

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.

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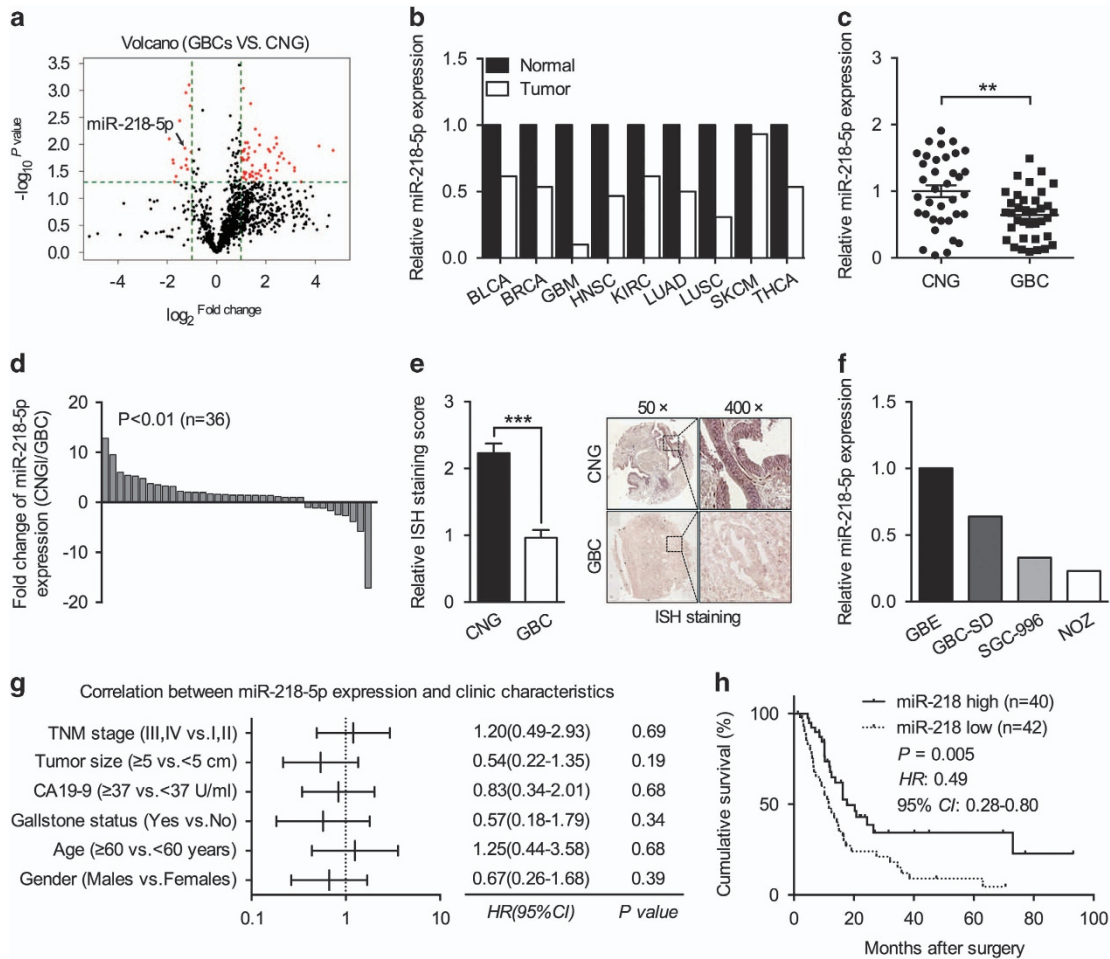


Figure 1 miR-218-5p is downregulated in GBC tissues and cell lines, and is associated with prognosis of GBC patients. (a) Volcano plot of microRNAs expression array from six pairs of GBC and CNG tissues. More detailed information can be found in Supplementary Materials. (b) miR-218-5p expression in other nine types of cancer from starBase v2.0 Pan-cancer Project database. (c) Validation of miR-218-5p differential expression in an independent cohort contained 36 pairs of GBC and CNG tissues. (d) Fold change of miR-218-5p expression in new 36 pairs of GBC and CNG tissues. (e) Comparison of miR-218-5p expression in 82 pairs of GBC and CNG FFPE tissues (left) and their representative ISH image stained by anti-miR-218-5p probe (right). (f) Relative expression of miR-218-5p in GBC cells and GBE cells. (g) Comparing different TNM stages, tumor size, CA19-9 level, gallstone status, age and gender ratio between miR-218-5p high and miR-218-5p low expression from 82 pairs of GBC FFPE tissues. Statistical significance was performed by the χ^2 -test. (h) Kaplan–Meier analysis of the correlation between miR-218-5p expression and POS in 82 GBC patients. Small nuclear RNA *U6* was used to normalize the Q-PCR results. bar, S.E.M., ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test. BLCA, urothelial bladder cancer; BRCA, breast cancer; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KIRC, clear cell kidney carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; SKCM, cutaneous melanoma; THCA, papillary thyroid carcinoma

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,¹⁸ 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).

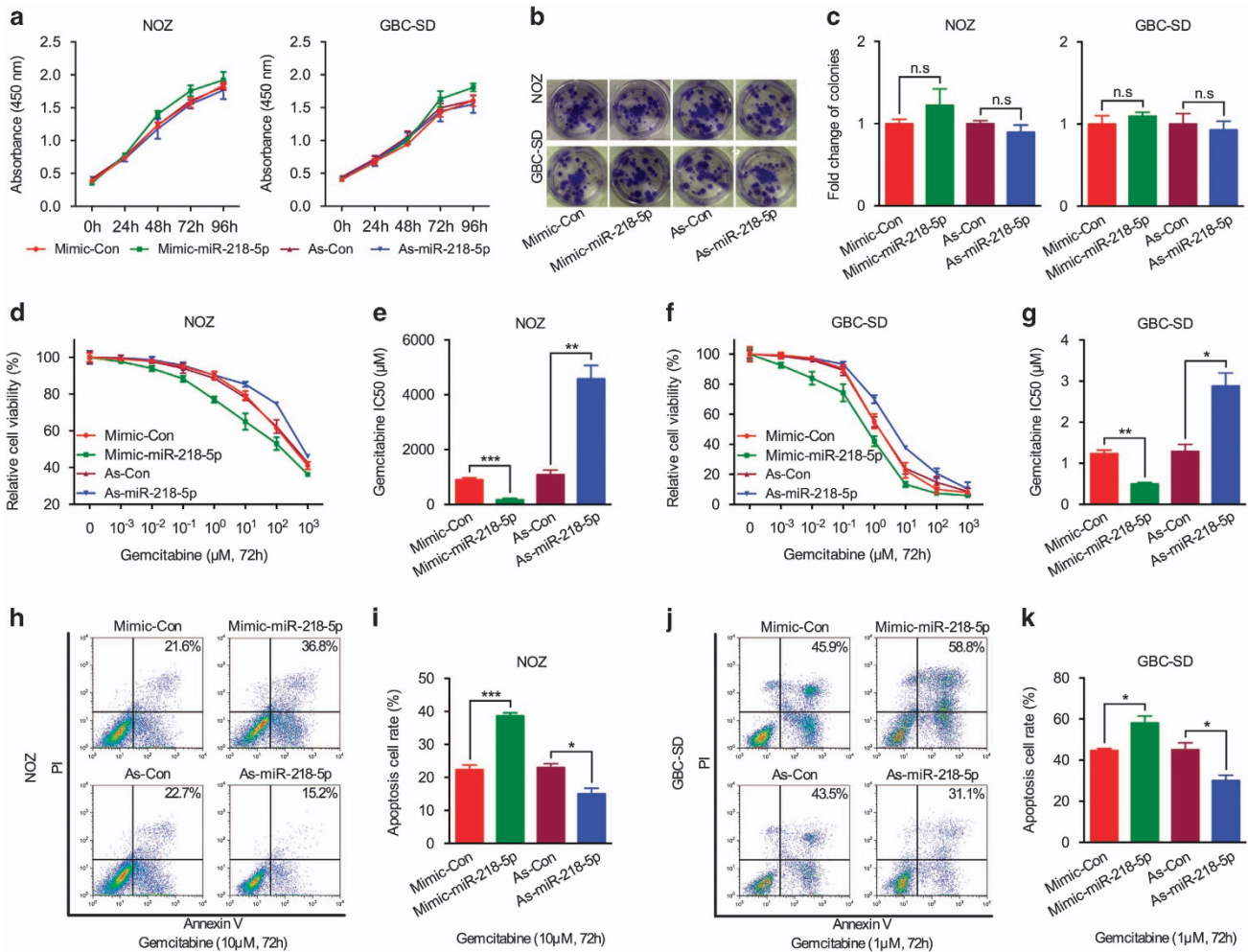


Figure 2 miR-218-5p promotes sensitivity of gemcitabine in NOZ and GBC-SD cell lines. (a) MTS analysis of GBC cells (NOZ and GBC-SD) transiently transfected with miR-218-5p mimic (Mimic-miR-218-5p), negative control mimic (Mimic-Con), miR-218-5p antisense (As-miR-218-5p), or negative control antisense (As-Con). (b–c) Representative images of colony formation and the mean fold change of colonies induced by miR-218-5p mimic or antagonist in GBC cells. (d–g) MTS assay and calculating the corresponding IC₅₀ of gemcitabine in GBC cells transfected with miR-218-5p mimic or antagonist. (h–k) Flow cytometric analysis of Annexin V/PI staining used to quantify apoptosis rate of GBC cells transfected with miR-218-5p mimic or antagonist, followed by exposure to gemcitabine (NOZ, 10 μ M; GBC-SD, 1 μ M) for 72 h. All $n = 3$; bar, S.E.M., NS, not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's t -test

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

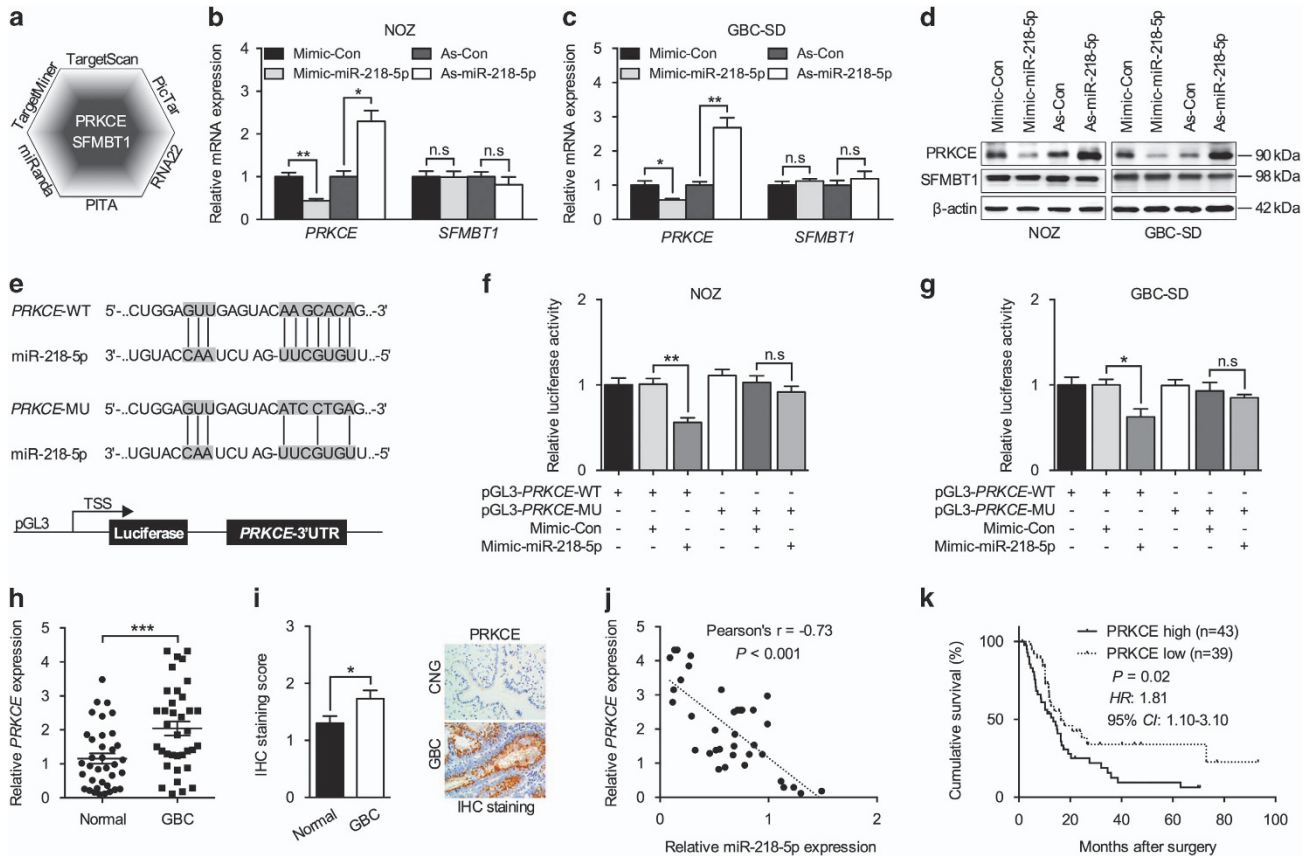


Figure 3 miR-218-5p directly targets the 3'-UTR of *PRKCE* and downregulated its expression. (a) *PRKCE* and *SFMBT1* were potential targets of miR-218-5p in all six miRNA target prediction algorithms. (b, c) Q-PCR to detect *PRKCE* and *SFMBT1* mRNA level in GBC cells when transfected with miR-218-5p 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 (x400) 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

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 pairing 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). *PRKCE* has been previously suggested to be involved in multiple kinds of tumors, but its involvement in GBC has never been investigated. We analyzed *PRKCE* expression in our 36 pairs

of CNG and GBC tumor samples, and found an increased expression in GBC (Figure 3h). Immunohistochemistry (IHC) staining further corroborated our results (Figure 3i). Interestingly, an inverse correlation between miR-218-5p and *PRKCE* mRNA expression was detected in GBC samples (Figure 3j). Moreover, higher *PRKCE* expression correlated with reduced cumulative survival rate, implying a poorer prognosis with *PRKCE* overexpression (Figure 3k).

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

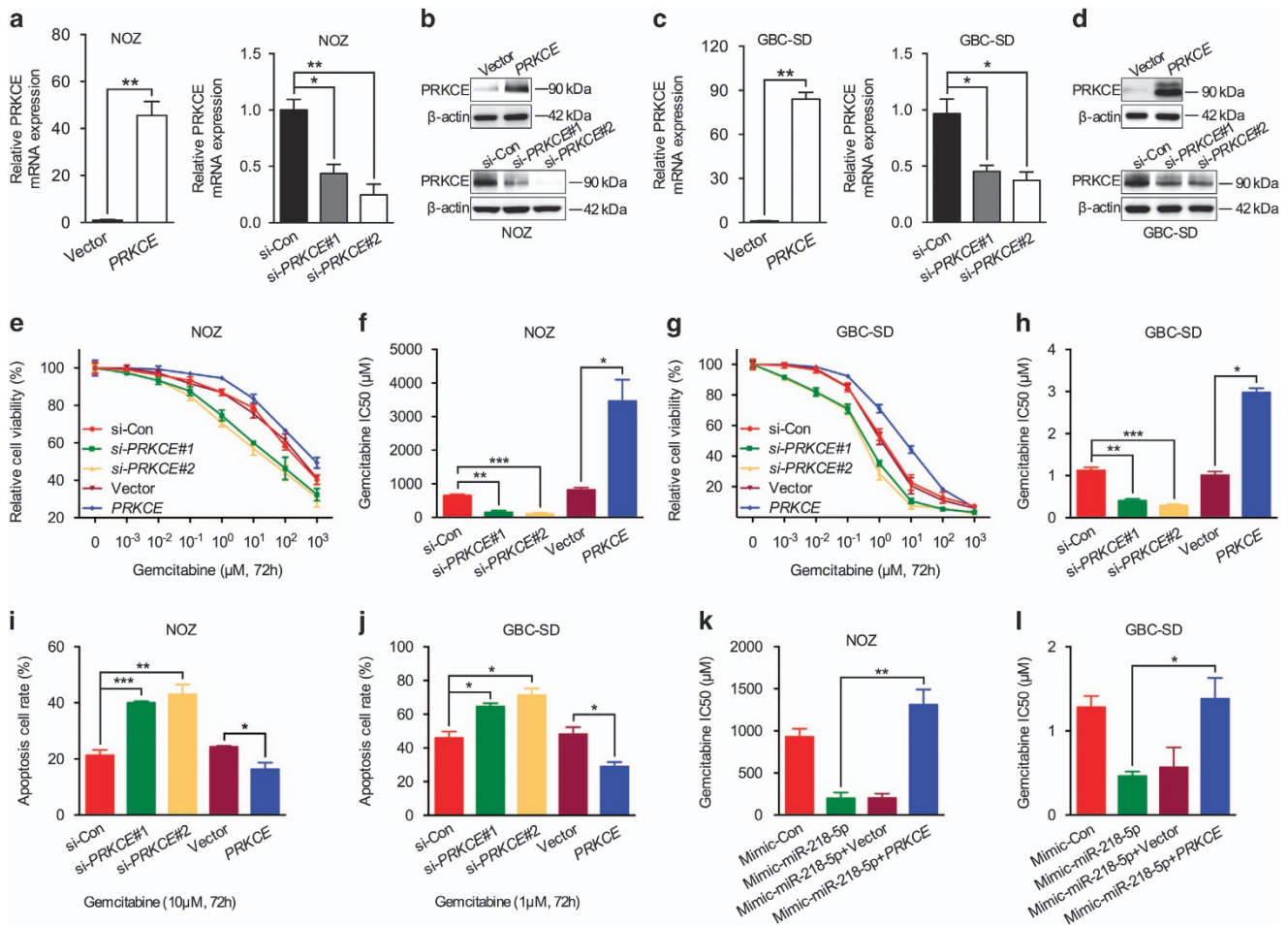


Figure 4 miR-218-5p-sensitized GBC cells to gemcitabine was mediated by PRKCE. (a–d) Efficiency testing of overexpression or interference of PRKCE in GBC cells by using Q-PCR and western blot. (e–h) MTS assay and calculating the corresponding IC50 of gemcitabine in GBC cells transfected with PRKCE construct or PRKCE siRNAs. (i, j) Flow cytometric analysis of Annexin V/PI staining used to quantify apoptosis rate of GBC cells transfected with PRKCE construct or PRKCE siRNAs, followed by exposure to gemcitabine (NOZ, 10 μ M; GBC-SD, 1 μ M) for 72 h. (k, l) IC50 of gemcitabine in GBC cells co-transfected with miR-218-5p mimic and PRKCE construct. GAPDH was used to normalize the Q-PCR results, and β -actin was the loading control in western blot assay. All $n = 3$; bar, S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; 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

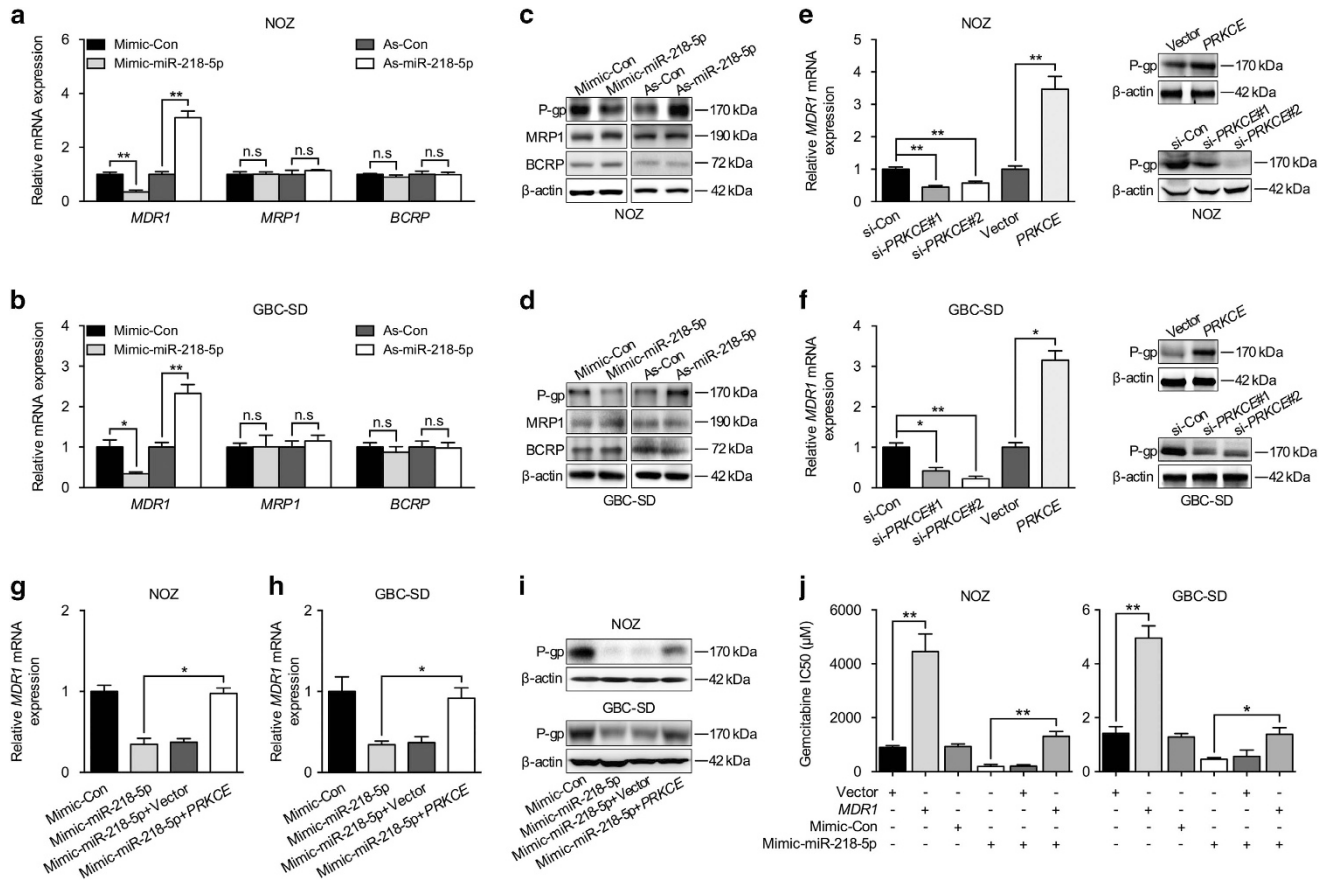


Figure 5 miR-218-5p negatively regulates resistance to gemcitabine through PRKCE/MDR1 axis in GBC cells. (a–d) Q-PCR and western blot to analyze *MDR1*, *MRP1*, and *BCRP* mRNA and protein expression, respectively, in GBC cell lines when transfected with miR-218-5p mimic or antagomir. (e, f) Q-PCR and western blot to analyse *MDR1* mRNA and P-gp expression, respectively, in GBC cell lines when transfected with *PRKCE* construct or *PRKCE* siRNAs. (g–i) Q-PCR and western blot to detect *MDR1* mRNA and P-gp expression, respectively, in GBC cell lines when co-transfected with miR-218-5p mimic and *PRKCE* construct. (j) IC₅₀ of gemcitabine in GBC cells when co-transfected with miR-218-5p mimic and *MDR1* construct. *GAPDH* was used to normalize the Q-PCR results, and β -actin was the loading control in western blot assay. All $n = 3$; bar, S.E.M. * $P < 0.05$; ** $P < 0.01$; Student's *t*-test

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–e, 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-HIF2 α 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

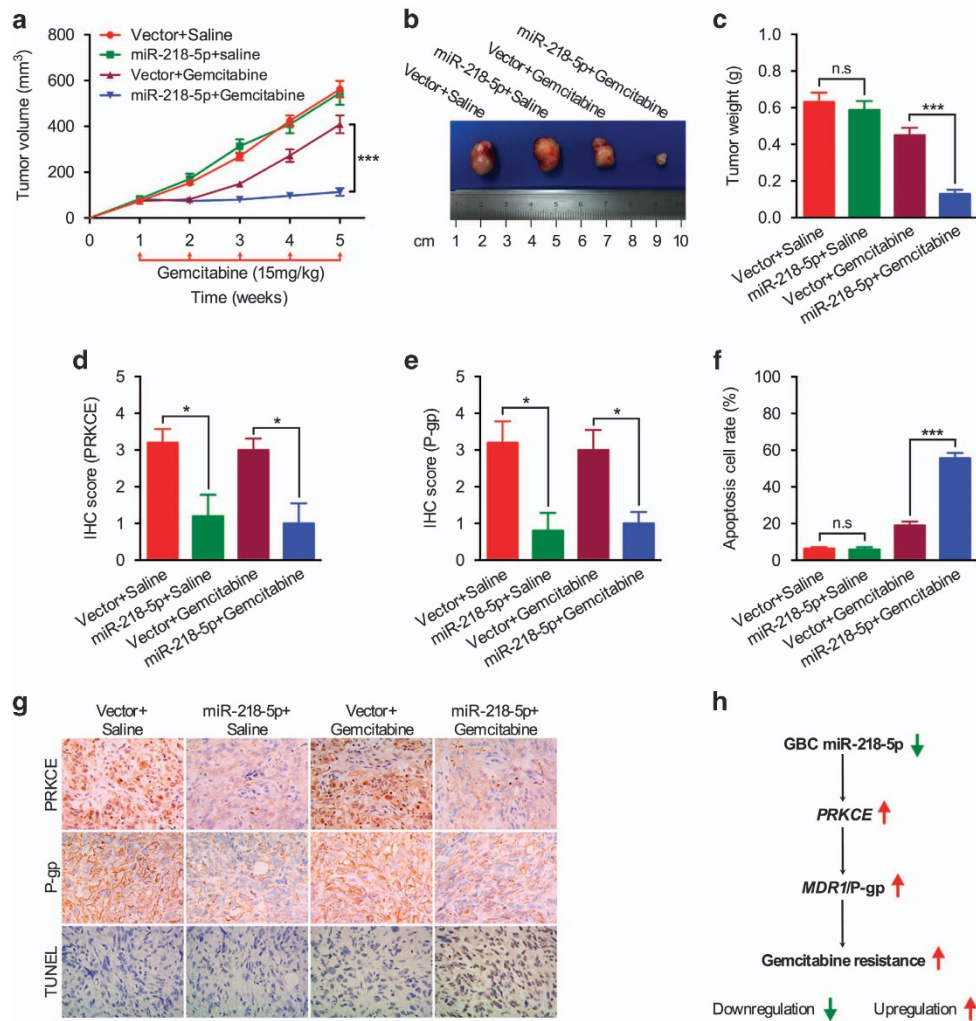


Figure 6 Effect of miR-218-5p overexpression on the gemcitabine sensitivity of GBC cells *in vivo*. (a) Tumor growth curves of NOZ cells transfected with miR-218-5p construct or empty vector treated with gemcitabine or saline *in vivo*. (b, c) Representative images and the mean tumor weight of the four 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

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.^{12,13,23–26} 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. Facilitated by further *in vitro* overexpression and site mutation-related luciferase assay, we then confirmed the regulatory role of *PRKCE* by miR-218-5p in GBC tumors. Emerging evidences suggested that *PRKCE* plays oncogenic role through increasing survival protein levels, which sustained cancer stem cell development.¹⁷ Our study reinforced the oncogenic role of *PRKCE* in increasing gemcitabine resistance and its correlation with a poor prognosis in GBC tumors. The inverse relationship between miR-218-5p and *PRKCE* was then confirmed in GBC tissues. Therefore, early examination of miR-218-5p and *PRKCE* expression in the tumors of GBC patients may aid in the prediction of chemotherapeutic effect and help in the development of new postoperative chemotherapy strategies. However, preclinical and clinical studies with

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.²⁷ 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 chemotherapeutics 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 William's E medium (Gibco, Grand Island, NY, USA), respectively, which supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 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 KSFM medium without FBS. After 30 min, adherent cells were discarded, and non-adherent cells were continually cultured at 37°C in 5% humidified CO_2 .

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 pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Palo Alto, CA, USA). Then recombinant lentiviruses produced in HEK293FT cells infect GBC cell lines in the presence of $4\ \mu\text{g/ml}$ polybrene (Sigma, St. Louis, MO, USA), followed by puromycin selection ($2\ \mu\text{g/ml}$) for stably overexpressing GBC cell lines. *PRKCE* and *MDR1* overexpression vector was generated by insertion of gene CDS into a pCDNA 3.1 vector (Invitrogen). GBC-SD and NOZ cells were transfected with the overexpression plasmid using Lipofectamine 2000 reagent. The pCDH-CMV-MCS-EF1-copGFP and pCDNA 3.1 empty vector were used as negative control (vector), respectively.

Cloning efficiency assays. For colony formation, cells in single-cell suspension were plated and grown in 24-well plates at a density of 100 per well for 24 h. After transfecting, GBC cells were incubated in the plate for 14 days until colonies were visible. The colonies were fixed for 15 min with 4% paraformaldehyde and stained with 0.1% crystal violet. The numbers of colonies were counted to assess the viability.

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.⁴ 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.⁴

Immunoblot analysis. Total proteins were extracted from GBC cells using cell lysates in RIPA buffer and protein expression was assessed by immunoblot analysis according to the procedure described previously. The primary mouse, rabbit, or goat antibodies used were as follows: *PRKCE* (1:500, sc-1681, Santa Cruz, Santa Cruz, CA, USA), *SFMBT1* (1:500, ab77419, Abcam, Cambridge, UK), *MDR1*/P-gp (1:500, sc-55510, Santa Cruz), *MRP1* (1:2000, sc-365635, Santa Cruz), *BCRP* (1:200, sc-25822, Santa Cruz), and β -actin (1:2000, A5316, Sigma).

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 using SYBR Premix Ex Taq (Takara, Shiga, Japan) in Applied Biosystems ViIATM 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed with $2^{-\Delta\Delta\text{CT}}$ method²⁸ 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.

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end-labeling assays. The IHC and TUNEL staining of FFPE tissue and its scoring system were performed, as described previously.⁴ The mouse primary antibodies used were as follows: *PRKCE* (1:200, sc-1681, Santa Cruz), *MDR1*/P-gp (1:200, sc-55510, Santa Cruz).

Dual-luciferase reporter assay. 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 miRNA Target Expression Vector (Promega) to form the reporter vector *MRP1*-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 (TransGen Biotech, Beijing, China). NOZ and GBC-SD cells were seeded into 96-well plates (1×10^4 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,²⁹⁻³² namely TargetScan Release 7.0 (http://www.targetscan.org/vert_71/), PicTar (http://www.pictar.org/cgi-bin/new_PicTar Vertebrate.cgi), RNA22 (<https://cm.jefferson.edu/rna22/>), PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), miRanda (<http://www.microrna.org/microrna/home.do>), and Targetminer (http://www.isical.ac.in/~bioinfo_miu/targetminer20.htm). The target genes were accepted only when they were positive in all six analyses.

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 $5\times$, $1\times$ and $0.2\times$ 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 2×10^6 NOZ/pCDH empty vector and NOZ/pCDH 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: $V=(\text{length}\times\text{width}^2)/2$.³³ All mice were killed at the 5th week, and the tumors were dissected out for IHC and TUNEL staining. The study was strictly performed accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University.

Statistics. Data were expressed as mean \pm 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 χ^2 -test was used to analyze the association of miR-218-5p expression with *PRKCE* expression. Dichotomous variables were compared using χ^2 -test. SPSS17.0 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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