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Stat3 inhibits WTX expression through up-regulation of microRNA-370 in Wilms tumor

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ABSTRACT

Wilms tumor (WT) is a genetically heterogeneous childhood kidney tumor. Several genetic mutations have been identified in WT patients, including inactivation of WTX, somatic stabilizing CTNNB1, and p53 mutations. However, the molecular mechanisms in tumorigenesis remain largely unexplored. Stat3 is a transcription factor that can promote oncogenesis. Stat3 activation is commonly viewed as crucial for multiple tumor proliferation and metastasis. We show that Stat3 is highly activated in Wilms tumor tissues compared to those in adjacent tissues. IL-6 treatment or transfection of a constitutively activated Stat3 in G401 cells promotes cell proliferation. At the molecular level, we further elucidate that Stat3 inhibits WTX expression through up-regulation of microRNA-370. Our results suggest that Stat3/miR-370/WTX regulatory axis might be a critical mechanism in Wilms tumor cells.

1. Introduction

Wilms tumor is a type of kidney cancer and affects very young children [1]. Although a number of Wilms tumors have been collected through international trials, the mechanisms remain challenging to determine [2]. Extensive research has identified somatic mutations at several loci in Wilms tumorigenesis, including WT1, CTNNB1, WTX and TP53 [2–4].

WTX has been characterized as a key tumor suppressor in Wilms tumor [5]. Inactivation of WT1 is the most frequent genetic event in sporadic Wims tumor, reported in up to 30% of cases [5]. Protein interaction studies have suggested that WTX associates with the APC complex and might negatively regulate β-catenin stability [6,7]. Besides, it could modulate the transcriptional activity of WT1, the first Wilms tumor suppressor that encodes a zinc finger transcriptional regulator of cellular differentiation program [8]. Moreover, recent studies have also suggested a positive effect on p53 signaling through enhancing CBP/P300-mediated acetylation of p53 at Lysine 382 [9]. Therefore, WTX may regulate Wilms tumor initiation and progression through multiple mechanisms. However, the molecular determinants of WTX expression remain largely unexplored.

Multiple lines of evidence suggest that aberrant Stat3 signaling play a central role in the initiation, development and progression of many human tumors [10,11]. Some of pro-inflammatory cytokines and growth factors secreted by cancer cells or inflammatory cells are activators of Stat3 [12]. For many cancers, elevated levels of activated Stat3 have been associated with a poor prognosis [13–15]. In addition to mediating tumor cell proliferation and survival in a tumor cell-autonomous manner, Stat3 signaling is also crucial for inflammatory cell-mediated transformation and tumor progression [16,17]. Therefore, inhibiting the Stat3 signaling pathway is implicated as a promising therapeutic target for developing anticancer drugs.

Here, we report a functional role of Stat3 in Wilms tumor proliferation, initially identified by the observation of activation in Wilms tumor tissues. At the molecular level, Stat3 promotes cell proliferation through up-regulation of microRNA-370, which directly targets WTX expression. Together, our results suggest a role of Stat3 in the regulation of WTX and Wilms tumor growth.

2. Material and methods

2.1. Tissue samples

16 Primary Wilms tumor tissues and adjacent tissues were collected from either therapeutic surgery or endoscopy at our

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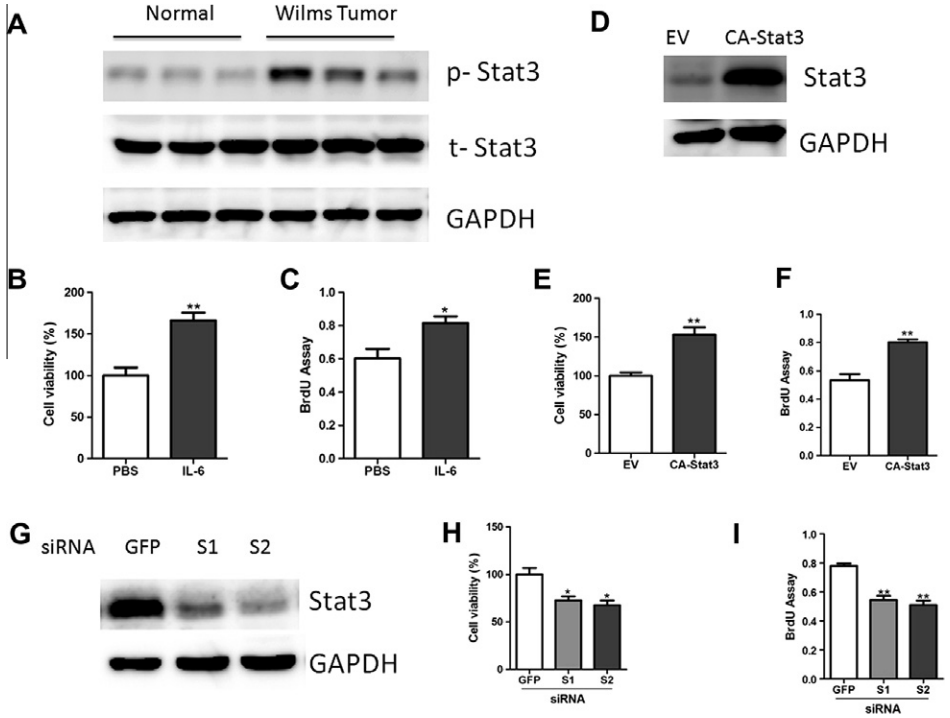

Fig. 1. Activation of Stat3 promotes Wilms tumor proliferation (A) Western Blot analysis of phosphorylated Stat3 (p-Stat3) expression in Wilms tumor and adjacent normal tissues. Total Stat3 (t-Stat3) and GAPDH were used as a loading control (B–C). The cell viability (B) and cell proliferative potential (C) were determined in G401 cells treated with control (PBS) or IL-6 (25 ng/ml). (D) Western Blot analysis of Stat3 protein expression in G401 cells transfected with empty vector (EV) or constitutively activated Stat3 (CA-Stat3). (E–F) The cell viability (E) and cell proliferative potential (F) were determined in G401 cells transfected with EV or CA-Stat3. (G) Western Blot analysis of Stat3 protein expression in G401 cells transfected with siRNA oligos targeting GFP or Stat3 (S1, S2). GFP siRNA was used as a negative control. (H–I). The cell viability (H) and cell proliferative potential (I) were determined in G401 cells transfected with siRNA oligos as indicated.

department. All samples were obtained with informed consent and approved by Childrens' Hospital Affiliated to Soochow University.

2.2. Reagents and cell culture

IL-6 was purchased from Merck, China. The following antibodies were purchased: anti-STAT3 (Cell Signaling), anti-WTX (Abcam), anti-$p21^{Cip1}$ (Santa Cruz), anti-Cyclin D1 (Cell Signaling), anti-acetylated Histone H3 and H4 (Abcam), anti-GAPDH (Santa Cruz). G401 cell lines were obtained from American Type Culture Collection (ATCC) cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin.

2.3. siRNA oligos

siRNA oligos targeting GFP or Stat3 were designed and synthesized by GenePharm (Shanghai, China).

2.4. RNA extraction, real-time analysis and Western blotting

Total RNAs were isolated from tissues or cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, China) following the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was perfomed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland). The primer sequences used are available upon request. β-Actin and small RNA U6 were used as a reference to normalize Real-time PCR data for mRNA and microRNA analysis respectively. For western blot analysis, tissues and cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM NaF, 1% NP40 and 0.1% SDS.

2.5. Plasmid construction

The miR-370 promoter (850 bp) was amplified from the human genomic DNA template and inserted into pGL4 vector (Promega). Mutant Stat3 binding site was generated using a PCR mutagenesis kit (Toyobo) with primer (mutation sites underlined) 5′-CCAATGCGTCAATCGACGTACCGCT-3′ and a reverse complement primer. To construct reporter vectors carrying miR-370 target sites, we synthesized the 3′UTR fragments containing the predicted target sites (Targetscan) for human WTX cDNA, and inserted the fragment of WTX cDNA into the multiple cloning sites in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion).

2.6. Transient transfections and luciferase assays

All the transient transfections were performed by Lipofectamin 2000 (Invitrogen), according to the manufacturer's instructions. For luciferase reporter assay, G401 cells were seeded in 24-well plates and transfected with the indicated plasmids. Cells were harvested 30 hours after transfection. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, USA).

2.7. MTT and BrdU assays

The viability of G401 cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenylte-trazolium bromide (MTT) to formazan. Absorbance was measured at