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A systems pathology model for predicting overall survival in patients with refractory, advanced non-small-cell lung cancer treated with gefitinib

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ABSTRACT

Purpose: To identify clinical and biometric features associated with overall survival of patients with advanced refractory non-small-cell lung cancer (NSCLC) treated with gefitinib.

Experimental design: One hundred and nine diagnostic NSCLC samples were analysed for EGFR mutation status, EGFR immunohistochemistry, histologic morphometry and quantitative immunofluorescence of 15 markers. Support vector regression modelling using the concordance index was employed to predict overall survival.

Results: Tumours from 4 of 87 patients (5%) contained EGFR tyrosine kinase domain mutations. A multivariate model identified ECOG performance status, and tumour morphometry, along with cyclin D1, caspase-3 activated, and phosphorylated KDR to be associated with overall survival, concordance index of 0.74 (hazard ratio (HR) 5.26, *p*-value 0.0002).

Conclusions: System-based models can be used to identify a set of baseline features that are associated with reduced overall survival in patients with NSCLC treated with gefitinib. This is a preliminary study, and further analyses are required to validate the model in a randomised, controlled treatment setting.

1. Introduction

The over-expression of epidermal growth factor receptor (EGFR) in a variety of solid tumours, including non-small-cell lung cancer (NSCLC), has made it an attractive target for selective molecular therapeutics, specifically for tyrosine kinase inhibitors (TKIs) such as gefitinib (IRESSA). Although initial results for gefitinib in pretreated patients were promising, in two pivotal Phase III trials patients treated with gefitinib did not demonstrate significantly better overall or progression-free survival compared with the placebo group.[1,2] Furthermore, in the Phase III, placebo-controlled IRESSA Survival Evaluation in Lung Cancer (ISEL) trial, gefitinib monotherapy was associated with some improvement in

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overall survival; however, the results did not reach statistical significance.[3]

An objective response to gefitinib has been linked to several molecular, demographic and clinical-pathologic factors including activating mutations in the ATP pocket of EGFR, tumour histology (adenocarcinoma – bronchoalveolar variant), amplification of the EGFR gene, Asian race, female gender, non-smoking history and good performance status.[4–7] Encouraging data from the recent iTARGET trial demonstrated an improved outcome when patients were treated with first-line gefitinib therapy based on their EGFR mutation status.[8] Although the results need to be compared with more traditional therapies, the study does provide some preliminary evidence for the future of 'genotype-based' treatment decision-making. In addition, evidence from the ISEL trial suggested that high EGFR gene copy number was predictive of clinical benefit and survival. Of note, a recent open-label Phase III study compares gefitinib with docetaxel in patients with locally advanced disease; however, this did not find an association with EGFR gene copy number and outcome.[9] Both the studies illustrate the importance of deriving base line characteristics from the patient's primary tumour sample when developing a comprehensive management and treatment plan, and indicate that different patients derive different degrees of clinical benefit from treatment with EGFR TKIs.

We previously developed models to predict disease progression and therapeutic outcome for patients with prostate and breast cancers using a systems pathology platform.[10–13] In this approach, conventional clinical-pathologic information is integrated with biometric features from the tumour specimen, using machine learning to interpret the complex data sets.[10,12] In the current study, we analysed 109 patients with refractory NSCLC, all treated with gefitinib using an Expanded Access Programme (EAP). We sought to determine EGFR mutation status in the patients' diagnostic tumour and to use systems pathology to identify a baseline phenotype predictive of overall survival.

2. Methods

2.1. Patients and tissues

This study was approved by the institutional review board of the Sarah Canon Cancer Research Centre, and where appropriate all patients provided informed consent. The initial cohort consisted of 284 US patients with advanced refractory NSCLC treated with 250 mg gefitinib orally each day. Six clinical variables were analysed: gender, smoking history, age at diagnosis, tumour histology, number of prior chemotherapies and ECOG performance status (a scale ranging from 0, healthy, to 5, death from disease). Unstained de-paraffinised slides (fine needle aspirates, cell pellets or cytospins) and/or paraffin blocks from the diagnostic specimen were evaluated with hematoxylin and eosin (H&E) for tumour content. All biomarkers were analysed without knowledge of clinical outcome.

2.2. EGFR mutation analysis

Two sequential 20-μm sections from each paraffin block or ⩾8 unstained sections from paraffin slides were analysed. Genomic DNA was obtained from de-paraffinised samples by incubation with proteinase K, then by chloroform extraction and ethanol precipitation. EGFR mutations were analysed primarily by DNA sequencing of exons 19, 20 and 21, and secondarily using the amplification refractory mutation system (ARMS), specifically allele-specific polymerase chain reaction (PCR) to detect the L858R mutation and del G2235-A2249. Patients were considered mutation positive if a mutation in the tyrosine kinase domain was detected by either ARMS or sequencing in both forward and reverse directions in at least two independent PCR products.

2.3. Histologic morphometry

H&E-stained slides were prepared from the original blocks or unstained sections. One to six images from representative areas of tumour were acquired with an Olympus bright-field microscope at 20× magnification using a SPOT Insight QE camera (KAI2000). Image analysis software[10] classified image objects as histopathological cellular elements, exhibiting particular colour channel values, generic shape features (e.g. area and length), and spatial relationship properties (e.g. amounts of lumen relative to total tissue), from which statistics were generated. Due to differences in sample preparation (i.e. cytospin, needle biopsy and tissue resection) fixation, staining, and tissue quality, several different scripts were developed for image segmentation.

2.4. EGFR immunohistochemistry

EGFR was analysed by immunohistochemistry using the EGFR pharmDX kit (DAKO, Glostrup, Denmark). A staining index with range 0–300 was calculated for each sample by multiplying each intensity level (0–3) by the percentage of cells at that intensity level.

2.5. Multiplex (M-Plex™) biomarker assessment

Fifteen antibodies were selected (Table 1). Each antibody was initially evaluated by immunohistochemistry on a series of cell lines and/or control lung cancer tissue samples with appropriate negative controls. To confirm specificity of the pEGFR and pERK antibodies, extracts of A431 NSCLC cells with or without EGF treatment were immunoprecipitated with these antibodies, followed by Western blotting. Similar tests were performed with pKDR in HUVEC cell lines activated with VEGF. In addition, for both EGF and VEGF, treated and non-treated cells were processed for routine immunohistochemistry. The 15 antibodies were organised into six multiplex formats (Table 1).

After de-paraffinisation and rehydration of tissue samples, slides were boiled in a microwave oven for 7.5 min in 1X Reveal Solution (BioCare Medical, Concord, CA) for antigen retrieval. After cooling for 20 min at room temperature, slides were washed twice for 3 min in phosphate-buffered saline (PBS).

To help permeate the cellular structures, samples were incubated in PBT (PBS with 0.2% Triton X-100) at room temperature for 30 min, followed by three rinses of 3 min each in PBS. To reduce autofluorescence, samples were incubated