

ORIGINAL ARTICLE

GNAS codon 201 mutations are uncommon in intraductal papillary neoplasms of the bile duct

Hanno Matthaei^{1,4*}, Jian Wu^{5,6*}, Marco Dal Molin¹, Marija Debeljak¹, Philipp Lingohr⁴, Nora Katabi⁷, David S. Klimstra⁷, N. Volkan Adsay⁸, James R. Eshleman^{1,2}, Richard D. Schulick^{1,3}, Kenneth W. Kinzler⁵, Bert Vogelstein^{5,9}, Ralph H. Hruban^{1,2} & Anirban Maitra^{1,2}

Departments of ¹Pathology, ²Oncology and ³Surgery, Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA, ⁴Department of Surgery, University of Bonn, Bonn, Germany, ⁵Ludwig Center for Cancer Genetics, Johns Hopkins University School of Medicine, Baltimore, MD, USA, ⁶State Key Laboratory of Cancer Biology, Cell Engineering Research Center & Department of Cell Biology, The Fourth Military Medical University, Xi'an, China, ⁷Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, ⁸Department of Pathology, Emory University School of Medicine, Atlanta, GA, USA and ⁹Department of Oncology, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Background: Activating point mutations of *GNAS* at codon 201 have been detected in approximately two thirds of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas. Intraductal papillary neoplasms of the bile ducts (IPNBs) morphologically resemble pancreatic IPMNs. This study sought to assess the mutational status of *GNAS* at codon 201 in IPNBs.

Methods: Thirty-four patients were included. DNA from microdissected IPNBs was subjected to a polymerase chain reaction and ligation method for the detection of *GNAS* mutations at codon 201 and of *KRAS* mutations at codon 12. Mutational status was compared with clinical and pathologic data.

Results: The IPNBs had a median diameter of 3.5 cm and were located intrahepatically ($n = 6$), extrahepatically ($n = 13$), both intra- and extrahepatically ($n = 4$) or in the gallbladder (intrahepatic papillary neoplasms, $n = 11$). Most exhibited pancreatobiliary differentiation ($n = 20$), high-grade dysplasia ($n = 26$) and an associated adenocarcinoma ($n = 20$). Analysis of *GNAS* codon 201 identified only one mutant sample in a multifocal intestinal subtype intrahepatic IPNB with high-grade dysplasia. Six lesions harboured a *KRAS* codon 12 mutation.

Conclusions: *GNAS* codon 201 mutations are uncommon in IPNBs, by contrast with pancreatic IPMNs. More comprehensive molecular profiling is needed to uncover the pathways involved in IPNB development.

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Correspondence

Anirban Maitra, Departments of Pathology and Oncology, Sol Goldman Pancreatic Cancer Research Center, 1550 Orleans Street, CRB II, Room 345, Baltimore, MD 21231, USA. Tel: + 1 410 955 3511. Fax: + 1 410 614 0671. E-mail: amaitra1@jhmi.edu

Introduction

Intraductal papillary neoplasms of the bile duct (IPNBs) are uncommon neoplasms arising in the biliary tree, which were first described more than a century ago.¹ In 1959, Caroli provided a rigorous anatomical description of this entity.² These neoplasms can arise as either solitary or multifocal lesions within the biliary tree; in the latter scenario they are termed in classical parlance as 'biliary papillomatosis'.^{3–6} Intraductal papillary neoplasms of the bile duct show considerable morphologic similarities to

intraductal papillary mucinous neoplasms (IPMNs) of the pancreas. Both entities present with a prominent intraductal papillary component within larger-diameter ducts, both are mucin-producing, both harbour varying grades of dysplasia in the lining epithelium, and both exhibit distinct patterns of differentiation, such as intestinal and pancreatobiliary manifestations.³ Finally, both IPMNs and IPNBs are *bona fide* precursor lesions of invasive adenocarcinomas at their respective anatomic sites. In view of these similarities, it has been proposed that IPNBs may be the biliary counterpart of pancreatic IPMNs,⁷ although there are also significant differences between IPMNs and IPNBs in incidence of mucin hypersecretion, relative prevalence of different papilla

*These authors contributed equally to this work.

phenotypes and the frequency of high-grade dysplasia. Reports of IPNBs in the Asian literature also cite aetiological associations (lithiasis, flukes) that differ from those of IPMNs.

Intraductal papillary neoplasms of the bile duct are predominantly diagnosed in the elderly population and show a slight male predominance. Characteristically, patients suffer from abdominal pain and recurrent cholangitis. The accompanying jaundice arises as a result of the physical obstruction of bile outflow by the neoplasm itself or the viscous inspissated mucin.⁵ There are no evident risk factors for IPNBs, although some studies have reported an association with longstanding biliary inflammation.^{5,8} Because of the underlying risk for malignant transformation to invasive adenocarcinomas (cholangiocarcinomas)⁵ and the obstructive nature of the lesions, resection with clear margins is the therapy of choice for IPNBs. Unfortunately, multifocal IPNBs may recur even after apparently curative resection and, in rare cases with diffuse intrahepatic disease, orthotopic liver transplantation may be considered.^{4,6}

Recently, Wu *et al.* analysed a large sample of 132 IPMNs, including an admixture of tissue specimens and cyst fluid samples for somatic alterations in 169 cancer-associated genes.⁹ They identified mutations of *GNAS* at codon 201 in two thirds (66%) of analysed cases.⁹ *GNAS*, located on chromosome 20q, encodes for the alpha subunit of a stimulatory G-protein, which activates a cyclic 3'-5'-cyclic adenosine monophosphate (cAMP)-mediated intracellular cascade, culminating in cellular proliferation and growth.¹⁰ Although this 'hotspot' codon 201 mutation has been previously described in endocrine neoplasms and, rarely, in colorectal carcinomas, this was the first report of involvement of this oncogenic pathway in pancreatic neoplasia. The high prevalence of *GNAS* mutations in IPMNs has been confirmed by others,¹¹ and a subsequent study demonstrated that amongst cystic neoplasms of the pancreas, *GNAS* mutations are restricted to IPMNs and are not observed in solid pseudopapillary neoplasms or mucinous cystic neoplasms.¹² In light of the morphologic similarities between IPMNs and IPNBs, and the shared embryologic origins of the pancreas and biliary tree,¹³ the present authors investigated the frequency of *GNAS* alterations in the latter using a sensitive polymerase chain reaction (PCR)/ligation methodology. Notably, the study found that *GNAS* codon 201 mutations are uncommon in IPNBs, which suggests that alternative mechanisms for the development of these neoplasms exist.

Materials and methods

Patients and tissues

The present study was approved by the Johns Hopkins University Institutional Review Board and the review boards of collaborating institutions, Memorial Sloan-Kettering Cancer Center, New York, NY, and Emory University, Atlanta, GA, USA. Papillary lesions located in the ampullary region were not included because it is sometimes difficult to group them according to anatomical origin (pancreatic vs. biliary vs. ampullary). The neoplasms were classified according to recent international guidelines.¹⁴ Reference slides

of all eligible cases were reviewed by pathologists at the participating institutes, who were experts in pancreatobiliary pathology (RHH, AM, NVA, NK and DSK) to confirm the diagnosis of IPNB, and corresponding formalin-fixed and paraffin-embedded (FFPE) blocks for the most suitable specimens were selected for subsequent molecular studies. A total of 34 IPNB specimens were collated from the three collaborating institutions. Detailed information about the patients included is shown in Table S1 (online).

Microdissection and DNA preparation

Twenty sections measuring 7 µm in thickness were cut from every FFPE block in PCR clean conditions and mounted on plus-charged glass slides, after which they were stained with hematoxylin and eosin (H&E). The first and last sections were used as references for needle microdissection. Thereafter, manual microdissection of the neoplastic epithelium was performed using a sterile needle on a stereoscopic zoom microscope SMZ1500 (Nikon Corp., Tokyo, Japan). Approximately 10 000 cells were harvested from each lesion with an estimated tumour cellularity of >80% and subjected to genomic DNA extraction using a QIAamp DNA FFPE Tissue Kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol.

PCR/ligation assay for the detection of *GNAS* and *KRAS* mutations

Polymerase chain reaction products containing codon 12 of *KRAS* and codon 201 of *GNAS* were amplified using the primers described in Table S2. Each 10-µl PCR contained 200 template molecules in 5 µl of 2× Phusion Flash PCR Master Mix (New England Biolabs, Inc., Ipswich, MA, USA), and final concentrations of 0.25 µM forward and 1.5 µM reverse primers. Note that the mutant-specific probes in some cases included locked nucleic acid residues (Exiqon Life Sciences A/S, Vedbaek, Denmark). The following cycling conditions were used: 98 °C for 2 min; three cycles of 98 °C for 10 s, 69 °C for 15 s, 72 °C for 15 s; three cycles of 98 °C for 10 s, 66 °C for 15 s, 72 °C for 15 s; three cycles of 98 °C for 10 s, 63 °C for 15 s, 72 °C for 15 s, and 41 cycles of 98 °C for 10 s and 60 °C for 60 s. Reactions were performed in at least quadruplicate and each was evaluated independently. Five µl of a solution containing 0.5 µl of Proteinase K, (18.8 mg/ml; Roche AG, Basel, Switzerland) and 4.5 µl of dH₂O was added to each well and incubated at 60 °C for 30 min to inactivate the Phusion polymerase and then for 10 min at 98 °C to inactivate the Proteinase K.

The ligation assay was based on techniques described previously, using thermotolerant DNA ligases.^{15–18} Each 10-µl reaction contained 2 µl of PCR product (unpurified), 1 µl of 10× Ampligase buffer (Epicentre Biotechnologies, Inc., Madison, WI, USA), 0.5 µl of Ampligase (5 U/µl; Epicentre Biotechnologies, Inc.), anchoring primer (final concentration 2 µM), wild-type specific primer (final concentration 0.1 µM), and mutant-specific primer (final concentration 0.025 µM). The sequences of these primers are listed in Table S2. The following cycling conditions were used: 95 °C for 3 min, and 35 cycles of 95 °C for 10 s, 37 °C for 30 s and

45 °C for 60 s. Five µl of each reaction was added to 5 µl of formamide and the ligation products were separated on a 10% Urea-Tris-Borate-EDTA gel (Life Technology, Inc., Grand Island, NY, USA) and imaged with an Amersham-GE Typhoon instrument (GE Healthcare Ltd, Waukesha, WI, USA).

Results

Clinical and pathologic characteristics of IPNBs

This study was conducted using archival IPNB cases collected from three large hepatopancreatobiliary surgery centres in the USA. The clinical and pathologic characteristics associated with each of the 34 individual IPNB samples are detailed in Table S1 and a summary tabulation is presented in Table 1. The median age of the 34 patients was 65 years (range: 26–88 years) and the sample included 20 men and 14 women. The non-invasive component of the IPNBs had a median diameter of 3.5 cm (range: 0.7–21.1 cm), and the lesions were located throughout the biliary tree, including in the intrahepatic ducts ($n = 6$), extrahepatic ducts ($n = 13$), both intra- and extrahepatic ducts ($n = 4$) and in the gallbladder ($n = 11$). An associated invasive adenocarcinoma was observed in 20 cases. Tumour–node–metastasis (TNM) staging showed most of the associated invasive cancers were T3 ($n = 9$) and had not yet spread to local lymph nodes ($n = 13$). Upon histopathologic examination, the non-invasive components uniformly exhibited an intraductal papillary growth pattern (Fig. 1). Based on the maximum grade of epithelial dysplasia, the lesions were classified into 26 high-grade, five intermediate-grade and three low-grade IPNBs.

Assessment of mutational status and clinicopathologic correlation

A sensitive PCR/ligation method was used to detect *GNAS* and *KRAS* mutations in DNA samples isolated from the 34 IPNBs. Only one case harboured a *GNAS* codon 201 mutation. The *GNAS* mutation was detected in a diffuse intrahepatic IPNB, of intestinal subtype and exhibiting high-grade dysplasia. The 65-year-old male patient in whom this neoplasm arose underwent a left lateral hepatectomy and was alive without evident disease at 60 months after resection.

KRAS mutations were found in six patients. When the cohort of patients with *KRAS* mutations in their IPNBs were compared with those without *KRAS* mutations for patient characteristics and clinical and pathologic features (age, gender, location, subtype, grade, maximum size, presence of invasive carcinoma and outcome), no significant differences between the groups were found (chi-squared test, $P > 0.05$).

Discussion

Invasive adenocarcinomas of the pancreas are postulated to arise via two major dichotomous pathways involving non-invasive precursor lesions: pancreatic intraepithelial neoplasia (panIN), and IPMNs.¹⁹ These two pathways are clinically, pathologically and

Table 1 Summary of clinical and pathologic data for 34 patients with intraductal papillary neoplasms of the bile duct

Age, years, median (range)		65 (54–76)
Gender, <i>n</i>	Male	20
	Female	14
Surgery, <i>n</i>	Hepatectomy	7
	Segmental bile duct resection	3
	Hepatectomy and segmental bile duct resection	5
	Tumour cholecystectomy	11
	Orthotopic liver transplantation	1
	Pancreatoduodenectomy (Whipple procedure)	7
Resection status, <i>n</i>	0	28
	1	2
	Unknown	4
Location, <i>n</i>	Intrahepatic bile ducts	6
	Extrahepatic bile ducts	13
	Intra- and extrahepatic bile ducts	4
	Gallbladder	11
Size, cm, median (range)		3.5 (0.7–21.1)
Subtype, <i>n</i>	Gastric	10
	Intestinal	2
	Pancreatobiliary	20
	Oncocytic	2
Grade of dysplasia, <i>n</i>	Low	3
	Intermediate	5
	High	26
Associated invasive cancer, <i>n</i>	Yes	20
	No	14
Tumour stage	T1	4
	T2	6
	T3	9
	T4	1
	N/A	14
Lymph node metastases, <i>n</i>	N0	13
	N1	3
	NX	4
<i>KRAS</i>	Mutation detected, <i>n</i>	6
<i>GNAS</i>	Mutation detected, <i>n</i>	1

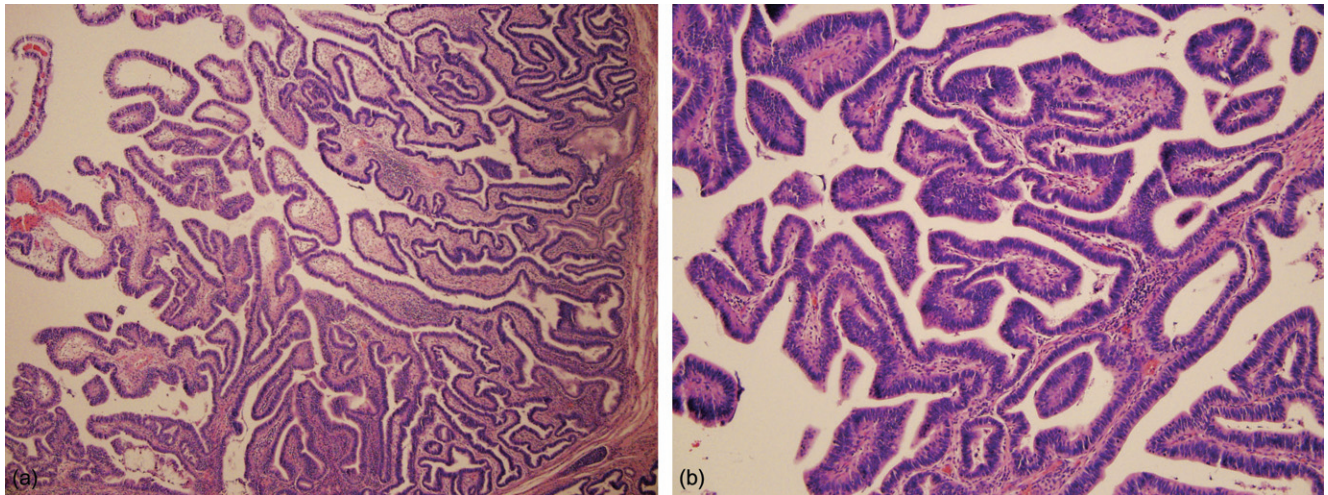


Figure 1 (a) Low- and (b) high-power photomicrographs obtained from an intraductal papillary neoplasms of the bile duct with high-grade dysplasia. [Haematoxylin and eosin stain; original magnification (a) $\times 10$, (b) $\times 20$]

genetically distinct. Analogous to the pancreas, two precursor pathways have been pathologically identified in the biliary tree, comprising non-papillary biliary intra-epithelial neoplasia (bilIN) lesions, and IPNBs.²⁰ Although molecular analyses of either of the two biliary precursor lesions are limited, the available information suggests underlying differences in terms of genetic alterations, as well as clinical distinctions in the invasive carcinomas arising in the respective contexts.^{21–23}

Emerging evidence suggests a number of similarities between IPMNs and IPNBs. For example, Zen *et al.* analysed both entities with respect to clinicopathologic features, expression of lineage-associated proteins and prognostic parameters.⁷ They compared a series of 32 IPNBs, including 22 with invasive cholangiocarcinomas, with a series of 31 pancreatic IPMNs (15 with associated invasive cancer); 15 non-IPNB-associated cholangiocarcinomas served as controls.⁷ The authors found that, similarly to those in IPMN, invasive adenocarcinomas arising from IPNBs were often of the mucinous type (five of 14, 35.7%). In addition, as seen in the pancreas, three predominant epithelial subtypes were observed in the biliary lesions, including gastric, intestinal and pancreatobiliary phenotypes. Immunohistochemically, IPNBs were characterized by the expression of apomucin MUC2, the intestinal differentiation factor CDX2, and the intermediate filament cytokeratin CK20, all of which are features prevalent in IPMNs, especially those of the intestinal subtype. Notably, these expression patterns were uncommon in cholangioadenocarcinomas not associated with IPNBs. The authors concluded that biliary papillary lesions may be regarded as the counterpart of IPMNs of the pancreas.⁷

Thus far, only limited information exists on the prevalent molecular alterations in IPNBs. Abraham *et al.* reported that IPNBs can have low levels of microsatellite instability in up to a third of cases, although these changes are not associated with loss

of hMLH1 function.²⁴ Furthermore, the same group of investigators assessed a panel of IPNBs for somatic alterations of *KRAS* and *CTNNB1* hotspot regions, and found *KRAS* codon 12 mutations in four of 14 (28.6%) IPNBs, whereas no *CTNNB1* mutations were detected.²⁵ Table 2 provides an overview of the available mutational data on IPNBs. The present study also suggests that the rate of *KRAS* mutations in IPNBs is lower (six of 34, 17.6%) than that previously reported in IPMNs (~80%),⁹ but comparable with that described previously in IPNBs.^{25,26} Notably, a recent genetically engineered mouse model of cholangiocarcinoma, generated by expression of mutant *KRAS* and Tp53 under an albumin promoter during development, also demonstrated non-invasive papillary lesions of the biliary tree histologically similar to the cognate precursor lesions in humans, underscoring the potential importance of mutant *KRAS* to the pathogenesis of some IPNBs.²⁷

The availability of massively parallel sequencing technologies has facilitated the identification of novel cancer-associated genes in several cancer types.^{12,28–30} Using a massively parallel approach, the present group and others identified activating mutations of *GNAS* at codon 201 in about two thirds of IPMNs.^{9,11} Point mutations of *GNAS* codon 227 have been reported in other tumour types, although not in IPMNs. Mutations at both codons 201 and 227 constitutively activate the encoded G-protein alpha subunit.³¹ The functional impact of these activating mutations is to confer cells with high adenyl cyclase activity and cAMP levels. *GNAS* mutations have predominantly been observed in association with endocrine disorders. For example, patients with McCune–Albright syndrome harbour germline *GNAS* mutations and present with a characteristic compendium of findings, including acromegaly, fibrous dysplasia and café au lait discoloration.^{32–34} Approximately 40% of pituitary somatotroph (growth hormone secreting) adenomas demonstrate *GNAS* mutations at either codon 201 or codon 227.³⁵ *GNAS* mutations are uncommon in

Table 2 Studies investigating the presence of distinct DNA mutations in intraductal papillary neoplasms of the bile duct (IPNB) located in the gallbladder and biliary ductal system

Author	Year	Location of IPNB	Gene locus	Involvement
Ohta <i>et al.</i> ⁴⁶	1993	Bile duct	<i>KRAS</i> codon 12	1 of 1
Yanagisawa <i>et al.</i> ⁴³	2001	Gallbladder	<i>β-catenin</i> exon 3	10 of 16
Kim <i>et al.</i> ⁴⁴	2001	Gallbladder	<i>KRAS</i> codon 12	0 of 3
			p53	0 of 3
Iwasaki <i>et al.</i> ⁴⁵	2002	Bile duct	<i>KRAS</i> codon 12	1 of 1
Abraham <i>et al.</i> ²⁵	2003	Bile duct	<i>KRAS</i> codon 12	4 of 14
			<i>β-catenin</i> exon 3	0 of 14
Pai & Mojtabeh ²⁶	2011	Gallbladder	<i>KRAS</i> codons 12 and 13	5 of 16
			<i>BRAF</i> codon 600	1 of 16

epithelial malignancies, occurring only in minor subsets of colorectal, prostate and breast cancers, renal cell carcinomas and small cell carcinomas of the lung, and these mutations are almost always restricted to codon 201.^{36–40} In the hepatobiliary region, *GNAS* mutations have been reported in only two of 245 (~1%) hepatocellular carcinomas (HCCs) and in four of 164 (2.4%) hepatocellular adenomas.⁴¹ Again, the mutations were all clustered at codon 201 and the corresponding HCCs were associated with an inflammatory infiltrate, postulated to reflect the activating of underlying proinflammatory pathways [interleukin-6 and STAT-3 (signal transducer and activator of transcription 3)] by the constitutively activated G-protein. Of note, *GNAS* mutations have not been identified in ‘usual’ pancreatic ductal adenocarcinomas arising outwith the context of IPMNs.^{9,30}

In the present study, *GNAS* mutations were detected only in a single intrahepatic IPNB and the remaining 33 lesions were wild-type at codon 201. These results are by stark contrast with the high prevalence (66%) previously reported in non-invasive IPMNs by Wu *et al.*⁹ In their study, *GNAS* mutations were observed at all grades of epithelial dysplasia, suggesting it was an ‘early’ genetic alteration. However, there was a tendency for subtype-specific prevalence, with 100% of intestinal IPMNs and only ~50% of gastric and pancreatobiliary IPMNs demonstrating *GNAS* codon 201 mutations. Interestingly, the single IPNB with a *GNAS* mutation in the present series was of the intestinal subtype. Overall, only two IPNBs showed intestinal differentiation in the present series, and one of the two was positive for *GNAS* mutation. Thus, the low prevalence of *GNAS* mutations in IPNBs may reflect the low prevalence of intestinal differentiation in these neoplasms. Nonetheless, it should be noted that ~50% of gastric and pancreatobiliary type IPMNs also harboured *GNAS* mutations,⁹ and all of the 31 IPNBs comprising these two subtypes were wild-type, suggesting a truly lower rate in the biliary counterparts.

There are several limitations to the present study. In this series, a subset of the IPNBs presented as multifocal lesions (biliary papillomatosis), but only the largest neoplastic foci were analysed. The present authors have previously shown that IPMNs can be

heterogeneous with regard to *GNAS* mutations,⁹ and this study’s failure to examine multiple synchronous lesions may have produced false negatives. The authors find this possibility less likely as *GNAS* mutations would confer a growth advantage to the index lesion and thus there is a high likelihood that the index lesion will be the most prominent neoplastic focus in a multifocal IPNB. Another caveat may be that the activating hotspot mutation in IPNBs is not at *GNAS* codon 201, but, rather, at codon 227, which was not examined in the current study. However, based on the data that *GNAS* mutations in epithelial neoplasms (including IPMNs) are always restricted to codon 201,^{9,35,42} the authors find this possibility unlikely. Finally, the issue of assay sensitivity, which is always a concern in a ‘negative’ study of this nature, should be addressed. The PCR/ligation assay used herein can detect a single mutant *GNAS* molecule amongst 200 wild-type templates (0.5% lower limit of detection)⁹ and has been successfully used for identifying mutant *GNAS* in highly biologically heterogeneous pancreatic cyst fluid samples. Thus, the present authors consider it quite unlikely that false negative results have been obtained using microdissected tumour tissue (with neoplastic cellularity of >80%).

In conclusion, *GNAS* codon 201 mutation, a newly discovered ‘mountain’ in the genetic landscape of IPMNs, is an uncommon alteration in IPNBs, despite the morphologic similarities between the two lesions arising at embryologically related sites. Additionally, *KRAS* mutations are also less common in IPNBs than in IPMNs. Based on the present series, it can be assumed that the signalling pathways involved in the pathogenesis of IPNBs are distinct from those in IPMNs. Larger, more comprehensive sequencing studies should help to elucidate the genetic landscape of IPNBs.

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Conflicts of interest

None declared.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1. Detailed clinical and histopathologic data for the study population.

Table S2. Oligonucleotides used for sensitive polymerase chain reaction ligation method for detecting *GNAS* and *KRAS* mutations.

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