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Clinicopathological Correlates of Activating *GNAS* Mutations in Intraductal Papillary Mucinous Neoplasm (IPMN) of the Pancreas

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Abstract

Background—Intraductal papillary mucinous neoplasms (IPMNs) are the most common cystic precursor lesions of invasive pancreatic cancer. The recent identification of activating *GNAS* mutations at codon 201 in IPMNs is a promising target for early detection and therapy. The purpose of this study was to explore clinicopathological correlates of *GNAS* mutational status in resected IPMNs.

Methods—Clinical and pathologic characteristics were retrieved on 54 patients in whom *GNAS* codon 201 mutational status was previously reported (“historical group”, Wu et al. Sci Transl Med 3:92ra66, 2011). In addition, a separate cohort of 32 patients (validation group) was included. After microdissection and DNA extraction, *GNAS* status was determined in the validation group by pyrosequencing.

Results—*GNAS* activating mutations were found in 64 % of the 32 IPMNs included in the validation group, compared with a previously reported prevalence of 57 % in the historical group. Overall, 52 of 86 (61 %) of IPMNs demonstrated *GNAS* mutations in the two studies combined. Analysis of both groups confirmed that demographic characteristics, tumor location, ductal system

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involvement, focality, size, grade of dysplasia, presence of an associated cancer, and overall survival were not correlated with *GNAS* mutational status. Stratified by histological subtype, 100 % of intestinal type IPMNs demonstrated *GNAS* mutations compared to 51 % of gastric IPMN, 71 % of pancreatobiliary IPMNs, and 0 % of oncocytic IPMNs.

Conclusions—*GNAS* activating mutations can be reliably detected in IPMNs by pyrosequencing. In terms of clinicopathological parameters, only histological subtype was correlated with mutational frequency, with the intestinal phenotype always associated with *GNAS* mutations.

Intraductal papillary mucinous neoplasm (IPMN) is a cystic neoplasm of the pancreas characterized by intraductal papillary proliferation of neoplastic mucinous cells.[1,2] IPMNs arise in the main pancreatic duct or its side branches (or both), can be unifocal or multifocal, and can involve the pancreatic head, body–tail, or, less frequently, the entire gland.[1,2] Varying subtypes of differentiation can be seen in the neoplastic cells of IPMNs including gastric, intestinal, pancreatobiliary, and oncocytic subtypes.[3] Among pancreatic cystic neoplasms, IPMNs are recognized precursor lesions of pancreatic adenocarcinoma, while others such as serous cystadenomas are benign and unless symptomatic generally do not require treatment.[4,5] The preoperative differentiation of IPMNs from other pancreatic cysts can be challenging, and in spite of meticulous assessment, changes to the preoperative diagnosis can be as high as 30 % upon subsequent pathological appraisal.[6,7] Even when an IPMN is correctly diagnosed, it is currently not possible to empirically predict its risk of developing an associated cancer; nor is it possible to assess the grade of dysplasia for that lesion unless resection and pathological examination are performed.

In a study by Wu et al.[8] a novel next-generation sequencing technology was applied to unravel molecular pathways important for the pathogenesis of IPMNs. Unexpectedly, activating *GNAS* mutations at codon 201 were identified in approximately two-thirds of these neoplasms. Of note, no *GNAS* mutations were identified in other pancreatic cystic neoplasms; nor were they identified in conventional pancreatic ductal adenocarcinoma. This suggests that *GNAS* mutations are specific for the IPMN phenotype.[8,9] In this study, we assessed the prevalence of *GNAS*-activating mutations in an additional set of surgically resected IPMNs using pyrosequencing and correlated *GNAS* mutational status with clinicopathological characteristics. Our results indicate that activating *GNAS* mutations are associated with the intestinal subtype morphology and are likely to be an early driver mutation in a significant subset of IPMNs.

MATERIALS AND METHODS

The present study was approved by the institutional review board of Johns Hopkins Medical Institutions. Previously published *GNAS* mutational status of patients included in the study by Wu et al.[8] was retrieved. For all patients included in the above-mentioned study (historical group), clinicopathological characteristics were available. In addition, we reviewed the prospectively maintained Johns Hopkins Department of Surgery clinical database and identified a separate cohort of patients who underwent pancreatic resection at the Johns Hopkins Hospital (validation group). In both cohorts of patients, case selection was further restricted to patients surgically resected in or after the year 1996, when the World Health Organization diagnostic criteria for IPMN were implemented at our institution. For all the patients included in the study, a minimum follow-up of 2 years was available. Patients with cysts in the pancreatic head, uncinate process, or neck underwent pancreaticoduodenectomy. Distal pancreatectomy and splenectomy was performed for neoplasms located in the pancreatic body–tail. In case of main duct IPMNs diffusely involving the duct of Wirsung, a total pancreatectomy was performed. IPMN can be multifocal, and independent clones have been demonstrated in at least a subset of branch

duct IPMNs.[10] To limit confounding factors related to the analysis of multiple cysts within the same surgical specimen, patients who underwent total pancreatectomy for multifocal branch duct IPMN were excluded. After *GNAS* mutational status was assessed in the validation group, the whole cohort of patients was dichotomized on the basis of the presence or absence of mutated *GNAS* in their surgically resected IPMNs. Clinicopathological correlations and survival analysis of *GNAS* mutational status in IPMNs were performed.

Histopathology

Two experts in pancreatic pathology (RHH and AM) confirmed all histological diagnoses using recent guidelines.[11] On the basis of the maximum cytoarchitectural atypia, IPMNs were classified as low grade, intermediate grade, or high grade. IPMNs were classified as gastric, intestinal, pancreatobiliary, or oncocytic subtypes with respect to the predominant phenotypic features of the neoplastic epithelium.[12] IPMNs were further classified as noninvasive or as having an associated invasive adenocarcinoma.

Laser-Capture Microdissection

Archival formalin-fixed, paraffin-embedded tissue blocks of selected IPMNs and matched normal pancreatic tissues were collected. For laser-capture microdissection, 10–30 sections (10 μm) were embedded onto UV-treated PALM membrane slides (Carl Zeiss MicroImaging, Inc., Thornwood, NY). After deparaffinization in xylene/ethanol and hematoxylin and eosin staining, small neoplasms or those with substantial inflammatory component were subjected to laser-capture microdissection on a PALM Micro Beam System (Carl Zeiss MicroImaging, Inc., Thornwood, NY). All other lesions were microdissected with a sterile needle under a stereomicroscope (SMZ1500, Nikon, Tokyo, Japan). Approximately 5,000–10,000 cells were collected from each lesion, with an estimated neoplastic cellularity >80 %. In addition, matched nonneoplastic pancreatic tissue was manually macrodissected. Subsequently, DNA was extracted using the QIAamp DNA Micro Kit (Qiagen Inc., Valencia, CA) per the manufacturer's instructions.

GNAS Pyrosequencing

On the basis of previously published findings, *GNAS* mutational analysis was restricted to the codon 201 hot spot contained within exon 8 because no mutations outside of this codon have been described in IPMNs.[13,14] Pyrosequencing was performed as published.[15,16] Briefly, DNA extracted from microdissected tissue was PCR amplified on GeneAmp9700 Thermal Cycler (Applied Biosystem) such that each reaction contained: 1× buffer (Qiagen), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 5 pmol of forward primer (5′-CCAGACCTTTGCTTTAG ATTGG-3′), 5 pmol of reverse primer (5′-biotin-TCCACC TGGAACTTGGTCTC-3′), 0.8 U of HotStart TaqDNA polymerase (Qiagen), 10 ng of DNA, and dH_2O for a 25 μl final volume. Cycling conditions were as follows: 95 °C for 15 min; 38 cycles of 95 °C for 20 s; 53 °C for 30 s; 72 °C for 20 s; and a final extension at 72 °C for 5 min. The amplicons were sequenced with the PyroMark Q24 (Qiagen) with PyroMark Gold reagents (Qiagen) containing 0.3 μmol/L sequencing primer (5′-TTTGTTTCAGGACCTGCTTCGC-3′) and annealing buffer. The nucleotide triphosphate dispensation order for codon 201 was TGCTAGTGTCTGACTCTGATCT.

Statistical Analysis

Patient characteristics were compared between those who had an IPMN that harbored a codon 201 *GNAS* mutation and those who had an IPMN that did not by Wilcoxon rank sum tests for continuous measures and Fisher's exact test for categorical measures. Pairwise comparisons between various clinical and pathological characteristics and their respective *GNAS* mutation frequency were performed by Fisher's exact test. Overall survival was

calculated as the date of surgery to the date of death or to the date last known alive and estimated using the Kaplan–Meier method. Hazard ratios estimated from Cox proportional hazards models were used to compare overall survival between patients with and without a codon 201 *GNAS* mutation while controlling for whether the patient had invasive cancer, age at surgery, and histological subtype. Analyses were completed by R software, version 2.15.1.[13]

RESULTS

On the bases of the time frame considered for this study (1996–2009) and the exclusion of total pancreatectomy specimens for multifocal disease, 54 IPMNs in the historical group were suitable for inclusion.[8] Within this cohort, 31 IPMNs (57 %) harbored a codon 201 *GNAS* mutation, as assessed by a sensitive PCR/ligation assay.[8] An additional cohort of 32 separate patients with IPMNs resected at Hopkins and not previously reported met the aforementioned inclusion criteria (validation group). A total of 21 IPMNs within this cohort (64 %) harbored *GNAS*-activating mutations (Fig. 1). After combining the two groups, a total of 86 IPMNs were suitable for clinicopathological analysis, of which 52 (61 %) harbored *GNAS* codon 201 mutations and 34 (39 %) were *GNAS* wild type.

Two independent statistical correlations were performed. First, the prevalence of any given clinical or pathological trait was compared in the *GNAS* mutant versus *GNAS* wild type subsets of IPMNs for statistically significant differences (Table 1). Second, we performed a series of pairwise comparisons between various clinical and pathological characteristics for significant differences in their respective *GNAS* mutation frequency in order to determine whether the proportion of mutated samples was different between samples belonging to a specific category (Table 2). We found no association with respect to age, gender, history of smoking, alcohol use, and the presence or absence of *GNAS* mutations (Table 1). Within the mutant *GNAS* group, 10 IPMNs (29 %) had low-grade dysplasia, while 10 IPMNs (19 %) had low-grade dysplasia within the *GNAS* wild type group ($P = 0.305$). Similarly, no statistical difference in the prevalence of either intermediate-grade or high-grade dysplasia was observed between *GNAS* mutant IPMNs and *GNAS* wild type IPMNs: 7 patients (21 %) vs. 12 patients (23 %) ($P > 0.99$), and 17 patients (50 %) vs. 30 patients (58 %) ($P = 0.514$), respectively. Further, by pairwise comparison of different grades of dysplasia with respect to their mutational status (Table 2), the prevalence of *GNAS* mutations in IPMNs with low-grade (LG) dysplasia was similar to that of IPMNs with intermediate-dysplasia (IG) or high-grade (HG) dysplasia. Ten of 20 LG (50 %) vs. 12 of 19 IG (63 %) ($P = 0.523$); and 10 of 20 LG (50 %) vs. 30 of 47 HG (64 %) ($P = 0.415$). Similarly, no difference was observed when comparing IG IPMNs (12 of 19, 63 %) with HG IPMNs (30 of 47, 64 %), ($P > 0.99$).

IPMNs with mutant *GNAS* and the IPMNs with wild type *GNAS* had a similar distribution among the main duct, mixed duct, and branch duct types (38, 12 and 50 % vs. 41, 11 and 48 %, respectively; $P = 0.948$) (Table 1). Nor were any differences observed in pairwise comparisons of the abovementioned categories; thus, 5 out of 9 (56 %) mixed duct IPMNs harbored *GNAS* mutations, compared with 18 out of 30 (60 %) of main duct IPMNs ($P > 0.99$) (Table 2). The prevalence of *GNAS* mutations in branch duct IPMNs (21 of 37, 57 %) was not different from the prevalence observed in main duct IPMNs (18 of 30, 60 %) or with that observed in mixed duct IPMNs (5 of 9, 56 %) ($P = 0.809$ and $P > 0.99$, respectively). Median cyst size was similar in the *GNAS* mutant group (2.2 cm), compared with the wild type *GNAS* group (2.5 cm). When considering a dimensional cutoff of 3 cm, no statistically significant difference was found between *GNAS* mutant IPMNs versus *GNAS* wild type IPMNs (Table 1). Nine IPMNs with mutant *GNAS* (26 %) had an associated invasive cancer, while 16 (31 %) IPMNs with wild type *GNAS* harbored an

associated invasive component ($P = 0.809$). Furthermore, IPMNs located in the head–neck–uncinate process had similar prevalence of *GNAS* mutations compared with those arising in the body or tail of the gland (Table 1).

We also examined the pattern of *GNAS* mutations in the different histologic subtypes of IPMNs. The pancreatobiliary subtype was observed in 7 % of IPMNs harboring a *GNAS* mutation compared with a prevalence of 11 % in the wild type group ($P = 0.694$) (Table 1). The gastric subtype was more frequently observed in IPMNs with wild type *GNAS* (87 %) compared to IPMNs harboring mutant *GNAS* (61 %); this difference was statistically significant ($P = 0.02$). Conversely, the intestinal subtype was frequently observed within the *GNAS* mutant group (27 %) but never observed in IPMNs with wild type *GNAS* alleles ($P = 0.001$). These statistically significant differences persisted in the pairwise comparison between histological subtypes, with 100 % of intestinal and only 51 % of gastric type IPMNs demonstrating *GNAS* mutations ($P = 0.001$). With the exception of borderline significance when the intestinal subtype was compared to the oncocytic ($P = 0.011$), the other pairwise comparisons did not reach statistical significance (Table 2). Finally, Kaplan–Meier survival analysis revealed no significant difference in survival between tumors harboring *GNAS* mutations and tumors wild type for *GNAS* (Fig. 2).

DISCUSSION

The entire exomes of the four most common cystic neoplasms of the pancreas have been sequenced, and mutations specific for each cyst type have been discovered, suggesting that the clinical evaluation of pancreatic cysts could greatly benefit from new gene-based biomarkers.[8,9,17] Among the genetic alterations identified in IPMNs, activating *GNAS* mutations harbor the most clinically relevant potential, as they are highly prevalent (approximately two thirds of cases) and are limited to a single hot spot codon, 201.

The *GNAS* gene, located on human chromosome 20q13.3, encodes the alpha subunit (Gsα) of a stimulatory G protein. Mutations in the codon 201 of *GNAS* result in the constitutive activation of Gsα and its effector adenylate cyclase, ultimately leading to autonomous synthesis of cyclic AMP (cAMP) and uncontrolled growth signaling.[14] Somatically acquired *GNAS*-activating mutations have been identified in several tumor types other than IPMN, such as kidney, thyroid, pituitary, liver, colorectal, and more recently in villous adenomas of the colorectum.[18–23]

In light of the recognition of *GNAS* as a definite mountain in the mutational landscape of IPMNs, we endeavored to determine the implications of this mutation on clinical and pathological traits in a series of well-characterized surgically resected IPMNs. We utilized data from our previously published series that assessed *GNAS* mutation status in IPMN cyst fluid samples using a sensitive PCR/ligation assay (historical group), as well as a separate cohort of IPMNs, in which *GNAS* codon 201 mutations were analyzed by pyrosequencing (validation group) in order to increase the sample size.[8] Overall, the combined mutational prevalence of codon 201 alterations in our series was 61 % (52 of 86 IPMNs). Despite this high prevalence of point mutations in GNAS, most clinicopathological factors examined, as well as survival analysis, did not correlate with either the presence or absence of a mutant allele. Notably, only the pattern of differentiation of the neoplastic epithelium was significantly associated with presence or absence of *GNAS* mutations. We confirmed that 100 % of the intestinal type IPMNs harbored a codon 201 mutation, while only 51 % of gastric IPMNs ($P = 0.001$) and 71 % of pancreatobiliary IPMNs ($P = 0.123$) had mutant GNAS. The association between *GNAS* mutations and an intestinal differentiation is not unique to IPMNs; a recent study has reported that 83 % of villous adenomas of the colorectum (which, by virtue of their lush fingerlike papillae, are the closest histological

mimic of intestinal-type IPMNs) also demonstrate *GNAS* mutations.[23] In that series, none of the tubular adenomas and only 3 % of tubulovillous adenomas/colorectal adenocarcinomas harbored *GNAS* mutations, emphasizing the notion that shared genetic traits might underlie the readily observed morphological similarities between colorectal villous adenomas and intestinal-type IPMNs.[23]

Some limitations of our study should be noted. First, the prevalence of *GNAS* mutations in our current series as well as in our prior publication is based on IPMNs that reached clinical criteria for surgical resection (Sendai criteria).[8] Because the majority of IPMNs are not resected, it is possible that we are overestimating the frequency of altered *GNAS* in IPMNs. This limitation could be overcome by including cysts that are sampled by endoscopy but not resected. In addition, the lack of correlation with natural history might also reflect the fact that all of the samples were surgically resected. A more biologically heterogeneous group of resected and unresected IPMNs might elucidate a tangible clinical correlate. Finally, we only assessed codon 201 for mutations in GNAS, as it has been historically reported as the unique hot spot codon for mutations in IPMNs.[8,9,17] Thus, although unlikely, IPMNs with a wild type codon 201 might harbor oncogenic mutations at other codons of GNAS, copy number alterations of chromosome 20q (the *GNAS* locus), or aberrant activation of downstream effectors of Gsa, such as protein kinase A, none of which were assessed in this study.[24–26]

In conclusion, the high prevalence of *GNAS* mutations in IPMNs, irrespective of grade of dysplasia, suggests that it is an early driver gene in IPMN pathogenesis. Furthermore, our data support the correlation of *GNAS* mutations and intestinal differentiation in IPMNs.

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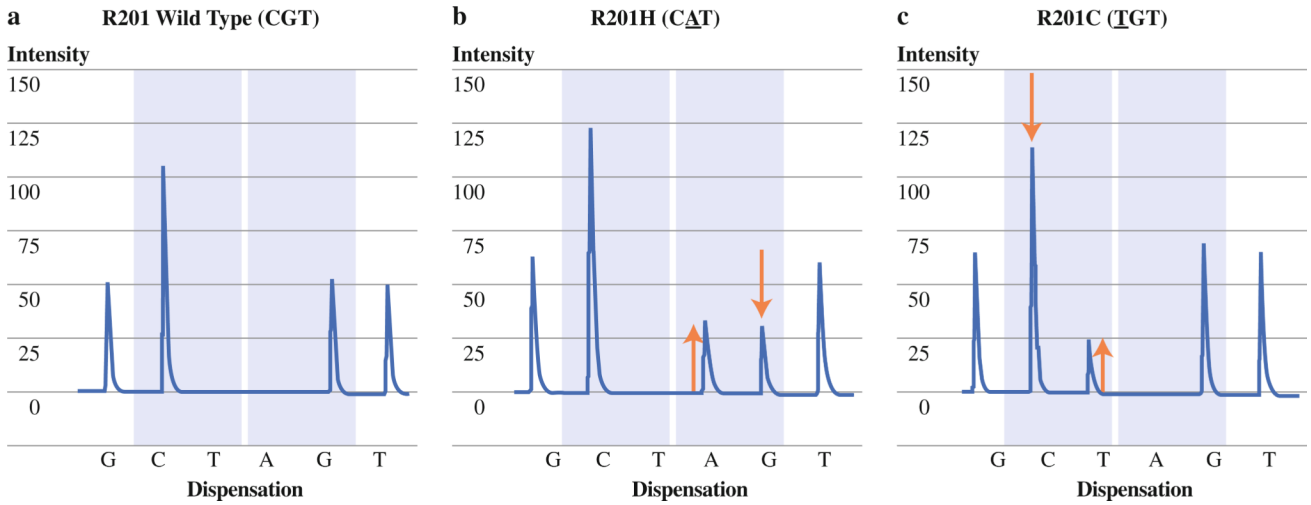

FIG. 1.

Pyrograms of *GNAS* mutational status. **a** Wild type *GNAS* codon 201 is retained. *GNAS* codon 201 exhibits missense mutations **b** CGT to CAT, resulting in amino acid substitution of an arginine with histidine (R201H) and **c** CGT to TGT, resulting in amino acidic substitution of an arginine with cysteine (R201C)

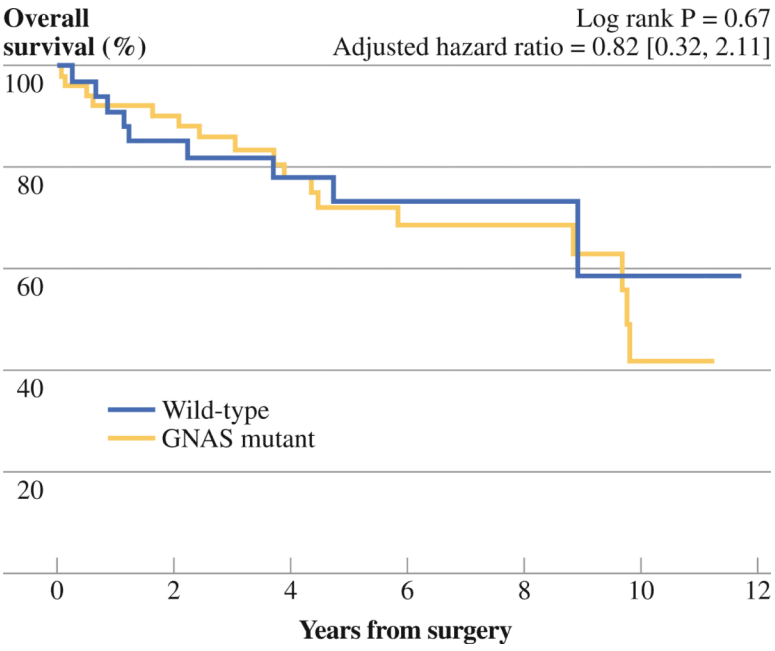

FIG. 2.
Kaplan–Meier survival analysis of patients with IPMN according to *GNAS* mutational status

TABLE 1

Characteristics of patients by *GNAS* mutation status

Characteristic	*GNAS* wild type (*n* = 34)	*GNAS* mutant (*n* = 52)	*P*
Age, y, median (range)	71 (48–85)	71 (35–91)	0.533
Gender			0.266
Female	17 (50 %)	19 (37 %)	
Male	17 (50 %)	33 (63 %)	
Smoking history			0.235
Never	25 (76 %)	31 (62 %)	
Ever	8 (24 %)	19 (38 %)	
Unknown	1	2	
Alcohol use			>0.99
None	7 (44 %)	11 (42 %)	
Some	9 (56 %)	15 (58 %)	
Unknown	18	26	
Dysplasia			
Low grade	19 (29 %)	10 (19 %)	0.305
Intermediate grade	7 (21 %)	12 (23 %)	>0.99
High grade	17 (50 %)	30 (58 %)	0.514
Focality			>0.99
Unifocal	23 (79 %)	37 (77 %)	
Multifocal	6 (21 %)	11 (23 %)	
Unknown	5	4	
Duct			0.948
Main	12 (38 %)	18 (41 %)	
Mixed	4 (12 %)	5 (11 %)	
Branch	16 (50 %)	21 (48 %)	
Unknown	2	8	
Cyst size			>0.99
<3 cm	17 (53 %)	28 (55 %)	
≥3 cm	15 (47 %)	23 (45 %)	
Unknown	2	1	
Size, cm, median (range)	2.2 (0.5–8)	2.5 (0.6–8)	0.35
Invasive cancer			0.809
No	25 (74 %)	36 (69 %)	
Yes	9 (26 %)	16 (31 %)	
Site			
Pancreatobiliary	2 (7 %)	5 (11 %)	0.694
Gastric	26 (87 %)	27 (61 %)	0.02
Intestinal	0	12 (27 %)	0.001
Oncocytic	2 (7 %)	0	0.161
Location			0.493

Characteristic	*GNAS* wild type (*n* = 34)	*GNAS* mutant (*n* = 52)	*P*
Distal	21 (62 %)	36 (69 %)	
Head–neck–uncinate process	13 (38 %)	16 (31 %)	

TABLE 2

Pairwise comparison of mutant *GNAS* samples by grade of dysplasia, epithelial differentiation, and duct involvement

Dysplasia grade	*n*	Mutant, *n* (%)
Low-grade dysplasia	20	10 (50)
Intermediate-grade dysplasia	19	12 (63)
P		0.523
Intermediate-grade dysplasia	19	12 (63)
High-grade dysplasia	47	30 (64)
P		>0.99
Low-grade dysplasia	20	10 (50)
High-grade dysplasia	47	30 (64)
P		0.415
Gastric	53	27 (51)
Pancreatobiliary	7	5 (71)
P		0.432
Intestinal	12	12 (100)
Pancreatobiliary	7	5 (71)
P		0.123
Oncocytic	2	0 (0)
Pancreatobiliary	7	5 (71)
P		0.167
Intestinal	12	12 (100)
Gastric	53	27 (51)
P		0.001
Oncocytic	2	0 (0)
Gastric	53	27 (51)
P		0.491
Oncocytic	2	0 (0)
Intestinal	12	12 (100)
P		0.011
Mixed duct	9	5 (56)
Main duct	30	18 (60)
P		>0.99
Branch duct	37	21 (57)
Main duct	30	18 (60)
P		0.809
Branch duct	37	21 (57)
Mixed duct	9	5 (56)
P		>0.99

P values are for testing whether the proportion of mutated samples is different between patient groups