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Original Article

Inhibition of bromodomain regulates cellular senescence in pancreatic adenocarcinoma

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Abstract: Background: Bromodomain and extra terminal domain (BET) proteins are important epigenetic regulators that promote the transcription of genes in the chromatin region associated with acetylated histones. Small molecule BET inhibitor JQ1 suppresses the biologic function of BET proteins in a variety of tumors and inhibits their proliferation. Methods: We investigated the effect of JQ1 in the treatment of pancreatic cancer. In addition, we evaluated the expression level of BRD4 protein in pancreatic cancer tissues using the Gene Expression Profiling Interactive Analysis (GEPIA) and the Human protein Altas databases and analyzed the correlation between BRD4 and the clinicopathologic features and immune checkpoints of pancreatic adenocarcinoma using UALACN and TIMER databases. Results: JQ1 significantly inhibited the proliferation of pancreatic adenocarcinoma (PAAD) cells and induced cell senescence but had little effect on Senescence-associated secretory phenotype (SASP). Interestingly, JQ1 inhibited the epithelial-mesenchymal transition (EMT) and Wnt signaling pathways. Conclusions: These results provide a theoretical basis for new targets in the treatment of pancreatic cancer.

Keywords: Pancreatic adenocarcinoma, JQ1, EMT, senescence, SASP

Introduction

Pancreatic adenocarcinoma (PAAD) is a highly malignant tumor, and the most dominant pathologic type of pancreatic cancer, which accounts for more than 80% of pancreatic tumors [1]. The five-year survival rate is only 8%. The main reason for the low overall survival rate is that it is difficult to make an early diagnosis [2]. Because of its occult onset, most patients are diagnosed at a late stage, resulting in only about 10%-20% of PAAD patients being eligible for radical surgery [3]. Furthermore, some patients may not benefit from surgery if they experience early tumor recurrence after radical tumor resection [4]. In addition, due to the lack of effective therapeutic targets for pancreatic cancer, targeted therapy and immunotherapy have little effect on pancreatic cancer. Therefore, chemotherapy plays the main role in the comprehensive treatment of pancreatic cancer.

Recently, targeting epigenetic regulatory proteins such as bromodomain and exodomain (BET) proteins have been considered as therapeutic targets for cancer, metabolic disorders, and inflammatory diseases [5]. Bromodomain proteins recognize histones and non-histones which are acetylated by their bromodomains as epigenetic readers [6]. It has been shown that BRD4, a member of the BET family of proteins, plays an important role in transcription, cell cycle control, inflammatory cytokine production, and cancer progression [7, 8]. Hematologic malignancies and solid tumors have been linked to BRD4, making it an attractive therapeutic target for cancer treatment [9]. BRD4 and other BET family proteins confer strong antitumor activity through their bromination domains, suppressing the proliferation and transformation potential of various cancers.

As a potent and selective inhibitor of the BET signaling pathway, JQ1 has been widely used in tumor biologic studies. JQ1 was able to effectively reduce cancer cell viability *in vitro* and *in vivo* [10, 11]. The underlying mechanisms include an effect on cell cycle arrest in the G1 phase and a decrease in the percentage of cells in the S phase [12]. Furthermore, JQ1 can alter cytokine expressions in T cells and den-

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dritic cells (DCs) [13]. Apoptosis of tumor cells was induced by JQ1 through the downregulation of E2f1 protein expression [14]. Phase I clinical studies have been completed in acute myeloid leukemia (NCT02308761) and some advanced solid tumors (NCT01987362). An essential role of BRD4 in cell senescence has been reported in several recent studies [15-18]. However, its role in pancreatic cancer has rarely been reported.

Senescence is a state in which cells stop dividing and enter a permanent, irreversible cell cycle arrest [19]. Generally, cell senescence when they experience a variety of stresses, including telomere shortening, activation of oncogenes, inactivation of tumor suppressors, deterioration of mitochondria, oxidative stress, and DNA damage [20]. Similarly, epigenetic regulation of gene expression regulates cellular senescence in cancer cells [21]. Senescence is usually accompanied by morphologic changes, including enlarged and flattened cells, increased senescence-associated β-galactosidase (SA-β-Gal) activity, and altered levels of it [22]. Cellular senescence is characterized by upregulation of the cyclin-dependent kinase inhibitors p16INK4A, p15INA4B, and p21 (also known as p21WAF1/Cip1 or CDKN1) [23, 24]. However, recent studies have found that the cellular senescence process is a double-edged sword. On the one hand, it can exert anti-tumor activity, because senescence is permanent in the stagnation of the cell cycle [20]. On the other hand, senescent cells secrete a variety of cytokines and chemokine-induced differentiation, thus increasing the cell division, even in adjacent cells. These include IL-1, -6, and -8, MCP-1, -2, -3, and -4, HCC-4, eotaxin-3, and GROα, in a phenomenon known as senescence-associated secretory phenotype (SASP) [25-27]. These SASP subsets, and some of their known paracrine effects on nearby cells, alter the ability of the tumor microenvironment to facilitate cancer progression [28].

This study demonstrates a novel pathway by which BRD4 affects pancreatic cancer growth through the cellular senescence pathway.

Materials and methods

Online database analysis

A publicly accessible online database, Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/), provides data from the cancer genome atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/) and genotype-tissue expression studies (GTEx; https://www.gtexportal.org/home/index.html). In the current study, we used GEPIA to graphically compare the differential expression of BRD4 between pancreatic adenocarcinoma (PAAD) and normal tissues. Then we used the Human Protein Altas database (https://www.proteinatlas.org/) to evaluate the BRD4 protein expression level in the pancreatic cancer tissue and normal tissue. The UALACN database (http://ualcan.path.uab.edu) was used to analyze the relationship between BRD4 gene expression and the clinicopathologic features of PAAD. The TIMER database (https://cistrome.shinyapps.io/timer/) was used to evaluate the correlation between BRD4 and immune checkpoints in PAAD.

Gene set enrichment analysis (GSEA)

In order to identify the pathways with enriched BRD4 expression, GSEA (version 4.2.3) was performed in both the C2 KEGG gene sets (c2.cp.KEGG.v7.4.symbols.GMT) and the C7 immunological gene sets (c7.all.v7.4.symbols.GMT) of the Molecular Signatures Database (MSigDB). Enrichment analyses were performed a thousand times with permutations of gene sets. FDRs of 0.05 and NOMs of 0.05 were considered significantly enriched pathways.

Drug

The JQ1 was purchased from ApexBio Biotechnology, with a purity > 95%.

Cell culture

Professor Jens Siveke, German Cancer Research Center provided mouse pancreatic cancer cell lines. Tumors (511950) and ascites (70202) from Kras+/LSL-G12D, Trp53+/LSL-R172H, Ptf1a+/Cre (KPC) mice. Previous research has described this in more detail [29].

Cells 70202 and 511950 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, China) supplemented with Gibco 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1% non-essential amino acids (Life Technologies Corporation, Carlsbad, CA, USA), and penicillin (50 U/mL)/streptomycin (50 μg/mL) at 37°C under a humidified 5% CO_2 atmosphere.

Cell growth detection using CCK-8

Cells were seeded in 96-well plates with an optimal 10% FBS in a total of 100 μL media. After 12 hours, 100 μL of sequentially diluted compounds or dimethyl sulfoxide (carrier) was added to the medium, and cells were incubated for 0, 24, 48, and 72 hours. Cell viability was assessed using the CCK-8 method (ApexBio Biotechnology, Hsinchu, Taiwan) as described by the manufacturer.

Senescence-associated beta-galactosidase staining

Senescent tumor cells were identified by SA-β-gal using a cellular senescence assay kit according to the manufacturer's protocol (Beyotime Biotechnology, Shanghai, China).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA), followed by cDNA synthesis using Prime-Script RT reagent kit (Takara Bio Inc., Kusatsu, Japan). Quantitative PCR was performed using the ABI QuantStudio3 Real-Time PCR system (Thermo Fisher Scientific) and SYBR Green Reagent (Takara Bio). Gene expression was normalized to GAPDH. The forward and reverse primers (5'→3') for real-time PCR were: p15, CCCTTCAAACGCCTGAACCTT, and ACTGGCTTGATTGTTGCCCTC; p21, GTACTTCCTCTGCCCTGCTG, and TCTGCGCTTGGAGTGATAGA; IL-6, TGGGGCTCTTCAAAAGCTCC, and AGGAACTATCACCGGATCTTCAA; GROα, CCTTGTCTCTTGCGTTCTTCC, and TCCAAAGTACCCTGCGGTATC; GAPDH, AGGTCGGTGTGAACGGATTTG, and TGTAGACCATGTAGTTGAGGTCA.

Western blotting

Cells were lysed with RIPA buffer (containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% (v/v) Triton X-100, 0.1% (m/v) SDS, protease inhibitor cocktail, and phosphatase inhibitor cocktail, was purchased from Sangon Biotech. Corp., Shanghai, China) for 30 min, and total protein was quantified by using BCA assays (Sangon, Shanghai, China). The lysates were separated by using 10% reducing SDS-PAGE, and protein bands were transferred to PVDF membranes, and incubated with corresponding primary antibodies [N-Cadherin, E-Cadherin, Non-phospho (Active) β-Catenin, Phospho-GSK3β (Ser9), and GSK3β (all from Cell Signaling) and GAPDH (Proteintech)] at the optimal dilution overnight at 4°C. The membranes were then incubated with secondary goat anti-rabbit or goat anti-mouse IgG-HRP antibodies (Proteintech). Immunoblotting images were captured by using a Chemiluminescent Imaging System (Thermo Fisher Scientific, USA).

Statistical analysis

Data were presented as the mean ± SEM from at least three separate experiments. The Student's test and one-way analysis of variance (ANOVA) were used for comparisons between groups as appropriate (GraphPad Prism 8.0). $P < 0.05$ was considered significant.

Results

Expression of BRDs mRNA and clinicopathologic changes in BRD4 in patients with pancreatic cancer

The GEPIA dataset (http://gepia.cancer-pku.cn/) was performed to compare BRDs mRNA expression in pancreatic cancer tissues with that in normal pancreatic tissues. According to our findings, the BRD1/2/3/4/7/89 expression levels (tumor sample: n=171 vs. normal sample: n=179) were upregulated within pancreatic cancer tissues relative to those of normal tissues (**Figure 1A**). We explored the protein expression levels of BRD4 by using the HPA database. As shown in **Figure 1B**, the protein expression levels of BRD4 in PAAD were higher than those in normal pancreatic tissues. We examined BRD4 expression and promoter methylation about several clinical-pathologic data in patients, including tumor stage (Normal, stage 1, 2, 3, and 4), tumor grade (normal, grade 1, 2, 3, and 4) by applying UALCAN (**Figure 1C**, **1D**).

Relationship between BRD4 expression and immune infiltration

Previous studies have shown that tumor-infiltrating lymphocytes are an independent predictor in tumors. Therefore, we used CIBERSORT to investigate whether the expression of BRD4 is correlated with tumor immune cell infiltration in PAAD by dividing the TCGA samples into high

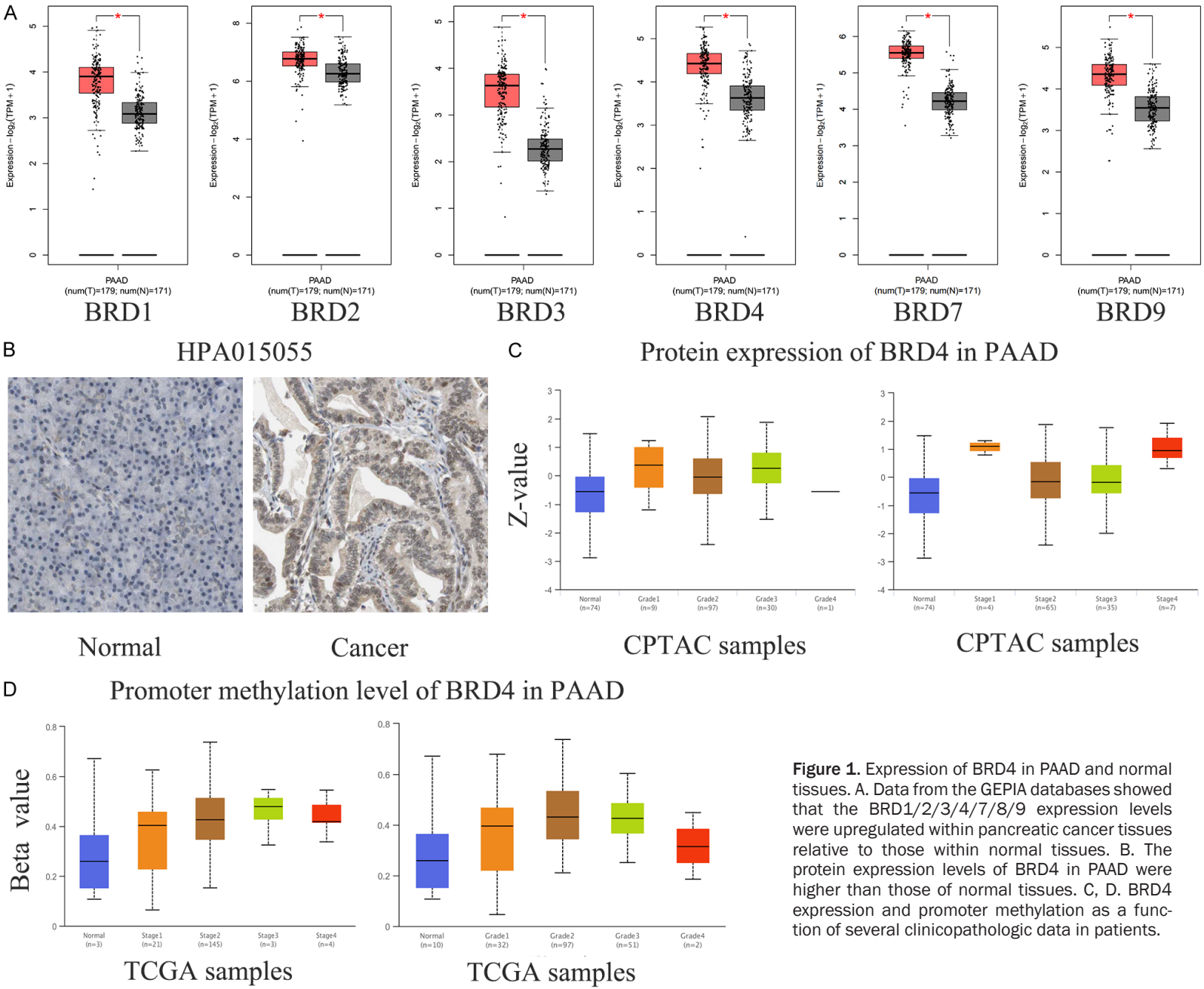

Figure 1. Expression of BRD4 in PAAD and normal tissues. A. Data from the GEPIA databases showed that the BRD1/2/3/4/7/8/9 expression levels were upregulated within pancreatic cancer tissues relative to those within normal tissues. B. The protein expression levels of BRD4 in PAAD were higher than those of normal tissues. C, D. BRD4 expression and promoter methylation as a function of several clinicopathologic data in patients.

and low groups according to the median expression of BRD4 and calculating the differences and correlations of 22 immune cell contents between the high and low groups (**Figure 2A**, **2B**). Subsequently, the TIMER database was used to show a correlation between BRD4 and immune checkpoint in pancreatic cancer, and the results showed that CTLA4, PDCD1, LAG3, and other molecules were positively correlated with BRD4 (**Figure 2C**).

Drug sensitivity analysis of BRD4 in pancreatic cancer

To advance the therapeutic efficacy in patients with PAAD, we further investigated the sensitivity difference of common chemotherapy drugs among the two groups. The IC_{50} values of BX-795, AT-7519, and other drugs in the group with high BRD4 expression in pancreatic cancer were lower than those in the group with low expression (**Figure 3A-L**).

BRD4 inhibitors can inhibit the proliferation of pancreatic cancer cells and promote senescence

Primary pancreatic cancer cells (70202 and 511950) derived from KPC mice were incubated with JQ1 (500 nM) for 72 hours, and the morphology of the cells changed significantly (**Figure 4A**). JQ1 concentrations ranging from 1 μM for 70202 and 511950 cells, significantly inhibited growth according to CCK-8 assays (**Figure 4B**). Then, through SA-β-gal staining, we found that JQ1 (500 nM) could significantly promote the senescence of pancreatic cancer cells (**Figure 4C** and **4D**) after incubation for 72 hours. At the same time, we found that the expression of senescence-related factors p15 and p21 increased (**Figure 4E** and **4F**) in the presence of JQ1 (100 nM-500 nM).

BRD4 inhibition alleviates the cellular senescence-associated secretory phenotype

Senescence is often thought to contribute to the death of tumor cells. However, studies have also shown that senescence can be a double-edged sword in tumors. Proteins secreted by senescent cells can create a pro-tumor environment, enhance the migration of tumor cells and thus form metastasis, and induce senescence of neighboring normal cells [30]. Therefore, we detected the expression of senescence-associated secretory phenotype (SASP) under different concentrations of JQ1 by qRT-PCR. It was found that the expressions of GROα and IL-6 were significantly decreased when JQ1 reached 100 nM (**Figure 5A-D**). The surprising results showed that JQ1 can promote cell senescence without causing the "side effects" of SASP.

JQ1 inhibits the Wnt signaling pathway and EMT in pancreatic cancer cells

GSEA was used to identify the underlying molecular mechanisms, specifically oncogenic pathways that may be influenced by BRD4 dysregulation. Several canonical pathways, including the Wnt signaling pathway, were associated with the BRD4 (**Figure 5E**). The western blot analysis further demonstrated that JQ1 inhibited N-cadherin expression at different concentrations of drug as drug concentration increased (**Figure 5F**, **5G**). Additionally, β-catenin, a critical Wnt signaling pathway locus, was decreased. This indicated that the Wnt signaling pathway and downstream epithelial-mesenchymal transition (EMT) were inhibited.

Discussion

We have shown that BET inhibitor JQ1 effectively inhibited the proliferation and senescence of pancreatic cancer cells but SASP expression was not promoted. Furthermore, the Wnt signaling pathway and EMT were inhibited.

Based on the high expression of BRD4 in pancreatic cancer, targeted BET is theoretically effective for pancreatic cancer. Previous studies have shown that JQ1 can effectively inhibit the proliferation of pancreatic tumors [29], and our evidence further confirms this conclusion. However, some studies also have found that JQ1 can activate additional oncogenic pathways and may affect epithelial-to-mesenchymal transition (EMT) [31]. The Wnt signal pathway is one of the major modulators of EMT [32]. In addition, the Wnt signaling pathway has been confirmed to be regulated by JQ1 in certain cancers [5, 33]. Notably, our study found that JQ1 inhibits the Wnt signaling pathway, a carcinogenic signal that helps cells maintain stem cell characteristics and self-renewal by regulating the balance of GSK3β Ser9 phosphorylation.

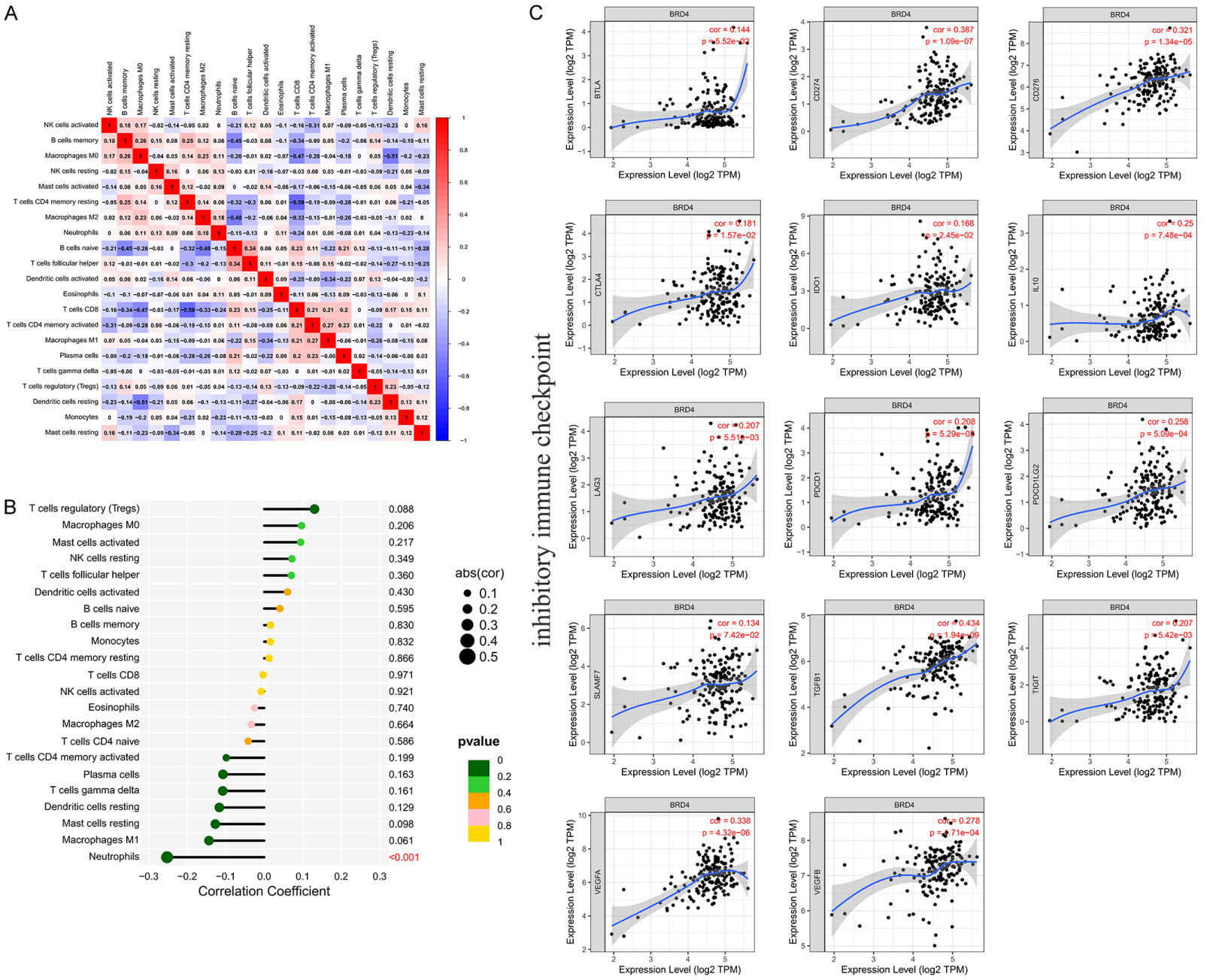
A
NK cells activated
B cells memory
Macrophages M0
NK cells resting
Mast cells activated
T cells CD4 memory resting
Macrophages M2
Neutrophils
B cells naive
T cells follicular helper
Dendritic cells activated
Eosinophils
T cells CD8
T cells CD4 memory activated
Macrophages M1
Plasma cells
T cells gamma delta
T cells regulatory (Tregs)
Dendritic cells resting
Monocytes
Mast cells resting
B
T cells regulatory (Tregs) 0.088
Macrophages M0 0.206
Mast cells activated 0.217
NK cells resting 0.349
T cells follicular helper 0.360
Dendritic cells activated 0.430
B cells naive 0.595
B cells memory 0.830
Monocytes 0.832
T cells CD4 memory resting 0.866
T cells CD8 0.971
NK cells activated 0.921
Eosinophils 0.740
Macrophages M2 0.664
T cells CD4 naive 0.586
T cells CD4 memory activated 0.199
Plasma cells 0.163
T cells gamma delta 0.161
Dendritic cells resting 0.129
Mast cells resting 0.098
Macrophages M1 0.061
Neutrophils <0.001
Correlation Coefficient
abs(cor)
pvalue
C
inhibitory immune checkpoint
BRD4
BTLA
CD274
CD276
CTLA4
IDO1
IL10
LAG3
PDCD1
PDCD1LG2
SLAMF7
TGFB1
TIGIT
VEGFA
VEGFB
Expression Level (log2 TPM)

Figure 2. The relationship between BRD4 expression and tumor-infiltrating immune cells. A. Intercellular associations of immune cells in pancreatic cancer. B. Comparison of immune cell infiltration levels between high and low BRD4 expression groups. C. TIMER database showed a correlation between BRD4 and immune checkpoint in pancreatic cancer.

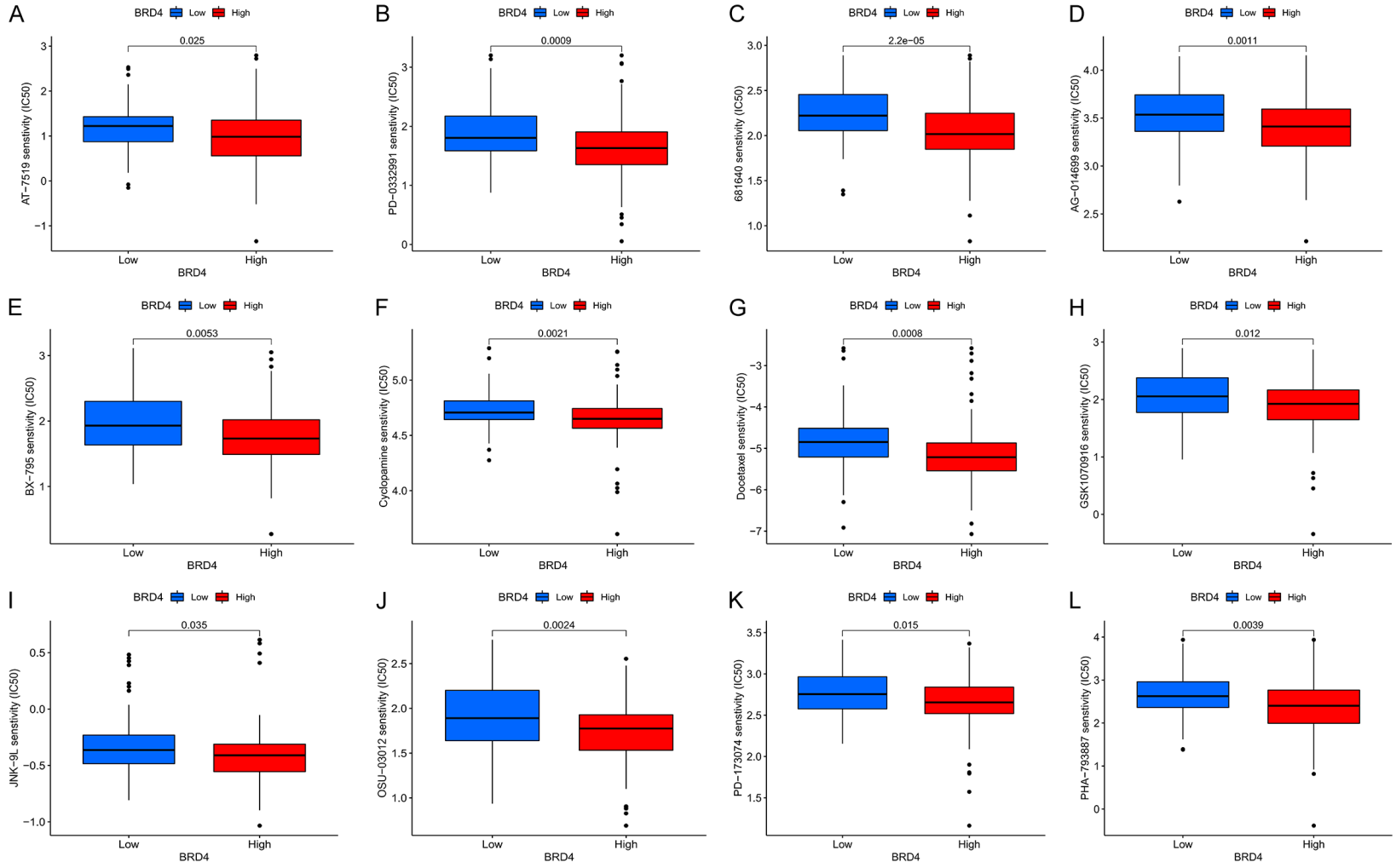

Figure 3. Differences in sensitivity of common chemotherapy agents between high and low BRD4 expression groups in pancreatic cancer. A-L. The IC_{50} values of BX-795, AT-7519, and other drugs.

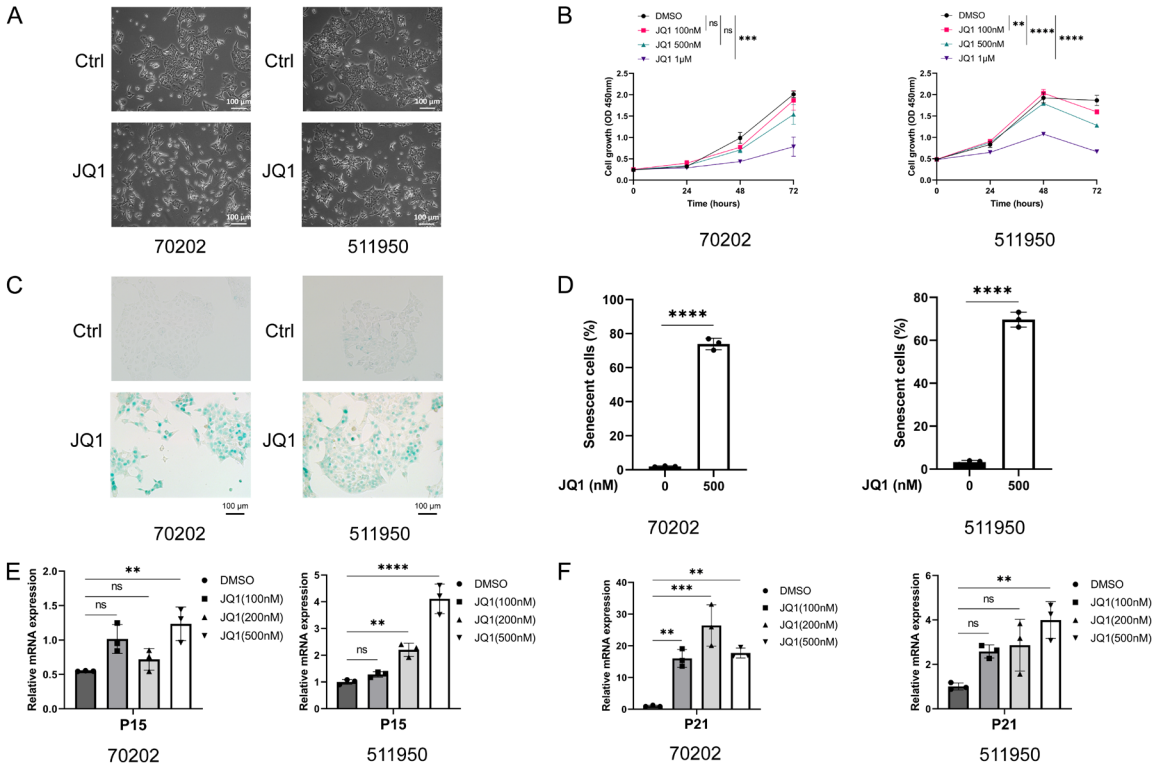

Figure 4. Effects of JQ1 on primary pancreatic cancer cells *in vitro*. (A) Changes in cell morphology before and after 72 h of JQ1 monotherapy (500 nM). Scale bars, 100 μm. (B) Primary pancreatic cancer cells were treated with the indicated concentrations of JQ1 for 72 h. Cell viability was determined using a CCK-8 assay. (C) SA-β-gal staining of cells treated with JQ1 (500 nM) for 72 h. Scales bars, 100 μm. (D) Quantification of the SA-β-Gal+ cell numbers stained in (C). **P < 0.01 by Student's t-test. Data are means ± SEM from three experiments. ****P < 0.0001 by Student's t-test. (E, F) mRNA expression of p15 and p21 was detected after JQ1 was treated at different concentrations for 72 h by quantitative PCR. Data are means ± SEM from three experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 and ns, not significant by Student's t-test.

For GSK3β, the target is located at the confluence of Wnt/β-catenin, RAS/RAF/MEK/ERK, PI3K-AKT, and AMPK signal pathways, therefore, GSK3β has become a potential target for cancer therapy. It is essential for maintaining cell signal transduction to maintain the delicate balance of phosphorylation and dephosphorylation by kinases and phosphatases [34]. When the Ser9 site of GSK3β is phosphorylated and inactivated, it will induce β-catenin dephosphorylation to the active form and translocate to the nucleus [35]. This is a typical mechanism of phosphorylation at the GSK3β Ser9 site, which activates the typical Wnt/β-catenin signal pathway [36]. It is also one of the most common ways of drug resistance in cancer treatment. However, although JQ1 up-regulated p-GSK3β (Ser9), it did not promote the expression of β-catenin. The results show that there may be other non-classical pathways in which JQ1 inhibits the Wnt/β-catenin signaling pathway. However, how JQ1 regulates β-catenin needs further verification. In addition, whether the inhibition of EMT induced by JQ1 is directly caused by the upstream Wnt/β-catenin signaling pathway, or whether there are other regulatory pathways, such as TGFβ/Smad signal pathway, needs to be further explored.

Cell senescence plays an important role in oncogenesis [30]. Our study found that JQ1 can promote the upregulation of p15 and p21 genes, the characteristic markers of cell senescence, and the expression of SA-β-Gal in pancreatic cancer cells. However, the mechanism of JQ1-induced p15 and p21 expression and related cell senescence is not clear. The down-regulation of oncogene c-Myc is considered to be the main mechanism of the anti-proliferative effect of BET inhibitors in many cancer cells [37]. Considering that c-yc negatively regulates the expression of p21, the up-regulation of p21

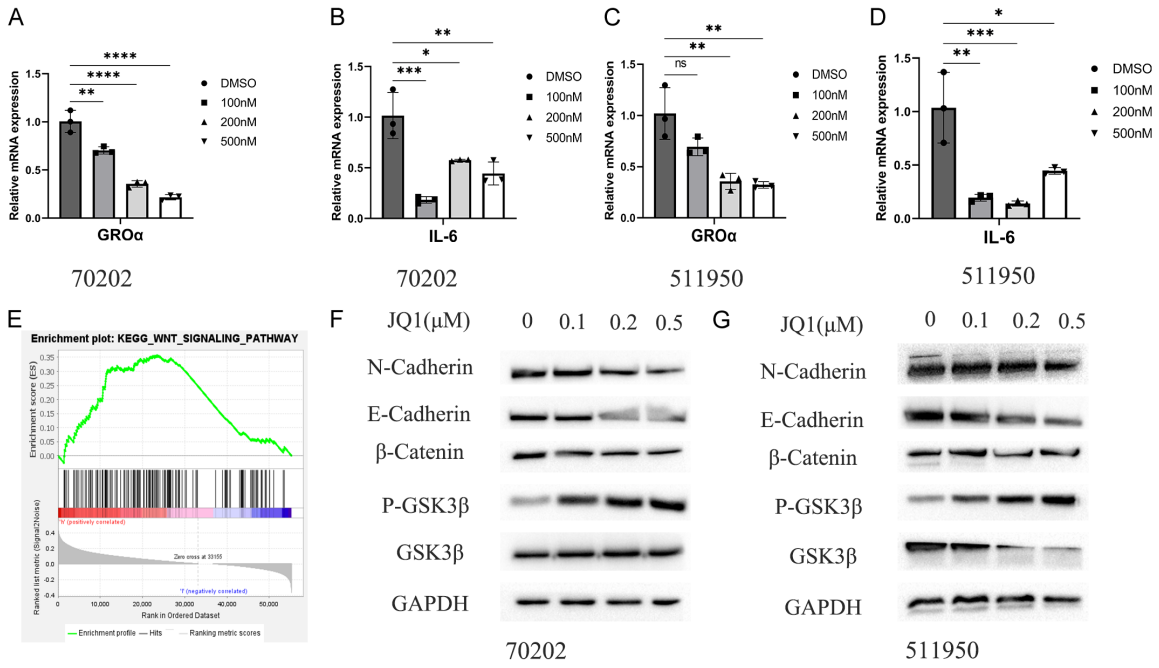

Figure 5. JQ1 inhibits SASP and activates the Wnt signaling pathway to promote EMT. A-D. mRNA expression of SASP (GROα and IL-6) was detected after JQ1 was treated at different concentrations for 72 h by quantitative PCR. Data are means ± SEM from three experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 and ns, not significant by Student's t-test. E. KEGG pathway enrichment analysis. F, G. Western blots of EMT changes and Wnt signaling pathway activation markers influenced by JQ1.

in pancreatic cancer cells treated with JQ1 may also be the result of the down-regulation of c-Myc. In addition, some studies have shown that JQ1 can down-regulate the transcription of CDK6, P15INK4B blocks the progress of the cell cycle by binding to CDK6, and the down-regulation of CDK6 can promote the expression of p15INK4B by negative feedback [39]. Nonetheless, the relevant mechanism still needs further confirmation.

Senescence-associated secretory phenotype (SASP), as the dark side of the senescence response, has the ability to promote tumor cell invasion and metastasis [30]. Interleukin-6 (IL-6) is a multipotent proinflammatory cytokine. IL-6 has been shown to be related to DNA damage and carcinogenic stress-induced senescence of keratinocytes, melanocytes, monocytes, fibroblasts, and epithelial cells in mice and humans [30]. GROα (CXCL-1) is a member of the CXC family and is highly expressed in most senescent cells [38]. Therefore, we studied whether JQ1 can induce the production of SASP while promoting the senescence of pancreatic cancer cells. However, the results showed that the mRNA expression of IL-6 and GROα was down-regulated. These results are of positive significance for the evaluation of JQ1 treatment in pancreatic cancer, and the reduced SASP response affects the immune surveillance of senescent cells. Nonetheless, the therapeutic effect of JQ1 requires validation in an *in vivo* model and clinical trials. We explored this more deeply in another study (unpublished data).

In summary, our study explored the anti-proliferative effect of JQ1 in pancreatic cancer cells and analyzed the possible mechanism of BRD4 inhibition regulating EMT by inhibiting the Wnt signal pathway. In addition, we confirmed that JQ1 can promote the senescence of pancreatic cancer cells but does not lead to the production of SASP. These studies provide new insight into the proliferation of cancer cells mediated by BRD4.

Conclusions

BRD4 is up-regulated and associated with immune infiltration in pancreatic cancer. High expression of BRD4 increases the sensitivity of pancreatic cancer cells to chemotherapeutic drugs. Inhibition of BRD4 inhibits proliferation and triggers cellular senescence in pancreatic

cancer cells. However, targeted BRD4 alleviates the senescence-associated secretory phenotype. Moreover, BRD4 inhibitor JQ1 inhibits the Wnt signaling pathway and EMT.

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Disclosure of conflict of interest

None.

Abbreviations

BET, bromine domain and extra terminal domain; SASP, senescence-associated secretory phenotype; EMT, epithelial-mesenchymal transition; GEPIA, the Gene Expression Profiling Interactive Analysis; PAAD, pancreatic adenocarcinoma; DCs, dendritic cells; SA-β-Gal, senescence-associated β-galactosidase; MSigDB, the Molecular Signatures Database; FDR, false discovery rate; KPC, Kras+/LSL-G12D, Trp53+/LSL-R172H, Ptf1a+/Cre.

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