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***BRAF* and *KRAS* Gene Mutations in Intraductal Papillary Mucinous Neoplasm/Carcinoma (IPMN/IPMC) of the Pancreas**

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Abstract

The Raf/MEK/ERK (MAPK) signal transduction is an important mediator of a number of cellular fates including growth, proliferation and survival. The *BRAF* gene is activated by oncogenic *RAS*, leading to cooperative effects in cells responding to growth factor signals. Our study was performed to elucidate a possible role of *BRAF* in the development of IPMN (Intraductal Papillary Mucinous Neoplasm) and IPMC (Intraductal Papillary Mucinous Carcinoma) of the pancreas. Mutations of *BRAF* and *KRAS* were evaluated in 36 IPMN/IPMC samples and two mucinous cystadenomas by direct genomic sequencing. Exons 1 for *KRAS*, and 5, 11, and 15 for *BRAF* were examined. Totally we identified 17 (47%) *KRAS* mutations in exon 1, codon 12 and one missense mutation (2.7%) within exon 15 of *BRAF*. The mutations appear to be somatic since the same alterations were not detected in the corresponding normal tissues. Our data provide evidence that oncogenic properties of *BRAF* contribute to the tumorigenesis of IPMN/IPMC, but at a lower frequency than *KRAS*.

Keywords

Intraductal papillary mucinous neoplasm; pancreas; *KRAS*; *BRAF*

Introduction

There has been an increase in the number of IPMN cases reported recently, although it is not clear if this represents a true increase in incidence or a manifestation of increased recognition and detection of their unique clinical, pathologic, and molecular features [1–6]. Most IPMNs are slow growing and less aggressive compared with conventional, ductal adenocarcinoma. An infiltrating adenocarcinoma, however, is not infrequently identified in pancreases affected by IPMNs, suggesting that IPMNs may evolve into invasive ductal adenocarcinomas [3,5,6]. IPMNs are subdivided into three groups based on increasing nuclear and architectural atypia: adenoma, borderline and intraductal papillary mucinous carcinoma (IPMC) [7]. IPMCs are

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further separated into invasive and noninvasive types depending on the absence or presence of neoplastic cells invading the pancreatic tissue surrounding the involved ducts [8]. The overall incidence of invasive carcinoma associated with an IPMN is 20% to 40% [9]. Although the majority of invasive carcinomas are associated with IPMC, invasive carcinoma coexisting with adenoma and borderline IPMN does occur [10]. In addition, invasive carcinoma is sometimes found distant from an IPMN, and small IPMNs have been detected incidentally in pancreases resected for conventional ductal pancreatic cancer [4].

In the quest to understand how oncogenic Ras proteins transmit extracellular growth signals, the MAP kinase (MAPK) pathway has emerged as an important link between membrane-bound Ras proteins and the nucleus. This key Ras effector pathway involves the kinase cascade Raf/MEK/ERK (*MEK*, MAPK/ERK kinase; *ERK*, extracellular signal-related kinase) [11–13]. Signalling through the MAPK cascade is transduced by GTP loading of Ras leading to the activation of Raf kinase. In mammalian cells, there are three isoforms of *RAF*: *ARAF*, *BRAF* and *CRAF/RAFI* [11,14]. Although all three of the Raf isoforms share a common function with respect to MEK phosphorylation, studies have shown that these proteins might be differentially activated by oncogenic Ras [11,14]. Recently, *BRAF* mutations have been described in about 15% of all human cancers, such as malignant melanomas, papillary thyroid cancer, lung cancer, and ovarian cancer [15–20].

Reported genetic alterations in IPMNs include mutations in the *KRAS* [21–26], *PIK3CA* [27], *TP53* [24], and *STK11/LKB1* genes [28,29] as well as loss of heterozygosity (LOH) of several chromosomal loci [28,30]. In addition to these genetic alterations, aberrant DNA methylation may contribute to the inactivation of a subset of tumor-suppressor genes in IPMNs [31,32]. Previous studies have found mutations in the exon 1 of *KRAS* in 31% to 86 % of IPMNs [21–26]. The genetic status of *BRAF* has not yet been evaluated previously. In the present study, we analysed the status of the *BRAF* gene together with *KRAS* to elucidate a possible role of these genes in the tumorigenesis of IPMNs and IPMCs.

Materials and Methods

Patients and Tissue Samples

Surgical paraffin embedded IPMN/IPMC and mucinous cystadenoma samples resected from 38 patients between 2000 and 2005 (female n=14, male n= 24, median age 68.1 years, range 41–84 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 (HIPPA) regulations. In detail, we analyzed three IPMN, adenoma (female n= 1, male n = 2, median age 62.7 years, range 53–77 years); four IPMN, borderline (female n= 1, male n= 3, median age 66.3 years, range 62–72 years), five IPMC without invasion (male n= 5, median age 69.2 years, range 59–81), 24 IPMC with invasive carcinoma (male n= 14, female= 10, median age 68.9 years, range 41–84 years), and two mucinous cystadenomas (female n=2, median age 57.5 years, range 53–62 years). Thirty-two of these lesions arose in the pancreatic head, one in the uncinate process, four within the transition from pancreatic head to the body, one within the body and one diffusely involved the entire gland. The maximum dimension of the lesions ranged from 0.4 to 7cm (mean: 4.2 cm). All samples were reviewed using the same criteria by two pathologists (N. T. C. and H. E. R.). Please see Table 1 for a more detailed register.

DNA Samples for Mutation Analysis

Paraffin embedded tumour samples were micro-dissected to ensure the highest possible amount of tumour cells. Surrounding non-tumorous tissue or tissue derived from a tumour free block

of the patient served as the corresponding normal control. Genomic DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Valencia, CA). The procedures were performed according to the manufacture's instructions for paraffin embedded tissues.

Exons 1 for *KRAS* and exons 5, 11, and 15 for *BRAF* were analyzed by PCR amplification of genomic DNA and direct sequencing of the PCR products. Genomic DNA (40ng per sample) was amplified with primers that had been designed to specifically amplify the codons 12 and 13 of *Kras* or each of the three exons and its exon/intron boundaries in the *BRAF* locus. The primers were adopted from those published in the literature to omit analysing the *BRAF* and *KRAS* pseudogenes [16,17,33]. Before sequencing, all PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacture's instruction. 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 mutation was then further verified by sequencing of a second PCR product derived independently from the original template.

Results

In the present study, 36 IPMN/IPMC and two mucinous cystadenoma specimens were analyzed for mutations in the *KRAS* and *BRAF* genes. We performed sequencing analyses of codons 12 and 13 in the exon 1 of *KRAS* and the entire exons 5, 11, and 15 of *BRAF* in all these specimens. These regions included the most common *KRAS* and *BRAF* mutations previously observed in human cancers [16,17,33]. We identified 17 (47%) mutations within the *KRAS* gene at codon 12 and one mutation (2.7%) in the exon 15 of *BRAF* at codon 615 (C1847T, TCC→TTC) in the 36 IPMN/IPMC specimens. The *BRAF* C1847T mutation leads to an amino acid change from serine to phenylalanine. All the mutations were sporadic, since none of the mutations was observed within the matching normal tissues (Figure 1). Of the 17 samples that harboured the *KRAS* codon 12 mutations, five were IPMN cases without associated invasive carcinoma (5/12 samples, 41.7%; one nuclear grade 1, two nuclear grade 2, and two nuclear grade 3) and 12 IPMC with associated invasive carcinoma (12/24 samples, 50%; all nuclear grade 3) (Table 1). The *KRAS* codon 12 mutation was detected in IPMN, adenoma (1/3), IPMN, borderline (2/4), IPMC without invasion (2/5), and IPMC with invasion (12/24) (Table 1). The *KRAS* codon 12 mutation spectrum includes G12D (7/17), G12V (6/17), and G12R (4/17) mutations. The exon 15 *BRAF* mutation was found in an IPMC sample with associated invasive carcinoma, which also harbored a *KRAS* (G12R) mutation (Table 1).

Discussion

Frequent *KRAS* gene mutations at codon 12 have been reported in several cancers, including those from colonic and pancreatic tissues [34–37]. Previous studies have found *KRAS* mutations, mainly at codon 12 in the exon 1, in 31% to 86 % of IPMNs (47% in our study) [21–26]. The wide variety of the reported frequencies most likely is due to the ongoing better definition of these lesions [7,38,39] and might also be dependent on the sensitivity of a chosen screening methodology [40,41]. For this study, we chose to use the plain PCR technique with direct genomic DNA sequencing to keep possible false positive results to a minimum.

In the present study the distribution of *KRAS* mutation showed a single mutation in all observed cases. Multiple mutations in the same sample were not observed. *KRAS* mutation is an early event in the tumorigenesis of IPMN- *KRAS* mutation was observed in IPMN, adenoma (1/3) and its mutation frequency remains consistent as IPMN progresses (2/4 for in IPMN, borderline; 2/5 in IPMC; and 12/24 in IPMC with invasion). There was no tumor size, gender, or age bias observed associated with *KRAS* mutation.

Unlike pancreatic ductal adenocarcinoma where *KRAS* is mutated at a frequency close to 100% [36,37], 14–69% (53% in our study) of IPMNs do not harbor an active *KRAS* mutation. This suggests that a relatively large percentage of IPMNs/IPMCs might use alternative ways other than *KRAS* mutation to stimulate this Ras-Raf-MEK-ERK-MAP kinase pathway. *BRAF*, a serine/threonine kinase located immediately downstream in Ras signalling, has been found frequently mutated in a variety of human malignant neoplasms [15–17,19,33,42,43]. Here we report a somatic *BRAF* mutation out of 36 cases of IPMN/IPMC examined (2.7%). While *BRAF* contributes to the tumorigenesis of IPMN, it is not a frequent event and certainly does not entirely explain the lower mutation rate of *KRAS* in IPMN/IPMC than in pancreatic ductal adenocarcinoma.

The *BRAF* mutation occurred at nucleotide 1847, a C to T change at codon 615 of the *BRAF* gene, leading to an amino acid change from serine to phenylalanine (S615F). Although located at exon 15, the S615F mutation is not the previously described hot-spot mutation at exon 15 (V599E) of the *BRAF* gene [15,16,33]. This mutation was also found to co-exist with a G12R mutation of *KRAS* in the same sample. It has been observed previously in colon and lung cancers that *BRAF* mutations, other than *BRAF* V599E, coexisted with *RAS* mutations [16]. The *BRAF* V599E mutation seems to uncouple cells from their proliferation requirement of *RAS*, and therefore mutation of *RAS* was not detected in any of the tumors carrying *BRAF* V599E mutation [16]. *In vitro* data indicated that *BRAF* V599E mutants can be further activated by mutant *RAS*, whereas other *BRAF* mutants remain dependent on *RAS* function [16]. A previous study on pancreatic ductal adenocarcinoma revealed that the *BRAF* hot-spot mutation was observed in two of nine tumors retaining wild-type copies of the *KRAS*, *NRAS*, and *HRAS* genes, but none in 72 adenocarcinomas with *KRAS* mutations within exons 11 and 15 [44]. In contrast, another study found both *KRAS* and *BRAF* V599E mutations coexisting in two cases of pancreatic ductal adenocarcinoma [45]. These two cases did not exhibit different clinicopathological characteristics from pancreatic cancers with *KRAS* mutation alone [45]. The novel S615F mutation observed here is also in the B-Raf activation segment [46], but its functional effect is unknown. 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 [16]. It is possible that tumors with both *BRAF* and *KRAS* mutations have an accelerated course in the development or progression of the tumors. Together, these observations suggest that different *BRAF* mutations can have distinct transforming potential in tumorigenesis, which would be worthy of further investigations in future studies.

This is the first mutational study of *BRAF* in IPMN/IPMC. Although the *BRAF* mutation frequency in IPMN/IPMC is low compared with those observed in malignant melanoma and colon cancers, our data suggests that alteration of the Ras-Raf-MEK-ERK-MAP kinase pathway by *BRAF* mutation together with *RAS* mutation may play an important role in the tumorigenesis of IPMN/IPMC.

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Figure 1. *BRAF* and *KRAS* mutations found in IPMNs/IPMCs. Representatives of the mutations identified in our cohort within the loci of *BRAF* and *KRAS*. All the mutations were confirmed to be somatic.

Table 1

Summary of the sample data and mutation status of the lesions investigated

Case Nr.	Sex	Age	Lesion analyzed	IPMN Nuclear Grade	Differentiation of invasive carcinoma, if present in resection	Location within pancreas	Maximum Dimension** (cm)	KRAS mutation	BRAF mutation
1	M	62	IPMN, borderline	2	N/A	body	-		
2	M	73	IPMC with invasion	3	moderate	head and body	4	+	
3	M	67	IPMC with invasion	3	moderate	head	-	+	
4	M	69	IPMC	3	N/A	head	3.5	+	
5	F	75	IPMC with invasion	3	moderate	head	5.5		
6	F	68	IPMC with invasion	3	moderate	head	5	+	
7	M	65	IPMN, borderline	2	poor (*)	head	5		
8	F	66	IPMN, borderline	2	N/A	uncinate process	2	+	
9	M	84	IPMC with invasion	3	moderate	head	5		
10	M	53	IPMN, adenoma	1	N/A	head	3.5	+	
11	M	71	IPMC with invasion	2-3	N/A	head	-		
12	M	81	IPMC	3	N/A	head	2.5		
13	M	63	IPMC	3	moderate to poor	head	2.3		
14	M	66	IPMC with invasion	3	moderate (*)	head	6		
15	F	70	IPMC with invasion	3	moderate to poor	head and body	7	+	+
16	F	70	IPMC with invasion	3	moderate	head	1.5		
17	M	72	IPMN, borderline	2	N/A	head	0.4		
18	F	53	mucinous cystadenoma	1	N/A	head	3		
19	M	79	IPMC with invasion	3	moderate to poor	head	6	+	
20	M	63	IPMC with invasion	3	moderate to poor	head	3.5	+	
21	M	77	IPMN, adenoma	1	N/A	head and body	2.2		
22	F	62	mucinous cystadenoma	1	N/A	head	2		
23	M	41	IPMC with invasion	3	moderate to poor	head	5		
24	M	71	IPMC with invasion	3	moderate to poor	head	1.5		
25	F	58	IPMN, adenoma	1	N/A	head	1.5		
26	M	49	IPMC with invasion	3	moderate	head	4.5		
27	M	71	IPMC with invasion	3	moderate to poor	head	5.5	+	
28	M	74	IPMC	3	well (*)	head and body	-		
29	M	59	IPMC	3	poor (*)	head	7	+	
30	M	81	IPMC with invasion	3	moderate to poor	head	3		
31	F	80	IPMC with invasion	3	moderate to poor	head	5	+	
32	F	66	IPMC with invasion	3	poor	head	3		
33	F	77	IPMC with invasion	3	poor	head	3		
34	M	73	IPMC with invasion	3	poor	head	5.5	+	
35	F	77	IPMC with invasion	3	well	head	3.2	+	
36	F	61	IPMC with invasion	3	well	head	1	+	
37	M	62	IPMC with invasion	3	moderate	head	2.2	+	
38	F	59	IPMC with invasion	3	moderate	head	3.4	+	

N/A, not applicable.

* Invasive carcinoma was associated with IPMN/IPMC in pancreatic resection, but lesion analyzed did not sample invasive carcinoma.

** Maximum tumor size includes both invasive and non-invasive components of tumor; -, not available. + Positive for mutation.