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Gap junction beta-4 accelerates cell cycle progression and metastasis through MET–AKT activation in pancreatic cancer

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Abstract

Despite continuing advances in the development of effective new therapies, including immunotherapies, the prognosis of pancreatic cancer remains extremely poor. Gap junction proteins have become attractive targets for potential cancer therapy. However, the role of gap junction beta-4 (GJB4) protein remains unexplored in pancreatic cancer. Through bioinformatic analyses we discovered pancreatic cancer tissues showed higher levels of *GJB4* transcripts compared to normal pancreatic tissues and this had a negative effect on overall survival in patients that had pancreatic cancer. The high expression of nuclear GJB4 was identified as a negative prognostic factor in such patients. Knockdown of *GJB4* in cultured pancreatic cancer cells resulted in G_0/G_1 arrest followed by decreased cell proliferation and suppression of metastatic potential. The overexpression of *GJB4* accelerated cell proliferation, migration, and invasion in a SUIT-2 cell line, whereas MET inhibitor canceled the acceleration. *GJB4* suppression with siRNA significantly inhibited tumor growth in a mouse xenograft model. Mechanistically, suppression of GJB4 inhibited MET–AKT activities. Such data suggest that targeting the GJB4–MET axis could represent a promising new therapeutic strategy for pancreatic cancer.

KEYWORDS
cell cycle arrest, GJB4, MET–AKT, metastasis, pancreatic cancer

1 | INTRODUCTION

Worldwide, pancreatic cancer is the seventh most common cause of cancer-related deaths, with approximately half a million deaths per year.[1] Of note, the number of deaths is almost the same as the incidence of pancreatic cancer due to a lack of progress in the development of effective therapeutics.[2] Despite recent advances in precision oncology,[3] pancreatic cancer continues to show a dismal prognosis: the 5-year survival late is approximately 10%.[4] Thus, the development of novel therapeutic approaches are urgently required.

Gap junction proteins, also known as connexins, are integral membrane proteins that consist of four transmembrane domains and two extracellular loops.[5] Gap junction proteins form channels

between neighboring cells that can exchange ions and small metabolites bidirectionally. This is called gap junction intracellular communication and it plays a crucial role in various cellular homeostatic mechanisms.[6] Gap junction proteins also show channel-independent activities, such as protein–protein interactions, phosphorylation, and nuclear localization, that contribute to cellular physiology.[7] Interestingly, accumulated evidence suggests that connexins are promising therapeutic targets for cancer metastases and resistance to chemotherapies because of their ability to promote growth, migration/invasion, and chemoresistance in cancer cells.[8]

Gap junction beta-4 protein (GJB4), also termed connexin 30.3, has been shown to be mutated and has been identified as one of the causes of erythrokeratodermia variabilis et progressiva, a rare hereditary skin disease characterized by red patches and keratotic plaques.[9] Recent studies have shown that GJB4 has oncogenic properties involved in multiplying proliferation, migration/invasion, and chemoresistance in lung, gastric, and bladder cancers.[10–12] However, any role GJB4 has in pancreatic cancer remains completely unexplored.

The hepatocyte growth factor (HGF)–mesenchymal–epithelial transition factor (MET) axis has an essential role in cell proliferation, metastasis, stem cell phenotype, and resistance to chemotherapy in pancreatic cancer.[13] Therefore, the HGF–MET pathway is an attractive target for therapeutics for this cancer.[14] However, clinical trials for this pathway in pancreatic cancer have not yet shown effective antitumor activities.[15] This underscores the need to explore alternative modes of action of the HGF–MET pathway to develop therapeutics for MET-driven pancreatic cancer.

Here, given the significance of GJB4 as a target for cancer therapy, our aim was to elucidate the biological functions of this protein in pancreatic cancer cells. The *GJB4* transcript level was significantly upregulated in pancreatic cancer tissue compared to its normal counterpart. The high expression of GJB4 in the nucleus conferred a poor prognosis in patients with pancreatic cancer. Suppression of *GJB4* resulted in G_0/G_1 arrest, reduced cellular proliferation, and suppressed invasion and migration because the MET–AKT pathway was inhibited, as identified by RNA sequencing (RNA-seq). Thus, impeding GJB4 represents a promising therapeutic tool for deadly pancreatic cancer.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analysis

Gene expression levels of *GJB4* in noncancer and pancreatic cancer tissues were derived from RNA-seq data that had been deposited in the NCBI Gene Expression Omnibus (GEO). The GEO accession numbers of the data used in the analysis were GSE 62452 and GSE 28735. Publicly accessible datasets from The Cancer Genome Atlas (TCGA) and GSE 36924, and an association of *GJB4* RNA expression with clinical data, were evaluated. We used a median value as the cut-off value and the public data were classified into two groups of either a high or low expression level of *GJB4*. Survival curves were described using the Kaplan–Meier method based on these groups.

2.2 | Patients and tissue samples

We enrolled patients with pancreatic cancer who were pathologically diagnosed using endoscopic ultrasound-fine needle aspiration (EUS-FNA) at our hospital, between 2019 and 2022. For patient selection, we randomly selected up to 10 cases for each clinical stage. As a result, we recruited and analyzed 10 cases of clinical stage II, 10 cases of stage III, and nine cases of stage IV. We carried out this study with the approval of the Institutional Review Board of Sapporo Medical University (IRB 342-192) and in accordance with the guidelines of the Declaration of Helsinki.

2.3 | Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were sliced, deparaffinized, and then subjected to heat-induced antigen retrieval.[16] Slides were incubated with anti-GJB4 Ab (#40–0900; Thermo Fisher Scientific) (1:50). Subsequently, slides were incubated with rabbit Ab and a BOND Polymer Refine Detection System (Leica Biosystems). Immunohistochemical analyses of GJB4 were undertaken by two independent pathologists who were blinded to patients' clinical data.[17] Nuclear GJB4 staining intensity was evaluated using a four-tiered system for staining intensity: 1, negative; 2, weak; 3, moderate; or 4, or strong. The extent of nuclear GJB4 staining was also scored using four classifications: 1, ≤10%; 2, 11%–50%; 3, 51%–75%; or 4, >75%. The total expression of nuclear GJB4 was defined by multiplying GJB4 staining intensity and staining extent scores. A nuclear GJB4 total expression score greater than 8 was designated as positive GJB4 expression. We used 29 patient tissue samples obtained from EUS-FNA for GJB4 immunohistochemistry (IHC); however, one clinical stage II sample was excluded from the analysis due to insufficient sample volume.

2.4 | Cell lines and cell culture

AsPC-1 and SUIT-2 cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank. RPMI-1640 (Sigma-Aldrich) with 10% FBS, penicillin–streptomycin, and L-glutamine (2 mM) was used to culture cell lines.

2.5 | Gap junction beta-4 expression inhibited by siRNA and increased by expression vector

Pancreatic cancer cell lines were transfected with nontargeting control siRNA (siControl) (D-001810-01-05; Dharmacon) and two independent siRNAs targeting human GJB4 (siGJB4_1, D-016916-18

and siGJB4_2, D-016916-19; Dharmacon) using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Twenty-four hours after transfection, the medium was replaced with medium containing 0.5% FBS, and cells were further incubated for 48 h. As insulin-like growth factor-1 is associated with serum-induced GJB4 expression,[10] we used 0.5% FBS for consecutive assays.

SUIT-2 cells were transfected with a negative control cDNA ORF clone (pCMV3-untagged Negative Control Vector; Sino Biological) and a cDNA ORF clone targeting human GJB4 (GJB4 cDNA ORF Clone, Human, untagged; Sino Biological) using Lipofectamine 3000 (Thermo Fisher Scientific).

2.6 | Cell proliferation analysis

Cells were seeded into 96-well plates at a density of 3×10^3 (SUIT-2) and 8×10^3 (AsPC-1) cells/well, then transfected with siRNAs 24 h later. Culture medium was replaced with that containing 0.5% FBS 24 h after transfection, and cells incubated for indicated times. Cell viability was evaluated using a WST-1 assay (Premix WST-1 Cell Proliferation Assay; Takara Bio). In an additional analysis using a GJB4 cDNA ORF clone and MET inhibitor (capmatinib; MedChemExpress), the concentration of capmatinib used was set at 500 nM based on previous reports and our analyses.[18] SUIT-2 cells were seeded into 6-well plates at a density of 3×10^6 cells/well; after 12 h, cells were then transfected with cDNA ORF. Culture medium was replaced with that containing 0.5% FBS 12 h after transfection, and cells seeded into 96-well plates at a density of 3×10^3 cells/well. Cells were incubated for a further 24 h, and exposed to capmatinib or DMSO.

2.7 | Western blot analysis

Protein samples were separated using SDS buffer and Mini-PROTEAN TGX Gels 4–20% (#4561096; Bio-Rad). Proteins were then transferred to PVDF membranes using a QBlot kit M (ATTO). Proteins were detected in membranes using primary rabbit Abs against: GJB4 (AV36633; Sigma-Aldrich), p27 (#3686), cyclin-dependent kinase 2 (CDK2; #2546), MET (#8198), phosphorylated MET (#3077), phosphorylated AKT (#4058), AKT (#4685), Src (#2123), or phosphorylated Src (#6943), all from Cell Signaling Technology; or actin (HRP; sc-1615; Santa Cruz Biotechnology). Anti-rabbit Ab linked to HRP was used as a secondary Ab.

2.8 | Flow cytometry analysis

Cells were stained with propidium iodide (FxCycle PI/RNase Staining Solution; Thermo Fisher Scientific) and analyzed in a BD FACSCanto II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). The status of cell cycles was determined with FlowJo software (Tree Star Inc.).[19]

2.9 | Migration and invasion analyses

Migration and invasion assays were undertaken as previously described (Cell Biolabs, Inc.).[20] A cell suspension was added to each upper chamber of 24-well Transwell plates. The medium was then added to the lower chamber. For invasion assays, the upper chambers were coated with ECM basement membrane. Invasion assays are related to migration assays, but can also evaluate ECM degradation and proteolysis. Migrating and invading cells, which lay on the underside of the membrane, were fixed with methanol, stained with methylene blue, and counted in five different randomly selected fields.

2.10 | RNA sequences

Total RNA from SUIT-2 cells transfected with non-targeting control siRNA and siRNA targeting human *GJB4* (siGJB4_1) was isolated. Raw paired-end sequence reads were assessed for quality using FastQC (version 0.11.7; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic software (version 0.38) was used for the trimming of low-quality (<20) bases and adapter sequences using the following parameters: ILLUMINACLIP: path/to/adapter.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:15 MINLEN:36. Trimmed reads were aligned to a reference genome using HISAT2 (version 2.1.0). FeatureCounts (version 1.6.3) estimated the amount of uniquely mapped reads.

2.11 | Differentially expressed gene analysis

We divided TCGA, GSE 28735, GSE 36924, and GSE 62452 datasets into two groups: the top 20 with the highest expression level of *GJB4* and the bottom 20 with the lowest expression level of *GJB4* (GJB4_high and GJB4_low, respectively). Differentially expressed genes between these two groups in these datasets were detected with thresholds of $|\log2\text{fold change [FC]}| > 1$ and an adjusted p value < 0.05 using an online tool, TCC-GUI.[21] Differentially expressed genes between SUIT-2 cells transfected with nontargeting control siRNA and siGJB4_1 were identified with the same criteria.

2.12 | Subcutaneous xenograft mouse models

Methods of animal experiments in Data S1.

2.13 | Statistical analyses

Statistical analyses were undertaken using EZR (version 1.55)[22] and GraphPad Prism (version 8.4.3; GraphPad Software). All experiments were carried out in triplicate or quadruplicate. The data

were subjected to Student's and Welch's *t*-tests, Fisher's exact test, the Mann–Whitney *U* test, log rank test, and Wilcoxon signed rank sum test, or one-way ANOVA then Bonferroni's post-hoc test where appropriate. A p value <0.05 was defined as statistically significant.

3 | RESULTS

3.1 | *GJB4* more highly expressed and associated with poor prognosis in pancreatic cancer patients

GJB4 expression in patients with pancreatic cancer was investigated using RNA-seq data deposited in public databases (GSE 62452 and GSE 28735). *GJB4* transcripts in pancreatic cancer tissues were expressed at a higher level than in non-cancer pancreatic tissues (Figure 1A,B). Furthermore, for patients with pancreatic cancer, we compared their prognosis with high and low *GJB4* expression using TCGA and GSE 36924 datasets. Those showing high *GJB4* expression had a significantly shorter overall survival (Figure 1C,D). This indicated that GJB4 might have oncogenic functions and that high GJB4 expression could be a poor prognostic factor in patients with pancreatic cancer.

3.2 | Gap junction beta-4 protein expression related to distant metastasis and advanced clinical stage in pancreatic cancer patients

To examine GJB4 protein expression in pancreatic cancer tissues and analyze its relationship with clinical characteristics, we undertook an IHC analysis of GJB4 protein expression in a series of 28 pancreatic cancer tissue samples. A previous report demonstrated that GJB4 was expressed within cellular boundaries and the nucleus.[23] Gap junction beta-4 immunostaining was noted in the nucleus (Figure 2A). Subsequently, we quantified GJB4 immunostaining in cell nuclei of pancreatic cancer tissues. As a result, 20 and 8 samples were allocated to negative- and positive-staining GJB4 groups, respectively (Table 1). The associations between clinicopathologic characteristics and GJB4 staining are presented in Table 1. Interestingly, positive GJB4 staining was significantly associated with distant metastasis ($p=0.0087$) and an advanced clinical stage ($p=0.043$). The two groups did not show differences with regard to other factors. Furthermore, we analyzed the correlation between the IHC score for GJB4 and metastasis. The IHC score for GJB4 (Table S1) was significantly associated with metastasis ($p=0.0017$; Figure 2B). In addition, the GJB4 positive-staining group showed a significantly poorer prognosis than the GJB4 negative-staining

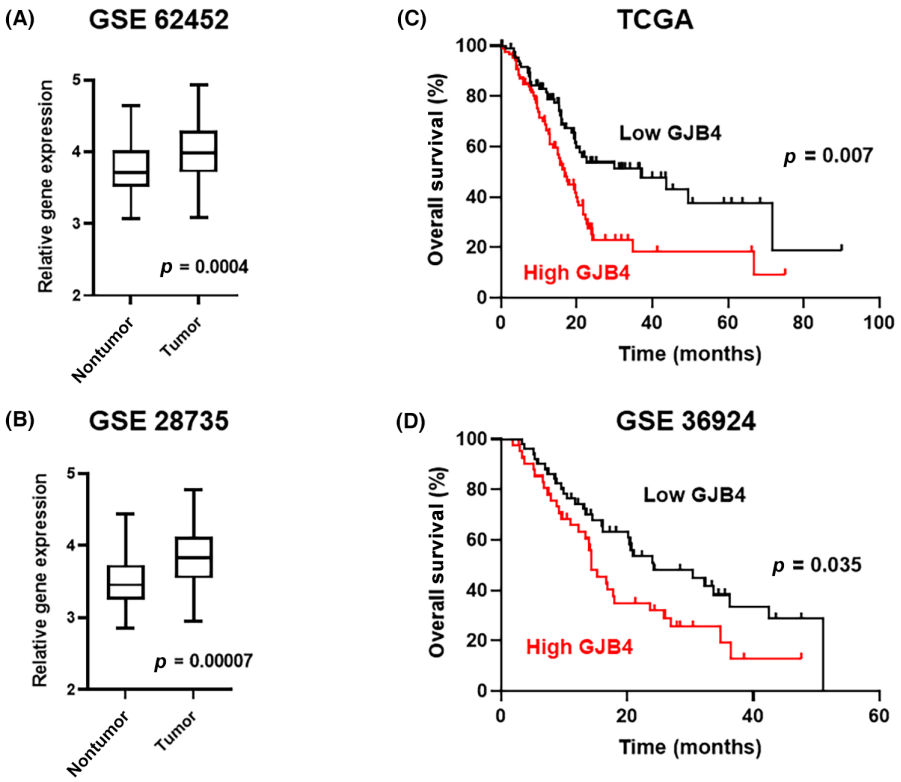

FIGURE 1 Upregulated gene expression levels of *GJB4* are associated with a poor clinical outcome in patients with pancreatic cancer. RNA expression data in the NCBI Gene Expression Omnibus (GEO) (A) GSE 62452 and (B) GSE 28735 datasets were used to assess *GJB4* expression in patients with pancreatic cancer. Any correlation of *GJB4* expression and overall survival in (C) The Cancer Genome Atlas (TCGA) and (D) GSE 36924 datasets was evaluated using Kaplan–Meier survival curves.

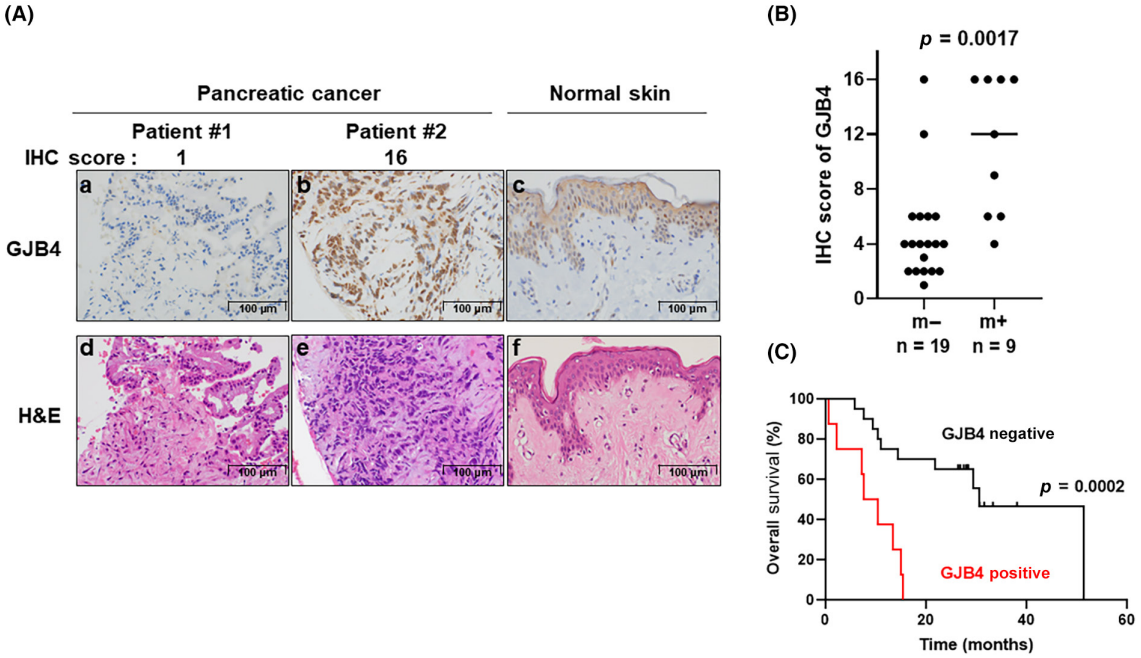

FIGURE 2 High of expression gap junction beta-4 (GJB4) is correlated with metastases and prognosis in patients with pancreatic cancer. (A) (a, b) Representative images of immunohistochemical (IHC) analyses of GJB4 expression in GJB4-negative and -positive staining pancreatic cancer tissues. (c) Normal skin tissue was used as a positive control. GJB4 scores were (a) 1×1=1 and (b) 4×4=16. (d–f) H&E staining of each sample. Scale bar, 100 μm. (B) IHC scores for GJB4 in 28 patients with or without metastases of pancreatic cancer. (C) Kaplan–Meier survival curves of IHC scores for GJB4-based overall survival in patients with pancreatic cancer. IHC score >8 is defined as positive GJB4. m–, distant metastasis-negative; m+, distant metastasis-positive.

group, with median overall survival rates of 8.5 and 22.4 months, respectively ($p=0.0002$; Figure 2C).

3.3 | *GJB4* knockdown suppresses cell growth by inducing cell cycle arrest at G_0/G_1 phase and metastatic potential in pancreatic cancer cells

Next, we evaluated the influence of GJB4 in pancreatic cancer using the authenticated pancreatic cancer cell lines, SUIT-2 and AsPC-1, in which *GJB4* was knocked down by two independent siRNAs (siGJB4_1 and siGJB4_2). The *GJB4* knockdown efficiency of these siRNAs was demonstrated using western blotting (Figure 3B,E) and RT–quantitative PCR (Figure S1). WST-1 assays showed *GJB4* knockdown significantly inhibited the in vitro proliferation of both pancreatic cancer cell lines (Figure 3A,D). To elucidate the mechanisms of growth inhibition caused by *GJB4* knockdown, we undertook apoptosis and cell-cycle assays in *GJB4* knockdown pancreatic cancer cell lines. *GJB4* knockdown did not trigger apoptosis (Figure S2); however, in both cell lines it significantly increased cell cycle arrest at the G_0/G_1 phase (Figure 3C,F). Western blot analysis of G_0/G_1-related proteins showed that *GJB4* knockdown induced increased p27 expression and decreased CDK2 expression in both cell lines (Figure 3B,E). These results indicated that inhibition of *GJB4* led to suppressed cell growth via G_0/G_1 arrest in pancreatic cancer cells. Using clinicopathologic analyses, we found that GJB4 likely increased the metastatic potential of pancreatic cancer (Figure 2). These results prompted us to evaluate cellular migration and invasion in vitro. Transwell assays revealed that the knockdown of *GJB4* significantly inhibited migration and invasion in both cell lines (Figure 4).

3.4 | *GJB4* knockdown suppressed the MET–AKT pathway in pancreatic cancer cells

To explore the regulatory mechanism of GJB4 participation in pancreatic cancer cell growth and migration/invasion, high-throughput RNA-seq, and differentially expressed gene (DEG) analyses were carried out between SUIT-2 cells transfected with nontargeting si-Control and siRNA targeting human *GJB4* (siGJB4_1) (Figure 5A). The DEG analysis of SUIT-2 cells showed 333 significantly downregulated genes ($|\log2FC|>1$ and adjusted p value <0.05) after siGJB4_1 transfection. In addition, we divided the RNA-seq data obtained from TCGA, GSE 28735, GSE36924, and GSE62452 datasets into GJB4_high and GJB4_low groups, and undertook DEG analyses in these settings (Figure 5A). The DEG analysis of TCGA, GSE 28735, GSE62452, and GSE36924 datasets showed 217, 34, 25, and 85 significantly downregulated genes ($|\log2FC|>1$ and an adjusted p value <0.05) in the GJB4_low group, respectively. We discovered that, except for *GJB4*, only one gene, *MET*, was significantly downregulated by all DEG analyses in SUIT-2 cells after siGJB4_1 transfection and TCGA, GSE 28735, GSE36924, and GSE63452 datasets of the GJB4_low group (Figure 5B). Next, we examine the

TABLE 1 Characteristics of 28 pancreatic cancer patients, grouped by gap junction beta-4 (GJB4) protein expression.

	GJB4 negative ($n=20$)	GJB4 positive ($n=8$)	p value
Age (years)	70 (50–83)	65 (46–82)	0.5600
Sex			1.0000
Male	9 (45)	4 (50)	–
Female	11 (55)	4 (50)	–
Pancreatic tumor location			0.5500
Ph	13 (65)	3 (37)	–
Pb	4 (20)	3 (37)	–
Pt	3 (15)	2 (25)	–
Size of tumor (mm)	29 (14–60)	33.5 (16–54)	–
Metastasis			0.0087
Positive	3 (15)	6 (75)	–
Metastatic lesions			–
HEP	2 (10)	6 (75)	–
PER	1 (5)	4 (50)	–
LYM	0 (0)	1 (12)	–
PUL	1 (5)	1 (12)	–
Clinical stage			0.0430
II	7 (35)	2 (25)	–
III	10 (50)	0 (0)	–
IV	3 (15)	6 (75)	–
Pathologic diagnosis			1.0000
PDAC	19 (95)	8 (100)	–
IPMC	1 (5)	0 (0)	–
CEA (ng/mL)	2.0 (1.1–133)	3.8 (0.6–579)	0.2900
CA19-9 (U/mL)	54 (10.1–4648)	59.2 (9.9–1383)	0.5800

Note: Data are shown as mean (range) or *n* (%).

Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; HEP, liver metastasis; IPMC, intraductal papillary mucinous carcinoma; LYM, lymph node metastasis; Pb, pancreatic body; PDAC, pancreatic ductal adenocarcinoma; PER, peritoneal metastasis; Ph, pancreatic head; Pt, pancreatic tail, PUL, pulmonary metastasis.

association between levels of *GJB4* and *MET* transcripts. An analysis of the Spearman correlation coefficient showed a significant positive relationship ($r=0.57$, $p=1.2\times10^{-16}$) between *GJB4* and *MET* mRNA expression in the TCGA datasets of patients with pancreatic cancer (Figure 5C). We hypothesized that GJB4 activates the MET signaling pathway, then induces cell cycle progression and upregulates metastatic properties in pancreatic cancer cells. AKT, which is recognized as a downstream target molecule of MET, is a positive regulator of the cell cycle, and of migration/invasion.[15,24] Additionally, Src has been identified to be involved in the MET signaling pathway in lung adenocarcinoma.[10] Therefore, we analyzed expression levels of GJB4 and MET, phosphorylated MET, AKT, phosphorylated AKT, Src, and phosphorylated Src using SUIT-2 and AsPC-1 cell lines into which nontargeting siControl and siRNAs targeting human *GJB4* (siGJB4_1 and siGJB4_2) were transfected. Western blot analysis revealed that MET, phosphorylated MET (Tyr-1234/1235), AKT, and phosphorylated AKT (Ser-473) were suppressed in both GJB4 knockdown pancreatic cancer cell lines (Figure 5D). In contrast, downregulated levels of phosphorylated Src (Thy416) were faint compared to changes in phosphorylated MET and phosphorylated AKT. This observation implied the existence of Src-independent pathways.

Then, to identify the plausible mechanism between GJB4 and MET expression, we investigated the localization of GJB4 and MET with immunofluorescence analyses in pancreatic cancer cell lines. GJB4 localized in the cytoplasm (Figure S3), which differed from the nuclear localization of GJB4 noted in clinical samples (Figure 2A). In addition, MET was expressed in the plasma membrane, suggesting that GJB4 did not bind to MET directly, at least in cultured cells.

3.5 | Overexpression of GJB4 activates the MET–AKT pathway in SUIT-2 cells

To further explore the relationship between GJB4 and the MET–AKT signaling pathway, we analyzed protein expression levels of GJB4 and MET, phosphorylated MET, AKT, and phosphorylated AKT using the

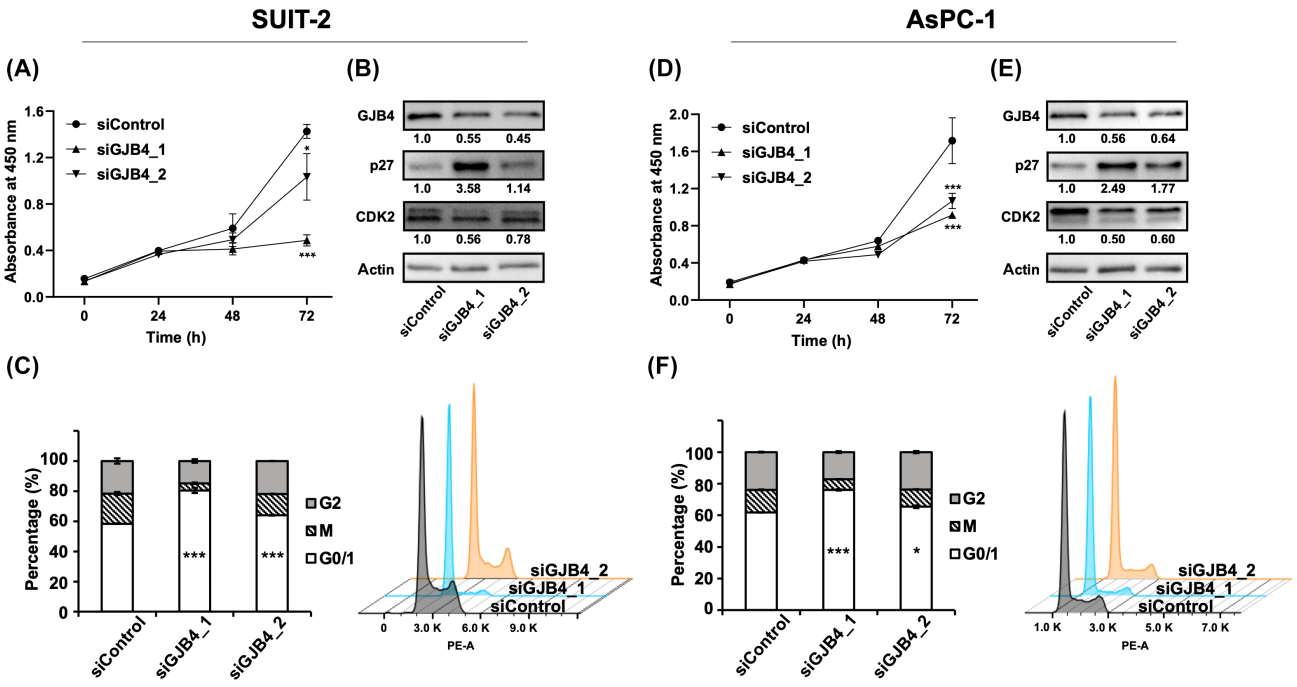

FIGURE 3 Knockdown of *GJB4* induces the inhibition of cell growth caused by cell cycle arrest at the G_0/G_1 phase in pancreatic cancer cell lines. Cell proliferation of (A) SUIT-2 and (D) AsPC-1 cell lines was assessed using a WST-1 assay after siRNA transfection. Cell cycle-related proteins were evaluated by western blotting of protein samples from (B) SUIT-2 and (E) AsPC-1 cell lines. Cell cycles in (C) SUIT-2 and (F) AsPC-1 cells were evaluated using a flow cytometer. A comparison was made of the percentage of G_0/G_1 cells transfected with siRNA targeting human *GJB4* with cells transfected with nontargeting siRNA (siControl). Values represent mean ± SE. $^{*}p < 0.05$, $^{***}p < 0.001$.

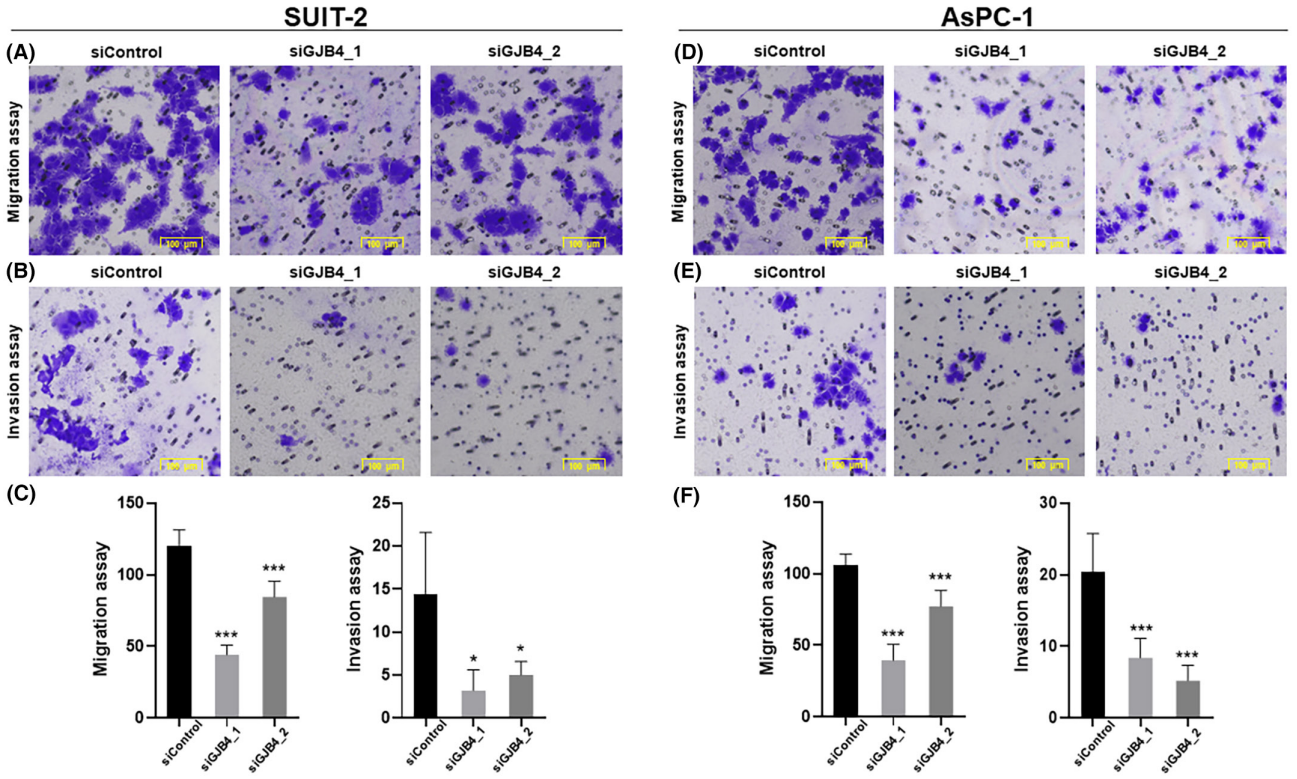

FIGURE 4 *GJB4* knockdown inhibited migration and invasion in pancreatic cancer cell lines. Migration/invasion of (A–C) SUIT-2 and (D–F) AsPC-1 cells was evaluated using a Transwell assay after siRNA transfection. Representative photographs of migration/invasion assays in (A, B) SUIT-2 and (D, E) AsPC-1 cells. Scale bar, 100 μm. (C, F) Summarized results of quintuplicate measurements from two independent experiments. Values represent the mean ± SE. $^{*}p < 0.05$, $^{***}p < 0.001$.

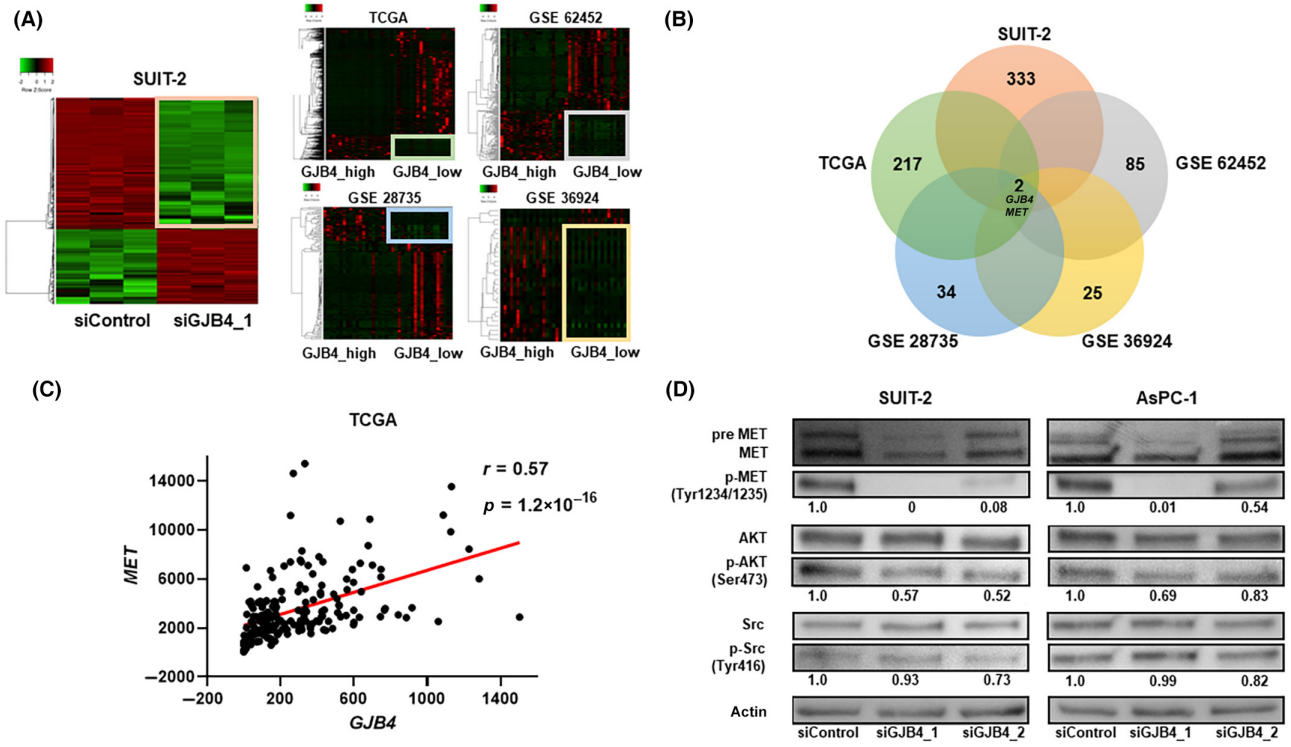

FIGURE 5 MET and AKT are downstream target molecules of gap junction beta-4 (GJB4). (A) Heatmap showing 191 upregulated and 333 downregulated genes from a DEG analysis of SUIT-2 cells transfected with nontargeting siRNA (siControl) and siRNA targeting human *GJB4* (siGJB4_1). The heatmap depicts the top 913, 99, 4, and 138, and bottom 217, 34, 25, and 85 genes from the differentially expressed gene analysis of samples with high and low *GJB4* expression in The Cancer Genome Atlas (TCGA), GSE 28735, GSE 36924, and GSE 62452 datasets. (B) Orange, green, blue, yellow, and gray circles represent genes downregulated by *GJB4* knockdown in SUIT-2 cells and genes downregulated in *GJB4*-low tumors in TCGA, GSE 28735, GSE 36924, and GSE 62452 datasets, respectively. Each colored circle represents the area enclosed by the respective colored square shown in (A). (C) Correlation between *GJB4* and *MET* in patients with pancreatic cancer using TCGA. (D) Western blot analysis showed pre-MET, MET, phosphorylated (p-)MET, AKT, p-AKT, Src, and p-Src proteins in SUIT-2 and AsPC-1 cells after transfection with nontargeting siRNA (siControl) and two independent siRNAs that targeted human *GJB4* (siGJB4_1 and siGJB4_2).

SUIT-2 cell line into which negative control cDNA ORF clone and a cDNA ORF clone targeting human GJB4 were transfected. These were then treated or not with the MET inhibitor, capmatinib. We confirmed that in pancreatic cancer cell lines, the phosphorylation of MET and AKT was inhibited by capmatinib (Figure S4). Western blot analysis revealed that GJB4 overexpression upregulated phosphorylated MET (Tyr-1234/1235) and phosphorylated AKT (Ser-473) proteins, whereas capmatinib impaired upregulated phosphorylated MET and AKT in GJB4 overexpressed cells (Figure 6A). Subsequently, we confirmed that GJB4 overexpression promoted cell proliferation. Notably, capmatinib canceled the acceleration of cell proliferation and migration/invasion induced by overexpressed GJB4 (Figure 6B–F). Collectively, these findings indicated that GJB4 activates the MET–AKT signaling pathway to promote cell growth and migration/invasion of pancreatic cancer cells.

3.6 | *GJB4* knockdown inhibits xenograft tumor growth

To examine the effects of GJB4 on pancreatic cancer cell lines in vivo, we used a subcutaneous xenograft mouse model injected with AsPC-1/CMV-Luc cells (Figure 7A). The size of tumors of GJB4 knockdown cells after using siRNA targeting human *GJB4* (siGJB4_1) was significantly smaller than those of controls (siControl) ($p = 0.031$), indicating *GJB4* knockdown inhibited cell growth (Figure 7B,C). In this model, GJB4 immunostaining was noted in the nucleus as well as seen in human specimens. Interestingly, GJB4 knockdown tumors after using siRNA targeting human *GJB4* showed markedly reduced nuclear GJB4 staining (Figure 7D).

4 | DISCUSSION

Here, we report that GJB4 has an essential role in pancreatic cancer cell growth, invasion, and migration through the MET–AKT pathway. Bioinformatic and clinicopathologic analyses reveal that high expression of GJB4 is associated with a poor prognosis and upregulated proliferative and metastatic potential. Collectively, these findings suggest GJB4 is a potential target for pancreatic cancer therapeutics.

Except for mutated *GJB4*, which has been identified as the causative gene for erythrokeratodermia variabilis et progressiva, the roles and signaling pathways of GJB4 have not been completely

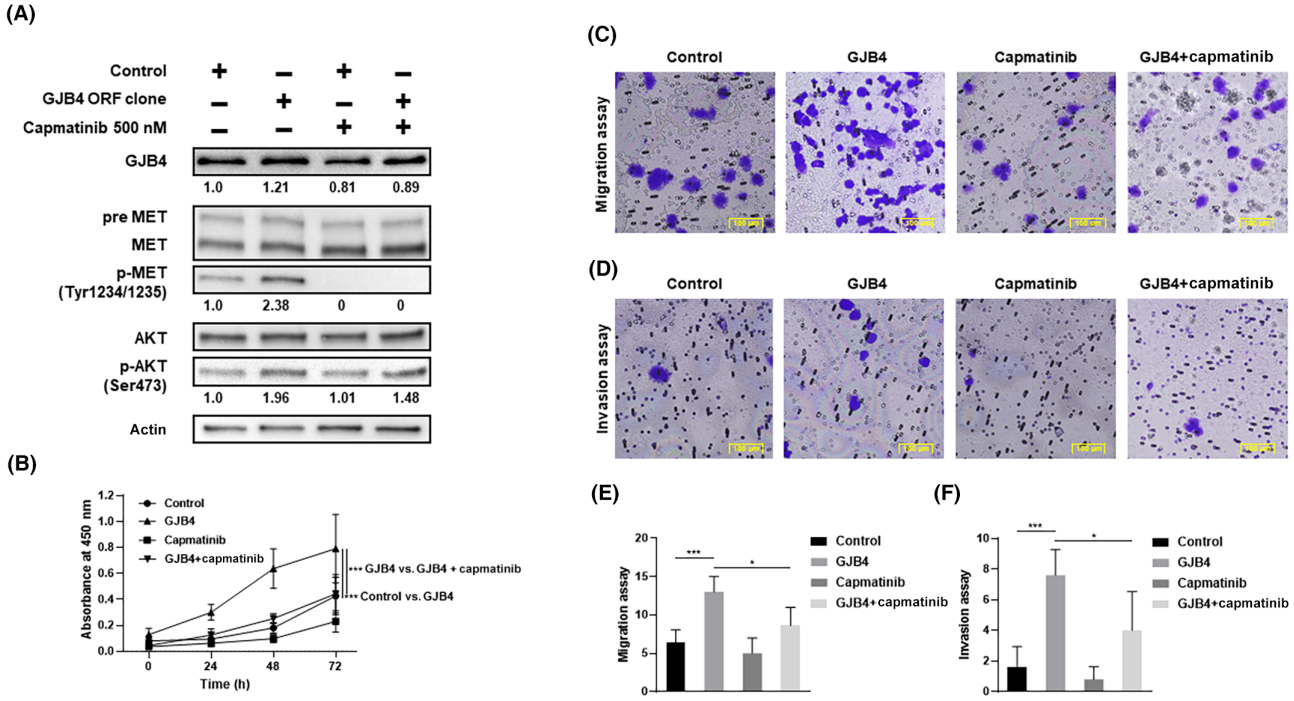

FIGURE 6 Gap junction beta-4 (GJP-4) activated the MET–AKT signaling pathway in pancreatic cancer cells. (A) Western blot analysis showed pre-MET, MET, phosphorylated (p-)MET, AKT, and p-AKT proteins in SUIT-2 cells after transfection with a negative control cDNA ORF clone (Control) and cDNA ORF clone targeting human GJB4 (GJB4). Treatment groups consisted of cells exposed or not to the MET inhibitor, capmatinib. (B) Growth of SUIT-2 cells was assessed after transfection with a cDNA ORF clone, and compared with cells exposed or not to capmatinib using a WST-1 assay. (C, D) Cell migration and invasion ability of SUIT-2 cells was assessed after transfection with a cDNA ORF clone, and compared to cells exposed or not to capmatinib. Scale bar, 100 μm. (E, F) Summarized results of quintuplicate measurements from two independent experiments. Values represent mean ± SE. $^{*}p < 0.05$, $^{***}p < 0.001$.

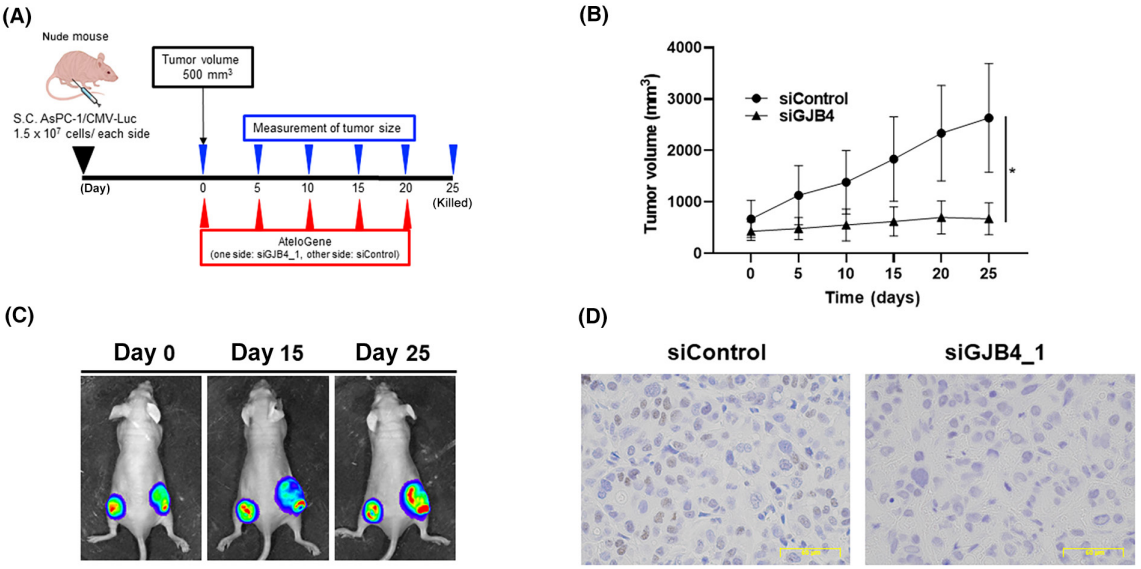

FIGURE 7 Effects of gap junction beta-4 (GJB4) on a pancreatic cancer cell line in a mouse model. (A) Schematic of xenograft mouse model. (B) Tumor volume changes in each tumor group using siRNA (siControl) and siRNA targeting human *GJB4* (siGJB4_1) subcutaneously transplanted with AsPC-1/CMV-Luc. (C) Representative in vivo bioluminescence imaging system (IVIS)-Lumina images of each siRNA tumor up to the end-point. Mice were injected with tumors with siGJB4_1 in their left side and with siControl in the right side. (D) Representative images of immunohistochemical analyses of GJB4 expression in *GJB4* knockdown and control tumors. Scale bar, 50 μm. Values represent mean ± SE. $^{*}p < 0.05$.

elucidated in either normal or cancer cells. With regard to the introduction of GJB4 inhibitors as cancer therapeutics, further functional analyses will be needed to avoid unexpected treatment-related adverse events.

As reported here, MET has emerged as a therapeutic target for patients with pancreatic cancer.[15] Selective MET inhibitors, such as tepotinib and capmatinib that are successful as cancer therapeutics, have been approved for patients harboring a *MET* exon 14 skipping mutation in non-small-cell lung cancer.[25,26] Regarding pancreatic cancer, Li et al. found that MET is a bona fide pancreatic cancer-specific receptor tyrosine kinase through comprehensive analyses and that high MET expression is related to a worse prognosis.[27] Although MET is a candidate for pancreatic cancer therapeutics, as mentioned above, to date, clinical trials of MET inhibitors for pancreatic cancer have not met clinical needs.

Immune checkpoint inhibitors (ICIs) and conventional chemotherapy plus ICIs are now successfully used in the clinic as standard therapies in many cancer clinical trials.[28–34] However, these have failed to show clinical benefits in pancreatic cancer[35] because of poor T cell infiltration into the tumor, a highly immunosuppressive tumor microenvironment (TME), and a low tumor mutational burden.[36] Li et al. also revealed that MET bound to and upregulated programmed death-ligand 1 (PD-L1) expression and then created an immunosuppressive TME, leading to resistance to immunotherapy.[27] Finally, the combination of a MET inhibitor plus PD-L1 blockade has been identified as a promising strategy for pancreatic cancer. Apart from the immune system, mounting evidence revealed that the TME accelerates cell proliferation, metastatic activities, and chemoresistance in pancreatic cancer.[37,38] Gap junction beta-4 could also affect the metastatic potential and functions of pancreatic cancer TME. Unfortunately, we could not evaluate whether GJB4 increased metastases and influenced the TME because it was neither a model of metastases nor of allogeneity. We will clarify this hypothesis in future. Thus, the GJB4–MET pathway should be investigated as a novel tool for changing the TME to improve immunotherapies.

An unresolved part of the current study is the mechanism by which GJB4 regulates MET expression. To elucidate this mechanism, we examined the localization of GJB4 in cultured cells, human samples, and a mouse xenograft model. As shown in Figure S3, GJB4 localized in the cytoplasm and MET was expressed in the plasma membrane, suggesting that GJB4 did not bind to MET directly in cultured cells. Interestingly, the localization of GJB4 was in the nucleus in human samples (Figure 2A) and a mouse xenograft model (Figure 7D). This suggests that GJB4 may translocate into the nucleus in vivo and may then act as an oncogenic protein. This discrepancy of GJB localization and the functions of nuclear GJB4 should be investigated as a next step in future research.

Several limitations exist in this study. We revealed that positive staining of GJB4 was a negative prognostic factor in our cohort of patients with pancreatic cancer. However, most patients showing GJB4-positive IHC were diagnosed with stage IV disease. Therefore, we should be careful in interpreting findings. Additionally, the small number of patients recruited from a single hospital and retrospective analyses caused selection bias, and the multivariate analyses used to identify independent prognosis factors were inappropriate because our cohort was small. We are presently planning a prospective multi-institutional observational study to confirm our results.

In summary, this work identified that GJB4 was more highly expressed in pancreatic cancer tissues, and could serve as a valuable biomarker for predicting patients with pancreatic cancer. Gap junction beta-4 promotes cell proliferation and metastatic activities through activation of the MET–AKT pathway. Our findings suggest GJB4 is a novel target for this deadly cancer.

AUTHOR CONTRIBUTIONS

Joji Muramatsu: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft. **Yohei Arihara:** Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft. **Makoto Yoshida:** Resources. **Tomohiro Kubo:** Data curation; formal analysis; investigation; methodology; resources; validation; visualization; writing – original draft. **Hajime Nakamura:** Data curation; investigation; validation. **Kazuma Ishikawa:** Resources. **Hiromi Fujita:** Data curation; formal analysis; investigation; validation. **Shintaro Sugita:** Data curation; formal analysis; investigation; methodology; validation; visualization. **Takumi Konno:** Data curation; investigation; methodology; validation; visualization; writing – original draft. **Takashi Kojima:** Data curation; investigation; methodology; validation; visualization. **Yutaka Kawano:** Data curation; investigation; methodology; software. **Masayoshi Kobune:** Conceptualization; methodology. **Kohichi Takada:** Conceptualization; data curation; investigation; methodology; project administration; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

K.T. received lecture fees from Daiichi Sankyo. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. We have submitted our RNA sequencing data to the DNA Data Bank of Japan (DDBJ) Repository. They are available in the DDBJ Sequenced Read Archive under the accession numbers DRR437000–DRR437005 and E-GEAD-590.

ETHICS STATEMENTS

Approval of the research protocol by an institutional review board: We had the approval of the Institutional Review Board of Sapporo Medical University (IRB 342-192) and conducted this study according to Declaration of Helsinki principles.

Informed consent: Patients were provided with opportunities to decline participation in this study using the "opt-out" option on our hospital website.

Registry and registration no. of the study/trial: N/A.

Animal studies: We had the approval of the Animal Research Committee of Sapporo Medical University (23–080) and the Genetic Recombination Experiments Committee (R5-211).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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