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Molecular Analysis of *PIK3CA*, *BRAF*, and *RAS* Oncogenes in Periapillary and Ampillary Adenomas and Carcinomas

Frank Schönleben,

Department of Otolaryngology/Head and Neck Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA; Department of General Surgery, University of Erlangen-Nuremberg, Erlangen, Germany

Wanglong Qiu,

Department of Otolaryngology/Head and Neck Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

John D. Allendorf,

Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

John A. Chabot,

Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Helen E. Remotti, and

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Gloria H. Su

Department of Otolaryngology/Head and Neck Surgery, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA; Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Gloria H. Su: gs2157@columbia.edu

Abstract

Background—Mutations of *KRAS* are known to occur in periapillary and ampillary adenomas and carcinomas. However, nothing is known about *NRAS*, *HRAS*, *BRAF*, and *PIK3CA* mutations in these tumors. While oncogenic *BRAF* contributes to the tumorigenesis of both pancreatic ductal adenocarcinoma and intraductal papillary mucinous neoplasms/carcinomas (IPMN/IPMC), *PIK3CA* mutations were only detected in IPMN/IPMC. This study aimed to elucidate possible roles of *BRAF* and *PIK3CA* in the development of ampillary and periapillary adenomas and carcinomas.

Methods—Mutations of *BRAF*, *NRAS*, *HRAS*, *KRAS*, and *PIK3CA* were evaluated in seven adenomas, seven adenomas with carcinoma in situ, and 21 adenocarcinomas of the periapillary duodenal region and the ampulla of Vater. Exons 1 of *KRAS*; 2 and 3 of *NRAS* and *HRAS*; 5, 11, and 15 of *BRAF*; and 9 and 20 of *PIK3CA* were examined by direct genomic sequencing.

Results—In total, we identified ten (28.6%) *KRAS* mutations in exon 1 (nine in codon 12 and one in codon 13), two missense mutations of *BRAF* (6%), one within exon 11 (G469A), and one V600E hot spot mutation in exon 15 of *BRAF*. *BRAF* mutations were present in two of five

periampullary tumors. All mutations appear to be somatic since the same alterations were not detected in the corresponding normal tissues.

Conclusion—Our data provide evidence that oncogenic properties of *KRAS* and *BRAF* but not *NRAS*, *HRAS*, and *PIK3CA* contribute to the tumorigenesis of periampullary and ampullary tumors; *BRAF* mutations occur more frequently in periampullary than ampullary neoplasms.

Keywords

Ampullary cancer; Periampullary; *KRAS*; *BRAF*; *PIK3CA*

Introduction

Ampullary cancers account for 5% of all gastrointestinal tract malignancies.¹ Tumors of the ampulla of Vater include tumors arising in the ampulla (intra-ampullary type), tumors arising in the periampullary region of the duodenum (periampullary type), or tumors involving both the intra-ampullary and periampullary region of the duodenum (mixed periampullary and intra-ampullary type).

Ampullary adenocarcinomas often are identified in association² with adenoma precursor lesions. Ampullary carcinomas represent approximately 10% of cancers resected via the Whipple procedure (pancreaticoduodenectomy).³ The peak age incidence is in the 8th decade, with men more commonly affected than women.² The 5-year survival rates of patients with resected ampullary carcinoma are reported to be 33–50%,⁴ and thus significantly better than the observed 10–20% 5-year survival rate of patients with resected conventional pancreatic ductal adenocarcinoma.⁵ These data suggest that differences in tumor biology may also be an explanation for the relatively good survival of patients with this disease. The biological characteristics of periampullary and ampullary adenocarcinomas have not been extensively studied. Genetic alterations of the *KRAS* oncogenes,⁶ the *p53*,⁷ *p16*,⁸ and *MADH4*(*SMAD4/DPC4*)⁹ tumor suppressor genes, all commonly altered in pancreatic cancer,¹⁰ have also been described in periampullary and ampullary cancer, although at lower frequencies.¹¹

Oncogenic point mutations in the three human *Ras* genes (*NRAS*, *HRAS*, *KRAS*) have been detected in a wide variety of human cancers. Since the discovery of the role of *RAS* oncogenes in tumorigenesis, an increasing focus has been set to define its oncogenic signal transduction pathway and the mitogen-activated protein kinase (MAPK) pathway has emerged as an important link between membrane-bound Ras proteins and the nucleus,^{12,13} involving the kinase cascade Raf–MEK–ERK (*MEK*, MAPK/ERK kinase; *ERK*, extracellular signal-related kinase).¹⁴ Signaling through the MAPK cascade is transduced by guanosine triphosphate loading of Ras leading to the activation of Raf kinase. *BRAF* mutations have been described in about 15% of all human cancers and are known to have a mutational hot spot at codon 600, which is reported to account for 91% of *BRAF* mutations in human cancers.¹⁵ We and others have previously shown that oncogenic *BRAF* contributes to the tumorigenesis of pancreatic ductal adenocarcinoma and IPMN/IPMC of the pancreas.^{16–18}

Phosphatidylinositol-3 kinases (PI3Ks) constitute a large and complex family of lipid kinases.^{19–21} They play an important role in several cellular functions, such as proliferation, differentiation, chemotaxis, survival, trafficking, and glucose homeostasis,¹⁹ activating diverse cellular target proteins such as the survival signaling kinase AKT/PKB.^{19,20,22} A tumorigenic role has been proposed for the *PIK3CA* gene that encodes the catalytic p110 α subunit of phosphatidylinositol 3-kinase belonging to the class IA of PI3Ks.^{19,21} Previously, Samuels et al.²³ reported mutations in *PIK3CA* in several tumor types. In the study by

Samuels et al.²³ three *PIK3CA* mutational hot spots were described and found to affect the helical (exon 9) and catalytic (exon 20) protein domains. Similar to colon tumors, *PIK3CA* mutations also clustered in the three hot spot regions (exons 9 and 20) in gastric carcinomas.^{23,24} Other independent studies in hepatocellular carcinomas, breast carcinomas, lung cancers, ovarian carcinomas, brain tumors, head and neck squamous cell carcinomas, and intraductal papillary mucinous neoplasms of the pancreas have since supported and emphasized the oncogenic potential of *PIK3CA* in the development of cancer.^{24–29} We and others have previously shown that *PIK3CA* mutations occur in ~10% of IPMN/IPMC but not in the pancreatic ductal adenocarcinoma.^{23,29,30} In the present study, we analyzed the mutational status of *BRAF*, *NRAS*, *HRAS*, *KRAS*, and *PIK3CA* to elucidate a possible role of these genes in the tumorigenesis of periampullary and ampullary adenomas and carcinomas.

Materials and Methods

Patients and Tissue Samples

Surgical paraffin-embedded adenoma and carcinoma samples from 31 patients (female $n=17$, male $n=14$, median age 63.6 years, range 40–85 years) were obtained from the archival tissue collection of the Columbia University Medical Center. Acquisition of the tissue specimens was approved by the Institutional Review Board of Columbia University Medical Center and performed in accordance with Health Insurance Portability and Accountability Act regulations. In detail, we analyzed 35 tumor areas including seven adenomas, seven adenomas with carcinoma in situ (CIS), and 21 carcinomas of the periampullary and ampullary region. The anatomic distribution of the tumors included 24 ampullary tumors, six tumors involving both ampulla and periampullary duodenal region, and five periampullary duodenal tumors. Of the five periampullary tumors analyzed, two tumors involved the papilla of Vater whereas three tumors were located within 2 cm of the papilla and were classified as periampullary tumors of duodenal origin (see Table 1 for a more detailed register.)

DNA Samples for Mutation Analysis

All tissue samples were handled in an environment free of polymerase chain reaction (PCR) products. Paraffin-embedded tumor samples were microdissected by hand. Surrounding nontumorous tissue or tissue derived from a tumor-free specimen of the same patient served as the corresponding normal control. Genomic DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). The procedures were performed according to the manufacturers' instructions for paraffin-embedded tissues.

Exons 1 of *KRAS*, 2 and 3 of *NRAS* and *HRAS*, exons 5, 11, and 15 of *BRAF*, and exons 9 and 20 of *PIK3CA* were analyzed by PCR amplification of genomic DNA and direct sequencing of the PCR products. Genomic DNA (40 ng per sample) was amplified with primers that had been designed to specifically amplify the codons 12 and 13 of *KRAS* or each exon and its exon/intron boundaries in the *NRAS*, *HRAS*, *BRAF*, and *PIK3CA* loci, respectively. The primers were adopted from those published in the literature to omit analyzing the *BRAF* and *KRAS* pseudogenes.^{31–33} Before sequencing, all PCR products were purified, using QIA-quick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing was performed with ABI's 3100 capillary automated sequencers at the DNA core facility of Columbia University Medical Center. All samples found to have genetic alteration in the target genes were subsequently sequenced in the reverse direction to confirm the mutation. The mutations were then further verified by sequencing of a second PCR product derived independently from the original template.

Results

In the present study, 35 periampullary and ampullary adenomas and carcinomas were analyzed for mutations in the *BRAF*, *NRAS*, *HRAS*, *KRAS*, and *PIK3CA* genes. We performed sequencing analyses of codons 12 and 13 of exon 1 of *KRAS*, the entire exons 2 and 3 of *HRAS* and *NRAS*, exons 5, 11, and 15 of *BRAF*, and exons 9 and 20 of *PIK3CA* in all these specimens. These regions included the most common *HRAS*, *NRAS*, *KRAS*, *BRAF*, and *PIK3CA* mutations previously observed in human cancers.^{23,31–33}

Two *BRAF* mutations (6% of all samples, 66% of periampullary tumors originating from the duodenum) were identified in our set of samples: one exon 15 hot spot mutation at nucleotide 1799 (GTG→GAG), leading to an amino acid change from valine to glutamic acid (V600E) and one exon 11 mutation at nucleotide 1406 (GGA→GCA), leading to an amino acid change from glycine to alanine (G469A), which has also been described previously (Fig. 1 and Table 1). Interestingly, both mutations were found in periampullary adenomas with CIS originating from the duodenal mucosa. We did not identify any mutation in exon 5.

KRAS mutations were found in ten (28.6%) of the 35 samples (Fig. 2 and Table 1). Nine tumors carried a mutation of codon 12 and one a mutation of codon 13. In detail, we identified four *KRAS* mutations within the 14 adenomas (28.6%) and six mutations in the 21 ampullary and periampullary carcinomas (28.6%). The distribution of *KRAS* mutations showed a single mutation in all observed cases. The coexistence of *KRAS* and *BRAF* mutations was observed in a periampullary adenoma sample (see Table 1). All mutations proved to be somatic since none of them was detected within the matching normal tissues (Fig. 1). No mutation was detected in the *HRAS*, *NRAS*, and *PIK3CA* loci.

Discussion

Carcinomas of the ampulla of Vater and the periampullary region are distinguished from conventional pancreatic ductal adenocarcinoma clinically and pathologically, but the relationship of these tumor types at the genetic level is still being investigated. *KRAS* gene mutations have been demonstrated in periampullary and ampullary adenomas, even in areas of low-grade dysplasia. In addition, there is a strong correlation (93%) between the *KRAS* gene mutation found in ampullary adenomas and their associated infiltrating carcinomas,^{34,35} indicating that *KRAS* gene mutations in ampullary cancer, when present, occur early in tumorigenesis. Mutant Kras constitutively activates the Raf–MEK–ERK–MAP kinase pathway, which mediates cellular response to various growth signals.^{36,37} Unlike pancreatic ductal adenocarcinoma, where *KRAS* is mutated at a frequency close to 100%.^{38,39} approximately 60% of ampullary carcinomas do not harbor an active *KRAS* mutation. This suggests that a relatively large percentage of periampullary and ampullary adenomas and carcinomas might use alternative ways to activate the RAS–RAF–MEK–ERK–MAP kinase pathway.

BRAF, a serine/threonine kinase located immediately downstream in RAS signaling, has been examined and found to be mutated in a variety of human malignant neoplasms. Here, we report two somatic *BRAF* mutations out of 35 (6%) periampullary and ampullary neoplasms examined. Our data are in concordance with the literature, which described a trend for *BRAF* mutations in cancer types harboring *KRAS* mutations.³¹ *BRAF* is known to have a mutational hot spot at nucleotide 1799, which is reported to account for 91% of *BRAF* mutations in human cancers^{15,31,33,40} (V600E). All mutations observed in our set of neoplasms were within exon 11 and 15. These mutations are not only the predominant type in melanoma but also in colon cancer and sarcoma.³¹ Transfection assays revealed that these

mutations were active *in vitro* and stimulate the activity of the ERK pathway *in vivo*.³¹ Davies et al. showed that Ras function was not required for the growth of cancer cell lines with the V600E mutation. Mutations at exon 11, codon 469, of *BRAF* have been found in several tumors and appear to be the second most frequent mutation of *BRAF* in human cancers.¹⁵ The G469A *BRAF* mutant in particular has been shown to have similar activity to V600E *BRAF* and is also generated through a single-nucleotide substitution but accounts for less than 1% of mutations.³¹ In our study, *KRAS* and the G469A *BRAF* mutation occur simultaneously in one periampullary adenoma of duodenal origin. It has been observed previously that *BRAF* mutations, other than *BRAF* V600E, coexisted with *RAS* mutations.³¹ The *BRAF* V600E mutation seems to uncouple cells from their proliferation requirement of *RAS*, and mutation of *RAS* was not detected in any of the tumors carrying this particular mutation.³¹ However, *in vitro* data indicated that V600E *BRAF* mutants can be further activated by mutant *RAS*, whereas other *BRAF* mutants remain dependent on *RAS* function.³¹ A previous study on pancreatic ductal adenocarcinoma revealed that the V600E mutation occurred in two of nine xenografted tumors retaining wild-type copies of the *KRAS*, *NRAS*, and *HRAS* genes, but none in 72 xenografted carcinomas with *KRAS* mutations within exons 11 and 15.¹⁶ In contrast, another study on pancreatic adenocarcinoma found both *KRAS* and *BRAF* V600E mutations coexisting in two cases.¹⁷ Previously, we were able to show that, in IPMN, *KRAS* mutations coexist with *BRAF* mutations, other than the V600 mutation.¹⁸ Cells with activating mutations in both *KRAS* and *BRAF* had a substantially higher B-Raf kinase activity and ERK 1/2 phosphorylation activities than those with *BRAF* mutation alone.³¹ So tumors with both *BRAF* and *KRAS* mutations might have an accelerated course in terms of development or progression. *BRAF* mutations have been detected in the early stages of colon cancer and melanoma development.^{41,42} These observations are in concordance with our results, where both *BRAF* mutations occurred in periampullary adenomas with CIS, indicating that *BRAF* mutation, when present, is an early event in tumorigenesis.

In summary, we found two *BRAF* and ten *KRAS* and no mutations of *HRAS*, *NRAS*, and *PIK3CA* in 35 periampullary and ampullary adenoma and carcinoma samples. The rarity of *PIK3CA* mutation in periampullary and ampullary adenoma and carcinoma is potentially significant, given that *PIK3CA* mutations was previously reported in 10% of IPMN but not in pancreatic ductal adenocarcinoma.^{23,29,30} There appears to be a divergent role for *PIK3CA* in the tumorigenesis of IPMN/IPMC and adenocarcinoma. Another intriguing aspect of our results is that both *BRAF* mutations were found in two of a total of three (66%) periampullary adenomas of duodenal origin and none in ampullary cancer. All mutations found proved to be somatic. This is the first mutational study of *BRAF*, in periampullary and ampullary neoplasms. Our data indicate that *BRAF* mutations do not play a major role in the tumorigenesis of ampullary carcinomas but are of importance in periampullary lesions, specifically those of duodenal origin. Targeted mutation of *BRAF* is an early event, which suggests that alteration of RAS–RAF–MEK–ERK–MAP kinase pathway by *BRAF* mutation together with *RAS* mutation plays an important role in periampullary tumorigenesis.

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Figure 1. Somatic *BRAF* mutations found in two duodenal periampullary lesions with their respective *KRAS* status (*WT*, wild type). One of the *BRAF* mutations (*V600E*) was a hot spot mutation. All mutations were confirmed to be somatic.

Figure 2.

Four KRAS mutant codons were identified in ten specimens. Nine tumors carried a mutation of codon 12 and one a mutation of codon 13. Representative of each mutant codon is shown here. All codon changes were somatic.

Table 1
Summary of the Sample Data and Mutation Status of the Lesions Investigated

Sample no.	Age	Sex	Lesion analyzed	Anatomic location Ampullary	Anatomic location Periampullary	pTNM	Stage	KRAS mutation	BRAF mutation
1	76	M	CA + CIS	X		pT3N0	II		
2	74	M	CA + CIS	X	X	pT2N0	II		
3	72	F	CA + CIS		X	pT3N1	III		
4	58	M	Adenoma	X	X	<i>a</i>	0	G13D	
5	49	M	CA + CIS	X	X	pT3N1	III	G12D	
6 ^a	63	F	CA, small cell	X		pT3N1	III		
6 ^a			Adenoma + CIS	X					
7	72	F	CA + CIS	X		pT2N0	II		
8	65	F	Adenoma	X		<i>a</i>	0		
9	66	F	Adenoma	X		<i>a</i>	0		
10	71	F	CA + CIS	X		pT3N1	III		
11	56	F	Adenoma + CIS	X		<i>a</i>	0		
12	65	F	CA + CIS	X		pT3N1	III		
13	40	M	Adenoma	X		pT2N0	II		
14	73	M	CA + CIS	X		pT3N1	III		
15	71	M	CA + CIS	X		pT2N0	II		
16	70	F	CA + CIS	X	X	pT3N0	II	G12D	
17	78	M	CA + CIS	X		pT1N0	I		
18	52	M	Adenoma + CIS		X-D	pT1N0	I	G12V	G469A
19	68	M	CA + CIS	X		pT1N0	I		
20	68	M	CA + CIS	X		pT3N0	II	G12D	
21 ^a	85	F	CA + CIS	X		pT2N0	II	G12R	
21 ^a			Adenoma + CIS	X				G12R	
22	44	F	Adenoma	X		<i>a</i>	0		
23	64	F	CA + CIS	X		pT3N1	III		
24	67	F	CA + CIS	X		pT3N0	II	G12V	
25 ^a	51	M	Adenoma + CIS		X-D	pTisN0	0		
25 ^a			Adenoma		X	<i>a</i>	0		

Sample no.	Age	Sex	Lesion analyzed	Anatomic location Ampullary	Anatomic location Periapullary	pTNM	Stage	KRAS mutation	BRAF mutation
26	44	F	Adenoma	X	X	a	0	G12D	
27	77	F	CA + CIS	X		pT2N0	II		
28 ^a	40	M	CA + CIS	X		pT3N0	II		
28 ^a			CA ^b	X					
29	71	F	Adenoma + CIS	X	X	pTisN0	0		
30	78	F	Adenoma + CIS		X-D	pT4N1	III		V600E
31	43	M	CA	X		pT3N1	III	G12V	

CA invasive carcinoma, CIS carcinoma in situ, Anatomic location of tumor: *ampullary* periapullary or both, *D* duodenal mucosa adjacent to ampulla not involving papilla of Vater, *TNM* stage of tumor in Whipple resection specimen (Tis: carcinoma in situ)

^aDifferent areas of same tumor were analyzed.

^bHigh-grade/giant cell