GNAS mutations in primary mucinous and non-mucinous lung adenocarcinomas

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GNAS mutations have been described in mucinous and non-mucinous epithelial neoplasms of the appendix, pancreas, and colon, with hotspot *GNAS* mutations found in up to two-thirds of pancreatic intraductal papillary mucinous neoplasms. Additionally, many *GNAS*-mutated tumors have concurrent mutations in the Ras/Raf pathway. The clinicopathologic features of *GNAS*-mutated lung carcinomas, however, have not yet been characterized. Primary lung carcinomas from Brigham and Women's Hospital ($n=1282$) or Massachusetts General Hospital ($n=1070$) were genotyped on a targeted massively parallel sequencing panel of oncogenes and tumor suppressor genes including *GNAS*. Clinical and pathological features were reviewed, and TTF-1 immunohistochemistry was performed when material was available. Nineteen lung adenocarcinomas with hotspot *GNAS* mutations were identified (19/2352, 0.8%) including 14 at codon 201 and 5 at codon 227. *GNAS*-mutated lung adenocarcinomas occurred predominantly in female patients (16/19, 84%). Ten (10) were classified as invasive mucinous adenocarcinomas (IMA), and nine (9) were non-mucinous adenocarcinomas. All IMAs had *GNAS* codon 201 mutations and concurrent Ras/Raf pathway mutations (9 *KRAS*, 1 *BRAF*). No tumors with *GNAS* codon 227 mutations had mucinous histological features. 86% of *GNAS*-mutated non-mucinous adenocarcinomas (6/7) were positive for TTF-1 immunohistochemistry, while only 25% of *GNAS*-mutated IMAs (1/4) were positive for TTF-1. Patients with *GNAS*-mutated non-mucinous adenocarcinomas were more likely to have a history of smoking (9/9, 100%) compared to patients with *GNAS*-mutated IMAs (2/10, 20%) ($P < 0.001$). Hotspot *GNAS* mutations can occur in primary lung adenocarcinomas. When associated with concurrent mutations in the Ras/Raf pathway, these neoplasms often present as IMAs. *GNAS* mutations are not specific to neoplasms of the gastrointestinal tract, and clinicopathologic correlation is necessary in *GNAS*-mutated adenocarcinomas in the lung to determine the primary site of origin.

Modern Pathology (2017) 30, 1720–1727; doi:10.1038/modpathol.2017.88; published online 4 August 2017

GNAS hotspot mutations have been described in various epithelial neoplasms, including appendiceal, pancreatic, and colonic tumors, and are found in up to two-thirds of intraductal papillary mucinous neoplasms (IPMNs).^{1–5} *GNAS* is a proto-oncogene originally described in pituitary adenomas that encodes for the G α subunit of heterotrimeric G-proteins that transduce signals via the G-stimulatory pathway and increase production of cyclic AMP (cAMP).⁶ Hotspot mutations have been identified in codons 201 and 227, with both hotspots resulting in

constitutive activation of the G-stimulatory pathway with increased cAMP production.^{6,7}

GNAS mutations in pancreatic cyst fluid have been shown to be a helpful biomarker in classifying pancreatic cystic lesions and are highly specific for IPMNs in that setting.^{1,2} *GNAS* mutations have also been identified in invasive pancreatic adenocarcinomas arising from IPMNs but rarely in invasive carcinomas without associated IPMNs.¹ *GNAS* mutations are present in ~20% of appendiceal mucinous tumors including low-grade appendiceal mucinous neoplasms and mucinous adenocarcinomas.³ Additionally, ~2% of colonic adenocarcinomas harbor hotspot *GNAS* mutations and have been associated with a villous morphology and right-sided location.⁴ Another study has found a correlation between *GNAS* mutations in colonic adenocarcinomas and a mucinous phenotype, with hotspot *GNAS* mutations seen in 20% of the mucinous colonic

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Received 20 March 2017; revised 8 June 2017; accepted 18 June 2017; published online 4 August 2017

adenocarcinomas in the study.⁸ *GNAS*-mutated neoplasms in the colon and pancreas frequently harbor concurrent mutations in *KRAS*, and are seen concurrently in ~90% of *GNAS*-mutated colon adenocarcinomas and in 50% of *GNAS*-mutated IPMNs.^{1,4}

Five to ten percent of lung adenocarcinomas are invasive mucinous adenocarcinomas (IMA).^{9–11} Mucinous neoplasms of the lung can be diagnostically challenging, as these tumors can be morphologically and immunohistochemically identical to metastatic mucinous tumors of the gastrointestinal tract.^{12–18} Primary IMA of the lung have distinctive molecular alterations, including frequent pathway-activating mutations in *KRAS*, occasional loss of function mutations in *NKX2-1*, and rare rearrangements in *ALK* and *NRG1*.^{19–24} Although *GNAS* mutations have been described in association with mucinous and non-mucinous tumors in multiple organs, pathway-activating *GNAS* mutations have yet to be characterized in lung carcinomas. Assessing whether *GNAS* mutations can also be associated with primary lung adenocarcinomas could be of potential use to determine primary site of origin of mucinous tumors in the lung. In this study, we interrogate 2352 lung carcinomas subjected to targeted somatic tumor sequencing panels from two institutions for the presence of activating *GNAS* mutations and evaluate their associated clinico-pathologic features.

Materials and methods

Case Selection

This study was approved by the institutional review boards at Dana Farber Cancer Institute and Massachusetts General Hospital and included patients with lung carcinoma undergoing a targeted next generation sequencing assay performed on tumor tissue at the Center for Advanced Molecular Diagnostics ($n=1282$) (Department of Pathology, Brigham and Women's Hospital) and the Center for Integrated Diagnostics ($n=1070$) (Department of Pathology, Massachusetts General Hospital). Only cases that were determined to be a primary lung carcinoma based on clinical history, radiologic findings, and pathologic features were included.

Clinicopathologic Features

The following features were recorded in the cases examined: sex, age at diagnosis, clinical presentation, radiographic findings, smoking history, and clinical stage. Histological slides were reviewed, and classification was performed according to the International Association for the Study of Lung Cancer recommendations.²⁵ IMA was defined by goblet or columnar cell morphology with abundant

intracytoplasmic mucin in greater than 90% of the tumor cells.

Immunohistochemistry

Primary antibody for TTF-1 was applied to five micron glass slides of formalin fixed, paraffin-embedded tumor tissue (clone 8G6G3/1 Dako, Inc., 1:300 dilution in citrate buffer, pressure cooker). Dako EnVision+ (Agilent Technologies, Santa Clara, CA) was used for signal detection. Strong nuclear immunohistochemical staining for TTF-1 in tumor cells was considered positive.

Targeted Tumor Genomic Sequencing ('OncoPanel') at the Center for Advanced Molecular Diagnostics

OncoPanel tumor profiling was performed as previously described.²⁶ Indexed sequencing libraries were prepared from a 50-ng sonically sheared DNA sample using Illumina TruSeq LT reagents (Illumina, Inc., San Diego, CA). A custom RNA bait set by Agilent SureSelect (Agilent Technologies, Santa Clara, CA) was used to enrich libraries for exons and select introns of 282 genes implicated in cancer biology by using solution-based hybrid capture (list of genes and transcripts, Supplementary Table 1). Massively parallel sequencing was performed using Illumina HiSeq2500 (Illumina, Inc.) with 100×100 paired-end reads to achieve a mean target coverage greater than $50 \times$. Sample reads were analyzed using a custom pipeline including Picard, BWA, and GATK (Broad Institute, Cambridge, MA) for sorting and alignment. Mutation analysis for single-nucleotide variants was performed using MuTect version 1.0.27200 (Broad Institute, Cambridge, MA). Insertions and deletions were called using Indelocator. Variants were filtered to exclude known germline variants in the Single-Nucleotide Polymorphism database by MuTect and variants that occur at a population frequency $>0.1\%$ in the Exome Sequencing Project database.

Targeted Tumor Genomic Sequencing at the Center for Integrated Diagnostics

Mutation hotspots and exons from 39 genes were targeted using Anchored Multiplex PCR.²⁷ Total nucleic acid containing total RNA and genomic DNA were extracted from formalin-fixed, paraffin-embedded tissue by using the Agencourt FormaPure Kit (Beckman Coulter, Indianapolis, IN, <https://www.beckmancoulter.com>) before undergoing end-repair, adenylation, and adapter ligation. Two hemi-nested PCR reactions were then performed using one primer specific to a sequence in the gene of interest and one specific to a universal sequence in the adapter, for each PCR. This library was then sequenced on the Illumina MiSeq (Illumina, San Diego, CA) and 2×150 base paired-end reads were

aligned to the human genome hg19 reference sequence with the BWA-MEM software package (version bwa-0.7.5a).²⁸ Single-nucleotide variants were detected with MuTect and indels with a custom indel-detection algorithm.²⁹ In regions with sufficient read coverage, this approach is validated to detect indels and single-nucleotide variants with allelic frequencies as low as 5%. The complete list of the genes and exons interrogated is listed in Supplementary Table 2.

Statistical Analysis

Categorical data was analyzed using Fisher's exact test. Statistical analysis was two-sided, and a *P*-value of < 0.05 was considered statistically significant.

Results

Clinicopathologic Features of GNAS-Mutated Lung Carcinomas

The combined cohort of lung carcinomas (*n* = 2352) included the following histotypes: 79% adenocarcinoma, 12% squamous cell carcinoma, 7% non-small cell lung carcinomas not otherwise specified, 1% large cell neuroendocrine carcinoma, and 1% adenosquamous carcinoma. Hotspot *GNAS* mutations involving codons 201 and 227 were identified in 11 of 1282 lung carcinomas (0.9%) at the Center for Advanced Molecular Diagnostics and in 8 of 1070 lung carcinomas (0.7%) at the Center for Integrated Diagnostics (total 19/2352, 0.8%), including 5 at codon 227 and 14 at codon 201 (Table 1). Abdominal and pelvic imaging study results were available for review in 18/19 patients with 15/18 (83%) having

completely negative findings, 1 with multiple liver and bony metastases (case 2), 1 with multiple liver, pancreas, and adrenal gland lesions thought to represent metastases (case 9), and 1 with a 1 cm cystic lesion in the pancreas (case 17). Follow-up MRI imaging was performed on the cystic pancreatic lesion, which revealed a side-branch IPMN. This side-branch IPMN was considered to be indolent in nature and unrelated to the patient's lung tumor. In all 19 *GNAS*-positive cases, the patients were clinically determined to have primary lung tumors.

Patients with *GNAS*-mutated lung carcinomas had a median age of 66 (range 24–78). *GNAS*-mutated lung carcinomas occurred predominantly in female patients (16/19, 84%). Ten of 19 *GNAS*-mutated lung carcinomas (53%) were classified as IMAs, whereas the remaining 9 tumors were adenocarcinomas without mucinous histological features (Figure 1). No cases met the criteria for colloid adenocarcinoma in the material that was available for review. TTF-1 immunohistochemistry was performed when tissue was available (*n* = 11) and was positive in 6/7 non-mucinous adenocarcinomas (86%) and 1/4 IMA (25%) (*P* = 0.09, Figure 2). Patients with *GNAS*-mutated non-mucinous adenocarcinomas were more likely to have a history of smoking (9/9, 100%) than those with IMAs (2/10, 20%) (*P* < 0.001) (Figure 3).

Clinical presentation of IMAs included upper respiratory infection with cough in 2 cases, incidentally identified lung nodule in 4 cases, abdominal pain in 1 case, follow-up imaging for a history of esophageal adenocarcinoma in 1 case, and chronic cough with shortness of breath in 1 case (Supplementary Table 3). Radiographic presentation included multiple bilateral lung nodules in 4 cases,

Table 1 Clinicopathologic and molecular features of *GNAS*-mutated lung adenocarcinoma

Case	Age	Sex	Smoking	Stage	Histotype	TTF1	<i>GNAS</i>	<i>Raf/Ras</i>	<i>TP53</i>	<i>CDKN2A</i>
1	60	F	Current	IV	ACA	+	p.Gln227His	–	p.Gly245Cys	c.457C>A
2	71	F	Former	IV	ACA	+	p.Gln227Leu	–	p.Tyr220Cys	–
3	66	M	Former	I	ACA	+	p.Gln227Leu	–	p.Gln100 ^a	–
4	74	F	Former	I	ACA	NA	p.Gln227Leu	–	–	–
5	67	F	Former	III	ACA	+	p.Gln227Leu	<i>KRAS</i> p.Gly12Cys	–	–
6	78	F	Former	III	ACA	NA	p.Arg201His	–	p.Arg249Gly	–
7	62	F	Former	IV	ACA ^a	–	p.Arg201Leu	<i>HRAS</i> p.Gln61Arg	c.783-1G>T	–
8	69	F	Former	IV	ACA	+	p.Arg201His	<i>BRAF</i> p.Gly469Ala	–	–
9	58	F	Former	IV	ACA	+	p.Arg201Ser	–	p.Arg273Leu	–
10	49	F	Never	I	IMA	+	p.Arg201His	<i>BRAF</i> p.Val600Glu	–	–
11	59	F	Former	I	IMA	–	p.Arg201Cys	<i>KRAS</i> p.Gly12Cys	–	–
12	70	M	Former	I	IMA	–	p.Arg201Cys	<i>KRAS</i> p.Gly12Asp	–	–
13	37	F	Never	IV	IMA	NA	p.Arg201His	<i>KRAS</i> p.Gly12Asp	p.Phe113Leu	–
14	24	F	Never	II	IMA	NA	p.Arg201Cys	<i>KRAS</i> p.Gly12Asp	–	–
15	55	F	Never	I	IMA	NA	p.Arg201Cys	<i>KRAS</i> p.Gly12Val	–	–
16	67	F	Never	I	IMA	NA	p.Arg201His	<i>KRAS</i> p.Gly12Asp	–	–
17	76	F	Never	I	IMA	NA	p.Arg201Cys	<i>KRAS</i> p.Gly13Asp	–	p.Asp68fs
18	75	F	Never	IV	IMA	NA	p.Arg201His	<i>KRAS</i> p.Gly12Asp	–	–
19	48	M	Never	II	IMA	–	p.Arg201Cys	<i>KRAS</i> p.Gly12Asp	–	p.Met54fs

Abbreviations: ACA, adenocarcinoma without mucinous features; IMA, invasive mucinous adenocarcinoma; NA, not available.

^aCytology specimen only available for morphologic review.

Figure 1 *GNAS*-mutated lung carcinoma histology. (a) Case 3 with *GNAS* p.Gln227Leu, non-mucinous adenocarcinoma histology with papillary and micropapillary architecture. (b) Case 4 with *GNAS* p.Gln227Leu, non-mucinous adenocarcinoma histology with acinar architecture. (c) Case 12 with *GNAS* p.Arg201Cys and *KRAS* p.Gly12Asp, invasive mucinous adenocarcinoma. (d) Case 17 with *GNAS* p.Arg201Cys and *KRAS* p.Gly13Asp, invasive mucinous adenocarcinoma.

cavitary or cystic nodules in 3 cases, and ground-glass opacities in 6 cases (Supplementary Table 3). Sixty percent of IMAs (6/10) were stage I at the time of diagnosis compared to 22% of non-mucinous adenocarcinomas (2/9); however, this correlation did not reach statistical significance ($P=0.17$).

GNAS codon 227 Mutated Lung Carcinomas Have Non-Mucinous Adenocarcinoma Histology

Five neoplasms had *GNAS* p.Gln227 mutations (4 p.Gln227Leu, 1 p.Gln227His) (Table 1). All of the *GNAS* p.Gln227 variants were transversion mutations ($A>T$ ($n=4$), $G>T$ ($n=1$)) and had non-mucinous adenocarcinoma histology (Figures 1a and b). The morphologies among these 5 cases included a mix of acinar, papillary, micropapillary, solid, and lepidic patterns. One case had a concurrent *KRAS* mutation (p.Gly12Cys), but did not show mucinous histological features.

GNAS codon 201 Mutated Lung Carcinomas with Concurrent Raf/Ras Pathway Mutations are Associated with Invasive Mucinous Adenocarcinoma Histology

Fourteen neoplasms had *GNAS* p.Arg201 mutations (6 p.Arg201Cys, 6 p.Arg201His, 1 p.Arg201Leu, 1 p.Arg201Ser). Additionally, a subset of these tumors (12/14, 86%) had concurrent mutations in the Ras/Raf pathway (9 *KRAS*, 1 *HRAS*, 2 *BRAF*) (Table 1). All IMA had *GNAS* p.Arg201 mutations and concurrent Ras/Raf pathway mutations (9 *KRAS*, 1 *BRAF*) (Figures 1c and d). A large percentage (78%) of *KRAS* mutations in IMAs with concurrent *GNAS* p.Arg201 mutations were G>A transitions. Similarly, all of the *GNAS* p.Arg201 variants seen in IMAs were also transitions ($G>A$ ($n=4$) or $C>T$ ($n=6$)). Case 7 had *GNAS* p.Arg201Leu and *HRAS* p.Gln61Arg mutations, and while there was only a cytologic specimen available for review, no mucinous features were present. Additionally, Case 8 had *GNAS* p.Arg201His and *BRAF* p.Gly469Ala mutations but did not have histological features of IMA.

Figure 2 TTF-1 immunohistochemistry in *GNAS*-mutated lung invasive mucinous adenocarcinomas. (a,b) Case 10 with *GNAS* p.Arg201His and *BRAF* p.Val600Glu with positive TTF-1 immunohistochemistry. (c,d) Case 11 with *GNAS* p.Arg201Cys and *KRAS* p.Gly12Cys with negative TTF-1 immunohistochemistry.

Figure 3 Clinicopathologic features of *GNAS*-mutated lung carcinomas. IMA, invasive mucinous adenocarcinoma.

Additional Genomic Alterations in *GNAS*-Mutated Lung Adenocarcinomas

Recurrent mutations in *TP53* and *CDKN2A* were identified in the cohort of 19 *GNAS*-mutated lung adenocarcinomas. *TP53* mutations were seen in 3 of

5 (60%) adenocarcinomas with *GNAS* p.Gln227 mutations and 4 of 14 (29%) adenocarcinomas with *GNAS* p.Arg201 mutations. *CDKN2A* mutations were seen in 1 of 5 (20%) adenocarcinomas with *GNAS* p.Gln227 mutations and 2 of 14 (14%) adenocarcinomas with *GNAS* p.Arg201 mutations. *TP53* variants included nonsense, splice site, and missense mutations, including *TP53* p.Tyr220Cys, p.Arg249Gly, and p.Arg273Leu hotspot mutations. *TP53* mutations were more likely to be seen in non-mucinous *GNAS*-mutated adenocarcinomas than in *GNAS*-mutated IMAs, 67 vs 10%, respectively ($P=0.02$). *CDKN2A* variants were frameshift or splice site variants, implying loss of tumor suppressor function (Table 1).

Discussion

GNAS mutations are most commonly associated with gastrointestinal neoplasms, including pancreatic IPMNs and invasive adenocarcinomas arising from IPMNs, colonic adenocarcinomas and mucinous appendiceal tumors.^{1–5,8} Our study

demonstrates that *GNAS* mutations are present in a small subset (0.8%) of primary lung carcinomas. As the genomic profiling of lung adenocarcinomas becomes more clinically utilized, it is important to recognize that *GNAS* variants can be identified in primary lung adenocarcinomas and do not necessarily indicate metastasis from a gastrointestinal site. Fifteen patients in this series underwent abdominal imaging without evidence of extrapulmonary disease. Although this study cannot absolutely exclude that these neoplasms may represent metastases from undiagnosed gastrointestinal sites, we consider this possibility unlikely given the patients' clinical evaluations and the known pathogenesis of pancreatic and other gastrointestinal mucinous neoplasms.

Mutations in both *GNAS* hotspots, p.Arg201 and p.Gly227, are seen in lung carcinomas. Approximately half of *GNAS*-mutated lung carcinomas have IMA histology, all of which have concurrent *GNAS* p.Arg201 and Ras/Raf pathway mutations. In contrast, *GNAS* p.Gly227 mutations are not associated with IMAs. The pathobiology behind this association is uncertain, as both p.Arg201 and p.Gly227 *GNAS* hotspot mutations have similar mechanisms of action, inhibiting GTPase activity, and thereby functioning as a dominant oncogene by constitutively activating Gs.⁶

Patients with IMA histology with concurrent *GNAS* p.Arg201 and Ras/raf pathway alterations are significantly less likely to have a history of smoking than those patients with *GNAS* p.Gln227 mutations and non-mucinous histology. Although *KRAS* mutations in lung carcinoma,^{30,31} particularly *KRAS* transversion mutations (G>T, G>C) have a strong association with smoking,^{32,33} 7 of the 9 IMAs with concurrent *GNAS* p.Arg201 and *KRAS* mutations in our cohort had *KRAS* G>A transition mutations, which is more commonly seen in non-smokers. The presence of these 'gastrointestinal-type' *KRAS* mutations (G>A transitions) has been previously described in primary lung IMAs and should not be taken as evidence that a tumor is a metastasis from a gastrointestinal primary site.³⁴ Additionally, it should be emphasized that the presence of a *GNAS* mutation cannot be used as a tool to distinguish primary lung adenocarcinomas from gastrointestinal or pancreaticobiliary metastases, as *GNAS*-mutated tumors from each of these sites have overlapping morphologic, immunophenotypic, as well as molecular findings. Therefore, careful clinicopathologic correlation is required in these scenarios to properly identify primary site of disease.

IMAs comprise a subset of lung adenocarcinomas with distinctive morphological and clinical features. IMAs are becoming an increasingly heterogeneous molecular group. In addition to common oncogenic mutations in *KRAS*, IMAs may infrequently harbor *ALK* and *NRG1* rearrangements.²⁴ Recurrent *NKX2-1* (also known as *TTF1*) mutations have been identified in conjunction with *KRAS* mutations, and have been

shown to promote pathogenesis and loss of TTF-1 expression in mucinous lung cancer.²³ This study shows an association between IMA histology and mutations involving *KRAS* and *GNAS* p.Arg201, suggesting a novel mechanism by which cooperative *KRAS* and *GNAS* mutations promote primary lung carcinogenesis toward a mucinous or gastrointestinal phenotype. Understanding the association between secondary mutations in *KRAS*-mutated lung adenocarcinoma and morphological phenotype requires additional experimentation and *in vivo* models.

The clinical and biological significance of *GNAS* mutations in lung carcinoma is currently unknown. Some data suggests that patients with pseudomyxoma peritonei and *GNAS* mutations have a significantly shorter progression free survival than patients without *GNAS* mutations.³⁵ Additionally, the same study has found that *GNAS*-mutated colorectal carcinomas have an aggressive clinical course. In contrast, *GNAS*-mutated IPMN-associated carcinomas follow a more indolent course than those with *GNAS* mutations.³⁶

Currently, therapies directly targeting *GNAS* mutations are not available. However, based on preclinical evidence, tumors with *GNAS* mutations may respond to inhibitors of the MAPK pathway.³⁷ More data is needed to determine whether *GNAS* mutations in lung carcinoma portend a particular clinical outcome or will be responsive to a specific targeted therapy. Since IMAs are heterogeneous on the molecular level, and the histological features of *GNAS*-mutated lung adenocarcinomas are not distinctive, broad testing of lung adenocarcinomas should be employed to identify such cases, and will become increasingly necessary should a therapeutic agent become available.

Although *GNAS* mutations are present in an infrequent subset of lung adenocarcinomas, the overall incidence of lung cancer is high, and the prevalence of *GNAS*-mutated lung adenocarcinomas is expected to be significant. This group of neoplasms is important to recognize and correctly classify the primary site based on clinicopathologic features, which can be challenging in the setting of IMA histology and negative TTF-1 immunohistochemistry. Further studies will be needed to determine their clinical course, biologic behavior, and whether they will be amenable to targeted therapies.

Disclosure/conflict of interest

AJI is a stockholder and scientific advisory board member of ArcherDx and is a consultant for Roche, Pfizer, DebioPharm, Chugai, and Constellation Pharmaceuticals. MM-K is a consultant for Merrimack pharmaceuticals, H3 biomedicine, ACD, and Roche. Authors LLR, MV, VN, LMS, and FD have no disclosures.

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Supplementary Information accompanies the paper on *Modern Pathology* website (<http://www.nature.com/modpathol>)