Biomarker Analyses

KRAS and *EGFR* Mutational Analysis

KRAS and EGFR mutational status were determined by direct sequence analysis of genomic DNA extracted from formalin-fixed, paraffin embedded tumor samples (Caris Life Sciences, Irving, TX). The limit of sensitivity for dideoxy sequencing of DNA isolated from formalin-fixed, paraffin embedded tissues is approximately 20% mutant DNA in a background of wild-type. For *KRAS*, custom primers were designed to flank, amplify and sequence codons 12, 13 and 61 in exons 2 and 3 of the *KRAS* gene. 9 nucleotides in these 3 regions were evaluated for the presence of non-synonymous alterations that would result in an amino acid changes. For *EGFR,* custom nested M13 linked primers were designed to flank, amplify and sequence exons 18-21 of the *EGFR* gene.

Gene Mutational Analysis

Gene mutational analysis was carried out on DNA extracted from archived tumor samples on the Sequenom MassARRAY platform (53 genes; 649 mutations) according to the manufacturer's protocol; assays were conducted by the Translational Research Laboratory at Oregon Health & Science University (OHSU), Portland, OR.

EGFR gene amplification

Fluorescent in situ hybridization (FISH) was performed on deparaffinized tissue sections using Abbott probes for *EGFR* (LSI EGFR 7p12, red) with an identifier probe for the chromosome 7 centromeric region (7p11.1-q11.1 Alpha Satellite DNA, green). 100 interphase cells were scored for each sample. Assays were conducted by the OHSU Research Cytogenetics Laboratory, Portland, OR.

ALK translocation assays

Tissue samples from 23 subjects with EGFR wild-type/KRAS wild-type NSCLC had sufficient tumor tissue available for analysis of *ALK* translocations. Three different assays for detection of *ALK* translocations were evaluated.

Fluorescent in situ hybridization. FISH was performed by the OHSU Research Cytogenetics Laboratory, Portland, OR. Briefly, archived tumor tissue samples were deparaffinized and analyzed using the Abbott LSI *ALK* (2p23) break-apart probe according to the manufacturer's protocol; 100 interphase cells were scored for each sample.

Quantitative nuclease protection array (qNPA). An allele-specific qNPA assay was performed using high throughput genomics (HTG Molecular Diagnostics, Inc., Tucson, AZ). The array included probes for measurement of EML4-ALK variants 1, 2, 3a, 3b, 4, 5a, 5b, and 6, as well as *KIF5B-ALK* and *TFG-ALK* fusions, total ALK, total EML4, and the ALK 3'/5' ratio. *ANT, DDX5, GAPDH, HPRT1*, and *ABL1* probes were used as controls. Briefly, formalin-fixed, paraffin embedded tumor sections were scraped directly into HTG lysis buffer containing gene-specific probes. Following probe binding, S1 nuclease was added to the reaction to remove all unbound nucleic acid and excess qNPA probes; the reaction was stopped by addition of HTG stop solution. Detection of hybridized probes was accomplished by hybridizing the qNPA lysate to gene-specific hybridization probes spotted on a universal array plate, followed by addition of universal detection probes and a streptavidin-horseradish peroxidase conjugate. Following addition of a chemiluminescent substrate, relative gene expression was measured on a ^{Super}CAPELLA imaging system.

Real-time quantitative PCR (RT-qPCR). The Insight ALK Screen (Insight Genetics, Nashville, TN; [1]), an RT-qPCR assay which detects all possible *ALK* fusions regardless of the fusion partner, was performed by Clarient Inc. (Aliso Viejo, CA). The assay used a three-tube reaction series (plus controls) to measure expression of the extracellular segment of *ALK* (*ALK* WT), ALK kinase domain (*ALK* Kinase), and expression of an internal reference gene, Cytochrome c oxidase subunit 5B (*COX5B*). All three reactions were run on each individual tumor specimen. RNA extracted from an *EML4-ALK* variant 3a/3b expressing cell line was used as a positive control for the entire sample series. A total of 10ng of total RNA was used for each reaction. Using a one-step qPCR platform, each sample was run in duplicate to obtain mean Ct values. When RNA input was insufficient as determined by the internal control, results were reported as Quantity Not Sufficient (QNS).

[1] Hout D, Xue L, Choppa P, Handshoe J, Bloom K, Ross D, et al. Insight ALK Screen™, a highly sensitive and specific RT-qPCR first-line screening assay for comprehensive detection of all oncogenic anaplastic lymphoma kinase (ALK) fusions: clinical validation. Cancer Res 2011; 71(suppl): 2220 [abstr]