

Short Communication

A short report on the tumor suppressor role of BIRC7 in pancreatic cancer

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ABSTRACT

Pancreatic cancer (PC) is accepted to be an aggressive malignancy among all type of cancers due to its poor prognosis and high cancer-led mortality ratio mostly affecting male community in older age. Multiple genes are involved in PC initiation, progression and metastasis including K-RAS, CDKN2A, p53, SMAD4. Baculoviral IAP repeat containing 7 (BIRC7) commonly known as Livin, an inhibitor of apoptosis protein (IAP) involved in the inhibition of cell death via apoptosis by preventing caspase activity through various approaches. The biological role of BIRC7 was previously identified in multiple cancers but ill investigated in PC. In this study, we investigate the function role of BIRC7 in PC. Multiple phenotypic tests including wound healing assay, CCK8 assay, trans-well assay and colony formation assay was run to rule out BIRC7 gene effect on PC genesis. We for the first time indicated that, overexpression of BIRC7 significantly reduced the proliferation, development, progression and metastasis of PANC-1 cell *in vitro*. Therefore, we anticipated that BIRC7 gene is a suppressor gene and might be a suitable candidate gene for therapeutic purposes in PC.

Keywords: BIRC7, Tumor suppressor, Pancreatic cancer, PADC, Gene mutation

INTRODUCTION

Pancreatic cancer (PC) is recognized as the most awful and deadliest malignancy among all types of cancer due to lack of timely clinical investigation, masking anatomy, and low overall 5-year survival rate that is less than 9%. PC is ranked the seventh most occurring malignancy around the globe while in the USA it is ranked in the third position due to high cancer-led mortality.¹ American cancer society 2019 has reported that a sudden increase in PC cases and mortality was observed which surpasses the breast cancer-led mortality rate and is anticipated to be the second leading cause of mortality due to cancer by 2030.^{2,3} Multiple factors are involved in the initiation, progression, and metastasis of PC including, family history, cigarette smoking, aberrant epigenetic mutation,

alcoholism, and most importantly genetic mutation in some tumor suppressor or oncogenes including, K-RAS, CDKN2A, p53, SMAD4, BRCA2, BRCA1, and ATM.⁴ Numerous genes are upregulated in PC, need to be evaluated for their possible interaction and role in PC initiation, progression, and metastasis.

Baculoviral IAP repeat containing 7 (BIRC7) commonly known as Livin, belong to the family of Inhibitor of apoptosis protein (IAP) involved in the inhibition of cell death via apoptosis by preventing caspase activity through direct or indirect approaches.⁵ However, these protein's involvement in other vital cell signalling pathways including cell cycle, cell survival, and cell migration is also reported.⁶ Inhibitor of apoptosis protein (IAP) has eight isoforms (BIRC1, BIRC2, BIRC3,

BIRC4, BIRC5, BIRC6, BIRC7, and BIRC8), all consist of at least a single BIR domain repeat, which is an 80 amino acid conserved sequence with zinc ion (Zn²⁺) in the centre. This domain is responsible for the protein-protein contact with caspases, vital for anti-apoptotic functions.^{7,8} The most striking feature of BIRC7 protein is that after a robust apoptotic stimulation this protein is sliced by caspases-3 and caspases-7 at asparagine residue 52 and gives rise to a condensed structure which in turn significantly promote cell death.⁹

Generally, the expression level of most of the IAPs particularly BIRC7 was not detectable in normal tissues except the spleen, developing embryonic tissues, placenta, and lymph nodes which represent high expression.^{5,10} BIRC7 over-expression has been reported in multiple cancers including thyroid carcinoma by promoting epithelial-mesenchymal transition along with metastasis, breast cancer, neuroblastoma, glioblastomas, neural crest, cervical cancer, colorectal cancer, liver cancer, lung cancer, ovarian and prostate cancer.¹⁰⁻¹⁵ Moreover, the high expression of BIRC7 was also detected in PC but its biological function in the initiation, progression, proliferation, and metastasis was still unclear and need further elaboration.

In this study, we have found the unexpected result of BIRC7 in PC, showing disconcertedness with studies done in other types of cancer. As BIRC7 represents an oncogenic role in all cancer describe earlier but here in this study we reported its tumor suppressor role in PC. This in vitro study of BIRC7 in PC cell lines will further enlighten the mechanism of this gene and pave a way for the researcher in the treatment of PC.

METHODS

TCGA database analysis

The gene of our interest was analysed through the cancer genome atlas cohort (TCGA) (<http://gepia.cancer-pku.cn/>) and (<http://tcga-data.nci.nih.gov/tcga/>) database portal for its differential expression level and survival rate.

Cell culture

The normal human pancreatic ductal epithelial cell line (HPDE6-C7) and three cancerous cell lines including human PC cell (PANC1), BxPC3, and ASPC1 cell lines were obtained from the cell bank of the Chinese academy of sciences. HPDE6-C, BxPC3, ASPC1, and PANC1 cell line growth and were maintained in high glucose DMEM (Biological industries: 01-052-1A) medium supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin (Hyclone, Logan, UT, USA), 10% Fetal bovine serum (FBS) (Biological industries: 04-007-1A). The culture condition was maintained at a humidified atmosphere of 5% CO₂ at a temp of 37°C.

Generation of stable cell lines

The BIRC7 overexpressed (BIRC7-OE) and BIRC7 knockdown (BIRC7-KD) stable cell lines were generated as described previously.¹⁶ The lentivirus was packaged with purified plasmid and then transfection into PANC1 cells was performed. After 36-48 hours of successful transfection, 5 µg/mL blasticidin S/2 µg/mL puromycin was added and after screening the positive BIRC7-KD and BIRC7-OE were selected after 10-15 days.

Quantitative real time-PCR (qPCR)

All the cultured cell lines were harvested for RNA extraction with TRIzol (Invitrogen, Carlsbad, CA, USA) and then cDNA was generated with cDNA Synthesis Kit (Vazyme Nanjing, China), and qPCR were performed as described previously.¹⁶ Beta-actin was used as an internal control. The forward and reverse primer used are: Human β-Actin, forward primer: TGACGTGGACATCCGCAAAG; human β-Actin, reverse primer: CTGGAAGGTGGACAGCGAGG; BIRC7-forward primer: GCTCTGAGGAGTTGCGTCTG; reverse primer: CACACTGTGGACAAAGTCTCTT.

Cell proliferation assay

The proliferative ability of cell lines with their corresponding controls was seeded and cultured in 96 well plates with a seeding capacity of 2×10³ cells per well and the signals were observed after every 24 hours. The proliferative signals were generated with the help of CCK8 cell counting Kit-8 (Solarbio, Beijing, China) according to the manufacture's instruction.

Colony formation assay

To check the colony formation ability of the cell lines 300 cells per well were seeded into a 6 well plate for 10-14 days in a humidified environment of 5% CO₂ at a temp of 37°C. After the required time the cell was washed with PBS and fixed with 4% formaldehyde and then stained with 1% crystal violet dye. The number of colonies were counted and photographs were taken.

Wound healing assay

The cell lines with their corresponding controls were seeding with a seeding density of 2×10⁵ in 12 well plates. After overnight incubation the cell attached to the surface of the well to form a monolayer. A straight line was scrapped with the help of a 200 µl sterile tip and place on incubation for the next 48 hours. After that the wound closure or healing were photograph and measured.

Transwell invasion assay

Transwell chamber and matrigel (BD biosciences, 8 µm Transwell inserts) were used for the evaluation of cell

invasion. The serum-free medium containing cell line with a seeding density of 1×10^4 in the upper chamber and in the lower chamber cell-free medium supplemented with 10% FBS as a cell attractant were added. After the required time the cell penetrated from the upper to the lower chamber were fixed with methanol (20%) and stained with crystal violet. Cells were visualized and photographed with the help of a microscope (Leica, USA). Three different fields were selected and the number of cells was counted.

Western blotting

Cell were harvested and protein was extracted from the cell lines and western blot were performed as described previously.¹⁶ The antibodies used in western blot are anti- β -Actin (ab8226, Abcam), BIRC7 antibody (CF500758, thermo fisher scientific).

Statistical analysis

The presented data are in the form of mean \pm SD from three independent biological experiments. All the quantitative results analysis was performed on GraphPad Prism 6.0 (GraphPad software, Inc., La Jolla, CA, USA). Significance of data are between two group were determined via two-tailed student's paired t-test. Involvements of more than two groups were analyzed by one-way or two-way ANOVA. Statistical significance was represented as * $p < 0.05$ or ** $p < 0.01$, *** $p < 0.001$.

RESULTS

BIRC7 is preferentially upregulated in human pancreatic cancer cells

Conferring the cancer genome atlas cohort (TCGA) (<http://gepia.cancer-pku.cn/>) and (<http://tcga-data.nci.nih.gov/tcga/>) TCGA database, it is confirmed that the expression level of BIRC7 was preferentially high in Pancreatic adenocarcinoma (PAAD)/PC (PC) tissues (n=179) as compared to its associated adjacent non-cancerous tissues (n=171) showing in (Figure 1A). Furthermore, the TCGA database also showed that the BIRC7 expression was high at the I and II stages of cancer while its expression level became reduced later in the III and IV (final stages) of the disease, indicating its expression is related to the tumor stage (Figure 1B). These indicated the BIRC7 may play an important role in PC. For confirmation of this data, the expression level of BIRC7 mRNA was quantified in three PC cell lines including ASPC1, BxPC3, PANC1, and one normal non-tumorous adjacent epithelial ductal cell line (HPDE6-C7) through quantitative polymerase chain reaction (qPCR). The qPCR data endorsed the upregulation of BIRC7 mRNA level in all three PC cell lines compared to adjacent normal tissue signifying the potential BIRC7 role in PC (Figure 1D).

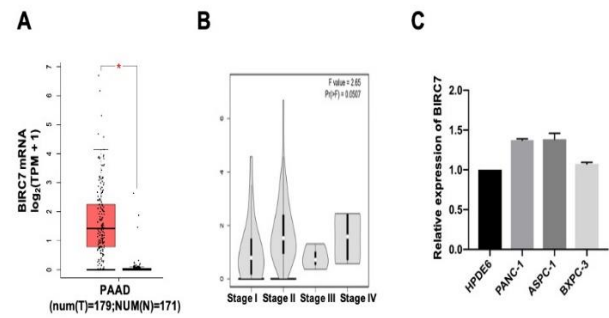


Figure 1: BIRC7 was preferentially upregulated in pancreatic cancer cell lines.

The BIRC7 expression level was investigated online via TCGA database and then confirmed. (A) Represent the upregulated mRNA expression level of BIRC7 in pancreatic ductal adenocarcinoma. (B) Stage wise (stage I, II, III and IV) expression level of BIRC7 in PC. (C) Upregulation of BIRC7 expression in multiple cancer cell lines including (ASPC-1, BxPC-3, PANC-1) and corresponding surrounding normal tissue (HPDE6).

BIRC7 functions as a tumor suppressor in PC

To investigate the functional role of BIRC7 in PC, we successfully established stably transfected cell lines including BIRC7 overexpressed (OE), knockdown (KD), and the corresponding controls in PANC-1 cell (PANC-1-Ctrl, PANC-1-BIRC7-OE, and PANC-1-shCtrl, PANC-1-shBIRC7-KD). Further, we confirmed the transfection efficiency by analyzing the mRNA level of BIRC7 in overexpressed and knockdown cell lines compared with their corresponding controls (Figure 2A). The CCK8 and clone formation assays showed that BIRC7 overexpression significantly reduced the proliferation, and colony formation ability of PANC-1 cells (Figure 2B, 2C) *** $p < 0.001$. Moreover, wound healing and transwell assays were performed to study the roles of BIRC7 in cell migration and invasion. The results show that the upregulation of BIRC7 in the PANC-1 cell line significantly reduces the migration distance of PANC-1 cells (Figure 2D). Besides, overexpression of BIRC7 dramatically decreased the number of invasive cells (Figure 2E) *** $p < 0.001$. Collectively, our result validates that BIRC7 upregulation significantly hamper the PANC-1 cell line proliferation, migration and invasion capacity and might be a possible target gene for therapeutic purposes.

Multiple phenotypic tests were performed to check the behavior of BIRC7 overexpression of PANC-1 cell line. (A) The BIRC7 overexpression level was confirmed through qPCR *** $p < 0.001$. (B) CCK8 proliferation assay representing the proliferative ability of cell showing that BIRC7 overexpression significantly reduce the proliferation of PANC-1 cell *** $p < 0.001$. (C) Representing that BIRC7 overexpression reduces the colony formation potential of PANC-1 cell *** $p < 0.001$.

(D) Wound healing assay showing that BIRC7 overexpression dramatically reduces the migration of cell*** $p < 0.001$. (E) Transwell migration assay represent that BIRC7 overexpression halt the invasion potential of PANC-1 cell*** $p < 0.001$.

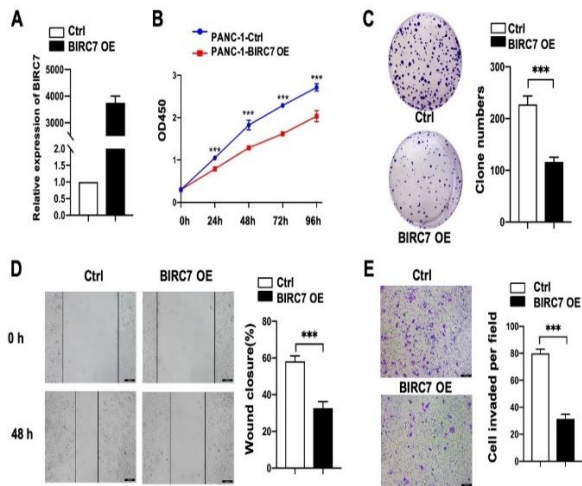


Figure 2: BIRC7 promote PANC-1 cell proliferation and invasion.

DISCUSSION

The current study mainly focuses on the tumor suppressor role and mechanism of BIRC7 in PC progression. In this study, we showed that BIRC7 overexpression in the PANC-1 cell line significantly reduced the invasion, proliferation, migration and tumorigenesis of the PC cell line. The data presented in this study demonstrating the essential tumor suppressor role of BIRC7 in PC.

Baculoviral Inhibitor of apoptosis (IAP) repeat-containing protein 7 (BIRC7) is a protein also known as Livin, encoded by BIRC7 gene in mammals. This gene encodes two different Livin protein including Lavin- α and Lavin- β due to its alternate splicing variants.¹⁷ A series of studies reported previously that BIRC7 aberrant expression is strongly associated with invasion, progression, development, and tumorigenesis of different types of human cancer.¹⁰⁻¹⁵ The most well-characterized role of BIRC7 in its interaction with caspases proteins and inhibit apoptosis while its zinc finger domain enhances BIRC7 antiapoptotic function.¹⁸ Conversely, the BIRC7 expression and its biological role or function in PC are still ill investigated. Besides BIRC7 oncogenic role is another type of cancer described earlier, we report here its tumor suppressor function.

The aim of this report is to highlight the unexpected behavior of BIRC7 in PC. The BIRC7 expression was high in various types of cancer and we confirmed through the TCGA database that the expression is also high in PC indicating its possible role. The expression is high in the first and second stages however, the expression start reducing in the third and fourth stages. To evaluate the

possible role of BIRC7 in PC first established BIRC7 overexpressed cell lines and then performed the different tests to check its possible effect on phenotypic expression. We unexpectedly found that the BIRC7 overexpression in the PANC-1 cell line significantly reduces the invasion, proliferation, progression, and tumorigenesis of PANC-1 cells. This data shows dissimilar behavior of BIRC7 in PC compared with its role in other types of cancers

In this study, we first time identified the tumor suppressor role of BIRC7 in PC besides its oncogenic function in different cancers. We think this short study will gain and divert the researcher's interest toward the tumor suppressor function of BIRC7 and will help in the further investigation of its biological role and molecular mechanism. We further expand and anticipate that BIRC7 might be a possible therapeutic gene of choice in the near future.

CONCLUSION

In this study, we first time reported that overexpression of BIRC7 significantly reduced the proliferation, development, progression, and tumorigenesis of PANC-1 cells in vitro. Therefore, we anticipated that the BIRC7 gene might be a suitable candidate gene for therapeutic purposes in PC.

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Conflict of interest: None declared

Ethical approval: Not required

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