CD74–NRG1 Fusions in Lung Adenocarcinoma

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ABSTRACT

We discovered a novel somatic gene fusion, CD74–NRG1, by transcriptome sequencing of 25 lung adenocarcinomas of never smokers. By screening 102 lung adenocarcinomas negative for known oncogenic alterations, we found four additional fusion-positive tumors, all of which were of the invasive mucinous subtype. Mechanistically, CD74–NRG1 leads to extracellular expression of the EGF-like domain of NRG1 III-B3, thereby providing the ligand for ERBB2–ERBB3 receptor complexes. Accordingly, ERBB2 and ERBB3 expression was high in the index case, and expression of phospho-ERBB3 was specifically found in tumors bearing the fusion (P < 0.0001). Ectopic expression of CD74–NRG1 in lung cancer cell lines expressing ERBB2 and ERBB3 activated ERBB3 and the PI3K–AKT pathway, and led to increased colony formation in soft agar. Thus, CD74–NRG1 gene fusions are activating genomic alterations in invasive mucinous adenocarcinomas and may offer a therapeutic opportunity for a lung tumor subtype with, so far, no effective treatment.

SIGNIFICANCE: CD74–NRG1 fusions may represent a therapeutic opportunity for invasive mucinous lung adenocarcinomas, a tumor with no effective treatment that frequently presents with multifocal unresectable disease. Cancer Discov; 4(4); 1–8. © 2014 AACR.

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INTRODUCTION

Lung adenocarcinomas of patients who have never smoked frequently bear kinase gene alterations, such as EGFR mutations and translocations affecting ALK, ROS1, and RET (1–6). These alterations cause “oncogene dependency” on the activated kinase and, thus, sensitivity of the tumor cells to kinase inhibitors. Patients whose tumors bear kinase gene alterations can be effectively treated with an ever-growing number of kinase inhibitors; for example, patients with EGFR-mutant lung cancer treated with EGFR receptor (EGFR) inhibitors have a significantly longer progression-free survival compared with patients treated with conventional chemotherapy (7). Similarly, ALK and ROS1 inhibition induces clinically relevant remissions in patients bearing the respective genomic fusion (8–10). Unfortunately, despite substantive cancer genome sequencing efforts, a majority of lung tumors still lack therapeutically tractable kinase alterations (1). We therefore sought to identify novel therapeutically relevant driver alterations in otherwise driver-negative lung adenocarcinomas.

RESULTS

We collected a cohort of 25 lung adenocarcinoma specimens of never smokers that lacked mutations in KRAS or EGFR, on which we performed chromosomal gene copy-number analysis as well as transcriptome sequencing with the aim of identifying new oncogenic driver alterations. We applied a novel computational data analysis strategy that combines split-read and read-pair analyses with de novo assembly of candidate regions containing potential breakpoints to achieve sensitive and accurate detection of fusion transcripts (see Methods; Fernandez-Cuesta and colleagues, published elsewhere). Of the 25 samples analyzed (Supplementary Table S1), 10 carried a known oncogene. One sample exhibited EGFR amplification, paralleled by overexpression of the gene (Fig. 1A and Supplementary Fig. S1). We also found 3 cases each of ALK, ROS1, and RET fusions (Fig. 1A and Supplementary Table S2). In addition, we detected one sample carrying a novel chimeric transcript fusing the first six exons of CD74 to the exons encoding the EGF-like domain of the neuregulin-1 (NRG1) III-β3 isoform (Fig. 1A and B and Supplementary Table S2). This fusion raised our interest because CD74 is part of recurrent fusions affecting the ROS1 (3) kinase in lung adenocarcinoma, and because NRG1 encodes a ligand of ERBB receptor tyrosine kinases, which are also frequently affected by genome alterations in this tumor type. NRG1 provides the ligand for ERBB3 and ERBB4 receptors (11). The NRG1 isoform present in our fusion transcript belongs to the type III and carries the EGF-like domain β3, which has higher affinity to the receptors than the α-type (12). NRG1 type III expression is mostly limited to neurons and is the only isoform displaying this degree of tissue-specific expression (13). Only the sample carrying the CD74–NRG1 fusion exhibited high expression of the NRG1 III-β3 isoform [74 fragments per kilobase per million reads (FPKM); Fig. 1C, top; Supplementary Table S3], and in this specimen there was no expression of the wild-type allele (Fig. 1C, bottom). In addition, NRG1 was generally not expressed in lung adenocarcinoma as shown by transcriptome sequencing data of our cohort of 25 lung adenocarcinomas of never smokers (Fig. 1C, top, and Supplementary Table S3), and of a cohort of 15 unselected lung adenocarcinomas (Fig. 1C, top, and Supplementary Table S4). The fusion resulted from a somatic genomic event as CD74–NRG1 fusion FISH and NRG1 break-apart FISH revealed rearrangements in the respective chromosomal regions in the tumor cells, but not in surrounding nontumoral cells (Fig. 1D and Supplementary Fig. S2). Furthermore, by applying hybrid-capture–based massively parallel genomic sequencing (Fig. 1D and Supplementary Table S5), we found five and two reads spanning and encompassing the chromosomal breakpoint (chr5:149,783,493 and chr8:32,548,502), respectively.

We next performed reverse transcriptase PCR (RT-PCR) using primers specific for the chimeric transcript to identify additional tumors bearing the fusion in a set of 102 pan-negative adenocarcinomas of never smokers (wild-type for EGFR, KRAS, BRAF, ERBB2, ALK, ROS, and RET genes). We identified four additional tumors carrying the fusion (Supplementary Table S6), which were also confirmed by break-apart FISH. All 5 cases (including the index case) occurred in invasive mucinous adenocarcinomas (IMA) of women who had never smoked (Fig. 2A). Invasive mucinous lung adenocarcinoma is highly associated with KRAS mutations (14). Indeed, out of 15 invasive mucinous lung adenocarcinoma specimens (all derived from an East Asian population), six carried a KRAS mutation (40%), and four carried the CD74–NRG1 fusion (27%; Fig. 2B; Supplementary Table S7). We additionally tested other lung tumor subtypes (63 cases), as well as four other cancer types (21 cases) and all were negative for the fusion gene (Supplementary Table S6), suggesting a strong link between the presence of CD74–NRG1 and invasive mucinous adenocarcinoma.

Characteristic features of type III NRG1 are cytosolic N-termini and membrane-tethered EGF-like domains (13, 15). In the case of CD74–NRG1, the part of CD74 is predicted to replace the transmembrane domain present in wild-type NRG1 III-β3, preserving the membrane-tethered EGF-like domain (Fig. 2C). To validate this prediction, we transduced NIH-3T3 cells with CD74–NRG1-encoding retroviruses, and performed flow cytometry analyses to determine the subcellular distribution of expression of the fusion protein. As expected, we observed a positive intracellular (but not extracellular) signal for CD74 (Fig. 2D, left) and a positive extracellular signal for NRG1 (Fig. 2D, right). Similar results were observed in H2052 cells (Supplementary Fig. S3). Furthermore, we were unable to detect the fusion in the supernatant of transduced cells with a polyclonal antibody raised against the EGF-like domain (data not shown). Thus, the fusion does not lead to secretion of the EGF-like domain, but probably generates a membrane-bound protein with the EGF-like domain presented on the outside of the cell.

We next analyzed the expression of ERBB receptors in the index case: ERBB1 (EGFR) was almost not expressed (FPKM = 1.9; Fig. 3A; Supplementary Table S8; Supplementary Fig. S4) and not phosphorylated (Supplementary Fig. S4). In contrast, ERBB2 was expressed (FPKM = 22.9; Fig. 3A; Supplementary Table S8) and phosphorylated (Fig. 3B, left), similar to ERBB2, ERBB3 was also expressed at relatively high levels.
CD74–NRG1 Fusions in Lung Adenocarcinoma

**RESEARCH BRIEF**

**Figure 1.** Identification of the CD74–NRG1 fusion gene. A, overview of driver genes detected in a cohort of 25 EGFR- and KRAS-negative lung adenocarcinomas of never smokers. B, detection of CD74–NRG1 fusion transcript by transcriptome sequencing. Schematic representation of the fusion transcript domains and some of the transcriptome sequencing reads spanning the fusion point. C, expression levels of NRG1 isoforms in 15 unselected and 23 pan-negative lung adenocarcinomas (AD; wild-type for EGFR, KRAS, BRAF, ERBB2, ALK, ROS, and RET), and, in the index case, inferred from transcriptome sequencing data. Average FPKM values are shown (top). RNAseq analysis for NRG1 reads to show where the breakpoint of CD74–NRG1 occurs. The dip in exon 4 represents reads of the fusion that could not be mapped. No reads could be mapped to exons 1–3 (bottom). D, top, the genomic intron/exon structure of the CD74 (in green) and the NRG1 locus (in orange) with the genomic breakpoints marked in red. Sequencing reads were obtained from hybrid-capture–based genomic sequencing of 333 genes using genomic DNA of the index case (see Methods). The breakpoint-spanning reads are shown by means of the Integrative Genomics Viewer (www.broadinstitute.org/igv/) focused on the CD74 gene (bottom). The gray area of the read is aligned to the CD74 reference sequence. Colored area on the right indicates bases not matching the CD74 reference sequence. Encompassing reads whose mate pairs are mapped to the NRG1 locus on chromosome 8 are displayed in dark purple. Bottom, a representative picture of NRG1 break-apart FISH. Arrows, break-apart signals.

### Table A

<table>
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*, Index case (Caucasian, invasive mucinous adenocarcinoma)
Figure 2. Association of CD74–NRG1 with invasive mucinous adenocarcinoma, and membrane localization of the fusion protein. A, clinical characteristics of the index case and the 4 additional cases found to harbor CD74–NRG1. B, frequency of KRAS mutations and CD74–NRG1 rearrangements in a cohort of 15 IMA tumors (East Asian population). C, schematic representation of wild-type NRG1 III−β3 and predicted CD74–NRG1 fusion protein in the cellular membrane. D, intracellular and extracellular staining of CD74 (left), and extracellular staining of NRG1 (right) in CD74–NRG1-transduced NIH-3T3 cells, detected by flow cytometry. The percentage of max is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells. e.v., empty vector control.

Table 1

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<th>Sample</th>
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<td>Case-B</td>
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<td>Case-C</td>
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</table>

EGFR, KRAS, BRAF, HER2, ALK, ROS, RET negative

To formally test this hypothesis, we transduced different cell lines with retroviruses encoding CD74–NRG1 and performed Western blot analyses under starving conditions. Because NIH-3T3 cells have low-to-absent expression of ERBB receptors, and NIH-3T3 cells ectopically expressing ERBB2 and ERBB3 are already oncogenic (Supplementary Fig. S5), we decided to use H322 and H1568 lung cancer cell lines expressing normal ERBB2 and ERBB3 levels instead. We transduced these cell lines with either an empty vector, a virus containing the full fusion transcript, or a virus containing a truncated version of the fusion lacking the EGF-like domain (Supplementary Fig. S6). We observed that H322 and H1568 cell lines ectopically expressing CD74–NRG1 showed increased levels of p-ERBB2, p-ERBB3, p-AKT, and p-S6K when compared with the empty vector control (Fig. 3D). Furthermore, both p-ERBB3 and...
Figure 3. Functional relevance of CD74–NRG1. A, expression levels of ERBB receptors in the index case inferred from transcriptome sequencing data. FPKM values are shown. B, levels of p-ERBB2 and p-ERBB3 detected by immunohistochemical analysis in a CD74–NRG1–positive case using an antibody directed against ERBB2 Tyr1221/1222 and ERBB2 Tyr1289. C, the same p-ERBB3 antibody was used to stain a tissue microarray composed of 241 lung adenocarcinomas. The frequency of p-ERBB3–positive cases in this cohort versus the five CD74–NRG1–positive samples is shown ($P < 0.0001$). D, activation of the PI3K–AKT pathway detected by Western blot analysis of H322 and H1568 lung cancer cells transduced with retroviruses encoding CD74–NRG1 or the empty vector control (e.v.). E, levels of p-ERBB3 and p-AKT measured by Western blot analysis in the presence of an empty vector, CD74–NRG1, or a truncated version lacking the EGF-like domain (CD74–NRG1_ΔEGF). F, anchorage-independent growth of H1568 cells expressing an empty vector, CD74–NRG1, or a truncated version lacking the EGF-like domain (CD74–NRG1_ΔEGF). Top, the average colony size for the three conditions, with error bars representing standard deviations. The experiment was performed with two independent transductions for a total of four times. **, $P < 0.01$; ***, $P < 0.001$. Bottom, representative pictures of the colony formation assay. Please note that H1568 cells are oncogenic and form small colonies without any manipulation.
p-AKT depended on the presence of the EGF-like domain of CD74–NRG1 in the fusion (Fig. 3E). In addition, coculture of NIH-3T3 cells ectopically expressing CD74–NRG1 with Ba/F3 cells genetically engineered to express normal ERBB2 and ERBB3 levels also led to activation of AKT (Supplementary Fig. S7). Finally, H1568 cells ectopically expressing CD74–NRG1 exhibited enhanced colony formation in soft-agar assays (Fig. 3F; Supplementary Table S9). Taken together, these data suggest that CD74–NRG1 leads to overexpression of the EGF-like domain of NRG1 III-B3 that acts as a ligand for ERBB3, inducing its phosphorylation and subsequent activation of the downstream PI3K–AKT pathway.

**DISCUSSION**

We have discovered CD74–NRG1, a novel recurrent fusion gene in lung adenocarcinoma that arises from a somatic genomic event. Taking into account the frequencies of mutations of EGFR (11.3%), KRAS (32.2%), BRAF (7%), ERBB2 (1.7%), or fusions affecting ALK (1.3%), ROS (1.7%), and RET (0.9%; refs. 17, 18) in lung adenocarcinomas, for which our cohort was never smokers, a tumor type that is otherwise associated with KRAS mutations (14). In our cohort of limited size (n = 15), CD74–NRG1 fusions accounted for 27% of invasive mucinous lung adenocarcinomas of never smokers, a tumor type that is otherwise associated with KRAS mutations (14). In our cohort of limited size (n = 15), CD74–NRG1 fusions accounted for 27% of invasive mucinous lung adenocarcinomas; together, KRAS mutations and CD74–NRG1 may therefore be considered the causative oncogenes in more than 60% of the cases. We provide evidence that CD74–NRG1 signals through induction of the downstream PI3K–AKT pathway.

**METHODS**

**Sample Preparation, DNA and RNA Extraction, and Illumina Sequencing**

Sample preparation and DNA and RNA extraction were performed as previously described (20). RNAseq was performed on cDNA libraries prepared from PolyA+ RNA extracted from tumor cells using the Illumina TruSeq protocol for mRNA. The final libraries were sequenced with a paired-end 2 × 100 bp protocol aiming at 8.5 Gb per sample, resulting in a 30x mean coverage of the annotated transcriptome. All the sequencing was carried on an Illumina HiSeq 2000 sequencing instrument (Illumina).

**Analysis of Chromosomal Gene Copy Number (SNP 6.0) and RNAseq Data**

Hybridization of the Affymetrix SNP 6.0 arrays was carried out according to the manufacturers’ instructions and analyzed using a previously described method (20). For the analysis of RNAseq data, we have developed a pipeline that affords accurate and efficient mapping and downstream analysis of transcribed genes in cancer samples (Fernandez-Cuesta and colleagues; published elsewhere). A brief description of the method was previously provided (20).

**Analysis of Targeted Enrichment Genome Sequencing**

Genomic DNA was isolated from fresh-frozen tumor tissue and subjected to CAGE Scanner analysis. This approach involves liquid-phase hybrid capture of genomic partitions enriched for genome alterations affecting 333 known cancer-associated genes (also including CD74). Subsequent to generation of genomic libraries from tumor DNA and capture, sequencing was performed on the Illumina platform according to the manufacturer’s instructions. Significant genomic alterations were identified using approaches described previously (20).

**Dideoxy Sequencing**

In case of validation, sequencing primer pairs were designed to enclose the putative mutation, or to encompass the candidate rearrangement or chimeric transcript as previously described (20). Sequencing was carried out, and electropherograms were analyzed by visual inspection using four peaks.

**Interphase FISH on Formalin-fixed, Paraffin-embedded Sections**

Two sets of probes were prepared. One was for break-apart FISH of which probes were mapped at centromeric and telomeric regions between the break point. The other was for fusion FISH that spanned the NRG1 and CD74 loci. To intensify the signals, each probe was made of two or three BAC clones as follows, and the probes were labeled with SpectrumGreen and SpectrumOrange (Abbott Molecular-Vysis). Centromeric probes for break-apart FISH were RP11-1002K11 and RP11-25D16. Telomeric probes for break-apart FISH were RP11-23A12 and RP11-715M18. NRG1 probes for fusion FISH were RP11-715H18, RP11-5713, and RP11-1002K11. CD74 probes for fusion FISH were PR11-759G10 and PR11-46B14.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (21). In brief, the tissue samples were stained with p-ERBB2 (Ty1211/1222; Cell Signaling Technology) and total ERBB1 (EGFR; Dako) at a dilution of 1:1,000 and 1:50, respectively. The Zeiss MIRAK DESK scanner was used to digitize the stained tissue. Staining for p-EGFR (Ty1068; Cell Signaling Technology) and p-ERBB3 (Ty1289; Cell Signaling Technology) was processed with an automated stainer (Autostainer; Dako), using the FLEX+ detection system (Dako).

**Cell Culture**

H2052, H322, and H1568 cells were obtained from the American Type Culture Collection and maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% penicillin–streptomycin (Gibco). The cells were cultured in a humidified incubator with 5% CO2 at 37°C. For Western blot analysis experiments, cells were serum starved for 24 hours. NIH-3T3 cells were maintained similarly but in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies). The cells were cultured in a humidified incubator with 5% CO2 at 37°C. For Western blot analysis experiments, cells were serum starved for 24 hours. NIH-3T3 cells were maintained similarly but in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies). The cells were confirmed to be wild-type for KRAS, EGFR, ERBB2, and ERBB3 by PCR amplification followed by Sanger sequencing of the PCR products. The cell lines have been authenticated via genotyping (SNP 6.0; Affymetrix) and tested for Mycoplasma contamination on a regular basis (MycAlert; Lonza).

**FACS Analysis**

NIH-3T3 mouse fibroblast cells were transduced with retrovirus containing empty vector, CD74–NRG1, ERBB2, ERBB3, and ERBB2+ERBB3. H2052 cells were transduced with retrovirus
containing empty vector or CD74-NRG1. Transduced cells (200,000) were washed in fluorescence-activated cell sorting (FACS) buffer (PBS, 2% FCS) and fixed in 4% paraformaldehyde for 30 minutes at room temperature. For permeabilization, cells were washed twice in Saponin buffer (PBS, 0.5% Saponin, and 2% FCS) and intracellular staining of CD74-NRG1 was performed with anti-human-CD74-PE (1:100; BioLegend). Intracellular staining of ERBB2 and ERBB3 was performed with anti-ERBB2 and anti-ERBB3 antibodies (1:50; Cell Signaling Technology). Binding of ERBB2 or ERBB3 was detected with goat-anti-rabbit-Alexa Fluor 488 (Life Technologies). Extracellular staining was performed before permeabilization with anti-human-CD74-PE and anti-NRG1 antibody (1:20; R&D Systems). Binding of the NRG1 part was detected with donkey-anti-goat-Alexa Fluor 488 (Life Technologies). Subsequently, cells were analyzed on a BD LSR II (Beckman Coulter) and quantification was assessed with Flowjo (TreeStar).

Western Blot Analysis

Immunoblotting was performed using standard procedures. The following antibodies were obtained from Cell Signaling Technology: p-AKT Ser473 (Catalog No. #9271), p-p70S6 (Catalog No. #9205), total ERBB2 (Catalog No. #2242), p-ERBB2 (Catalog No. #2243), total ERBB3 (Catalog No. #4754), and p-ERBB3 (Catalog No. #4791). Anti-human CD74 was obtained from Abcam (Catalog No. #ab22603), and anti-polyclonal NRG1 (Catalog No. #4779). The antibodies were diluted in 5% BSA/TBST and incubated at 4°C overnight. Proteins were detected with HRP-conjugated anti-mouse, anti-goat, or anti-rabbit antibodies (Millipore) using enhanced chemiluminescence (ECL) reagent (GE Healthcare).

Colony Formation Assay

On a layer of bottom agar (1%), NIH-3T3 cells were suspended at low density in top agar (0.6%) containing 10% FCS (final concentration), supplemented with 1% penicillin–streptomycin. p-BABE retroviral vector inserts were confirmed via Sanger sequencing. The cells were generated by at least two independent transductions with retrovirus containing empty vector, CD74-NRG1, or CD74-NRG1ΔEGF. After selection for 7 days with puromycin (3 μg/mL), cell lysates were taken for Western blot analysis, and cells were also used for colony formation assays as follows: on a layer of bottom agar (1%) cells were suspended at low density in top agar (0.6%) containing 10% FCS (final concentration), and were grown for 14 days. Subsequently, pictures were taken with a Zeiss Axiosvert 40 CFL microscope at ×100 magnification, and colony size was assessed with ImageJ (http://rsbweb.nih.gov/ij/).

Generation of Bo/F3 ERBB2-ERBB3 Cells

The ERBB2 and ERBB3 open reading frames were amplified by PCR and cloned into the MSCV-puromycin or MSCV-neomycin vectors, respectively (Clonetech). Ba/F3 cells expressing ERBB2 and ERBB3 were generated by retroviral transduction and subsequent puromycin or/and neomycin selection. We verified the expression of the correct proteins by Western blot analysis. Ba/F3 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1 ng/mL mouse interleukin-3.

Statistical Analyses

In Fig. 3C and F, we used a two-tailed Fisher exact test.
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