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The biological features of PanIN initiated from oncogenic *Kras* mutation in genetically engineered mouse models ☆

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ABSTRACT

Pancreatic intraepithelial neoplasia (PanIN) is the most common premalignant lesion of the pancreas. Further understanding of the biological behavior and molecular genetic alterations in the stepwise progression of PanINs is necessary toward the development of pancreatic ductal adenocarcinoma (PDAC) interventions. In this study, we analyzed the morphological characteristics, molecular alterations, and biological behavior of pancreatic wild-type and neoplasia tissues, including analysis of PanIN cell line SH-PAN (isolated from *Pdx-1-Cre*; *LSL-Kras$^{G12D/+}$* mouse) and PDAC cell line DT-PCa (isolated from *Pdx1-Cre*; *LSL-Kras$^{G12D/+}$*; *LSL-Tp53$^{R172H/+}$* mouse). Results show that *Kras^{G12D}* induces ductal lesion PanINs. Increased expression of *EGFR*, *Her-2/Neu*, *p-MAPK and β-Catenin* was observed in low-grade PanINs. *Tp53* was not expressed in wild-type and low-grade PanINs, however, increased expression was observed in high-grade PanINs. Furthermore, SH-PAN cells did not exhibit any colony formation and showed significantly lower migration and invasion ability compared with DT-PCa cells. Notably, we first found *PPP2R2A* (protein phosphatase 2, regulatory subunit B, alpha) expression was significantly higher in SH-PAN cells than DT-PCa cells, and was high in 96 of 172 peritumoral normal human pancreatic tissues and 20 of 36 human low- or middle-grade PanIN tissues, whereas, was weak or negligible in 12 of 20 human high-grade PanIN tissues and 124 of 172 human PDAC tissues post-operation. The expression of *PPP2R2A* appears to be correlated with clinical survival. Taken together, *Kras^{G12D}* – driven PanIN showed the tumorigenic ability, however, did not undergo a malignant transformation, and decreased expression of *PPP2R2A* in PDACs may provided a new target for pancreatic carcinoma intervention.

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1. Background

Pancreatic cancer is the fourth leading cause of cancer death in the world. With an overall 5-year survival rate of less than 5%, pancreatic cancer has one of the poorest prognoses among all cancers [1]. Therefore, it is of paramount importance to better understand its precursor lesions and the pathological mechanisms regulating the genetic progression from normal cells to pancreatic ductal adenocarcinomas (PDAC).

Pancreatic intraepithelial neoplasia (PanIN) is the most common pancreatic precursor lesion. Activating *Kras* mutations are almost uniformly present in the early stages of PanIN, whereas subsequent inactivating mutations in *p16*, *p53*, and *Smad4* occur in advanced lesions [2–8]. However, because it is difficult to isolate and establish PanIN cell lines from the pancreatic tissue of pancreatic cancer patients, the previous studies of PanIN were mainly conducted using a hybrid of PanIN and pancreatic cancer tissues.

The development of genetically engineered mouse models with pancreatic cancer [9,10], specifically the progress in PanIN and PDAC mouse models [11–13], has significantly contributed to our understanding of the genetics of pancreatic neoplasia [14,15]. Previous studies have isolated cell lines from the pancreas of genetically engineered mutant mice with PanIN and PDAC [11,12]. In this study, mouse pancreatic neoplasia tissues and PanIN cell line isolated from the mutant mouse were employed to study the biological features and molecular genetic alterations of PanIN.

Abbreviations: PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; *PPP2R2A*, protein phosphatase 2, regulatory subunit B, alpha.

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2. Materials and methods

2.1. Patient and tissue samples

PDAC and peritumoral normal tissue samples were obtained from 86 patients at Ruijin Hospital in Shanghai. None of the patients had previously received radiotherapy or chemotherapy. After surgery, each tissue sample was fixed in formalin and embedded in paraffin. Histological diagnoses were performed by two independent senior pathologists in the Department of Pathology at Ruijin Hospital.

2.2. Mouse strains and cell lines

Genetically engineered mouse models were generated as previously described [11,12]. Briefly, *LSL-Kras^{G12D}* mice, which are functionally heterozygous for the wild-type allele (*Kras$^{+/-}$*), were crossed with *Pdx-1-Cre* mice to produce *Kras^{G12D}* (*Pdx1-Cre*;
LSL-Kras$^{G12D/+}$) mice that develop ductal lesions identical to the stages of PanIN. The classification of PanIN stage was determined by the age of mice and tissue HE staining results. *LSL-Kras^{G12D}* and *LSL-Tp53^{R172H}* mice were crossed with *Pdx-1-Cre* transgenic mice to develop *Kras^{G12D}* and *Tp53^{R172H}* (*Pdx1-Cre*; *LSL-Kras$^{G12D/+}$*; *LSL-Tp53$^{R172H/+}$*) mice that developed invasive and metastatic pancreatic ductal adenocarcinomas. The PanIN cell line (SH-PAN) was isolated from a *Pdx1-Cre*; *LSL-Kras$^{G12D/+}$* mutant mouse, and PDAC cell lines (DT-PCa) were isolated from the *Pdx1-Cre*;
LSL-Kras$^{G12D/+}$; *LSL-Tp53$^{R172H/+}$* compound mutant mouse as previously reported [11,12]. Early passage SH-PAN and DT-PCa cells were used in all experiments and were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and streptomycin.

2.3. Cell proliferation, soft agar, invasion and migration assays

Detailed procedures were followed as previously described [16]. Briefly, MTT was used to assess cell proliferation, and a spectrophotometer measured the absorbance at 490 nm.

For the soft agar assay, each well of six-well plate contained a bottom layer of 1.2% agarose, a middle layer of 0.64% agarose that included 5000 cells, and a top layer of medium. The medium in the top layer was changed every six days. After 28 days, colonies were counted by using Quantity One analysis software (BioRad Inc., Hercules, CA, USA) .

An invasion assay was performed with a Millicell invasion chamber (Millipore, Billerica, MA, USA). The 8 μm pore inserts were coated with 15 μg of Matrigel (Becton Dickinson Labware, Bedford, MA, USA), and 1×10^5 cells were seeded in the top chamber. The Matrigel invasion chamber was incubated for 20 h in a humidified tissue culture incubator. Noninvasive cells were removed from the top of the Matrigel with a cotton-tipped swab, and invading cells on the bottom surface of filter were fixed in methanol and stained with crystal violet solution. Invasion ability was determined by counting the stained cells.

To assess cell migration, experimental procedures of a general cell invasion assay were followed, with the exception that the porous polycarbonate membrane was not Matrigel. Cell migration ability was determined by counting the stained cells.

2.4. Mutation analysis of Kras, Tp53, p16 Ink4A, Smad4 and Cdk4

The main genetic mutations related to the progression of pancreatic carcinoma in SH-PAN and DT-PCa cell lines were analyzed. Total RNA was extracted from wild-type murine pancreatic and SH-PAN cell lines using the RNeasy Mini Kit (Qiagen), and cDNA was prepared using the First-Strand cDNA Synthesis Kit (Amersham Biosciences) according to manufacturers' recommendations. PCR using Ampli Taq polymerase (Applied Biosystems, Roche) mixed with PfuUltra HF DNA polymerase (Stratagene, Cedar Creek, TX) was performed to amplify the *K-ras*, *Tp53*, *p16Ink4A*, *Smad4* and *Cdk4* genes. Following electrophoresis, PCR products were excised from 1% agarose gel and purified using the QIAquick gel extraction kit (Qiagen Science, Maryland, USA). Purified PCR fragments were digested with EcoRI/XhoI for *DPC4*/*Smad4*, EcoRI/BamHI for *K-ras*, *Tp53* and *Cdk4* or PstI/BamHI for *p16Ink4A*, and were subcloned into pBluescript II KS+ cloning vectors. After transformation into DH10B cells by electroporation, the transformants were selected using LB plates with ampicillin, X-gal and IPTG. A total of 8 white colonies for each cDNA were selected and amplified. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen Science, Maryland, USA). Sequencing for each colony was performed at a DNA sequencing facility using standard universal T3 and T7 primers for *K-ras*, *Tp53*, *p16Ink4A*, *Cdk4* and *Smad4*, additional primers for *Smad4*. To exclude the pseudomutations arising from PCR, only mutation that were found in two or more out of the eight clones were recognized as valid mutations. PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min for a total of 30 cycles. The PCR primers used in this study are as follows:

Kras:

5′-CGCGGAATTCGGCCTGCTGAAAATGACTGAG-3′
5′-GACAGGATCCTCACATAACTGTACACCTTGTCC-3′

Tp53:

5′-GTCTGAATTCACTGGATGACTGCCATGGAGG-3′
5′-CGTTGGATCCAGGCAGTCAGTCTGAGTCAGG-3′

p16INK4A:

5′-CATACTGCAGATGGAGTCCGCTGCAGACAGACTGG-3′
5′-CTATGGATCCTTAGCTCTGCTCTTGGGATTGGC-3′

Cdk4:

5′-GGCTAGAATTCATGGCTGCCACTCGATATGAACC-3′
5′-GCTTGGATCCTCACTCTGCGTCGCTTTCCTCC-3′
5′-CAATCTCGAGCGCGGAGCCCAGGTCATCCTGC-3′
5′-GCGTGAATTCTCAGTCTAAAGGCTGTGGGTCCG-3′

T3 and T7 primers for sequencing each plasmid DNA:

5′-AATTAACCCTCACTAAAGGG-3′
5′-GTAATACGACTCACTATAGGGC-3′

Primers for sequencing *Smad4*:

5′-CGTTCACGACTTTGAAGGACAGC-3′
5′-CGTTTAAGGTCCCTTCAAGCTGC-3′
5′-CGATTACTTGGCGGGTGTTGG-3′
5′-GTGGACATTGGAGAGTTGACC-3′

2.5. Subcutaneous nude mouse models

For subcutaneous nude mouse models, BALB/c nude mice were maintained under specific pathogen-free conditions in the Shanghai Experimental Animals Center of the Chinese Academy of Sciences, and cared for in accordance with institutional guidelines. Mice were randomly assigned into 2 groups (5 mice/group) and 5×10^6 DT-PCa or SH-PAN cells were implanted subcutaneously in nude mice. Tumor volume (V) was estimated 4 weeks later post implantation using the formula $V = LW2\pi/6$ (L: length of tumor; W: width of tumor).

2.6. Histology and immunohistochemistry

Detailed procedures are provided as we have previously described [12]. Briefly, tissues were fixed in 10% formalin overnight and then embedded in paraffin. Serial 5-μm thick sections were cut from the tissue blocks, and dried overnight in an oven at 60 °C. The sections were dewaxed in xylene, dehydrated using a series of alcohol gradations to water, and stained with HE for histological verification. Immunohistochemical analysis was performed on formalin-fixed, paraffin wax-embedded sections. All primary antibodies used were either rabbit or goat polyclonal anti-mouse antibodies, and included antibodies for Tp53 (NCL-p53-CM5P, NovoCastra, 1:500), phospho-p44/42-MAPK (Cell Signaling, 1:100), Neu antibody (C-18,Snata Cruz, 1:200), EGFR (Cell Signaling, 1:50), Smad4 (B-8, Santa Cruz, 1:50), β-Catenin (Santa Cruz, 1:200) and PPP2R2A (ab18136, Abcam,1:1000). As a negative control, sections were incubated with PBS instead of primary antibody. Signals were detected with biotinylated anti-rabbit/anti-goat secondary antibodies (1:500, Vector) using the Elite Vectastain ABC kit and peroxidase substrate DAB kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris Hematoxylin. Immunohistochemical staining of PPP2R2A in the cytoplasm and/or perinuclear region of PDAC and peritumoral normal tissues was evaluated and semi-quantitatively estimated from the staining intensity and assigned grade 0 (no staining), grade 1 (weak staining), grade 2 (moderate staining) and grade 3 (strong staining). The percentage of positive cells was scored from grades 0 to 3 as follows: grade 0, <1% of cancer cells was negatively stained; grade 1, 1–49% positive expression; grade 2, 50–70% positive expression; and grade 3, >70% positive expression.

2.7. Quantitative real-time PCR

To validate the expression of *PPP2R2A* (NM_002717.3), quantitative real-time PCR was employed. The primer sequences used were F: 5′-CACTACGAGTGCCAGTCTTTAGG-3′, and R:
5′-CTGCTGCTGTAATCAC CTCTGT-3′, and the product size was 246 bp. The assays were completed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative quantification of lncRNA and mRNA expression was determined using the comparative C_T method. The expression of lncRNAs and mRNAs in DT-PCa cells relative to SH-PAN cells was calculated using the following formulas: $\Delta\Delta CT = \Delta CT$ DT-PCa $- \Delta CT$ SH-PAN, where fold change = $2^{-\Delta\Delta CT}$.

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