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ORIGINAL ARTICLE

GNAS mutation is a frequent event in pancreatic intraductal papillary mucinous neoplasms and associated adenocarcinomas

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Abstract In contrast to pancreatic ductal adenocarcinomas (PDAs), intraductal papillary mucinous neoplasms (IPMNs) frequently harbour *GNAS* mutations. To characterise *GNAS*-mutated pancreatic carcinomas, we examined mutations of *GNAS* and *KRAS* in 290 pancreatic adenocarcinomas and 77 pancreatic intraepithelial neoplasias (PanINs). In 64 % (39/61) of IPMNs and 37 % (11/30) of IPMN-associated adenocarcinomas, a *GNAS* mutation was found. *GNAS* mutations were frequent (78 %, 7/9) in mucinous carcinomas, with or without associated IPMN. In contrast, *GNAS* mutations were rarely observed in PDAs (1 %, 1/88) and PanINs (3 %, 2/77), and not at all in mucinous cystic neoplasms (MCNs) (0/10), neuroendocrine neoplasms (0/52), acinar cell neoplasms (0/16), serous cystadenomas (0/10), and solid-pseudopapillary neoplasms (0/14). We found *GNAS* mutations in 55/91 IPMNs with or without associated invasive carcinoma, solely in intestinal-type (78 %, 21/27) and gastric-type (62 %, 34/55) IPMNs. Of the IPMN-associated adenocarcinomas, mucinous-subtype tumours harboured *GNAS* mutations more frequently (83 %, 5/6) than tubular-subtype tumours (25 %, 6/24) (p=0.02). We separately analysed *GNAS* in the adenocarcinoma and the IPMN component in the IPMN-associated adenocarcinomas. In all mucinous-subtype tumours, the two components exhibited identical genotypes. In contrast, the two components in 8 of 24 tubular-subtype tumours exhibited different genotypes, indicating intratumour heterogeneity. In conclusion, mucinous carcinomas with or without associated IPMN as well as IPMNs frequently harbour a *GNAS* mutation, reinforcing the notion that these constitute a spectrum of pancreatic tumours. Clinically and pathologically, these tumours are associated, but *GNAS* mutation sheds further light on this spectrum.

Keywords Pancreas · *GNAS* · *KRAS* · Mucinous · Tubular · IPMN-associated adenocarcinoma

Introduction

The cyclic AMP-mediated signalling pathway is responsible for a wide variety of physiological processes and for maintaining homeostasis in the majority of human cells. The *GNAS* gene encodes the stimulatory G-protein α-subunit (Gsα) of the heterotrimeric G-proteins. When activated by the exchange of GDP for GTP and the dissociation from G-protein β- and γ-subunits, Gsα stimulates the cyclic AMP-generating enzyme adenylyl cyclase and other effectors. Gsα is turned off by returning to the GDP-bound form through the intrinsic GTPase activity and reassociation with G-protein β- and γ-subunits [1, 2]. Activating *GNAS* mutations have been described in a wide variety of tumours; early studies identified mutations in pituitary adenomas [3, 4], thyroid adenomas [4] and fibrous dysplasias of bone [5, 6], followed by studies in tumours of the gonads (Leydig cell tumours) [7], soft tissue (intramuscular myxomas) [8], pancreas [9, 10], stomach/

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duodenum [11], colon [12, 13], bile duct [14, 15], uterine cervix [16] etc. Hot spot missense mutations were noted in codon 201 and codon 227 [2]. These amino acid residues lie in the catalytic domain of the GTPase, and substitution mutations of these codons cause constitutive activation of the Gsα protein and persistent stimulation of their downstream signal transduction [1, 17, 18].

As for pancreatic tumours, two research groups first investigated pancreatic ductal adenocarcinomas (PDAs) and cystic neoplasms, and found recurrent R201H and R201C mutations in intraductal papillary mucinous neoplasms (IPMNs) (41–66 %) but none in PDAs or other cystic neoplasms [9, 10]. A subsequent study reported that pancreatic intraepithelial neoplasias (PanINs), a putative precursor of PDA, harboured infrequent *GNAS* mutations (10 %) [19]. These results suggest that *GNAS* mutation is highly correlated with IPMN. However, because *GNAS* mutation in non-ductal tumours of the pancreas has not been investigated fully, and mutations of *GNAS* have been documented not only in epithelial tumours but also in endocrine and certain rare tumours in many other organs, investigation of a wide variety of histological types is necessary to address the distribution of *GNAS* mutation in the pancreas.

IPMN is the most common cystic tumour of the pancreas, characterised by intraductal proliferation of mucinous cells with varying degrees of atypia, leading to cystic dilatation of the pancreatic ducts and the formation of clinically detectable masses. IPMN is considered another putative precursor of invasive adenocarcinoma that develops through the process of multi-step tumourigenesis. Although accumulating evidence suggests that IPMN-associated adenocarcinoma has several biological and prognostic characteristics different from conventional PDA [20, 21], there have been no markers available that can reliably separate *IPMN-derived* adenocarcinoma from conventional PDA.

In this study, we attempted to specify the distribution of *GNAS* mutation in the pancreatic tumours and reveal the characteristics of *GNAS*-mutated carcinomas to obtain insight from a genetic classification of tumours of the pancreas.

Materials and methods

Patients and tissues

We selected 290 surgically resected pancreatic tumours from 284 patients from the database of the Department of Pathology and Molecular Diagnostics, Aichi Cancer Centre Hospital, Nagoya, Japan. The tumours were classified according to the 2010 WHO classification [22]. The cohort consisted of 88 tubular adenocarcinomas, 4 adenosquamous carcinomas, 2 undifferentiated carcinomas, 3 mucinous carcinomas (with no apparent findings of an associated IPMN), 30 IPMN-associated adenocarcinomas (including 6 mucinous subtypes and 24 tubular subtypes), 61 IPMNs, 10 mucinous cystic neoplasms (including 1 with high-grade dysplasia and 9 with low-grade dysplasia), 52 neuroendocrine neoplasms, 16 acinar cell neoplasms (including 15 acinar cell carcinomas and 1 acinar cell cystadenoma), 10 serous cystadenomas, and 14 solid-pseudopapillary neoplasms. In addition, we analysed 77 PanIN lesions (35 of PanIN-1, 25 of PanIN-2, and 17 of PanIN-3) that were microdissected from 57 resected specimens with invasive ductal carcinomas, neuroendocrine neoplasms, mucinous cystadenomas, and serous cystadenomas. Of these, 17 lesions of PanIN-3 were obtained from 14 cases of invasive ductal carcinoma. Each lesion was microdissected from the cut sections at a distance from those containing invasive cancer cells. It is important to distinguish between PanIN, small-sized IPMN, and intraductal extension of invasive carcinoma cells (so-called cancerization of pancreatic duct epithelium). We therefore excluded from our study PanIN lesions occurring in the pancreas along with IPMN or with invasive ductal carcinoma with a carcinoma in situ component extending into the surrounding main pancreatic duct. All tissues were fixed in 10 % formalin and embedded in paraffin. This study was part of a comprehensive research programme of the tissue bank in Aichi Cancer Centre, which has been approved by the institutional review board.

Pathological assessment of IPMN-associated adenocarcinoma and IPMN

As IPMN-associated adenocarcinoma, we only included tumours of which the invasive carcinoma component was in contiguity with the IPMN component. Tumours were excluded from this study if invasive carcinoma nodules and cysts of IPMN were topologically discontinuous. Invasive carcinoma components were subcategorised into 6 mucinous and 24 tubular subtypes. In this study, we included five IPMN-associated adenocarcinomas of which the IPMN component showed low-grade dysplasia, in order to assess the relationship between gene abnormalities in adenocarcinoma and IPMN.

Regarding epithelial subtype of IPMN, we classified all IPMNs into four subtypes (gastric, intestinal, pancreatobiliary, and oncocytic) based on their morphology and immunohistochemical reactivity against antibodies of MUC6, MUC2, MUC5AC, and CDX2, according to previously described criteria [23, 24].

Immunohistochemistry

Immunohistochemical staining was performed using primary antibodies directed against MUC6 (clone CLH5, mouse, 1:200, Novocastra), MUC2 (clone Ccp58, mouse, 1:200, Novocastra), MUC5AC (clone CLH2, mouse, 1:100,

Novocastra), and CDX2 (clone DAK-CDX2, mouse, ready to use, Dako). Staining was carried out using an Autostainer Link 48 (Dako, Copenhagen, Denmark) according to the manufacturer's instruction. The antigens were retrieved by PT Link (Dako) for 30 min in a high-buffer solution (pH 9.0, Dako).

Mutation analysis

To detect mutations of *GNAS* and *KRAS*, we applied direct sequencing and Cycleave PCR methods, as described previously [25, 26]. For direct sequencing of *GNAS* exon 8, we used the following primer set: 5′-TTCCAAACTACTCCAG ACC-3′ as the forward primer and 5′-AAAGGTAACAGTTG GCTTAC-3′ as the reverse primer. For the Cycleave PCR method to detect codon 201 mutations of *GNAS*, the sequences of the primer set and the probes used were as follows: PCR forward primer, 5′-CAGACCTTTGCTTTAGATTG-3′; PCR reverse primer, 5′-GTAACAGTTGGCTTACTGGA-3′; wild-type probe, 5′-ACGGCAGC-3′; mutant probes, 5′-GACACAGCA-3′ (for the R201C mutation); and 5′-ATGGCAGCG-3′ (for R201H mutation) (the underlines represent codon 201). *KRAS* mutations were analysed using the Cycleave PCR method, the details of which we described in the previous report [26].

Statistical analysis

The Fisher's exact test and the Mann–Whitney *U* test were used to compare categorical data. The unpaired *t* test was used to compare continuous variables. For survival analysis, the Kaplan–Meier method was used to assess survival time distribution, and the log-rank test was applied using the SYSTAT software (SYSTAT Software, Inc., Richmond, CA, USA). $P<0.05$ was considered statistically significant.

Results

GNAS and *KRAS* mutations in pancreatic tumours and precursor lesions

We examined mutations in *GNAS* and *KRAS* in 290 cases of surgically resected pancreatic tumours using Cycleave PCR assay and direct sequencing. It has been reported by us and others that the Cycleave PCR assay has a higher detection sensitivity (that is, samples containing mutant alleles of more than 5 % in the total DNA are sufficient for detection), compared with direct sequencing (mutant alleles need to comprise at least 10–25 % of the total DNA) [25, 27]. Accordingly, all of the lesions microdissected from the formalin-fixed paraffin-embedded tissue sections were analysed by the Cycleave PCR assay, with direct sequencing used to confirm the results of the Cycleave PCR assay, particularly at the initial stage of this study. In IPMN-associated adenocarcinomas, DNA was extracted separately from both adenocarcinoma and IPMN components. The results of the mutation analyses are summarised in Table 1. IPMNs and IPMN-associated adenocarcinomas frequently displayed a *GNAS* mutation, in 64 % (39/61) and 37 % (11/30) of the cases, respectively. Conversely, *GNAS* mutation was rarely observed in PDAs (1 %, 1/88), and mutations were not detected in mucinous cystic neoplasms, neuroendocrine neoplasms, acinar cell neoplasms, serous cystadenomas, or solid-pseudopapillary neoplasms. In contrast, *KRAS* mutations were distributed evenly among PDAs (92 %, 81/88), IPMN-associated adenocarcinomas (77 %, 23/30), cystic neoplasms, including IPMNs (59 %, 36/61), and mucinous cystic neoplasms (40 %, 4/10).

We examined clinicopathological and genetic features of 91 IPMNs with or without an associated invasive carcinoma (Supplementary Table 1). *GNAS* mutations were more frequently detected in low- and intermediate-grade IPMNs than in high-grade IPMNs (76 % (25/33), 80 % (8/10), and 46 % (22/48), respectively) (for low- and intermediate-grade IPMNs combined versus high-grade IPMNs, $p=0.003$). Additionally, *GNAS* mutations were frequent in intestinal-type IPMNs (78 %, 21/27), followed by gastric-type IPMNs (62 %, 34/55), while *GNAS* mutations were not detected in the oncocytic and pancreatobiliary types. It is noteworthy that

Table 1 *GNAS* and *KRAS* mutation analysis of 290 pancreatic tumours and 77 PanINs

		GNAS		*KRAS*	
	Total (n)	Mutant (n)	Prevalence (%)	Mutant (n)	Prevalence (%)
Ductal adenocarcinomas	88	1	1	81	92
Adenosquamous carcinomas	4	0	0	4	100
Undifferentiated carcinomas	2	0	0	2	100
Mucinous carcinomas without associated IPMN	3	2	67	1	33
Invasive carcinomas with associated IPMN	30	11	37	23	77
Tubular subtype	24	6	25	20	83
Mucinous subtype	6	5	83	3	50
IPMNs	61	39	64	36	59
Mucinous cystic neoplasms	10	0	0	4	40
Neuroendocrine neoplasms	52	0	0	1	2
Acinar cell neoplasms	16	0	0	0	0
Serous cystadenomas	10	0	0	0	0
Solid-pseudopapillary neoplasms	14	0	0	0	0
PanINs (total)	77	2	3	60	78
PanIN-1	35	0	0	27	77
PanIN-2	25	1	4	18	72
PanIN-3	17	1	6	15	88

no correlation was observed between the mutation status of *GNAS* or *KRAS* and the presence of an associated invasive carcinoma. In total, 92 % (84/91) of IPMNs harboured mutations in either *GNAS* (27 %, 25/91) or *KRAS* (32 %, 29/91), or in both genes (33 %, 30/91).

Next, we examined the distribution of gene mutations in IPMNs according to histological grade and epithelial subtype. A combination of low- and intermediate-grade IPMNs, consisting of 37 gastric and 6 intestinal types, was studied, and *GNAS* mutations were detected in 73 and 100 % of the cases, respectively (Supplementary Table 2). In contrast, high-grade IPMNs, consisting of 18 gastric, 21 intestinal, 2 oncocytic, and 7 pancreatobiliary types, were analysed, and *GNAS* mutations were found in 39, 71, 0, and 0 % of the cases, respectively. The lower rate of *GNAS* mutation in high-grade IPMNs compared with low- and intermediate-grade IPMNs appears to be attributed to (1) the rarity of *GNAS* mutation in oncocytic and pancreatobiliary types, which constituted 19 % of high-grade IPMNs, and (2) the lower incidence of *GNAS* mutation in gastric-type IPMNs with high-grade dysplasia (39 %, 7/18), compared with those with low- and intermediate-grade dysplasia (73 %, 27/37) (p=0.01).

We also examined *GNAS* and *KRAS* mutations in 77 PanIN lesions, including 35 PanIN-1, 25 PanIN-2, and 17 PanIN-3. The rarity of *GNAS* mutation (3 %, 2/77) was in marked contrast to the common incidence of *KRAS* mutation, which was identified in 78 % of the lesions.

To summarise the spectrum of gene mutations among the pancreatic tumours, *KRAS* mutation was prevalent in ductal neoplasms, including precursor PanINs, whereas IPMN-related neoplasms frequently harboured *GNAS* mutation.

More frequent *GNAS* mutation in adenocarcinoma with mucinous features

In IPMN-associated adenocarcinomas, the prevalence of *GNAS* mutation was different between mucinous and tubular subtypes: almost all mucinous carcinomas showed a *GNAS* mutation (83 %, 5/6), in contrast to the relatively small proportion of tubular carcinomas (25 %, 6/24) (p=0.02).

In this study, we included three cases of mucinous carcinoma histologically similar to mucinous-subtype IPMN-associated adenocarcinoma but not accompanied by any discernible IPMN. We designated these cases as mucinous carcinoma without associated IPMN and discussed them separately from mucinous-subtype IPMN-associated adenocarcinoma in order to distinguish the features of IPMN-associated adenocarcinoma more clearly. In doing so, we found that two of three mucinous carcinomas without associated IPMN exhibited *GNAS* mutations as well.

Table 2 shows clinicopathological characteristics and genetic features of IPMN-associated adenocarcinomas and PDAs in this study. We found that IPMN-associated adenocarcinomas and PDAs differed in several aspects: patients with mucinous-subtype IPMN-associated adenocarcinoma were more likely to be female and presented at an early stage compared with those with PDA. Molecularly, mucinous-subtype IPMN-associated adenocarcinomas were more likely to have mutated *GNAS* and less likely to have mutated *KRAS*

Table 2 Clinicopathological characteristics of patients with IPMN-associated adenocarcinoma and PDA

				p	
	IPMN-Ad (mucinous) (n=6)	IPMN-Ad (tubular) (n=24)	PDA (n=88)	IPMN-Ad (mucinous) vs PDA	IPMN-Ad (tubular) vs PDA
Age (years)					
Range	50-74	57-83	47-83	0.604	0.001
Median	70	75	67		
Gender					
Male	3	13	81	0.015	<0.001
Female	3	11	7		
Location					
Head	4	12	59	1.000	0.225
Body or Tail	2	11	29		
Diffuse	0	1	0		
Size of invasive portion					
<10 mm	4	9	0	0.336	0.592
10-20 mm	2	8	68		
>20 mm	0	7	20		
pT					
T1	1	10	1	0.235	<0.001
T2	0	0	2		
T3	5	14	85		
pN					
N0	5	10	21	0.006	0.121
N1	1	14	67		
pM					
M0	6	22	80	0.658	1.000
M1	0	2	8		
Stage (UICC)					
IA	1	8	1	0.003	0.042
IB	0	0	2		
IIA	4	2	18		
IIB	1	12	59		
III	0	0	0		
IV	0	2	8		
Median survival (months)	36	43	24	0.549	0.668
GNAS					
Mutant	5	6	1	<0.001	<0.001
Wild-type	1	18	87		
KRAS					
Mutant	3	20	81	0.015	0.245
Wild-type	3	4	7		

IPMN-Ad IPMN-associated adenocarcinoma

compared with PDAs. Similarly, tubular-subtype IPMN-associated adenocarcinomas and PDAs showed differences in some features: patients with tubular-subtype IPMN-associated adenocarcinoma were more likely to present at a median of 8 years later, be female, present at an early stage, and have mutated *GNAS*, compared with those with PDA. These differences in patient characteristics are in agreement with past studies that have shown differences between IPMN-associated adenocarcinomas and conventional PDAs [28–32]. Taken together, these data support the view that IPMN-associated adenocarcinoma is distinct from PDA.

We subsequently focused on the epithelial subtypes of IPMN components in IPMN-associated adenocarcinomas. Mucinous-subtype IPMN-associated adenocarcinomas consisted of IPMNs with intestinal phenotype (5/6) and that with oncocytic phenotype (1/6) (Table 3). Of these, *GNAS* mutations were detected in all IPMNs with intestinal phenotype (100 %, 5/5). In contrast, tubular-subtype IPMN-associated adenocarcinomas were composed of IPMNs with gastric phenotype (16/24), those with pancreatobiliary phenotype (5/24), and those with intestinal phenotype (3/24). *GNAS* mutations were identified in three IPMNs with gastric phenotype (19 %, 3/16) and three with intestinal phenotype (100 %, 3/3).

Intratumour genetic divergence in tubular-subtype IPMN-associated adenocarcinoma

Lastly, we examined the gene status of adenocarcinoma and IPMN components in IPMN-associated adenocarcinoma to assess if the components shared the same features. We microdissected multiple portions of IPMN components and examined both *GNAS* and *KRAS* genes using the Cycleave PCR assay. Of the 30 IPMN-associated adenocarcinomas, we examined tumours whose invasive portions were more than 5 mm in diameter (n=23).

Mucinous-subtype IPMN-associated adenocarcinomas showed a consistent result: All cases (6/6, 100 %) displayed an identical mutation pattern of *GNAS* and *KRAS* in the mucinous adenocarcinoma and the associated IPMN (Table 3 and Fig. 1). In contrast, tubular-subtype IPMN-associated adenocarcinomas showed an identical gene status in the adenocarcinoma and IPMN components in 9 of 17 cases (Fig. 1), the remaining 8 displaying a discrepant mutation status of *GNAS*, *KRAS*, or both genes between components (Supplementary Fig. 1). Among the discrepant tumours, 5 cases displayed different mutations (for example, G12V versus G12D of *KRAS* in case no. 25), suggesting a collision of a tubular adenocarcinoma and an IPMN. When clinicopathological traits (age, gender, location, macroscopic type of IPMN, and mutation status of adenocarcinoma) were compared, the 8 tumours with a discordant mutation pattern tended to accompany branch-type IPMN, in comparison with the remaining 16 tumours without discordant results (p=0.02).

International consensus guidelines for the management of IPMN and MCN of the pancreas have been published recently [33]. The guidelines recommend that IPMN-associated adenocarcinoma be subdivided into carcinoma derived from and concomitant with an IPMN, based on the topological relationship and histological transition between the adenocarcinoma and IPMN components. We compared the result of our mutation analysis with this proposed stratification in order to assess to what extent the mutation pattern was in agreement with the histological evaluation. We classified 5 cases of adenocarcinoma with an associated low-grade IPMN as carcinoma concomitant with IPMN because of a lack of histological transition with a high-grade IPMN component, although both adenocarcinoma and IPMN were topologically contiguous. We found that in a total of 15 IPMN-associated adenocarcinomas with concordant mutational status, the majority were categorised into carcinoma derived from IPMN (14 of 15 cases). One case was carcinoma with concomitant IPMN. As for IPMN-associated adenocarcinoma with discrepant genetic results (n=8), 4 cases were carcinoma with concomitant IPMN and 2 were carcinoma of undetermined relationship with IPMN. We found 2 IPMN-associated adenocarcinomas with discrepant genetic results that were histologically categorised into carcinoma derived from IPMN (Table 3, Supplementary Fig. 1).

Discussion

We performed mutation analysis of *GNAS* and *KRAS* using a series of 290 pancreatic tumours of various histological types and 77 PanINs. This study confirmed previous results that (1) *GNAS* mutation is commonly detected in IPMNs and rare in PDAs [9, 10, 34], and (2) *KRAS* is frequently mutated in ductal neoplasms, particularly in the vast majority of ductal adenocarcinomas. In our study, *GNAS* mutations were prevalent in low- and intermediate-grade IPMNs. In addition, we confirmed the absence of *GNAS* mutations in mucinous cystic neoplasms and other non-ductal tumours, including neuroendocrine neoplasms, acinar cell neoplasms, serous cystadenomas, and solid-pseudopapillary neoplasms. Taken together, our study suggests that *GNAS* mutation is a molecular hallmark of IPMN-related neoplasms and plays a role in the tumourigenesis of IPMN during its early stages.

IPMN-associated adenocarcinomas comprise 10–40 % of surgically resected IPMNs [28–30] and account for 1–10 % of resected invasive ductal carcinomas of the pancreas [28, 29, 35]. IPMN-associated adenocarcinoma has been considered to develop via an IPMN to adenocarcinoma sequence. This notion is founded on the histopathological observation that IPMN commonly contains a varying degree of atypia in a

Table 3 Concordance of genetic mutations within IPMN-associated adenocarcinomas

Case ID	Size of invasion (mm)	Grade of IPMN	Macroscopic type of IPMN	Epithelial subtype of IPMN	Int. guidelines of IPMN-Ad	Genetic status		
						IPMN component (*GNAS*/*KRAS*)	Adenocarcinoma component (*GNAS*/*KRAS*)	Concordance
Mucinous subtype								
1	6	High	M	Intestinal	Derived	R201C/Q61H	R201C/Q61H	Concordant
2	6	High	M	Oncocytic	Derived	WT/G12R	WT/G12R	Concordant
3	7	High	M	Intestinal	Derived	R201C/Q61H	R201C/Q61H	Concordant
4	7	High	M	Intestinal	Derived	R201C/WT	R201C/WT	Concordant
5	37	High	M	Intestinal	Derived	R201H/WT	R201H/WT	Concordant
6	30	High	M	Intestinal	Derived	R201C/WT	R201C/WT	Concordant
Tubular subtype								
7	1	High	M	Gastric	Derived	WT/G12V	n.d.	
8	1	High	M	Pancreatobiliary	Derived	WT/G12R	n.d.	
9	2	High	M	Gastric	Derived	R201H/G12V	n.d.	
10	2	High	M	Intestinal	Derived	R201C/WT	n.d.	
11	3	High	B	Intestinal	Derived	R201C/G12D	n.d.	
12	2	High	B	Pancreatobiliary	Derived	WT/G12V	n.d.	
13	4	High	B	Gastric	Derived	WT/WT	n.d.	
14	8	High	B	Intestinal	Derived	R201C/WT	R201C/WT	Concordant
15	14	High	B	Gastric	Derived	R201C/G12D	R201C/G12D	Concordant
16	8	High	M	Gastric	Derived	WT/G12V	WT/G12V	Concordant
17	12	High	M	Pancreatobiliary	Derived	WT/G12R	WT/G12R	Concordant
18	24	High	M	Pancreatobiliary	Derived	WT/G12V	WT/G12V	Concordant
19	25	High	M	Gastric	Derived	WT/G12V	WT/G12V	Concordant
20	18	High	B	Pancreatobiliary	Derived	WT/G12V	WT/G12V	Concordant
21	35	High	B	Gastric	Derived	WT/G12D	WT/G12D	Concordant
22	17	Low	B	Gastric	Concomitant	R201H/G12D	R201H/G12D	Concordant
23	21	High	B	1) High: gastric 2) High: gastric 3) Interm.: gastric 4) Interm.: gastric	Derived	1) high: R201H/G12D 2) high: R201C/G12D 3) interm.: WT/G12V * 4) interm.: WT/G12D	WT/G12V	Discrepant
24	35	High	B	Gastric	Derived	R201H/WT	WT/WT	Discrepant
25	20	High	B	1) High: gastric 2) High: gastric	Undetermined	1) high: R201H/G12D 2) high: WT/G12D	WT/G12V	Discrepant
26	30	High	B	Gastric	Undetermined	R201C/G12D	WT/Q61H	Discrepant
27	17	Low	B	Gastric	Concomitant	WT/WT	WT/G12D	Discrepant
28	24	Low	B	Gastric	Concomitant	WT/G12V	WT/G12R	Discrepant
29	25	Low	B	Gastric	Concomitant	WT/WT	WT/G12D	Discrepant
30	28	Low	B	Gastric	Concomitant	R201H/G12V	WT/G12D	Discrepant

M main-duct (or mixed) type, *B* branch-duct type, *interm*., intermediate, *Int. guidelines* the international consensus guidelines for IPMN [33], *n.d*. not determined, *WT* wild-type.

*In case 23, a focus of intermediate-grade IPMN showed the same genetic pattern as the invasive carcinoma. However, because the foci of the representative, high-grade IPMNs displayed different patterns, and WT/G12V is a common combination of *GNAS* and *KRAS* mutations in invasive ductal carcinoma and IPMN, we did not classify this case as concordant.

single lesion, and that minimally invasive carcinoma is often identified in connection with high-grade IPMN. Several molecular studies have supported the notion by showing that mutations of *KRAS*, *p16*, and *TP53* genes and epigenetic alteration of various genes accumulate as IPMNs progress from low-grade to high-grade lesions [21]. Furthermore, it has been postulated that IPMN-associated adenocarcinoma is biologically different from conventional PDA. Clinical studies have substantiated this hypothesis: Patients with surgically resected IPMN-associated adenocarcinoma had a better 5-year survival (40–60 %) than those with PDA (10–25 %), although controversy still remains as to whether this favourable outcome is due to its biologic characteristics or to the early-stage manifestation [28–30, 32]. Molecular studies

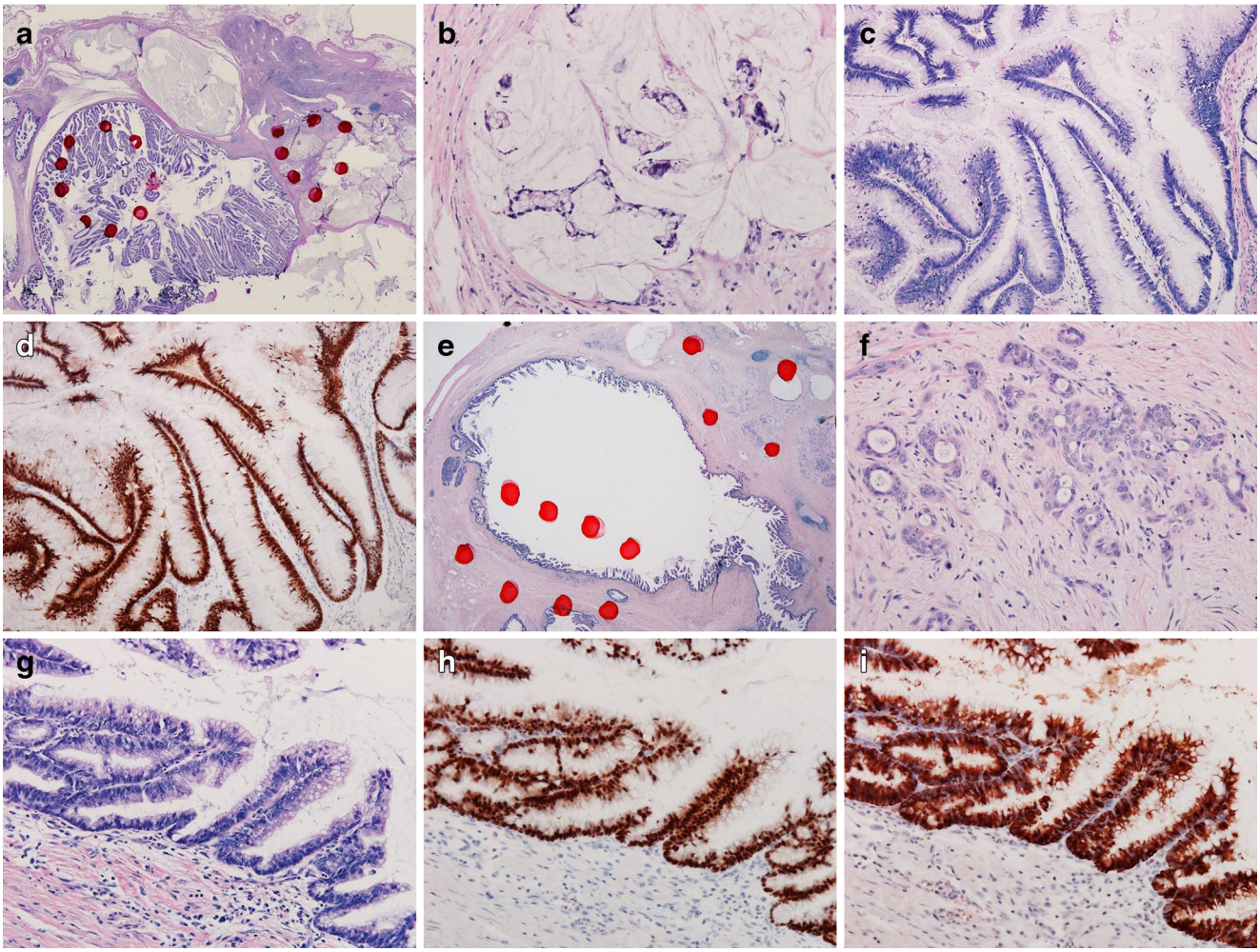

Fig. 1 Histology of IPMN-associated adenocarcinoma with concordant genetic status between adenocarcinoma and IPMN components. Mucinous-subtype IPMN-associated adenocarcinoma (**a**), which consisted of a mucinous carcinoma (**b**) and an intestinal-type IPMN marked by villous morphology (**c**) and diffuse and strong CDX2 immunostaining (**d**), had identical *GNAS* and *KRAS* mutations between the components (R201H *GNAS*; wild-type *KRAS*). Tubular-subtype IPMN-associated adenocarcinoma (**e**), consisting of a tubular adenocarcinoma (**f**) and an intestinal-type IPMN (**g**) with diffuse and strong immunostaining of CDX2 (**h**) and MUC2 (**i**), exhibited shared genetic status of *GNAS* and *KRAS* between the components (R201C *GNAS*; wild-type *KRAS*). The *red marks* indicate the microdissected portions

have also supported this notion, by showing that DPC4 expression is absent in half of PDAs, but retained in the majority of IPMN-associated adenocarcinomas [36]. Promoter methylation of several cancer-related genes, including *p16*, *E-cadherin*, *hMLH1*, and *MGMT*, was reported to be differentially involved in IPMN-associated adenocarcinomas and conventional PDAs [37, 38]. The recent discovery of the recurrent mutation of *GNAS* in IPMN has paved the way for the evaluation of this hypothesis. In this study, *GNAS* mutations were seen almost exclusively in IPMN-associated adenocarcinomas as well as IPMNs. This result strongly supports the view that this type of adenocarcinoma develops by multi-step progression of IPMN.

In IPMN-associated adenocarcinomas, two distinct subtypes of invasive adenocarcinomas, mucinous and tubular, have been described. Mucinous-subtype IPMN-associated adenocarcinoma shows a close association with intestinal-type IPMN, characterised by villous morphology and diffuse and strong MUC2 and CDX2 immunostaining. In contrast, tubular-subtype IPMN-associated adenocarcinoma typically develops in association with IPMN of gastric or pancreatobiliary types, both of which are marked by their specific morphology and negative MUC2 and CDX2 immunostaining [39]. Patient prognosis is also different, as mucinous-subtype tumours tend to be more indolent and carry a favourable prognosis, compared with tubular-subtype tumours [28–30, 32]. Based on these results, the terms 'intestinal and indolent pathway' and 'pancreatobiliary and aggressive pathway' were coined to represent the clinicopathological features of mucinous and tubular subtypes, respectively [20,

39]. In this study, we show the difference between mucinous and tubular subtypes in terms of genetic status of *GNAS* and *KRAS*. Specifically, mucinous-subtype IPMN-associated adenocarcinoma was characterised by a high prevalence of *GNAS* mutation and a strong association with intestinal-type IPMN, which was also characterised by frequent mutation of *GNAS*. In addition, by demonstrating the identical mutation status between adenocarcinoma and IPMN components, we confirmed that all mucinous-subtype IPMN-associated adenocarcinomas were clonal. Our results are in line with a study by Dal Molin et al., who reported *GNAS* mutations in all intestinal-type IPMNs ($n=12$), although the authors did not examine IPMN-associated adenocarcinomas [40]. In contrast, tubular-subtype IPMN-associated adenocarcinomas appear to be heterogeneous. Tubular-subtype tumours consisted of IPMNs with epithelial subtypes of varying kinds (gastric, pancreatobiliary, and intestinal). Six of 24 tubular-subtype tumours had *GNAS* mutations in both adenocarcinoma and IPMN components, and this result reliably qualified them as adenocarcinoma that had developed from IPMN. However, in 8 of 24 tubular-subtype IPMN-associated adenocarcinomas, mutation status of the adenocarcinoma was different from that of the IPMN component, indicating genetic divergence of this subtype.

In this study, we distinguished mucinous-subtype IPMN-associated adenocarcinoma from mucinous carcinoma lacking an apparent associated IPMN and examined them separately. Mucinous (or colloid) carcinoma is currently categorised in the 2010 WHO classification as a variant of ductal carcinoma, with a description that 'it almost always arises in association with an intestinal-type IPMN'. Seidel et al. reported that extensive sampling of colloid carcinomas enabled them to reveal the presence of IPMN in most cases in their study and concluded that colloid carcinoma develops from IPMN [41]. Practically, however, identifying an associated IPMN in a colloid carcinoma tends to be arbitrary among pathologists, particularly in tumours that are exclusively predominated by neoplastic cysts with a pushing border and embedded in dense fibrosis. In our study, a diagnosis of mucinous carcinoma without an associated IPMN was made in three cases. These tumours were large, measuring 50, 50, and 70 mm in diameter and proliferated in such a destructive manner that the associated IPMNs were not clearly discernible. However, our results are in support of the view that although an apparent IPMN component was not identified, mucinous carcinoma is highly linked to IPMN and IPMN-associated adenocarcinoma in terms of *GNAS* mutation. Because of the limited number of mucinous carcinoma without an associated IPMN analysed in this series, further studies are warranted.

In summary, we confirm that *GNAS* mutation is almost exclusively confined to IPMN-related tumours, which supports the notion that carcinoma with mucinous features and IPMN, particularly that of intestinal type, constitute a spectrum of pancreatic tumours sharing common pathological and genetic features.

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Conflict of interest The authors declare no conflict of interest.

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