CASE REPORT

Subclonal Therapy by Two EGFR TKIs Guided by Sequential Plasma Cell-free DNA in *EGFR*-Mutated Lung Cancer

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Received 19 October 2016; revised 12 February 2017; accepted 15 February 2017
Available online - 7 March 2017

Case Report

We present the case of a patient with multiple *EGFR* mutations that highlights tumor heterogeneity leading to a mixed response to osimertinib and emphasizes the complexity of *EGFR*-driven lung cancer. Subclonal tumor evolution was proved by tissue biopsy, monitored by serial analyses of cell-free circulating tumor DNA (cfDNA) at disease progression, and addressed using EGFR tyrosine kinase inhibitor (TKI) combination therapy.

In October 2013, stage IIIA adenocarcinoma was diagnosed in a 49-year-old white female never-smoker and treated with definitive chemoradiotherapy. In April 2014, she progressed with multiple brain, liver, and bone

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Disclosure: Dr. Peled serves as an adviser to and receives honoraria from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Lilly, Merck Sharp and Dohme, Novartis, Pfizer, Roche, Foundation Medicine, NovellusDx, and Gaurdant360. Dr. Roisman reports personal fees (honoraria for talks) from Roche and Pfizer during the conduct of the study. Dr. Miron was an employee of NovellusDx during the conduct of the study. Dr. Lanman reports being an employee with stock options of Guardant Health, Inc., during the conduct of the study. Dr. Ilouze reports personal fees from Pfizer outside the submitted work. Dr. Dvir reports other from Teva Pharmaceuticals, outside the submitted work. Dr. Soussan-Gutman reports being an employee of Oncotest-Teva during the conduct of the study. Dr. Barlesi reports personal fees from Astra-Zeneca, Bristol-Myers Squibb, Boehringer-Ingelheim, Clovis Oncology, Eli Lilly Oncology, F. Hoffmann-La Roche Ltd., Novartis, Merck, Merck Sharp and Dohme, Pierre Fabre, and Pfizer outside the submitted work. Dr. Tarcic reports personal fees from NovellusDx outside the submitted work. Dr. Edelheit is an employee of NovellusDx during the conduct of the study. Dr. Gandara reports receiving grants from and serving as a consultant to AstraZeneca, Clovis, Genentech, Lilly, Merck, and Novartis; receiving grants from Bristol-Myers Squibb and Johnson and Johnson; and serving as a consultant to Bayer, Boehringer Ingleheim, Celgene, Guardant Health, Liquid Genomics, Mirati, Peregrine, Pfizer, Synta, and Trovogene during the conduct of the study. Dr. Elkabetz reports nonfinancial support from French Friends of Tel Aviv University during the conduct of the study and nonfinancial support from Ramot-IP Company of Tel Aviv University outside the submitted work. Dr. Elkabetz is the brother of the patient reported in the study; however, apart for simply increasing the author's interest and enthusiasm in helping the group decipher the mechanisms underlying the progression of disease in order to bring relief, seek for a cure, and help other patients down the road, this relationship has not been affected by any conflicts of interest. The process of obtaining scientific findings (plasma cell-free DNA analysis) was funded privately by the family, and the findings were provided in parallel to clinicians/authors and were, in any case, not affected by the family. Similarly, the recording and processing of clinical disease measures were funded by insurance companies and/or privately by family. A complete separation was made between these and the decisions on treatments made by clinician and patient. The remaining author declares no conflict of interest.

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ISSN: 1556-0864

http://dx.doi.org/10.1016/j.jtho.2017.02.023

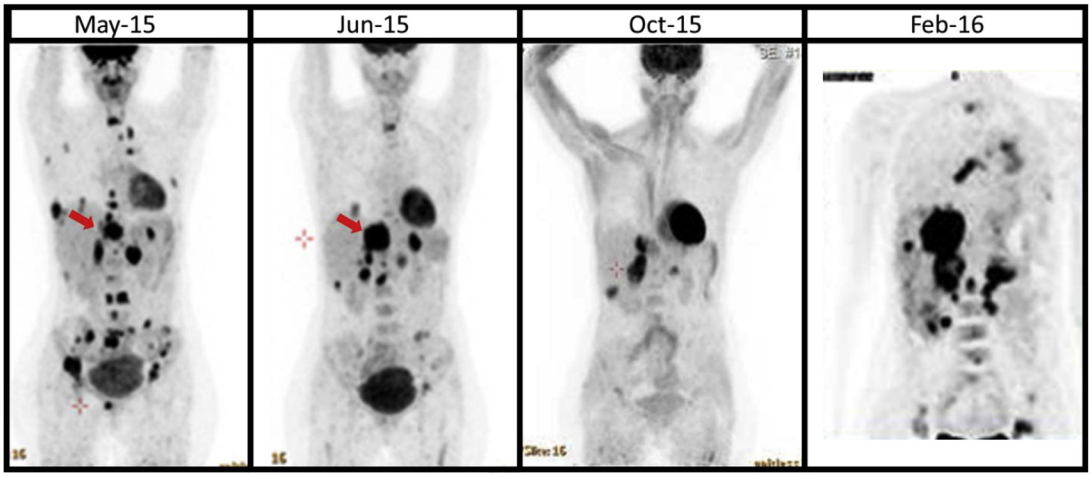

Figure 1. Response to treatment through positron emission tomography scan. Fludeoxyglucose F 18 positron emission tomography scans along the treatment journey, showing a rapid decrease in fludeoxyglucose F 18 uptake in most of the bone lesions within 2 weeks after initiation of osimertinib therapy. The liver lesion (*red arrow*), which was T790M negative, did not respond; a partial response to the drug combination and subsequent severe progression followed.

lesions and an activating *EGFR* exon 19 deletion (ex19del) mutation was identified by bronchoscopy and polymerase chain reaction (PCR). Whole brain radiotherapy was administered and gefitinib, 250 mg/d, was started, with a good response for 1 year. In March 2015, visceral and skeletal progression was noted and liver biopsy showed *EGFR* ex19del negative for T790M by PCR (Amoy Diagnostics, Xiamen, People's Republic of China). Immediately thereafter, complete exon sequencing of plasma cfDNA (Guardant360 [Guardant Health, Inc., Redwood City, CA]) was examined and showed multiple *EGFR* mutations: ex19del (mutant allele frequency [MAF] 19.5%), T790M (MAF 6.5%) and G724S (MAF 2.9%), which is a rare mutation. To confirm the results from the PCR liver biopsy, the liver specimen was further analyzed by hybrid capture–based

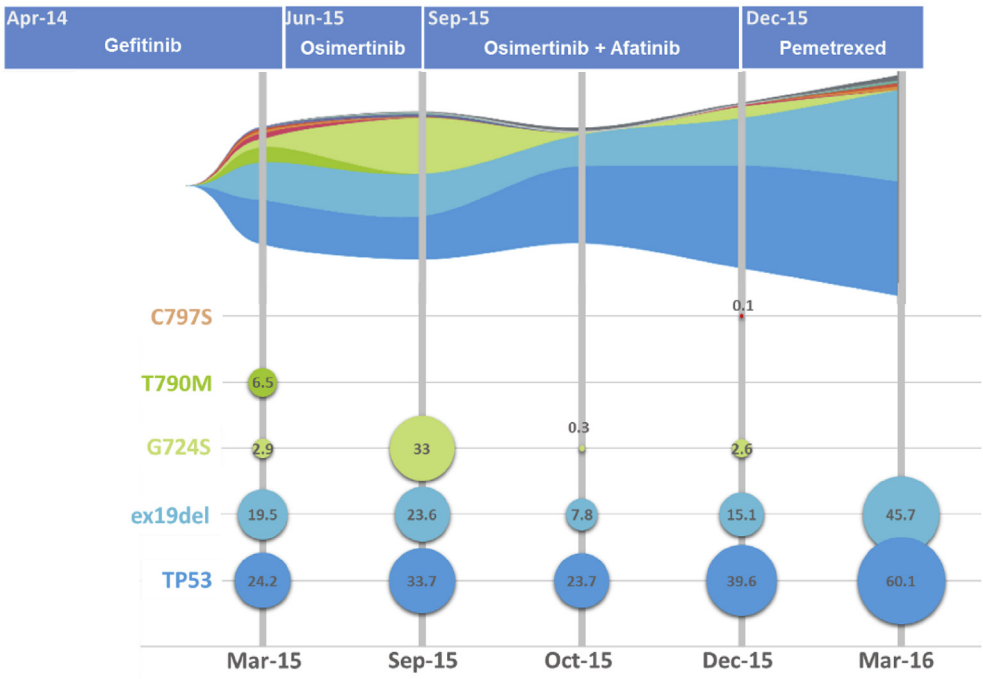

Figure 2. Cell-free DNA tumor response. Cell-free DNA analysis of total somatic alteration burden detected over five time points and the *EGFR* variant-specific results over time reflect responses to changes in matched therapy. TP53, tumor protein p53.

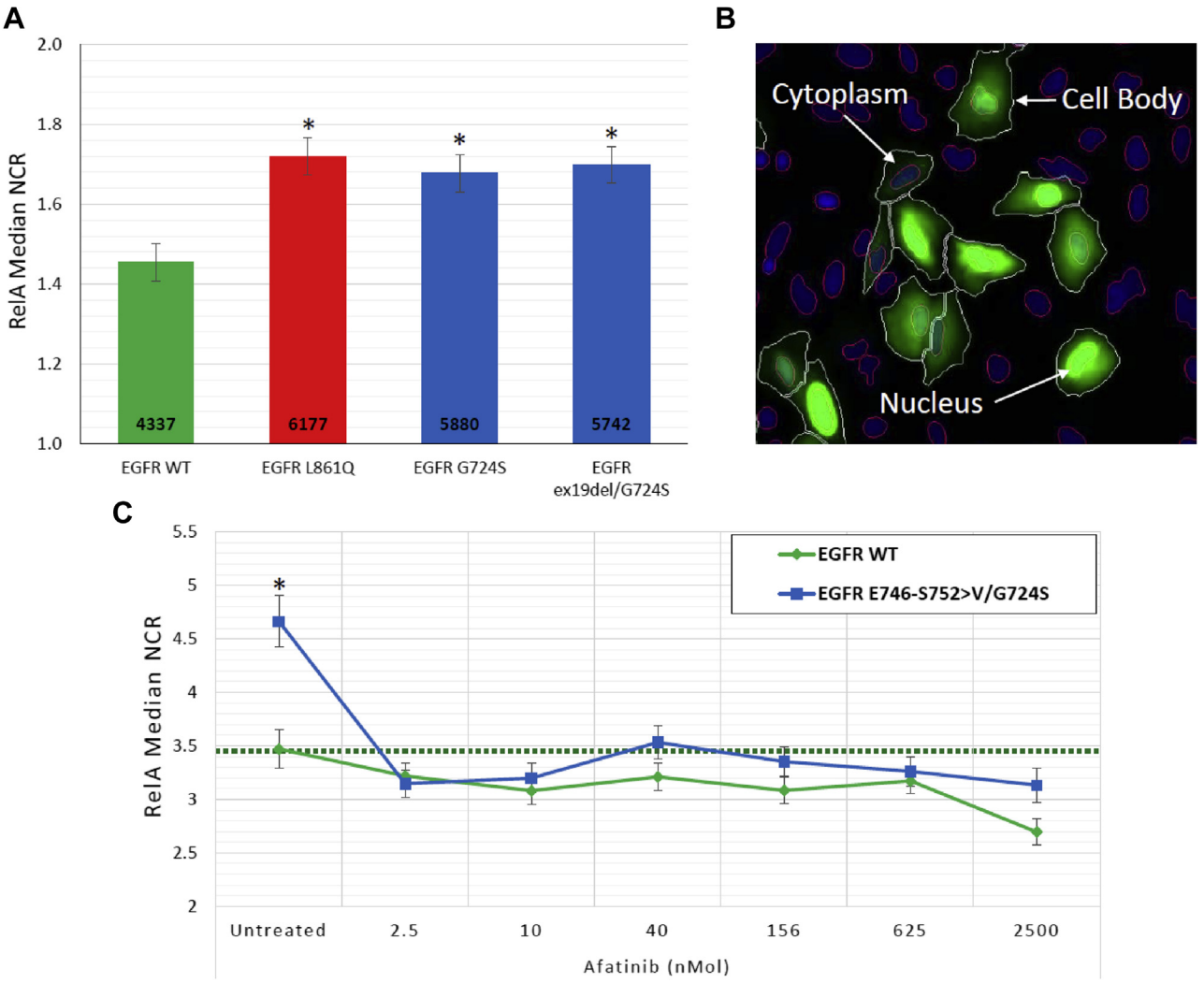

Figure 3. Functional analysis of EGFR alterations identified from cell-free DNA. Oncogenic activity of *EGFR* mutations was measured by using signaling pathways reporter for the nuclear factor kappa B subunit 1 (NFkB) pathway (RelA). Activation levels of the *EGFR* mutation G724S alone and in combination with E746-S752>V (exon 19) were significantly greater than wild-type (WT) activation ($p < 0.05$) and comparable to a known activating oncogenic mutation in *EGFR* (L861Q) (*A*). NFkB activation is represented in both graphs by the median nuclear-to-cytoplasmic ratio (NCR) for each condition. NCR values were measured by using a fluorescent microscope and an accompanying image analysis algorithm that is able to identify and segment the nuclei and cell border of the imaged cells. NCR is a ratio of the measured fluorescent reporter (RelA) in the nuclear (active) state versus in the cytoplasm. Presented is a representative segmented microscopy image with relevant labels (*B*). NFkB activation of the *EGFR* double-mutant E746-S752>V/G724S NCR was also measured after incubation with increasing concentrations of afatinib (in nmol). At baseline, activation by the *EGFR* double-mutant E746-S752>V/G724S was significantly greater than by the WT ($p < 0.05$). NFkB activation was inhibited to WT levels with 2.5 nmol afatinib, which persisted with escalating doses (*C*). Functional analysis of EGFR activity was characterized by using an in vitro platform. The analyzed mutations were synthesized on a WT expression vector backbone and transfected into HeLa cells along with signaling pathway reporters. Presented in both panels is one representative experiment with multiple repeats. Significance was calculated by Student's *t* test and is indicated with an asterisk accompanied by SE bars. The number of the cells analyzed is shown on the graph (*A*) at the base of each bar.

next-generation sequencing (FoundationOne [Foundation Medicine, Cambridge, MA]), indicating *EGFR* amplification, ex19del, and existence of the rare EGFR mutation G724S and negativity for T790M mutation.

In June 2015 osimertinib, 80 mg/d, was initiated, with a rapid clinical improvement and improvement on fludeoxyglucose F 18 positron emission tomography/computed tomography imaging. However, the liver lesion did not respond (Fig. 1) and notable lack of T790M mutation in this lesion was confirmed both by PCR and hybrid capture–based next-generation sequencing.

During osimertinib therapy the T790M clone was abrogated but the G724S clone became dominant according to plasma cfDNA testing (Fig. 2 [Sep-15]).

To target this rare mutation, on the basis of previous reports,[1,2] afatinib, 20 mg/d, was added to osimertinib, 80 mg/d, with a significant reduction in the G724S clone according to plasma cfDNA testing (from 33.7% to 0.3%) (Fig. 2 [Oct-15]) and the accompanying response on fludeoxyglucose F 18 positron emission tomography/computed tomography imaging after 1 month of treatment (see Fig. 1).[3] The drug combination was very well tolerated, with grade II skin toxicity.

After continued progression of the liver lesion (see Fig. 1), repeat plasma cfDNA testing revealed a new clone (C797S [0.1%]) typical of acquired resistance to osimertinib that was accompanied by an increase in the level of all the other mutations, including G724S. The patient was then treated with a reduced dose of pemetrexed, which efficiently reduced the levels of C797S and G724S mutations according to plasma cfDNA (see Fig. 2), but her total cfDNA increased and there was no clinical benefit; she died as a result of disease progression and pneumonia in April 2016.

To understand the significance of these uncommon mutations, functional studies were conducted (NovellusDx, Jerusalem, Israel) and revealed that the G724S mutation alone and in combination with ex19del overactivated nuclear factor kappa B subunit 1 signaling (Fig. 3*A*). This assay was then used to model the activity of afatinib, which showed effective inhibition and restored wild-type levels of nuclear factor kappa B subunit 1 signaling (Fig. 3*C*), supporting the response and cfDNA dynamics seen in this patient.

This patient exemplifies tumor heterogeneity, subclonal evolution under EGFR TKI therapy, integration of cfDNA into clinical practice, and combination therapy by two EGFR TKIs. Here we present how, in contrast to tissue biopsy (which reflects only one specific disease site), cfDNA testing can provide a global summary of multiple metastatic lesions in oncogene-driven NSCLC by highlighting distinct *EGFR* variant clones and revealing subclonal tumor evolution under the influence of targeted therapy. Furthermore, this case demonstrates how this emerging technology can be used to facilitate an individualized and noninvasive approach to therapeutic decision making.

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