Original Article

Curcumin suppresses the WTX expression via Stat3 signaling in G401 cells

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Abstract: Backgrounds: Wilms’ tumor gene on the X chromosome (WTX) has been considered as a key tumor suppressor in Wilms’ tumor. This study aimed to assess the effects of curcumin on WTX expression in G401 cells and explore its underlying mechanism. Methods: The G401 Wilms’ tumor cell line were treated with curcumin for 48 h. The effects of curcumin on G401 cell proliferation was analyzed using CCK8 assay. In addition, the effects of curcumin on G401 cell apoptosis was detected by flow cytometry and TUNEL staining. Total RNA and protein was collected for qRT-PCR and Western blot analysis, respectively. Results: Curcumin inhibited growth and increased the expression of apoptosis protein in these cells. At the molecular level, curcumin treatment led to a significant increase of WTX expression. Moreover, curcumin also markedly inhibited the phosphorylation level of Signal Transducer and Activator of Transcription-3 (Stat3). In addition, Stat3 siRNA was administered, and it significantly decreased WTX expression in G401 cells. Conclusion: Curcumin exerts anti-cancer effects and increases WTX expression via suppressing Stat3 signaling pathway in G401 cells.

Keywords: Curcumin, WTX, Stat3, G401 cells, Wilms’ tumor

Introduction

Wilms tumor, the most regular malignant neoplasm of the kidney in children, is an embryonic tumor that is composed of blastemal, stromal and epithelial cells [1]. Although international trials have been established, which have collected a number of Wilms’ tumor samples; the mechanisms underlying its pathogenesis and effective treatment strategies remain to be clearly revealed [2]. Rapidly growing research has identified somatic mutations at several loci in Wilms tumorigenesis, including WT1, CTNNB1, WTX and TP53 [3-5].

WTX has been deemed as a vital tumor suppressor in Wilms’ tumor [6, 7]. Inactivation of WTX is the most common genetic episode in sporadic Wilms’ tumor, reported in up to 30% of cases [3]. Protein interaction studies have suggested that WTX associates with the APC complex and might negatively regulate β-catenin stability [8]. In additional, previous studies have also uncovered a positive effect on p53 signaling through enhancing CBP/P300-mediated acetylation of p53 at Lysine 382 [9]. Accordingly, WTX may involve in Wilms tumor initiation and progression through several mechanisms. However, the molecular determinants of WTX expression remain largely unexplored.

Curcumin, a diferuloylmethane derived from the rhizomes of turmeric, have been extensively proved to be a strong anti-cancer agent against a wide range of cancer cells while being nontoxic normal cells [10-12]. Moreover, curcumin hinders cancer cell proliferation and promotes cancer cell differentiation [13]. These effects contain multiple pathways and intracellular targets, including DNA, mRNA, and proteins [14]. Therefore, the results implying that the mechanisms underlying curcumin’s anticancer effects are distinct among tumor cell types suggest a cell-type specific effect of curcumin on the inhibition of tumor progression.
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However, it remains unclear whether curcumin directly inhibits proliferation in the G401 human Wilms' tumor cell line. Thus, the aim of the present study was to investigate the effect of curcumin on G401 cell proliferation and the underlying molecular mechanisms, to potentially provide results which may aid in the development of effective drugs for the clinical treatment of Wilms' tumor.

Materials and methods

Cell cultures

The G401 Wilms' tumor cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin (all obtained from Gibco-BRL, Carlsbad, CA, USA). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability assay

The CCK-8 (Dojindo Laboratories, Japan) assay was used to assess effects of curcumin on proliferation and viability of G401 cells. Cells were seeded onto 96-well plates at a concentration of 1 x 10⁴ per well and treated with curcumin or diluent. After treatments, absorbances at 450 nm were registered using a microplate reader (Bio-Rad, USA).

RNA isolation and qPCR

Total RNA was isolated from cells by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and reverse transcription was conducted using a Takara RNA PCR kit (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. In order to analyze the transcripts of the genes of interest, qPCR was performed using an SYBR-Green Premix Ex Taq (Takara Biotechnology) on an ABI 7300 machine (Invitrogen Life Technologies).

Western blotting

Western blotting was performed similarly as described previously [18]. In brief, membrane protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% milk and incubated in 4°C overnight with WTX, pStat3, t-Stat3, Bax, Bcl-2, GAPDH (Santa Cruz, CA, USA) (1:500 dilution in TBST). Proteins were visualized with ECL procedure (Bio-Rad, USA). The results were analyzed with Quantity One software (Bio-Rad).

Transient transfections

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. (Promega, USA).

Flow cytometry analysis

G401 cells were seeded at a concentration of 1x10⁵/ml onto 6-well culture plates. When the cells reached about 70% degree confluency, the medium was changed, and curcumin (10 μM) or diluent was added. Then, G401 cells
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were trypsinized to obtain a single cell suspension. One million cells were double-stained with APC-labeled annexin V and propidium iodide (Becton-Dickinson, USA). Percentage of apoptotic cells was determined by flow cytometry (Becton-Dickinson, USA).

**TUNEL staining**

TUNEL staining was performed with the use of the In Situ Cell Death Detection kit (Roche) according to the manufacturer's instructions. Propidium iodide staining was performed to visualize nuclei after TUNEL reaction, and the percentage of TUNEL-positive nuclei was quantified using Image J software. The sections were dehydrated, mounted and observed at 400 × magnification.

**Statistical analysis**

Statistical analysis was performed with a paired Student's t-test or two-way analysis of variance test. Numerical data are presented as the mean ± SEM. *P < 0.05 was considered to indicate a statistically significant difference.

**Results**

Curcumin treatment inhibits cell growth in a dose-dependent manner

To the best of our knowledge, the effects of curcumin on Wilms' tumor cells has not been previously analyzed. Thus, G401 cells were selected to investigate whether curcumin exhibits potential anti-proliferative functions. G401 cells were treated with curcumin at several concen-
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A

Control  DMSO  Cur 10µM

DAPI

TUNEL

MERGE

B

Apoptotic cell (%)

Control  DMSO  Cur 10µM

C

Relative mRNA expression of WTX (fold change)

Control  DMSO  Cur 10µM

D

WTX

GAPDH

E

Relative level of WTX (fold of control)

Control  DMSO  Cur 10µM

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After 48 h of treatment, growth was inhibited in a dose-dependent manner as determined by CCK-8 assays (Figure 1A). Moreover, these results suggested that the concentration of curcumin at 10 μM was optimal in G401 lines. Therefore, 10 μM of curcumin was selected for the further analysis of gene expression in G401 cells.

Effect of curcumin on apoptosis in G401 cells

We further determined the effect of curcumin on G401 cell apoptosis. As shown in We assayed for annexin V/PI to show that the relative percent of apoptotic cells and the number of annexin V positive cells were significantly lower in curcumin treated cells (Figure 2A). Treatment with curcumin at 10 μM profoundly attenuated apoptotic morphology and decreased the number of apoptotic cells as evidenced by TUNEL staining (Figure 3A, 3B). Furthermore, curcumin treatment increased the expression of several important pro-apoptotic proteins Bax and decreased the expression of antiapoptotic Bcl-2 (Figure 2B, 2C).

Curcumin increases WTX expression in G401 cells

WTX activation plays a critical role in tumor development and progression. We determined the effect of curcumin treatment on this protein. It was observed that WTX expression was markedly increased following curcumin treatment in G401 cells (Figure 3C, 3E).

Curcumin downregulates Stat3 activity in G401 cells

Several studies have indicated that the antiproliferative effects of curcumin involve the Stat3

Figure 3. Effects of curcumin on apoptosis with TUNEL assay and the WTX expression in G401 cells. A. Fluorescent microscopic images showing TUNEL and nuclear staining. B. Quantification of apoptotic cells. C. The expression of WTX mRNA was determined by real-time PCR using β-actin as the internal reference. D. The protein level of WTX was examined by Western blot analysis. E. Densitometric analysis was used to quantify the expression of WTX. The results represent the mean ± SEM for three experiments. *P < 0.05 vs. Control group.

Figure 4. Curcumin downregulates Stat3 activity in G401 cells. A, B G401 cells were pretreated with curcumin for 48 h. The phosphorylation level of Stat3 was determined by Western blot analysis. C-E. G401 cells strains with stable expression of Stat3 siRNA or negative control (NC) siRNA were established. The phosphorylation level of Stat3 and protein level of WTX were examined by Western blot analysis. The results represent the mean ± SEM for three experiments. *P < 0.05 vs. Control group.
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pathway [15]. We first determined the effect of curcumin treatment on this pathway. For this, we documented expression of phosphorylated versus total Stat3 in G401 cells exposed to curcumin. As expected, curcumin did not affect expression of total Stat3 (Figure 4A). In contrast, phosphorylation of Stat3 was markedly reduced in G401 cells treated with curcumin (Figure 4A, 4B). Thereby, we demonstrated that curcumin negatively modulates the Stat3 signaling pathway.

Stat3 inhibits WTX expression in G401 cells

At the molecular level, Stat3 modulates the transcription of a variety of genes involved in the regulation of critical functions, including cell differentiation, proliferation, apoptosis, angiogenesis, metastasis and immune responses [16, 17]. However, whether Stat3 involved in the roles of WTX remain poorly understood. To determine whether the reduction of WTX by Stat3 is required for the anti-proliferative effect in G401 cells, Stat3 knockdown experiments using siRNA were conducted (Figure 4C, 4D). As a result, the siRNA increase the expression of WTX in G401 cells (Figure 4C, 4E). Therefore, the results indicate that curcumin can inhibit the WTX expression in G401 cells via Stat3 signaling.

Discussion

In this study, the involvement of curcumin and its molecular mechanism in Wilms’ tumor cells was investigated. Curcumin was shown to inhibit cell proliferation in G401 cells as demonstrated by CCK8, flow cytometry and TUNEL assays. At the molecular level, the results demonstrated that curcumin activated Stat3 activation. In addition, Stat3 was identified to be a novel molecular target of curcumin. Stat3 invalidation, using siRNA, reduced the WTX expression in G401 tumor cells. Collectively, the data suggested that curcumin may be beneficial in the treatment of Wilms’ tumor.

Accumulating evidence indicates that curcumin inhibits tumor progression via multiple cellular signaling pathways [18-20]. It has been documented that curcumin suppresses pancreatic cancer migration and invasion through the inhibition of the ROS/ERK/NF-κB signaling pathway [21]. Zhang et al demonstrate that curcumin exerts anti-cancer effects by negative modulation of the PI3K/AKT signaling pathway [22]. In the present study, curcumin treatment could markedly exacerbate the apoptosis of G401 cells, and boost up the expression of WTX. Collectively, our findings indicate that curcumin treatment may exert the anti-cancer effect in G401 cells through up-regulation WTX expression.

Stat3, a member of the STAT family, is capable of regulating the expression of target genes implicated in cell cycle progression, apoptosis, promotion of cellular transformation, and aberrant cell proliferation [23, 24]. Previous studies have suggested that the antiproliferative effects of curcumin involved the Stat3 pathway [15, 25, 26]. Therefore, Stat3 represents an attractive target for therapeutic intervention. Early in vitro work reported that structurally modified curcumin analogs inhibit Stat3 phosphorylation and promote apoptosis of human renal cell carcinoma [27]. Moreover, Cao et al uncovered that Stat3 inhibits WTX expression through up-regulation of microRNA-370 in Wilms tumor [28]. In the current work, we disclosed that the phosphorylation level of Stat3 was distinctly suppressed by curcumin in G401 cells. Besides, Stat3 siRNA markedly decreased the expression of WTX. Together, we suggested the protective effects of curcumin to WTX activation by regulating the Stat3 signaling in G401 cells.

In conclusion, the results suggest the underlying mechanisms that may contribute to the antineoplastic effects of curcumin. Further studies are required to investigate the potential of curcumin as a therapy for Wilms’ tumor prevention and treatment.

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Disclosure of conflict of interest

None.

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References

[1]  Breslow NE, Beckwith JB, Perlman EJ and Reeve AE. Age distributions, birth weights, ne-


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