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Full Paper

CA9 and PRELID2; hypoxia-responsive potential therapeutic targets for pancreatic ductal adenocarcinoma as per bioinformatics analyses

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ABSTRACT

A strong hypoxic environment has been observed in pancreatic ductal adenocarcinoma (PDAC) cells, which contributes to drug resistance, tumor progression, and metastasis. Therefore, we performed bioinformatics analyses to investigate potential targets for the treatment of PDAC. To identify potential genes as effective PDAC treatment targets, we selected all genes whose expression level was related to worse overall survival (OS) in The Cancer Genome Atlas (TCGA) database and selected only the genes that matched with the genes upregulated due to hypoxia in pancreatic cancer cells in the dataset obtained from the Gene Expression Omnibus (GEO) database. Although the extracted 107 hypoxia-responsive genes included the genes that were slightly enriched in angiogenic factors, TCGA data analysis revealed that the expression level of endothelial cell (EC) markers did not affect OS. Finally, we selected *CA9* and *PRELID2* as potential targets for PDAC treatment and elucidated that a CA9 inhibitor, U-104, suppressed pancreatic cancer cell growth more effectively than 5-fluorouracil (5-FU) and PRELID2 siRNA treatment suppressed the cell growth stronger than CA9 siRNA treatment. Thus, we elucidated that specific inhibition of PRELID2 as well as CA9, extracted via exhaustive bioinformatic analyses of clinical datasets, could be a more effective strategy for PDAC treatment.

Abbreviations: CA, carbonic anhydrase; CAF, cancer-associated fibroblast; ERK-5, extracellular signal-regulated kinase 5; EC, endothelial cell; GEO, Gene Expression Omnibus; GO, Gene Ontology; HIFs, hypoxia-inducible factors; OS, overall survival; PDAC, pancreatic ductal adenocarcinoma; PRELI, the proteins of relevant evolutionary and lymphoid interest; PRELID2, PRELI domain containing 2; TCGA, The Cancer Genome Atlas; VEGF, vascular endothelial growth factor.

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1. Introduction

Five-year overall survival (OS) rate in pancreatic cancer patients is lower than that of patients with other cancers. Although early stage of pancreatic cancer is asymptomatic and surgical treatment cannot be applied for advanced stage of pancreatic cancer, no chemotherapy is completely effective to heal it. Metastatic pancreatic cancer has much poor OS also. A strong hypoxic environment exists in pancreatic ductal adenocarcinoma (PDAC), and it contributes to drug resistance, tumor progression, and metastasis.[1–7] Although several studies have proposed new strategies targeting hypoxia in the tumor microenvironment for PDAC treatment, none of them have been completely effective. Hypoxia-inducible factors (HIFs) are the key transcription factors that respond to hypoxia; HIF-1α is a key factor responsible for malignancy in the tumor microenvironment.[8] In our previous studies, we reported the roles of HIFs in vascular remodeling[9–12] and that the cancer-associated fibroblast (CAF)-derived extracellular signal-regulated kinase 5 (ERK5) deficiency–HIF–1α induction axis changes tumor vasculature and contributes to colon cancer progression.[13]

HIF-1α is a key regulator of tumor angiogenesis, and the vascular endothelial growth factor gene is one of its targets.[8] Previous studies have suggested that angiogenesis can be targeted for suppressing PDAC growth,[14–16] and tumor vasculature is essential for tumor metastasis, which is a crucial factor for PDAC malignancy. In the present study, we selected candidate target genes from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets and screened them for angiogenic factor-coding genes to find a potential therapeutic target in the hypoxic tumor microenvironment of PDAC. We identified genes related to worse OS in TCGA database and selected the genes that overlapped with the genes that were upregulated due to hypoxia in pancreatic cancer cells in the GEO database. We analyzed 107 hypoxia-responsive genes using Gene Ontology (GO) analysis and found that "angiogenesis-related genes" was the fourth highest enriched term for some genes. However, the expression level of endothelial cell (EC) markers did not affect OS as per TCGA analysis. This unfortunately means that angiogenesis may not be able to a therapeutic target for PDAC. Finally, we selected two hypoxia-responsive genes, namely carbonic anhydrase IX (CA9) and the proteins of relevant evolutionary and lymphoid interest (PRELI) domain containing 2 (PRELID2), as targets for PDAC treatment. Then, we investigated whether acetazolamide administration, a carbonic anhydrase (CA) inhibitor used at the bedside, can reduce the incidence of pancreatic cancer via the Food and Drug Administration (FDA) Adverse Events Reporting System (FAERS) database analysis, whether CA9 inhibition can suppress pancreatic cancer cell growth effectively in *in vitro* experiments, and whether PRELID2 siRNA treatment also suppressed the cell growth as well as CA9 siRNA treatment in this study.

2. Materials and methods

2.1. TCGA analysis

The University of California Santa Cruz Xena browser was used to obtain the pancreatic cancer data from TCGA (TCGA-PAAD dataset).[17] For survival analysis, all gene expression values ($\log_2$ of the normalized count of 19,913 genes that were assigned gene symbols in the dataset) of 178 pancreatic cancer samples with "sample_type" as "Primary Tumor" were extracted from the PAAD dataset. For each gene, the samples were divided into two groups, i.e., high and low expression groups, based on the median of each gene expression value. The survival probability of each group was calculated using Kaplan–Meier analysis, and significance was analyzed using the log-rank test. The hazard ratios for the high expression group were calculated relative to the low expression group. We extracted 1450 protein-coding genes with significantly poor survival probability in the high expression group as the genes related to poor survival probability. The protein-coding genes were defined with reference to GRCh38.p13 of GENCODE (https://www.gencodegenes.org/). Kaplan-Meier survival curves were shown for five genes (VWF, PECAM1, KDR, TEK and CDH5). Statistical analyses were performed using R (version 3.4.1), and significance was assumed at $p < 0.05$.

For gene expression analysis, gene expression changes of all genes in 178 pancreatic cancer samples with "sample_type" as "Primary Tumor" from the PAAD dataset were measured based on the gene expression level of four normal tissue samples with "sample_type" as "Solid Tissue Normal" using the R package edgeR (version 3.34.0). The fold change log-value ($\log_2$FC), count per million log-value ($\log_2$CPM), and p-value of all genes were calculated. 603 protein-coding genes were defined as significantly upregulated in tumors with $\log_2\text{FC} \geq 1$, $\log_2\text{CPM} \geq 1$ and $p < 0.05$. The protein-coding genes were defined with reference to GRCh38.p13.

2.2. Gene Expression Omnibus (GEO) database analysis

We used the Genevestigator software (https://genevestigator.com/, version: 9.5.0, Release Date: 2022-10-06) to analyze the data retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), i.e., dataset GSE9350.[18] Gene expression changes in "hypoxia study 5" (three samples) compared to those in "untreated pancreatic carcinoma cell line (FG) sample" (three samples) were calculated by "Differential Expression" analysis, and the $-\log_{10}p$-value and $\log_2$FC for each gene were obtained. We defined 641 genes as significantly upregulated based on a false discovery rate (FDR) < 0.05 and $\log_2\text{FC} > 0.6$. In addition, 556 genes were extracted as protein-coding genes by referring to GRCh38.p13.

2.3. Gene ontology (GO) analysis

For GO analysis, enrichment analyses of GOTERM_BP_DIRECT and KEGG_PATHWAY genesets were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (http://david.ncifcrf.gov/). The p-value for each term was obtained, and the $-\log_{10}p$-value was calculated as the enrichment score.

2.4. Food and Drug Administration (FDA) Adverse Events Reporting System (FAERS) database analysis

FAERS is a database used for post-marketing drug safety surveillance and contains data on drug safety, including adverse events, patient demographics, and medication errors. The adverse event terms reported in this study are based on the Medical Dictionary for Regulatory Activities v18.1. Brand names were changed to generic names as per the DrugBank database[19] (https://www.drugbank.ca/). Duplicate reports were excluded following FDA recommendations. The MySQL Community Server (version 5.7.39) software was used to build a database by integrating information from the FAERS database.

The reporting odds ratio (ROR) was calculated to detect drug-associated adverse events in a spontaneous reporting database.[20,21] To calculate ROR, the reported adverse events associated with pancreatic cancer (13 terms in total; Table 2) were defined as "cases," and all other reported adverse events were defined as "non-cases." The RORs were calculated using two-by-two

Table 1
Details of the 12 candidate genes presented in Fig. 2A and B.

Gene Symbol	Description
CA9	carbonic anhydrase 9
OVOL1	ovo like transcriptional repressor 1
GJB3	gap junction protein beta 3
EREG	epiregulin
EPHA2	ephrin type-A receptor 2
SCEL	sciellin
BCAR3	breast cancer anti-estrogen resistance 3
MGLL	monoglyceride lipase
TUFT1	tuftelin 1
LONRF3	LON peptide N-terminal domain and ring finger 3
PRELID2	PRELI domain containing 2
CHMP4C	charged multivesicular body protein 4C

contingency tables of counts indicating the presence or absence of acetazolamide administration and pancreatic cancer incidence in the reports. These values are expressed as point estimates with 95% confidence intervals (CIs). When the upper limit of the 95% CI of an ROR signal was less than 1.0 and there were two or more cases, the signal was considered statistically significant. The ROR and 95% CI for all adverse events and individual adverse events associated with pancreatic cancer were calculated. The subgroup analysis calculated the ROR in male or female cases after excluding cases with unknown gender. Moreover, ROR was calculated in cases over and under the age of 60 years after excluding cases with unknown age.

2.5. Cell culture

MIA PaCa-2 cells,[22,23] a human PDAC cell line, were purchased from JCRB Cell Bank (JCRB0070; Ibaraki City, Osaka, Japan)[24] and maintained in Dulbecco's modified Eagle medium (high glucose concentration) with L-glutamine and phenol red (044–29765; Wako, Osaka, Japan) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% MEM non-essential amino acid solution (139–15651; Wako). PANC-1 cells, another human PDAC cell line, were provided by the RIKEN BRC through the National BioResource Project of the MEXT, Japan (RCB2095; Tsukuba City, Ibaraki, Japan) and maintained in RPMI1640 (30264-14; nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured in 5% CO_2 and 95% room air (21% O_2) at 37 °C for the normoxic condition (Nx) or in 5% CO_2 and 1% O_2 for the hypoxic condition (Hx).

2.6. Quantitative real-time PCR

The cells were seeded in 6-well plates one day before stimulation. The cells were incubated under the Hx or Nx for 24 h. Total RNA from MIA PaCa-2 cells or PANC-1 cells was extracted as per the modified Acid Guanidinium-Phenol-Chloroform method using RNAiso Plus (9109 TaKaRa Bio; Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Total RNA (1000 ng) was used for cDNA synthesis with ReverTra Ace qPCR RT Master Mix (FSQ-301; TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The PCR mixture contained cDNA (equivalent to 33 ng total RNA), forward and reverse primer mix, and THUNDERBIRD SYBER qPCR Mix (QPS-201; TOYOBO). PCR was performed in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 1 min at 95 °C and 45 cycles of 10 s at 95 °C and 40 s at 60 °C. Human β-actin gene expression was used to normalize sample amplification. Four independent samples in each group were used for real-time PCR. The human-specific primers used in this study are listed in Table 3[10,25–32]. Statistical significance was analyzed using Unpaired *t*-test (n = 6) using GraphPad Prism 9.4.1.

2.7. MTT assay

The cells were seeded in 24-well plates one day before stimulation. We used 5-fluorouracil (5-FU; 064–01403; Wako) or CA9 inhibitors, U-104 (S2866; Selleck chemicals, Houston, TX, USA) and acetazolamide (A2598; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), under the hypoxic condition or normoxic condition, and the cells were incubated for 48 h. After stimulation, MTT reagent (349–01824 DOJINDO; Kumamoto, Japan) dissolved in PBS was added (0.5 mg/mL final), and the cells were incubated for 4 h. After incubation, the medium was removed, and dimethyl sulfoxide was

Table 2
Reporting odds ratio (ROR) of adverse events related to the use of acetazolamide for pancreatic carcinoma treatment as per the (Food and Drug Administration Adverse Events Reporting System) FAERS database analysis.

	ROR	95% CI	With drug		Without drug	
			Case	Non-case	Case	Non-case
Pancreatic cancer	**0.16**	**0.04–0.65**	2	6308	28588	14540318
Pancreatic carcinoma	–	–	1	6309	22590	14546316
Pancreatic carcinoma metastatic	–	–	1	6309	2312	14566594
Pancreatic carcinoma stage IV	–	–	0	6310	1618	14567288
Adenocarcinoma pancreas	–	–	0	6310	1048	14567858
Pancreatic carcinoma stage III	–	–	0	6310	353	14568553
Pancreatic carcinoma stage II	–	–	0	6310	312	14568594
Pancreatic carcinoma stage I	–	–	0	6310	224	14568682
Pancreatic carcinoma recurrent	–	–	0	6310	138	14568768
Ductal adenocarcinoma of pancreas	–	–	0	6310	114	14568792
Intraductal papillary-mucinous carcinoma of pancreas	–	–	0	6310	49	14568857
Pancreatic carcinoma stage 0	–	–	0	6310	29	14568877
Acinar cell carcinoma of pancreas	–	–	0	6310	17	14568889
Mucinous cystadenocarcinoma of pancreas	–	–	0	6310	7	14568899
Subgroup						
Gender						
Male	–	–	1	2114	13058	5089807
Female	–	–	1	3602	11194	7867450
Age						
<60	–	–	1	2635	5054	4497791
≥60	–	–	1	1958	9504	3891033

Bold indicates a statistically significant.

Table 3
Human oligonucleotide primers used in quantitative Real-Time PCR.

(Human)	Forward	Reverse
CA9	5'-GATGAGAAGGCAGCACAGAAGG-3'	5'-CTCTGGCTGGCTTCTCACATTC-3'
OVOL1	5'-ACGATGCCCATCCACTACCTG-3'	5'-TTTCTGAGGTGCTGGTCATCATTC-3'
GJB3	5'-GCCCCCTGCCCCAACATCGTG-3'	5'-GTGGCAGCGGCAGGTGGAAGC-3'
EREG	5'-CTGCCTGGGTTTCCATCTTCT-3'	5'-GCCATTCATGTCAGAGCTACACT-3'
EPHA2	5'-TGGCTCACACACCCGTATG-3'	5'-GTCGCCAGACATCACGTTG-3'
SCEL	5'-TGGTCTCTGGCTAGAGTTAGCAATAA-3'	5'-CCACCACTCACAGCCAACAT-3'
BCAR3	5'-GCGGTGGAACTGAAGGATTC-3'	5'-TGGCAGTTTGGGTGTACTGG-3'
MGLL	5'-GGAAACAGGACCTGAAGACC-3'	5'-ACTGTCCGTCTGCATTGAC-3'
TUFT1	5'-CCAAGCAGAACGAGAAAAGG-3'	5'-TGGCTCTTGAGCATGTCATC-3'
LONRF3	5'-TGCATCTTTCGACGCATCTG-3'	5'-TGCACAATGGACACTTTGCG-3'
PRELID2	5'-TGCCAGCACATTCTTACGAC-3'	5'-CACCACACTGTTCCTTTAGCAG-3'
CHMP4C	5'-AGGTTCGAGAAACAGCTCACTC-3'	5'-TTGCTGCAAAGCCCATGTTC-3'
β-actin	5'-GCGGGAAATCGTGCGTGACATTA-3'	5'-ATGGAGTTGAAGGTAGTTTCGTG-3'

added to dissolve the generated formazan crystals. To evaluate cell proliferation, absorbance was measured at 538 nm. Statistical significance was analyzed using GraphPad Prism 9.4.1 via a one-way analysis of variance (ANOVA) followed by Tukey's test for the comparison of multiple groups (n = 12).

2.8. siRNA treatment

For the small interfering RNA (siRNA) assay, the cells were transiently transfected with control siRNA (sc-37007; SANTA CRUZ BIOTECHNOLOGY, INC.) as a control, human CA9-specific siRNA (sc-29869; SANTA CRUZ BIOTECHNOLOGY, INC.) or human PRELID2-specific siRNA (sc-91841; SANTA CRUZ BIOTECHNOLOGY, INC.) (10 nmol/L) by Lipofectamine™ RNAiMAX (13778030; Thermo Fisher Scientific) in antibiotics-free medium according to the manufacturer's instructions (reverse transfection method).[10] After 24 h from transfection, the medium was changed to complete media and hypoxic or normoxic stimulation for 24 h was started as described in the figure legends.

2.9. Western blotting

The cell lysate was prepared in lysis buffer containing 20 mM HEPES-Na, 5 mM EDTA-Na, 5 mM EGTA-Na, 50 mM NaF, 50 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 250 mM sucrose, 1.5% Triton-X100, 1 mM Na_3VO_4, 50 mg/L soybean trypsin inhibitor, 25 mg/L aprotinin, 0.15 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride and 0.1% sodium dodecyl sulfate (SDS). A volume of cell lysate corresponding to 40–60 μg of total protein were resolved on SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes as described previously.[10] The membranes were blocked in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) containing 5% milk powder for 90 min at room temperature and then incubated overnight at 4 °C with one of the following primary antibodies: anti-CA9 antibody (NB-100-417SS; Novus) at a dilution of 1:500 or anti-PRELID2 antibody (HPA042486-25UL; Sigma Aldrich) at a dilution of 1:250. The membranes were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (#7074; Cell signaling Technology) for 1 h at room temperature, and the signals were developed using Pierce ECL Western Blotting Substrate (32106; Thermo Fisher Scientific). Immunoreactive bands were detected using ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare) and were quantified by Image J1.52a software (National Institutes of Health, Bethesda, MD, USA). After the stripping the antibodies, every membrane was reprobed with mouse anti-β-actin antibody (sc-47778; SANTA CRUZ BIOTECHNOLOGY, INC.). The densitometry of β-actin bands was used to normalize the expression level of samples.

2.10. Statistical analysis

Detailed statistical analysis methods are described along with each method in this section and in figure legends.

3. Results

3.1. TCGA analysis combined with GEO transcriptome analysis picked up hypoxia-responsive 107 genes related to poor survival probability, but the expression level of EC markers did not affect on survival probability

We analyzed the TCGA-PAAD dataset, which revealed that a higher expression level of 1450 protein-coding genes was related to poor survival probability in PDAC patients (Fig. 1A). Moreover, since the hypoxic environment in pancreatic cancer contributes to resistance to chemotherapy and malignancy, we identified 556 protein-coding genes (674 genes containing 118 genes which are outside of protein-coding genes (Fig. 1B)) that were significantly upregulated by hypoxia in FG cells,[18] via GEO transcriptome analysis of the GSE9350 dataset (Fig. 1A and B). We observed that 107 genes were common between the 1450 genes identified in the TCGA analysis and the 556 genes identified in the GEO analysis (Fig. 1A). Next, we hypothesized that the overlapping genes could be potential therapeutic targets for PDAC and performed GO analysis (Fig. 1C), which revealed that "Response to hypoxia" was the most enriched term (Fig. 1C), whereas "regulation of angiogenesis" was the fourth-most enriched term; moreover, the expression level of five EC-specific markers, namely, von Willebrand factor (*VWF*), PECAM1 (*CD31*), KDR (*VEGFR2*), TEK (*TIE2*), and CDH5 (*VE-cadherin*), did not affect the survival probability rate (Fig. 1D). This suggests that tumor angiogenesis does not affect OS rate in PDAC patients, and other crucial factors are responsible for the poor OS.

3.2. Twelve genes were extracted as candidates for potential therapeutic targets for PDAC from the 107 genes

To identify potential therapeutic candidates among the common genes identified in Section 3.1, we analyzed the TCGA-PAAD dataset, which revealed that 603 genes were higher expressed in Primary Tumor than Solid Tissue Normal in PDAC patients (Fig. 2A). We observed that 12 genes were common between the 603 genes and the 107 genes identified in Fig. 1A (Fig. 2A and Table 1). The 12 extracted genes were ranked based on the expression level in the tumor (log_2FC, TCGA-PAAD), relativity to lower OS (Hazard ratio, TCGA-PAAD), and expression level by hypoxia (log_2FC, GSE9350), and sorted and colored based on the ranks (Fig. 2B). The top gene of the ranks was *CA9*, which was also contained in the top enriched

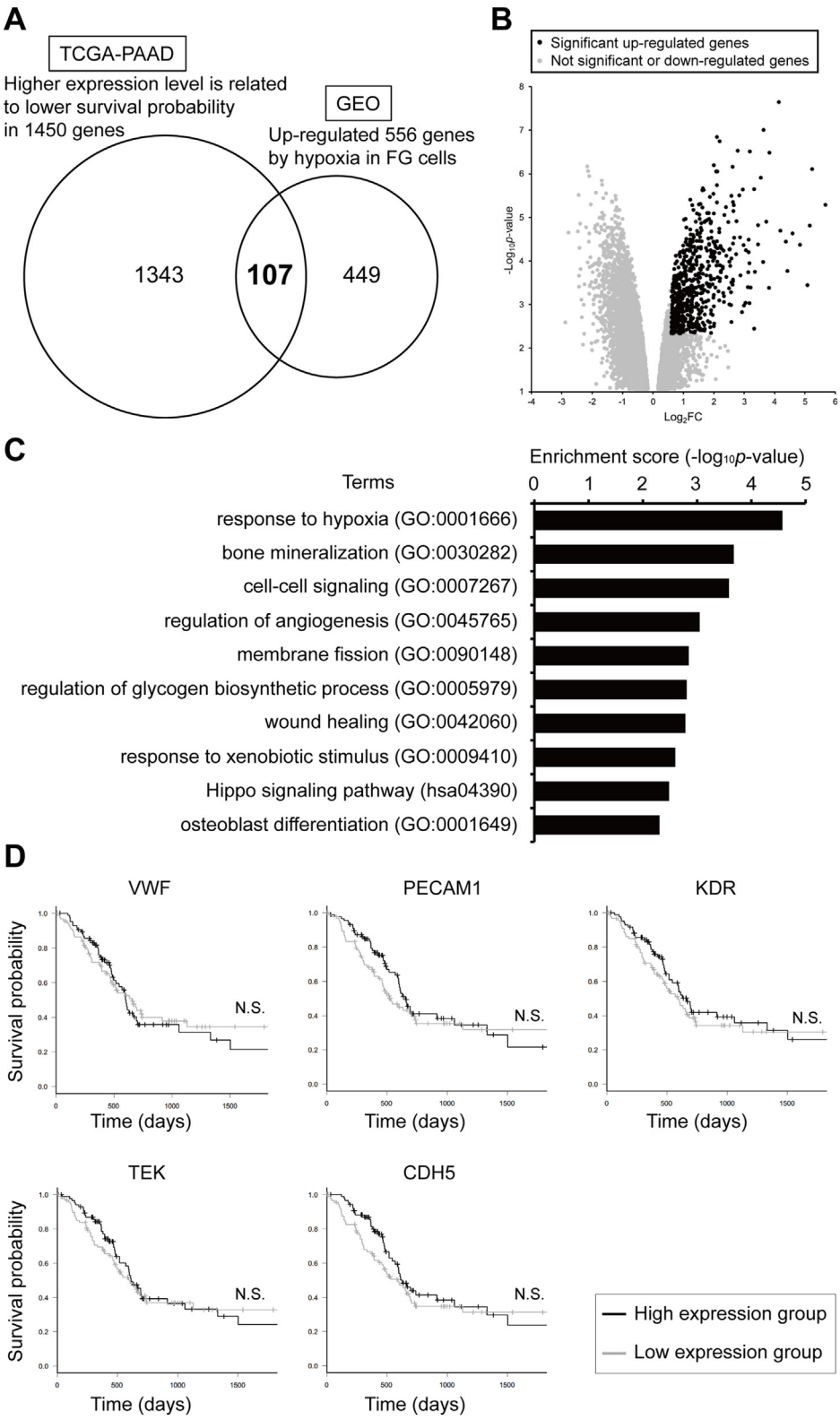

Fig. 1. Analysis of Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets revealed 107 hypoxia-responsive genes related to poor survival probability. **(A)** Overlap between 1450 protein-coding genes related to poor survival probability from TCGA analysis and 556 hypoxia-responsive protein-coding genes from GEO analysis. **(B)** Volcano plot developed using data from GEO analysis. Significantly upregulated genes (674 genes containing 118 genes which are outside of protein-coding genes) are indicated as black dots, and those not significant or downregulated genes are indicated as gray dots. **(C)** Top 10 enriched terms as per Gene Ontology (GO) analysis of the 107 genes. The bar graph indicates each enrichment score (-$\log_{10}p$-value). **(D)** Kaplan–Meier plots based on the expression of each endothelial cell (EC)-marker gene (*VWF*, *PECAM1*, *KDR*, *TEK*, and *CDH5*) (higher or lower expression than the median) from TCGA analysis. Data were analyzed using the Log-rank test; N.S: not significant.

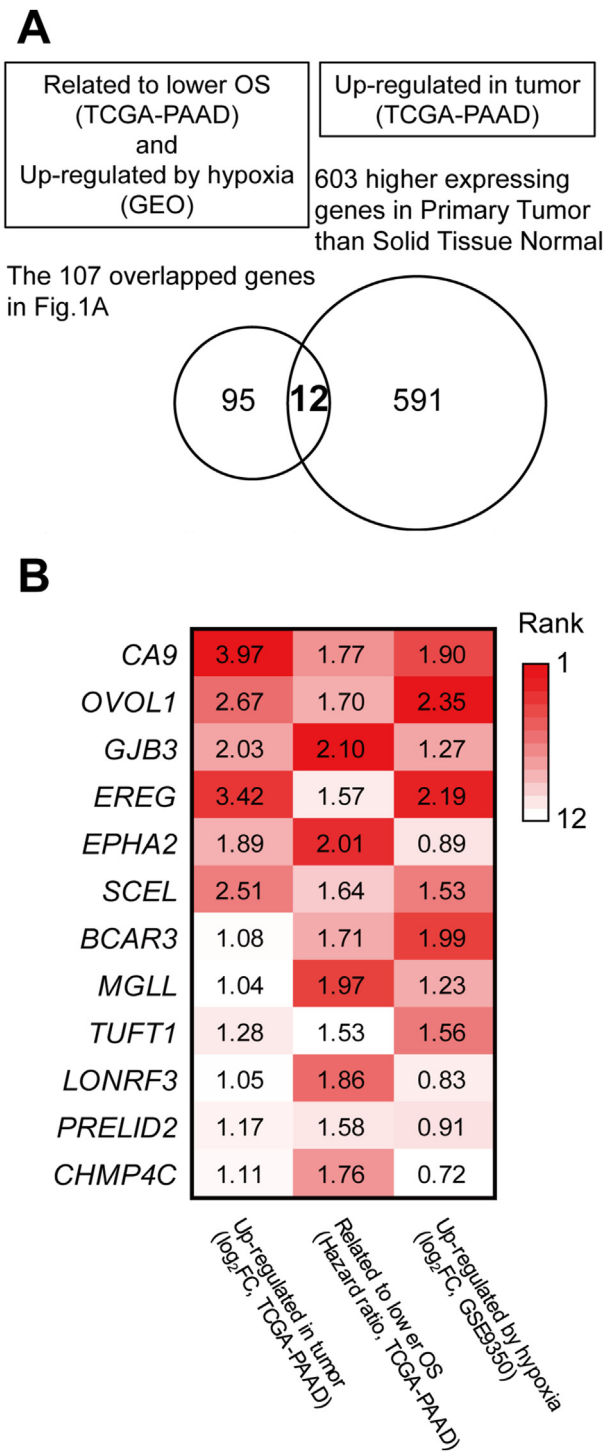

Fig. 2. Twelve potential therapeutic candidates were identified among the 107 common genes. **(A)** Twelve genes were common between the 603 genes which were higher expressed in Primary Tumor than Solid Tissue Normal in PDAC patients from TCGA analysis and the 107 genes identified in Fig. 1A. **(B)** Heatmap of the extracted 12 genes. Each gene was ranked based on the expression level in the tumor (log_2FC, TCGA-PAAD), relativity to lower OS (Hazard ratio, TCGA-PAAD), and expression level by hypoxia (log_2FC, GSE9350), and sorted and colored based on the ranks.

term "response to hypoxia" as per GO analysis (Fig. 1C; genes classified in each term are not shown). These results suggest that CA9 is a crucial target for suppressing pancreatic cancer cell growth. CA9 is a membrane-associated CA, and many studies have already reported that CA9 inhibition could suppress cancer cell growth or lead to chemoradiation therapy sensitivity in some kinds of other cancer cells.[33,34] Moreover, phase II clinical trials of a CA9 inhibitor, U-104 in combination with gemcitabine are under progress for the treatment of metastatic pancreatic ductal cancer.[35,36] These reports strengthen that the candidates extracted from our bioinformatics analyses can be crucial therapeutic targets for PDAC.

3.3. Five genes in the 12 candidates were significantly induced by hypoxia in MIA PaCa-2 cells

We checked hypoxia-induced mRNA expression level of the 12 candidate genes extracted in Fig. 2 in MIA PaCa-2 pancreatic cancer cells. The five genes; *CA9*, *OVOL1*, *GJB3*, *MGLL* and *PRELID2* are also significantly induced by hypoxia in our *in vitro* study (Fig. 3). *CA9* had the top fold change by Hx, and *PRELID2* had the second largest change, although the other 3 genes had lower fold change than 2-fold, so we focused on *CA9* and *PRELID2* from the 12 candidates in our *in vitro* system. Hypoxia-induced mRNA expression of those two genes was also observed in another human PDAC cell line, PANC-1 cells (Supplementary Figure 1).

3.4. Administration of a CA inhibitor, acetazolamide significantly reduced the incidence of pancreatic cancer in FAERS analysis

Acetazolamide, a non-specific inhibitor of CAs, is clinically used as a diuretic agent or for treating patients with glaucoma. To investigate the effect of the CA inhibitor on pancreatic cancer in real-world clinical practice, we used the FAERS database to estimate the occurrence of pancreatic cancer in patients treated with acetazolamide. The FAERS database contained 14,575,216 reports from January 2004 to March 2022, including 28,590 reports related to pancreatic cancer (13 terms) and 6310 cases treated with acetazolamide. We determined that any adverse event related to pancreatic cancer was not reported or reported only once in the case of acetazolamide administration, and a significant inverse association was observed between overall pancreatic cancer adverse events and acetazolamide administration (ROR: 0.16; 95% CI: 0.04–0.65) (Table 2). Thus, CA inhibition could reduce pancreatic cancer incidence.

3.5. A CA9 inhibitor, U-104 suppressed PDAC cell proliferation more than 5-FU, and it enhanced 5-FU-induced suppressive effect of pancreatic cancer cell growth

U-104, also known as SLC-0111, is a CA9 inhibitor. In the present study, U-104 suppressed the proliferation of MIA PaCa-2 cells under Hx at a lower concentration (10 μM) than that of 5-FU (30 μM) (Fig. 4A). Also under Nx, U-104 showed the stronger suppression effect than 5-FU, and both U-104 and 5-FU suppressed the cell proliferation under Nx more than Hx (Supplementary Figure 2). Although 5-FU (30 μM) alone could suppress cell proliferation, a combination of U-104 (10 μM) and 5-FU promoted this effect under Hx (Fig. 4B). Thus, CA9 inhibition can enhance the pharmacotherapeutic effects of 5-FU and be a potential therapeutic strategy for PDAC. U-104 and 5-FU did not have the ability to suppress hypoxia-induced mRNA expression level of *CA9* and *PRELID2* (Fig. 4C).

3.6. PRELID2 siRNA treatment suppressed pancreatic cancer cell growth stronger than CA9 siRNA treatment

PRELID2 was also contained in the 12 candidates as well as *CA9* and its mRNA expression change induced by hypoxia was the second highest in MIA PaCa-2 cells. The protein expression level of CA9 and PRELID2 was also induced by hypoxia in MIA PaCa-2 cells (Fig. 5A and B). CA9 siRNA treatment reduced both CA9 protein

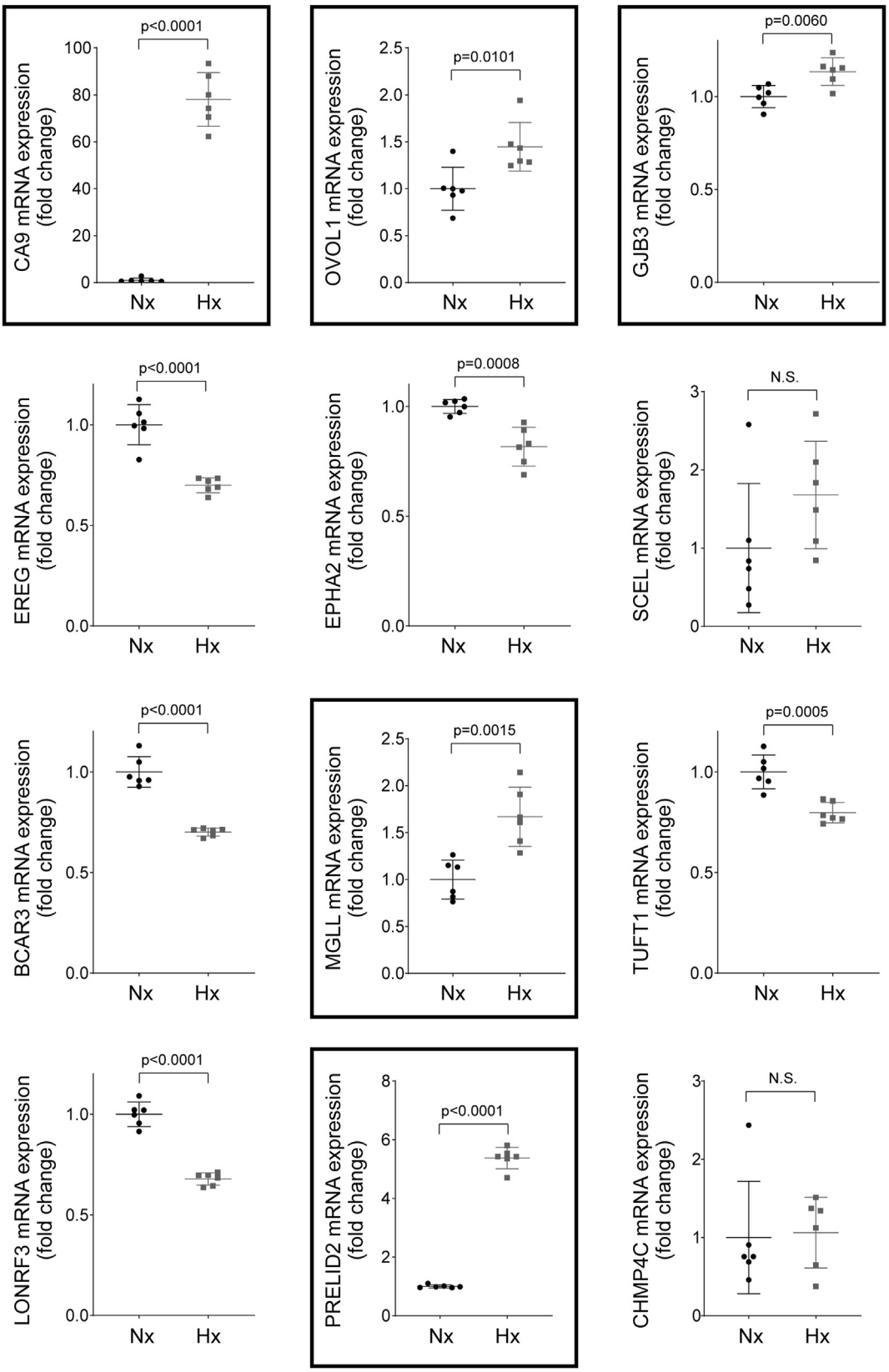

Fig. 3. mRNA expression level of Five genes (*CA9*, *OVOL1*, *GJB3*, *MGLL* and *PRELID2*; surrounded by squares) in the 12 candidates was induced by Hx for 24 h in MIA PaCa-2 cells. Data were analyzed using Unpaired *t*-test; N.S: not significant. Data are expressed as mean ± SD, n = 6.

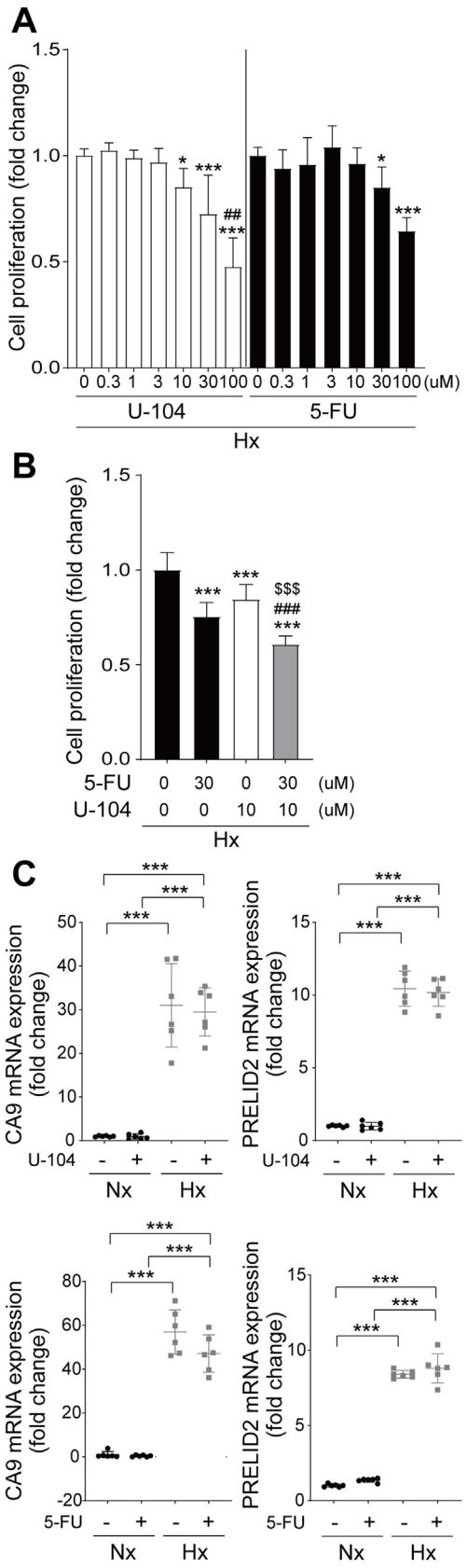

Fig. 4. The suppressive effect of U-104 compared to that of 5-fluorouracil (5-FU) on MIA PaCa-2 cell proliferation and the combined effect of U-104 with 5-FU under hypoxic conditions (Hx). **(A)** The effect of U-104 or 5-FU on cell proliferation under Hx for 48 h as per MTT assay. The cell viability by U-104 (white bar graph) or 5-FU (black bar graph) was indicated as the ratio compared to each control. Data were analyzed using one-way ANOVA followed by the Tukey test; *$p < 0.05$ vs. control, ***$p < 0.001$ vs. control, and ##$p < 0.01$ vs. 5-FU. Mean ± SD, n = 12. **(B)** U-104 (10 μM), in combination with 5-FU (30 μM), suppressed cell proliferation more than that by 5-FU alone or U-104 alone under Hx for 48 h. Data were analyzed using one-way ANOVA followed by the Tukey test; ***$p < 0.001$ vs. control, ###$p < 0.001$ vs. 5-FU alone, and \$\$\$$p < 0.001$ vs. U-104 alone. Data are expressed as mean ± SD, n = 12. **(C)** The effects of U-104 (10 μM) or 5-FU (30 μM) on hypoxia (24 h)-induced CA9 and PRELID2 mRNA expression level. U-104 or 5-FU stimulation was started 1 h before hypoxia. Data were analyzed using one-way ANOVA followed by the Tukey test; ***$p < 0.001$. Data are expressed as mean ± SD, n = 6.

level and mRNA level, and PRELID2 siRNA treatment reduced both PRELID2 protein level and mRNA level successfully (Fig. 5C–F), then those siRNA treatment significantly suppressed MIA PaCa-2 cell proliferation under Hx for 24 h (Fig. 5G). The suppression effect of PRELID2 siRNA treatment was stronger than that of CA9 siRNA treatment, and the double-knockdown enhanced the effects of CA9 siRNA treatment and PRELID2 siRNA treatment under Hx (Fig. 5G). Under Nx, PRELID2 siRNA treatment still had the stronger suppression effect than CA9 siRNA treatment (Supplementary Figure 3). Thus, PRELID2 can be a new more effective therapeutic target than CA9. To the best of our knowledge, this is the first study to report that PRELID2 could be a potential target for PDAC treatment using bioinformatics analyses.

4. Discussion

In the present study, hypoxia-responsive 12 genes were extracted as candidates for potential therapeutic targets for PDAC by our bioinformatics analyses (TCGA-PAAD dataset and GEO dataset). In our *in vitro* experiments with MIA PaCa-2 cells, five genes out of the 12 candidates were induced significantly by Hx. *CA9* and *PRELID2* had more than 2-fold mRNA expression change by Hx, so we checked the effects of their specific inhibition on MIA PaCa-2 cell growth in our *in vitro* systems. A real-world database, FAERS analysis strengthened CA inhibition can be a therapeutic strategy for PDAC, then our *in vitro* studies showed that a CA9 inhibitor, U-104 and CA9 siRNA treatment suppressed MIA PaCa-2 cell proliferation (U-104 was more effective than 5-FU.). These results will contribute to developing new therapeutic strategies using CA9 inhibitors such as U-104. Moreover, phase II clinical trials of U-104 in combination with gemcitabine are under progress for the treatment of metastatic pancreatic ductal cancer.[35,36] Our results showed that U-104 or CA9 siRNA treatment were also effective under Nx. Actually, U-104 inhibits not only CA9 but also carbonic anhydrase XII (CA12), so this might be a reason why U-104 suppressed the cell proliferation under Nx more than Hx. Many papers have also showed that CA9 knockdown suppressed cancer cell proliferation under Hx,[25,37–39] and Hyuga et al. showed that CA9 knockdown reduced cell viability even under Nx in HepG2 cells.[25] CA9 might still have key roles in cell viability also under Nx in some cancer cell lines. Although another CA9 inhibitor, acetazolamide did not suppress MIA PaCa-2 cell proliferation under Hx (Supplementary Figure 4), we supposed that specific inhibition of CA9 may be more effective to suppress pancreatic cancer cell growth. Moreover, although several papers have examined the effect of acetazolamide on pancreatic cancer cell growth,[40,41] its suppression effect was limited depending on the kind of cell lines.

In our previous studies, we investigated the role of HIFs in vascular remodeling[9–13] and elucidated that the CAF-derived ERK5 deficiency–HIF–1α induction axis contributes to colon cancer progression with tumor vasculature changes in a mouse model.[13] In the present study, we focused on targeting the hypoxic environment in pancreatic cancer, and based on the combined analyses of the two datasets, we identified 107 hypoxia-responsive genes that correlated with lower OS rates in patients with PDAC. GO analysis

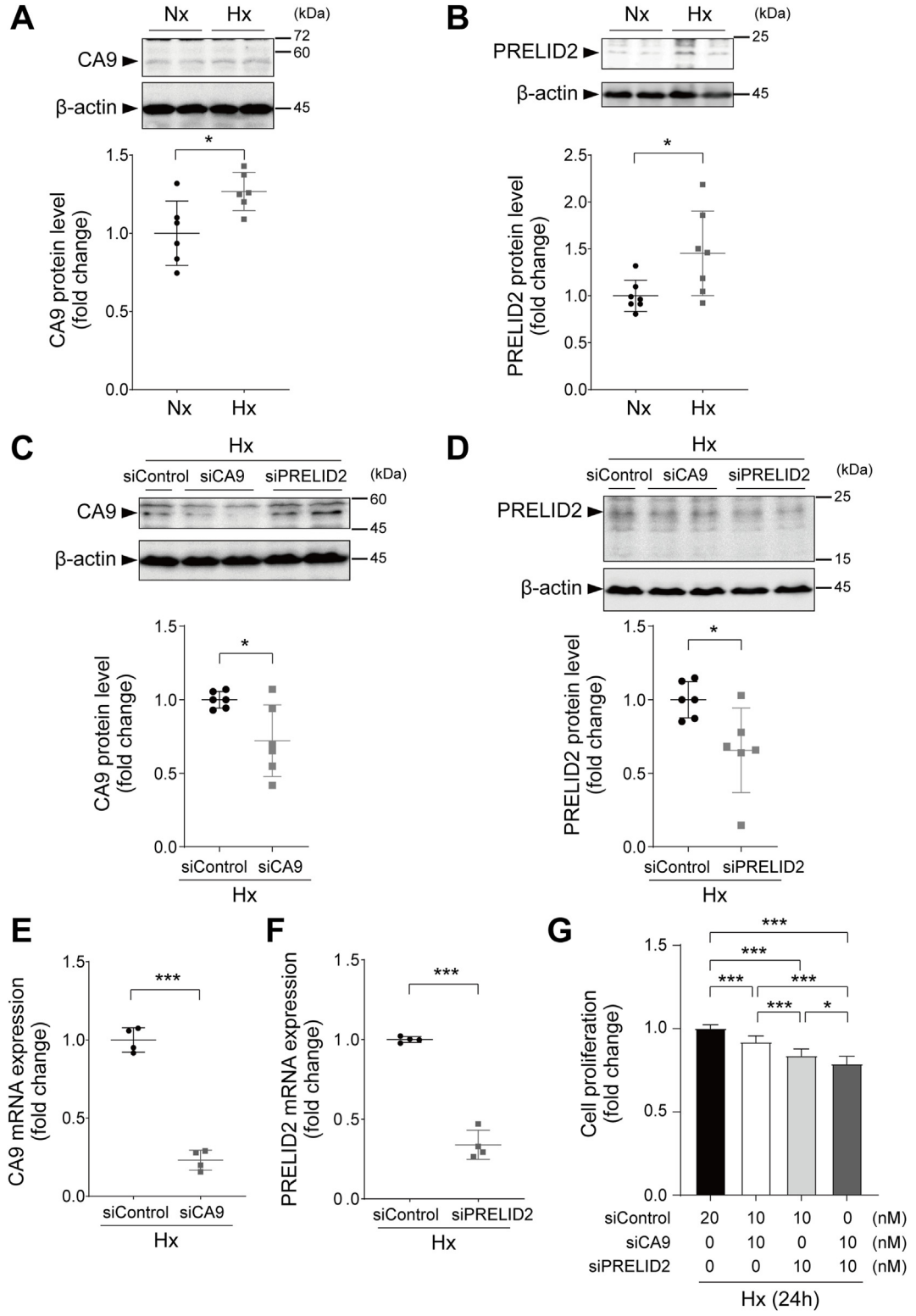

Fig. 5. The effects of CA9 siRNA treatment or PRELID2 siRNA treatment on MIA PaCa-2 cell proliferation. **(A and B)** Western blotting analysis for checking hypoxia (24 h)-induced CA9 protein expression level **(A)** and PRELID2 protein expression level **(B)**. Representative immunoblots show CA9 bands of two independent Nx samples and two independent Hx samples **(A)** or PRELID2 bands of two independent Nx samples and two independent Hx samples **(B)**. Densitometric analysis showed that both CA9 protein expression level and PRELID2 protein expression level were induced by Hx. Data were analyzed using Unpaired *t*-test; $*p < 0.05$, Mean ± SD, n = 6 **(A)** or n = 7 **(B)**. **(C and D)** Western blotting analysis for checking CA9 protein expression level **(C)** and PRELID2 protein expression level **(D)** due to CA9 siRNA or PRELID2 siRNA treatment under Hx. After 24 h from the siRNA transfection, the medium was changed to complete media and the cells were incubated for 24 h under Hx, followed by preparing cell lysate. Densitometric analysis showed that CA9 protein expression level was suppressed by CA9 siRNA treatment **(C)** and PRELID2 protein expression level was suppressed by PRELID2 siRNA treatment **(D)**. Data were analyzed using Unpaired *t*-test; $*p < 0.05$, Mean ± SD, n = 6. **(E and F)** Real-time PCR analysis for checking CA9 mRNA expression level **(E)** and PRELID2 mRNA expression level **(F)** due to CA9 siRNA or PRELID2 siRNA treatment under Hx (24 h). Data were analyzed using Unpaired *t*-test; $***p < 0.001$, Mean ± SD, n = 4. **(G)** MTT assay revealed that both CA9 siRNA treatment and PRELID2 siRNA treatment suppressed the cell proliferation under Hx for 24 h. The suppression effect of PRELID2 siRNA treatment was stronger than that of CA9 siRNA treatment, and the double-knockdown enhanced the effects of CA9 siRNA treatment and PRELID2 siRNA treatment. After 24 h from the siRNA transfection, the medium was changed to complete media and hypoxic stimulation for 24 h was started. Data were analyzed using one-way ANOVA followed by the Tukey test; $*p < 0.05$, $***p < 0.001$. Mean ± SD, n = 12.

revealed that "regulating angiogenesis" was the fourth-highest enriched term for the four genes (Fig. 1C). However, the expression level of EC-specific markers did not affect OS (Fig. 1D). In our previous study,[13] we reported that higher expression of CAF markers correlated with worse OS and also with that of an EC-specific marker, *PECAM1* in colon and rectal cancer (CRC). Although we suggested an interaction mechanism between CAFs and ECs in CRC, the expression level of CAF markers did not affect OS in TCGA-PAAD dataset analysis of the present study (data not shown). Tumor angiogenesis may not be important for cancer growth in PDAC like that in CRC, and these results agree with the fact that angiogenesis inhibitors are clinically used for treating patients with colon cancer but not for patients with pancreatic cancer.

Finally, we selected *CA9* and *PRELID2* as the candidate therapeutic targets. CA9 is a membrane-associated CA and is a target gene of HIF-1α.[8,42] McDonald et al. reported that the HIF-1α/CA9 axis regulates pH and glycolysis, which results in tumor cell survival in PDAC.[43] Alkaliptosis, a pH-dependent form of regulated cell death, has been recently identified as a new strategy for cancer therapy,[44] and many studies have suggested that the HIF-1α/CA9 axis contributes to malignancy in some types of highly hypoxic cancers.[45–48] Moreover, we examined the correlation of the expression level of *CA9* with those of the five EC marker genes (*VWF*, *PECAM1*, *KDR*, *TEK*, and *CDH5*) in the "Primary Tumor" group of TCGA-PAAD dataset, but could not find any strong positive correlations (data not shown). This suggests that CA9 do not regulate tumor angiogenesis. Thus, CA9-specific inhibition might have suppressed PDAC cell growth in our study via alkaliptosis instead of suppressing angiogenesis during hypoxia. As we described in the Results section or the Discussion section, there are already many reports which suggest that CA9 can be a new therapeutic target for PDAC. However, we extracted the 12 candidates which contain CA9. Those reports strengthen that the candidates extracted from our bioinformatics analyses can be crucial therapeutic targets for PDAC, and the specific inhibition of PRELID2, for example, by siRNA treatment suppressed PDAC cell growth more effectively than CA9 siRNA treatment in our *in vitro* experiments. PRELID2 is a PRELI domain containing family protein which plays an important role of embryonic and development lymphocyte differentiation and they have been proposed to involve apoptosis, cellular lipid metabolism and cellular signaling.[49] Very few reports have suggested that PRELI-like family proteins regulate phospholipids transport in mitochondria and mitochondrial reactive oxygen species signaling as well as tumor progression.[50–52] However, to the best of our knowledge, this is the first study to elucidate PRELID2 as a potential target for PDAC using bioinformatics analyses. In addition, our 12 candidates contained several lipid metabolism-related genes such as *MGLL*. Further investigation is required to elucidate the underlying mechanism involved in the contribution of lipid metabolism, containing PRELID2, to PDAC progression during hypoxia.

Author contributions

MI, KF, and RN performed bioinformatics analyses. MI, TI, RY, MN, KK, and YG performed experiments and analyzed data. HT and KG supported the experiments and interpretation of the data. LM, MF, MD, KI, AO, HF, YI, and KT contributed to the interpretation of the data and edited the manuscript. MI, TI, and KF interpreted the data and wrote the manuscript. MI and KT planned and generated the study design and obtained funding.

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Declaration of competing interest

The authors have no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors did not use any generative AI or AI-assisted technologies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2023.10.003.

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