS for the study population measured 12 weeks (range 3 – 112 weeks). The median OS was 38.5 weeks (range 7 – 135 weeks), and, to date, 20 patients have died. The estimated 1 year OS rate is 41% (95% CI: 20% – 62%). Table 4 demonstrates that survival outcomes did not differ by prior lines of treatment. Similarly, administration of a loading dose of fulvestrant to the final four enrollees did not impact survival. Finally, treatment efficacy (response and survival) did not correlate with histology, smoking history, prior lines of treatment, or the presence or absence of EGFR mutations (data not shown).

3.5 Results from Correlative Studies

Twelve patients supplied archived tumor available for full correlative analyses. One additional patient had tumor sufficient only for IHC staining, but not for EGFR mutation or amplification studies. Twenty-two patients submitted peripheral blood samples for ABCB1 C3435T genotyping.

One of the 3 responding patients had an L858R missense mutation in exon 21 of the EGFR gene; the 2 other responders did not have sufficient tissue for analysis. Two additional patients with SD had EGFR gene mutations: 1 with a mutation at G2591A in exon 21 (A864V) and the other with a 746–750 deletion in exon 19. The median PFS and median OS of the patients with (n=3) and without (n=9) EGFR gene mutations did not differ statistically (21 weeks and 78 weeks, vs, 6 weeks and 26 weeks, respectively). The L858R point mutation and the 746–750 deletion mutations are the most frequently reported mutations found in the EGFR TK domain. However, the A864V mutation occurred in a previously unreported residue.

In general, EGFR staining per IHC was low in the 13 samples tested. Immunohistochemical staining for EGFR, phospho-MAPK, and p-Akt expression did not correlate with response to treatment, histology, or smoking history (data not shown). None of the 12 tumors analyzed displayed EGFR gene amplification.

Immunohistochemical staining for ER α and ER β in terms of total overall cellular expression and the percentage of positive cells in the nucleus was recorded in tumor samples from 13 patients. There was no correlation between ER α and ER β staining (either total or nuclear) with either histology or smoking history. Overall ER β staining exceeded 50% (IHC score of at least 3) in all samples assayed, while ER α staining was low. Fewer than 10% of cells in all samples analyzed contained nuclear ER α staining. An exploratory post-hoc analysis was performed to explore any relationship between the percentage of cell nuclei staining positive for ER β per IHC and treatment efficacy. As Table 5 and Figure 1 demonstrate, the OS in the 8 patients whose tumors exhibited at least 60% staining measured 65.5 weeks, while that of the 5 patients with ER β staining of less than 60% was 21 weeks. These survival durations are not statistically distinct, but this study was not powered to determine efficacy outcomes. In contrast, no association of ER α staining with survival endpoints was found.

Tumor samples from 11 patients were analyzed for EGFR single nucleotide polymorphism (SNP) in exon 20 of the tyrosine kinase (TK) domain. Zhang et al. previously detected that a polymorphism at nucleotide 2607, codon 787 (Gln), which changed nucleotide 2607 from G

to A, without amino acid substitution, was associated with lung cancer and occurred independently of EGFR-TK mutations (47). Table 6 lists the SNP frequencies found in our 11 patients and their associated survivals. Statistical analyses of these findings are limited by the small number of patients tested. Median overall survival with the low lung cancer risk allele (GG) has not yet been reached due to the continued survival of one participant.

Genotyping for ABCB1 and CYP3A4*1B revealed wild-type alleles at the expected frequencies, as shown in Table 7. Variants did not correlate with toxicity or response.

4.0 Discussion

The primary endpoint of this pilot study was safety and tolerability in this population. Combination treatment was tolerated well, with minimal grade 2 treatment-related toxicities, one of which resulted from an inadvertent over-dosage of fulvestrant. The single patient with grade 4 toxicity (dyspnea) possibly related to treatment was found to have disease progression in the liver 2 weeks following this adverse event. Therefore, her underlying disease may have contributed to her dyspnea. Overall treatment-related toxicities with this novel combination compare favorably to the experience seen with each single agent (37,48,49). The prevalence of grade 3 toxicities related to disease progression underscores the fact that 14 of the 22 participants were receiving second- or greater-line treatment for their disease.

Although efficacy outcomes were secondary, the response rate, PFS, and OS from our pilot study population, over half of whom were pretreated, compare reasonably with survival durations from first- and second-line treatment trials using cytotoxics or single agent targeted therapies (31,50–53). Comparison of our efficacy results with those from larger studies is limited by the biases inherent in a small single-institution pilot trial, especially one in which prior lines of treatment were not limited. Confirmation of possible efficacy with this combination requires validation in a population of both male and female patients, restricted to predetermined lines of pretreatment.

However, the 76 week duration of response in the 3 participants with a PR is provocative. One of these individuals was found to have a missense mutation in exon 21 of the EGFR gene, while the remaining two responders did not have tissue available for mutation analysis. Of the 12 patients who underwent EGFR mutation testing, the PFS and OS of the 3 patients with EGFR mutations (1 responder and 2 with SD) were not statistically superior compared to those without. This comparison is sharply limited by the small number of individuals tested. Although not entirely conclusive, clinical data to date suggest that the presence of the most common EGFR mutations correlates with improved anti-tumor response rates and survival (32,33,54–56). The lengthy duration of response in the 3 responding patients suggests that the other 2 (non-tested) responders may well have had EGFR somatic mutations.

Immunohistochemical staining for ER β was found in all 13 lung tumor samples tested, and did not correlate with histology or smoking history. Retrospective surgical series have been inconclusive regarding any relation between the presence of ER β and sex, histology, or smoking history (20,27–30). These analyses have identified ER β as a favorable prognostic variable although in some studies the relationship was only significant in males. Nuclear localization of ER β was found in 45–69% of NSCLC cases in these reviews (27–30). ER β immunoreactivity was also observed in the cytoplasm in cases that also express nuclear ER β . Cytoplasmic receptor expression could be responsible for the rapid nongenomic estrogen signaling observed in lung cancer cells (23). Our post-hoc demonstration of a non-significant trend toward prolonged survival with a threshold of nuclear ER β expression of at

least 60% raises the hypothesis that this variable serves as a favorable predictive factor with the use of anti-estrogen therapy. Exploration of the predictive value of ER β expression as a biomarker with anti-estrogen therapy requires evaluation in a larger clinical protocol.

Zhang et al. previously identified a germline EGFR SNP (G2607A) that was associated with lung cancer risk and occurred independently of somatic EGFR-TK mutations (47). It is not known if this EGFR SNP affects EGFR expression or function. Based on the data presented here, the low lung cancer risk allele (GG) correlated with better survival. This correlation may not be treatment related. Larger populations are required before survival comparisons between the homozygous (G2607G) and either the homozygous mutant (A2607A) or heterozygous mutant (G2607A) can be validated.

Our data demonstrate that despite being a p-glycoprotein substrate, the efficacy and toxicity of gefitinib are not influenced by ABCB1 genotype. Although formal pharmacokinetic analyses were not performed in this pilot study, this result suggests that gefitinib concentrations are not an important determinant of outcome in this population. All patients studied in this trial were wild-type for CYP3A4. This is likely due to the small sample size of this study and the low frequency of CYP3A4 polymorphisms. As such, an assessment of CYP3A4 genotype influence efficacy and toxicity could not be performed.

Preclinical work suggests that dual inhibition of the ER and EGFR pathways represents a relevant therapeutic target (44). Nelson et al. showed that EGF-blocking antibody prevents estrogen-induced vaginal and uterine growth, implying that cross-talk between the ER and EGFR pathways may be physiologically important in reproductive tissue (57). Filardo et al. showed in breast cancer cells that estrogen rapidly acts to stimulate the transactivation of EGFR, leading to cAMP and ERK up-regulation (58). Using MDA-MB231 and SKBr3 breast cancer cells, which are ER-negative and overexpress human EGF family receptors, Boerner et al. showed that estradiol suppressed basal STAT5-mediated transcription, as well as EGF-stimulated STAT5b tyrosine phosphorylation, STAT5-mediated transcription, and EGF-induced DNA synthesis (43). Marquez-Garban et al. showed that both estrogen and EGF rapidly activate MAPK and promote SRC-3 phosphorylation in the NIH-H23 NSCLC cell line, and that exposure to fulvestrant and erlotinib, an EGFR TKI, counteracts this phosphorylation (41). Lastly, treatment of human xenograft mouse models of NSCLC with both fulvestrant and an EGFR TKI, either gefitinib or erlotinib, reduced tumor volume greater than treatment with either drug alone, with histologic evidence revealing increased apoptosis, decreased phosphor-p44/p42 MAPK expression, and increased Ki-67 expression, compared to treatment with either drug alone (23,41).

Taken in sum, this work, and that from others, suggests that ER and EGFR interact in a biologically relevant manner in NSCLC. As recently reviewed by Pietras and Marquez-Garban, data now show that both nuclear and nonnuclear ERs interact in a cooperative fashion with transmembrane growth factor receptor signaling pathways in breast and other cancers that have ERs, including NSCLC tumors, and that this cross-talk between estrogen and growth factor receptors promotes downstream signaling for tumor cell proliferation, survival, and endocrine resistance (59). ER β has been shown to localize in caveolae from lung cancer cells, where EGFR often concentrate in the plasma membrane, thus promoting possible interaction between downstream effectors (42). The potential to block compensatory effects between the two pathways is the therapeutic objective of this novel combination in NSCLC. Estrogen receptor-negative breast tumors have been found to overexpress EGFR; MCF-7 breast cancer cells resistant to tamoxifen and fulvestrant demonstrate increased basal activation of EGFR/HER2/MAPK signaling clinically (60,61). Clinically, breast tumors that overexpress HER family receptors typically have a poorer prognosis and do not respond as well to antiestrogen therapies (62). Epidermal growth factor

receptor expression in A549 lung cancer cells decreased following exposure to estrogen and increased following treatment with fulvestrant; correspondingly, ER β expression in these cells rose following exposure to gefitinib (23).

Our pilot study represents an initial attempt to inhibit dual growth stimulatory pathways in NSCLC. A phase II study combining erlotinib with fulvestrant is underway, and we are developing a dose escalation study of vandetanib, a multitargeted EGFR and VEGFR TKI, with fulvestrant (63). Both of these trials are enrolling pre- and post-menopausal women and men patients in order to more fully understand the role of estrogen blockade in NSCLC.

5.0 Conclusions

Combination therapy with gefitinib and fulvestrant was well-tolerated in our population of post-menopausal women with advanced NSCLC. No unexpected toxicities were seen. Efficacy data in this population of treatment-naïve and pre-treated patients were promising. The ability of correlative assays to elucidate predictors of efficacy was limited by the small number of enrollees. Preclinical work documenting interactions between the ER and EGFR pathways, as well as the good tolerability of this combination, should provide further insight into this novel treatment paradigm in NSCLC.

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Figure 1. Percentage of Tumor Cell Nuclei Staining Positive for ERβ by Immunohistochemistry and Survival

Kaplan-Meier curves of progression-free survival and overall survival in post-hoc analysis of patients with tumor cells with nuclear IHC ERβ staining at least 60% (N=8) versus ERβ staining less than 60% (N=5). Numerical values are listed in Table 5.

Table 1**Patient Characteristics**

Patients, N	22
Age, year	
Median	66
Range	56–81
Performance status, N	
0	10
1	9
2	3
Lines of prior chemotherapy	
0	8
1	6
≥ 2	8
Histology	
Adenocarcinoma	8
NSCLC-NOS	6
Squamous cell	4
Bronchioalveolar	4
Smoking history	
Never smoked	7
Former smoker	13
Current smoker	2

NSCLC-NOS = non–small cell lung cancer – not otherwise specified.

Table 2**Grade 3 Toxicities**

Grade 3 Toxicity	Attribution
Confusion and weakness	Increased vasogenic edema from prior whole brain irradiation
Dizziness	Acute embolic stroke
Erythema multiforme	Allergic dermatologic reaction to phenytoin
Pneumonia	PD
Dehydration, rectal bleeding	New diagnosis of rectal adenocarcinoma
Dyspnea, pleural effusion, hypoxia	PD with a new large pleural effusion
Pain, nausea/vomiting, fatigue, and cough	PD with a new large pleural effusion and new progression of the tumor into the brachial plexus
Pain	Post-operative pain from a seroma formed at a site of resected axillary metastasis
Nausea, anorexia, malaise spinal cord compression	PD with a new metastasis at the T7 vertebral body

PD = progressive disease

Table 3**Treatment Outcomes**

Evaluable patients, N	20
Median follow-up time, wk	38
Number of cycles of treatment	
Median	2.5
Range	1->21
Progression-free survival, wk	
Median	12
Range	3->112
Overall survival, wk	
Median	38.5
Range	7->135
Estimated 1-year overall survival, %	41
95% confidence interval, %	20-62
Overall response rate, %	15
95% confidence interval, %	5-36
Complete response, N	0
Partial response	
N	3
Median duration, wk	76
Stable disease	
N	11
Median duration, wk	9.5
Progressive disease	
N	6
Median duration, wk	28.5

wk = week

Table 4

Survival by Lines of Prior Treatment and Fulvestrant Dose

	Lines of prior treatment			Fulvestrant dose		
	0 (N=8)	1 or 2 (N=14)	P-value	Loading dose absent (N=18)	Loading dose present (N=4)	P-value
PFS, wk						
median	10.5	12		12.5	6.5	0.88
range	4 – 80	3 – 111	0.98	3 – 111	4 – 80	
OS, wk						
median	57	36.5	0.74	38.5	70	0.42
range	8 – 124+	7 – 135		8 – 135	7 – 124+	

PFS = progression free survival
OS = overall survival
wk = week

Percentage of Tumor Cell Nuclei Staining Positive for ERβ by Immunohistochemistry and Survival

Table 5

Intensity of ERβ Staining, %	N	Progression-Free Survival, wk	P value	Overall Survival, wk	P value
≥ 60	8	8.5	0.73	65.5	0.52
< 60	5	14		21	

wk = week

Table 6**EGFR Polymorphisms and Survival**

EGFR Polymorphism	Median PFS (wk)	Median OS (wk)
AA (n=2)	9	10.5
GA (n=7)	11	53
GG (n=2)	44.5	NBR

Comparison	p-value (PFS)*	p-value (OS)*
AA vs GA	0.7650	0.0019
AA vs GG	0.4328	0.2253
GA vs GG	0.2781	0.5700

* nominal p-values (i.e., no adjustment for multiple comparisons due to the exploratory nature of these analyzes)

PFS = progression free survival

OS = overall survival

wk = week

NBR = not yet been reached

Table 7

Frequencies of ABCB1 and CYP3A4*1B Genotypes

Allele	Wild-type	Heterozygote	Variant
ABCB1 C1236T	CC	CT	TT
N=20	0.40	0.45	0.15
ABCB1 G2677T	GG	GT	TT
N=21	0.33	0.48	0.19
ABCB1 C3435T	CC	CT	TT
N=22	0.27	0.55	0.18
CYP3A4*1B	AA	A/G	GG
N=20	1.0	0	0

CYP = cytochrome P450