Novel Preclinical Patient-Derived Lung Cancer Models Reveal Inhibition of HER3 and MTOR Signaling as Therapeutic Strategies for NRG1 Fusion-Positive Cancers

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ABSTRACT

Introduction: NRG1 rearrangements produce chimeric ligands that subvert the ERBB pathway to drive tumorigenesis. A better understanding of the signaling networks that mediate transformation by NRG1 fusions is needed to inform effective therapeutic strategies. Unfortunately, this has been hampered by a paucity of patient-derived disease models that faithfully recapitulate this molecularly defined cancer subset.

Methods: Patient-derived xenograft (PDX) and cell line models were established from NRG1-rearranged lung adenocarcinoma samples. Transcriptomic, proteomic, and biochemical analyses were performed to identify activated pathways. Efficacy studies were conducted to evaluate HER3- and MTOR-directed therapies.

Results: We established a pair of PDX and cell line models of invasive mucinous lung adenocarcinoma (LUAD) (LUAD-0061AS3, SLC3A2-NRG1), representing the first reported paired in vitro and in vivo model of NRG1-driven tumors. Growth of LUAD-0061AS3 models was reduced by the anti-HER3 antibody GSK2849330. Transcriptomic profiling revealed activation of the MTOR pathway in lung tumor samples with NRG1 fusions. Phosphorylation of several MTOR effectors (S6 and 4EBP1) was higher in LUAD-0061AS3 cells compared with human bronchial epithelial cells and the breast cancer cell line MDA-MB-175-VII (DOC4-NRG1 fusion). Accordingly, LUAD-0061AS3 cells were more sensitive to MTOR inhibitors than MDA-MB-175-VII cells and targeting the MTOR pathway with rapamycin blocked growth of LUAD-0061AS3 PDX tumors in vivo. In contrast, MDA-MB-175-VII breast cancer cells had higher MAPK pathway activation and were more sensitive to MEK inhibition.

Conclusions: We identify the MTOR pathway as a candidate vulnerability in NRG1 fusion-positive lung adenocarcinoma that may warrant further preclinical evaluation, with the eventual goal of finding additional therapeutic options for patients in whom ERBB-directed therapy fails. Moreover, our results uncover heterogeneity in downstream
oncogenic signaling among NRG1-rearranged cancers, possibly tumor type-dependent, the therapeutic significance of which requires additional investigation.

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Keywords: NRG1 fusion; HER3 antibody; Lung adenocarcinoma; GSK2849330

Introduction

NRG1 fusions are recurrent but uncommon clinically actionable somatic alterations identified in 0.1% to 0.2% of all tumors. In the largest published study that looked at the distribution of NRG1 fusions among different cancer types, 41 of 21,858 tumors had a fusion and the incidences varied by tumor type—0.5% of gallbladder cancer, 0.5% of renal clear cell carcinoma, 0.5% of pancreatic cancer, 0.4% of ovarian cancer, 0.2% of sarcoma, and 0.2% of breast cancer. NRG1 fusions have also been identified in uterine cancer and head and neck cancer. Nevertheless, in absolute numbers, NSCLC accounts for the largest number of NRG1 fusion-positive cancers, representing 0.3% of NSCLC cases tested.

Although NRG1 fusions were first described in the 1990s, it was not until the recent emergence of the possibility of targeting NRG1 fusion-driven cancers that the biology of these fusions gained attention. Nearly all NRG1 gene fusions retain the EGF-like domain, which is essential for transformation by this chimeric oncogene. Binding of the EGF-like domain of NRG1 to ERBB3 in an autocrine or paracrine manner leads to formation and activation of an ERBB2-ERBB3 heterodimer, which then activates downstream signaling by means of the PI3K/AKT and RAS/MAPK pathways. Moreover, NRG1 has diverse physiological functions in different tissues, and it is unknown if tissue-specific dependencies exist in cancers arising from NRG1 fusions. One important limitation in studying NRG1 fusion-positive cancers is a paucity of patient-derived disease models that faithfully recapitulate the disease and are available to academic laboratories. To the best of our knowledge, there is only one NRG1 fusion-positive cell line that is widely available to researchers with a confirmed fusion (DOCA-NRG1 fusion in MDA-MB-175-VII).

All efforts made to date to identify and develop new therapies for NRG1-driven tumors have primarily focused on agents that block activation of the most upstream components of NRG1-activated pathways, such as ERBB3 and ERBB2. Our group has revealed that targeting ERBB3 with the anti-ERBB3 antibody GSK2849330 achieved a durable response in one patient with invasive mucinous adenocarcinoma of the lung harboring a CD7-NRG1 fusion. The pan-ERBB tyrosine kinase inhibitor (TKI), afatinib, has been used to treat NRG1 fusion-positive cancers with mixed outcomes. To date, more NRG1 fusion-positive patients have received afatinib than any other ERBB-targeted agents, with at least four patients revealing a partial response of more than 18 months. Several anti-ERBB2 agents were reported to partially reduce growth of xenograft tumors developed from an artificial system in which an NRG1 fusion was expressed in transformed lung cells. These studies suggest that there are multiple potential therapeutic targets for NRG1 fusion-driven lung cancer. Nevertheless, evaluating these therapies in more appropriate preclinical patient-derived models would yield more appropriate data that can determine the translational potential of specific agents.

Here, we report the generation and characterization of the first paired in vitro and in vivo model of NRG1-rearranged (SLC3A2-NRG1) lung cancer. We leveraged this model to study activated cell signaling pathways in a lung-specific context and report novel therapeutic vulnerabilities that can be explored for treatment of NRG1-driven malignancies in the context of resistance to current experimental anti-ERBB therapies.

Materials and Methods

Materials and Cell Lines

Cell culture growth media, antibiotics, and phosphate-buffered saline (PBS) were prepared by the MSK Media Preparation Core Facility. Fetal bovine serum (FBS) was procured from Atlanta Biologicals (Flowery Branch, GA). Primocin was purchased from InvivoGen (San Diego, CA). The immortalized, nontransformed breast epithelial cell line MCF10A was obtained from the American Type Culture Collection (Manassas, VA). All studies were conducted within 6 months of thawing the purchased cells, and cells were routinely tested for mycoplasma. Human bronchial epithelial cells (HBECS) were immortalized by overexpression of CDK4 and TERT (HBECC-3KT cell line) and were obtained from Dr. John Minna (UT South Western, TX). A p53 C-terminal mutant was then introduced into HBECC-3KT (HBECC-DNp53) as described previously, and these cells were used in this study. Details of antibodies raised against total or phosphorylated proteins used for Western blotting and immunohistochemistry (IHC) are given in Supplementary Table 1. Small molecule inhibitors were obtained from Sellckchem (Houston, TX). Reagents for polymerase chain reaction (PCR), Promega’s ApoOne Homogenous Caspase 3/7 activity assay kit, AlamarBlue viability dye, keratinocyte serum-free medium, tissue culture plastic wares,
and Western blotting reagents not listed elsewhere were obtained from Thermo Fisher Scientific (Waltham, MA). Precision plus protein kaleidoscope precasted molecular weight markers used for Western blotting was obtained from Bio-Rad (Hercules, CA). Mammary epithelial cell growth medium and supplements were purchased from Lonza (Portsmouth, NH). Protease inhibitor cocktail, radioimmunoprecipitation assay lysis buffer (×10), and all other chemicals not listed previously were purchased from EMD-Millipore Sigma (St. Louis, MO). All oligonucleotides used for PCR assays were custom designed by the authors and obtained from Integrated DNA technologies (Coralville, IA). The monoclonal anti-ERBB3 antibody GSK2849330 was provided by GlaxoSmithKline.

**Generation of Patient-Derived Xenograft Models and Cell Lines and Efficacy Studies**

Tissue samples were collected under an institutional review board–approved biospecimen collection protocol, and informed consent was obtained. All animals were monitored daily and cared for in accordance with guidelines approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee and Research Animal Resource Center. Pleural effusion fluid samples were obtained from thoracentesis procedures, and samples were collected in a sterile container in which heparin was added (10 USP units of heparin/mL fluid). Cells in the sample were collected by centrifugation (300 rcf for 5 min) in a tabletop centrifuge, washed twice with cold PBS, centrifuged again, and resuspended in Dulbecco’s modified Eagle’s medium (DMEM):F12 (1:1 ratio) growth medium supplemented with 10% FBS and 1% antibiotics (complete growth media). Then, 40 million cells were pelleted, mixed with 50% Matrigel (vol/vol), and then injected at 20 million cells per subcutaneous flank of female NSG mice to generate xenografts. After randomizing into groups of five, tumor-bearing animals were treated with vehicle, afatinib (5 mg/kg once daily [QD] on a 5 d on, 2 d off schedule), immunoglobulin G (IgG) (25 mg/kg twice daily [BIW]), GSK2849330 (25 mg/kg BIW), or rapamycin (1–4 mg/kg QD, on a 5 d on, 2 d off schedule) when tumors reached approximately 100 to 150 mm³ volume. GSK2849330 and IgG were diluted in PBS and administered by means of intraperitoneal injection. Afatinib was resuspended in 0.5% methylcellulose and 0.1% Tween-80 and administered by oral gavage. Rapamycin was resuspended in 2% dimethyl sulfoxide, 30% polyethylene glycol 300 and 5% Tween-80, and administered by means of intraperitoneal injection. Tumor size and body weight were measured twice weekly, and tumor volume was calculated using the modified ellipsoid formula $V = \frac{1}{2} \times \text{length} \times \text{width}^2 \times 0.52$.16

**Genomic Characterization of Preclinical Models**

Cell lines and PDX were profiled by the Memorial Sloan Kettering Cancer Center-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) platform, which is a large panel next-generation sequencing assay designed to detect mutations, copy-number alterations, and select fusions involving up to 505 cancer-associated genes.17 Paired analysis of PDX tissue or cell line and matched-normal sample was performed to unambiguously identify somatic mutations. MSK-IMPACT does not identify the SLC3A2-NRG1 fusion, and therefore, reverse-transcriptase PCR (RT-PCR) was used to confirm the presence of the fusion mRNA.

**Growth and Propagation of Cell Lines**

All cell lines were maintained in a humidified incubator infused with 5% carbon dioxide and subcultured when stock flasks reached approximately 75% confluence at a 1:3 dilution. The LUAD-0061AS3 cell line was maintained in DMEM:F12 growth medium supplemented with 10% FBS and 100 µg/mL primocin. MDA-MB-175-VII cells were maintained in DMEM:F12 growth medium supplemented with 20% FBS and 100 µg/mL primocin. MCF10A cells were maintained in mammary epithelial cell growth medium containing 52 µg/mL bovine pituitary extract, 500 ng/mL hydrocortisone, 10
ng/mL EGF, 5 μg/mL insulin, and 100 μg/mL primocin. HBEC-DNp53 cells were maintained in keratinocyte serum-free media supplemented with 50 μg/mL bovine pituitary extract, 5 ng/mL recombinant EGF, and 100 μg/mL primocin. For determination of growth kinetics and how it was affected by treatment, cells were plated at a density of 10,000 cells per well in 12-well plates, and 24 hours after plating (d0), the cells were placed in standard growth media with or without therapeutic compounds. The cells were counted on day 0 and then every 24 to 48 hours thereafter. Data points were fitted to an exponential growth equation using Prism 8 (GraphPad Software, San Diego, CA) to establish a growth curve.

**Tumor Type and IHC**

Hematoxylin and eosin-stained slides from the original patient tumor and PDX were closely inspected and compared. Unstained tissue sections on slides were deparaffinized and rehydrated. After antigens were retrieved, IHC staining for the following antibodies was performed: TTF1, p-HER3, Napsin, thyroglobulin, PAX8, WT1, PR, and ER. The antibody clones and suppliers are listed in Supplementary Table 1. A relevant positive control tissue was concurrently stained for each antibody.

**Cytology**

For cytologic examination of cell suspension, the cells were spread on a glass slide to form a thin and even monolayer, and the suspension was left to air-dry for 2 to 5 minutes. The slide was then immersed in 100% methanol for 2 minutes at room temperature to fix the sample. Fixed slides were stained by immersion into Liu solution A (30 s) followed by Liu solution B (90 s). Stained slides were gently rinsed with water and air-dried.

**Viability and Caspase 3/7 Assays**

For viability assays, the cells were plated in clear-bottom, white 96-well plates at a density of 7500 cells per well and incubated with compounds for 96 hours. The relative number of viable cells was determined using alamarBlue viability dye, and fluorescence was measured using a Molecular Devices SpectraMax M2 multimodal plate reader (Ex: 485 nm, Em: 530 nm). Data were analyzed by nonlinear regression and curves fitted using Prism 8 to generate concentration that inhibits 50% values (IC50). For caspase 3/7 activity, the cells were plated at a density of 20,000 cells per well directly into inhibitors in white, clear-bottom 96-well plates, grown for 48 hours and then caspase 3/7 enzymatic activity determined using Apo-One Homogenous caspase 3/7 activity assay kit (Promega) following the manufacturer’s instructions. All data are expressed relative to control values and an average of two to five independent experiments in which each condition was assayed in triplicate determinations.

**Preparation of Whole-Cell Extracts and Western Blotting**

Protein levels and phosphorylation state were detected by Western blotting. Cells were lysed in 1× radioimmunoprecipitation assay lysis buffer containing phosphatase and protease inhibitors. Lysates were denatured in 2× sample buffer at 55°C for 15 minutes, resolved on 4% to 12% NuPAGE gels (Invitrogen), and transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 3% bovine serum albumin in Tris-buffered saline supplemented with 0.1% Tween-20 (vol/vol) for 1 hour at room temperature and probed with primary antibodies with specificity as outlined in Supplementary Table 1. Bound antibodies were detected with peroxidase-labeled goat antibody raised to mouse or rabbit IgG (R&D Systems, Minneapolis, MN) and imaged with enhanced chemiluminescence Western blotting detection reagent (GE Healthcare). Images were captured on radiograph films. Western blotting was conducted at least two times from independently prepared samples. Films with bands in the linear range of exposure were scanned, and bands quantitated by densitometry using ImageJ software (http://imagej.nih.gov/ij/) and all values are expressed relative to vehicle-treated controls.

**Proteome Profiling Arrays**

We used a human proteome profiling array system (R&D Systems) that contain duplicate validated positive and negative controls and capture antibodies that can simultaneously detect the phosphorylation state of 43 human kinases (Proteome Profiler Human Phospho-Kinase Array kit). A total of 5 million cells were plated in 10-cm dishes and grown for 48 hours. Cells were deprived of serum by culturing for 24 hours in growth media supplemented with 0.05% FBS and then treated with 1 μM GSK2849330 or afatinib for 0.5 hours; detection of protein phosphorylation was carried out according to the manufacturer’s instructions. In brief, the array membranes were blocked, incubated with 350 μg total cellular protein per array overnight at 4°C on a rocking platform, washed, and incubated with phospho-specific detection antibodies. Captured phosphorylated proteins were detected by enhanced chemiluminescence and imaged on X-ray films. The average pixel densities of duplicate spots were measured using ImageJ software.
RT-PCR and Quantitative PCR Experiments

For detection of the SLC3A2-NRG1 fusion transcript and RPTOR expression in the cell lines and PDX, RNA was extracted using a Qiagen RNA mini kit, and complementary DNAs (cDNAs) were synthesized using SuperScript IV VILO (Thermo Fisher Scientific) according to the manufacturer’s instructions. The SLC3A2-NRG1 fusion was detected by RT-PCR using 5'-ATGCTTGCTGGTGCGTGGTCA-3' (forward, SLC3A2 exon 4) and 5'-GGTCTTTCCACCATGAAGCACTCCTCC-3' (reverse, NRG1 exon 6) primers. For quantitative PCR (qPCR) of RPTOR and GAPDH, the TaqMan assays Hs00375332_m1 and Hs02786624_g1 were used, respectively. Three replicates were produced for each cell line and probe combination. RPTOR expression values were normalized to GAPDH expression level and compared with HBE-DNP53. For detection of SLC3A2-NRG1 mRNA expression, RNA was isolated from rapamycin-treated cells (24 h), cDNAs were synthesized as described above, and then qPCR was performed using the primers listed above for SLC3A2 and NRG1 and SYBR Green PCR master mix (Thermo Fisher Scientific).

Analysis of RNA Expression Data

Gene expression data from The Cancer Genome Atlas (TCGA) were retrieved from a public genomics data repository Gene Expression Omnibus (accession GSE62944).22 Data were normalized and analyzed using DESeq2 package in the R programming environment. Gene Set Enrichment Analysis was conducted and visualized using clusterProfiler R package. In addition, ggplot2 R package was used for data visualization.

Statistical Analysis

Student’s t test was used to compare caspase activity or protein phosphorylation. For animal studies, area under curve (AUC) analysis was used to compare the average tumor volume between groups. Briefly, AUC values and their SEs were computed as an estimation of the surface area between baseline values (mean value of the tumor volumes at the beginning of the treatment) and growth curves for vehicle and each treatment conditions. Regression of tumor volume below the baseline value is indicated by negative AUC values. Treatment response was compared with the vehicle group using one-way ANOVA test. All data were plotted and analyzed using Prism 8. p value less than 0.05 was considered significant.

Results

Patient History

The treatment history of the patient from which the models were generated is depicted in Figure 1A. This 37-year-old female never-smoker presented with hemoptysis and back pain. She was found to have a right upper lobe mass, extensive mediastinal and hilar adenopathy, right pleural effusion, and distant disease, including widespread osseous lesions, peritoneal carcinomatosis with ascites, and a large cystic adrenal mass, on cross-sectional imaging. Biopsy results of the right upper lobe and the iliac bone were consistent with those of LUAD with acinar and cribriform patterns. IHC analysis revealed that the tumor was positive for TTF1 and napsin-A and negative for PAX8 and WT1, confirming the diagnosis. An SLC3A2-NRG1 fusion was detected by means of targeted RNA sequencing using MSK-Fusion Solid.20,21 In addition, a DNA-based exon capture assay (MSK-IMPACT) was performed to profile the tumor sample.22,23 Results are summarized in Figure 1A. The patient underwent palliative intent radiation to painful skeletal lesions followed by systemic therapy with four cycles of carboplatin, pemetrexed, and pembrolizumab with a mixed response. She was continued on maintenance pemetrexed and pembrolizumab for four months. Therapy was then switched to afatinib with docetaxel and ramucirumab. Docetaxel was discontinued after two cycles owing to an infusion reaction, and ultimately, she continued on afatinib and ramucirumab for four months followed by widespread progression, including multiple new brain metastases. The patient was started on adotrastuzumab emtansine (T-DM1) but passed away after one cycle at 11.4 months after initial diagnosis, likely owing to disease progression.

Establishment of Patient-Derived Lung Cancer Models With NRG1 Fusion

Five attempts were made to establish a PDX from the tissues obtained from this patient. Although three attempts (two from circulating tumor cells and one from ascites fluid) failed, we successfully established two different PDX models from pleural effusion samples, LUAD-0061AS3pdx and LUAD-0061AS4pdx. These samples were collected approximately 6 weeks apart. Both models grew robustly, as found in Figure 1B and C. The models took approximately 40 (LUAD-0061AS3pdx) and 60 (LUAD-0061AS4) days before palpable tumors were detected, respectively, after the first implantation. Nevertheless, after the first transplant, the tumors grew much faster. To date, LUAD-0061AS3pdx has continuously grown for 14 passages, with tumors cryopreserved at every transplant, and this model was used in this
Figure 1. Generation of a lung cancer PDX model with NRG1 fusion. (A) Patient history revealing major therapeutic interventions, results of molecular diagnostics, and time points of PDX establishment. (B, C) Growth curves of the first five serial passages revealed continuous growth of the xenograft models (B) LUAD-0061AS3 and (C) LUAD-0061AS4. CT, computed tomography; LUAD, lung adenocarcinoma; MSK-IMPACT, Memorial Sloan Kettering Cancer Center-Integrated Mutation Profiling of Actionable Cancer Target; PDX, patient-derived xenograft; RT, radiotherapy.
Figure 2. Histopathologic characterization of the LUAD-0061AS3 PDX and sensitivity to ERBB therapy. (A) HE-stained samples revealed a typical appearance of mucinous adenocarcinoma cells with abundant eosinophilic cytoplasm and eccentric nuclei forming gland-like structures filled with mucin. IHC staining of TTF1 was performed to compare with the original patient sample. (B) Cytopathologic characterization was performed for an attached cell culture of LUAD-0061AS3cl (unstained, phase contrast microscopy) and stained cell suspension (Liu stain). (C) SLC3A2-NRG1 fusion-specific PCR was performed using cDNA.
study. LUAD-0061AS4pdx was cryopreserved after five serial passages and not used for any studies.

To validate the LUAD-0061AS3 PDX model, we performed histopathological characterization of the xenograft tissue. Hematoxylin and eosin-stained samples revealed a typical appearance of mucinous adenocarcinoma cells with abundant eosinophilic cytoplasm and eccentric nuclei forming gland-like structures filled with mucin (Fig. 2A, left). This histopathology closely recapitulated that of the original patient sample (Fig. 2A, right). Furthermore, we performed IHC staining for common differential diagnostic markers, and as expected, thyroglobulin, WT1, PAX8, PR, and ER were negative, whereas TTF1 displayed a strong positive signal (Fig. 2A and Supplementary Fig. 1). Staining for napsin-A, which was weakly positive in the original patient sample, was negative in the PDX tissue, likely owing to suboptimal activity of the mouse antinapsin-A antibody in murine xenograft tissue (Supplementary Fig. 1). Phospho-ERBB3 staining revealed membranous positivity, consistent with previous reports of this immunostaining pattern in NRG1-rearranged invasive mucinous adenocarcinoma (Supplementary Fig. 1).

In addition to the two PDX models, we established a cell line from the LUAD-0061AS3 PDX tissue. The cell line has shown continuous growth without any signs of senescence for the past 15 months. Inspection of unstained attached cell line culture by phase-contrast microscopy revealed highly atypical cells with foci of multilayer growth in spots of high cellular density, a sign of tumorigenicity. Atypical cells were also observed on the stained cytology slide (Fig. 2B). RT-PCR confirmed the presence of the SLC3A2-NRG1 fusion in the PDX tissue and the cell line (Fig. 2C). Genomic profiling of the cell line and PDX models were performed using the latest version of MSK-IMPACT, which profiles 505 genes for somatic alterations, and compared. The three disease models retained the TP53 and CDKN2A mutations that were detected in the patient sample (Supplementary Table 2). The SLC3A2-NRG1 fusion is not detected by MSK-IMPACT but, as noted previously, was confirmed by RT-PCR (Fig. 2C).

**GSK2849330 Efficiently Reduces Growth of the NRG1 Fusion-Positive Models In Vitro**

Here, we examined the effect of GSK2849330 on growth of LUAD-0061AS3cl and MDA-MB-175-VII cells in time-course experiments. As a control for growth inhibition, we used the pan-ERBB TKI afatinib. Cells were treated with either GSK2849330 (0.1 or 1 μM) or afatinib (0.05 μM) for 9 days and the number of cells counted at the time points indicated in Figure 2D and E. GSK2849330 treatment inhibited growth to a similar extent in both cell lines (Fig. 2D and E). This inhibition of growth was equivalent to that observed after afatinib treatment (Fig. 2D and E). The high sensitivity of the LUAD-0061AS3 cell line to higher dose afatinib in vitro contrasts with the poor response of the patient to the standard clinical dosing. Studies performed in vitro are not subject to systemic toxicity constraints; hence, higher dose of afatinib seems as an effective agent in vitro. Alternatively, given that the PDX and cell line models were developed in the absence of afatinib in the course of several months, it is possible that afatinib-sensitive clones could have re-emerged to dominate the culture.

**Characterization of Activated Intracellular Signaling Pathways in NRG1 Fusion-Positive Cells**

Little is known about the signaling pathways activated in NRG1-rearranged tumors. Although some studies have attempted to address this, all studies were performed in either murine cells or transformed lung cells, in which NRG1 fusions were ectopically expressed, likely at supraphysiological levels. As changes in intracellular signaling are reflected downstream in specific transcriptional signatures, we decided to analyze available gene expression data to define prominent gene expression signatures in NRG1-rearranged lung tumors. We compared three NRG1-rearranged lung carcinomas (two squamous carcinomas, one adenocarcinoma) with normal lung tissue RNA sequencing data from TCGA data set. Using principal component analysis, we found that NRG1-rearranged tumors were grouped separately from healthy lung tissue samples as expected (Fig. 3A). Differential gene expression analysis found 6455 (29%) and 4852 (22%) significantly up-regulated and down-regulated transcripts, respectively, with NRG1 being in the top of the list of up-regulated genes (log2 fold change: 4.1, adjusted p < 0.0001). To classify this output, we ranked the list of differentially expressed genes and performed Gene Set Enrichment Analysis. Figure 3B illustrates the top 40 most significant expression signatures (ranked by p value). This analysis revealed...
Figure 3. Identification and characterization of activated signaling pathways in NRG1 fusion-driven lung cancers. (A) Principal component analysis revealed distinctive grouping of NRG1-rearranged lung tumor samples. (B) GSEA analysis was performed to compare NRG1-rearranged lung cancer samples and healthy lung tissue gene expression (TCGA LUAD and LUSC...
activation of multiple facets of oncogenesis: G2-M progression and mitotic spindle, E2F and MYC, glycolysis, etc. As for intracellular kinase signaling, the AKT/MTOR pathway was the most prominent expression signature present, with consistent up- and down-regulation of corresponding gene sets (Fig. 3C and Supplementary Fig. 2B).

As noted previously, most studies to date on NRG1 fusion-driven biology have used isogenic cancer cell lines that overexpress cDNAs encoding NRG1 fusions. Indeed, the only patient-derived cell line in which NRG1 signaling has been investigated is the breast cancer cell line MDA-MB-175-VII that expresses a DOC4-NRG1 fusion. It is believed that the EGF-like domain of NRG1 directly interacts with ERBB3 and ERBB4 to activate ERBB-family receptors and trigger a downstream cascade of phosphorylation and activation of effector proteins. Here, we sought to validate the data obtained in Figure 3A and C and to identify additional activated signaling pathways in NRG1 fusion-positive cell lines, using LUAD-0061AS3 and MDA-MB-175-VII cells. As comparisons, we used immortalized, untransformed HBECs (HBEC-DNP53) and MCF10A breast epithelial cells. Western blotting analysis with phosphospecific antibodies revealed that EGFR, ERBB2, ERBB3, and ERBB4 were all highly phosphorylated in the two NRG1 fusion-positive cell lines compared with the respective tissue-matched control lines (Fig. 3D). Similarly, the NRG1 fusion-positive cell lines had higher level of phosphorylation of multiple effectors of the RAS-MAPK (ERK, MEK, BRAF), AKT-PI3K (AKT, p70S6K, 4EBP1), and STAT pathways (STAT3). The increased phosphorylation of AKT, p70S6K, 4EBP1, and RICTOR, a negative regulator of PI3K, in LUAD-0061AS3 cells compared with the HBEC-DNP53 cells confirmed the signaling changes inferred from the transcriptomic profiling data. The LUAD-0061AS3 cells had lower levels of phosphorylated AKT and phospho-BRAF than MDA-MB-175-VII 0061AS3 cells.

Phosphoproteomic Profiling of NRG1 Fusion-Associated Intracellular Signaling Downstream of ERBB3

Uncontrolled activation of the ERBB-family receptors and downstream signaling presents a target for biology-oriented therapy of NRG1 fusion-driven tumors. Currently, multiple ERBB-family TKI or antibodies are available for the treatment of NRG1-rearranged tumors, with some being tested in clinical trials (NCT04100694, NCT02912949, NCT03805841). Despite this, little is known on how downstream signaling is affected by therapy in lung malignancies with NRG1 fusions. We have previously reported that the anti-ERBB3 antibody GSK2849330 was efficacious in a patient with lung cancer with a CD74-NRG1 rearrangement. Here, we used GSK2849330 to attempt to elucidate the signaling networks that are used by ERBB3 to drive growth in the SLC3A2-NRG1–expressing LUAD-0061AS3 cell line. To this end, we used proteomic arrays (a schematic outline of the experimental design is given in Fig. 4A), as reported previously.14 This uncovered significant inhibition of multiple effectors in the AKT-PI3K pathway (Fig. 4B). In contrast, phosphorylation of ERK was inhibited less prominently (phospho-ERK, p = 0.035; 0.1 μM, p = 0.081). The complete data are found in Supplementary Figure 3. To confirm the results obtained by phosphoproteomic profiling, we performed Western blotting analysis of cell extracts obtained from cells treated with multiple concentrations of GSK2849330. We observed dose-dependent inhibition of multiple kinases, including ERBB2, ERBB3, ERBB4, AKT, p70 S6K, STAT3, and ERK (Fig. 4C). Some PI3K effectors, such as p70 S6 kinase, S6, and 4EBP1, were less readily inhibited in LUAD-0061AS3 cells compared with MDA-MB-175-VII, perhaps owing to higher intrinsic activity of PI3K in these cells. Of note, the inhibition of ERK and STAT3 phosphorylation after GSK2849330 treatment was less prominent than inhibition of AKT in both cell lines.

Inhibition of PI3K But Not MEK Decreases Growth of LUAD Cells With NRG1 Fusion

On the basis of the proteomic and transcriptomic evidence of activation of AKT-PI3K pathways described previously, we hypothesized that PI3K inhibition can be effective in suppressing growth of tumor cells with NRG1 rearrangements. As revealed in Figure 5A and B, rapamycin and everolimus, two PI3K inhibitors, decreased viability of LUAD-0061AS3 cells at low nanomolar concentrations, achieving 75% to 90% reduction in growth. In contrast, rapamycin and everolimus only partially inhibited the growth of MDA-MB-175-VII cells. The two...
cell lines exhibited similar sensitivity to two PI3K inhibitors, alpelisib and pictilisib (Fig. 5E and F). We next tested the effect of two pan AKT inhibitors on growth of the NRG1 fusion-positive cell lines. Growth of LUAD-0061AS3 and MDA-MB-175-VII cells was only affected at very high concentrations of AKT inhibitors (Supplementary Fig. 4) beyond that which was found to inhibit growth of AKT-dependent cells. Rapamycin treatment induced higher levels of caspase 3/7 activation in LUAD-0061AS3 cells than in the MDA-MB-175-VII cell line (Fig. 5C). Although a low-level RPTOR copy-number gain was identified in the patient sample by MSK-IMPACT analysis, we did not detect RPTOR overexpression at the transcriptomic or proteomic level (Supplementary Fig. 4A and Fig. 3D, respectively), so it is unlikely to explain the increased sensitivity to MTOR inhibitors. LUAD-0061AS3 cells had lower sensitivity to the MEK inhibitor trametinib (Fig. 5D) compared with MDA-MB-175-VII, and this correlated with the lower level of MAPK pathway activation (Figs. 3D and 5G).

Rapamycin Effectively Inhibits Growth of LUAD-0061AS3 Models In Vivo

To begin to translate our in vitro findings revealing increased sensitivity of LUAD-0061AS3 cells to MTOR inhibitors, we evaluated the efficacy of rapamycin in the LUAD-0061AS3 PDX model in comparison to afatinib. The data are presented in Figure 6 with tumor volumes

Figure 4. Phosphoproteomic profiling of intracellular signaling pathways. (A) Schematic outline of the experiment. (B) Volcano plot of phospho-kinase array data obtained in LUAD-0061AS3 cells treated with 1 µM GSK2849330 for 3 hours. The points that reached significance (α = 0.05) are highlighted in red. AKT-MTOR and SRC pathway effectors are labeled. (C) Western blot validation of protein kinase array results in LUAD-0061AS3 (left) and MDA-MB-175-VII (right) cell lines. Cells were treated for 3 hours with GSK2849330, and then extracts were prepared for Western blotting. Representative immunoblots from two independent experiments are shown. LUAD, lung adenocarcinoma; p-, phospho.-
in the course of treatment found in Figure 6A and the percent change in volume of each tumor found in Figure 6B for afatinib and rapamycin. All doses of rapamycin (1, 2, and 4 mg/kg, QD) were more effective at controlling tumor growth than afatinib when used at a dose in mice (5 mg/kg QD), which is similar to the clinical dose (Fig. 6A). The highest dose of rapamycin tested resulted in regression of two of the five tumors (Fig. 6B). We used AUC to compare the effect of treatment between the groups as this analysis considers the magnitude and duration of the treatment effect (Supplementary Fig. 6A). As revealed, all treatments caused a significant decrease in tumor growth (Supplementary Fig. 6A) when compared with the vehicle (calculated on d11 when all tumor-bearing animals were alive). To better compare afatinib to rapamycin, the AUC values were calculated on d19, which was the last treatment with afatinib (Fig. 6B, numbers
Figure 6. Rapa treatment inhibits growth of LUAD-0061AS3 PDX tumors and reduce expression of the SLC3A2-NRG1 fusion. LUAD-0061AS3 PDX tumors were implanted into a subcutaneous flank of NSG mice and treated as indicated on the graphs. Tumor volume and animal weight were measured twice weekly. Animal weight is given in Supplementary Figure 6. (A, C) Tumor volume over time and (B, D) change in tumor volume are shown. Each group consisted of five animals, and data represent the mean ± SE of tumor volumes. AUC analysis was used to compare treatment effects, and the AUC ± SE values are shown above the bars in B and D for days 19 and 20, respectively. Additional AUC data are provided in Supplementary Figure 6 for days 11 and 12, respectively. (E-G) LUAD-0061AS3 cells were treated with the indicated concentrations of Rapa for 24 hours and then either (E) whole-cell extracts were prepared for Western blotting. (F) SLC3A2-NRG1 levels in comparison to cell viability and phosphorylation of p70S6K as a function of Rapa concentration. Immunoblots were quantitated by
above the bars). This analysis revealed that the three doses of rapamycin were significantly better than afatinib at blocking growth of LUAD-0061AS3 PDX tumors. Although afatinib caused a significant reduction in tumor volume compared with vehicle-treated tumors, all tumors continued to grow slowly in the afatinib-treated group (Fig. 6A). A higher dose of afatinib (25 mg/kg QD) was more effective than the 5 mg/kg QD dose at inhibiting tumor growth (89.4 ± 0.7% tumor shrinkage, p < 0.0001); however, this dosage resulted in 12% loss in animal weight (p = 0.004) and is unlikely to be achieved clinically. There was no reduction in animal weight, and no toxicity was observed in any of the treatment groups (Supplementary Fig. 6C).

We next attempted to determine if the antitumor effect of rapamycin might be enhanced by combination with the anti-HER3 therapeutic antibody GSK2849330. The data are presented in Figure 6C and D with tumor volumes in the course of treatment shown in Figure 6C and the percentage change in volume of each tumor given in Figure 6D for GSK2849330, rapamycin, or the combination of the two agents. As discussed previously, we used AUC to compare the effect of treatment between the groups. Administration of a control IgG did not significantly alter growth of LUAD-0061AS3 PDX tumors compared with PBS vehicle treatment (Fig. 6C and Supplementary Fig. 6B revealing AUC for d12). GSK2849330 (25 mg/kg BIW) treatment resulted in a significant reduction in tumor growth compared with IgG-treated tumors (Supplementary Fig. 6B, p < 0.0001). Nevertheless, rapamycin (2 mg/kg QD) was more effective at blocking growth of LUAD-0061AS3 PDX tumors than GSK2849330 (Fig. 6D). A combination of rapamycin (2 mg/kg QD) and GSK2849330 (25 mg/kg BIW) was not more effective than rapamycin treatment alone (Fig. 6D and Supplementary Fig. 6B). There was no reduction in animal weight, and no toxicity was observed in any of the treatment groups (Supplementary Fig. 6D).

Expression of SLC3A2-NRG1 Fusion Is Dependent on MTOR Activity

To explore potential mechanisms by which rapamycin treatment inhibited growth of the LUAD-0061AS3 cell line and PDX tumors, we evaluated the effect of the drug on expression of the SLC3A2-NRG1 fusion. Cells were treated for 24 hours with various concentrations of rapamycin, and then the level of SLC3A2-NRG1 fusion protein (Western blotting) or SLC3A2-NRG1 mRNA (qPCR) was determined. Rapamycin treatment caused a significant reduction in expression of SLC3A2-NRG1 fusion protein at concentrations that correlated with inhibition of P70S6K and S6 phosphorylation (Fig. 6E). Quantitation of the immunoblots by densitometry illustrated that down-regulation of SLC3A2-NRG1 expression after rapamycin treatment also correlated with loss of cell viability (Fig. 6F). Treatment of cells with rapamycin did not cause a reduction in SLC3A2-NRG1 fusion mRNA level (Fig. 6G).

Discussion

Although an NRG1 fusion was first described in 1999 in a breast cancer cell line, it was not until 2014 that the first observation of lung cancers harboring this genetic alteration was reported. The subsequent discovery of numerous tumor types with NRG1 rearrangements using next-generation sequencing-based techniques prompted the evaluation of targeted therapies for this subset of molecularly defined tumors. As the ERBB-family receptors represent direct binding partners of NRG1, multiple agents that antagonize the ERBB-family are being evaluated, some with promising results. In the two largest analyses of data looking for cancer drivers, NRG1 fusions were identified in approximately 0.3% of NSCLC. On the basis of an estimated diagnosis of 2.1 million lung cancer cases in 2018 (1.76 million NSCLC), more than 5000 patients with NSCLC per year would benefit from therapy for this group of lung cancers. In addition, NRG1 fusions have been found to cooccur with ALK fusion, and in one study, the NRG1 fusion was found to arise after resistance to ALK inhibitor treatment, suggesting there are additional patients who may benefit from therapeutically targeting NRG1 fusions in the setting of resistance to targeted therapy.

Investigation of the mechanisms of NRG1-induced tumorigenesis is lagging behind the therapeutic work, largely owing to a paucity of preclinical models that faithfully represent this molecular subset of lung cancers. Fernandez-Cuesta et al. reported expression of CD74-NRG1 fusions in invasive mucinous adenocarcinomas of lung. Ectopic expression of the NRG1 fusion in two lung cancer cell lines (H322 and H1568) revealed increased levels of phospho-ERBB2, phospho-ERBB3, phospho-AKT, and phospho-P70S6K. H1568 cells ectopically expressing a CD74-NRG1 fusion exhibited...
enhanced colony formation in soft-agar assays.\textsuperscript{5} Coculture of NIH-3T3 cells ectopically expressing CD74-NRG1 with Ba/F3 cells genetically engineered to overexpress human ERBB2 and ERBB3 also led to activation of AKT. Likewise, Murayama et al.\textsuperscript{26} reported activation of PI3K/AKT/NF-κB signaling pathways and induction of an IGF2-IGF1R autocrine/paracrine circuit on ectopic expression of a CD74-NRG1 transcript in lung (H322) and breast cancer (BT20) cell lines. Nevertheless, expression of the CD74-NRG1 cDNA in H322 cells did not enhance tumor growth in mice. The limitation of interpreting these published results is that NRG1 fusions were introduced into systems that already had fully activated transforming genetic alterations, perhaps blunting the effect of an additional oncogene, thereby making it difficult to delineate NRG1 fusion-specific biology. Although these isogenic comparisons in cancer cell lines have been somewhat informative, further confirmation of the findings has been hampered by a lack of patient-derived models with NRG1 rearrangements. To address this urgent need, we established and characterized a novel paired in vitro and in vivo model, LUAD-0061AS3, of NRG1 fusion-positive lung invasive mucinous adenocarcinoma harboring an SLC3A2-NRG1 fusion. We show efficacy of the therapeutic anti-ERBB3 antibody GSK2849330 in vitro and in vivo in the LUAD-0061AS3 models. The LUAD-0061AS3 cell line was sensitive to afatinib, in contrast to the poor response of the patient and the LUAD-0061AS3 PDX model, which revealed no tumor regression when treated with 5 mg/kg QD afatinib. We estimate that this dose of afatinib in mice is equivalent to a human dose of 32 mg/day, derived by allometric scaling on the basis of Food and Drug Administration draft guidelines.\textsuperscript{33,34} A higher dose of afatinib (25 mg/kg) was able to cause tumor shrinkage in the PDX model, but this was accompanied by toxicities, as reflected by substantial weight loss of tumor-bearing animals. This dose is not achievable clinically as most published cases of patients with NRG1 fusion-driven cancers were treated with 40 mg and several had dose reductions.\textsuperscript{9,35} The discordant responses to afatinib of the cell line compared with the patient and the PDX model are a reminder that caution should be exercised in interpreting data obtained only \textit{in vitro}.

As NRG1 fusions are identified across a variety of cancers, and numerous tissue-dependent functions of physiological NRG1 isoforms are described, the question of cross-organ differences of NRG1-induced tumorigenesis arises. Our study identified some differences between the breast cancer cell line MDA-MB-175-VII and our novel lung cancer model, LUAD-0061AS3. We observed higher phosphorylation of BRAF and ERK1/2 in the MDA-MB-175-VII cell line, concomitantly with increased sensitivity to MEK inhibitors. In contrast, MTOR (but not PIK3CA) inhibitors displayed relatively higher activity in LUAD-0061AS3 cells than in the breast cancer cell line. In support of these findings, transcriptomic profiling of lung tumors with NRG1 chimeric transcripts revealed an activated MTOR signature and two proteins that function downstream of MTOR, such as p70S6K and 4EBP1, had higher phosphorylation in the LUAD-0061AS3 cell line compared with nontumor HBEC cells and the MDA-MB-175-VII cell line. These findings prompted us to evaluate the therapeutic capacity of rapamycin for NRG1 fusion-positive lung cancers. Administration of rapamycin to mice bearing LUAD-0061AS3 PDX tumors resulted in a significant reduction in tumor growth compared with afatinib or GSK2849330 with the magnitude and duration of the antitumor effect of rapamycin being superior. The doses of rapamycin that we tested in mice are clinically achievable.\textsuperscript{46} Combination of GSK2849330 and rapamycin did not provide any additional benefit compared with rapamycin alone. These results support our hypothesis that MTOR is an important effector of NRG1-dependent tumorigenesis in lung cancers with NRG1 fusions. Given that we used only one lung cancer PDX model to evaluate the efficacy of rapamycin, caution should be exercised in interpreting these encouraging data. Future studies should evaluate the efficacy of rapamycin and other MTOR inhibitors in vivo in additional preclinical disease models of NRG1 fusion-driven lung cancers.

Importantly, we found that rapamycin treatment down-regulated SLC3A2-NRG1 fusion protein levels in the LUAD-0061AS3 cell line. To our knowledge, this is the first observation of a targeted agent causing down-regulation of an oncogenic fusion protein and helps to explain the exquisite sensitivity of the LUAD-0061AS3 cell line to rapamycin. It remains unclear which additional pathways downstream of MTOR may also be co-opted by NRG1 fusions to drive tumorigenesis. Inhibition of AKT and PI3K was not sufficient to block cell growth, either because compensatory signaling pathways exist or that antagonizing these pathways are not very effective in cells in which these effectors are not constitutively activated by genetic alterations.

Although we report some differences between the cell lines of different tissue origins, caution should be applied in interpreting these results as these models harbor different 5’ partners of NRG1. It is possible that different partners of NRG1 in the chimeric fusion protein may affect lipid raft localization, dimerization, extracellular cleavage, etc., events that can influence the mechanism and intensity of their oncogenicity. Nevertheless, anti-ERBB therapy was effective in both tumor types, and therefore, we believe that it would be more practical to use such therapy for patients with NRG1 fusion-
dependent cancers given the low prevalence of this oncogene. Our results suggest that the use of MTOR inhibitors in combination with HER3 antibodies (or other effective anti-ERBB therapy) may not provide additional benefit over inhibition of MTOR alone. Perhaps, therapeutically targeting MTOR alone may be possible in unique situations where an anti-ERBB agent no longer offer substantial benefit, for example, if resistance to therapy occurs. Nevertheless, as noted previously, further studies are required to clarify differences between tissues and fusion partners using additional patient-derived disease models with NRG1 fusions as they can substantially guide and transform therapy in this patient subpopulation.

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Supplementary Data
Note: To access the supplementary material accompanying this article, visit the online version of the Journal of Thoracic Oncology at www.jto.org and at https://doi.org/10.1016/j.jtho.2021.03.013.

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