Deficient Rnf43 potentiates hyperactive Kras-mediated pancreatic preneoplasia initiation and malignant transformation

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Abstract

Background: Largely due to incidental detection, asymptomatic pancreatic cystic lesions (PCLs) have become prevalent in recent years. Among them, intraductal papillary mucinous neoplasm (IPMN) infrequently advances to pancreatic ductal adenocarcinoma (PDAC). Conservative surveillance versus surgical intervention is a difficult clinical decision for both caregivers and PCL patients. Because RNF43 loss-of-function mutations and KRAS gain-of-function mutations concur in a subset of IPMN and PDAC, their biological significance and therapeutic potential should be elucidated.

Methods: Pancreatic Rnf43 knockout and Kras activated mice (Rnf43−/−; KrasG12D) were generated to evaluate their clinical significance in pancreatic pre-neoplastic initiation and malignant transformation.

Results: Loss of Rnf43 potentiated the occurrence and severity of IPMN and PDAC in oncogenic Kras mice. The Wnt/β-catenin signaling pathway was activated in pancreatic KrasG12D and Rnf43 knockout mice and the PORCN inhibitor LGK974 blocked pancreatic IPMN initiation and progression to PDAC accordingly.

Conclusions: Rnf43 is a tumor suppressor in the prevention of pancreatic malignant transformation. This genetically reconstituted autochthonous pancreatic Rnf43−/−; KrasG12D preclinical cancer model recapitulates the pathological process from pancreatic cyst to cancer in humans and can be treated with inhibitors of Wnt/β-catenin signaling. Since the presence of RNF43 and KRAS mutations in IPMNs predicts future development of advanced neoplasia from PCLs, patients with these genetic anomalies warrant surveillance, surgery, and/or targeted therapeutics such as Wnt/β-catenin inhibitors.

KEYWORDS
intraductal papillary mucinous neoplasms, KRAS, pancreatic ductal adenocarcinoma, RNF43, Wnt
1 | INTRODUCTION

With a pooled prevalence of 8% in adults, pancreatic cystic lesions (PCLs) are often asymptptomatically and incidentally discovered on routine cross-sectional abdominal imaging. PCLs are a heterogeneous group of benign tumors and precursor lesions of pancreatic ductal adenocarcinoma (PDAC). PDAC is a major type of pancreatic malignant tumor, with the worst prognosis among cancers. Even though the malignant potential of PCL is very low, accurate diagnosis and correct assessment of malignant potential are often hard to achieve. Since it is a daunting task to distinguish tumors with or without malignant potentials, perceived dire prognosis of PDAC, evolved from incidentally detected PCLs, often forces both caregivers and patients to choose between major pancreatic resection, with substantial morbidity and mortality, and lifelong surveillance, with associated financial burdens, anxiety, and the risk of missed malignancy.

Intraductal papillary mucinous neoplasm (IPMN) is a major type of PCL, which may progress from low-grade dysplasia (LGD) to high-grade dysplasia (HGD), and eventually to PDAC. Therefore, IPMN is a precursor lesion of PDAC. Progression from precancerous lesions to invasive pancreatic cancer may take about 20 years. It is a curable disease if detected and treated before it proceeds to invasive carcinoma. In clinical practice, LGD can potentially be managed conservatively while HGD and invasive carcinoma would require surgical resection. The surgical indication for these patients is based on the presence of indirect predictors in diagnostic images. Given the lack of information on the natural history of IPMN malignant transformation, whether noninvasive carcinoma or HGD should be operated on or observed remains an unsolved practical issue. Therefore, analysis of these precancerous lesions is crucial to understand the earliest events of pancreatic tumorigenesis.

Recent studies have shown that the most frequently mutated genes in IPMNs are KRAS, GNAS, and RNF43. We and others identified that the pooled prevalence of RNF43 inactivating mutation in IPMN was 24% by next generation sequencing assessment. RNF43 is a member of the RING finger protein family, and an E3 ubiquitin ligase. It exerts negative feedback regulation of the Wnt/β-catenin signaling pathway by (a) mediating the ubiquitination and degradation of the Wnt receptor complex component Frizzled; and (b) tethering TCF4 to the nuclear membrane for silencing TCF4 transcriptional activity. Loss of RNF43 activates Wnt/β-catenin signaling pathway. To dissect potential interplaying roles and underlying mechanisms of aberrant RNF43 and KRAS in IPMN initiation and malignant transformation, we simulated human IPMN and PDAC in mice by establishing a pancreatic Kras deficient preclinical model. We also developed a noninvasive therapeutic regimen for the unmet needs of this disturbing disease.

2 | METHODS

2.1 | Generation of mouse models

All animal experiments were approved by the Animal Care and Use Committee at Peking Union Medical College and were performed in accordance with international guidelines. Pdx1-cre; LSL-KrasG12D mice have been described previously. Conditional Rnf43loxP/loxP mice were established through a contract with Beijing Biocytogen. Mice were kept on a C57BL/6 genetic background. LoxP sites were inserted into the introns before exon 7 and after exon 8 of Rnf43 alleles. To generate pancreatic Kras activation and Rnf43 knockout mice, we crossed Pdx1-cre mice (B6.FVB-Tg (lpf1-cre)1Tuv/Nc), LSL-KrasG12D mice and Rnf43loxP/loxP mice to produce Pdx1-cre; LSL-KrasG12D; Rnf43loxP/loxP (Rnf43ΔT; KrasG12D) mice, Pdx1-cre; LSL-KrasG12D (KrasG12D) mice, Pdx1-cre; Rnf43loxP/loxP (Rnf43ΔT) mice, and Rnf43loxP/loxP (Rnf43ΔT) mice. These mice were sacrificed at the ages of 16 days, 1 month, and 6 months.

Histological and immunohistochemical analyses

After fixing in 10% formalin for more than 24 hours, tissues were embedded in paraffin and cut to 4-μm sections. H&E and immunohistochemical staining was performed according to standard procedures. Antibodies utilized are listed in Table 1. Photos were taken with Leica DM18 microscope, with identical adjustments and exposure times between genotypes and treatment groups. Immunohistochemical staining images were captured using CaseViewer software (3DHISTECH) and quantified with Image-Pro Plus 6.0 software (Media Cybernetics).
Membrane bound O-acyl transferase porcupine (PORCN) inhibitor LGK974 treatment

LGK974 (#S7143, Selleck) was formulated in 0.5% MC/0.5% Tween-80 (MC: low viscosity, #BB12BA0026, BBI Life Sciences; Tween-80: #CB01BA0035; BBI Life Sciences).

Rnf43⁻/⁻; KrasG12D mice were randomly assigned to control (21 mice in total, 12 ♂ 9 ♀) or treatment groups (22 mice in total, 14 ♂ 8 ♀). Treatment started at 20 days after birth. LGK974 was administered by oral gavage at a dosage of 5 mg/kg animal body weight, twice daily, for 14 consecutive days. Controls were given corresponding volumes of 0.5% MC/0.5% Tween-80. Body weight was monitored twice daily for 14 treatment days. Mice were observed daily for survival and sacrificed at the age of 6 months. Tissues were fixed in 10% formalin for 24 hours and then paraffin embedded for histological and immunostaining.

Immunoblotting

Pancreatic tissues were ground in liquid nitrogen and then sonicated (power 20%, 5 seconds on, 5 seconds off, total time 5 minutes) in lysis buffer (2% SDS, 10% glycerol, 10 mM Tris (pH 6.8) and 100 mM DTT). The tissue suspension was then centrifuged for 10 minutes (4°C, 12,000 rpm). The supernatant was removed and boiled for 10 minutes. Protein lysates were resolved on NuPAGE™ 4%-12% Bis-Tris Mini Protein Gel (#NP0336BOX; Invitrogen) and transferred to 0.45 μm nitrocellulose membranes (#HATF00010; Merck). Protein samples were normalized to GAPDH. Antibodies utilized are listed in Table 1. Imaging was taken using LI-COR Odyssey CLx.

cBioPortal analysis of gene mutations in human pancreatic adenocarcinoma

Pancreatic cancer genomics data deposited in ICGC, QCMG, UTSW and TCGA database were analyzed using the cBioPortal for Cancer Genomics (http://cbioportal.org) for genetic alterations of KRAS, TP53 and RNF43.

2.6 | Statistical analysis

The Kaplan-Meier log-rank test was used for analysis of mouse survival and body weight differentiation using GraphPad Prism software (Version 7). All quantitative data are reported as means ± SD unless otherwise indicated in the figure legends. A p-value of <0.05 was considered as significant.

3 | RESULTS

3.1 | Pancreatic Rnf43-deficient mice have enlarged pancreata

We and others identified mutated RNF43 in tumor tissues of IPMN patients.11,12 To check whether loss of RNF43 causes IPMN, we generated pancreatic Rnf43 knockout mice. First, conditional Rnf43 knockout mice were created by inserting LoxP and neo cassette into the introns flanking exon 7 and exon 8 of Rnf43 gene (Figure 1A). Rnf43LoxP−/neo+ mouse (#64) was identified by genotyping analysis (Figure 1B). Neo cassette was then removed by crossing Rnf43LoxP−/neo+ with Flp-deleter mice (Figure 1C,D). Lastly, Rnf43Lox/Lox mice were mated with pancreatic specific Cre (Pdx1-cre) mice to produce pancreatic Rnf43 knockout mice (Pdx1-cre; Rnf43LoxP/LoxP (Rnf43−/−)) (Figure 1C). Knockout of Rnf43 in the pancreata of mice was detected by genotyping analysis (Figure 1E). DNA sequencing confirmed that exons 7 and 8 were removed from Rnf43 gene (Figure 1F).

Rnf43+ and Rnf43−/− mice were viable and born at the expected Mendelian ratio. Pancreas weight of Rnf43−/− mice was heavier than that of Rnf43+ mice at both 1 month and 6 months after birth (Figure 1G). Histopathological analysis did not reveal obvious alterations in the architecture of the pancreata in Rnf43−/− and Rnf43+/+ mice at both time points (Figure 1H). Survival of Rnf43−/− mice was
no worse than that of wild type mice (Figure 1I). Even though the loss of Rnf43 led to bigger pancreata, this was not sufficient to cause tumor.

3.2 | Pancreatic Kras\(^{G12D}\) and Rnf43 deficient mice develop intraductal papillary mucinous neoplasms

Oncogenic KRAS mutations are the earliest driver gene alterations in IPMNs.\(^{21}\) Mutations of KRAS and RNF43 often concur in a subset of IPMNs.\(^{11,12}\) To decipher the causative relationship between concomitant RNF43/KRAS mutations and pancreatic carcinogenesis, we generated pancreatic Rnf43 knockout and Kras activation mice (Rnf43\(^{-/-}\); Kras\(^{G12D}\)) through cross-mating of Pdx1-cre mice with Rnf43\(^{loxP/loxP}\) and Kras\(^{loxP/+}\) mice (Figure 2A,B). KRAS\(^{G12D}\) protein was only detected in the pancreata of Rnf43\(^{-/-}\); Kras\(^{G12D}\) and Pdx1-cre; Kras\(^{loxP/+}\) (Kras\(^{G12D}\)) mice but not in that of Rnf43\(^{+/+}\) and Rnf43\(^{-/-}\) mice (Figure 2C).

Rnf43 deficiency could not be verified at the protein level due to lack of an effective antibody against mouse Rnf43. Rnf43\(^{-/-}\); Kras\(^{G12D}\) mice had shortened lifespans compared to Rnf43\(^{+/+}\), Rnf43\(^{-/-}\), or Kras\(^{G12D}\) mice (Figure 2D).

Although Kras\(^{G12D}\) mice develop mouse pancreatic intraepithelial neoplasia (PanIN) lesions, these lesions rarely progress to invasive carcinoma within 1 year.\(^{20}\) Consistent with this previous report,
Kras\textsuperscript{G12D} mice showed modest proliferation of duct-epithelial cells with low-grade atypia that corresponded to acinar-to-ductal metaplasia (ADM) by 16 days of age. Rnf43\textsuperscript{-/-}; Kras\textsuperscript{G12D} mice began to develop pancreatic cystic tumors 16 days after birth. Tumors consisted of dilated ducts with prominent proliferation of epithelial cells. Around 6 months after birth, all Rnf43\textsuperscript{-/-}; Kras\textsuperscript{G12D} mice had widespread IPMN and PanINs embedded in fibrotic stroma with almost no normal acinar cells while Kras\textsuperscript{G12D} mice had duct-epithelial cell atypia that corresponded to PanINs (Figure 3). These data suggest that deficient Rnf43 and mutant Kras synergistically promote mouse pancreatic tumorigenesis, which mimics the cardinal features of human IPMN.

3.3 Pancreatic Kras\textsuperscript{G12D} and Rnf43 deficient mice develop invasive and metastatic PDAC

At about 6 to 7 months after birth, several Rnf43\textsuperscript{-/-}; Kras\textsuperscript{G12D} mice had abdominal distension owing to the accumulation of malignant ascites (Figure 4A). Large firm tumors in the head of the pancreas were almost invariably seen (Figure 4B). Possible invasion of neighboring structures by pancreatic head tumors resembles the classic features of human pancreatic cancer. Pathologic analysis demonstrated PDAC and its invasion to adjacent bile duct, small bowel, and lymph nodes (Figure 4C). Since the tumor nodules in Rnf43\textsuperscript{-/-}; Kras\textsuperscript{G12D} lungs were positive for duct marker CK19 and negative for...
pulmonary adenocarcinoma marker thyroid transcription factor 1 (TTF-1) (Figure 4D,E), they are likely to be metastatic PDAC but not spontaneous lung tumor. Therefore, our data show that deficient Rnf43 and mutant Kras mice develop invasive and metastatic PDAC.

### 3.4 | Biliary obstruction in pancreatic KrasG12D and Rnf43 deficient mice

Unlike KrasG12D mice which did not develop obvious lesions, 6-month-old Rnf43−/−; KrasG12D mice harbored biliary obstruction and gallbladder distension (Figure 5A). Pathological analysis demonstrated hyperplasia in bile duct epithelia of Rnf43−/−; KrasG12D mice (Figure 5B) and major duodenal papilla (Figure 5C). These manifestations are commonly seen and have devastating consequences in pancreatic cancer patients.

### 3.5 | Mutation co-occurrence of RNF43 and KRAS in human pancreatic adenocarcinoma

To determine the clinical relevance of our findings from mice, we analyzed the genomic data of 1034 pancreatic adenocarcinoma samples deposited in ICGC, QCMG, UTSW and TCGA databases with cBioPortal for KRAS, TP53 and RNF43 alterations. The most common aberration of KRAS is missense mutation. Missense mutation and truncating mutation are frequent in TP53. Truncating mutation and missense mutation are common in RNF43. The mutation frequencies of KRAS, TP53, and RNF43 are 83%, 58%, and 6%, respectively. KRAS and TP53 mutations occur together, while, in contrast, RNF43 and TP53 mutations do not co-exist. Mutant RNF43 almost exclusively occurs along with abnormal KRAS (Figure 6) and therefore, KRAS mutation is likely required for RNF43 mutation-associated pancreatic cancer development.

### 3.6 | Intraductal papillary mucinous neoplasm development requires activation of Wnt/β-catenin pathway

β-Catenin and its downstream target glutamine synthetase were highly expressed in the pancreata of 6-month-old Rnf43−/−; KrasG12D mice with IPMN and PanIN (Figure 7A), suggesting that deficient Rnf43 and mutated Kras activate Wnt/β-catenin signaling. To investigate whether Wnt/β-catenin signaling activation is indispensable to IPMN development, we treated Rnf43−/−; KrasG12D mice with PORCN inhibitor LGK974 which blocks Wnt secretion. Because Rnf43−/−; KrasG12D mice initiated pancreatic IPMN at 16 days and began to die by 21 days after birth, we chose 20-day-old Rnf43−/−; KrasG12D mice for treatment. These mice were randomly assigned to experimental (LGK974) or control (MC/T-80) groups. Mice were then administered with either MC/T-80 or LGK974 in MC/T-80 by oral gavage at a dosage of 5 mg/kg animal
body weight, twice daily, for 14 consecutive days (Figure 6B). The body weight loss in LGK974 treated mice was not statistically significant (Figure 6C). While ~50% control mice died, none of LGK974-treated mice died within the 14-day treatment period (Figure 6D). After discontinuation of the treatment, mice from the experimental treatment group began to die, but higher mortality was still observed in sham-treated mice than in LGK974-treated mice (Figure 6D). The surviving mice were sacrificed at the age of 6 months. No gender difference was observed in response to the treatment (Figure 6D). Taken together, our data show that Wnt/β-catenin signaling activation is necessary for pancreatic IPMN development and malignant transformation.

4 | DISCUSSION

Loss-of-function mutation of RNF43 coexists with gain-of-function mutation of KRAS in human IPMN and PDAC.11,12,14 To study the pathological effect of deficient Rnf43 and active Kras on mouse pancreas, we first generated Rn$^{43^{-/-}}$; Kras$^{G12D}$ mice and then found that these mice developed cystic papillary lesions and invasive/metastasizing PDAC with aberrant activation of the Wnt/β-catenin signaling pathway. Accordingly, PORCN inhibitor LGK974 blocked pancreatic tumor development and prolonged the survival of Rn$^{43^{-/-}}$; Kras$^{G12D}$ mice.
Identification of the patients who do not require follow-up and unnecessary surgery may help reduce the burden and societal costs for the majority of patients. To differentiate the cysts with malignant lesions from those without malignant potential, we need to dissect the molecular events underlying the malignant transformation. Although RNF43 is the 3rd most frequently mutated gene in pancreatic tumor tissues of IPMN patients, the causative relationship between loss of RNF43 and IPMN is yet to be established. To study the effect of RNF43 deficiency on pancreatic oncogenesis, we generated pancreatic Rnf43 knockout mice. As deficient RNF43 caused bigger pancreata without obvious alterations of pancreas architecture, deficiency of RNF43 alone is not sufficient to cause pancreatic cysts. Mutant KRAS is thought to be the earliest driver gene in IPMNs, and mutant RNF43 often coexists with mutant KRAS. These mutations are evolutionally selected in a subset of IPMNs.

We observed development and progression of IPMN and PanIN in Rnf43−/−; KrasG12D mice.

Concurrent activation of KRAS and GNAS simulated human IPMN lesions in mice. However, GNAS does not accelerate KRAS-mediated development of PDACs. In contrast, our Rnf43−/−; KrasG12D mice developed invasive and metastatic PDAC much sooner than KrasG12D mice, resulting in reduced survival. Coexistence of mutated RNF43 and KRAS in PDAC also supports a collaboration between RNF43 and KRAS in the development of human pancreatic cancer. Based on these findings, we suggest that close monitoring of lesion progression and more aggressive intervention such as surgery and novel therapeutics should be applied to IPMN with concomitant mutations of KRAS and RNF43.

Current management of pancreatic cysts is life-long surveillance, endoscopic ultrasound-guided fine-needle aspiration, or surgery. No drugs are available for the treatment of pancreatic cysts. Single-cell transcriptome sequencing of IPMN and PDAC revealed that many signaling pathways are altered, including Wnt/β-catenin signaling, during progression from noninvasive dysplasia to invasive malignancy. We found that the Wnt/β-catenin signaling pathway was activated in pancreatic KrasG12D and Rnf43 null mice. RNF43 is one of the negative feedback regulators of the Wnt/β-catenin signaling pathway. Oncogenic KRAS can also promote Wnt/β-catenin signaling, even though the exact mechanism of how they synergistically activate this signaling cascade is yet to be dissected.

PORCN-catalyzed palmitoylation of WNT is critical for WNT secretion into the cytoplasm. The PORCN inhibitor LGK974 selectively blocked the growth of Rnf43 mutant PDAC cell line-derived xenografts. This inhibitor is now in a phase 1 clinical trial (NCT01351103) for patients with Wnt ligand-dependent malignancies. We found that LGK974 reduced β-catenin, abrogated pancreatic IPMN initiation and progression into PDAC, and dramatically prolonged the survival of Rnf43−/−; KrasG12D mice without significant weight loss of the mice. Wnt/β-catenin signaling activation is thus necessary for pancreatic IPMN initiation and development in

PDAC may arise covertly from noninvasive precursor lesions, including PCLs, after long latency. Therefore, opportunities do exist for early detection and intervention of malignant transformation. Since the majority of PCLs are asymptomatic, and thus detected incidentally, and may never progress to advanced neoplasia, they do not need surgery or surveillance. We should thus stratify patients with distinct genetic alterations in their tumors and analyze their clinical features such as disease progression, treatment outcome, and survival. However, there are no reliable measurements to stratify PCLs for malignant potential. Consequently, almost all PCL patients have to undergo frequent surveillance with abdominal imaging and endoscopy which only benefits a small fraction of the patients. Even though pancreatectomy is technical challenge with significant morbidity and mortality, surgical resection is the only widely accepted treatment for IPMN with high-grade dysplasia or invasive cancer. However, more than 60% of resected mucinous PCLs are found to have low or intermediate dysplasia. Consequential overdiagnosis and overtreatment may have dire consequences, with significant risks of mortality and morbidity including exocrine and endocrine pancreatic insufficiencies.
ZHOU et al.  Rnf43−/−; KrasG12D mice. We suggest that a PORCN inhibitor would be effective and safe in the treatment of IPMN with Rnf43−/−; KrasG12D.

In summary, Rnf43 is a tumor suppressor in the prevention of pancreatic precancerous lesions and PDAC development. Loss of RNF43 accelerates oncogenic KRAS-driven IPMN and PDAC in mice. Activation of the Wnt/β-catenin pathway as a result of the synergistic effect of RNF43 deficiency and KRASG12D promotes IPMN initiation and progression into PDAC, which can be abolished by LGK974. Mutated KRAS and RNF43 detected from tumors, cyst fluid, pancreatic juice, and blood cell-free DNA may serve as novel biomarkers of advanced neoplasm in the molecular surveillance and stratification for IPMN patients. Since the presence of KRAS and RNF43 mutations in...
IPMNs may predict future development of advanced neoplasia from PCLs, these PCLs should be closely monitored and treated with surgery and/or targeted therapeutics. Our findings may thus provide a window of opportunity and a rationale to prevent the progression of benign pancreatic tumors to malignant ones through surgery and/or medicine and thereby transform the care of a subset of the patients with PCLs. In addition, the biomimetic human disease model we have constructed should serve as a useful platform for further study of pancreatic tumorigenesis and drug screening.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

HZ, XZ, ZS, and JW conceived and designed the experiment. XZ, ZS, MZ, XQ, SY, LW, and LL performed the experiments and analyzed the data. YJ, WD, FL, and DJ assisted the study. JC analyzed the data. HZ, XZ, ZS and SY drafted and revised the manuscript.
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