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Clinicopathological significance of somatic *RNF43* mutation and aberrant expression of ring finger protein 43 in intraductal papillary mucinous neoplasms of the pancreas

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Mutations in *RNF43*, which encodes the ubiquitin E3 ligase ring finger protein 43, were recently found in intraductal papillary mucinous neoplasms of the pancreas. We evaluated somatic mutations of *RNF43* and the expression of ring finger protein 43 as well as their associations with the molecular and clinicopathological features in 176 surgically resected intraductal papillary mucinous neoplasms. Frozen tissues were available for 57 cases and were used for next-generation sequencing analysis of the entire coding exons of *RNF43*. Formalin-fixed and paraffin-embedded tissues from all 176 cases were used for the immunohistochemical analysis of the expression of ring finger protein 43. Mutations detected with the next-generation sequencing analysis were validated by using Sanger sequencing. Statistical analysis was used to evaluate the associations between *RNF43* aberrations and molecular and clinicopathological features including *GNAS* mutations, *KRAS* mutations, loss of SMA and MAD4 homologue expression, tumor protein 53 overexpression, tumor grade, histological type, mural nodule detection, macroscopic type, stage, recurrence, and survival. Somatic *RNF43* mutations were found in 8 (14%) of the 57 examined cases, and included 5 frameshift mutations (p.F69fs, p.S264fs, p.L311fs, p.R363fs, and p.V490fs), 1 non-sense mutation (p.Q153X), and 2 missense mutations (p.I164N and p.P310A). The expression of ring finger protein 43 was downregulated in 52 (29.5%) of the 176 examined cases. *RNF43* mutations were significantly associated with the downregulated expression of ring finger protein 43 ($P=0.011$), *GNAS* mutation ($P=0.020$), and mural nodule detection ($P=0.038$). The expression of ring finger protein 43 was not associated with any clinicopathological features except *RNF43* mutation. These results indicate that *RNF43* mutation might cause downregulation of the expression of ring finger protein 43 and play a crucial role and associate synergistically with *GNAS* mutation during development of intraductal papillary mucinous neoplasm of the pancreas.
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Intraductal papillary mucinous neoplasm of the pancreas is a primarily non-invasive tumor characterized by a dilated, mucin-filled duct.[1] The dilated duct is lined with neoplastic epithelial cells exhibiting a papillary growth pattern with varying degrees of atypia and abundant mucin secretion; this appearance differs greatly from that of a conventional pancreatic cancer such as ductal adenocarcinoma that forms a highly invasive, firm mass.[2] However, intraductal papillary mucinous neoplasms often accompany or progress to ductal adenocarcinoma, resulting in a poor prognosis; therefore, intraductal papillary mucinous neoplasm

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is regarded as a precursor of ductal adenocarcinoma.[1] Currently, given the lack of appropriate biomarkers to monitor the disease phenotype, diagnosis and treatment of intraductal papillary mucinous neoplasms rely heavily on imaging studies in accordance with a guideline for the management of patients with these neoplasms,[3] thus indicating a pressing need to develop molecular markers on the basis of the molecular phenotypes. In 2011, it was discovered that intraductal papillary mucinous neoplasms often harbored mutations in *GNAS* and *RNF43*.[4–6] Most *GNAS* mutations found in intraductal papillary mucinous neoplasms are either R201H or R201C, which are known to induce gain of function of the encoded protein guanosine nucleotide-binding protein alpha.[7] In contrast, *RNF43* mutations are mostly frameshift or nonsense mutations that are expected to induce a loss-of-function of the encoded protein ring finger protein 43.[4,5] Among pancreatic neoplasms, *GNAS* and *RNF43* mutations are almost exclusively found in intraductal papillary mucinous neoplasms, and therefore such mutations are thought to play crucial roles in the characteristic phenotypes of intraductal papillary mucinous neoplasms.[4,5]

Ring finger protein 43 is a ubiquitin E3 ligase that targets the frizzled receptor via interactions with the R-spondin protein.[8] Ubiquitination of the frizzled receptor results in the blocking of signal transduction through the wingless-type MMTV integration site (Wnt) pathway; accordingly, loss of function of ring finger protein 43 leads to Wnt signaling activation and tumorigenesis in the colon.[8] *RNF43* mutations have also been shown to confer Wnt dependency in pancreatic cancer cells.[9] However, the clinicopathological relevance of *RNF43* mutations in intraductal papillary mucinous neoplasms remains unclear.

Herein, we evaluated *RNF43* mutations and expression as well as the associations with the molecular and clinicopathological features of intraductal papillary mucinous neoplasms.

Materials and methods

Tissues

A total of 176 patients with intraductal papillary mucinous neoplasms who were surgically treated between 2001 and 2010 at the Tokyo Women's Medical University Hospital were evaluated. Formalin-fixed and paraffin-embedded tissues from the 176 patients were used for the immunohistochemical examination. Frozen tissues from 57 of the 176 patients were used for next-generation sequencing analysis. The clinicopathological features of the included cases are listed in Table 1. This study was approved by the Ethics Committee of the Tokyo Women's Medical University.

Table 1 Clinicopathological features of studied cases

Total	*176*
GNAS	
Mutant	80
Wild	96
KRAS	
Mutant	96
Wild	80
SMAD4 expression	
Retain	154
Loss	22
TP53 expression	
Overexpression	25
Normal	151
Grade and invasion	
Low grade	91
High grade	37
Invasive	48
Type	
Gastric	101
Intestinal	56
Oncocytic	8
Pancreatobiliary	11
Mural nodule	
Detected	129
Not detected	47
Macroscopic type	
Branch	83
Main	51
Mixed	42
Stage[a]	
0a	81
0	35
1	8
2	10
3	19
4a	8
4b	3
Recurrence	
Recurred	31
Not recurred	145
Prognosis	
5-year survival rate	0.904

[a]Stage according to Japan Pancreas Society Staging system with modification with Stage 0a indicating low-grade tumor (Japan Pancreas Society. Classification of Pancreatic Carcinoma, 3rd edn. Kanehara & Co. Ltd.: Tokyo, 2011).

Next-Generation Sequencing

Methanol-fixed and hematoxylin-stained frozen sections were prepared from frozen tissues. Tumor tissues were collected from each section by manual dissection under microscopic guidance. In non-invasive cases, a representative intraductal lesion in each case showing the highest grade of atypia was

Table 2 Primers used for validation of *RNF43* mutations

GRCh37/hg19	*Exon*	*Label*	*Forward*	*Reverse*	*Annealing* (°C)
Ch17:56435671	Exon 9	G25-26	CCAGCCAGTGACTCCAGCTC	GCTGGGGATCCCCTTTAGGG	62
Ch17:56436050	Exon 9	G27-28	ACCAGGTCGAAGACTCCACC	TGATGCCGAGGGCCCATGCC	64
Ch17:56437534	Exon 8	G29-30	GCTACGGGTCATTTCCTGCC	TGTCTGCCTACACAGAGGGG	62
Ch17:56438207	Exon 7	G17-18	CCGCTTCAGCAGAGAACAGC	TGGTCATGGAGGTGAACCAC	62
Ch17:56440727	Exon 5	G31-32	AAGTCACAGCAGCCCTGTG	GCTCAATCCTCACATGGGCC	62
Ch17:56440761	Exon 5	G19-20	AGATAAAGCTCTCAGGGGAG	GGTTCTTGTACACAAACTCC	60
Ch17:56492734	Exon 2	G21-22	GGAGTCTGAAAGATCAGCAG	CATATTTCAAACAGATGGAAAGTG	55
Ch17:56492734	Exon 2	G13-14[a]	TTATCAGAGTGATCCCCTTG	CTTGCCTGCATTAATTTTCC	58

[a]Nested primers.

dissected and collected. In invasive cases, ie, cases with intraductal papillary mucinous neoplasm with an associated invasive carcinoma, an area containing invasive carcinoma was dissected and collected. Genomic DNA was extracted from the collected tissues by using the Charge Switch® gDNA Micro Tissue kit (Life Technologies, Carlsbad, CA, USA). The extracted DNA was used for semiconductor sequencing analysis with an Ion AmpliSeq™ Primer Pool custom designed for all *RNF43* coding regions, Ion AmpliSeq™ Library Kit, Ion Xpress™ Library Barcode Adaptors, Ion One Touch™ 2, and an Ion PGM™ sequencer according to the manufacturers' instructions (Life Technologies). One case was examined by using a massively parallel sequencer, the SOLiD system (Life Technologies), as reported previously.[4]

Sanger Sequencing

DNA samples obtained from frozen tissues or formalin-fixed and paraffin-embedded tissues were used in a Sanger sequencing analysis to validate the *RNF43* mutation statuses and detect *GNAS* and *KRAS* mutations as described previously.[4] The primers used to validate the *RNF43* mutation statuses are listed in Table 2. The primers used to detect *GNAS* and *KRAS* mutations were described previously.[4]

Immunohistochemical Analysis

Indirect streptavidin-biotin immunohistochemical staining of paraffin-embedded tissues was performed by using a rabbit polyclonal anti-ring finger protein 43 antibody (Atlas Antibodies, Stockholm, Sweden) and a Histofine SAB-PO kit (Nichirei Bioscience Inc., Tokyo, Japan) according to the manufacturers' instructions. To evaluate the staining specificity, a negative control staining condition (no primary antibody) was performed. The immunohistochemical results were evaluated as either reduced or retained expression by comparing the staining intensity of each sample with that of an islet of Langerhans in the same section because the islet of Langerhans showed consistent staining that was easy to identify and evaluate compared with normal ducts that were often devastated by obstructive pancreatitis cause by the neoplasm. The staining was evaluated blindly from sequencing results at lesions with the highest grade of atypia or invasive area in each case. SMA and MAD4 homolog (SMAD4) and tumor protein 53 (TP53) expression were examined by using a mouse monoclonal anti-SMAD4 antibody (B-8; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and a mouse monoclonal anti-p53 antibody (DO-7; Dako, Glostrup, Denmark) as described previously.[10]

Statistics

The statistical analysis incorporated the chi-squared test for comparisons between the molecular and clinicopathological data, and Kaplan–Meier analysis with the log-rank test for survival analyses. Analyses were performed using the PASW Statistics software package (version 18.0; SPSS Inc., Chicago, IL, USA). *P*-values of <0.05 were considered as statistically significant.

Results

We performed sequencing analyses of all coding exons of *RNF43* in the 57 patients for whom frozen tissues were available; the analysis was conducted via semiconductor sequencing in 56 samples and via massively parallel sequencing as a whole exome analysis in 1 sample. We obtained the sequence data at an average read depth of 2664 with semiconductor sequencing. The called variations were validated by using tumor DNA, and the somatic state was examined via Sanger sequencing analysis of normal DNA. The massively parallel sequencing data obtained from the single case had been reported previously.[4] As a result, we found that 8 (14%) of the 57 intraductal papillary mucinous neoplasms harbored somatic *RNF43* mutations, including 5 frameshift mutations, 1 non-sense mutation, and 2 missense mutations (Figure 1; Table 3). Of these mutations, 3 of the 5 frameshift mutations, specifically p.F69fs, p.S264fs, and p.L311fs, as well as the non-sense mutation p.Q153X occurred upstream of

or within the ring finger domain (residues 272–316 according to the Conserved Domain Database)[11] of ring finger protein 43 (Figure 1). The remaining two frameshift mutations, p.R363fs and p.V490fs, occurred immediately downstream of the ring finger domain. Of the two missense mutations, p.I164N involved a conserved residue in the protease-associated domain (residues 87–186), and p.P310A involved a conserved residue of the ring finger domain, according to the Conserved Domain Database[11] (Figure 1). SIFT (http://sift.jcvi.org/)[12] and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml),[13] the online programs used to predict the functional significance of the missense mutations, predicted that both these missense mutations would have damaging effects.

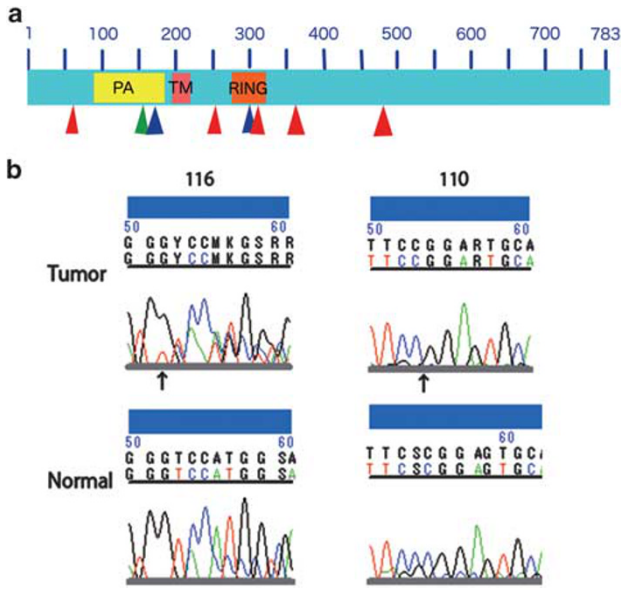

Figure 1 Somatic *RNF43* mutations in intraductal papillary mucinous neoplasms. (**a**) Mutation locations in the ring finger protein 43. Red triangles indicate the frameshift mutations, a green triangle indicates the non-sense mutation, and blue triangles indicate the missense mutations. PA, protease-associated domain; TM, transmembrane domain; and RING, ring finger domain. (**b**) Representative images of the Sanger sequencing validation. Arrows indicate the c.1087delC mutation in #110 and the c.1466delG mutation in #116.

Next, we investigated the expression of ring finger protein 43 in 176 formalin-fixed and paraffin-embedded tissues of intraductal papillary mucinous neoplasms via immunohistochemical analysis. Because intraductal papillary mucinous neoplasm is intrinsically a heterogeneous lesion, some cases showed heterogeneity of staining, so that we evaluated the staining at areas showing the highest grade of atypia or invasive area with comparison with staining of islet cells. As a result, the expression of ring finger protein 43 was reduced in 52 (29.5%) of the 176 examined tissues relative to the normal islet cells (Figure 2). This reduced expression was significantly associated with the presence of somatic mutations in all but two cases, specifically the case with the p.R363fs frameshift mutation and the case with the p.P310A missense mutation ($P=0.011$).

We evaluated the association between *RNF43* mutations or the expression of ring finger protein 43 and the molecular and clinicopathological features, including *GNAS* mutations, *KRAS* mutations, SMAD4 downregulation, TP53 overexpression, tumor grade, histological type, mural nodule detection, macroscopic type, stage, recurrence, and survival. We found that *RNF43* mutation was significantly associated with *GNAS* mutations ($P=0.020$) and mural nodule detection ($P=0.038$; Table 4). RNF43 expression was not associated with any of the features (Table 4).

Discussion

In this study, we found that 8 (14%) of the 57 intraductal papillary mucinous neoplasms harbored somatic *RNF43* mutations. Among these mutations, 3 of 5 frameshift mutations and 1 non-sense mutation were expected to yield truncated proteins lacking the protease-associated domain and/or the ring finger domain, whereas 2 missense mutations affected conserved residues in the protease-associated domain or the ring finger domain and would likely result in the functional abrogation of ring finger protein 43. The remaining two frameshift mutations would yield truncated ring finger protein 43 immediately downstream of the ring finger

Table 3 Somatic mutations of *RNF43* in intraductal papillary mucinous neoplasms

GRCh37/hg19	*Mutation*	*Exon*	*Nucleotide*	*Protein*	*Histopathology of IPMN*	*Type of IPMN*
ch17:56492734	Frameshift deletion	Exon 2	c.204_205delG,T	p.F69CfsX5	Invasive	Gastric
ch17:56440761	Nonsense	Exon 5	c.C457T	p.Q153X	Low grade	Gastric
ch17:56440727	Misssense	Exon 5	c.T491A	p.I164N	High grade	Intestinal
ch17:56438207	Frameshift deletion	Exon 7	c.786delA	p.S264AfsX155	Low grade	Gastric
ch17:56437534	Misssense	Exon 8	c.C928G	p.P310A	High grade	Intestinal
ch17:56437530	Frameshift insertion	Exon 8	c.931_932insC	p.L311PfsX132	Invasive	Intestinal
ch17:56436050	Frameshift deletion	Exon 9	c.1087delC	p.R363GfsX56	High grade	Intestinal
ch17:56435671	Frameshift deletion	Exon 9	c.1466delG	p.V490SfsX12	Low grade	Gastric

Abbreviation: IPMN, intraductal papillary mucinous neoplasm.

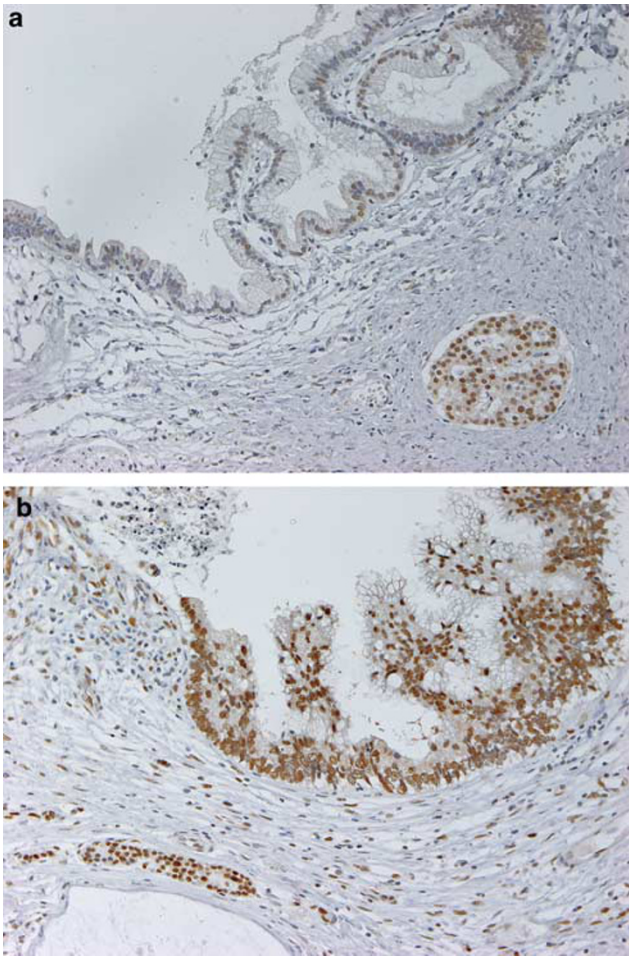

Figure 2 Immunohistochemical analysis indicates the reduced (**a**) or retained (**b**) expression of ring finger protein 43 in intraductal papillary mucinous neoplasms. Original magnification, ×100.

Table 4 Associations between *RNF43* mutations and clinicopathological features

	RNF43			*RNF43 expression*		
Variable	*Mutant*	*Wild*	P-*value*	*Retain*	*Reduced*	P-*value*
RNF43 expression						
Reduced	6	14	0.011	—	—	
Retain	2	35		—	—	
GNAS						
Mutant	8	28	0.020	60	20	0.36
Wild	0	21		66	30	
KRAS						
Mutant	7	34	0.290	66	30	0.36
Wild	1	15		60	20	
SMAD4 expression						
Retain	7	45	0.688	114	40	0.058
Loss	1	4		12	10	
TP53 expression						
Overexpression	1	12	0.454	14	11	0.062
Normal	7	37		112	39	
Histopathology						
Low grade	3	27	0.448	66	25	0.633
High grade	3	9		28	9	
Invasive	2	13		32	16	
Type						
Gastric	4	30	0.495	71	30	0.133
Intestinal	4	13		45	11	
Oncocytic	0	2		4	4	
Pancreatobiliary	0	4		6	5	
Mural nodule						
Detected	8	31	0.038	91	38	0.378
Not detected	0	18		35	12	
Macroscopic type						
Branch	5	26	0.843	62	21	0.688
Main	2	13		35	16	
Mixed	1	10		29	13	
Stage[a]						
0a	3	21	0.493	57	24	0.553
0	3	7		26	9	
1	2	6		5	3	
2	0	2		7	3	
3	0	6		11	8	
4a	0	6		6	2	
4b	0	1		3	0	
Recurrence						
Recurred	0	8	0.218	23	8	0.723
Not recurred	8	41		103	42	
Prognosis						
5-year survival	—	—		0.926	0.908	0.723

[a]Stage according to Japan Pancreas Society Staging system with modification with Stage 0a indicating low-grade tumor (Japan Pancreas Society. Classification of Pancreatic Carcinoma. 3rd edn. Kanehara & Co. Ltd.: Tokyo, 2011).

domain and would not affect the known functional domains; however, the carboxyl-terminal regions downstream of the ring finger domain were also highly conserved among mammals, according to HomoloGene (http://www.ncbi.nlm.nih.gov/homologene). Moreover, according to the Catalog of Somatic Mutations in Cancer database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/),[14] 8 of the 27 reported frameshift mutations in *RNF43* involved residues downstream of the ring finger domain, indicating a commonality and suggesting some level of functional significance regarding such mutations. Indeed, Jiang *et al*[9] reported the identification of a frameshift mutation, p.R330fs, that led to the truncation of the encoded protein immediately downstream of the ring finger domain in a pancreatic cancer cell line; this mutation appeared to induce Wnt dependency in the cell, thus indicating the functional abrogation of ring finger protein 43. Nevertheless, most somatic mutations identified in intraductal papillary mucinous neoplasms are loss-of-function mutations, indicating that *RNF43* might function as a pancreatic tumor suppressor gene affecting susceptibility linked to intraductal papillary mucinous neoplasm. Recently, Amato *et al* published a report in which 6 (14%) of

42 intraductal papillary mucinous neoplasms harbored *RNF43* mutations, including 3 frameshift mutations resulting in premature stop codons upstream of the ring finger domain, 2 missense mutations affecting the protease domain and the ring finger domain, and 1 non-sense mutation affecting a residue downstream of the ring finger domain,[15] thus demonstrating a similar distribution of mutations to that observed in our samples. Wu *et al* reported that 6 of 8 intraductal papillary mucinous neoplasms harbored *RNF43* mutations, all of which were non-sense mutations; 5 of the 6 mutations would generate premature stop codons upstream of the ring finger domain and the remaining mutation, p.R371X, would generate a stop codon after the ring finger domain.[5] Jiang *et al*[9] identified one non-sense mutation, p.E174X, and one missense mutation, p.F69C, as well as a frameshift mutation, p.R330fs, in pancreatic cancer cell lines, and noted that these mutations appeared to confer Wnt dependency on the cells, thus indicating the functional abrogation of ring finger protein 43 by these mutations. These reports and our current results suggest that loss-of-function *RNF43* mutations might play an imperative role in intraductal papillary mucinous neoplasms, possibly by conferring Wnt dependency on the neoplastic cells.

The *RNF43* mutations were associated with *GNAS* mutations and mural nodule detection. *GNAS* mutations have been found in 40–60% of intraductal papillary mucinous neoplasms, and are known to be exclusive to these tumors among the various pancreatic neoplasms.[4–6] Komatsu *et al*[16] reported that *GNAS* mutations induced the upregulation of cyclic adenosine monophosphate and overexpression of mucin protein genes in some pancreatic duct-lineage cells; therefore, *GNAS* mutation appears to play a key role in the secretion of abundant mucin, the most prominent characteristic phenotype of intraductal papillary mucinous neoplasms. Interestingly, the same authors also demonstrated that the exogenous expression of mutated *GNAS* did not confer a proliferative advantage on pancreatic cancer cells.[16] These results regarding the association between *RNF43* and *GNAS* mutations might indicate that the loss-of-function of ring finger protein 43 would confer an advantage with respect to proliferation and/or maintenance upon pancreatic ductal cells with *GNAS* mutations in intraductal papillary mucinous neoplasms. Amato *et al*[15] also indicated that *RNF43* mutation was associated with *GNAS* mutation. The mural nodule indicates the protuberance of a neoplasm into the pancreatic ductal lumen; this is considered to indicate a high-grade lesion.[3] The association between the mural nodule and *RNF43* mutation suggests that ring finger protein 43 might confer proliferative advantages that lead to protruding neoplasms. However, no associations were identified between *RNF43* mutation and various clinicopathological features including the histological type, tumor grade, stage, and prognosis, possibly indicating that *RNF43* mutation is an early developmental event with no significant impact on the progression of the histological type, dysplastic grade, or tumor stage of intraductal papillary mucinous neoplasms.

We found that 52 (29.5%) of the 176 intraductal papillary mucinous neoplasms exhibited reduced expression of ring finger protein 43. To the best of our knowledge, this is the first report to reveal aberrant expression of ring finger protein 43 in intraductal papillary mucinous neoplasms. The reduced expression of ring finger protein 43 was associated with *RNF43* mutation, which indicates that *RNF43* mutation may lead to reduced expression of ring finger protein 43. Indeed, most *RNF43* mutations are truncating mutations and as a result, protein expression will likely be reduced. However, because the reduced expression of ring finger protein 43 was observed more frequently than were mutations, epigenetic alterations might be responsible for this reduced expression in addition to somatic mutations, a possibility that will be addressed in a future study. The reduced protein expression was not associated with any clinicopathological features, indicating that the aberrant expression of ring finger protein 43 might play a role in the development but not in the progression of intraductal papillary mucinous neoplasms.

In conclusion, the results of this study indicate that *RNF43* mutation might cause reduced expression of ring finger protein 43 and play a crucial role and associate synergistically with *GNAS* mutation in development of intraductal papillary mucinous neoplasms of the pancreas.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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