Clin. Lab. 2026;72:XXX-XXX

ORIGINAL ARTICLE

CLCA1 Modulates Pancreatic Cancer Proliferation via SIRT1-HIF-1α Pathway

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SUMMARY

Background: **Pancreatic ductal adenocarcinoma (PDAC) is a form of cancer known for its aggressive behavior, poor survival rates, and high resistance to chemotherapy. Our previous study has found that low calcium-activated chloride channel regulator 1 (CLCA1) expression is associated with worse survival of patients with PDAC, yet its underlying function in PDAC is not clear.**
Methods: **We constructed CLCA1-overexpressed Pancreatic Adenocarcinoma Cell Line 1 (PANC-1) cells and investigated its effect on the cell growth and invasion by Cell Counting Kit-8 (CCK-8) Assay and Transwell Migration Assay. Sensitivity of PANC-1 cells to gemcitabine and hypoxia environment was also studied. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) based-proteomics was employed to discover key molecular cause based on CLCA1-overexpression. Further mechanistic insights into the SIRT1/HIF-1α axis were obtained using Ribonucleic Acid (RNA) interference, qPCR, and western blotting.**
Results: **CLCA1 expression was significantly reduced in PANC-1 cells. Overexpression of CLCA1 inhibited proliferation and invasion while enhancing sensitivity to gemcitabine. Proteomic study suggested that SIRT1 expression was dramatically downregulated in CLCA1-over expression PANC-1 cells compared with control PANC-1 cells. Mechanistic studies revealed that CLCA1 downregulates SIRT1 expression, leading to reduced stabilization of HIF-1α and subsequent suppression of its downstream hypoxia-responsive genes (GLUT1, LDHA). Hypoxia partially reversed this suppression. Synergistic effects were observed with CLCA1 overexpression and SIRT1 knockdown, significantly reducing tumor cell proliferation and HIF-1α expression.**
Conclusions: **We found that CLCA1 modulates the SIRT1/HIF-1α pathway, suppressing tumor proliferation, and might enhance gemcitabine sensitivity in pancreatic cancer cells. This investigation points to CLCA1 as a viable therapeutic target for tackling hypoxia-induced chemoresistance and enhancing treatment success in PDAC. Further exploration of CLCA1-based therapies could offer new opportunities for clinical translation.**
(Clin. Lab. 2026;72:xx-xx. DOI: 10.7754/Clin.Lab.2025.250721)

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Manuscript accepted August 6, 2025

KEYWORDS

CLCA1, pancreatic cancer, gemcitabine, SIRT1, HIF-1α pathway

INTRODUCTION

Pancreatic cancer ranks the third most deadly cancers in men and women combined [1], with PDAC being the most common and aggressive form [2-4]. The condition has the worst five-year survival rate of only 13% [1,5, 6]. Most patients, around 80-85%, are diagnosed at an advanced stage with unresectable tumors due to the absence of effective screening strategies and reliable biomarkers [5,7]. Even with chemotherapy, such as gemcitabine, its efficacy is significantly hindered by the development of resistance, emphasizing the urgent need to address this challenge [8-10].

One of the primary contributors to chemoresistance in pancreatic cancer is the hypoxic microenvironment, a hallmark of solid tumors [11-13]. Not only does hypoxia modify cellular metabolism, but it also reprograms key signaling pathways, largely driven by hypoxia inducible factor-1 alpha (HIF-1α), to foster resistance to chemotherapeutic drugs [14-16]. HIF-1α, activated under hypoxic conditions, drives the expression of genes that support cell survival, angiogenesis, and metabolic adaptation, thereby exacerbating tumor progression and chemoresistance [17].

In addition, silent information regulator 1 (SIRT1), a NAD+-dependent deacetylase, plays a pivotal role in hypoxia-driven resistance by stabilizing HIF-1α through direct deacetylation, enhancing its transcriptional activity [18,19]. The hypoxic response is intensified by this interaction, which promotes the expression of HIF-1α target genes associated with angiogenesis and metabolic flexibility, consequently decreasing chemotherapy's effectiveness [20].

Our previous study has found that low calcium-activated CLCA1 expression is associated with worse survival of patients with PDAC [21]. Recent research has also underscored the growing importance of CLCA1 in controlling tumor development and treatment response in different cancer forms [21,22]. For example, in liver cancer, CLCA1 suppresses the formation of new blood vessels by acting on the TGFβ1/SMAD/VEGF signaling pathway, thereby slowing tumor growth and increasing the sensitivity of cells to sorafenib, a leading targeted therapy [23]. Additionally, CLCA1 modulates chloride currents by interacting with transmembrane protein 16A (TMEM16), a calcium-dependent chloride channel, stabilizing its cell surface expression and influencing cellular signaling pathways relevant to tumor progression [24]. In pancreatic cancer, low CLCA1 expression has been associated with shorter disease-free survival, suggesting its potential as an independent prognostic marker [21]. Additionally, CLCA1 has been found to decrease the aggressiveness of colorectal cancer by hindering the Wnt/β-catenin signaling pathway [25].

This study investigates the function of CLCA1 in pancreatic cancer, focusing on its role in modulating gemcitabine sensitivity. Accordingly, CLCA1 expression is low in the pancreatic cancer cell line known as PANC-1. The overexpression of CLCA1 leads to a substantial decrease in tumor cell proliferation and invasion, while also heightening gemcitabine sensitivity. Using proteomics approach, SIRT1 expression was found most evidently downregulated in CLCA1-over expression PANC-1 cells. Mechanistic investigations confirmed that CLCA1 reduces SIRT1 expression, which in turn diminishes the expression of HIF-1α and its associated genes for hypoxia adaptation. These mechanisms not only control the growth patterns of pancreatic cancer cells but also greatly improve the effectiveness of gemcitabine treatment. By elucidating how CLCA1 modulates the biological behavior of pancreatic cancer cells via the SIRT1/HIF-1α signaling pathway, this study underscores CLCA1's critical role in overcoming chemoresistance and highlights its potential as a novel therapeutic target for pancreatic cancer.

MATERIALS AND METHODS

Cell culture

The Shanghai Institute of Cell Biology, the Chinese Academy of Sciences, provided the PANC-1 human pancreatic cancer cell line. The cells were cultured in DMEM (Gibco, USA) with an addition of 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2, and the culture medium was routinely replaced to keep them in logarithmic growth phase.

siRNA transfection

PANC-1 cells in the logarithmic phase were collected, and the culture medium was taken away. After being washed twice with sterile PBS, the cells were incubated with 2 mL of trypsin at 37°C for 1 - 3 minutes until detachment occurred. To stop the reaction, 4 mL of medium with serum was added, and the cells were pipetted to disperse them before being placed in a centrifuge tube and spun at 1,000 rpm for 5 minutes. Following resuspension, the cells were placed in a 6-well plate, with each well receiving 2 mL of medium without antibiotics, and incubated at 37°C.When the PANC-1 cells were about 70 - 80% confluent as observed under the microscope, the culture medium was removed, the cells were rinsed with PBS, and fresh basic culture medium was added. Lipofectamine 2000 (5 µL per well) and the corresponding siRNAs (100 pmol per well) were each diluted in 250 µL of Opti-MEM, incubated for 5 minutes, then mixed and left to stand for 20 minutes. The cells were incubated at 37°C for 4 - 6 hours after adding the transfection complexes, followed by a replacement with fresh complete culture medium. Observations and recordings were made 48 hours after transfection. Then samples were collected for further analysis.

Hypoxia model establishment

Logarithmic-phase PANC-1 cells were digested and resuspended in culture medium, with serum-free and antibiotic-free medium replacing the original medium 1 - 2 hours before transfection. A total of 60 µL of Lipofectamine 2000 per well was combined with Opti-MEM to reach a final volume of 1,500 µL, then gently mixed and left to incubate at room temperature for 5 minutes. Afterward, 24 µg of the CLCA1 overexpression plasmid and the empty vector were diluted in Opti-MEM to reach a total volume of 1,500 µL, mixed gently, and left to incubate at room temperature for 5 minutes. The plasmid dilution was mixed with the diluted Lipofectamine 2000 solution and left to incubate at room temperature for 20 minutes. The cell culture plates received the transfection complexes, and the cells were kept at 37°C for 4 - 6 hours before the medium was replaced with fresh complete culture medium. After 48 hours of transfection, the cells were placed in a hypoxic environment containing 1% O_2, 5% CO_2, and 94% N_2 for 8 hours. Samples were observed under a microscope and collected.

Construction of overexpression vector and stable CLCA1-expressing cell line

The CLCA1 gene, with specific sticky ends, was constructed using a full gene synthesis approach. The pcDNA3.1-C-EGFP vector was digested and ligated with the purified CLCA1 fragment. Competent bacteria were transformed with the ligation products, and single colonies were picked for sequencing to ensure the sequence was correct. The successfully constructed plasmid, pcDNA3.1-EGFP-CLCA1, was subsequently subjected to ultra-purification to remove endotoxins and obtain sufficient plasmid.

PANC-1 cells were routinely cultured to 50% - 60% confluence and then switched to serum-free and antibiotic-free medium for transfection, following the same protocol as previously described. Following 48 hours, the cells were exposed to an environment with low oxygen levels (1% O_2, 5% CO_2, and 94% N_2) for 8 hours. Samples were observed under a microscope and collected. Prior to conducting additional experiments, the expression of related markers was assessed using RT-PCR, qRT-PCR, and WB.

Transfection of PANC-1 cells with overexpression plasmids

The procedure was performed as previously described. A final volume of 25 µL of Lipofectamine 2000 was prepared by diluting 0.25 µL per well in Opti-MEM medium, gently mixed by pipetting up and down 3 - 5 times, and left to incubate at room temperature for 5 minutes. In each well, 0.2 µg of plasmid DNA was diluted in Opti-MEM medium to a total volume of 25 µL, gently mixed by pipetting 3 - 5 times, and left at room temperature for 5 minutes. The dilutions of Lipofectamine 2000 and plasmid were combined, gently mixed, and then allowed to rest at room temperature for 20 minutes. The transfection complexes were subsequently introduced to PANC-1 culture flasks, and the cells were kept at 37°C for 4 - 6 hours before the medium was replaced with fresh complete culture medium.

CCK-8 assay to evaluate the effects of different concentrations of gemcitabine and exposure times on PANC-1 cells

PANC-1 cells in the logarithmic phase were gathered into one centrifuge tube and then resuspended in the culture medium. Following the count, the cell concentration was set to 5 × 10^4 cells/mL and 100 µL was added to each well of a 96-well plate. The experimental groups consisted of three replicate wells and control wells, and the plates were left in a culture incubator overnight. Following incubation, the drug was added at varying concentrations based on the experimental groups, and the cells were incubated for an additional 24 or 48 hours. The plasmid transfection group involved incubating cells for a designated duration post-transfection. Afterward, the culture medium was changed, and 10 µL of CCK-8 solution was introduced to each well.

Assessment of cell migration and invasion ability

The capabilities of cell migration and invasion were evaluated using Transwell chambers that have a diameter of 6.5 mm and pores measuring 8 µm (Corning Incorporated, 2 Alfred Road, Kennebunk, ME 04043, USA). In the invasion assay, Matrigel from BD Biosciences was used to coat the top of the Transwell membrane, forming a basement membrane [26]. Cells on the bottom of the Transwell chamber were examined with a phase-contrast microscope (Leica) after being cultured for 24 to 48 hours, then photographed and counted. To guarantee the reliability of the results, each experiment was conducted three times.

RNA isolation and real-time qPCR

Cell lines and tissues are used to isolate total RNA. According to the manufacturer's instructions, the RNAiso reagent is employed to extract total RNA, and the RNA's quality and concentration are assessed using a microvolume spectrophotometer as per the manufacturer's guidelines. Using 2 µg of RNA as a template, cDNA is synthesized. Random primers are used to reverse transcribe RNA into cDNA, followed by the design of specific primers for target genes such as HIF-1α and SIRT1 for qPCR detection, utilizing SYBR Green I for real-time monitoring.

The primers used for mRNA quantification are shown below for PCR amplification and gene expression normalization:

CLCA1-F: AACACAGCCACCAAATCCCA
CLCA1-R: ATTGAGGCGGTTACCAGTC
SIRT1-F: ACAGGTTGCGGGAATCCAAA
SIRT1-R: GTTCATCAGCTGGGCACCTA

HIF1α-F: ATTACAGCAGCCAGACGATCA
HIF1α-R: ATTGATTGCCCCAGCAGTCT
SLC2A1-F: TTGGCTCCGGTATCGTCAAC
SLC2A1-R: GGCCACGATGCTCAGATAGG
LDHA-F: GGAGATTCCAGTGTGCCTGT
LDHA-R: TGTATCTGCACTCTTTCCACTGT
GAPDH(homo)-F:
AGATCCCTCCAAAATCAAGTGG
GAPDH(homo)-R: GGCAGAGATGATGACCCTTTT

A melt curve analysis is performed for all amplification products.

Western blotting (WB)

RadioImmunoprecipitation Assay (RIPA) buffer with protease and phosphatase inhibitors (from Beyotime Biotechnology) was used to lyse PANC-1 cells. The protein concentration of the extracted samples was quantified using a BCA assay, ensuring equal loading of protein for each sample. SDS-PAGE was performed on a Polyvinylidene Fluoride (PVDF) membrane (12% Bis-Tris gel, from Invitrogen, Thermo Fisher Scientific) followed by transfer [27].

The membrane was blocked at room temperature for 1 hour in 5% non-fat milk and then incubated overnight at 4°C with primary antibodies: CLCA1 (ab180851, Abcam), HIF-1α (CST8469, Cell Signaling Technology, USA), SIRT1 (CST48085, Cell Signaling Technology, USA), and GAPDH (Abcam, USA) at a dilution of 1:1,000. Subsequently, the membrane underwent an one-hour incubation with HRP-conjugated goat anti-mouse IgG secondary antibody, diluted 1:1,000, sourced from Biyuntian, China. The ECL detection solution was created by combining solution A and solution B in equal parts and spreading it uniformly over the whole membrane for 2 minutes. The membrane was placed in a chemiluminescence imaging system, and Image Lab software from Bio-Rad Laboratories was used for quantification.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The proteomic investigation of SiRNA-NC and SiRNA-CLCA1 in PANC-1 cells involved extracting proteins from the samples and quantifying them using the Micro BCA Kit. Once reduction and alkylation were completed, acetone was used to precipitate the samples, and centrifugation was employed to remove the supernatant. Ammonium bicarbonate along with acetonitrile was used to dissolve the pellet. Trypsin was introduced at a mass ratio of 70:1, and the digestion process took place overnight at 37°C. Using Oasis HLB columns, desalting was carried out, and peptides were separated on a C18 capture column with a flow rate of 300 nL/minute employing a nonlinear gradient. An Orbitrap Fusion Lumos instrument was used for mass spectrometry analysis, employing a data-dependent acquisition method to target the top 10 ions within a mass-to-charge ratio range of 350 - 1,500 for full mass scans. The analysis of data was performed with Protein Discoverer 2.4, while the false discovery rate was observed via a reverse database. Student's *t*-test was used to compare the protein intensity between the two groups.

Statistical analyses

Statistical analyses were conducted using SPSS software (version 25, IBM SPSS Statistics for Windows: IBM Corp., Armonk, NY, US) or GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). To compare data with normal or non-normal distribution, either Student's *t*-test or Mann-Whitney U test was used. An ANOVA test was conducted on several groups of continuous variables, with $p < 0.01$ indicating statistical significance.

RESULTS

Overexpression of CLCA1 in PANC-1 Cells inhibits tumor cell proliferation and invasion while enhancing sensitivity to gemcitabine chemotherapy

qPCR analysis revealed that the transcription level of CLCA1 in PANC-1 cells was significantly lower than that of GAPDH, indicating low baseline expression. A plasmid for CLCA1 (pcDNA3.1-EGFP-CLCA1) was constructed and confirmed by gel electrophoresis (Figure 1A). Following transfection into PANC-1 cells, qPCR results demonstrated effective expression of pcDNA3.1-CLCA1, with a significant increase in CLCA1 mRNA levels (Figure 1B). Overexpression of CLCA1 protein in transfected PANC-1 cells was also confirmed by proteomic analysis (normalized expression ratio 3.68, $p < 0.05$). We used the CCK-8 assay to study the impact of CLCA1 overexpression on cell proliferation (Figure 1D), and evaluated the invasive ability of cells through Transwell assays (Figure 1F). The results showed that the overexpression of CLCA1 in pancreatic cancer cells (PANC-1 cells) could significantly inhibit the proliferation and invasion of tumor cells.

To evaluate the effects of different concentrations of gemcitabine (0, 0.5 μM, 1 μM, 2 μM) on PANC-1 cell proliferation at 24 and 48 hours, the CCK-8 assay was employed. Notably, 2 μM gemcitabine significantly affected PANC-1 cell proliferation, resulting in an average survival rate of 68.8% after 48 hours of co-culture (Figure 1C).

Additionally, to assess the impact of gemcitabine on the biological behavior of pancreatic cancer cells, we investigated the combined effect of CLCA1 overexpression and gemcitabine on cell proliferation using the CCK-8 assay (Figures 1D and 1E), and evaluated cell invasion capability through Transwell assays (Figure 1F). The results showed that overexpression of CLCA1 in PANC-1 cells significantly inhibits tumor cell proliferation and invasion while enhancing sensitivity to gemcitabine chemotherapy.

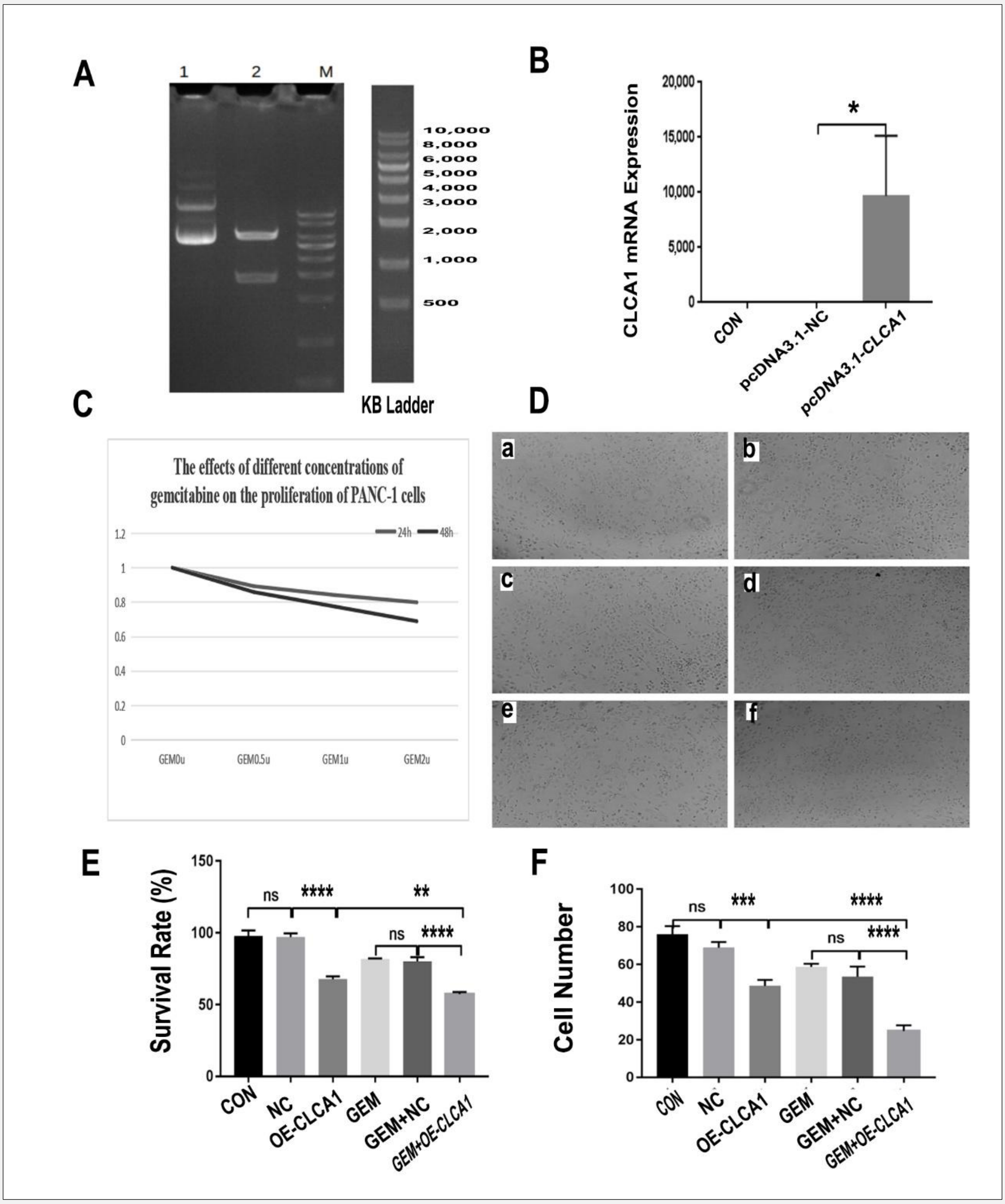

Figure 1. Overexpression of CLCA1 in PANC-1 cells inhibits tumor cell proliferation and invasion while enhancing sensitivity to gemcitabine chemotherapy.

A) Gel electrophoresis of the digestion products of the CLCA1 plasmid (pcDNA3.1-EGFP-CLCA1); Lane 1: pcDNA3.1-EGFP-CLCA1 before digestion, Lane 2: pcDNA3.1-EGFP-CLCA1 after digestion, M: KB Ladder; B) qPCR analysis of CLCA1 mRNA expression following transfection of PANC-1 cells with pcDNA3.1-EGFP-CLCA1; C) Effects of different concentrations of gemcitabine at varying time points on PANC-1 cell viability as assessed by CCK-8 assay; D) Images of PANC-1 cells under different treatment conditions (100 x magnification). a: Human pancreatic cancer PANC-1 cells; b: Gemcitabine + human pancreatic cancer PANC-1 cells; c: NC + human pancreatic cancer PANC-1 cells; d: Gemcitabine + NC + human pancreatic cancer PANC-1 cells; e: CLCA1 overexpression + human pancreatic cancer PANC-1 cells; f: Gemcitabine + CLCA1 overexpression + human pancreatic cancer PANC-1 cells; E) Effect of different treatment groups on PANC-1 cell survival rate measured by CCK-8 assay; F) Effect of different treatment groups on the invasion capability of PANC-1 cells as assessed by invasion assays. Group designations: CON, NC, OE-CLCA1, GEM, GEM+NC, GEM+OE-CLCA1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns indicates no statistically significant difference.

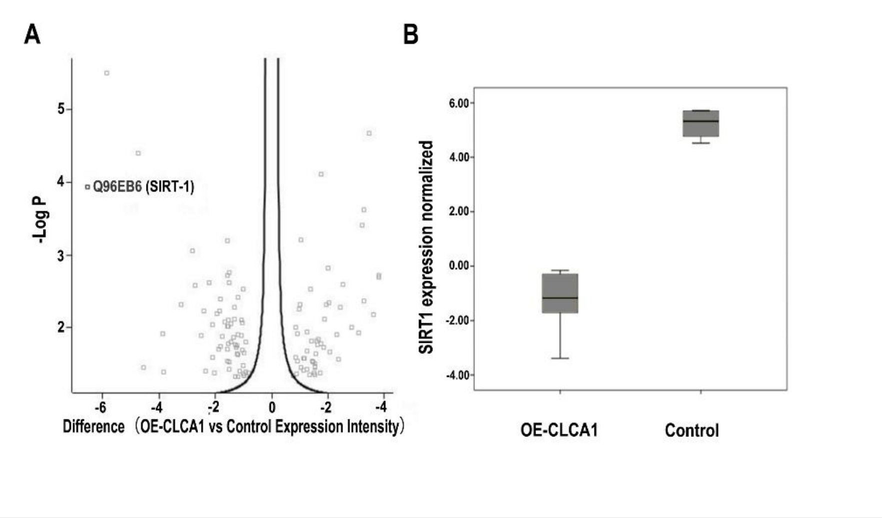

Figure 2. High expression of CLCA1 in PANC-1 cells significantly reduces SIRT1 expression.

A) Volcano plot illustrating the differentially expressed proteins in CLCA1 overexpressed PANC1 cells, discovering SIRT1 (Q96EB6) as the most significant down-regulated protein compared to control PANC1 cells; B) Boxplot depicting SIRT1 expression levels in the CLCA1 overexpressed group compared to the control group in PANC-1 cells.

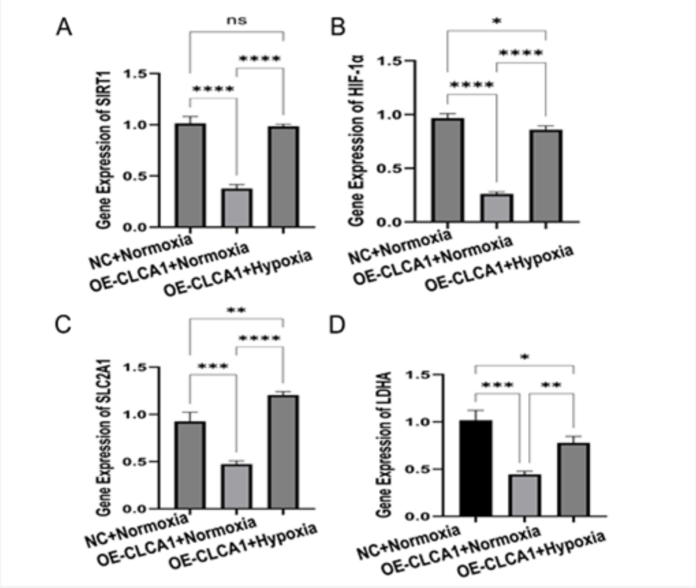

Figure 3. Expression profiles of various genes following CLCA1 overexpression in PANC-1 cells with or without hypoxia condition.

The expression level of A) SIRT-1 gene, B) HIF-1α gene, and its target genes C) SLC2A1 and D) LDHA were decreased in PANC-1 cells in CLCA1 overexpression group compared to the empty vector control group in normal oxygen conditions. Expression of these genes in in CLCA1 overexpression PANC-1 cells were all increased in hypoxic conditions compared to normal oxygen conditions. (NC: empty vector control group; OE-CLCA1: CLCA1 overexpression group; Normoxia: normal oxygen conditions; Hypoxia: hypoxic conditions) Data are presented as mean ± standard deviation from three independent experiments. Comparisons to the control group showed significance at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns indicates no statistically significant difference.

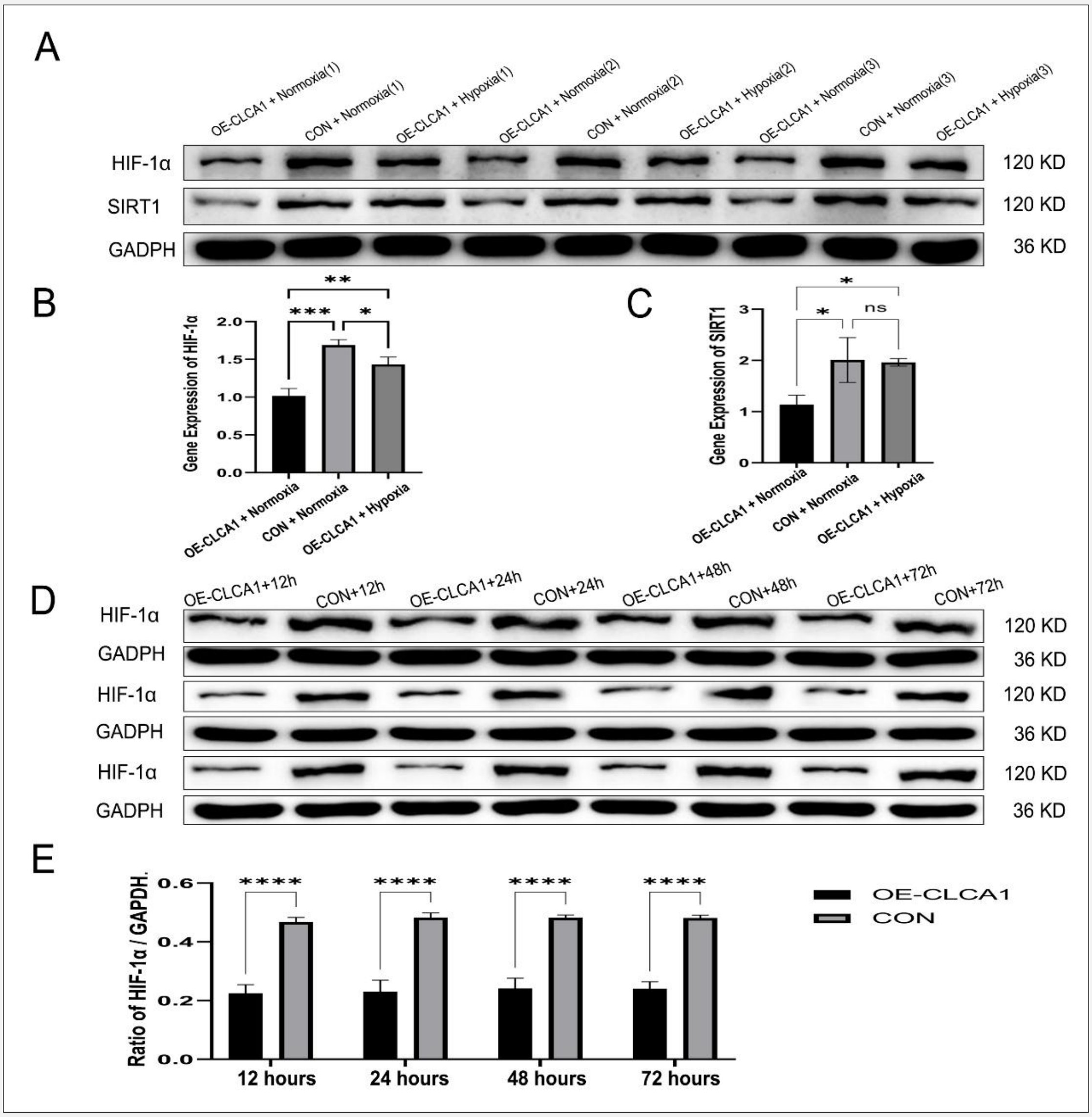

Figure 4. Regulatory effects of CLCA1 overexpression on HIF-1α and SIRT1 under different oxygen conditions

A) Protein expression levels of HIF-1α and SIRT1 were assessed using western blot analysis in different treatment groups. Samples were categorized into three groups: cells overexpressing CLCA1 under normoxic conditions; cells transfected with the empty vector under normoxic conditions; and cells overexpressing CLCA1 in a hypoxic environment; B) Effects on HIF-1α expression across different treatment groups; C) Effects on SIRT1 expression across different treatment groups; D) Expression levels of HIF-1α and SIRT1 in PANC-1 cells were evaluated at various time points (12 hours, 24 hours, 48 hours, 72 hours) in normoxic conditions to assess the regulatory effect of CLCA1 overexpression on HIF-1α; E) The ratio of HIF-1α to GADPH in the control group versus the CLCA1 overexpression group at different time points (12 hours, 24 hours, 48 hours, 72 hours). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns indicates no statistically significant difference.

Proteomics study revealed significant down-regulation of SIRT1 in CLCA1-overexpressed PANC-1 cells

We employed LC-MS/MS to examine the protein profiles of the CLCA1 high-expression PANC-1 cell group (pcDNA3.1-CLCA1, n = 3) and the control PANC-1 cell group (pcDNA3.1-nc, n = 3). A total of 4,669 proteins were identified, among which 115 exhibited differential expression between the two groups ($p < 0.05$, with a normalized expression ratio > 2 or < 0.5). Notably, SIRT1 was identified as the protein with the most down-regulated in the CLCA1 high-expression group

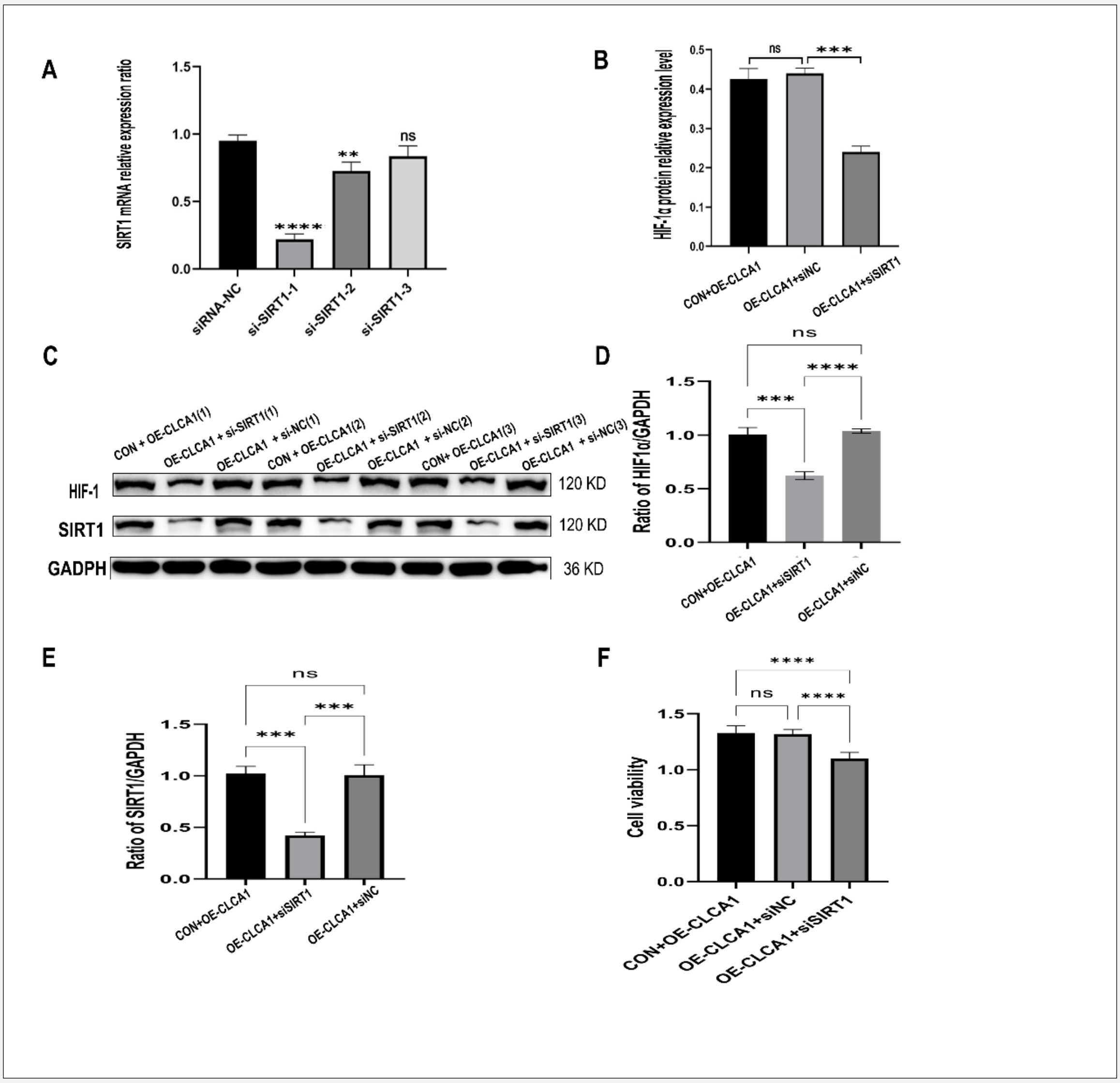

Figure 5. CLCA1 regulates HIF-1α stabilization and pancreatic cancer cell proliferation via a SIRT1-mediated mechanism.

A) Screening of three siRNA sequences for their interference efficiency against the SIRT1 gene using qPCR; B), C) WB analysis demonstrating the effects of CLCA1 overexpression and SIRT1 interference on the protein expression levels of HIF-1α and SIRT1 in PANC-1 cells. The CLCA1 overexpression combined with SIRT1 interference (CLCA1+si-SIRT1 group) significantly downregulated HIF-1α expression, indicating the important role of SIRT1 in CLCA1-mediated regulation of HIF-1α; C) Expression levels of HIF-1α and SIRT1 across different groups (CON+OE-CLCA1, OE-CLCA1+si-SIRT1, OE-CLCA1+si-NC); D) Ratio of HIF-1α to GAPDH among different groups; E) Ratio of SIRT1 to GAPDH among different groups; F) The CCK-8 assay showed that Figure 1E. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns indicates no statistically significant difference.

(normalized expression ratio = 0.009175, p = 0.000116) (Figure 2A - B).

Oxygen-dependent regulation of HIF-1α and SIRT1 expression by CLCA1 overexpression

PCR analysis showed that under normal oxygen conditions, the CLCA1 overexpression group had significantly lower levels of HIF-1α and SIRT1 compared to the empty vector control group (Figure 3A - B). Furthermore, the expression of the target genes of HIF-1α, GLUT1 (Solute Carrier Family 2 Member 1/SLC2A1) and LDHA (Lactate dehydrogenase A/GSD11), was downregulated in the CLCA1 overexpression group (Figure 3C - D). On the other hand, hypoxic conditions led to elevated levels of HIF-1α and SIRT1, as well as increased expression of the target genes GLUT1 (SLC2A1) and LDHA (GSD11) (Figure 3A - D).

We further assessed the expression levels of HIF-1α and SIRT1 across various treatment groups through WB analysis. The findings showed that in normoxic conditions, the CLCA1 overexpression group had significantly lower levels of HIF-1α and SIRT1 compared to the empty vector control group. An increase in the expression of HIF-1α and SIRT1 was noted under hypoxic conditions (Figure 4A - C). This indicates that overexpressing CLCA1 decreases the levels of HIF-1α and SIRT1 in normal oxygen conditions, although low oxygen levels might partially counteract this suppression. Moreover, we examined the prolonged regulatory impact of CLCA1 overexpression on HIF-1α by measuring its expression at different intervals (12 hours, 24 hours, 48 hours, 72 hours) in PANC-1 cells in normoxic conditions. HIF-1α levels were found consistently lower in the CLCA1 overexpression group compared to the control group at every time point (Figure 4D - E). The results suggest that CLCA1 overexpression can consistently reduce HIF-1α levels, implying that CLCA1 might negatively regulate HIF-1α expression through a potential signaling pathway rather than direct breakdown of HIF-1α.

CLCA1 regulates HIF-1α stabilization and pancreatic cancer cell proliferation via a SIRT1-mediated mechanism

We screened three siRNA sequences for their interference efficiency against the SIRT1 gene using qPCR. The experimental results indicated that si-SIRT1-1 exhibited the most significant inhibitory effect on SIRT1, with a marked reduction in mRNA expression levels compared to the other two siRNAs, thus it was selected as the interference sequence for subsequent experiments (Figure 5A).

The impact of CLCA1 overexpression and SIRT1 interference on the protein levels of HIF-1α and SIRT1 in PANC-1 cells was assessed using western blot analysis. In the experimental group with CLCA1 overexpression and SIRT1 interference (CLCA1+si-SIRT1 group), HIF-1α expression was significantly downregulated. These results indicate that SIRT1 plays a crucial role in CLCA1's regulation of HIF-1α (Figure 5B - E). Additionally, results from the CCK-8 assay demonstrated that CLCA1 overexpression combined with SIRT1 interference (CLCA1+si-SIRT1 group) significantly inhibited PANC-1 cell proliferation. This indicates that CLCA1 might reduce the expression of HIF-1α by inhibiting SIRT1, which in turn significantly decreases the proliferation of pancreatic cancer cells (Figure 5F).

In summary, CLCA1 downregulates HIF-1α expression through SIRT1 regulation and significantly inhibits pancreatic cancer cell proliferation.

DISCUSSION

This research highlights the essential function of CLCA1 as a tumor suppressor in pancreatic cancer and its influence on chemosensitivity. In PANC-1 pancreatic cancer cells, we noted a significant decrease in the expression level of CLCA1. Overexpression of CLCA1 not only markedly inhibited cell proliferation and invasion but also enhanced chemosensitivity to gemcitabine. Proteomic approach suggested that SIRT1 expression was dramatically downregulated in CLCA1-over expression PANC-1 cells. Mechanistically, CLCA1 downregulates SIRT1 expression, thereby suppressing the activity of HIF-1α and its downstream target genes. The continuous suppression of HIF-1α by CLCA1, along with its cooperative effect with SIRT1 disruption, highlights the pivotal role of CLCA1 in influencing the biological behavior of pancreatic cancer. These findings not only deepen our understanding of the molecular pathology of pancreatic cancer but also lay a foundation for the development of novel therapeutic strategies targeting CLCA1, offering a promising avenue to overcome chemoresistance in pancreatic cancer.

The tumor-suppressive role of CLCA1 revealed in this study for pancreatic cancer aligns with its functions in other cancer types, further solidifying its status as a broad-spectrum tumor suppressor. We demonstrated that CLCA1 inhibits pancreatic cancer cell proliferation and invasion while enhancing chemosensitivity by modulating the SIRT1-HIF-1α signaling pathway. This mechanism has not been addressed by other studies on the CLCA1's roles in other cancers: in colorectal cancer, CLCA1 suppresses the Wnt/β-catenin signaling pathway [25]; in hepatocellular carcinoma, it regulates angiogenesis through the TGFβ1/SMAD/VEGF axis [23]; and in ovarian cancer, CLCA1 modulates the TMEM-16A calcium-activated chloride channel, impacting cellular signaling and metabolic adaptation [24]. These findings collectively highlight the central tumor-suppressive role of CLCA1 across multiple cancers. In pancreatic cancer, CLCA1's control over the SIRT1/HIF-1α pathway highlights its distinct role in enhancing chemosensitivity, offering valuable insights for creating new treatment approaches.

In the low-oxygen environment of pancreatic cancer, the stability and function of HIF-1α are crucial for the survival of tumor cells and their resistance to chemotherapy [27]. HIF-1α enhances resistance to therapy by promoting the expression of genes associated with survival, angiogenesis, and metabolic adaptation [15], increasing DNA repair capacity, suppressing apoptosis, and facilitating autophagy [28].

The activity of the HIF pathway is modulated by not only hypoxia, but also other inputs including histone modifiers, post-translational modifications, and non-coding RNAs [29,30]. HIF-1α has been reported to interact with several histone modifiers, thus leading to an important connection with epigenetic regulation. Hydroxylation of HIF-1α has been shown to impair the recruitment of histone acetyltransferases such as P300 and CBP, thereby influencing transcriptional activity [31]. Moreover, the interplay between HIF-1α and cancer cells exacerbates therapeutic resistance [32] and modulates the tumor microenvironment by influencing mesenchymal stem cells and immune responses [33,34]. As a result, HIF-1α has become an important therapeutic target to enhance the effectiveness of chemotherapy in pancreatic cancer [35].

As a deacetylase, SIRT1 not only modifies gene expression through histone deacetylation, but also acts on non-histone substrates, such as tumor suppressor p53, HIF1-α, NFKB and PPARγ [36]. HIF-1α is a direct target of SIRT1. The overexpression of SIRT1 has been shown to improve the stability of the HIF-1α protein via deacetylation in pancreatic cancer [37]. The SIRT1-HIF-1α axis is reported to mediate immune responses [38]. A recent study has also indicated that SIRT1-HIF-1α axis may potentially act as a therapeutic target in bladder cancer organoids [39].

This study is the first to uncover a novel mechanism by which CLCA1 attenuates HIF-1α function through the downregulation of SIRT1, thereby disrupting hypoxia-induced tumor adaptive responses. These findings not only identify a potential new therapeutic target for pancreatic cancer but also elucidates the multifunctional tumor-suppressive mechanisms of CLCA1. Specifically, CLCA1's regulation of the SIRT1-HIF-1α axis highlights its unique role in the hypoxic microenvironment, which may explain its significant effect in enhancing chemosensitivity.

Collectively, our findings demonstrate the multifaceted role of CLCA1 in suppressing tumor progression, enhancing chemotherapy efficacy, and overcoming resistance. These results provide a theoretical basis for developing novel therapeutic strategies targeting CLCA1, such as designing CLCA1 mimetics or activators, which could become effective approaches to improve pancreatic cancer treatment outcomes. Upcoming research should delve deeper into how CLCA1 interacts with other elements of the tumor microenvironment to create more accurate combination therapy approaches.

The main clinical importance of this research is in uncovering the exact molecular mechanism by which CLCA1 boosts the effectiveness of gemcitabine by regulating the SIRT1-HIF-1α pathway. In pancreatic cancer's low-oxygen environment, the increased levels of HIF-1α play a crucial role in promoting resistance to chemotherapy [15,18]. Our findings demonstrate that CLCA1 overexpression downregulates SIRT1 expression, subsequently reducing the activity of HIF-1α. This regulation also reduces the expression of HIF-1α downstream target genes including GLUT1 and LDHA, greatly diminishing the ability of pancreatic cancer cells to adapt to low oxygen conditions and increasing their sensitivity to gemcitabine.

This multi-layered regulatory mechanism of CLCA1 not only highlights its potential as an ideal target for chemosensitization therapies but also offers a novel perspective for overcoming chemoresistance in pancreatic cancer. For example, the development of CLCA1 activators or small-molecule compounds mimicking its function could serve as adjuvants to gemcitabine treatment. Furthermore, the expression level of CLCA1 may be a potential biomarker for predicting chemotherapy response in pancreatic cancer patients, enabling the formulation of individualized therapeutic strategies.

Unlike other methods to boost gemcitabine sensitivity, such as blocking the PI3K/AKT or NF-κB signaling pathways, CLCA1 targets metabolic changes and oxygen sensing in low-oxygen environments. By downregulating the SIRT1-HIF-1α axis, CLCA1 markedly reduces the expression of key genes involved in tumor metabolic adaptation, such as GLUT1 and LDHA [14, 18]. Moreover, in hepatocellular carcinoma, CLCA1 has been shown to enhance the efficacy of sorafenib by inhibiting angiogenesis [23], suggesting that it may exert broad anti-tumor effects across multiple cancers by modulating the tumor microenvironment. Overall, these results highlight the extensive potential of CLCA1 as a target for therapy.

However, this study has certain limitations. The experiments were conducted solely on the PANC-1 cell line and have yet to be validated in other pancreatic cancer cell lines or animal models. Besides, this study cannot rule out the potential “trigger” role of hypoxia on CLCA1-SIRT1-HIF-1α axis. Additionally, our study only provides preliminary evidence regarding the role of CLCA1 in gemcitabine sensitivity. More studies are needed to solidify these conclusions. Further exploration across a more extensive array of cell lines and *in vivo* models is needed to fully understand the therapeutic potential of CLCA1 in pancreatic cancer due to these limitations.

In summary, this study highlights the critical role of CLCA1 in pancreatic cancer, offering new insights into the biological characteristics of the disease while providing novel indications for overcoming chemoresistance and optimizing therapeutic strategies. With more validation and additional mechanistic studies, CLCA1 shows potential as a major therapeutic target for pancreatic cancer, offering significant clinical advantages to patients.

CONCLUSION

Our study reveals the anti-oncogenic role of CLCA1 in PDAC and its mechanism of regulating tumor cell behavior through the SIRT1-HIF-1α pathway. Targeting the CLCA1-SIRT1-HIF-1α interaction may pave new paths for developing innovative cancer treatments. Our research provides crucial insights into the molecular mechanisms of PDAC and the development of therapeutic strategies.

Acknowledgment:
The authors would like to thank all participants who contributed to the study.

Source of Funds:
This study was supported by The Zhejiang Provincial Medical and Health Science and Technology Plan (2021KY802), Wenzhou Science and Technology Bureau Project (Grant No. Y2023228) and the Zhejiang Provincial Basic Public Welfare Research Program (Grant No. LGF21H160035).

Declaration of Interest:
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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