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The gene RAD51AP1 promotes the progression of pancreatic cancer via the PI3K/Akt/NF-κB signaling pathway

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Pancreatic cancer is one of the most lethal tumors due to its rapid proliferation and aggressiveness. RAD51AP1 is a protein-coding gene with critical functions in many cancers but few studies have assessed RAD51AP1 in pancreatic cancer. Bioinformatics methods and cell function experiments were performed to reveal the functions of RAD51AP1 *in vitro*. Gene Expression Profiling Interactive Analysis (GEPIA) was used to explore key proteins and their relationships with RAD51AP1 in the PI3K/AKT/NF-κB signaling pathways. Western blotting (WB) was conducted to detect the expression of key proteins after the downregulation of RAD51AP1. Co-Immunoprecipitation (Co-IP) was applied to confirm the binding of RAD51AP1 and PI3K. In addition, the lentivirus was used to construct subcutaneous tumors in nude mice to verify the function of RAD51AP1 *in vivo*. The Kaplan-Meier curves illustrated that elevated expression levels of RAD51AP1 were significantly correlated with reduced overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI) in pancreatic cancer patients. The results of WB showed that several key proteins in the PI3K/AKT/NF-κB signaling pathway (including PI3K, AKT, IKK1, IKK2, P65, P50, C-FLIP, and XIAP) exhibited a significant knockdown upon reducing the expression of RAD51AP1. Co-IP suggested that RAD51AP1 could directly bind to PI3K. *In vitro*, CCK-8, wound healing, and Transwell assays revealed that high RAD51AP1 expression was significantly correlated with increased cell proliferation, migration, and invasion. *In vivo*, mouse tumor formation experiments showed that RAD51AP1 inhibition significantly inhibited tumor growth. RAD51AP1 plays an important role in fostering cellular proliferation, invasion, metastasis, and tumor enlargement via the PI3K/AKT/NF-κB signaling pathway.

Key words: RAD51AP1; pancreatic cancer; mutation; prognostic; progression

Pancreatic cancer is regarded to be one of the most fatal and invasive tumors in the world, and the incidence is still rising significantly according to some studies [1]. Despite the progress of current treatment methods, the 5-year overall survival rate is just around 10% due to recurrence, early metastasis, local invasion, and a lack of methods for early inhibition [2].

The aberrant expression of genes is interrelated with several biological characteristics of cancer cells, which may lead to a bad survival expectancy and an insensitive response to therapy in many cancer types, including pancreatic cancer [3, 4]. In addition, many signaling pathways are involved in this physiological process, especially the PI3K/AKT/NF-κB pathways [5–7].

RAD51-associated protein 1 (RAD51AP1), also known as PIR51, is a protein-coding gene located on chromosome 12p13 and has a significant role in regulating DNA replication stress. Some studies have indicated that ectopic expression of RAD51AP1 can lead to an adverse prognosis in many malignant tumors, such as hepatic carcinoma [8], mammary malignancy [9], cholangiocarcinoma [10], ovarian neoplasms [11] as well as non-small cell lung cancer [12]. Comprehensively, ectopic expression of RAD51AP1 is generally associated with quick disease progression, low operation rates, unfavorable prognosis, etc. In different tumors, the pathways and mechanism of RAD51AP1 are not entirely the same, which refers to different research plans and treatment options. Although a bioinformatics study indicates that RAD51AP1 could be a prospective biomarker in pancreatic cancer, further exploration and verification are still needed to find the underlying mechanism [13].

In our work, we further explored the influence and mechanism of RAD51AP1 in pancreatic cancer cases in order to provide a new perspective on the pathogenesis and therapeutic measures.

Materials and methods

Bioinformatics analysis of databases. Open-source pancreatic adenocarcinoma (PAAD) gene expression information of RAD51AP1 was downloaded via TCGA database (https://portal.gdc.cancer.gov/). In this process calculation, with the assistance of the UCSC Xena network (http://xena.ucsc.edu/), patients' OS, DSS, and PFI data were analyzed in order to obtain survival information. The threshold of the p-value was selected as 0.05. After integrating two sets of data, redundant data were eliminated. Based on the median expression value of RAD51AP1, the cohorts were classified into low- and high-level groups.

The box plot analysis of the GEPIA database (http://gepia.cancer-pku.cn/) was used to present the mRNA expression levels of RAD51AP1 in pancreatic cancer cases. In addition, for interactive analyses of proteins corresponding to genes, GEPIA was also used to analyze the relationship between two protein factors. Key factors of PI3K/AKT signaling pathway are PI3K, AKT, and IKK1. IKK1 can stimulate the NF-κB pathway through crosstalk interaction. The key factors in the the NF-κB pathway are IKK2, P50, P65, C-FLIP, and XIAP. The key protein factors above make up the PI3K/Akt/NF-κB signaling axis.

Tissue acquisition and cell culture. 5 paired pancreatic cancer along with corresponding adjacent normal tissues were adopted from randomly selected patients diagnosed with pancreatic cancer by postoperative pathology at East Hospital. These tissues served to test RAD51AP1 expression levels in actual pancreatic cancer patients. All patients agreed to join our study by signing the consent form for incorporation of their samples into the biological sample library. The following five pancreatic cancer cell lines (PANC-1, AsPC-1, PaCa-2, SW1990, and BxPC-3) were provided by Procell Life Science & Technology Co., Ltd. (Wuhan, China). The normal immortalized pancreatic cell line hTERT-HPNE was purchased from ICell Bioscience Inc (Shanghai, China). AsPC-1 and BxPC-3 cells were incubated in 10% fetal bovine serum (FBS), which was mixed with RPMI-1640. SW1990 cells were cultured in Leibovitz's L-15 medium with 10% FBS. PANC-1 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. MIAPaCa-2 cells were cultured in DMEM, 2.5% HS and 10% FBS.N (hTERT-HPNE) were propagated in 70.5% sugar-free DMEM, 10 ng/ml EGF, 23.5% M-3 base medium, 5% FBS, 2.5 g/l glucose, and 750 ng/ml puromycin. The environment of the humidified incubator was set to 37 °C with 5% CO_2.

Table. 1. The primer sequences of GAPDH and RAD51AP1.

Primer name		
GAPDH	Forward (5'–3')	CATCATCCCTGCCTCTACTGG
	Reverse (5'–3')	GTGGGTGTCGCTGTTGAAGTC
RAD51AP1	Forward (5'–3')	CTAGGAAACCATTAGAAATACGC
	Reverse (5'–3')	CAAAGGTTTAACTCGTGCTAATC

RNA extraction and real time quantitative PCR (RT-qPCR). All the tissue RNA was extracted with TRIpure Total RNA Extraction Reagent (EP013, ELK Biotechnology, Wuhan, China). Synthesis of cDNA was completed with the M-MLV reverse transcriptase kit (EQ002, ELK Biotechnology, Wuhan, China). RT-qPCR was carried out by a StepOne Real-Time PCR System (Life Technologies, Carlsbad, USA) using the QuFast SYBR Green PCR Master Mix Kit (EQ002; ELK Biotechnology, Wuhan, China) with conditions as follows: pre-denaturation at 95 °C for 60 s, next 40 cycles of 95 °C for 15 s and 58 °C for 20 s, then 72 °C for 45 s. The reference gene was GAPDH. Primer sequences of RAD51AP1 and GAPDH are shown in Table 1. The results were calculated using the formula $2^{-\Delta\Delta}$CT.

Small interfering RNA (siRNA) transfection. siRNA specially targeted RAD51AP1 (si-RAD51AP1) and control siRNA (si-NC) was obtained from GeneCreate Biological Engineering Company (Wuhan, China). The sequences of si-RAD51AP1 and si-NC were as follows: siRNA1, 5'-ACAGTTTGACCACTCTGACAGTGAT-3'; siRNA2, 5'-GCCTTGTACAAAGATGGCTTTAGAT-3'; siRNA3, 5'-CCTCATATCTCTAATTGCAGTGTAG-3'; control, 5'-ACAAGTTCACCGTCTGACATTGGAT-3'. The cell lines AsPC-1 and BxPC-3 were incubated in 6-well plates and were transfected with 50 nmol/l si-RAD51AP1 or si-NC for 6 hours. Cells were further incubated for 72 h preparing for the following test, including RT-qPCR, CCK-8, wound healing, and Transwell experiment.

Cell proliferation assay. The selected pancreatic cancer cell line was incubated in 96-well plates at 1×10^4 cells/well and transfected with si-RAD51AP or si-NC. CCK-8 solution (C0038, Beyotime, Shanghai, China) was chosen to check and measure the status of cell proliferation. The cell was incubated for 2 hours in the culture medium containing 10 μl/well CCK-8 reagent, the absorbance of each well was measured using a DR-200B enzyme labeling analyzer (Wuxi, China) at 450 nm from day 1 to day 4.

Wound healing assay. The selected pancreatic cancer cell line was plated onto 6-well plates. The wound was scratched with a pipette tube. The width of the wound was measured at 0 and 24 h. Photographs were taken in several premarked spots with an IX51 inverted microscope (Olympus, Tokyo, Japan) and a MicroPublisher imaging system (Q-Imaging, Surrey, BC, Canada).

Transwell assay. A Transwell invasion assay was executed with 24-well plates (Corning, NY, USA). A 200 μl suspension of cells (1.0×10^5 cells/ml) was added to the upper chamber of the Transwell, while the lower chamber was filled with medium containing 10% FBS. For staining, crystal violet dye was diluted to 0.1%. Cells in chambers above were erased with

a cotton bud, those in the below were considered the invasive cells. 4% paraformaldehyde and crystal violet were used to fix and stain the cells respectively. Then the lower chamber was placed in an inverted microscope IX51 (Olympus, Japan).

Western blot analysis. The cells were fully lysed by RIPA total protein lysis solution (AS1004, ASPEN). The total protein was identified by the BCA assay kit (AS1086, ASPEN). Forty micrograms of protein per sample were subjected to SDS-PAGE, and 5× protein loading buffer (AS1011, ASPEN) was used in a boiling water bath. The electrophoretic voltages were set at 120 V. Then, the samples were diverted onto polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, USA) and blocked for 1 hour under normal temperature. Below is the information of the primary antibodies selected for the experiment: rabbit anti-GAPDH (dilution 1:10,000; ab181602; Abcam), mouse anti-RAD51AP1 (dilution 1:1,000; ab88370; Abcam), rabbit anti-PI3K (dilution 1:1,000; 20584-1-AP; Proteintech), rabbit anti-AKT (dilution 1:3,000; #75692; CST), rabbit anti-IKK1 (dilution 1:3,000; ab32041; Abcam), rabbit anti-IKK2 (dilution 1:1,000; #8943; CST), rabbit anti-P50 (dilution 1:1,000; #13586; CST), rabbit anti-P65 (dilution 1:3,000; #8242; CST), rabbit anti-CFLIP (dilution 1:1,000; ab8421; Abcam), and rabbit anti-XIAP (dilution 1:1,000; 10037-1-Ig; Proteintech). The secondary antibodies were HRP-goat anti-rabbit (dilution 1:10,000, AS1107, ASPEN) and HRP-goat anti-mouse (dilution 1:10,000, AS1106, ASPEN). After incubation, the membranes were treated by freshly prepared enhanced chemiluminescence (ECL) mixed solution (AS1059, ASPEN) and exposed in a darkroom. The gray values were assessed with AlphaEaseFC software system.

Construction of overexpression vector. A full-length cDNA clone of H-RAD51AP1 with the open reading frame (ORF) and restriction enzyme site was constructed at ELK Biotechnology (Wuhan, China). NheI and BamHI were used for dual enzyme digestion, and electrophoresis was performed to confirm the digestion. The colony was selected and inoculated in a liquid nutrient medium for 16 h at 37 °C, followed by centrifugation at 1200× g for 1 min. After incubation, *E. coli* DH5α with the pcDNA3.1-RAD51AP1-HA plasmid was obtained using the EndoFree Plasmid Miniprep Kit (EP004, Elk Biotechnology), and the RAD51AP1 fragment was amplified. The plasmid was extracted using the Gel DNA Purification Kit (EP006, ELK Biotechnology), and the entire sequencing was performed using the EntiLink™ PCR Master Mix (Elk Biotechnology, China).

Co-IP assay. For protein collection, IP lysis buffer (AS1003, ASPEN) and protease inhibitor cocktail (AS1005C, ASPEN) were used to fully lyse the cells. The samples were incubated with 20 μl of prewashed SureBeads™ Starter Kit Protein A (#1614813, BIO-RAD) at 4 °C for 30 min. Then, the IP antibodies were mixed with the pre-prepared lysates and incubated at 4 °C for 1 h. Subsequently, 20 μl of beads was added to each sample, which were incubated overnight at 4 °C. After thorough centrifugation and cleaning, the obtained Co-IP products were mixed with 2× SDS sample loading buffer containing β-mercaptoethanol. The mixture was boiled for 10 min and subjected to SDS-PAGE. The separated proteins were then electrophoretically transferred to a PVDF membrane. After blocking, diluted primary and secondary antibodies were added sequentially and incubated simultaneously. Chemiluminescence detection photos were taken after thorough washing with TBST Buffer.

Lentiviral construction and transduction. The shRNA fragment targeting RAD51AP1 was designed and provided by Aspen (Wuhan, China) using the commercial lentivirus vector pLVX shRNA2 (P0425, MiaoLingBio). Lentivirus packaging was performed in 293FT/293T cells using Lipofectamine2000 (Invitrogen). After packaging, the lentivirus was collected and concentrated using a lentivirus concentration solution (ELK, EP0132). For transduction, cells were seeded into a 24-well plate and the selection of resistant cells was initiated when the cell confluence reached 80–90%. Puromycin was chosen as the antibiotic at an appropriate concentration of 1 μg/ml.

Logarithmic growth phase cells (1×10^4 cells/ml) were inoculated into 96-well plates (100 μl/well). When the cell density reached approximately 40%, the lentivirus was added to the target cells for transduction.

Transduced cells with stable integration were selected using the previously determined optimal concentration of puromycin. The antibiotic maintenance concentration was further reduced to 1/4 of the screening concentration for subsequent screening and expansion. The resistant cells were maintained and expanded using T25 culture flasks and preserved in liquid nitrogen.

***In vivo* tumorigenesis assay.** After stably expressed cells were constructed, nine 5-week-old male nude mice (Animal Disease Control Center, Hubei, China) were randomly and evenly divided into three groups (Blank control group, NC-LV negative control group, and RAD51AP1-shRNA-LV group), which were injected with pancreatic cancer cells transfected with lentivirus (sh-NC lentivirus and RAD51AP1-shRNA lentivirus, respectively). The experiment was completed in a specific pathogen-free environment, which complies with laboratory animal law and the guidelines of the institute. 5×10^6 cells for each sample were injected subcutaneously into the middle axilla of the mice in the corresponding groups. Diameters of the tumor were recorded every 7th day. The calculation formula for tumor volume was: volume = a*b*b/2 (a, long diameter; b, short diameter). The mice were sacrificed under anesthesia after 28 days.

Immunofluorescence staining. Fixed with 4% paraformaldehyde, cells were incubated with the corresponding primary antibody at 4 °C overnight. The primary antibodies used were as follows: rabbit anti-RAD51AP1 (dilution 1:200; 11255-1-AP; Proteintech), mouse anti-PI3K (dilution 1:200; 67071-1-Ig; Proteintech). The following incubation was with the targeted fluorescently labeled secondary antibodies for 40 min in the darkroom at 37 °C. The secondary antibodies used

were as follows: Alexa Fluor 488-conjugated AffiniPure goat anti-rabbit IgG (dilution 1:100; SA00013-2; Proteintech), Cy3-conjugated AffiniPure goat anti-mouse IgG (dilution 1:100; AS-1111; Aspen). Staining of the cell nucleus was performed with DAPI. The fluorescence images of the cells were photographed by the luminescence microscope.

Statistical analysis. Statistical data were recorded in the form of mean ± SD. For the CCK-8, Transwell, and wound healing assays, Student's t-test was chosen to calculate the differences between experimental groups. For western blotting and tumorigenicity analysis in nude mice, nonparametric tests were used. The KM survival curve was adopted to compare survival differences. The p-value <0.05 meant significant differences. The experimental results were evaluated with SPSS 22.0 software.

Results

RAD51AP1 is elevated in pancreatic cancer and correlated with poor prognosis. RAD51AP1 plays an important role in DNA repair by homologous recombination. To further explore the clinical effect of RAD51AP1 in pancreatic cancer cases, we calculated the RAD51AP1 expression data of TCGA-PAAD cohort. Compared with normal cases, the mRNA expression level of RAD51AP1 in cancer cases was illustrated to be obviously elevated in tumor ones (Figure 1A). To verify the results, qPCR was performed to detect the mRNA expression of RAD51AP1 in five pairs of tissues from pancreatic cancer cases. The results demonstrated a significant increase in RAD51AP1 mRNA expression in pancreatic cancer tissue compared to adjacent normal tissues. (Figure 1B). Subsequently, the cases from TCGA-PAAD dataset were divided into high- and low-expression groups based on RAD51AP1 mRNA levels. The results of the KM plot showed that RAD51AP1 was significantly related to OS, DSS, and PFI in pancreatic cancer patients ($p<0.05$, Figure 1C), revealing that patients with high RAD51AP1 expression suffered a poorer prognosis ($p<0.05$). We analyzed the expression of RAD51AP1 in some commonly used pancreatic cell lines in research. The box plots indicated that RAD51AP1 was significantly higher in AsPC-1 and BxPC-3 cells compared with hTERT-HPNE cells, which was commonly used as a model for normal pancreatic cells ($p<0.05$, Figure 1D).

Downregulation of RAD51AP1 restrained the pancreatic cancer cell proliferation and invasion ability *in vitro*. For further exploration of the RAD51AP1 biological function *in vitro*, three types of siRNA were constructed. AsPC-1 and BxPC-3 cells were chosen to verify the interference effect of siRNA. The results revealed that

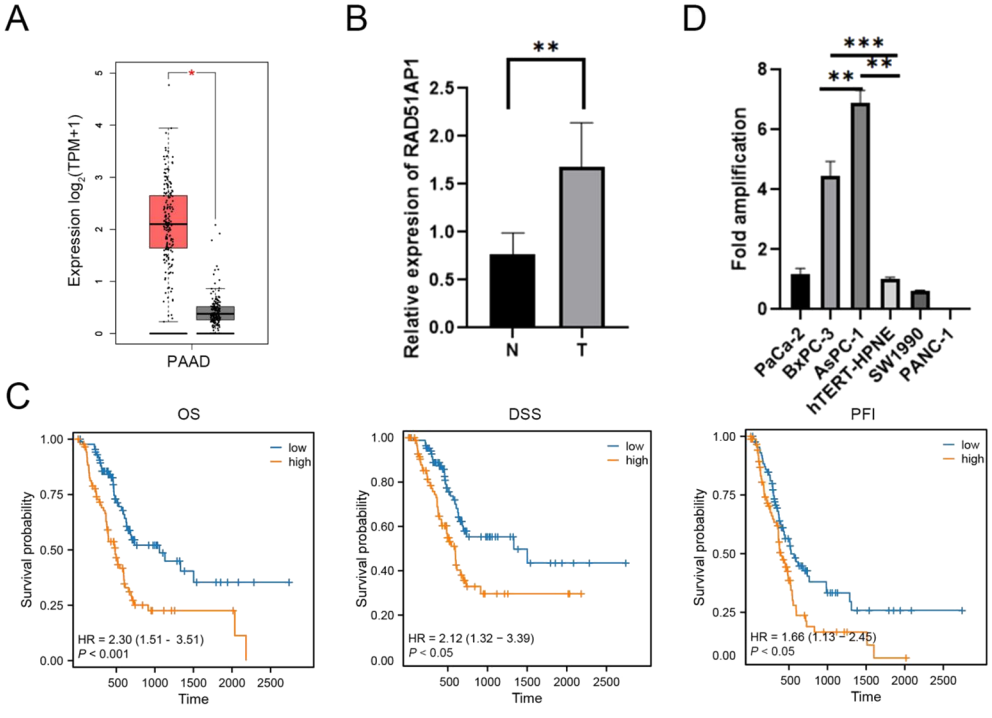

Figure 1. RAD51AP1 was elevated in pancreatic cancer and relevant to poor prognosis. A) Level of RAD51AP1 expression in pancreatic cancer and normal cases from GEPIA, *p<0.05. The red bars represent pancreatic cancer samples and the gray bars represent normal samples. B) RAD51AP1 expression in human pancreatic cancer tissues, **p<0.01. The gray bars represent pancreatic cancer samples and the black bars represent normal samples. C) KM curves revealed the relationships between the expression of RAD51AP1 and the prognosis of patients. The blue curves indicated the low-level groups; the red curves indicated for the high-level groups. The survival times were measured in days. D) RAD51AP1 expressions in different pancreatic cell lines, **p<0.01, ***p<0.001.

si-RAD51AP1-2 had the most obvious inhibitory effect ($p<0.05$, Figure 2A). In addition, there was no significant difference between the si-NC groups and the control groups. In the CCK-8 experiment, cells were classified into the si-NC group and the si-RAD51AP1 group. The results were observed for four days with six repeats of each group. The observed optical density (OD) values of the si-RAD51AP1 groups were significantly lower than that of the si-NC group ($p<0.05$), indicating that cell proliferation was inhibited after knockdown of RAD51AP1 ($p<0.05$, Figure 2B). Compared with si-NC groups, the wound healing rates were significantly lower in the si-RAD51AP1 groups at the 24th hour ($p<0.05$, Figures 2C, 2D). The result of the wound-healing assay indicated that a high level of RAD51AP1 expression could increase the metastatic ability of pancreatic cancer cells. In addition, the number of transmural cells in the si-RAD51AP1 groups was significantly lower than that in the si-NC groups ($p<0.001$), suggesting that the invasive ability was significantly decreased in the si-RAD51AP1 groups ($p<0.05$, Figures 2E, 2F).

Signaling pathway and mechanism of RAD51AP1 exploration in bioinformatics. To further analyze the underlying mechanisms, GEPIA was utilized to explore the relationships between RAD51AP1 and key factors of the PI3K/AKT/NF-κB axis in pancreatic cancer. The results displayed a positive interaction between the mRNA expression of RAD51AP1 and the key factors, which included PI3K, AKT, IKK1, IKK2, P50, P65, C-FLIP, and XIAP ($p<0.05$, $R>0$, Supplementary Figures S1A–S1H). As a downstream protein of the PI3K/AKT signaling pathway, IKK1 was significantly associated with RAD51AP1. Moreover, IKK1, IKK2, P65, and P50 are the key factors in the NF-κB pathway, indicating that there was a crosstalk conjunction between PI3K/AKT and NF-κB pathway via the factor IKK1. These findings signified that RAD51AP1 could regulate the cytoactive of pancreatic cancer cells through the PI3K/AKT/NF-κB signaling axis.

RAD51AP1 induces the key protein factor of PI3K/AKT and NF-κB pathways in pancreatic cancer cells. Considering that the PI3K/Akt/NF-κB signaling axis may be a potential action pathway of RAD51AP1, western blot

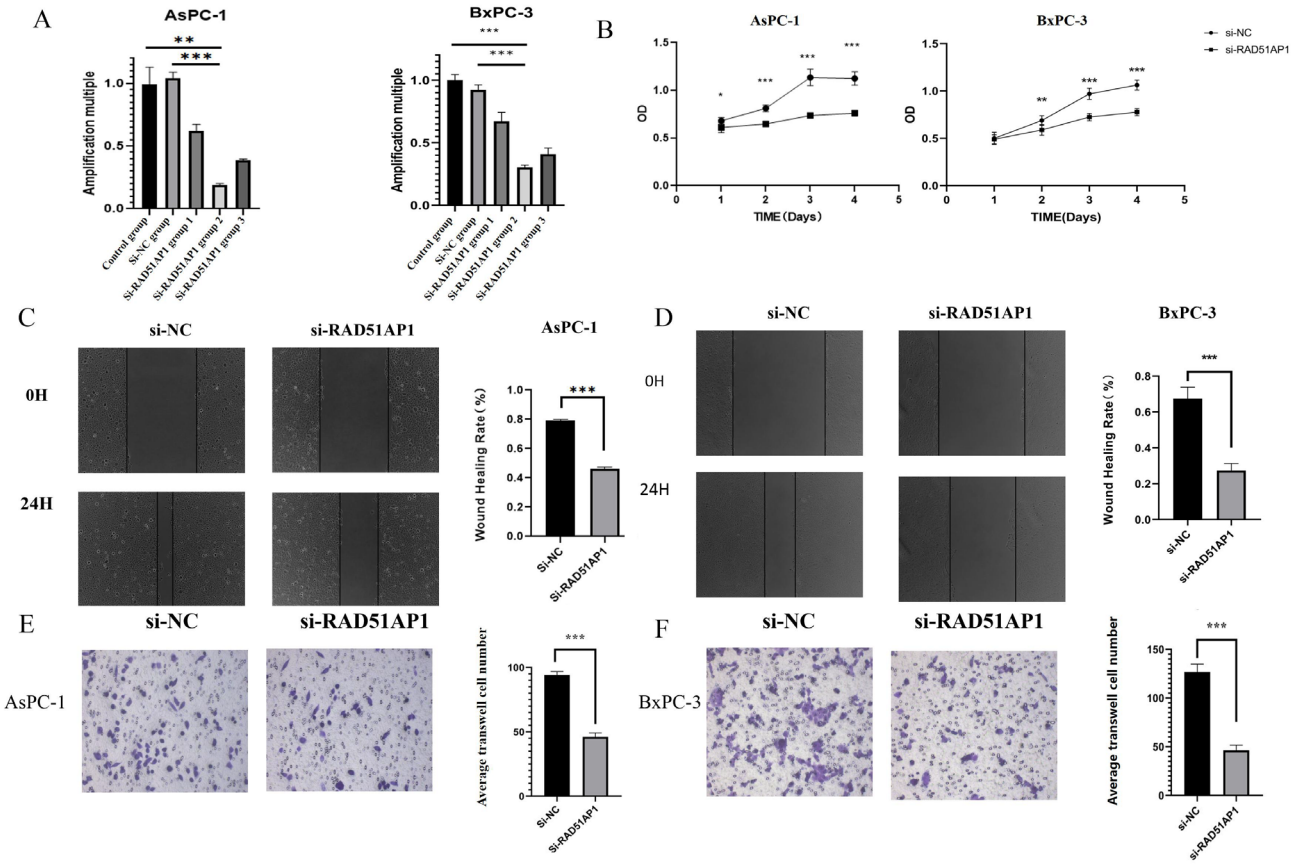

Figure 2. Knockdown of RAD51AP1 could restrain the pancreatic cancer cell proliferation and invasion ability *in vitro*. A) Histogram plots showed the inhibitory efficiency of RAD51AP1 siRNA-1/2/3 in AsPC-1 and BxPC-3 cells by qPCR. Results illustrated that siRNA-2 had the most obvious inhibitory effect, **$p<0.01$, ***$p<0.001$. B) From day 1 to day 4, the OD value of the CCK-8 assay was significantly lower in the si-RAD51AP1 groups than in the si-NC groups, *$p<0.05$, ***$p<0.001$. C–D) The migratory distances were significantly reduced in the si-RAD51AP1 groups, ***$p<0.001$. E–F) There were fewer invasive cells in the si-RAD51AP1 groups than in the si-NC groups, ***$p<0.001$.

assays were arranged to verify the protein expressions of key genes. For the PI3K/AKT pathway, PI3K, AKT, and IKK1 were chosen as the key genes and IKK1 is an activator of the NF-κB pathway. The protein bands and histograms showed that the protein abundances of PI3K, AKT, and IKK1 were significantly decreased after the knockdown of RAD51AP1 ($p<0.05$, Figures 3A, 3C). For the NF-κB pathway, the key genes IKK2, P65, and P50 were also significantly downregulated in the si-RAD51AP1 group ($p<0.05$, Figures 3B, 3D). In particular, the downstream proteins C-FLIP and XIAP are two important antiapoptotic proteins, and their reduced expression can make pancreatic cancer cells more prone to apoptosis with synergistic effects [14, 15]. These proteins are pivotal molecules that form the core of the PI3K/AKT/NF-κB axis, and their alterations impact the entire signaling pathway and its target molecules.

Co-IP and immunofluorescence assay to explore the correlation between RAD51AP1 and PI3K. In the Co-IP assay, the input and IgG samples were set as positive and negative control groups, separately. Protein bands revealed the direct interaction between endogenous RAD51AP1 and PI3K (Figure 4A), which was further confirmed by immunofluorescence assay (Figure 4B). Additionally, immunofluorescence could further present the most remarkable interaction location between the two proteins in the cell. It could be concluded that RAD51AP1 could directly bind to PI3K in pancreatic cancer, and this is a potential way to initiate the PI3K/AKT/NF-κB signaling pathways.

RAD51AP1 knockdown inhibits tumor proliferation *in vivo*. To study the impact of RAD51AP1 *in vivo*, nude mice were injected with lentivirus-treated AsPC-1 cells that had RAD51AP1 knocked down, thereby establishing a subcutaneous xenograft tumor model. Meanwhile, corresponding control groups were established (Figure 5A). The subcutaneous tumors of nude mice in the RAD51AP1-shRNA-LV group showed a significant reduction when compared with the blank control and NC-LV groups, suggesting that the tumor-promoting effect of RAD51AP1 *in vivo* ($p<0.05$, Figure 5B). Next, we performed HE staining on the subcutaneous tumor tissue of nude mice. Compared with the control and NC-LV groups, the RAD51AP1-shRNA-LV group showed multifocal cell necrosis, indicating that RAD51AP1 can promote cancer cell proliferation but when inhibited it can retard the tumor cell growth (Figure 5C).

Discussion

Pancreatic cancer is a fatal cancer with high aggressiveness and poor survival worldwide [16], and its incidence has increased significantly. We found that the excessive abnormal expression of RAD51AP1 can lead to a poor prognosis of pancreatic cancer patients.

RAD51AP1 is a critical protein for DNA repair by homologous recombination (HR) [17, 18]. Research has demonstrated the profound impact of DNA stability and repair fidelity on fundamental aspects such as cancer cell proliferation and metastasis. Although primarily localized in the nucleus, it also sometimes exhibits functional significance in the cytoplasm [19]. The validation and analysis of such observations may necessitate additional experimental investigations and in-depth discussions tailored to the unique characteristics of the protein. Given the crucial role of specific repair proteins in mitigating endogenous and exogenous replication stress to preserve genomic integrity, they possess substantial research value [20–22]. Thus, the related genes and underlying mechanisms are still being explored to improve the prognosis of cancer [23, 24].

RAD51AP1 defects can cause a range of problems, such as genomic instability and telomere erosion [25, 26]. Previous research has shown that upregulation of RAD51AP1 can lead to short survival time in a series of cancers, including liver cancer, cholangiocarcinoma, breast cancer, ovarian cancer, and lung cancers, but studies in pancreatic malignant tumors still need further exploration.

In the current study, we utilized bioinformatics and gene function technology platforms together to identify the important role of RAD51AP1 as a novel potential marker for pancreatic cancer. Bioinformatics is a tool that can be used to analyze large datasets and provides preliminary data for further study. Researchers have gained extensive expertise in this field, which has expanded significantly in recent years. Moreover, the application of bioinformatics has enabled scientists to refine their focus in scientific research by predicting specific targets [27].

Initially, we focused on RAD51AP1 through bioinformatics analysis, and RAD51AP1 was found to be overexpressed in pancreatic cancer. Based on the KM plotter, the curves revealed that overexpression of RAD51AP1 can lead to bad OS, DSS, or PFI time in patients with pancreatic malignancy. Subsequently, extensive research was conducted on the pathways and mechanisms of RAD51AP1 at a deeper level, delving into its complex working mechanisms. Based on bioinformatics analysis, we focused on the mechanism of RAD51AP1 on the PI3K/AKT/NF-κB axis.

The PI3K/AKT/NF-κB axis is always been a focus pathway in cancer research, in which both the PI3K and NF-κB signaling pathways are involved [28–30]. It can act on a sequence of cellular pathophysiological processes, including cell proliferation, apoptosis, differentiation, migration, invasion, and adhesion [31–33]. Key factors affecting the PI3K/AKT/NF-κB pathway are also important targets for the treatment of cancers, including pancreatic tumors [34–39]. In our study, the correlation of key proteins between RAD51AP1 and signaling pathways was analyzed by bioinformatics. This finding was further corroborated by cell function experiments, providing additional evidence to support the observed pathway and mechanism of RAD51AP1. When RAD51AP1 was inhibited, its protein expression level changed significantly. Moreover, Co-IP and immunofluorescence assay revealed direct binding between

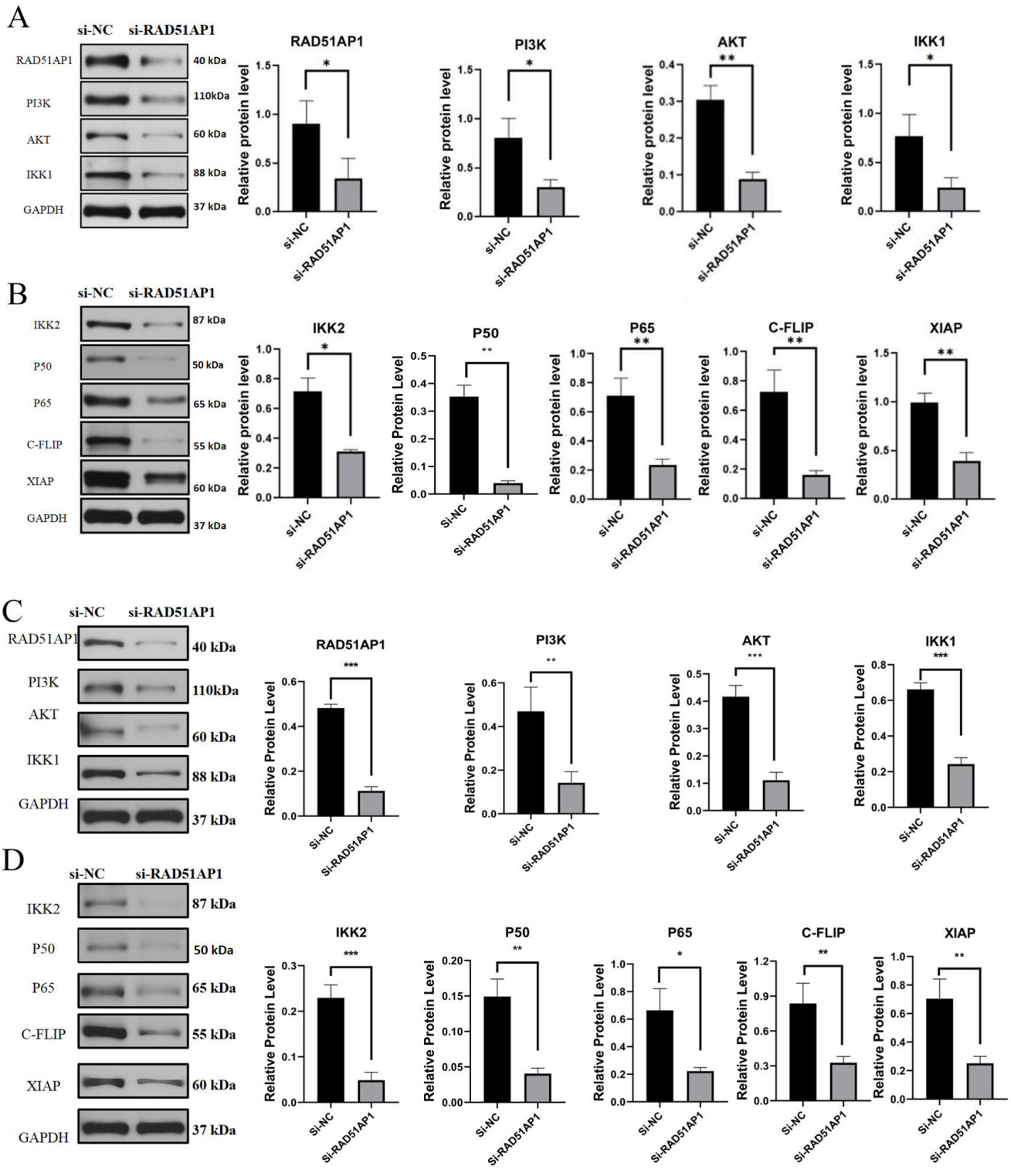

Figure 3. RAD51AP1 induces the proliferation of pancreatic cancer through PI3K/AKT and NF-κB pathways. A, C) Downregulation of RAD51AP1 inhibited PI3K, AKT, and IKK1 in the si-RAD51AP1 groups. B, D) Similarly, IKK2, P50, P65, C-FLIP, and XIAP protein expression was also decreased in the si-RAD51AP1 groups. PI3K, AKT, IKK1, IKK2, P50, P65, C-FLIP, and XIAP are important signal transduction factors that constitute the integrated PI3K/AKT/NF-κB axis. In detail, figures A and B showed the changes of protein levels in the AsPC-1 cell line, while C and D showed the changes in the BxPC-3 cell line.

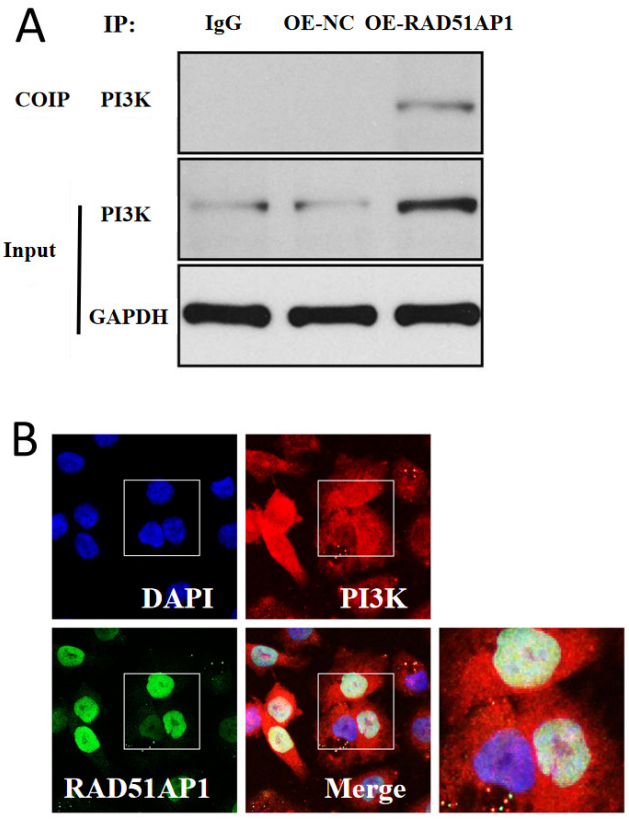

Figure 4. Co-IP and immunofluorescence assay to explore the correlation between RAD51AP1 and PI3K. A) Co-IP assay revealed the direct combining between PI3K and RAD51AP1. The input and IgG groups were set in the assay as positive and negative controls, respectively. OE-NC: overexpression normal control; OE-RAD51AP1: overexpression RAD51AP1. B) The binding of the two proteins was further visualized using an immunofluorescence assay. The highlighted yellow areas illustrate the most remarkable interaction location between the two proteins within the cell. The colors and corresponding labeling information are as follows: RAD51AP1 (green), PI3K (red), and nuclei (blue).

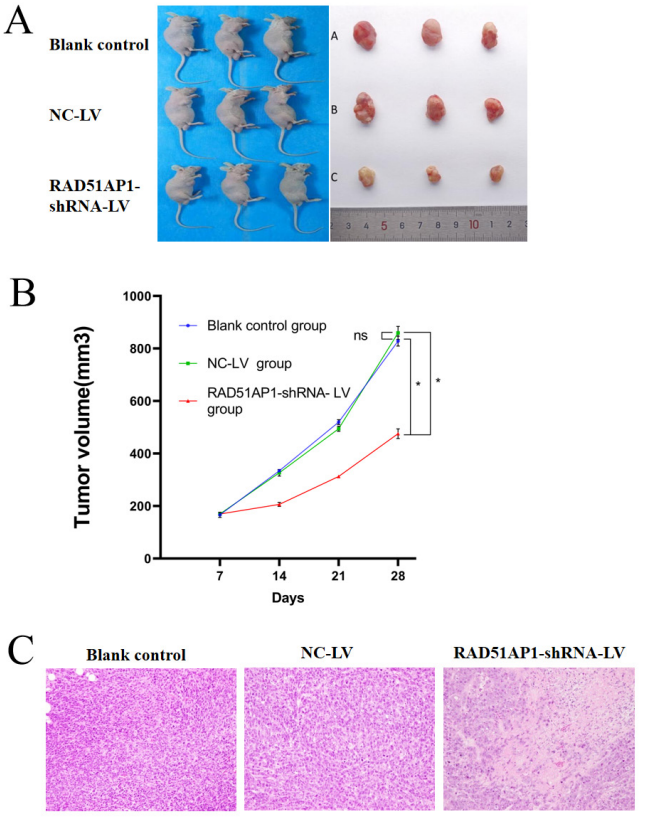

Figure 5. RAD51AP1 knockdown inhibits tumor proliferation *in vivo*. A) Photographs of subcutaneously transplanted tumors in different groups of nude mice. Tumors in theh RAD51AP1-shRNA-LV group were obviously smaller than tumors in blank control and NC-LV groups. B) Line chart showing the tumor growth curve. The result showed that the tumor volume of the RAD51AP1-shRNA-LV group shrank significantly than that of blank control and NC-LV groups. *p<0.05; 'ns' means 'no significance' (p > 0.05). C) Hematoxylin & eosin staining. Magnification: 200×. More necrosis cells were detected in the RAD51AP1-shRNA-LV group.

RAD51AP1 and PI3K, which initiated the PI3K/AKT/NF-κB axis in pancreatic cancer. For organisms, when the expression of RAD51AP1 was knocked down by lentivirus-mediated shRNA, the tumor size decreased significantly.

We found that RAD51AP1 could mediate the two important downstream effectors of the PI3K/AKT/NF-κB signaling pathway, C-FLIP, and XIAP. These two factors exhibit antiapoptotic properties and have the potential to synergistically contribute to the inhibition of apoptosis [14, 15, 40, 41]. Therefore, the downregulation of RAD51AP1 expression holds promise in inhibiting the progression and course of pancreatic cancer, suggesting potential therapeutic benefits.

RAD51AP1 is an important HR-related gene. Studies on these types of genes are helpful in identifying future directions and clinical opportunities. Some studies have indicated that tumor cells can recognize DNA damage and activate related repair mechanisms. Some specific drugs or biological agents are expected to intervene in the self-identification and repair process [42, 43]. Currently, some drugs, especially those targeting DNA damage response (DDR) pathways, are being analyzed for their ability to improve the tolerance or treatment response to drugs, such as chemotherapy and radiotherapy, which are traditional methods in tumor treatment [44–46]. Homologous recombination deficiency (HRD) related drugs, such as PARP inhibitor, was proven effective against hepatitis B virus-associated hepatocellular carcinoma [47]. In addition, it can also be used for gastrointestinal cancer, lung cancer, ovarian cancer, etc. Cancers with HR gene disorders may be more responsive to this kind of treatment [48–50]. Therefore, studies on the mechanism between RAD51AP1 and pancreatic cancer have certain clinical significance for improving the progression of pancreatic cancer patients in the future.

Our study has some limitations. This study was exploratory, and more assays and samples are needed to reveal the mechanism and significance of this gene. In addition, the

number of mouse tumorigenesis experiments was limited, however, the difference was apparent and significant. More studies with clinical cases and experimental animals will be beneficial. AsPC-1 and BxPC-3 cell lines, derived from primary pancreatic cancer tumors and characterized by their diffuse and metastatic behavior, have been widely utilized as standard models to investigate gene function in numerous studies related to pancreatic cancer [51–53]. However, additional research is required to explore the use of other types of pancreatic cancer cells.

In summary, because of the poor prognosis, researchers are still aiming to discover valuable molecular signatures associated with pancreatic cancer. We believe that RAD51AP1 could be an influential gene in the progression and metastasis of pancreatic cancer. In the future, research can focus on inhibiting RAD51AP1 expression and corresponding signaling pathways. Related drugs or genetic modifications could improve the survival time and quality of life.

Looking forward, by conducting research and investigating the function of RAD51AP1 and its potential mechanisms in pancreatic cancer, we can potentially develop targeted anticancer treatment options that aim to prolong the survival time of patients with pancreatic cancer.

To summarize, we performed an exploratory analysis on RAD51AP1 in pancreatic cancer. This study indicates that RAD51AP1 has an important influence on the development and metastasis of pancreatic cancer. We found that overexpression of RAD51AP1 was significantly associated with a poor prognosis in pancreatic cancer patients. Additionally, we demonstrated a potential mechanism of RAD51AP1. We discovered that the upregulation of this gene can significantly enhance the malignant characteristics of cells, including proliferation, invasion, and migration, which play crucial roles in metastasis and tumorigenesis. The gene RAD51AP1 plays a role via the PI3K/AKT/NF-κB signaling pathway. Finally, bioinformatics and cell function experiments were organically combined in our study to screen valuable genes and explore their function.

Supplementary information is available in the online version of the paper.

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