miR-218-5p restores sensitivity to gemcitabine through PRKCE/MDR1 axis in gallbladder cancer

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Gallbladder cancer (GBC) is one of the most common malignancy of the biliary tract characterized by its high chemoresistant tendency. Although great progresses have been made in recent decades for treating many cancers with anticancer drugs, effective therapeutics methods for anti-GBC are still lacking. Therefore, investigations into identifying the mechanisms underlying the drug resistance of GBC are greatly needed. In this study, we show that miR-218-5p plays a critical role in gemcitabine resistance of GBC. miR-218-5p levels were significantly lower in GBC than adjacent non-cancer tissues, and which were also associated with patient prognosis. While miR-218-5p overexpression abrogated gemcitabine resistance of GBC cells, silencing of which exhibited the opposite effects. Via six microRNA targets prediction algorithms, we found that PRKCE is a potential target of miR-218-5p. Moreover, miR-218-5p overexpression repressed the luciferase activity of reporter constructs containing 3′-UTR of PRKCE and also reduced PRKCE expression. Further studies revealed that miR-218-5p promotes sensitivity of gemcitabine by abolishing PRKCE-induced upregulation of MDR1/P-gp. Taken together, our results imply that an intimate correlation between miR-218-5p and PRKCE/MDR1 axis abnormal expression is a key determinant of gemcitabine tolerance, and suggest a novel miR-218-5p-based clinical intervention target for GBC patients.

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Gallbladder cancer (GBC) accounts for about 40% of all biliary tract carcinomas, and is one of the most common malignancy of the biliary tract.1,2 Although associated with poor prognosis, the mechanism underlying the aggressive malignancy of GBC remains largely unknown. Moreover, unlike several other tumors, it is highly resistant to the currently available standard adjuvant therapy, which makes its treatment even more challenging.3 Although several potential targets and signaling pathways underlying GBC chemoresistance have been revealed, the precise mechanisms are still elusive.4–6 Thus deeper unraveling the molecular mechanisms of drug resistance is of vital importance and urgently needed.

MicroRNAs (miRNAs), 22 nucleotides on average, are a group of evolutionarily conserved, small, endogenous, single-stranded non-coding RNAs.7 Through binding the 3′-untranslated regions (3′-UTRs) of their target genes, they could regulate specific genes expression at the posttranscriptional level. Multiple miRNAs have been found implicated in various kinds of cancers. miRNAs are capable of modulating cell proliferation, differentiation, metabolism, apoptosis, and thus actively participate in regulating tumorigenesis and tumor progression.8,9 Evidence suggests that miRNA can modulate the therapeutic efficacy in several cancer types,10 but it is still unclear whether and how deregulated miRNAs are involved in the chemoresistance of GBC. miR-218-5p, a tumor suppressor miRNA, has been found to be downregulated in several cancer types such as GBC, cervical cancer, colon cancer, and prostate cancer.11–14 In this study, we assessed the involvement of miR-218-5p in GBC chemosensitivity.

Permeability glycoprotein (P-gp), also known as multidrug resistance 1 (MDR1), is a well-known membrane transporter, which is able to transport various kinds of toxic substances and exerts a protective effect under physiological conditions. However, this function when utilized by cancer cells, may lead to the effluxing of many drugs and enhanced chemoresistance.15 PRKCE, a member of protein kinase C (PKC) family, is a serine- and threonine-specific protein kinase that actively participate in promoting drug resistance through phosphorylating a variety of protein targets, such as P-gp, ATF2, PI3K, Stat3, and Erk.16–17

Here, using a genome-wide miRNA expression profiling in six pairs of GBC and the corresponding non-cancerous gallbladder (CNG) tissues, we found that miR-218-5p is significantly downregulated in GBC. Further, we show that miR-218-5p regulates gemcitabine sensitivity in GBC cells by simultaneously repressing PRKCE expression. Due to the ability of PRKCE to activate MDR1/P-gp, repression of miR-218-5p causes abolishment of PRKCE inhibition and leads to increased MDR1/P-gp levels, and then enhances gemcitabine resistance of GBC cells. Moreover, we show that miR-218-5p and PRKCE are prognostic markers in GBC patients receiving chemotherapy. This adds to the possibility that strengthening of GBC chemoresistance might be countered by restoring expression of miR-218-5p, a notion that can be tested in the clinic.
Results

Reduced miR-218-5p expression in GBC well correlated with tumor prognosis. To identify transcripts that potentially drive malignance of GBC, a miRNA expression profile was determined by microarray analysis. The volcano plot and heat map showed systematic variations in transcript expression levels of miRNAs between GBC tissues and CNG tissues from six GBC patients (Figure 1a, Supplementary Figure 1, and Supplementary Table 1). Further analyzing via our miRNA expression profile data and other microarray databases (starBase v2.0; http://starbase.sysu.edu.cn/) of different tumors, we found that miR-218-5p is one of the common target reduced in many kinds of tumors (Figure 1b and Supplementary Figure 2). For validating the possible involvement in GBC, we further assessed its expression in 36 pairs of fresh GBC tissues and CNG tissues. Indeed, reduced miR-218-5p expression was detected in GBC tumors samples (Figures 1c and d). In situ hybridization (ISH) staining also confirmed a remarkably lower miR-218-5p expression in GBC paraffin sections (Figure 1e). Moreover, we also noticed a reduced miR-218-5p expression in different GBC cell lines compared with normal gallbladder epithelial cell (GBE) cells (Figure 1f). Intriguingly, further correlation analysis manifested no obvious relationship between miR-218-5p expression and patients’ clinic characteristics such as TNM stage, tumor size, CA 19-9 level, gallstone status, age, and gender (Figure 1g; Supplementary Table 2). However, an inverse relationship between miR-218-5p and cumulative survival rate was observed (Figure 1h).
Enforcing miR-218-5p expression accelerated tumor cell death and improved chemotherapeutic efficacy. To further explore the biological effect of miR-218-5p, we overexpressed and reduced miR-218-5p in GBC cell lines (NOZ and GBC-SD) by transfecting miR-218-5p mimics and antisense, respectively. However, no obvious difference in proliferation and colony formation ability was found (Figures 2a–c). GBC is well known for its higher chemoresistance than other kinds of tumors. Therefore, we wondered if miR-218-5p expression influences GBC patients’ prognosis through influencing the chemotherapeutic efficacy. To verify this hypothesis, we treated miR-218-5p overexpressing or downregulated NOZ and GBC-SD cell lines with various concentrations of gemcitabine and analyzed the viability at 72 h post treatment. Surprisingly, enforced miR-218-5p expression improved the antitumor effect of gemcitabine in NOZ and GBC-SD cells, with a significant reduction in IC50. In contrast, reduced miR-218-5p expression enhanced gemcitabine resistance ability, with a remarkable increase in IC50 (Figures 2d–g). To validate these results, the effect of miR-218-5p was examined by flow cytometry analysis of Annexin V positive cells after gemcitabine addition. Consistently, similar results were obtained showing that miR-218-5p overexpression greatly accelerated cell death, and downregulation inhibited cell apoptosis after gemcitabine treatment (Figures 2h–k). However, miR-218-5p itself has no effect on the apoptosis of GBC cells (Supplementary Figure 3). In conclusion, our results demonstrate that miR-218-5p can sensitize GBC cells to chemotherapeutic treatment.

miR-218-5p targets 3'-UTR of PRKCE and suppresses its expression. miRNA usually regulate specific gene expression by binding the 3'-UTR of their target genes. Through prediction analysis using six different databases, two common targets of miR-218-5p were found (PRKCE and SFMBT1) (Figure 3a). We then analyzed the mRNA
expression of PRKCE and SFMBT1 in miR-218-5p mimics or antisense-transfecting NOZ and GBC-SD cells. Surprisingly, only PRKCE showed reduced expression in miR-218-5p overexpressing cells, and increased expression upon miR-218-5p downregulation (Figures 3b and c). No obvious alterations in SFMBT1 mRNA expression were observed (Figures 3b and c). Further, we analyzed PRKCE and SFMBT1 protein expression by immunoblotting analysis, and found that miR-218-5p reduced PRKCE protein expression in both NOZ and GBC-SD cells. No obvious alterations in the SFMBT1 protein amount were detected (Figure 3d).

We then mutated the predicted complementary paring region of the 3'-UTR of PRKCE-WT (5'-AAGCACA-3') to PRKCE-MU (5'-ATCCTGA-3') (Figure 3e), and cloned into luciferase reporter vector. miR-218-5p addition reduced the luciferase activity carrying PRKCE-WT reporter but failed to do so with PRKCE-MU transfection in NOZ cells (Figure 3f). Similar results were obtained in GBC-SD cells (Figure 3g).

The chemotherapeutic sensitizer function of miR-218-5p is PRKCE dependent. To find out whether PRKCE is involved in regulating chemosensitivity of GBC, we overexpressed and knocked down PRKCE in GBC cell lines using overexpression vector and siRNA, respectively. Quantitative-PCR (Q-PCR) and immunoblotting analysis confirmed the overexpression and knock down efficiency of PRKCE (Figures 4a–d). Indeed, downregulation of PRKCE expression reduced IC50 of gemcitabine and increased tumor-killing effect of gemcitabine in NOZ and GBC-SD cells. On the other hand, PRKCE overexpression had the opposite effect and remarkably increased IC50 of gemcitabine, and enhanced

Figure 3 miR-218-5p directly targets the 3'-UTR of PRKCE and downregulated its expression. (a) PRKCE and SFMBT1 were potential targets of miR-218-5p in all six miRNA target prediction algorithms. (b, c) Q-PCR to detect PRKCE and SFMBT1 mRNA level in GBC cells when transfected with miR-218-5p mimic or antagonist. n = 3; bar, S.E.M. (d) Western blot to analyze PRKCE and SFMBT1 protein levels in GBC cell lines when transfected with miR-218-5p mimic or antagonist. (e) A schematic diagram showing the predicted miR-218-5p binding sites and the designed mutant sequence in the 3'-UTR of PRKCE (up), and the luciferase reporter constructs (down). (f, g) Firefly luciferase activity analysis of PRKCE 3'-UTR performed after co-transfection with PRKCE-wild type or PRKCE-mutant pGL3 constructs and miR-218-5p mimic GBC cell lines. n = 3; bar, S.E.M. (h) Q-PCR analysis of PRKCE mRNA levels in 36 pairs of GBC and CNG tissues. (i) Semi-quantitative analysis and the representative images (×400) of IHC staining for PRKCE protein in 82 paired GBC and CNG FFPE tissues. (j) The correlation between miR-218-5p and PRKCE expression in 36 GBC tissues measured by Q-PCR. (k) POS analysis based on PRKCE protein expression levels in 82 GBC patients. GAPDH was used to normalize the Q-PCR results, and β-actin was the loading control in western blot assay. NS, not significant, *P < 0.05; **P < 0.01; ***P < 0.001; Student's t-test.
chemoresistance (Figures 4e–j). Next for confirming PRKCE the potential downstream effector executing the anti-therapy effect with miR-218-5p deficiency, PRKCE were overexpressed in concomitant with miR-218-5p mimic transfection in NOZ and GBC-SD cell lines in parallel. As expected, PRKCE overexpression abolished the increased efficacy of gemcitabine with the addition of miR-218-5p mimic (Figures 4k–l). Our results demonstrate that miR-218-5p directly targeted PRKCE and involved in the chemotherapeutic efficacy.

miR-218-5p/PRKCE targeted MDR1 involved in the chemoresistance of GBC. Our previously study showed that MDR1, MRP1, BCRP are involved in the chemoresistance mechanisms of GBC.4–6 Several studies have implicated PKC family in regulating several chemotherapeutic-resistant protein such as P-gp and BCRP. In order to find out whether miR-218-5p is also capable of modulating these proteins, MDR1, MRP1, BCRP mRNA, and protein expression were analyzed in NOZ and GBC-SD cells transfected with miR-218-5p mimic or antisense by Q-PCR and western blot. Among these three genes, only MDR1/P-gp exhibited an altered expression. miR-218-5p overexpression inhibited MDR1/P-gp expression, while reduced miR-218-5p increased MDR1/P-gp expression (Figures 5a–d). On the other hand, our results also showed that MDR1/P-gp expression was regulated by PRKCE in NOZ and GBC-SD cells. Knockdown of PRKCE decreased MDR1/P-gp levels, whereas overexpression of PRKCE increased MDR1/P-gp expression (Figures 5e–f). Interestingly, when PRKCE is overexpressed simultaneously with miR-218-5p mimic, MDR1/P-gp expression was restored (Figures 5g–i). In addition, MDR1 overexpression not only strengthened the chemoresistance of GBC cells to gemcitabine, but also abrogated the effect of miR-218-5p overexpression on promoting gemcitabine sensitivity as shown by restored IC50 (Figure 5j). All the above results demonstrated that miR-218-5p-PRKCE-MDR1 axis is a potential candidate target in GBC chemotherapeutic treatment.

The improved chemotherapeutic efficacy of miR-218-5p addition in vivo. In order to confirm the relationship of miR-218-5p with chemotherapeutic effect in vivo, we utilized a tumor xenograft mouse model. NOZ cells were stably
transfected with a miR-218-5p plasmid construct or control plasmid and subcutaneously injected into male nude mice. A week later, one group was treated with gemcitabine (15 mg/kg per week) administered by intraperitoneal administration, and the other with the carrier (Saline). Consistently, miR-218-5p addition had no effect on tumor size in mouse group with no drug treatment (Figures 6a–c). However, tumor sizes and weight were greatly reduced in miR-218-5p overexpression mouse treated with gemcitabine (Figures 6a–c). IHC analysis of PRKCE and P-gp both exhibited reduced expression with miR-218-5p overexpression (Figures 6d–g). Transferase dUTP nick end-labeling (TUNEL) analysis of tumor specimens showed that the antitumor effect of gemcitabine was greatly enhanced in miR-218-5p overexpression mouse due to increased apoptosis (Figures 6f–g). These results from tumor xenograft mouse model provided further evidence that miR-218-5p enhances gemcitabine-induced apoptosis by targeting PRKCE and MDR1.

Discussion

The high chemoresistance of GBC made its treatment even more difficult and prognosis more pessimistic. Our study identified a novel miR-218-5p-PRKCE-MDR1 pathway implicated in modulating GBC chemosensitivity (Figure 6h). Reduced miR-218-5p expression in GBC, disabled its ability in degrading PRKCE, which eventually led to increased MDR1/P-gp expression. P-gp, one of the MDR-related proteins, played important role in orchestrating chemoresistant effect of gemcitabine.19,20 Thus, future treatment targeting miR-218-5p-PRKCE-MDR1 pathway would provide promising prospect for GBC patients.

Increasing evidence suggests that miR-218-5p exhibits antitumor effect in the development and progression of various human cancers through influencing tumor proliferation, invasion, and metastasis progression.11–13,21 Moreover, recent studies suggest that miR-218-5p also exert critical role on regulating chemosensitivity.22–24 For example, miR-218-5p improved 5-fluorouracil therapeutic efficacy through inhibiting BIRC5 and TS expression, both of which are important contributors to chemoresistance.22 Another investigation in glioblastoma multiforme cells found that elevating miR-218-5p increased sensitivity to cisplatin via inactivating RTK-HIF2α signaling axis.23 More remarkably, altered miR-218-5p expression in breast cancer cells modulated the sensitivity of cisplatin through regulating BRCA1 expression.24 However, whether
and how miR-218-5p might be involved in GBC has not been studied. Consistent with previous studies, we also found a significantly reduced miR-218-5p expression of GBC tissues compared with CNG tissues and correlated with a poor prognosis. Simultaneously, an increased sensitivity to gemcitabine was observed in vitro miR-218-5p overexpression. But no obvious effects on GBC cell growth and apoptosis were detected. This implied that miR-218-5p specifically influenced GBC chemosensitivity and thus changed GBC patients' prognosis.

So far, miR-218-5p has been reported to bind to various targets such as ARAF, PIK3C2A, LASP1, BRCA1, SFMBT1, DCUN1D1, and PXN to repress corresponding gene translation. However, the function of miR-218-5p and the association of its target gene with gemcitabine resistance in GBC remains unclear. Further elucidating the precise mechanism inside, in silico analysis of putative miRNA-binding sites in 3'-UTR of the target genes by using different algorithms (TargetScan, PicTar, RNA22, PITA, miRanda, and TargetMiner) was performed. PRKCE and SFMBT1 were finally identified, both harboring evolutionarily conserved targeting sequence of miR-218-5p. Downregulation of PRKCE expression in GBC cells can elevate gemcitabine resistance. This increases the expression of MDR1/P-gp, resulting in gemcitabine resistance of GBC. All n = 5; bar, S.E.M. NS, not significant, *P < 0.05; **P < 0.001; Student's t-test.

**Figure 6** Effect of miR-218-5p overexpression on the gemcitabine sensitivity of GBC cells in vivo. (a) Tumor growth curves of NOZ cells transfected with miR-218-5p construct or empty vector treated with gemcitabine or saline in vivo. (b, c) Representative images and the mean tumor weight of the four paired groups formed at the 5th week after subcutaneous transplantation. (d-f) Semi-quantitative analyses of IHC results for PRKCE and P-gp expression, and percentage of apoptotic tumor cells from the four paired NOZ tumor xenografts. (g) Representative images of PRKCE and P-gp IHC staining, and TUNEL staining in paraffin sections from the four paired NOZ tumor xenografts (×400). (h) Schematic representation of pathway modulated by miR-218-5p in GBC cells. Downregulation of miR-218-5p in GBC cells can elevate PRKCE expression. This increases the expression of MDR1/P-gp, resulting in gemcitabine resistance of GBC. All n = 5; bar, S.E.M. NS, not significant, *P < 0.05; **P < 0.001; Student's t-test.
large sample sizes and longer follow-up times are required to confirm the utility of these potential biomarkers.

Reducing intracellular drug accumulation is one of the most important mechanisms utilized by cancer cells in chemoresistance. Elevated drug efflux pump-related proteins such as P-gp, MRP1, MRP2, and BCRP expression significantly promotes cancer chemoresistance. Consistent with the previous report in renal carcinoma cells that reduce the expression of PRKCE lowered MDR1/P-gp, which affects the cancer stem cell potential of sorted side population cells and suppresses proliferation potential, resistance to chemotherapy and in vivo tumor formation ability.17 Our identification of the regulatory role of miR-218-5p-PRKCE-MDR1 axis in GBC cells further confirmed its importance.

Taken together, our study demonstrated that miR-218-5p is a potent tumor chemoresistance suppressor in the GBC and that its chemosensitization effects are mediated, at least in part, via downregulation of the PRKCE/MDR1 pathway. Loss of miR-218-5p expression, leading to the induction of PRKCE and its downstream MDR1/P-gp expression, appears to be a critical event in the development of gemcitabine resistance.

Materials and Methods

Patients and tissue samples. This study was approved by the Ethical Committee of Renji hospital, Shanghai Jiao Tong University School of Medicine. A total of 82 pairs of formalin-fixed, paraffin-embedded (FFPE) GBC tissues and CNG tissues were retrieved from GBC patients who underwent surgical resection and postoperative adjuvant chemotherapy from the Department of Pathology of Renji Hospital. Among the 82 GBC patients, 36 pairs of fresh GBC tissues and CNG tissues were also collected after the surgical removal and snap-frozen in liquid nitrogen immediately, then stored at −80 °C until total RNA was extracted. All 82 GBC patients were retrospectively followed up until December 2014. Postoperative survival (POS) was defined as the interval between the dates of surgery and last follow-up or death. All the subjects provided written informed consent in this study.

Cell culture. Three different kinds of human GBC cell lines were used in this study. GBC-SD was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). SGC-996 was provided by the academy of life sciences, Tong Ji university (Shanghai, China). NOZ were obtained from the Health Science Research Resources Bank (Osaka, Japan). GBC-SD, SGC-996, and NOZ were cultured in DMEM, RPMI-1640, and Williams’s E medium (Gibco, Grand Island, NY, USA), respectively, which supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO2 at 37 °C. All cell lines were ensured mycoplasma-negative cultures by monthly mycoplasma tests. For primary GBE culturing, cells from digested gallbladder epithelial tissue were cultured in KSN medium without FBS. After 30 min, adherent cells were discarded, and non-adherent cells were continually cultured at 37 °C in 5% humidified CO2.

Cell transfection. The miR-218-5p mimic and a non-specific mimic control, miR-218-5p antisense and a non-specific antisense control, PRKCE siRNA, and a negative control siRNA were all purchased from GenePharma (Shanghai, China), and were transfected into GBC cell lines by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to instructions of the manufacturer. Human miR-218-5p expression construct was generated by insertion of the coding sequence (CDS) of miRNA into pcDNA3.1 (V5-His A) to generate miR-218-5p overexpression vector. The expression plasmid was cotransfected with pCDH-CMV-MCS-EGFP (System Biosciences, Palo Alto, CA, USA), which supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO2 at 37 °C. After transfection for 48 h, the viability of GBC cell was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI, USA) assay. The results of firefly luciferase activity were normalized against Renilla luciferase activity.

Immunostaining. The 3′-UTR of PRKCE containing the predicted miR-218-5p binding site was amplified by PCR using cDNA of GBC cell as a template and then subcloned into a pmirGLO Dual-Luciferase Targeting Vector (Promega) to form the reporter vector pmirGLO-3′-UTR wild type. The mutant 3′-UTR of PRKCE, which contained point-mutated sequence in the binding region of miR-218-5p, was generated by using a site-directed mutagenesis kit from Fast Mutagenesis System (T ransGen Biotech, Beijing, China). NOZ and GBC-SD cells were seeded into 96-well plates (1 × 104 cells per well) and then co-transfected with miR-218-5p mimics and the luciferase reporter construct. After transfection for 48 h, the Renilla and firefly luciferase activities were analyzed using the Dual-Luciferase Assay System (Promega). The results of firefly luciferase activity were normalized to the Renilla luciferase activity.

Bioinformatics. The target genes of miR-218-5p were predicted by six computer-aided algorithms for quantification of the relative mRNA expression levels. Expression values of genes and miRNA were first normalized against GAPDH and small nuclear U6 RNA, and then compared to experimental controls. The primers were purchased from Sangon Biotech (Shanghai, China) and the sequences are listed in Supplementary Table 3.

Quantitative real-time PCR analysis. TRI reagent (Sigma) was used to isolate total RNA for mRNA analysis, and miRNeasy kit (Qiagen, Valencia, CA, USA) for miRNA analysis from the snap-frozen tissues or cultured cells. After determining RNA concentration and purity by using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, MA, USA), the cDNAs were synthesized by using Reverse Transcriptase M-MLV kit (Invitrogen). The expression levels of miRNA and mRNA were analyzed by using SYBR Premix Ex Taq (Takara, Shiga, Japan) in Applied Biosystems Viia7 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed with 2−ΔΔCT method for quantification of the relative mRNA expression levels.

Cell viability and apoptosis assays. After transfecting with miRNA mimic or antisense and treating with concentration gradient of gemcitabine (Selleck, Houston, TX, USA), the viability of GBC cell was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega, Madison, WI, USA) assay (MTS), as described previously. After transfecting with gemcitabine for 48 h, cells were stained with FITC-conjugated Annexin V (BD Biosciences, Heidelberg, Germany) and propidium iodide (5 mg/ml) and analyzed by fluorescence-activated cell sorting analysis, as described previously.

In situ hybridization. ISH of GBC FFPE tissues were performed using a human miR-218-5p-specific digoxigenin-labeled locked nucleic acid probe. Briefly, following dewaxing and rehydration, tissue sections were treated with proteinase K at 37 °C for 15 min. We then washed specimens with PBS and dehydrated through a graded series of ethanol (70, 96 and 100%). After incubation under 50 °C
overnight with miR-218-5p probe in hybridization buffer, the slides were washed in pre-warmed 5 x, 1 x and 0.2 x SSC at 50 °C for 30 min. The slides were blocked with blocking reagent (Roche, Mannheim, Germany) and then incubated with antibody against digoxigenin (1:1000, Roche) at room temperature for 30 min, respectively. Finally, the substrate NBT/BCIP (Roche) was added on specimens and incubated for 15 min in dark until the specific blue signal was observed followed by stopping further reaction using KTB buffer.

**Tumor formation assay in a nude mouse model.** Twenty male BALB/c nude mice (4 weeks old, 15-25 g) were randomly divided into two groups. A total 2 x 10^6 NOZ/cPDH empty vector and NOZ/cPDH miR-218-5p overexpression cells in 100 μl medium were subcutaneously transplanted into the mouse left flank of two groups, respectively. One week later, each group was randomly divided into two subgroups (n = 5) and subjected to intraperitoneal injection of gemcitabine (15 mg/kg) or saline (100 μl; negative control) weekly. Tumor growth was measured using external caliper once every week and were calculated based on the equation: \( V = (l_{\text{length}} \times w_{\text{width}}^2)/2.33 \). All mice were killed at the 5th week, and the tumors were dissected out for H&E and TUNEL staining. The study was strictly performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University.

**Statistics.** Data were expressed as mean ± S.E.M. Group comparisons of normally distributed data were performed with unpaired Student's t-test (two-tailed) or one-way ANOVA. For multiple comparisons, the Tukey-Kramer honestly significant difference was applied following ANOVA. Kaplan–Meier analyses log-rank test were used to determined POS. The Pearson's \( r^2 \)-test was used to analyze the association of miR-218-5p expression with PRKCE expression. Dichotomous variables were compared using \( r^2 \)-test. SPPS17.0 software (IBM, Chicago, IL, USA) was used for all statistical analysis. \( P < 0.05 \) was considered statistically significant.

**Conflict of Interest**

The authors declare no conflict of interest.

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