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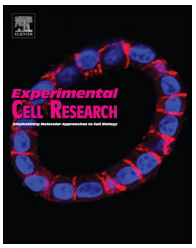

Research Article

ANKHD1, a novel component of the Hippo signaling pathway, promotes YAP1 activation and cell cycle progression in prostate cancer cells

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ABSTRACT

ANKHD1 is a multiple ankyrin repeat containing protein, recently identified as a novel member of the Hippo signaling pathway. The present study aimed to investigate the role of ANKHD1 in DU145 and LNCaP prostate cancer cells. ANKHD1 and YAP1 were found to be highly expressed in prostate cancer cells, and ANKHD1 silencing decreased cell growth, delayed cell cycle progression at the S phase, and reduced tumor xenograft growth. Moreover, ANKHD1 knockdown down-regulated YAP1 expression and activation, and reduced the expression of CCNA2, a YAP1 target gene. These findings indicate that ANKHD1 is a positive regulator of YAP1 and promotes cell growth and cell cycle progression through Cyclin A upregulation.

Introduction

The Hippo signaling pathway was initially characterized in *Drosophila melanogaster* as a mechanism that controls tissue growth and organ size, and the core signaling components of this pathway are evolutionarily conserved and play a role of a tumor suppressor in mammals [1]. The most important human components of the Hippo signaling pathway are Yes-associated protein 1 (YAP1; ortholog of Yorkie), Large tumor suppressors 1 and 2 (LATS1/2; ortholog of Wts), Mammalian

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STE-20 kinases 1 and 2 (MST1/2; ortholog of Hpo) and Msp-one-binder (MOB1; ortholog of Mats) [2].

YAP1, the nuclear effector of the Hippo signaling pathway, acts as a transcriptional coactivator and YAP1 serine phosphorylation leads to cytoplasmic sequestration and/or degradation [2]. YAP1 binds to several transcription factors, which includes ErbB4 [3], p53BP-2 [4], RUNX2 [5], TEAD1-4 [6,7] and p73 [8], regulating the expression of diverse genes [2]. Aberrations in the Hippo signaling pathway have been described in a large number of solid tumors, including prostate cancer. Transgenic mice with YAP1 overexpression present liver overgrowth and cancer [9], and YAP1 ectopic expression promotes *in vitro* cell growth and oncogenic transformation [10]. In primary prostate tumors, YAP1 is highly expressed [11], whereas LATS2, a negative regulator of YAP1, was found to be underexpressed [12].

Ankyrin Repeat and KH Domain Containing 1, ANKHD1, was first identified in LNCaP prostate cancer cells [13]. The presence of multiple ankyrin repeats suggests a role for ANKHD1 as a scaffolding protein, bringing together a number of signaling molecules [14]. The overexpression of ANKHD1 has been reported in acute leukemias [15] and multiple myeloma cells [16], and has been found to be associated with a significantly decreased survival in breast cancer patients [17]. The recent identification of ANKHD1 as a novel member of the Hippo signaling pathway has provided new possibilities for investigation [17,18].

In the present study, we aimed to further characterize the involvement of ANKHD1 in the Hippo signaling pathway and malignant phenotype of prostate cancer cell lines. Thus, we investigated the effects of ANKHD1 silencing in cell growth, cell cycle progression, and YAP1 activation in LNCaP and DU145 cells.

Material and methods

Cell culture, transient transfection and transduction

LNCaP, DU145, PC3, K562 and HeLa cells were obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in an appropriate medium containing 10% fetal bovine serum (FBS) and glutamine with penicillin/streptomycin and amphotericin B, and maintained at 37 °C, 5% CO_2. ANKHD1 silencing was performed in prostate cancer cells using specific siRNAs from ThermoFisher Scientific (Lafayette, CO, USA). Briefly, the cells were plated at 70% confluence and transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were analyzed 72 h after transfection. For xenograft tumor models, LNCaP cells were transduced with lentivirus-mediated shRNA Control or lentivirus-mediated shRNA targeting ANKHD1; named shControl and shANKHD1, respectively. Briefly, 2×10^5 cells were transduced with lentivirus by spinoculation at multiplicity of infection equal to 3 and selected by blasticidin (10 μg/mL).

Lentiviral vectors

The preparations of lentiviral vectors expressing the human short hairpin RNA (shRNA) target *ANKHD1* (shANKHD1; 5′-TGTCCGAGGTTGAATCATTTT-3′) or *LacZ* (shControl; 5′-CTACACAAATCAGCGATTT-3′) were performed using the BLOCK-It Lentiviral RNAi Expression System (Invitrogen; Carlsband, CA, USA), following the manufacturer's instruction.

Quantitative polymerase chain reaction

Quantitative PCR (qPCR) was performed with an ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) with specific primers. Primer sequences and concentrations are described in Supplementary Table 1. The relative gene expression was calculated using the equation $2^{-\Delta\Delta CT}$ [19]. A negative 'No Template Control' was included for each primer pair. Three replicas were run on the same plate for each sample.

Western blot analysis and immunopreciptation

Equal amounts of protein were used for total extracts or for immunoprecipitation with specific antibodies followed by SDS-PAGE and Western blot analysis with the indicated antibodies and the ECL Western Blot Analysis System (Amersham Pharmacia Biotech, UK). Antibodies against ANKHD1 (sc-160960), YAP1 (sc-101199), Cyclin A (sc-271645), Histone H4 (sc-25260), OP18 (sc-55531), LAST1 (sc-130429), LAST2 (sc-23065), p21 (sc-6246), CDK2 (sc-6248), CDK4 (sc-166373) and Actin (sc-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against ANKHD1 (A303-307A) was from Bethyl Laboratories (Montgomery, TX, USA); the antibody against Phosphoserine (612547) was from BD Biosciences (San Jose, CA, USA) and antibodies against phosho-MST1/2 (#36815), MST1 (#39525) and MST2 (#38825) were from Cell Signaling Technology (Beverly, MA, USA).

Confocal immunofluorescence microscopy

Confocal imaging was carried out using primary antibodies against ANKHD1 or YAP1 (diluted 1:200), as previously described [15].

Subcellular fractionation

DU145 cells were resuspended in buffer 1 (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 10 mM Na_3VO_4 and 2 mM PMSF). Cells were chilled on ice for 10 min and then lysed by the addition of 0.1% Nonidet P-40 and homogenization by 10 passages through a 26.5-gauge needle. The extracts were centrifuged at $12{,}000 \times g$ for 10 min at 4 °C. The supernatant was used as a cytoplasm and membrane fraction. The pellet was resuspended with buffer 2 (20 mM HEPES pH 7.9, 25% Glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 10 mM Na_3VO_4 and 2 mM PMSF). The homogenate was incubated on ice for 30 min at 4 °C and centrifuged at $12{,}000 \times g$ for 10 min at 4 °C. Supernatant was used as the nuclear fraction. Equal amounts of protein were used for Western blotting analysis and antibodies against Histone H4 and OP18 were used for controls for nucleus and cytoplasm, respectively.

Analysis of cell proliferation

Cell proliferation was measured by methylthiazoletetrazolium (MTT) assay. Twenty-four hours after transfection, 9×10^3 cells per well were plated in a 96-well plate in RPMI containing 10% FBS and incubated for 48 hours. To evaluate cell viability, 10 μL of