Lung adenocarcinoma is comprised of distinct mutational subtypes characterized by mutually exclusive oncogenic mutations in RTR/KAS pathway members KRAS, EGFR, BRAF and ERBB2, and translocations involving ALK, RET and ROS1. Identification of these oncogenic events has transformed the treatment of lung adenocarcinoma via application of therapies targeted toward specific genetic lesions in stratified patient populations. However, such mutations have been reported in only ~55% of lung adenocarcinoma cases in the United States, suggesting other mechanisms of malignancy are involved in the remaining cases. Here we report somatic mutations in the small GTPase gene RIT1 in ~2% of lung adenocarcinoma cases that cluster in a hotspot near the switch II domain of the protein. RIT1 switch II domain mutations are mutually exclusive with all other known lung adenocarcinoma driver mutations. Ectopic expression of mutated RIT1 induces cellular transformation in vitro and in vivo, which can be reversed by combined PI3K and MEK inhibition. These data identify RIT1 as a driver oncogene in a specific subset of lung adenocarcinomas and suggest PI3K and MEK inhibition as a potential therapeutic strategy in RIT1-mutated tumors.

Keywords: lung adenocarcinoma; cancer genetics; RAS pathway; signal transduction; oncogene; GTPase

INTRODUCTION

Lung adenocarcinomas contain characteristic, mutually exclusive mutations in receptor tyrosine kinase (RTK) and RAS pathway oncogenes including frequent mutation of KRAS or EGFR in ~30 or 10% of US cases, respectively.1–13 Rarely, translocations are seen involving EML4 and ALK (1–4%), RET (1%) or ROS1 (2%).2,5 (The Cancer Genome Atlas, manuscript submitted). Additional rare oncogenic mutations are seen in HRA, NRAS, BRAF, ERBB2, MET and MAP2K1.1,2 Together these oncogenic events are found in ~55% of lung adenocarcinomas in the US population.2 The importance of identifying such ‘driver’ mutations is exemplified by the success of targeted therapies directed against these events— the prototypical example being the use of the small molecule EGFR inhibitors Iressa (gefitinib) and Tarceva (erlotinib) in EGFR-mutated lung adenocarcinoma.14 Among patients with EGFR mutations, single-line EGFR-targeted therapy significantly extends progression-free survival compared with conventional chemotherapy.7,8 Patients without EGFR mutations, however, typically do not benefit from targeted therapy, underscoring the need to genotype patient tumor resections and/or biopsies to guide appropriate treatment decisions. Modeling this paradigm, clinical trials of the ALK inhibitor crizotinib for treatment of tumors with EML4-ALK translocation were rapidly reported15 only 3 years after the identification of the EML4-ALK event.9 Therefore, continued identification of oncogenic driver genes in the remaining ~45% of ‘oncogene-negative’ lung adenocarcinoma cases may allow further patient stratification and targeted therapies.

Recent genomic analyses of lung adenocarcinoma have identified novel mutations in diverse genes, but few additional oncogenic mutations have been identified that display mutual exclusivity with known lung adenocarcinoma driver oncogenes.1,6 Here we report the genomic and functional characterization of somatic RIT1 mutations that we identify as novel driver mutations in lung adenocarcinoma.

RESULTS

To identify rare oncogenic driver mutations, we and others in The Cancer Genome Atlas (TCGA) Research Network performed a targeted analysis of ‘oncogene-negative’ tumors that lack mutations in the known lung adenocarcinoma driver oncogenes KRAS, EGFR, BRAF, ERBB2, MAP2K1, MET, HRAS, NRAS and/or fusions involving ALK, RET or ROS1 (TCGA, manuscript submitted). In 87 ‘oncogene-negative’ samples lacking mutations/fusions in the known driver genes, RIT1 (Ras-like in all tissues) was among the top significantly mutated genes. 5/87 samples in this group harbored somatic RIT1 mutations. In contrast, no mutations in RIT1 were found in the 143 samples containing known driver oncogenic mutations (P < 0.01 by Fisher’s exact test). Moreover, we identified five additional RIT1-mutant samples in sequence data from an independent cohort of 183 lung adenocarcinomas.16

In total, non-synonymous somatic mutations of RIT1 were identified in 10/413 (2.4%) lung adenocarcinomas subjected to whole exome sequencing (Figure 1a, Supplementary Table 1). Mutations in RIT1 consisted of missense mutations and small in-frame insertion/deletions. In all, 7/10 RIT1 mutations were clustered near the switch II domain region (Figures 1a and b), including three recurrent p.M90I mutations. The switch II domain in RIT1 is homologous to the switch II region in RAS genes that contains glutamine 61 (Supplementary Figure 1), which is directly involved in GTP hydrolysis and also a hotspot of mutation in RAS genes.7 This switch II domain hotspot is distinct from the P29 hotspot in RAC genes recently reported in melanoma and other cancers18–20 (Supplementary Figure 1).
studies harbored the RIT1 p.M90I variant seen recurrently in lung adenocarcinoma. In line with these observations, somatic RIT1 mutation was recently reported in myeloid malignancies. The mutations observed in myeloid malignancies overlap with the mutations we report in lung adenocarcinoma (F82L, M90I) and include additional mutations clustering in the switch II domain.

To test whether mutated RIT1 is capable of inducing cellular transformation, we expressed wild-type or mutated RIT1 cDNA constructs in NIH3T3 cells and assayed the ability of these cells to form colonies in soft agar. A constitutively active form of RIT1, RIT1 Q79L, was used as a positive control. With the exception of RIT1 Q40L, all other RIT1 mutations robustly induced colony formation of NIH3T3 cells in soft agar (Figure 2a). To confirm that these cells were indeed transformed, we injected RIT1-transduced cells subcutaneously into nude mice and assessed tumor-forming capability. Six of six switch II domain mutations induced significant tumor formation by 3 weeks post-injection, comparable to transformation induced by KRAS G12V or EGFR L858R (Figure 2b and Supplementary Figure 3), whereas transformation capability of non-switch II domain mutations varied. RIT1 Q40L, although consistently less active than other RIT1 variants, showed intermediate transforming capability in the xenograft assay (Supplementary Figure 3). It remains possible that RIT1 Q40L is weakly activating, and should be noted that RIT1 Q40 is homologous to KRAS Q22, which is found mutated in Noonan syndrome and rarely in somatic cancers.

In addition to the lung adenocarcinoma mutations, we tested the transforming capability of RIT1 mutations reported in myeloid malignancies or curated in COSMIC from diverse tumor types (Supplementary Table 3). Consistent with the lung adenocarcinoma data, transforming mutations were mainly clustered in the switch II domain. We identified transforming RIT1 mutations in salivary gland carcinoma, endometrial carcinoma, myeloid malignancies and melanoma. These data indicate that rare RIT1 mutations may have a role in the pathogenesis of diverse cancers.

RIT1 mutation is mutually exclusive with mutations in other RAS/RTK-pathway genes, so we hypothesized that RIT1 may activate PI3K and MEK. To investigate the signaling changes induced by mutated RIT1, we expressed wild-type or mutated RIT1 in PC6 cells and analyzed downstream signaling changes by western blot. Transformation potential of RIT1 mutants correlated with their ability to activate MEK and ERK pathways; all RIT1 mutations with the exception of RIT1 Q40L induced phosphorylation of MEK and ERK (Figure 2c). Treatment of RIT1-expressing cells with the specific MEK inhibitor, PD98059, completely inhibited transformation induced by mutated RIT1, whereas PI3K inhibition almost completely abrogated colony formation (Figure 2f). To test whether mutated RIT1 activates PI3K and MEK, we hypothesized that RIT1 may activate PI3K and MEK. To determine if activation of MEK and PI3K was responsible for RIT1-induced transformation, we assayed the ability of RIT1 mutants to form colonies in soft agar in the presence or absence of different kinase inhibitors. Either MEK inhibition with 1 μM AZD-6244 or PI3K/mTOR inhibition with 1 μM GDC-0941 significantly impaired colony formation (Figure 2f), whereas treatment with the EGFR inhibitor erlotinib (1 μM) showed no effect. Combination MEK and PI3K inhibition almost completely abrogated colony formation (Figure 2f).

Given the prevalence of RIT1 mutation in primary human lung adenocarcinomas, we hypothesized that human lung adenocarcinoma cell lines may also harbor mutations in RIT1 and that identification of these cell lines would facilitate study of RIT1
function in human cancer pathogenesis. On the basis of the pattern of RIT1 mutation mutual-exclusivity with driver mutations in primary samples, we focused our efforts on 'oncogene-negative' human non-squamous non-small cell lung cancer cell lines. We curated the mutational status of all known lung adenocarcinoma driver genes from non-squamous non-small cell lung cancer lines in the Cancer Cell Line Encyclopedia. Among 35 'oncogene-negative' cell lines, NCI-H2110 showed the highest level of RIT1 mRNA expression (Supplementary Figure 4 and Supplementary Table 4). Next, we sequenced the switch II domain of RIT1 in cDNA from 19 'oncogene-negative' cell lines and 3 'oncogene-positive' cell lines (Supplementary Table 5). In all, 1/19 'oncogene-negative' and 0/3 'oncogene-positive cell lines' had a non-synonymous change in RIT1; NCI-H2110 cells harbored the p.M90I mutation also seen in human primary lung adenocarcinomas (Figure 3a).

To determine if RIT1-mediated PI3K and MEK activation are involved in RIT1-mutated human lung adenocarcinoma, we investigated the role of these pathways in NCI-H2110 cells. Using lentiviral delivery of RIT1-targeting (shRIT1-1, shRIT1-2 and shRIT1-3) and non-targeting (shLacZ) short hairpin RNA, we knocked down RIT1 levels in NCI-H2110 and NCI-H1299 cells, the latter of which express low or no RIT1 (Figure 3b). Knockdown of RIT1 in NCI-H2110 cells resulted in loss of AKT phosphorylation and a reduction in MEK and ERK phosphorylation, whereas expression of the same hairpins in NCI-H1299 cells had no consistent effect. Therefore, RIT1 regulates PI3K and MEK signaling in NCI-H2110 cells.

Figure 2. Mutated RIT1 induces cellular transformation via activation of MEK and PI3K. (a) Soft agar transformation assay in NIH3T3 cells. Cells were transduced with retrovirus to ectopically express wild-type (WT) or mutated RIT1 constructs or empty vector (control), then plated in soft agar (Methods). Colonies were visualized at 14 days and quantified using CellProfiler. Top panel, data shown is mean ± s.e.m. of triplicate wells. Data shown are representative of at least three independent experiments. *P < 0.05 by two-tailed t-test. Bottom panels, western blot showing expression of RIT1 or vinculin (loading control). 'INS', T76_insTLDT. (b) Tumor growth of xenografts of NIH3T3 cells with or without expression of RIT1. Data shown is mean ± s.e.m. of nine replicates per construct. *P < 0.01 by two-tailed t-test. Data shown are representative of at least three independent experiments per construct. (c) Western blot of PC6 cell lysates following transfection of wild-type or mutant RIT1 or vector control ('Emp'). Data shown is representative of at least three independent experiments. (d) Western blot of PC6 lysates following transfection of FLAG-RIT1 constructs or vector control ('Emp') in the presence or absence of 10 μM PD98059. Cells were serum starved for 5 h prior to lysis. (e) Western blot of PC6 cell lysates generated after transfection of FLAG-RIT1 mutant constructs in the presence or absence of 10 μM LY294002. Cells were serum starved for 5 h prior to lysis. (f) Soft agar colony formation of NIH3T3 cells stably expressing RIT1 M90I or RIT1 Q79L in the presence or absence of 1 μM erlotinib, GDC-0941, AZD-6244 or GDC-0941/AZD-6244 or vehicle control (dimethylsulfoxide). 5 × 10³ cells were suspended in a top agar solution together with each respective drug to a final concentration of 1 μM in triplicate. After 15 days, colonies were photographed and quantified using CellProfiler. *P < 0.05.
xenografts were generated via subcutaneous injection into the flanks of nude mice. After tumors were established, mice were dosed daily with 150 mg/kg GDC-0941 as previously described.28 GDC-0941 significantly impaired tumor growth (Figure 3c), resulting in markedly smaller tumor masses in the GDC-0941-treated group at the experimental end point (Figure 3d).

DISCUSSION

RIT1 encodes a RAS-family small GTPase24,29 with significant domain and sequence homology to KRAS, HRAS and NRAS (Figures 1a and b). Although RIT1 is ubiquitously expressed, expression of its closest homolog, RIT2/RIN, is restricted to the nervous system.29 Like RAS proteins, RIT1 possesses intrinsic GTP hydrolysis activity,24 which can be inactivated by construction of a point mutation, Q79L, at the glutamine homologous to Q61 in RAS.24 Expression of Q79L RIT1 is sufficient to transform NIH3T3 cells30 and induces activation of p38, ERK or AKT signaling pathways depending on cellular context.31,32 Interestingly, the codon for Q79 also provides the 5' splice site for intron 4–5 of RIT1; mutation of glutamine 79 to leucine would be predicted to disrupt the splice site, which may make natural Q79L mutation incompatible with RIT1 expression.

In PC6 cells, a derivative of the PC12 pheochromocytoma line commonly used for study of RAS/MAPK signaling and neuronal differentiation,33,34 RIT1 activates MEK/ERK through direct binding with BRAF.31 Moreover, RIT1 activity is induced by stimulation with EGF or NGF in PC6 cells.33,34 Recently, studies in Rit knockout mice demonstrated that Rit promotes survival after oxidative stress through activation of a p38-AKT-BAD signaling cascade.35 Rit−/− cells are hypersensitive to apoptosis after exposure to reactive oxygen species (ROS) whereas cells expressing Q79L Rit1 are protected.35

The RIT1 switch II domain mutations identified here in ~2% of lung adenocarcinomas define a new subset of lung adenocarcinoma. RIT1 switch II domain mutations are mutually exclusive with all other known lung adenocarcinoma oncogenes and rapidly induce transformation in vitro and in vivo. RIT1 mutation induces activation of PI3K and MEK signaling and these pathways are required for Rit1-mediated cellular transformation. These experimental and observational data indicate that Rit1 likely acts in the RTK/MAPK pathway to promote tumorigenesis. In agreement with this notion is the recent identification of germline RIT1 mutations in Noonan syndrome, a developmental disorder caused by mutations in RAS-pathway genes.36 In cancer, targeted inhibition of PI3K and MEK should be explored as a possible therapeutic strategy for patients with Rit1 mutations.

MATERIALS AND METHODS

Identification of mutations

Somatic RIT1 mutations were identified in two independent cohorts of lung adenocarcinoma sequence data from Imielinski et al.1 or The Cancer Genome Atlas (http://cancergenome.nih.gov, manuscript submitted).

Construct generation and virus production

pDONR223-RIT1 was obtained from the Broad Institute's ORF collection37 and confirmed to be wild-type by Sanger sequencing. Mutations were introduced into pDONR223-RIT1 by site-directed mutagenesis using the Quikchange II kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Primer sequences are available upon request.
RIT1 mutations were then subcloned from pDONR223 into pBabe-puro for generation of retrovirus or p3xFlag-CMV10 (Sigma-Aldrich, St Louis, MO, USA) for transient transfection experiments. Retrovirus was generated by co-transfection of pBabe-puro and a pCCL-Eco packaging vector into 293T cells using Fugene6 (Promega, Madison, WI, USA). Forty-eight hours post-transfection, virus was harvested and filtered through a 0.45 μm syringe filter before transduction of exponentially growing NIH3T3 cells. Forty-eight hours post-infection, cells were selected with 2 μg/ml puromycin for 2–3 days before proceeding with downstream soft agar assays or other experiments.

Cell line sequencing
We considered ‘known’ adenocarcinoma oncogenes as previously defined, with the addition of the recently identified MET exon 14 skipping variant. We curated the genotypes of these genes in 64 non-squamous cell lines (Supplementary Table 5) from previously generated Oncomap data. Nineteen cell lines lacking known lung adenocarcinoma oncogenes and three control cell lines were selected for RIT1 cDNA sequencing. Cell line culturing and cDNA preparation was as previously described. The switch II domain region of RIT1 was PCR amplified using the following primers: RIT1-c-ex3-F, TCATCAGCCACCG ATTCC; RIT1-c-ex5-R, CCGTGACCTGAAATAGAAGT. PCR was performed using the HotStarTaq mastermix (Qiagen, Valencia, CA, USA) with the following thermocycling conditions: 5 min at 95 °C, 38 cycles of 15 s at 94 °C, 1 min at 50 °C, 1 min at 72 °C and an additional extension time of 10 min at 72 °C. PCR products were purified using the Qiagquick PCR purification kit (Qiagen) and sequenced by Sanger sequencing (Genewiz, Cambridge, MA, USA) using the same primers as before. Sequin (DNAStar, Madison, WI, USA) was used to align sequencing traces and identify single nucleotide polymorphisms. The p.M90I mutation identified in NCI-H2110 was validated in an independent cDNA sample from an independent aliquot of cells obtained directly from ATCC.

Soft agar assay
NIH3T3 cells were transduced with retrovirus generated with pBabe-puro-RIT1 plasmids or pBabe-puro-empty vector. Forty-eight hours post-infection, cells were selected with 2 μg/ml puromycin. After 48 h of selection, cells were split for seeding in a soft agar assay or lysis for verification of protein expression by western blotting. For the soft agar assay, 5 × 10^5–5 × 10^6 cells were suspended in 1 ml of 0.33% agarose. Agar wells were hydrated weekly with media containing 1% agarose. Xenograft assays
Xenografts were performed as previously described. For transformation assays, RIT1- or empty vector-transduced NIH3T3 cells, generated as above, were harvested by trypsinization, washed in PBS and resuspended at 10^5 cells/ml in PBS. Two hundred microlitres (2 × 10^3 cells) were injected into each injection site, n = 9 sites per cell line, in 4–6-week-old female nu/nu mice (Jackson Laboratory, Bar Harbor, ME, USA). Cells were allowed to engraft for 1 week, then tumors were measured every 3 days using a digital caliper (VWR, Radnor, PA, USA). Tumor volume was calculated with the formula 0.5 × L × W^2 where L is the longest diameter and W is the diameter perpendicular to L. NCI-H2110 studies, cells were prepared as above and then resuspended at 10^5 cells/ml. Two hundred microlitres (2 × 10^3 cells) were injected per site into flanks of nu/nu mice. Tumors were monitored until ~100 mm^3, at which time inhibitor dosing was initiated. GDC-0941 (Selleckchem) was resuspended at 20 mg/ml in 0.5% methylcellulose/0.2% Tween-80 and 150 mg/kg administered daily by oral gavage. All animal experiments were carried out in accordance with the DFCI Institutional Animal Care and Use Committee guidelines.

Protein from each lysate was resolved by 10% SDS–PAGE and subjected to immunoblot analysis with the indicated antibody as described previously. For inhibitor experiments, cells were pre-treated with 10 μM PD98059 or LY294002 or dimethylsulfoxide for 30 min prior to transfection. 60 h post-transfection, cells were serum-starved for 5 h in the presence of the inhibitor or dimethylsulfoxide before lysis in kinase buffer. Commercial antibodies were used to total- and phospho-specific MEK, ERK, AKT (Cell Signaling, Danvers, MA, USA), Flag (Sigma-Aldrich) and RIT1 (Abcam).

Knockdown experiments
Hairpins targeting RIT1 (shRIT1-1, -2 and -3) and a non-targeting hairpin (shLacZ) were obtained from The RNAi Consortium (TRC) in the pLKO lentiviral backbone. Virus was generated by co-transfection of pLKO, VSVG and A8.9 packaging vectors in 293T cells. Lentivirus was harvested in DMEM + 30% FBS and filtered or frozen before application to exponentially growing NCI-H1299 or NCI-H2110 cells. Cells were selected in 2 μg/ml puromycin for 3 days and then expanded for 1–2 weeks before analysis. Hairpin TRC clone IDs and target sequences were as follows: shLacZ/TRCN0000072223, TTGTCCGATTTCGAAACTT; shRIT1-1/TRCN0000047888, CGCTCATATTTGATGTTT; shRIT1-2/CGTCGAAGTTTCCGAAATGGT, CGTCGAAGTTTCCGAAATGGT; shRIT1-3/TRCN0000047890, CGAGAATTCAGCTCCCTT.

CONFLICT OF INTEREST
MM is a founder and equity holder of Foundation Medicine, a for-profit company that provides next-generation sequencing diagnostic services.

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