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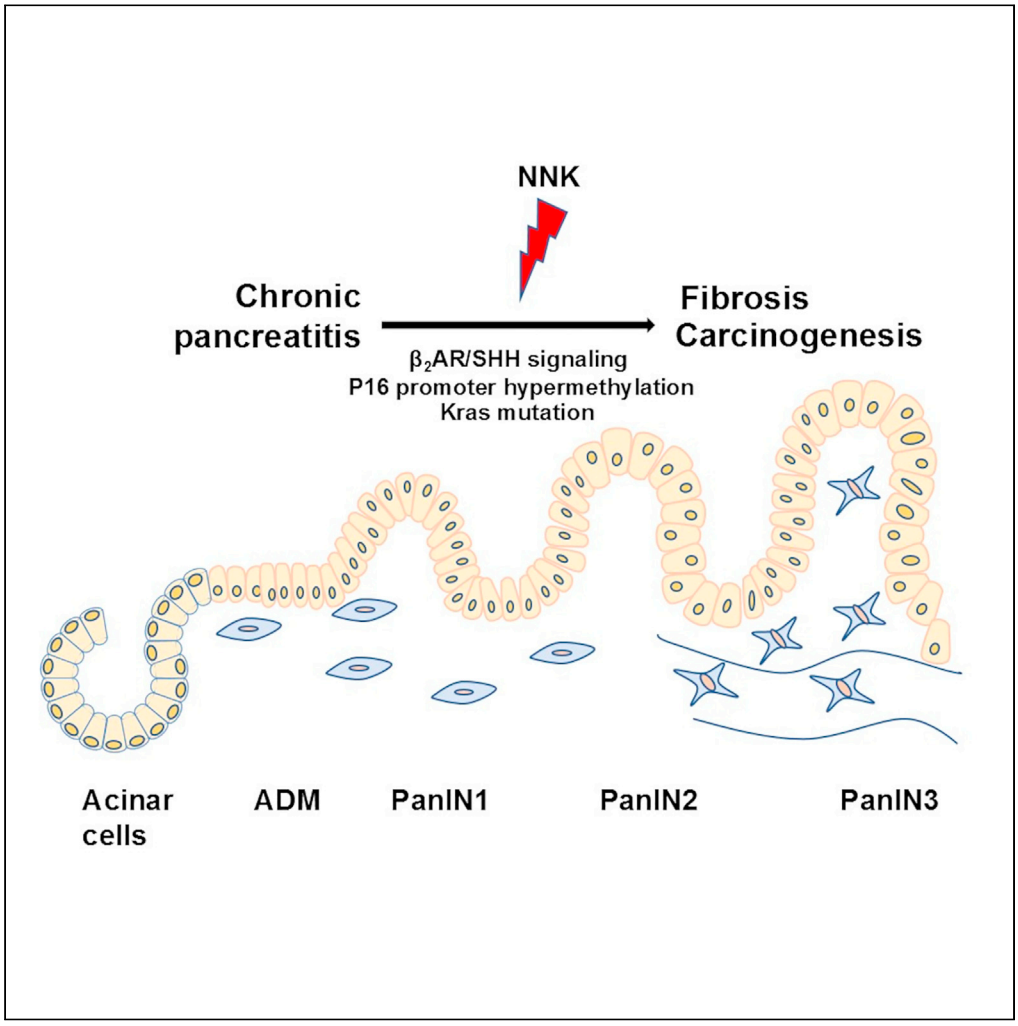

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Highlights

Smoking is positively correlated with *Kras* mutation and P16 hypermethylation

NNK promotes acinar-to-ductal metastasis and preneoplasia lesions in rats

NNK promotes desmoplastic reaction and Gli1 expression in chronic pancreatitis

NNK facilitates the growth and migration of cancer via Sonic Hedgehog signaling

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone provokes progression from chronic pancreatitis to pancreatic intraepithelial neoplasia

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SUMMARY

The risk of pancreatic cancer is higher among people who are cigarette smokers than among non-smokers; however, the action mechanisms of cigarette metabolites are not yet fully understood. In this study, we investigated the effect of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in cigarette smoking on chronic pancreatitis and pancreatic cancer as well as the biological mechanism of NNK causing malignant transformation. We show that smoking may promote Kras mutation and P16 promoter methylation from clinical samples and NNK markedly facilitates the growth and migration of pancreatic cancer cells via the activation of Sonic Hedgehog signaling. We demonstrate that NNK promotes acinar-to-ductal metastasis and pancreatic intraepithelial neoplasia in rats with chronic pancreatitis, accompanied by desmoplastic reaction and Gli1 overexpression. Together, we here present evidence that NNK provokes the progression of chronic pancreatitis toward pancreatic cancer and highlight potential strategies and targets for early prevention of pancreatic cancer and its therapeutics.

INTRODUCTION

Pancreatic cancer is a highly lethal cancer with a 5-year survival rate of less than 10% (Siegel et al., 2021). Most patients are asymptomatic until the tumor has progressed to an advanced stage, at which point there are few effective treatment options. Therefore, there is a critical need to understand the etiology and pathogenesis of pancreatic cancer using different approaches in order to lower its risk and achieve better prevention and treatment of this malignancy.

Accumulating studies support the fact that there is a correlation between cigarette smoking and pancreatic cancer (Yuan et al., 2017). It is estimated that smoking contributes to the causes of around one-third of pancreatic cancer cases (Lowenfels and Maisonneuve, 2004; Qiu et al., 2005). The risk of getting pancreatic cancer is approximately twice as high among people who are cigarette smokers compared with those who have never smoked (ACS, 2020). In addition, smoking is associated with a reduction in survival among patients with pancreatic cancer (Yuan et al., 2017). Tobacco carcinogens can enter the pancreas through blood, duodenal juice, and bile, which may be one of the reasons why the pancreas head is the most common site of pancreatic cancer. In addition, cigarette smoking is associated with chronic pancreatitis and pancreatitis itself is also a risk factor for pancreatic cancer (Ye et al., 2015). Cigarette smoking was first reported to induce pancreatic cancer *in vivo* in 1988 (Rivenson et al., 1988). Using lifelong administration of tobacco-specific nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-3-pyridyl)-1-butanol (NNAL), and areca-derived N-nitrosoguvacoline (NG) in the drinking water for F344 rats, Rivenson et al. found that these compounds induced exocrine pancreatic cancer as well as lung cancer (Rivenson et al., 1988). Recently, cigarette smoking was found to not only accelerate pancreatic cancer progression but also induce pancreatic cancer stemness (Kovi et al., 2018; Nimmakayala et al., 2018).

Cigarette products contain more than 4,000 chemicals, including nicotine, NNK, aldehydes, butadiene, and a large number of free radicals, of which more than 60 are carcinogenic (Edderkaoui and Thrower, 2013). Among multiple tobacco-specific chemicals, NNK is considered as a potent carcinogen and mutagen and is the most carcinogenic (Foiles et al., 1992; Hecht, 1998; Hecht et al., 1983; Keohavong et al., 2011; Kumar et al., 2015; Loprieno, 1975; Momi et al., 2012; Ronai et al., 1993; Secretan et al.,

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2009; Srinivasan et al., 2018). NNK is naturally synthesized in tobacco leaves when exposed to light. It is easily formed when the pyrrolidine ring in nicotine opens (Adams et al., 1983). NNK can be converted from nicotine and metabolized into the nitrosamine NNAL, another potent carcinogen (Zimmerman et al., 2004). A higher level of NNK has been found in the pancreatic juice of cigarette smokers compared with non-smokers (Prokopczyk et al., 2002). NNK has been shown to stimulate cell proliferation in normal human bronchial epithelial cells and small airway epithelial cells via NF-κB signaling (Ho et al., 2005). NNK and nicotine were also shown to stimulate survival of normal lung epithelial cells (West et al., 2003). In the case of pancreas, NNK was shown to mediate β-adrenergic receptor (β-AR) transactivation of the epidermal growth factor receptor (EGFR) and phosphorylation of extracellular-signal-regulated kinase (Erk) 1/2 in immortalized human pancreatic duct epithelial cells (Askari et al., 2005).

Several research groups, including our own, have focused on NNK in cancer development and progression (Kumar et al., 2015; Nimmakayala et al., 2018; Shan et al., 2013; Srinivasan et al., 2018; Zhang et al., 2011, 2016). NNK is known as a high-affinity agonist of β-AR (Schaal and Chellappan, 2014). Our previous work found that β2-adrenergic receptor (β_2-AR) is expressed in the several pancreatic cancer cell lines (BxPC-3, MIA PaCa-2, and Panc-1) and that β_2-AR expression levels are higher in smokers compared with non-smokers and increased along with tumor-node-metastasis (TNM) staging progression (Zhang et al., 2011). Also, we demonstrated that stressors such as smoking and restraints can cause tumor growth via β-AR (Shan et al., 2013; Zhang et al., 2016). Kumar et al. showed that nicotine exposure significantly increased the number of pancreatic intraepithelial neoplasms in a mouse model (Kumar et al., 2015). More recently, Srinivasan et al. revealed that tobacco carcinogens such as NNK might induce the generation of granulocyte-macrophage colony-stimulating factor (GM-CSF), which could consequently activate AKT/CREB signaling and promote pancreatic cancer in a mouse model (Srinivasan et al., 2018). Nimmakayala et al. reported that exposure of pancreatic cancer cell lines to NNK led to increased expression of stemness-related markers via RNA polymerase II-associated factor (PAF1) (Nimmakayala et al., 2018). These research efforts suggest that the effect of NNK in tumorigenesis may be exerted through multiple cellular signaling pathways. However, the involvement of NNK in malignant progress from chronic pancreatitis still remains obscure.

In this study, we employed clinical samples and experimental animals as well as pancreatic cancer cell lines to investigate the effect of smoking on chronic pancreatitis and pancreatic cancer and further dissected the biological mechanism of NNK causing chronic pancreatitis to pancreatic intraepithelial neoplasia (PanIN).

RESULTS

Cigarette smoking correlates with the early onset of pancreatic cancer

Clinical demographic data from 93 patients with pancreatic cancer were summarized in Table 1. Patients were separated into two groups: smokers and non-smokers. Fisher tests were used to assess correlations of cigarette smoking with age, gender, clinical stage, tumor grade, lymph node metastasis, and serum levels of CEA (carcinoembryonic antigen) or CA19-9 (carbohydrate antigen 19-9). As illustrated in Table 2, the age of onset is significantly lower in smokers compared with non-smokers and there is a positive correlation between smoking and age of onset ($p = 0.0003$), suggesting that there may be a correlation between cigarette smoking and pancreatic cancer. The analysis results also show that cigarette smoking is positively correlated with lymph nodes metastasis ($p = 0.032$). No significant correlation was observed between smoking and other factors including gender, clinical stage, tumor grade, and serum levels of CEA or CA19-9 (p values >0.05).

Kras mutation and P16 methylation occur in the examined patient cohort with smoking

It has been known that a constitutive activation of Kras is present in more than 90% of pancreatic tumors and is thought to be the earliest genetic event in pancreatic cancer (Buscail et al., 2020b; Yachida and Iacobuzio-Donahue, 2013). Thus, we analyzed the correlation between Kras mutations on exon 2 (codon 12 and codon 13) and smoking habit in the same patient cohort. Two different sets of primer pairs were used to propagate exon 2 and to determine the Kras mutation status among patients.

Of the 93 patients, 72 (77.42%) had a point mutation in the Kras gene, among which 49 (68.06%) occurred on codon 12 of exon 2 and 23 (31.94%) occurred on codon 13 of exon 2. The representative sequencing results were shown in Figure S1. Among the 72 patients with Kras point mutations, the normal GGT sequence was mutated to GAT in 29 (40.28%) patients. In the remaining 43 of the 72 patients, 8 (11.11%) showed GGT→GTT mutation; 12 (16.67%)

Table 1. The clinical-pathologic characteristics of 93 patients with pancreatic cancer

Character	Cases
Sex	
Male	55
Female	38
Age	
≤55years	48
>55years	45
Position	
Head	40
Body and tail	53
Serum CA19-9	
<39.5ng/mL	47
≥39.5 ng/mL	46
Serum CEA	
<3.4ng/mL	38
≥3.4 ng/mL	55
Clinical stage	
I	26
II	18
III	29
IV	20
Grade	
High	24
Moderate	32
Poor	37
Lymph nodes	
Positive	39
Negative	54

showed GGT→GCT mutation, and 23 (31.94%) showed GGC→GAC mutation. By contrast, only one (14.29%) of the seven chronic pancreatitis tissues harbored a point mutation on codon 12 of the Kras gene (Table 3). By statistical analysis, we found that mutations that occurred in codon 12 did not show a significant correlation with smoking habit ($p > 0.05$); however, mutations that occurred in codon 13 showed a positive correlation with cigarette smoking ($p = 0.001$) in patients with pancreatic cancer (Table 4).

P16 alteration is considered as an early molecular signature in pancreatic tumorigenesis (Nakashima et al., 1999). Loss of an allele and promoter hypermethylation are the most common alterations of P16 (Rozenblum et al., 1997). To determine whether these patients lost a P16 allele, DNA was extracted from the pancreatic cancer tissues and PCR was performed using primers for exon 1 and exon 2 of P16. The result demonstrated that none of the 93 patients with pancreatic cancer harbored an allelic loss of P16; the representative results are shown in Figure 1A. We further detected the hypermethylation of P16 promoter in patient tissues using methylation specific PCR; we found that 52 (55.91%) of 93 patients with pancreatic cancer contained hypermethylation in the P16 promoter (Table S1). In addition, 73.33% (33 of 45) smokers with pancreatic cancer harbored P16 promoter hypermethylation, whereas P16 hypermethylation was only observed in 39.58% (19 of 48) of the non-smokers (Table S1). The percentage of hypermethylation of the P16 promoter was significantly higher in cigarette smokers compared with non-smokers. We next examined P16 alteration in patients with chronic pancreatitis including one smoker and found no allelic loss of P16. Two of seven patients with chronic pancreatitis harbored hypermethylation of the P16 promoter; one was completely hypermethylated, and the other one was partially hypermethylated (Figure 1B).

Table 2. The correlation between smoking and clinical-pathologic characteristics in cancer patient cohort

	Smoking cases	Non-smoking cases	*p* value
Sex			
Male	31	24	0.064
Female	14	24	
Age			
≤55	32	16	0.0003
>55	13	32	
Position			
Head	15	25	0.0682
Body and Tail	30	23	
Clinical stage			
I & II	17	27	0.075
III & IV	28	21	
Head			
Stage I & II	6	14	0.3272
Stage III & IV	9	11	
Body and tail			
Stage I & II	11	13	0.1501
Stage III & IV	19	10	
Grade			
Poor	19	18	0.642
Moderate/Well	26	30	
Lymph nodes			
Positive	24	15	0.031
Negative	21	33	
Serum CA19-9			
<39.5ng/mL	21	26	0.470
≥39.5 ng/mL	24	22	
Serum CEA			
<3.4ng/mL	20	18	0.496
≥3.4 ng/mL	25	30	

Furthermore, the mRNA levels of P16 were found to be decreased in the chronic pancreatitis group compared with the normal pancreas group (Figures 1C and 1D). Taken together, these results suggest that cigarette smoking may promote hypermethylation of the P16 promoter.

β-ARs play a role in the NNK-mediated promotion of cancer cell proliferation

We then evaluated whether NNK affects the proliferation of pancreatic cancer cells. Panc-1 cells were treated with 0, 5, 10, 25, 50, 75, 100, or 200 μM NNK for 24, 48, or 72 h. With the increase of NNK's concentration, the proliferation of Panc-1 cells gradually increased (Figure 2A), indicating that NNK promotes the proliferation of Panc-1 cells and this happens in a dose- and time-dependent manner. Since there was no significant difference between 100 and 200 μM, we selected 100 μM for further experiments.

Next, we tested whether the β-ARs play a role in the NNK-mediated promotion of cancer cell proliferation. Cells were incubated with 5, 10, 25, 50, or 100 μM propranolol, a β-AR blocker, before NNK treatment. The mRNA levels of β2-AR were measured by real-time PCR under different treatment conditions. As shown in Figure S2, NNK increases cellular β2-AR levels and the increased effect was rescued by propranolol treatment. In addition, the ability of NNK to promote cell proliferation was reduced with the increased

Table 3. Kras mutation of 93 patients with pancreatic cancer and 7 patients with chronic pancreatitis

	Site of mutation	Type of mutation	Number of cases	Total
PDAC (Total 93 cases)	Codon12	GGT→GAT	29	72
		GGT→GCT	12	
		GGT→GTT	8	
	Codon13	GGC→GAC	23	
CP (Total 7 cases)	Codon12	GGT→GTT	1	1

concentration of propranolol treatment (Figure 2B). As no significant difference was found between 50 and 100 μM propranolol, we selected 50 μM for further experiments. Moreover, treatment with cyclopamine, a Sonic Hedgehog (SHH) pathway inhibitor, significantly inhibited cell growth induced by NNK (Figures 2B–2D). In our previous research, we identified that 10 μM of cyclopamine is sufficient to inhibit SHH signaling in pancreatic cancer cells even with 2 μg/mL recombinant SHH treatment (Li et al., 2014). Therefore, we selected 10 μM of cyclopamine here to perform our experiments. We also performed cell growth assay under cyclopamine and propranolol co-treatment. We observed that NNK promoted pancreatic cancer cell growth, which could be inhibited by cyclopamine or propranolol and did not see a further reduction when cells were co-treated with cyclopamine and propranolol, indicating that their activities were synergistic (Figure S3). Collectively, these results suggest that NNK may promote the proliferation of pancreatic cancer cells via regulating the β-AR and SHH pathways.

NNK stimulates the migration of pancreatic cancer cells through SHH signaling

Next, we tested the effect of NNK on the migration of BxPC-3 (Kras wild type) and Panc-1 (Kras G12D) cells using Transwell migration assay. Compared with the control group, NNK can significantly increase the migration of pancreatic cancer cells. Intriguingly, we also found that cyclopamine could inhibit the migration of pancreatic cancer cells. To address if NNK promotes pancreatic cancer cell migration via the SHH pathway, we pretreated cells with cyclopamine and the antagonist of β-ARs, propranolol, before NNK treatment. As shown in Figures 2E and 2F, both cyclopamine and propranolol significantly reduced the number of migrated cells even with NNK treatment.

Recent studies revealed that chronic β-AR activation could enhance GRK-2 expression in the heart (Oyama et al., 2005), whereas GRK-2 can activate SMO and its downstream transcriptional factors such as Gli1 (Chen et al., 2004; Meloni et al., 2006). Thus, to address the possibility that NNK promotes cancer cell migration through this pathway we detected the expression level of GRK-2 and several SHH signal related proteins. Western blotting results (Figures 2G and 2H) showed that NNK treatment increased the expression of GRK-2, SMO, and Gli1 without influencing SHH and PTCH. Pretreatment of propranolol inhibited the expression of GRK-2, SMO, and Gli1; meanwhile cyclopamine-pretreated cells exhibited lower expression levels of SMO and Gli1. Taken together, these data suggest that NNK promotes the migration of both pancreatic cancer cell lines through β-AR, which then trans-activates SHH signaling. This also aligns with the statistical data in Table 4 that there is no significant correlation seen between smoking and Kras codon 12 mutation in patients with pancreatic cancer.

Table 4. The correlation between Kras mutation and smoking

	Smoking (n/Total cases)	Non-smoking (n/Total cases)	*p* value
Mutation			
Positive	38/45	34/48	0.117
Codon12 mutation			
Positive	27/45	22/48	0.171
Codon13 mutation			
Positive	18/45	5/48	0.001
G→A mutation			
Positive	24/45	28/48	0.627

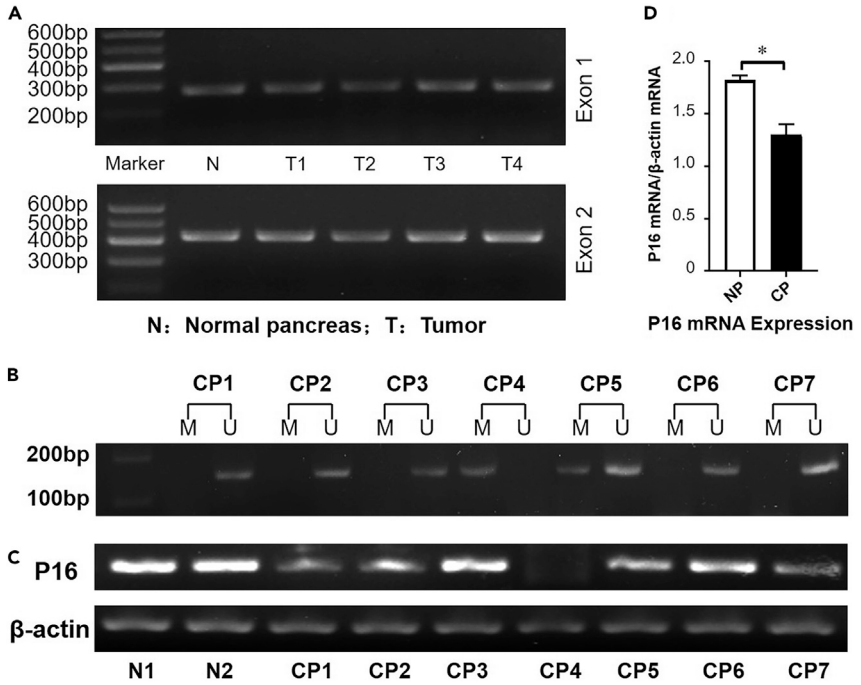

Figure 1. Hypermethylation (A) and expression (B) of P16 in chronic pancreatitis

(A) Representative image of PCR for exon 1 and exon 2 of P16 in indicated pancreatic cancer tissues.

(B) DNA was extracted from chronic pancreatitis (CP) tissues and the methylation (M) of P16 promoter was analyzed. Two (CP4 and CP5) of seven patients with chronic pancreatitis harbored methylation of the P16 promoter; CP4 was completely hypermethylated and CP5 was partially hypermethylated. (U was abbreviation of un-methylation).

(C and D) Real-time PCR was used to assess the mRNA level of P16 in chronic pancreatitis (CP) and normal pancreas (NP) tissues. Results represent the mean ± SD. We found a decreased expression of P16 in chronic pancreatitis tissues. *, $p < 0.05$ by Student's t test.

Dibutyltin dichloride induces chronic pancreatitis *in vivo*

In this study, we built a chronic pancreatitis animal model using dibutyltin dichloride (DBTC) based on a previous report (Sparmann et al., 1997). We divided 70 rats into two groups; group A was treated with 8 mg/kg DBTC and group B treated with control solvent. The body weight of the rats was monitored daily. Compared with the control group, the weight of the rats in the chronic pancreatitis animal model group increased slowly and even slightly declined during the first 2 weeks (Figure 3A). Anatomical evaluation revealed atrophic pancreatic edema. Pathological examination showed pancreatic fibrosis and inflammatory cell infiltration. Two weeks after DBTC treatment, five of six rats were diagnosed with mild chronic pancreatitis, five of six rats were diagnosed with moderate chronic pancreatitis after 4 weeks, four of six rats were diagnosed with slightly severe chronic pancreatitis after 8 weeks, and all six rats were diagnosed with severe chronic pancreatitis after 20 weeks (Figure 3B). An evaluation of pancreatic injury was performed taking structural abnormality, acinar atrophy, fibrosis, and inflammatory cell infiltration into consideration. Compared with the control group, the severity of pancreatic injury in DBTC-treated rats was significantly higher in a time-dependent fashion. Together, these results suggest that DBTC treatment is able to induce chronic pancreatitis in SD rats (Figure 3C).

NNK exacerbates fibrosis and acinar-to-ductal metastasis in chronic pancreatitis

Next, we tested if NNK has any effects on fibrosis and acinar-to-ductal metastasis (ADM) in SD rats. Rats were separated into eight groups, groups A1-A4 and groups B1-B4, and were treated as stated in the STAR Methods section.

Twenty weeks later, 10 of 12 rats treated with additional NNK (group A1) showed severe fibrosis, characterized by collagen deposits around the acinar and interlobular areas, and some areas were replaced by

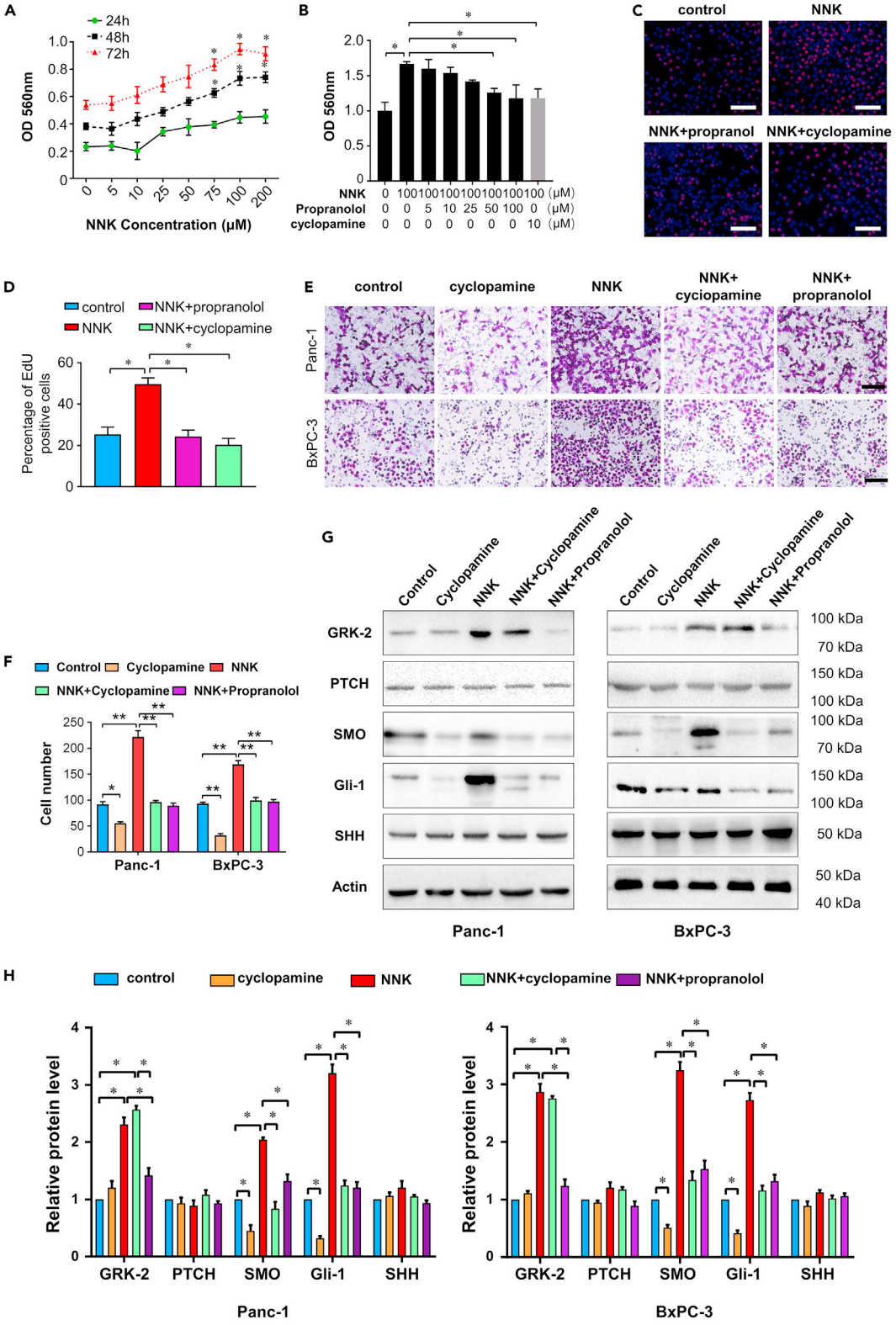
A
24h
48h
72h
OD 560nm
NNK Concentration (μM)
B
OD 560nm
NNK
Propranolol
cyclopamine
C
control
NNK
NNK+propranol
NNK+cyclopamine
D
control
NNK
NNK+propranolol
NNK+cyclopamine
Percentage of EdU positive cells
E
control
cyclopamine
NNK
NNK+ cyciopamine
NNK+ propranolol
Panc-1
BxPC-3
F
Control
Cyclopamine
NNK
NNK+Cyclopamine
NNK+Propranolol
Cell number
Panc-1
BxPC-3
G
Control
Cyclopamine
NNK
NNK+Cyclopamine
NNK+Propranolol
GRK-2
PTCH
SMO
Gli-1
SHH
Actin
100 kDa
70 kDa
150 kDa
100 kDa
100 kDa
70 kDa
150 kDa
100 kDa
50 kDa
50 kDa
40 kDa
Panc-1
BxPC-3
H
control
cyclopamine
NNK
NNK+cyclopamine
NNK+propranolol
Relative protein level
GRK-2
PTCH
SMO
Gli-1
SHH
Panc-1
BxPC-3

Figure 2. NNK promotes the migration of pancreatic cancer cells through Sonic Hedgehog signaling and GRK-2

(A) Panc-1 cells were seeded in 96-well plate, then cells were treated with increased concentrations of NNK (0, 5, 10, 25, 50, 75, 100, 200 μM), cell growth was tested using MTT assay after 24, 48, 72 h.

(B) Panc-1 cells seeded in 96-well plate were treated with NNK, propranolol, and cyclopamine, respectively, at indicated concentration. Cell growth was then measured using MTT assay after 48 h.

(C and D) Cell proliferation was assessed for Panc-1 cells treated with NNK (100 μM), propranolol (50 μM), cyclopamine (10 μM) by EdU staining. DNA (blue) was stained with DAPI. Purple cells show EdU/DAPI-positive cells (scale bar, 100μm). *, $p < 0.05$ by one-way ANOVA test.

(E and F) Cells with different treatment were seeded into a Transwell chamber for 24 h (scale bar, 100 μm). Migrated cells were quantified by counting the number of cells in 10 random fields. *, $p < 0.05$; **, $p < 0.05$ by one-way ANOVA test.

(G–H) Western blot to analyze the protein level in each group. *, $p < 0.05$ by one-way ANOVA test. All results shown by bar graphs are represented as mean ± SD.

collagen completely. To determine whether NNK augments fibrosis during chronic pancreatitis, we detected the fibrotic proportion in the two groups. The percent of the fibrotic area in DBTC-treated rats (group A4) was 27.8 ± 1.77, which increased to 39.6 ± 3.75% when rats received additional NNK treatment (group A1) (Figures 4A and 4B).

ADM is considered as a pre-malignant lesion of pancreatic cancer (Dey et al., 2014; Kumar et al., 2015). Normal acini lose their characteristics and acquire ductal properties, which may result in pancreatic ductal adenocarcinoma. Using H&E sections, we found approximately five ADM per surface area in DBTC-treated rats, which increased significantly to approximately 38 per surface area when rats received an additional NNK treatment (Figures 4C and 4D). We also performed immunohistochemistry (IHC) staining of acinar marker amylase and ductal marker cytokeratin 19 (CK19) (Figure S4) to confirm ADM. Collectively, NNK promotes not only the fibrosis of chronic pancreatitis but also ADM progression.

NNK induces PanINs in rats with chronic pancreatitis

PanINs, characterized by columnar cells with varying amounts of mucin and varying degrees of cytological and architectural atypia, is another pre-neoplasia lesion (LaConti et al., 2015). In this study, we did not find any PanINs lesions in the rats with DBTC-induced chronic pancreatitis. However, six rats showed PanINs pathology when they were treated with NNK and DBTC (Figures 4E and 4F). To testify whether these lesions on pancreas were metastasis from NNK-induced lung malignancy (Kovi et al., 2018), we performed immunohistochemical staining for type II pneumocytes marker SP-C and bronchioles marker CC-10. No reactive staining was found in the pancreatic lesions (Figure S5). Collectively, these data suggest that NNK could promote the primary malignant progression from chronic pancreatitis.

Gene expression of Kras, P16, P53, and Gli1 is affected by NNK in the progression of chronic pancreatitis

Clinical studies have revealed that cigarette smoking may influence the expression of genes such as Kras, P16, and P53 (Curtin et al., 2009). We thus detected if NNK affects the levels of these genes using immunohistochemistry in rats. In normal pancreatic tissue, P16 and P53 were expressed at a high level in both acinar and ductal cells. In contrast, a high level of Kras expression was found in acinar cells but a low level in ductal cells. No differences were observed in the expression levels of these three genes in acinar cells between DBTC-induced chronic pancreatitis concomitant with ADM and normal pancreas. However, when ADM was accompanied by NNK-induced PanINs, P16 expression was significantly lower and Kras expression increased, whereas P53 expression decreased in acinar cells (Figure 5A). Also, we detected the expression of Kras, P16, and P53 in ductal cells and PanINs cells. We found a lower level of P16 and a higher level of Kras in the PanINs cells in comparison with normal ductal cells (Figure 5B).

In addition, in chronic pancreatitis, Gli1 was expressed at a low level in both acinar and ductal cells. Compared with chronic pancreatitis, there was a significantly increased expression of Gli1 in ADM and PanINs. Both the low-grade and high-grade PanINs had a higher level of Gli1 expression compared with ADM. However, no difference was noted for the level of Gli1 between low-grade and high-grade PanINs (Figure 5C).

DISCUSSION

In this study, we broadened the landscape of the evidence supporting that cigarette smoking involves pancreatic cancer. We demonstrate that NNK promotes chronic pancreatitis to PanINs and have elucidated its underlying mechanism using a combination of clinical samples, experimental animals, and cell lines. We show that there is a correlation between cigarette smoking and the major molecular signatures

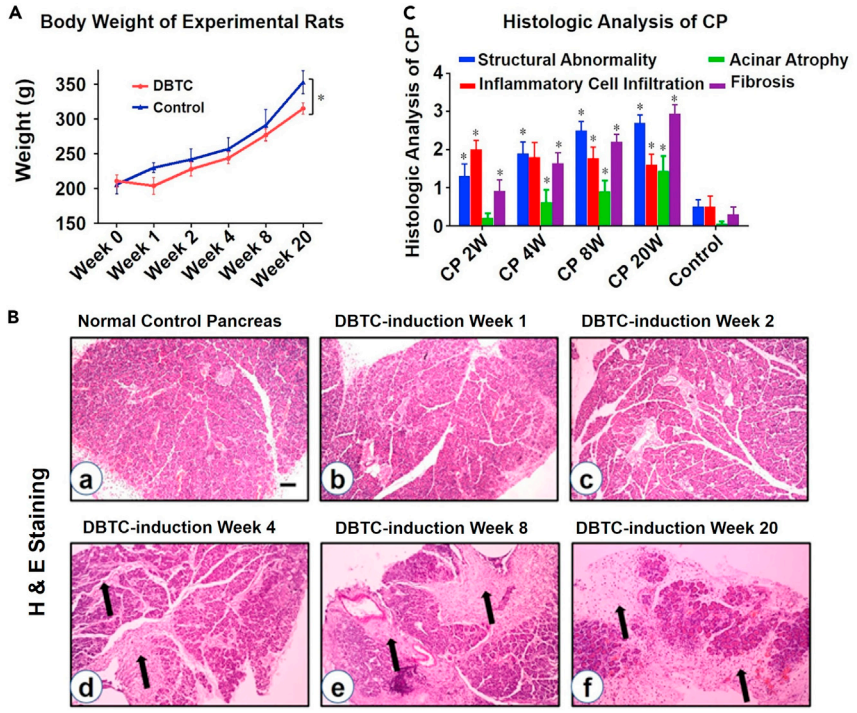

Figure 3. DBTC induces chronic pancreatitis in SD rats

(A) The body weight of rats in the chronic pancreatitis group and control group was measured at indicated time points. Results represent the mean ± SD. *, p <0.05 by Student's t test.

(B) Representative H&E staining of normal control pancreas (a) and pancreas of chronic pancreatitis SD rats at indicated time points, 1 week (b), 2 weeks (c), 4 weeks (d), 8 weeks (e), and 20 weeks (f). The pathological results show pancreatic fibrosis (arrow) and inflammatory cell infiltration (scale bar, 100 μm).

(C) Histologic analysis of chronic pancreatitis of SD rats at indicated time points taking structural abnormality, acinar atrophy, fibrosis, and inflammatory cell infiltration into consideration. All results shown by bar graphs are represented as mean ± SD. *, p <0.05 compared with control group by one-way ANOVA test.

of pancreatic cancer. NNK facilitates the formation of PanINs, the earliest stages of pancreatic cancer, from chronic pancreatitis *in vivo*. Moreover, the nuclear factor Gli1, a key effector in SHH pathway is expressed higher during this process. NNK treatment markedly enhances the expression of GRK-2 and Gli1, which can be reversed by propranolol and cyclopamine treatment, suggesting that NNK promotes the malignant progression of chronic pancreatitis through trans-activating GRK-2 and Gli1. Therefore, our study highlights potential strategies and targets for early pancreatic cancer prevention and therapeutics.

β-ARs and SHH signaling proteins play an important role in pancreatic cancer cells, such as promoting cells growth, angiogenesis, and perineural invasion (Guo et al., 2013; Schaal and Chellappan, 2014; Schuller, 2013; Schuller and Al-Wadei, 2012; Shan et al., 2013; Zhang et al., 2011). Inhibition of the SHH pathway is reported to mitigate the progression of pancreatic cancer (Thayer et al., 2003). As a β-AR agonist, NNK can promote the migration of pancreatic cancer cells through GRK-2-induced activation of SHH signaling. In this study, our data in Figure 2 show that β-AR antagonist, propranolol, abolished the elevated expression of SMO and Gli1 caused by NNK treatment, suggesting that β-AR mediated the transactivation of SHH signaling. From animal study we show that Gli1 is highly expressed in NNK-induced PanINs, indicating a promoting role for the SHH pathway in the carcinogenesis of NNK. Chronic pancreatitis and pancreatic cancer are both characterized by a progressive fibrosis. In this study, we reveal that smoke carcinogen NNK could aggravate the desmoplastic reaction of chronic pancreatitis. As activating stellate cells are the main source of pancreatic fibrosis, NNK has been found to induce proteomic changes in both fibroblasts and cancer cells, probably through activating the nicotinic acetylcholine receptors or inducing oxidative stress (Chaudhary et al., 2013; Prins and Wang, 2012). Whether the desmoplastic reaction in our study is attributed to the activation of the SHH pathway by NNK treatment needs further investigation.

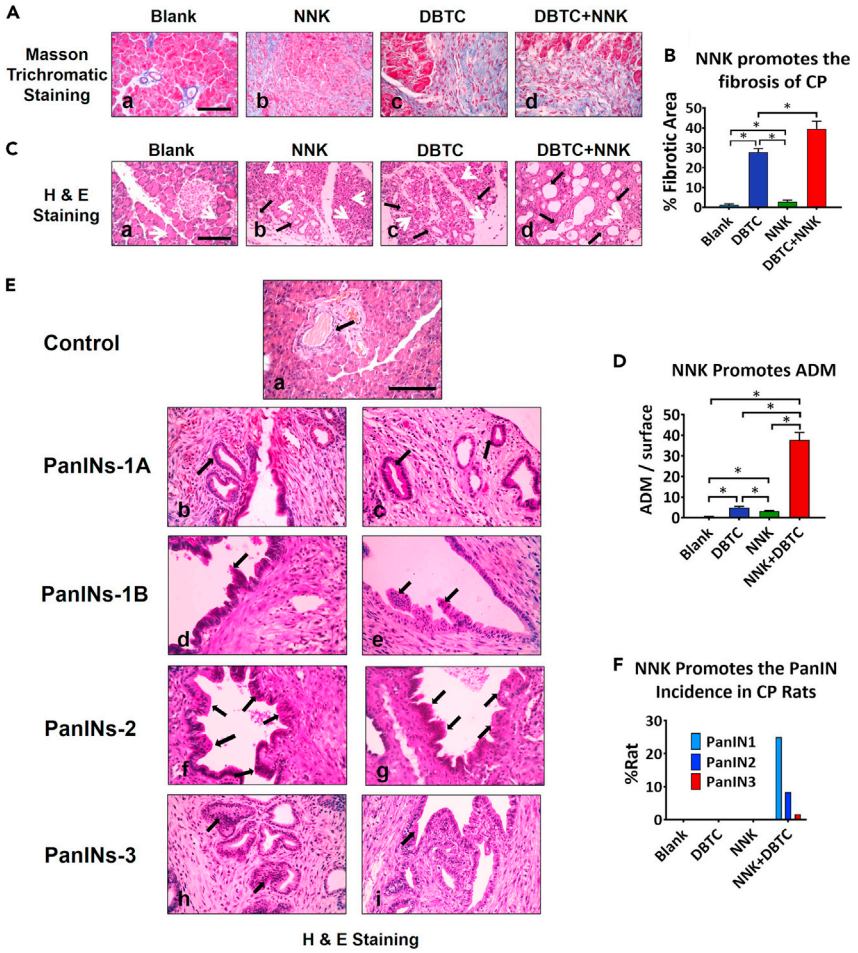

Figure 4. Promotion of the fibrosis, ADM, and PanINs in chronic pancreatitis by NNK

(A) Representative Masson trichromatic staining to detect the fibrosis of pancreas in different groups: blank group (untreated control group) (a), NNK group (b), DBTC group (c), and DBTC+NNK group (d) (scale bar, 100 μm).

(B) The histogram shows the percent of fibrotic area in each group *, $p < 0.05$ by one-way ANOVA test.

(C) H&E staining shows the promotion of ADM process in NNK additional treatment SD rats. Rats were divided into four groups, blank group (a), NNK group (b), DBTC group (c), and DBTC+NNK group (d). Normal acinus (white arrow) loses its characteristic and acquires a ductal property (black arrow) (scale bar, 100 μm).

(D) The histogram shows the number of ADM per visual field in each group. *, $p < 0.05$ by one-way ANOVA test.

(E) H&E staining shows PanINs formation in NNK+DBTC treatment SD rats: normal pancreas(a), PanINs-1A (b, c), PanINs-1B (d and e), PanINs-2 (f and g), PanINs-3 (h and i); the precancerous lesion is characterized by columnar cells with varying amounts of mucin and varying degrees of cytological and architectural atypia (arrow) (scale bar, 100 μm). Three rats had PanIN 1 lesion, one rat had PanIN 2 lesion, and two rats had PanIN 3 lesion.

(F) The histogram shows the percent of rats that harbors precancerous lesions in each group. All results shown by bar graphs are represented as mean ± SD.

In human pancreas, about 13 ± 1 DNA adducts/10^8 nucleotides were found in non-smokers, whereas it increased to about 102 ± 21 in smokers (Wang et al., 1998), which may induce gene mutation and then result in pre-neoplastic lesions. In patients with lung cancer, smokers harbor as many as 49 mutations, whereas only five mutations are found in non-smokers (Ding et al., 2008). Our clinical study of patients with pancreatic cancer also reveals a positive correlation relationship between smoking and Kras mutations. Kras mutations, especially codon 12 mutations, are widely seen in patients with pancreatic cancer. In patients with chronic pancreatitis, the frequency is lower than that in patients with pancreatic cancer. By using samples from chronic pancreatitis with ductal hyperplasia, a study found codon 12 mutations in 18% samples but not codon 13 mutations. The frequency from the literature is comparable with our present study (Rivera et al., 1997). The codon 12 GGT→GTT mutation of Kras gene, which is also known as Kras

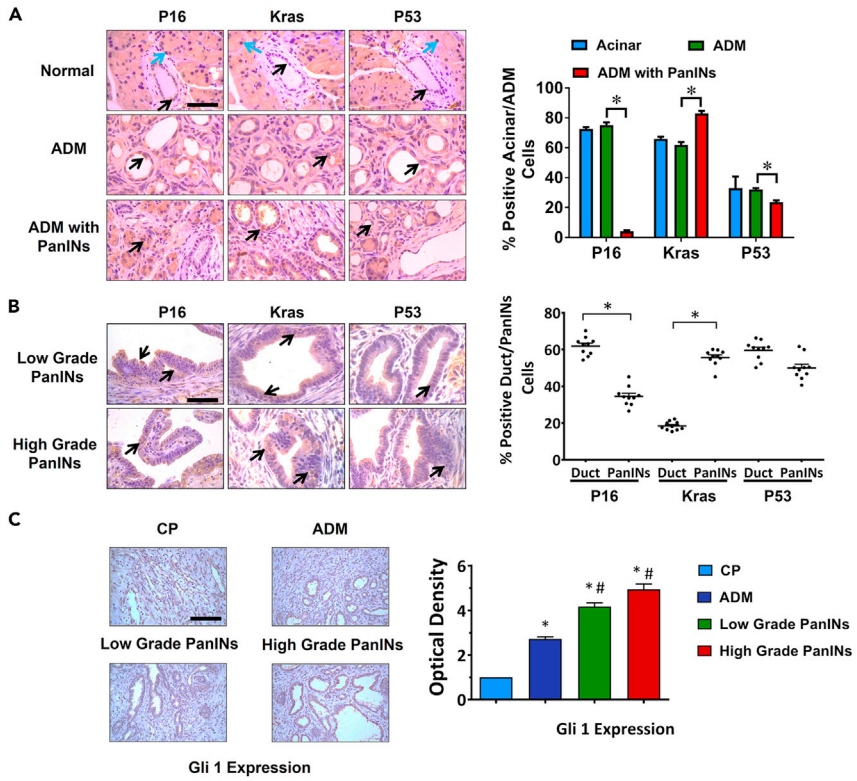

Figure 5. Expression of Kras, P16, P53, and Gli1 in chronic pancreatitis and PanINs

(A) IHC staining for P16, Kras, and P53 in normal pancreas and pancreas harboring ADM or ADM with PanINs (scale bar, 25 μm). Blue and black arrows show the positive staining normal acinar cells and normal ductal cells, respectively. The right histogram shows the percentage of tissues cores displaying P16, Kras, and P53 staining in normal acinar cells, ADM lesion, or ADM with PanINs lesions. *, $p < 0.05$ by one-way ANOVA test.

(B) IHC staining for P16, Kras, and P53 in pancreas harboring low-grade or high-grade PanINs. The arrow shows the positive staining cells (scale bar, 25 μm). The right histogram shows the percentage of tissues cores displaying P16, Kras, and P53 staining in duct cells or PanINs lesions. *, $p < 0.05$ by one-way ANOVA test.

(C) IHC staining for Gli1 in pancreas from chronic pancreatitis to high-grade PanINs. The image shows chronic pancreatitis, ADM lesion, low-grade PanINs, and high-grade PanINs (scale bar, 100 μm). Gli1 expression is increased in precancerous lesions compared with chronic pancreatitis. Both of the low-grade PanINs and high-grade PanINs have higher expression levels of Gli1 compared with ADM. Blank group means the group receiving control vehicle treatment. *, $p < 0.05$ compared with CP group by one-way ANOVA test; #, $p < 0.05$ compared with ADM with PanINs group by one-way ANOVA test. All results shown by bar graphs are represented as mean ± SD.

G12V, was observed in one patient with chronic pancreatitis and seven patients with pancreatic cancer from our clinical study. A study showed that Kras G12V in the HPV16-E6E7 immortalized human pancreatic duct epithelial cells successfully activated its downstream targets, such as AMPL, AKT, and NF-κB, further leading to tumorigenic transformation *in vivo* (Qian et al., 2005), whereas in mice with Kras G12V mutant expression, carcinogenesis only occurs upon induction of chronic or acute pancreatitis (Guerra and Barbacid, 2013). However, to our knowledge and from our present study, this point mutation does not show a positive correlation in smoking-related pancreatic cancer or chronic pancreatitis.

A study on smoker groups with lung cancer shows a higher frequency of codon 12 mutations than codon 13 mutations (Riely et al., 2008); our study shows a positive correlation of codon 13 mutation with smoking-related pancreatic cancer. Kras codon 13 mutations were reported to have a frequency of ~7% in pancreatic ductal adenocarcinoma cases (Buscail et al., 2020b), including the one (GGC→GAC) shown in this study. Besides our present work, Kras codon 13 mutations were also observed in some other tumor types, such as gastric cancer, lung cancer, colorectal cancer, ovarian cancer, prostate cancer, and pancreatobiliary tumor (Ayatollahi et al., 2018; Egeli et al., 2016; Silan et al., 2012). A meta-analysis of eight research papers suggests that codon 13 mutations of Kras gene seem to correlate with a worse overall survival of patients

with colorectal cancer, but the overall survival is similar to those with Kras wild type in patients receiving anti-EGFR therapy (Kwak et al., 2017). Kras codon 13 mutations were also significantly associated with high plasma cholesterol in colorectal cancer cases (Kwak et al., 2018). In addition, Kras codon 13 mutations, but not codon 12 mutations, were associated with a higher risk for overall extrahepatic recurrence and a lung-specific recurrence in patients after surgery for colorectal liver metastasis (Margonis et al., 2016). These pieces of research suggest that Kras codon 13 mutations may be a potential target for these cancers. Whether the point mutation on Kras codon 13 we report here plays significant roles in the process of smoking-induced pancreatic cancer or not surely justifies further mechanistic studies.

Even though Kras mutation is a key genetic event in pancreatic cancer (Buscail et al., 2020a), Kras mutation alone fails to transform normal cells into malignant cancer (Singh and Ellenrieder, 2013). Cell senescence is an obstacle that must be stepped over in the neoplastic transformation of pancreas, whereas P16 and P53 play a critical role in cell senescence (Singh and Ellenrieder, 2013). In the animal experiments from this study, NNK treatment not only induced the alteration of Kras but also resulted in expression changes of P16 and P53. We found an increased proportion of Kras-positive ductal cells and a decreased number of P53 and P16-positive ductal cells in NNK-induced PanINs lesions, a similar molecular transformation to human pancreatic cancer.

In our previous reports, we demonstrated that β_2-AR-HIF-1α signaling is a regulatory axis for stress-induced pancreatic tumor growth and angiogenesis and β_2-adrenogenic signaling regulates NNK-induced pancreatic cancer progression via upregulation of HIF-1α (Shan et al., 2013; Zhang et al., 2016). In this study, we further show that NNK promotes the proliferation of pancreatic cancer cells through β-ARs. A study showed previously that adult knockout mice (β_2-AR −/−) appear grossly normal and are fertile. The primary physiologic consequences of the β_2-AR gene disruption are observed only during the stress of exercise (Chruscinski et al., 1999). Therefore, from our work and several related reports from other groups (Momi et al., 2012; Srinivasan et al., 2018), we consider that NNK promotes pancreatic cancer via multiple mechanisms. Cigarette smoking could also be regarded as a stressor leading to pancreatic cancer. HIF-1α has been reported to be a regulator for carcinogenesis, and the SHH pathway has been shown to be a modulating pathway for the HIF-1α (Chen et al., 2015; Lei et al., 2013; Onishi et al., 2013). Further studies on the cross talk of the HIF-1α pathway and SHH pathway would be warranted.

In summary, we established a new animal model to evaluate the effects of NNK on progression from chronic pancreatitis to pancreatic intraepithelial neoplasia. We found that there is a positive correlation between cigarette smoking and gene alteration, such as mutation on codon 13 of Kras exon 2, hypermethylation of P16 promoter. As a potent carcinogen, NNK, on the one hand, has an ability to form DNA adducts attributing to its genotoxicity, which may result in gene mutations (Peterson, 2017). On the other hand, by acting as an agonist for both β-ARs and nicotinic acetylcholine receptors (nAChRs) (Schaal and Chellappan, 2014), it can activate the SHH pathway through GRK-2 in the progression of pancreatic lesions. Thus, our results show that "Mutual cooperation" between these two actions may contribute to the malignant progression of chronic pancreatitis and highlight potential targets for early pancreatic cancer detection and therapeutics.

Limitation of the study

This study included a total of 93 patients with pancreatic cancer to study the correlation of smoking and pancreatic cancer, and 7 patients with chronic pancreatitis were included. A study of Kras mutation frequency in a large cohort of patients with chronic pancreatitis and their smoking record may further validate the evidence of the correlation between smoking and malignant transformation from chronic pancreatitis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS

- Cells and culture conditions
- Patients and specimens
- Animal models
- METHOD DETAILS
  - *Kras* mutation analysis
  - Detection of P16 promoter methylation
  - MTT assay
  - Transwell assay
  - Animal treatments
  - Immunohistochemistry (IHC)
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

X.C., L.S., J.M., and D.Q. performed all the experiments, analyzed the data, and drafted the manuscript. X.L., Z.W., and Z.W. contributed to patient recruitment and data analysis. J.H.H. and L.W. contributed to data analysis and manuscript editing. Q.M., E.W., and D.Z. conceived and supervised the project, provided the resources, and contributed to manuscript editing. All authors have given approval to the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GRK-2	Proteintech	Cat# 13990-1-AP; RRID:AB_2225833
shh	Proteintech	Cat# 20697-1-AP; RRID:AB_10694828
PTCH	Proteintech	Cat# 55091-1-AP; RRID:AB_10858392
SMO	Proteintech	Cat# 66851-1-Ig; RRID:AB_2882191
β-actin	Proteintech	Cat# 66009-1-Ig; RRID:AB_2687938
Kras	Proteintech	Cat# 12063-1-AP; RRID:AB_878040
Gli1	Bioss	Cat# BS-1206R; RRID:AB_10856462
P53	Abcam	Cat# ab32389; RRID:AB_776981
P16	Abcam	Cat# ab186932; RRID:AB_2895712
Biological samples		
93 pancreatic cancer tumor specimens, 2 normal pancreas tissue specimens, and 7 chronic pancreatitis tissue specimens	Department of Hepatobiliary Surgery, the First Affiliated Hospital of Medical College, Xi'an Jiaotong University	n/a
Experimental models: Cell lines		
Human cell line: Panc-1	The Cell Bank of Type Culture Collection of Chinese Academy of Sciences	SCSP- 535
Human cell line: BxPC-3	The Cell Bank of Type Culture Collection of Chinese Academy of Sciences	TCHu012
Experimental models: Organisms/strains		
Rat: Sprague Dawley (SD) rat	Animal Experiment Center of Xi'an Jiaotong University	SD rat
Chemical, peptides, and recombinant proteins		
NNK	TRC	M325751
Cyclopamine	Sigma Aldrich	239806
propranolol	Sigma Aldrich	P0884
Software and algorithms		
SPSS	IBM	Version 13.0

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Qingyong Ma (qyma56@xjtu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This study did not generate new sequencing data.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells and culture conditions

Human pancreatic cancer cell lines Panc-1 and BxPC-3 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CBTCCCAS, Shanghai, China). Panc-1 is a pancreatic cancer cell line derived from a 56-year-old male's adenocarcinoma of the head of the pancreas with Kras codon 12 GGT→GAT mutation and a homozygous deletion of p16 (Deer et al., 2010; Gradiz et al., 2016). BxPC-3 is a pancreatic cancer cell line cultured from a 61-year-old woman's adenocarcinoma of the body of the pancreas with wild type Kras and a homozygous deletion of p16 (Caldas et al., 1994; Deer et al., 2010). Panc-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) and BxPC-3 cells were cultured in RPMI-1640 medium (HyClone, Logan, USA). The media for both cell lines were supplemented with 10% fetal bovine serum (FBS), 100 μg/mL ampicillin and 100 μg/mL streptomycin. NNK was purchased from Toronto Research Chemicals (TRC, Canada) and was dissolved in dimethyl sulfoxide (DMSO) at the stock concentration of 10 mM. Antibodies detecting G Protein-Coupled Receptor Kinase 2 (GRK-2), the sonic hedgehog protein (SHH), the patched protein (PTCH), the smoothened protein (SMO) and β-actin were purchased from Proteintech (Wuhan, China). Glioma-associated oncogene homolog 1 (Gli1) antibody was purchased from Bioss (Beijing, China). Cyclopamine and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

Patients and specimens

One hundred and two pancreatic tissues, including 93 pancreatic ductal adenocarcinoma samples (N(male)/N(female) = 55/38), seven chronic pancreatitis samples (N(male)/N(female) = 4/3), and two normal pancreas tissues (both male), resected from surgical operations were collected by the Department of Hepatobiliary Surgery, the First Affiliated Hospital of Medical College, Xi'an Jiaotong University. The clinical demographic data is shown in Table 1. Patients who had undergone chemotherapy or radiation therapy before the operation were excluded. Diagnosis was confirmed by pathological examination of tissue sections. The TNM status was assessed using the TNM staging system of the Union for International Cancer Control (2002). Samples were stored in liquid nitrogen immediately after collection and total DNA was extracted later. Ethical approval of our experiment was granted by the Internal Research Board of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, China. Written informed consents were obtained from the patients participating in the study.

Animal models

Eight-week old female Sprague Dawley (SD) rats were purchased and housed in a pathogen-free Animal Experiment Center at the Medical College, Xi'an Jiaotong University. Rats were treated either by dibutyltin dichloride (DBTC) or control solvent (40% ethanol and 60% glycerine). Animal experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animal operation protocol was approved by the Animal Care and Experiments Committee of the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, China.

METHOD DETAILS

Kras mutation analysis

Kras mutations were detected by Sanger sequencing. Two different pairs of primers were designed to amplify exon 2 of Kras; primer pair 1: forward 5′-ATTATAAGGCCTGCTG-AAAATGACT-3′ and reverse 5′-ATGATTCTGAATTAGCTGTATCGTC-3′, and primer pair 2: forward 5′-GTTTGTATTAAAAGGTACTGG TGGA-3′ and reverse 5′-TGCCCGATGACATACCCCA-3′. The predicted lengths of the PCR products were 100 bp using primer pair 1 and 422 bp using primer pair 2, respectively. The primer sequences used to amplify exon 3 of Kras were forward 5′-TCCAGACTGTGTTTCTCCCT-3′ and reverse 5′-TGCATGG CATTAGCAAAGACTC-3′, and the predicted length of the product was 287 bp. PCR products were sequenced using the Sanger sequencing method with radioactively or fluorescently labeled primers purchased from the Ding Guo company (Beijing, China). The results were analyzed using the BioEdit Sequence Alignment Editor Software.

Detection of P16 promoter methylation

P16 promoter methylation was assessed by methylation-specific PCR. DNA was extracted from the samples and then bisulfite modified using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Two

different pairs of primers were designed to amplify DNA fragments; one pair was specific for methylated DNA and the other pair was specific for unmethylated DNA. The primer sequences used for methylated DNA were as follows: forward 5′-TTATTAGAGGGTGGGGCGGATCGC-3′ and reverse 5′-TTATTAGAGGGTGGGGTGGATTGT-3′. The predicted length of the PCR product for the methylated DNA sequence was 150 bp. The primer sequences used for unmethylated DNA were as follows: forward 5′-TTATTAGAGGGTGGGGTGGATTGT-3′ and reverse 5′-CAACCCCAAACCACAACCATAA-3′. The predicted length of the PCR product for the unmethylated DNA sequence was 151 bp. Products were run on a 2% agarose gel and stained with ethidium bromide. The results were visualized under UV illumination.

MTT assay

Panc-1 and BxPC-3 cells were seeded into a 96-well plate at a density of 5000 cells/well with a culture medium containing 10% FBS. After 24 h, cells were retreated with a culture medium containing 1% FBS and drugs at different concentrations. In this experiment, cells were treated with 0, 5, 10, 25, 50, 75, 100, or 200 μM NNK and the combination of 100 μM NNK with 5, 10, 25, 50, or 100 μM propranolol. At various time points (24, 48 and 72 h), cells were further treated with MTT and incubated for another 4 h. Cells were treated with DMSO for 10 minutes before the absorbance was measured at 490 nm using a multi-well microplate reader (BIO-TEC Inc., VA).

Transwell assay

Pancreatic cancer cells were divided into five groups: control group, cyclopamine treatment group, NNK treatment group, NNK plus cyclopamine treatment group, and NNK plus propranolol treatment group. After 36 h treatment, cells were collected and resuspended in culture FBS-free medium. Then 5×10^4 cells were seeded in the upper chamber of the transwell chambers (pore size, 8.0 μm; Millipore, Billerica, USA). 500 μL culture medium containing 10% FBS were added to the lower chamber. 24 h later, migrated cells on the bottom side of the chamber were washed with PBS and then fixed with 4% paraformaldehyde. Cells were stained with crystal violet staining solution (Boster Biological Technology Ltd., Wuhan, China). Cell migratory ability was determined by counting the numbers of stained cells in ten randomly selected fields.

Animal treatments

Rats were randomly divided into two groups, group A (40 rats, chronic pancreatitis group) and group B (30 rats, control group). Rats in group A were tail-vein injected with 8 mg/kg DBTC to induce chronic pancreatitis. Two weeks later after rats were treated either with DBTC or the control vehicle (100% ethanol + glycerol (2:3)), chronic pancreatitis was assessed in a random selection of six rats from each group. From the third week of the experiment, rats within each group (A and B) were subdivided into four groups, groups A1-A4 and B1-B4. Group A1 has 12 rats and other groups have six rats each. Rats in group A1 and B1 were given 100 mg/kg NNK via intraperitoneal injection and were sacrificed at the end of the experiment (week 20). The total dosage was divided into 20 portions, which were given once every other day. Rats in groups A2 and B2, A3 and B3, A4 and B4 were given saline as a control and were sacrificed at weeks 4, 8, and 20, respectively. Tissue samples from the rats were collected and fixed with 4% paraformaldehyde and then embedded in paraffin for further research.

Immunohistochemistry (IHC)

Immunohistochemical staining for Kras (Proteintech), P16 (Abcam), P53 (Abcam) and Gli1 was performed using an SABC kit (Maxim, Fuzhou, China) according to the manufacturer's protocol. Briefly, 5 μm sections were incubated with primary antibodies for Kras (1:100), P16 (1:100), P53 (1:100) and Gli1 (1:50) overnight at 4°C in a hybridization chamber. Then, sections were incubated with anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. After rinsing, sections were visualized with 3, 3-diaminobenzidine (DAB) for 5 minutes and counterstained with hematoxylin for 1 minute.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS13.0 software. The results are presented as the means ± standard deviations (SD) of triplicate assays. Differences between the groups were assessed by Student's t-test, one-way analysis of variance (ANOVA), Fisher test and Chi square test. *P*-values less than 0.05 were considered to be statistically significant.