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Krüppel-like factor 9 suppressed tumorigenicity of the pancreatic ductal adenocarcinoma by negatively regulating frizzled-5

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ABSTRACT

Krüppel-like factor 9 (KLF9) has been implicated in mediating a diverse range of biological processes. However, the expression pattern and biological functions of KLF9 in pancreatic ductal adenocarcinoma (PDAC) are still unknown. Here, we evaluated the role of KLF9 in pancreatic ductal adenocarcinoma (PDAC). Overexpression of KLF9 significantly inhibited proliferation and clone formation in PDAC cells, while silencing KLF9 expression dramatically promoted this effect *in vitro*. Knocking down the expression of KLF9 also promoted the tumorigenesis in the PDAC mouse xneograft model. In *in vitro* mechanism study, KLF9 negatively regulated the activity of wnt/beta-catenin pathway in Top/Fop reporter assay. Frizzled-5, a key component involving in this pathway, was sharp inhibited by KLF9 both in mRNA and protein level. Furthermore, a KLF9-binding site (BTE) was identified in the promoter region of Frizzled-5. Mutation or deletion of this BTE strongly disrupted the KLF9's regulatory effect on Frizzled-5. More importantly, the expression level of KLF9 was significantly lower in clinical PDAC tissue compared to matched normal tissues and inversely associated with survival of the patients. Together, our findings indicated that KLF9 suppressed tumorigenicity of the pancreatic ductal adenocarcinoma by negatively regulating frizzled-5.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC), with a prevalence of 2%–3% of new cancer cases annually in the United States [1], is the fifth leading causes of cancer deaths in the United States and global. PDAC is a highly aggressive malignancy and also exhibits a profound resistance to current chemotherapies, radiotherapy or immunotherapy treatment [2]. The prognosis of PDAC is extremely dismal and the 5 years survival rate is approximately 5% [3]. Stepwise accumulation of multiple genes mutation is one major cause in the development of the malignant tumor. Mutations of K-ras and p53 [4], PTEN [5], the methylation of SPARC promoter [6], aberrant expression of TGF-β [7] and other factors have been observed in the PDAC. However, the underlying mechanism of PDAC at the molecular level is still uncovered.

KLF9 is an evolutionary conserved member of the KLF family of transcriptional regulators [8], which also belonged to a ubiquitously expressed member of the Sp1 C2H2-type zinc finger family of transcription factors [9]. KLF9 was first identified as a transcriptional repressor of the rat Cyp1a1 (previously P-4501A1) gene and originally named as basic transcription element-binding protein 1 (BTEB1) [10]. Human KLF9 gene, localized on human chromosome 9q13 [11], has been implicated in mediating a diverse range of biological processes including stem cell maintenance [12], differentiation of T-and B-lymphocytes, and animal development [13].

More important, KLF9, with its abnormal expression, is also involved in the multiple carcinomas development and progression. It has been reported KLF9 was down-regulated in human colorectal tumors, breast cancer and hepatocellular cancer [14–17]. Down-regulated KLF9 was associated with the estrogen-mediated growth regulation in the endometrial carcinoma [18,19]. Notably, KLF9 may have a special role in the cancer stem cells for its further down-regulation in ovarian cancer stem cell and glioblastoma-derived neurospheres [12,20–22]. However, the role of KLF9 in

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PDAC is not clear.

In our previous research, we found that KLF9 is a prognostic indicator in human PDAC [23]. In this study, we investigated the underlying mechanism of anti-oncogenic role of KLF9 in PDAC cells and demonstrated that KLF9 significantly inhibited PDAC cells proliferation and clone formation in *vitro* and *in vivo* by the suppression of Frizzled-5.

2. Materials and methods

2.1. Reagents and antibodies

Human polyclonal anti-rabbit KLF9 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-mouse GAPDH antibody was purchased from Applied Biosystems (Foster City, CA). Human polyclonal anti-rabbit Frizzled-5 antibody was purchased from Abcam(Cambridge, MA). Matrigel was purchased from BD Biosciences (Bedford, MA). All other chemicals were obtained either from Sigma (St. Louis, MO) or Fisher Scientific (Houston, TX).

2.2. Tissue samples

Archived, 4% formalin-fixed, paraffin-embedded primary pancreatic adeno-carcinoma samples were obtained from patients treated at Zhongshan Hospital of Fudan University (Shanghai, China) from 2005 to 2009 after their written informed consent, and none of the patients received any neoadjuvant therapy. RNA extraction, cDNA synthesis was essentially the same as that previously described [24]. Our study was approved by the Institutional Review Board of Zhongshan Hospital of Fudan University.

2.3. Cell culture

All Pancreatic cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 units/ml streptomycin (Sigma) at 37 °C incubator in the presence of 5% CO_2.

2.4. Quantitative real-time PCR

Total RNA was extracted from tissue sample using TRIZOL (Invitrogen, Carlsbad, CA). cDNA was prepared from total RNA by using Taqman Reverse Transcription kit. Real-time PCR was performed from cDNA products using Taqman universal PCR and Taqman assay kit by Applied Biosystem Step One real-time PCR system. CT values for KLF9 were normalized to human β-actin by subtracting the average CT value for each sample. Relative quantification (RQ) values for KLF9 in each sample were determined using the $2^{-\Delta\Delta CT}$ method. Each PCR reaction was performed in triplicate. Sequences of quantitative real-time PCR primers are listed as follows:

KLF9 Forward primer: 5′-ACAGTGGCTGTGGGAAAGTC -3,
KLF9 Reverse primer: 5′- TCACAAAGCGTTGGCCA GCG-3′,
β-actin Forward primer:5′-GATCATTGCTCCTCCTGAGC-3′,
β-actin Reverse primer: 5-ACTCCTGCTTGCTGATCCAC-3.

2.5. Western blot analysis

Cell lysates from different pancreatic cell lines were prepared for Immuno-Western blotting according previous method [25]. Briefly, cells were lysed in RIPA buffer and homogenized. The proteins were subjected to 10% SDS-PAGE, and the gel-fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad) and reacted with appropriate antibodies. Signals were detected with ECL kit (Pierce, Rockford, IL, USA) by using one dimensional Image analysis.

2.6. Plasmid construction and transfection

The human KLF9 cDNA was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) and the insert was confirmed by sequencing. The KLF9 expression vector and empty pcDNA3.1 (as wild-type control) were transfected using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were selected with G418 at the concentration 600 μg/ml, and resistant clones were further confirmed by Western blotting. The small interfering RNAs (siRNAs) against KLF9 were designed and synthesized by Sigma-Aldrich and transfected with Lipofectamine 2000 reagent according to the manufacturer's protocol.

2.7. MTT assay

Cell growth was measured *in vitro* using an MTT assay as previously described [25]. The measurement process was performed every 48 h for 5 or 7 days to generate a cell growth curve.

2.8. Soft agar assay

3×10^3 cells per well suspended in 200 μl of 1% agar-DMEM/25% FBS were plated in 24-well plate overlying a 1.25% agar-DMEM/20% FBS bottom layer and cultured at 37 °C with 5% CO_2 as described earlier [26]. All the experiments were repeated at least three times using triplicate plates per experimental point.

2.9. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [27]. The chromatin (~250bp) was subjected to immunoprecipitation using 5 μg of anti-KLF9 antibody or control IgG. Immunoprecipitated DNA was analyzed by PCR using specific primers for the region encompassing the GCGG sequences of the Frizzled-5 gene promoter. Coding region Frizzled-5 gene primers were used as negative control.

2.10. Tumorigenesis assay in vivo

KLF9 si con, KLF9 siRNA knockdown cells sorted by FACS were injected in 4-week-old nude mice, purchased from Shanghai Laboratory Animal Center, CAS (Shanghai, China). 1×10^7 cells suspended in DMEM and Matrigel (1:1) were subcutaneously injected on opposite flanks of the same mouse and treated in accordance with the American Association for the Accreditation of Laboratory Animal Care guidelines. The resulting tumors were measured once a week and tumors volume (mm^3) were calculated using the standard formula: length × width × height × 0.5236. Tumors were harvested 4 weeks after injection and individually weighed. Data were presented as tumor volume (mean ± S.D.) and tumor weight (mean ± S.D.)

2.11. Statistical analysis

The results of each experiment were the representative of at least three sets of experiments performed in triplicate. All data were expressed as the mean ± S.D. and statistically significant differences between groups were determined by using the non paired Student's two-tailed *t*-test or one-way ANOVA. A value of $p < 0.05$ was considered as statistically significant.

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