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Glycogen synthase kinase-3β participates in acquired resistance to gemcitabine in pancreatic cancer

Masahiro Uehara[1] | Takahiro Domoto[1] | Satoshi Takenaka[1,2] | Dilireba Bolidong[1] | Osamu Takeuchi[3] | Tomoharu Miyashita[2,4] | Toshinari Minamoto[1]

[1]Division of Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

[2]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Japan

[3]Biomedical Laboratory, Department of Research, Kitasato University Kitasato Institute Hospital, Tokyo, Japan

[4]Department of Surgical Oncology, Kanazawa Medical University, Ishikawa, Japan

Correspondence
Toshinari Minamoto, Division of Translational and Clinical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan.
Email: minamoto@staff.kanazawa-u.ac.jp

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Abstract

Acquisition of resistance to gemcitabine is a challenging clinical and biological hallmark property of refractory pancreatic cancer. Here, we investigated whether glycogen synthase kinase (GSK)-3β, an emerging therapeutic target in various cancer types, is mechanistically involved in acquired resistance to gemcitabine in human pancreatic cancer. This study included 3 gemcitabine-sensitive BxPC-3 cell-derived clones (BxG30, BxG140, BxG400) that acquired stepwise resistance to gemcitabine and overexpressed ribonucleotide reductase (RR)M1. Treatment with GSK3β-specific inhibitor alone attenuated the viability and proliferation of the gemcitabine-resistant clones, while synergistically enhancing the efficacy of gemcitabine against these clones and their xenograft tumors in rodents. The gemcitabine-resensitizing effect of GSK3β inhibition was associated with decreased expression of RRM1, reduced phosphorylation of Rb protein, and restored binding of Rb to the E2 transcription factor (E2F)1. This was followed by decreased E2F1 transcriptional activity, which ultimately suppressed the expression of E2F1 transcriptional targets including *RRM1*, *CCND1* encoding cyclin D1, *thymidylate synthase*, and *thymidine kinase 1*. These results suggested that GSK3β participates in the acquisition of gemcitabine resistance by pancreatic cancer cells via impairment of the functional interaction between Rb tumor suppressor protein and E2F1 pro-oncogenic transcription factor, thereby highlighting GSK3β as a promising target in refractory pancreatic cancer. By providing insight into the molecular mechanism of gemcitabine resistance, this study identified a potentially novel strategy for pancreatic cancer chemotherapy.

KEYWORDS

acquired resistance, gemcitabine, glycogen synthase kinase-3β, pancreatic cancer, ribonucleotide reductase M1

Abbreviations: CDK, cyclin-dependent kinase; dCK, deoxycytidine kinase; dFdC, 2′-2′-difluoro-2′-deoxycytidine; dFdCDP, dFdC diphosphate; E2F1, E2 transcription factor 1; EMT, epithelial-mesenchymal transition; FOLFIRINOX, combination of folate, 5-FU, irinotecan, and oxaliplatin; FU, fluorouracil; GSK3β, glycogen synthase kinase-3β; hENT1, human equilibrative NT1; IHC, immunohistochemistry; IP, immunoprecipitation; nab, nanoparticle albumin-bound; NT, nucleoside transporter; PS, performance status; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Rb, retinoblastoma gene/tumor suppressor protein; RRM1, ribonucleotide reductase M1; S, serine; TK1, thymidine kinase 1; TP53INP1, tumor protein p53 inducible nuclear protein 1; TS, thymidylate synthase; WB, Western blotting; Y, tyrosine.

1 | INTRODUCTION

Pancreatic cancer is one of the most intractable cancer types, with little improvement in the survival rate over the past decades.[1,2] Patients with locally advanced tumors and those with metastatic tumors account for approximately one-third and one-half, respectively, of all pancreatic cancer cases.[1] Due to the extreme difficulty in obtaining an early diagnosis,[3] only 10%-15% of newly identified pancreatic cancer patients present with resectable or borderline resectable tumor (stage I or II). Most patients have local disease recurrence and/or distant metastasis following surgery. Therefore, most patients undergo nonsurgical therapies including conventional chemotherapy, radiation, and molecular-targeted therapies.[1] More recently, clinical trials with immune checkpoint blockades and precision therapies have also been conducted.[4,5] Despite multimodal combinations of these therapies, the 5-year overall survival rate for pancreatic cancer is at most 3%-15%.[2,6]

Chemotherapeutic agents used for pancreatic cancer patients include gemcitabine alone or in combination with nanoparticle albumin-bound (nab)-paclitaxel, TS-1 (a fluorouracil [FU] prodrug), FOLFIRINOX (sequential combination of folate, 5-FU, irinotecan, and oxaliplatin) and nanoliposomal irinotecan with 5-FU and folate.[7] Currently, gemcitabine-based regimens are the most frequently prescribed in the first-line setting and remain one of the standard therapies for pancreatic cancer.[7,8] However, most patients are initially resistant or soon acquire resistance to gemcitabine, therefore obtaining little survival benefit from this treatment.[9] Therefore, the identification of mechanism-based strategies and therapeutic targets to overcome gemcitabine resistance has become an active area for biological and clinical research.[10]

In the present study we hypothesized that glycogen synthase kinase (GSK)-3β was a candidate molecular target that may affect the efficacy of chemotherapeutic agents. GSK3β is an isoform of the multifunctional GSK3 family serine/threonine kinases and regulates a diverse array of fundamental cellular pathways by phosphorylation and interaction with dozens of structural and functional molecules.[11] GSK3β activity is finely controlled by differential phosphorylation of serine (S)9 (inactive) and tyrosine (Y)216 (active) residues. It is constitutively active in normal cells, but in many circumstances negative regulation of its activity allows cells to maintain vital activity and homeostasis in response to various stimuli.[12] Deregulation of GSK3β expression and activity has been implicated in the pathogenesis and progression of common diseases including diabetes mellitus, neurodegenerative disorders, and various inflammatory and immunological conditions.[12,13] Such diverse roles in normal cells and in diseases have highlighted GSK3β as a potentially attractive drug target and has led to the development of inhibitors.[14] At present, several early-phase clinical trials have been evaluating some of these inhibitors and lithium (an ATP non-competitive and non-specific GSK3 inhibitor) for neurodegenerative disorders and various cancer types, but none of them has been approved for clinical use (reviewed in Ref.15,16).

Based on its known functions against pro-oncogenic pathways (eg, Wnt/β-catenin, hedgehog, Notch signaling) and epithelial-mesenchymal transition (EMT) in untransformed cells, GSK3β has long been hypothesized to suppress tumor development and progression.[13] Previous studies on the putative tumor-suppressive roles of GSK3β have shown that it is inactivated by S9 phosphorylation in various oncogenic pathways. However, there has been no evidence showing that active GSK3β suppresses tumorigenesis, or that GSK3β inhibition promotes tumor development and progression (reviewed in Ref.15). In contrast, many studies by our group and others have demonstrated direct tumor-promoting roles for GSK3β as well as therapeutic effects following its inhibition in at least 25 different cancer types[15-17] including pancreatic cancer.[18] In addition to its therapeutic effect, we and others have shown that GSK3β inhibition sensitizes pancreatic cancer cells to gemcitabine through the impairment of DNA damage repair and cell cycle regulation.[19-21] However, none of the earlier studies modeled the acquired resistance to gemcitabine in pancreatic cancer that is frequently encountered in the clinical setting. To more directly investigate the role of GSK3β in the acquisition of gemcitabine resistance, we established cell clones derived from a gemcitabine-sensitive human pancreatic cancer BxPC-3 cell line that had acquired stepwise resistance to gemcitabine. This system models the development of gemcitabine resistance observed in clinical pancreatic cancer.[22]

2 | MATERIALS AND METHODS

2.1 | Cell lines

This study investigated the human pancreatic cancer cell line BxPC-3 and its derivative clones that acquired stepwise resistance to gemcitabine (BxG30, BxG140, and BxG400 in increasing order of resistance).[22] The cloned cells were confirmed to sustain their resistance without supplementation of gemcitabine in the medium for at least 2 months. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 unit/mL penicillin G and 0.1 mg/mL streptomycin) (Gibco) at 37°C with 5% CO_2. All subsequent experiments, except for the mouse xenograft study, were completed within 2 months after initiation from frozen cell cultures.

2.2 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using ISOGEN (Wako). Complementary DNA was synthesized from total RNA using a Reverse Transcription Kit (Promega). qRT-PCR was performed using the Stratagene Mx3000P system (Agilent Technologies) and SYBR Premix Ex *Taq* (TaKaRa Bio) with sets of sense and anti-sense primers (FASMAC) for the respective genes, as shown in Supporting Information Table S1. Relative mRNA expression of each gene was calculated using the ΔCt method and calibrated against the expression of GAPDH as an internal control.

2.3 | Western blotting (WB)

Cellular protein was extracted from cultured cells using a lysis buffer (CelLytic-MT; Sigma-Aldrich) with a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). A 20 μg aliquot of protein extract was subjected to WB for the proteins of interest, as reported previously.[20] The expression of β-actin was monitored as a control for the loading amount of protein sample. Primary antibodies used at their respective dilutions are shown in Table S2.

2.4 | Analyses for cell survival, proliferation, and apoptosis

BxPC-3 cells and cells from the gemcitabine-resistant clones (BxG30, BxG140, BxG400) were seeded into 96-well plates and treated with gemcitabine (Sigma-Aldrich) at different concentrations. Two other batches of the same cells were passaged in the absence of gemcitabine for 1 or 2 months, respectively, and then treated with gemcitabine. In these 3 sets of experiments, the relative numbers of viable cells at designated time points following treatment with gemcitabine were determined using the WST-8 assay kit (Cell Counting Kit-8; Dojindo). These cells were also treated with DMSO (Sigma-Aldrich) or with one of the GSK3β inhibitors: AR-A014418 (Calbiochem)[23] or SB-216763 (Sigma-Aldrich)[24] at the indicated final concentration in the medium. The relative number of viable cells at the designated time points was measured as described above. The concentrations of GSK3β inhibitors used in this study are within the reported range of pharmacologically relevant doses.[23,24] After treatment with DMSO or GSK3β inhibitor, the relative numbers of proliferating and apoptotic cells were determined using the Click-iT EdU Proliferation Assay for Microplates kit (Thermo Fisher Scientific) and the Cellular DNA Fragmentation ELISA kit (Roche Diagnostics), respectively. The mean numbers of viable cells, EdU-labeled proliferating cells, and apoptotic cells from triplicate experiments were calculated together with their standard deviations (SDs). They were compared statistically to the same cells treated with gemcitabine at different concentrations, as well as to cells treated with DMSO or GSK3β inhibitors.

2.5 | RNA interference (RNAi)

siRNA specific for human GSK3β (GSK3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were obtained from Invitrogen. The specificity of GSK3β-specific siRNA was validated in our previous study.[20] Cells were transfected with 20 nmol/L of either siRNA using Lipofectamine RNAiMAX reagent (Invitrogen). The efficiency of RNA interference was evaluated by WB with an antibody that recognized both GSK3α and GSK3β (Table S2). The effects of GSK3β knockdown on the relative numbers of surviving, proliferating, and apoptotic cells were examined and compared with the same cancer cells transfected with control or GSK3β-specific siRNA for 72 h as described above.

2.6 | Effects of GSK3β inhibitor on the susceptibility of cancer cells to gemcitabine

IC_{50} values at 72 h following treatment with gemcitabine and AR-A014418 were measured by WST-8 assay for BxPC-3, BxG30, BxG140, and BxG400 cells. These cells were then treated with gemcitabine at the doses close to the respective IC_{50} in combination with DMSO or AR-A014418 at the concentrations shown in Table S3. The combined effects of gemcitabine and AR-A014418 on the viability of cells were determined as being additive, synergistic, or antagonistic using the isobologram method.[25]

2.7 | Immunoprecipitation (IP) analysis

BxG400 cells were treated with DMSO or 25 μmol/L AR-A014418 for 24 h. The nuclear fraction of these cells was isolated using Nuclear Complex Co-IP Kit (Active Motif) and pre-cleaned with protein G magnetic beads slurry (Cell Signaling Technologies). Nuclear extracts from the respective cells were divided into aliquots and immunoprecipitated with a mixture of non-immune mouse and rabbit IgG, mouse anti-Rb, and rabbit anti-E2F1 antibodies, respectively, according to our previous study.[26] An aliquot of nuclear extract (input) and the immunoprecipitated material was analyzed by WB using anti-Rb and anti-E2F1 antibodies. The primary antibodies used at the respective dilutions are shown in Table S2.

2.8 | Luciferase reporter assay

Transcriptional activity of E2F1 was determined by luciferase reporter assay. The respective cancer cells were co-transfected with E2F1 firefly luciferase vector (E2F1(3) Luciferase Reporter Vector, Panomics) and internal control *Renilla* luciferase vector (pRL-SV40 Vector, Promega) in accordance with the manufacturers' protocols. At 72 h after transfection, the cells were treated with DMSO or 25 μmol/L AR-A014418 for 24 h. The cells were then examined for activities of both luciferases using the Dual-Luciferase Reporter Assay System (Promega) and Fluoroscan ascent FL instrument (Dainippon Sumitomo Pharmaceutical). The relative transcriptional activity of E2F1 was determined by normalizing firefly luciferase activity with *Renilla* luciferase activity in the same cells.

2.9 | Animal study

The therapeutic effects of gemcitabine and GSK3β inhibitor, either alone or in combination, were examined in gemcitabine-sensitive BxPC-3 cells and in the most gemcitabine-resistant BxG400 cells grown as xenografts in athymic mice. In total, 1×10^6 BxPC-3 or BxG400 cells suspended in 50 μL of phosphate buffered saline were subcutaneously inoculated into each of 53 athymic mice (Charles River Laboratories, Japan). The mice were randomly assigned to 4

groups and given intraperitoneal injections of 100 μL of 75% DMSO, gemcitabine (20 mg/kg body weight), or AR-A014418 (2 mg/kg body weight) alone or in combination, respectively, twice a week for 7-10 wk. Assuming that total body fluid in mice accounted for *c*. 60% of their body weight, the AR-A014418 dose of 2 mg/kg body weight corresponded to a concentration of *c*. 10 μmol/L in culture medium, which was within the known pharmacological dose range for this agent.[23] The dose of gemcitabine corresponded closely to the standard clinical dose (1000 mg/m^2 body surface area). Throughout the experiment, all mice were carefully observed every day for adverse events and their body weight was monitored. Tumors were measured in 2 dimensions, twice a week. Tumor volume (cm^3) was calculated using the formula: $0.5 \times S^2 \times L$, where S is the smallest tumor diameter (cm) and L is the largest (cm). The design and protocol of the animal experiment and changes in body weight of mice during treatment are shown in Figure S1. All animal experiments were undertaken in accordance with the Japanese animal ethics guidelines.[27] The protocol was approved by the Institute for Experimental Animal Work, Kanazawa University Advanced Science Research Center.

At necropsy, tumors were removed, fixed in 10% paraformaldehyde and embedded in paraffin for histopathological and immunohistochemical staining. Representative paraffin sections of the tumors were stained with H&E and immunostained using the avidin-biotin-peroxidase complex (ABC) method as described previously.[28] The primary antibodies used at the dilutions for immunohistochemistry (IHC) are shown in Table S2.

2.10 | Statistical analysis

The results are presented as mean ± SD. Data were analyzed using Student *t* test in a two-tailed analysis. Statistical significance was defined as values of $P < .05$.

3 | RESULTS

3.1 | Efficacy of gemcitabine on BxPC-3 cell-derived clones with stepwise acquired resistance to gemcitabine

The IC_{50} values for gemcitabine in BxPC-3 cells and for the gemcitabine-resistant BxG30, BxG140, and BxG400 cells were 1.28 ng/mL, 39 ng/mL, 370 ng/mL, and 2000 ng/mL, respectively (Figure 1A). Consistent with our previous study,[22] the results confirmed that the persistent and stepwise gemcitabine resistance was retained during long-term frozen storage of these resistant clones. We also determined the IC_{50} values of gemcitabine in the same cells following continuous culture in the absence of gemcitabine for 1 or 2 months, respectively (Figure S2). This indicated that gemcitabine resistance was present for at least 2 months after frozen cell culture, thus allowing subsequent experiments. mRNA and protein expression of ribonucleotide reductase M1 (RRM1), a known biomarker for gemcitabine resistance,[9,29] increased significantly in the BxG140 and BxG400 cells as the level of resistance to gemcitabine increased (Figure 1B,C).

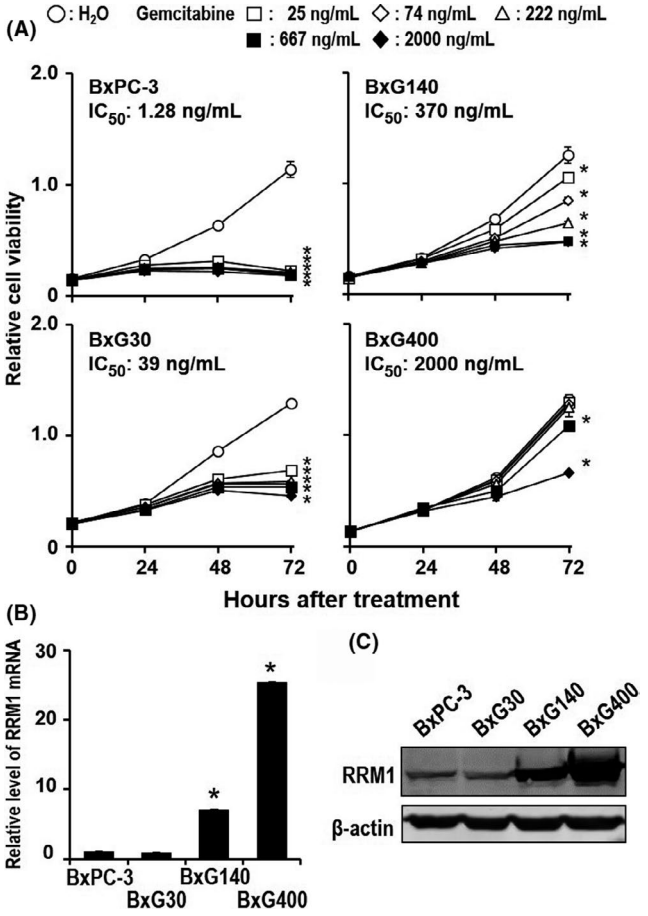

FIGURE 1 Efficacy of gemcitabine and expression of RRM1 in BxPC-3 cells and cells from gemcitabine-resistant clones (BxG30, BxG140, BxG400). A, Respective cells were treated with H_2O or the indicated concentrations of gemcitabine for the designated times in triplicate. The relative mean number of viable cells at each time point is shown with SDs. *$P < .05$. B, Relative expression of RRM1 mRNA in BxPC-3 and the gemcitabine-resistant clones. Values shown are the means ± SDs of triplicate measurements. *$P < .05$. C, Comparison of RRM1 expression between BxPC-3 cells and the gemcitabine-resistant clones by Western blotting. β-actin expression was monitored as a loading control in each sample

3.2 | Effects of GSK3β inhibition on the gemcitabine-resistant clones

We previously reported the constitutive activation of GSK3β in human pancreatic cancer cells, including BxPC-3, and the therapeutic effect of GSK3β inhibition against these cancer cells.[19,20] We therefore evaluated the effects of GSK3β inhibition on 3 gemcitabine-resistant clones. Gemcitabine-resistant cells showed a higher level of cell survival and proliferation and a lower frequency of apoptosis compared with their parent BxPC-3 cells (Figures 2 and S3). The

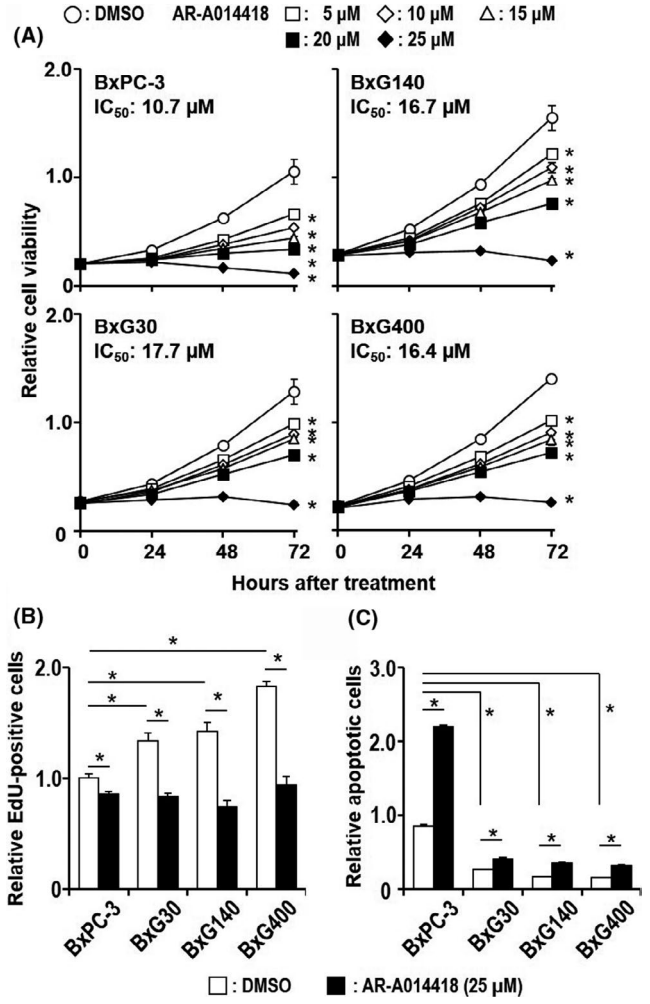

FIGURE 2 Effects of GSK3β inhibition on viability, proliferation, and apoptosis in BxPC-3 cells and the gemcitabine-resistant clones. A, Respective cells were treated with DMSO or the indicated concentration of AR-A014418 for the designated times. The relative number of viable cells at each time point was measured by WST-8 assay. B,C, EdU-positive proliferating cells (B) and the relative number of apoptotic cells (C) were compared between the respective cells treated with DMSO or AR-A014418. A-C, The mean value from triplicate experiments was scored with SDs. $^*P < .05$

GSK3β inhibitors AR-A014418 and SB-216763 reduced the viability of BxPC-3 cells and the gemcitabine-resistant clones in a dose-dependent and time-dependent manner, with similar IC_{50} values irrespective of gemcitabine resistance (Figures 2A and S3A). The GSK3β inhibitors also suppressed the proliferation of all cancer cells and induced their apoptosis (Figures 2B,C and S3B,C). Similar effects were observed in these cancer cells following depletion of GSK3β (Figure S4). These results indicated that GSK3β sustained the survival and proliferation of BxPC-3 cells and of derived gemcitabine-resistant clones.

Compared with the parental BxPC-3 cells that are prone to going apoptosis following inhibition of GSK3β, the effect of GSK3β inhibition on cell proliferation was more prominent than on apoptosis in the BxPC-3-derived gemcitabine-resistant clones (Figures 2B,C, S3B,C, and S4B,C). Such difference suggested that these resistant clones might also acquire the phenotype invulnerable to apoptosis-inducing stimuli. Alternatively, they might have more dependence on the cell proliferation signal mediated by cyclin D1 and cyclin-dependent kinase (CDK)4/6 as we previously observed in pancreatic cancer cells that were primarily unresponsive to gemcitabine.[20] The effects of GSK3β RNAi on cell survival, proliferation, and apoptosis (Figure S4) were less marked than the GSK3β inhibitors (Figures 2 and S3). In many cases, the effect of an enzyme largely depends on its biochemical or catalytic activity rather than its level of expression. The biological effect of RNAi depends on the efficiency of siRNA transfection as well as on the subsequent knockdown of the target molecule. In line with these notions, our previous study using an in vitro kinase assay showed that the GSK3β inhibitor AR-A014418 inactivated GSK3β in human pancreatic cancer cells (including BxPC-3 cells) within 1 h after treatment, while the GSK3β-specific siRNA took longer than 48 h to efficiently, but not completely, deplete GSK3β expression in the same cells.[20] Therefore, it is conceivable that pharmacological GSK3β inhibitors more promptly and efficiently inhibit GSK3β in cells, leading to a more prominent biological effect on cells compared with GSK3β RNAi.

3.3 | Combined effect of gemcitabine and GSK3β inhibitor

Molecular-targeted therapeutics are preferably prescribed in combination with conventional chemotherapeutic agents and/or radiation and with other targeted agents. This allows the optimization of therapeutic efficacy and the minimization of undesired effects, as well as preventing the acquisition of therapy resistance.[30] We previously showed that deregulated GSK3β renders pancreatic cancer cells intrinsically unresponsive to gemcitabine via the tumor protein p53 inducible nuclear protein (TP53INP)1-mediated DNA damage repair machinery and the impairment of Rb-mediated cell cycle regulation.[19,20] Therefore, we investigated whether GSK3β inhibition affected cancer cells that had acquired resistance to gemcitabine.

AR-A014418 at 25 μmol/L alone showed adequate and similar therapeutic effects against BxG30, BxG140, and BxG400 cells (Figure 2). We therefore examined the therapeutic effects of all combinations of AR-A014418 at a dose range of 1.56-25 μmol/L and of gemcitabine at different doses in accordance with the IC_{50} of the respective gemcitabine-resistant clones (Table S3). When BxG30, BxG140, and BxG400 cells were treated with gemcitabine at increasing doses, the combination with AR-A014418 reduced the IC_{50} of gemcitabine in a dose-dependent fashion (Figure S5 and Table S4). Analysis of the data using the isobologram method[25] showed that AR-A014418 in combination with gemcitabine was synergistic against cancer cells from all gemcitabine-resistant clones (Figure 3A). Knockdown of GSK3β also significantly enhanced the effects of gemcitabine against these cancer cells (Figure 3B), although the combined effects were not amenable to isobologram

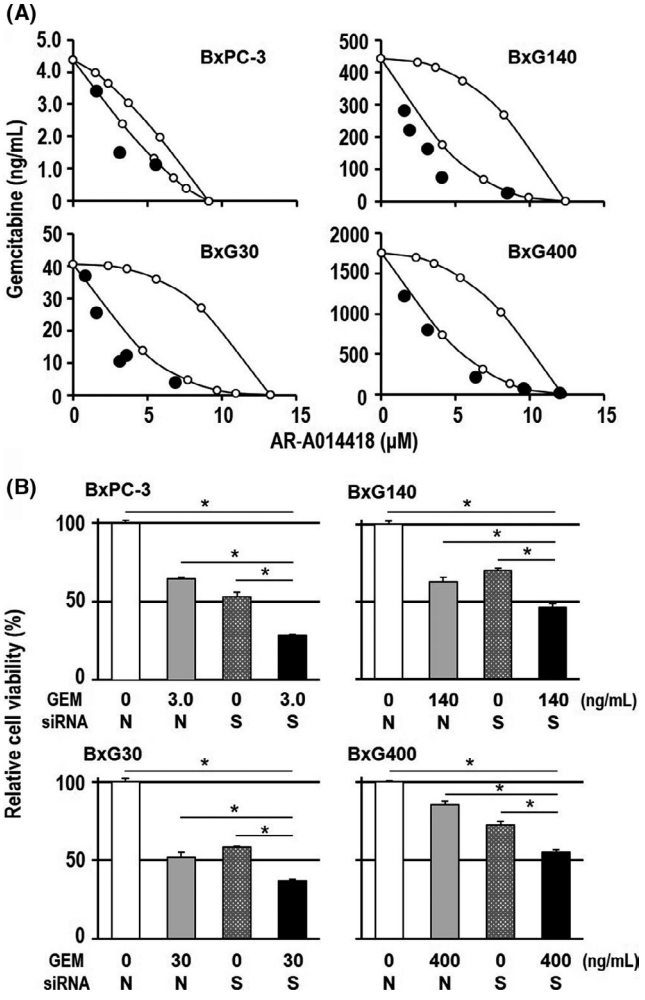

FIGURE 3 Effect of gemcitabine in combination with GSK3β inhibitor or GSK3β-RNAi on BxPC-3 cells and gemcitabine-resistant clones. A, The respective cells were treated with various combinations of gemcitabine and/or AR-A014418 as shown in Table S3. The combined effect at 72 h after treatment was measured by WST-8 assay and analyzed by the isobologram method at respective doses (closed circle). B, The respective cells were transfected with non-specific (N) or GSK3β-specific siRNA (S) and treated with the indicated concentrations of gemcitabine (GEM) for 72 h. Mean relative number of viable cells in triplicate were scored with SDs and compared between the same cells with different treatments. *$P < .05$

analysis. These results indicated that GSK3β inhibition could circumvent acquired resistance to gemcitabine in pancreatic cancer cells.

3.4 | Changes in molecular characteristics of gemcitabine-resistant cancer cells following GSK3β inhibition

To improve the treatment of refractory pancreatic cancer, it is important to clarify the molecular mechanism by which GSK3β inhibition alters the acquired resistance of pancreatic cancer cells to gemcitabine. Strikingly, inhibition of GSK3β expression and activity decreased the expression of RRM1 at both mRNA and protein levels in gemcitabine-resistant cancer cells (Figure 4A,B), although the levels of RRM1 expression in BxG140 and BxG400 cells were still higher than in BxPC-3 and BxG30 cells at 72 h after treatment with AR-A014418 (Figure S6). RRM1 is a transcriptional target for E2F1. The Rb tumor suppressor protein normally traps E2F1, thereby repressing its transcriptional activity.[31,32] We therefore hypothesized that GSK3β may interfere with the Rb-mediated negative regulation of E2F1 during acquisition of resistance to gemcitabine in pancreatic cancer cells.

We have previously shown that expression of cyclin D1 and CDK4 decreased in pancreatic cancer cells (including BxPC-3) concurrently with their sensitization to gemcitabine via GSK3β inhibition.[20] Treatment with AR-A014418 consistently decreased the expression of cyclin D1 and CDK4 in cells from BxPC-3-derived gemcitabine-resistant clones (Figure 4B). As the cyclin D1-CDK4/6 complex negatively regulates the tumor suppressor function of Rb via its phosphorylation, we next investigated the expression and phosphorylation of Rb in these cells. Phosphorylation of Rb ($pRb^{S807/811}$) is known to affect its ability to bind to E2F1[32] and was progressively higher in the gemcitabine-resistant clones compared with BxPC-3 cells. Treatment with AR-A014418 decreased the level of $pRb^{S807/811}$ but did not affect the expression of Rb and E2F1 in the same cells (Figure 5A). This result suggested that GSK3β-mediated loss of Rb function may be involved in the acquisition of resistance to gemcitabine in pancreatic cancer.

3.5 | Changes in E2F1 transcriptional activity following GSK3β inhibition in gemcitabine-resistant cancer cells

Further to the results shown above, we next examined whether GSK3β affected the ability of Rb to bind to E2F1, as well as evaluating the transcriptional activity of E2F1 in gemcitabine-resistant cancer cells. Following treatment with DMSO or 25 μmol/L AR-A014418, nuclear extracts from the most resistant BxG400 cells were immunoprecipitated with antibodies to Rb and E2F1. WB of these immunoprecipitates showed that GSK3β inhibition increased binding between Rb and E2F1 in the cell nuclei (Figure 5B). Luciferase reporter assay showed that transcriptional activity of E2F1 in BxG140 and BxG400 cells was significantly higher than in BxPC-3 cells. Treatment with GSK3β inhibitor reduced the transcriptional activity of E2F1 (Figure 5C) and the expression of its target genes including *RRM1* (Figure 4A), *CCND1* (encoding cyclin D1), *TS* and *thymidine kinase 1* (*TK1*) in both BxG140 and BxG400 cells (Figure 5D). These results suggested that GSK3β may alter the functional interaction between Rb and E2F1 during the acquisition of gemcitabine resistance in pancreatic cancer.

3.6 | Effects of GSK3β inhibitors on BxPC-3 and BxG400 xenografts in mice

As a prerequisite for the clinical translation of GSK3β-targeted therapy for pancreatic cancer patients with resistance to gemcitabine,

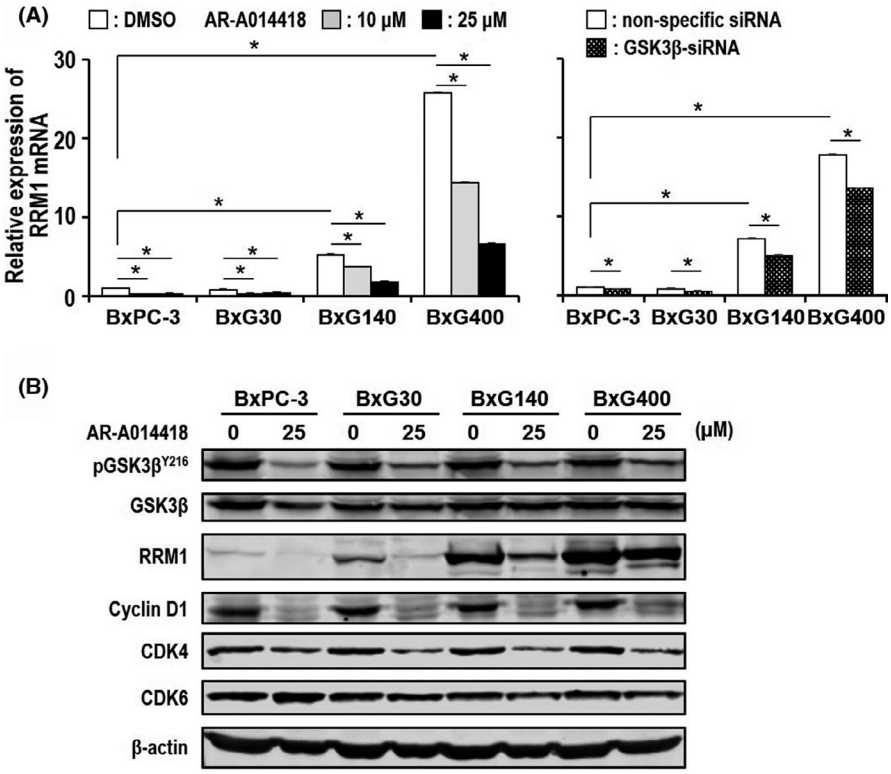

FIGURE 4 Changes in expression of RRM1, GSK3β and cell cycle regulatory molecules in BxPC-3 cells and gemcitabine-resistant clones following GSK3β inhibition. A, The relative expression of RRM1 mRNA was compared between the respective cells treated with DMSO or AR-A014418 for 24 h (left panel), and between cells transfected with non-specific or GSK3β-specific siRNA (right panel). The mean value from triplicate experiments was scored with SDs. *$P < .05$. B, Western blotting analysis for expression of GSK3β, RRM1 and cell cycle regulatory molecules (cyclin D1, CDK4, CDK6) and for GSK3β phosphorylation (pGSK3β^{Y216}) in the respective cells treated with DMSO or AR-A014418 for 24 h. β-actin expression was monitored as a loading control in each sample

we tested the efficacy of gemcitabine and AR-A014418, either alone or in combination, against BxPC-3 and BxG400 cell xenograft tumors in athymic mice (Figure S1A). Xenograft tumors of BxG400 cells grew faster than BxPC-3 tumors and were unresponsive to gemcitabine at 20 mg/kg, which was almost equivalent to the standard clinical dose. Due to animal ethics issues, we euthanized the sham (DMSO)-treated mice with BxG400 xenografts and those treated with gemcitabine at 6 and 8 wk, respectively, after treatment. Compared with treatment with either AR-A014418 or gemcitabine alone, treatment of mice with the 2 agents in combination significantly reduced tumor growth in a time-dependent manner (Figure 6). We observed no serious adverse events in the 4 groups of mice during treatment, and there were no statistically significant differences in mean body weight between the groups (Figure S1B). At necropsy, gross observation and histological examination showed no lesions, primary tumors, or metastatic tumors in the major vital organs of all mice.

IHC examination of the tumors removed from sham (DMSO) and gemcitabine-treated mice showed higher levels of active GSK3β (pGSK3β^{Y216}) and RRM1 expression in BxG400 tumors than in BxPC-3 tumors. Treatment with AR-A014418 alone or in combination with gemcitabine decreased the pGSK3β^{Y216} level and RRM1 expression (Figure 7). Similar to the results from cell culture studies (Figures 4B and 5A), cyclin D1 expression and Rb phosphorylation (pRb$^{S807/811}$) in BxG400 tumors treated with DMSO or gemcitabine alone were higher than in BxPC-3 tumors treated with the same agents, but were reduced following treatment with AR-A014418 alone or in combination with gemcitabine (Figure S7).

4 | DISCUSSION

Current first-line chemotherapy for locally advanced and metastatic pancreatic cancers consisted of 2 combination protocols, FOLFILINOX and nab-paclitaxel with gemcitabine. Both have been shown to improve the efficacy of gemcitabine monotherapy.[33,34] The only approved second-line regimen for patients who failed gemcitabine-based therapy is nanoliposomal irinotecan with 5-FU and folate.[35] Importantly, however, no study has yet shown a significant improvement in outcome from any of the combination regimens over gemcitabine alone in patients with poor (≥2) PS as defined by the Eastern Cooperative Oncology Group. As the vast majority of pancreatic cancer patients present with a PS ≥ 2, gemcitabine therefore remains the standard of treatment for pancreatic cancer.[7,8] This in turn has attracted growing attention to the problem of gemcitabine resistance.[9,10]

Putative biochemical mechanisms of gemcitabine resistance include the decreased expression of human equilibrative nucleoside transporter-1 (hENT1) that is indispensable for cellular uptake of gemcitabine,[36] the inactivation of deoxycytidine kinase (dCK) that is a late-limiting enzyme for metabolic activation of gemcitabine,[37] and the overexpression of RRM1 that sustains DNA synthesis, thus counteracting the pharmacological action of gemcitabine.[29] These molecular alterations found in the tumors were associated with poor survival of pancreatic cancer patients undergoing treatment with gemcitabine in various clinical settings.[38,39] Recent experimental approaches aimed at overcoming the acquired resistance to gemcitabine include targeting of hENT1 expression by TS inhibitor and bypassing nucleoside transporters by prodrugs. Other approaches

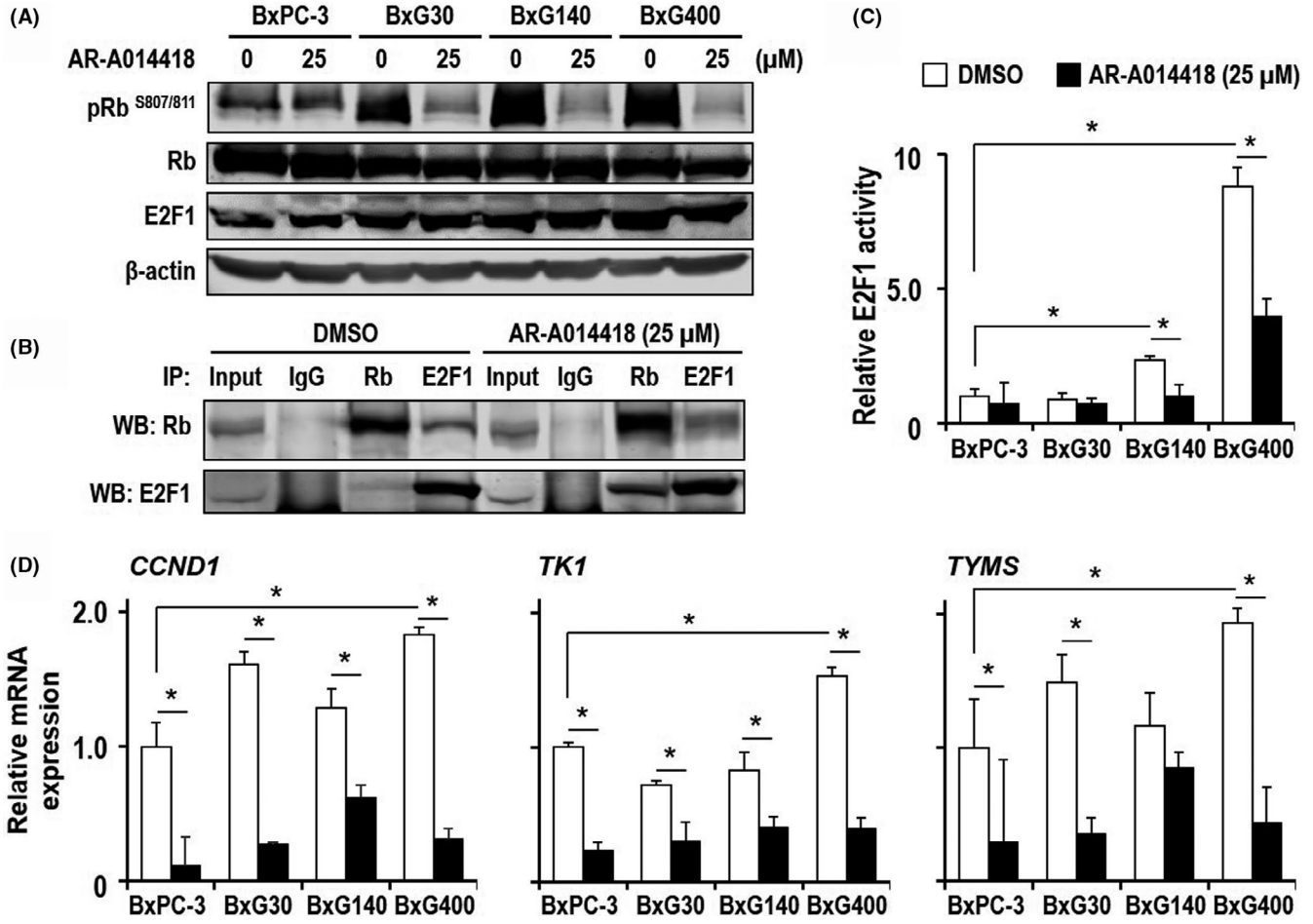

FIGURE 5 Effects of GSK3β inhibitor on the expression and phosphorylation of Rb, its binding to E2F1 and the transcriptional activity of E2F1. A, Western blotting (WB) analysis for expression of Rb, E2F1 and phosphorylated of Rb ($pRB^{S807/811}$) in cells treated with DMSO or AR-A014418 for 24 h. β-actin expression was monitored as a loading control in each sample. B, Immunoprecipitates (IP) from nuclear extracts of DMSO-treated or AR-A014418-treated BxG400 cells with non-immune mouse/rabbit IgG and the antibody to Rb or E2F1 were analyzed by WB with the indicated antibodies. C, Relative transcriptional activity of E2F1 in the respective cells treated with DMSO or AR-A014418 was measured by luciferase reporter assay. D, Relative expression of CCND1 (cyclin D1), TK1, and TS mRNA in the respective cells treated with DMSO or AR-A014418. C, D, Data are the mean values with SDs of triplicate experiments. *$P < .05$

included improving the intracellular delivery of gemcitabine by conjugating it with nanocarriers, as well as molecular targeting of the pro-oncogenic Wnt, hedgehog and Notch signaling that are reactivated during acquired resistance to gemcitabine (reviewed in Ref. 9,10). However, there have been few attempts at targeting RRM1 to enhance the efficacy of gemcitabine, even though it is recognized as a potentially strong target for overcoming gemcitabine resistance.[40]

The gemcitabine-resistant clones (BxG30, BxG140, BxG400) used in this study were established from gemcitabine-sensitive pancreatic cancer BxPC-3 cells and were characterized by overexpression of RRM1, but only minor alterations in the expression of hENT1, dCK, RRM2, and Bcl2.[22] This suggested that there may be dependency of these resistant clones on RRM1 for the acquisition of resistance to gemcitabine. We showed that GSK3β inhibitors alone exerted therapeutic effects against BxPC-3 cells as well as against gemcitabine-resistant clones with a similar IC_{50}. Notably, when combined with various concentrations of gemcitabine, the GSK3β inhibitor synergistically enhanced the efficacy of gemcitabine against all of the resistant clones in culture and against the most resistant BxG400 xenografts in mice. These effects of GSK3β inhibition were associated with decreased expression of RRM1, suggesting that impairment of the transcriptional activity of E2F1 may be responsible for the "resensitization" of resistant clones to gemcitabine. GSK3β inhibition consistently attenuates E2F1 transcriptional activity, resulting in decreased expression of its transcriptional targets including *RRM1*, *CCND1*, *TS*, and *TK1*. As we previously reported,[20] GSK3β inhibition decreases the expression of cyclin D1 and CDK4 and phosphorylation of Rb, thereby restoring the binding of Rb to E2F1 in tumor cell nuclei. Consequently, our results suggest that the mechanism whereby GSK3β confers acquired resistance to gemcitabine in pancreatic cancer is via disturbance of the physiological (tumor-suppressive) interaction between Rb and E2F1.

The levels of RRM1 expression in the gemcitabine-resistant BxG140 and BxG400 cells decreased during longer treatment

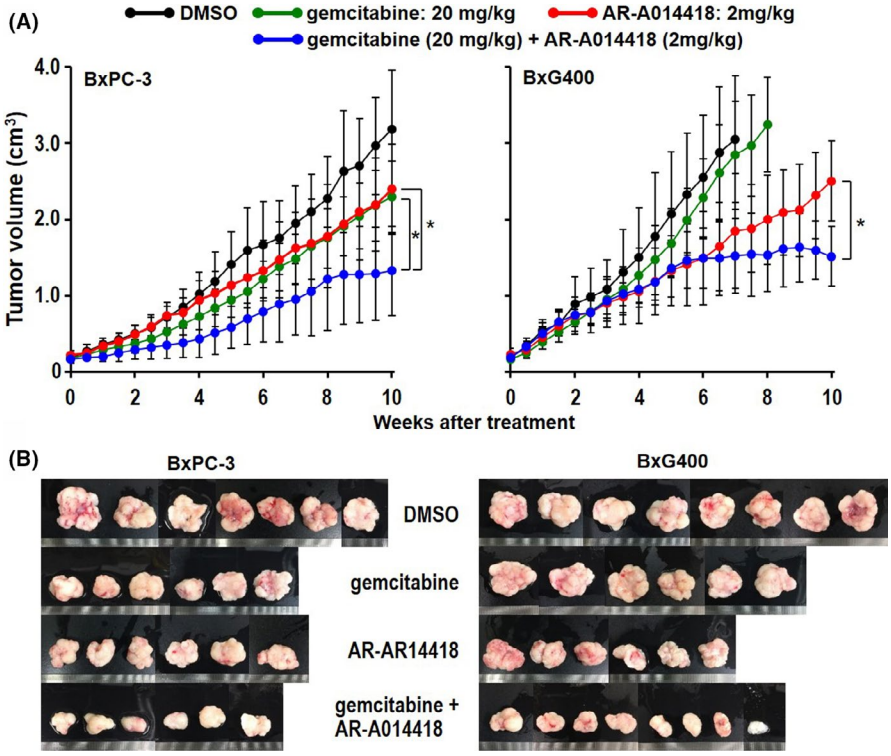

FIGURE 6 Efficacy of gemcitabine and GSK3β inhibitor on BxPC-3 and BxG400 xenograft tumors in athymic mice. A, Time course of mean xenograft tumor volume with SDs in mice treated with DMSO, gemcitabine alone, AR-A014418 alone, or the gemcitabine/AR-A014418 combination. Mice with the BxG400 xenograft and treated with DMSO or gemcitabine were euthanized at 6 and 8 wk, respectively, after treatment because of animal experiment ethical issue. *$P < .05$. B, Gross appearance of xenograft tumors removed at autopsy from the mice

with AR-A014418, but they were still higher than in BxPC-3 and BxG30 cells (Figure S6). Isobologram analysis showed synergistic therapeutic effects of gemcitabine and AR-A014418 in combination against both resistant clones (Figure 3A). Nevertheless, AR-A014418 could substantially, but not completely, reverse the resistance to gemcitabine in BxG140 and BxG400 cells (Figure S5) and BxG400 xenograft tumors in rodents (Figure 6). An intermediate metabolite of gemcitabine, dFdCDP (2′-2′-difluoro-2′-deoxycytidine diphosphate), potently binds to and inhibits RRM1, thereby exerting its therapeutic effect via decrease of competing deoxyribonucleotide pools necessary for DNA synthesis (reviewed in Ref.9). Our results may therefore imply that the amount of RRM1 in the resistant clones treated with both gemcitabine and AR-A014418 exceeded the dose of gemcitabine. Collectively, it is suggested that the remaining RRM1 in the resistant clones after treatment with GSK3β inhibitor may still contribute to gemcitabine resistance. As we previously reported,[22] resistance to gemcitabine in these clones was not only dependent largely on RRM1, but also on the other known factors (such as hENT1, dCK, RRM2, and Bcl2) and probably on unknown factors. Accordingly, future systematic analysis of RRM1 and these factors is necessary to clarify whether any remaining RRM1 in the resistant clones (BxG140, BxG400) following the treatment with GSK3β inhibitor contributes to the gemcitabine resistance in these cells.

As previously reported, the gemcitabine-resistant clones examined in this study were also resistant to 5-FU, cisplatin, irinotecan (CPT-11), and docetaxel.[22] As TS and TK1 are known biomarkers for the efficacy of 5-FU and ionizing radiation, respectively, GSK3β may participate in cross-resistance to multiple chemotherapeutic agents. Furthermore, combination with GSK3β inhibitor may potentially enhance the efficacy of FORFIRINOX, nab-paclitaxel with gemcitabine, nanoliposomal irinotecan with 5-FU, and folate, as well as ionizing radiation. In addition to pancreatic ductal adenocarcinoma, a previous study reported that GSK3β participates in tumor progression and resistance to everolimus, an inhibitor of mechanistic target of rapamycin complex 1, in pancreatic neuroendocrine neoplasm.[41] Collectively, GSK3β may potentially play broader pathologic roles in pancreatic malignancy.

The proposed biological mechanisms for gemcitabine resistance in cancer cells include pro-invasive capacity and cancer stemness phenotypes (reviewed in Ref. 9,10). Previous studies have shown that gemcitabine-resistant pancreatic cancer cells acquire a pro-invasive phenotype such as EMT,[42] thus contributing to acquired resistance.[43,44] Based on the notion of an interconnection between cancer invasion and therapy resistance,[45] we previously showed that GSK3β facilitates both pro-invasive capacity and resistance to chemotherapy in pancreatic cancer[20] and glioblastoma.[46,47] It has also been reported that gemcitabine treatment promotes pancreatic cancer stemness through a distinct molecular pathway.[48] In light of the mounting evidence for tumor-promoting roles of GSK3β, we propose that GSK3β functions as a molecular hub that integrates therapy resistance, pro-invasive capacity, and the cancer stemness phenotype in refractory cancer, as represented by pancreatic cancer.[15,16] This cancer type is also characterized biologically by a desmoplastic and immunosuppressive tumor microenvironment that has emerged as a robust barrier to various therapeutic agents and radiation.[49,50] Recent evidence has suggested that GSK3β plays an active role in establishing the

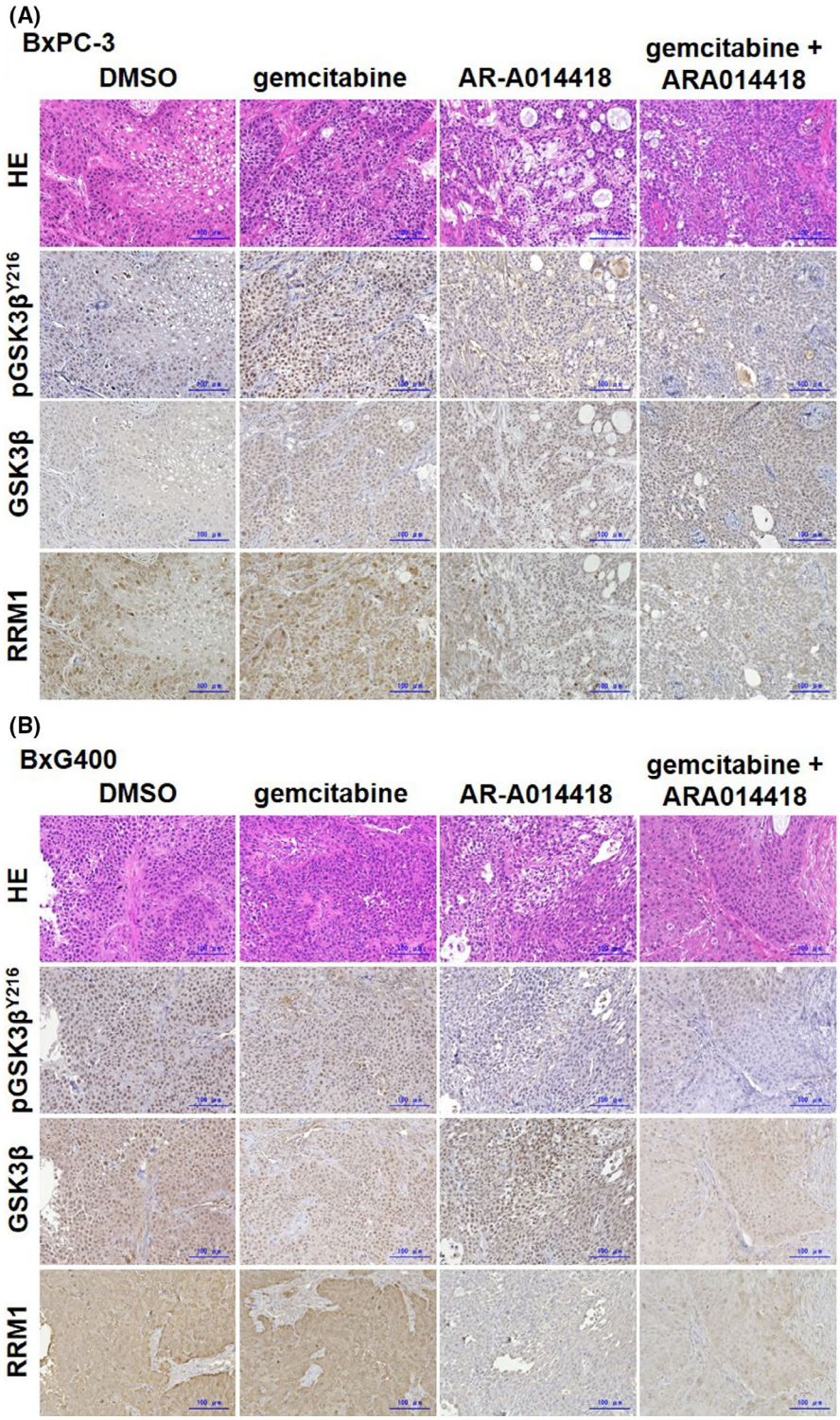

FIGURE 7 Histological and immunohistochemical findings of xenograft tumors of BxPC-3 (A) and BxG400 (B) cells in mice treated with DMSO, gemcitabine alone, AR-A014418 alone or the gemcitabine/AR-A014418 combination. Serial sections of the tumors were stained with H&E and immunostained for GSK3β, pGSK3β^{Y216}, and RRM1. Scale bars, 100 μm

immunosuppressive tumor environment (reviewed in Ref. 16). Therefore, further research in this area is important for understanding the broader biological mechanisms of GSK3β-mediated therapy resistance in pancreatic cancer.

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DISCLOSURE

The authors have no conflict of interest.

ORCID

Masahiro Uehara https://orcid.org/0000-0002-9874-2672
Takahiro Domoto https://orcid.org/0000-0002-8040-7630
Osamu Takeuchi https://orcid.org/0000-0003-3826-8654
Toshinari Minamoto https://orcid.org/0000-0003-4168-996X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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