AXIN1 protects against testicular germ cell tumors via the PI3K/AKT/mTOR signaling germ pathway

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Abstract. Axis inhibition protein 1 (AXIN1) is characterized as a tumor suppressor in numerous types of cancer. However, the functional role of AXIN1 in the testicular germ cell tumors (TGCTs) remains unclear. The human embryonal carcinoma-derived cell line NTERa2 was transfected with a recombinant AXIN1 expression vector (pcDNA3.1-AXIN1) and/or a small interfering RNA (siRNA) directed against AXIN1 (siAXIN1). Following transfection, the mRNA and protein levels of AXIN1 were determined via reverse transcription-quantitative polymerase chain reaction analysis and western blotting, respectively. In addition, cell viability, apoptosis and the expression of apoptosis-associated proteins [apoptosis regulator Bax (Bax) and B-cell lymphoma (Bcl)-2] and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) mammalian target of rapamycin (mTOR) signaling pathway proteins [phosphorylated (p)-mTOR, mTOR, p-AKT, AKT, P-70S ribosomal protein S6 (S6) and S6] were assessed. AXIN1 mRNA and protein levels were increased following transfection with pcDNA3.1-AXIN1 and decreased following transfection with siAXIN1 compared with their respective control groups. After overexpression of AXIN1, NTERa2 cell viability and expression of Bcl-2, p-mTOR p-AKT and p-S6 protein was decreased, while apoptosis and Bax protein levels were increased, compared with the control group. However, there was no significant difference in AXIN1 mRNA expression, apoptosis or Bax/Bcl-2 protein expression when NTERa2 cells were simultaneously transfected with pcDNA3.1-AXIN1+siAXIN1. In conclusion, the results of the present study indicate that overexpression of AXIN1 protects against TGCTs via inhibiting the PI3K/AKT/mTOR signaling pathway, suggesting that AXIN1 may be a potential target for gene therapy in TGCTs.

Introduction

Testicular germ cell tumors (TGCTs) are relatively rare, representing ~1-1.5% of all malignancies diagnosed in males and ~5% of urological tumors in males in general (1). However, TGCTs are the most frequently observed solid tumor among men aged between 15 and 35 years old (2). Additionally, it has been reported that the incidence of TGCTs has increased over the last 30-40 years (3). Despite this, the 5-year disease-free survival of patients with TGCTs is the highest amongst any other solid malignancy in males due to effective modern therapy, including platinum-based combination chemotherapy regimens, and close surveillance following surgery. In addition, the cure rate for patients with TGCTs is 95-96% (4,5). However, ~5% of patients with TGCTs still develop treatment resistance (6). Therefore, a better understanding of the pathogenesis of TGCTs is important in order to develop novel treatments.

The functional role of axis inhibition protein 1 (AXIN1), a multi-domain scaffold protein, has been associated with the tumorigenesis and progression of a number of diseases, including hepatitis B virus-related hepatocellular carcinoma and gastrointestinal cancer (7-10). AXIN1 acts as a tumor suppressor, and thus mutations of AXIN1 serve a significant role in carcinogenesis (8,11). Additionally, in combination with several different protein complexes AXIN1 has been reported to be involved in the Wnt, transforming growth factor (TGF)-β, stress activated protein kinase JNK1 (JNK) and cellular tumor antigen p53 (p53) signaling pathways (12-15). Furthermore, activation of the Wnt/β-catenin signaling pathway in human germ cell tumors has been reported (16), a cancer that AXIN1 mutation has been associated with (17). However, little information is available regarding the association between the functional role of AXIN1 and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) mammalian target of rapamycin (mTOR) signaling pathway in TGCTs.

The aim of the present study was to determine the functional role of AXIN1 in TGCTs. Furthermore, whether AXIN1 functions through the PI3K/AKT/mTOR signaling pathway, suggesting that AXIN1 may be a potential target for gene therapy in TGCTs.
pathway was investigated. The results of the current study may contribute to the identification and development of novel drugs for the treatment of TGCTs.

Materials and methods

Cell lines and culture. The human embryonal carcinoma (EC)-derived cell line NTera2 was purchased from the Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal calf serum (BD Biosciences, San Jose, CA, USA), 58.5 mg/ml L-Glutamine (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an incubator with 5% CO2.

Transfection. When the cells reached 70% confluence, they were subjected to transfection with small interfering RNA (siRNA) or an expression construct using Lipofectamine® 2000 reagent (1x10⁶ cells/well) (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. AXIN1 was knocked down using a siRNA (siAXIN1) and a scrambled siRNA (siControl) was used as a control. The sequence of siRNAs was listed as following: siAXIN1, 5’GGGAUAAGCCUGUUCAGGAATTT3’ and siControl, 5’AAAATCGACTCGTTTTTGCTC3’. The target sequences were designed and constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China). In addition, an AXIN1 expression vector (pcDNA3.1-AXIN1) was constructed and confirmed by sequencing (7). As a control, the empty construct pcDNA3.1 was transfected into NTera2 cells. For the cells simultaneously transfected with siAXIN1 and pcDNA3.1-AXIN1, the cells simultaneously transfected with scrambled siRNA and empty construct were considered as a control.

MTT cell viability assay. The survival of NTera2 cells was determined using the MTT colorimetric assay. Briefly, cells were collected, washed with PBS seeded into a 96-well plate (1x10⁵ cells/well) and incubated at 37°C. After 24, 48, 72 and 96 h incubation, 10 µl MTT was added to each well, and the cells were cultured for a further 4 h at 37°C. The absorbance at 595 nm was measured with a Synergy™ 4 Hybrid Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Apoptosis assay. Cell apoptosis was assessed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s protocol. Briefly, the cells were harvested (2x10⁶ cells/well), washed with PBS and re-suspended with binding buffer. Subsequently, the mixture was incubated with 5 µl Annexin V-FITC and 5 µl propidium iodide (PI). After incubation at room temperature in the dark for 15 min, the cells were analyzed with a flow cytometer (BD LSR Flow Cytometer; BD Biosciences). The collected data were analyzed using CellQuest™ software (version 3.0; BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. AXIN1 mRNA levels in NTera2 cells transfected with pcDNA3.1-AXIN1 or siAXIN1 for 48 h was determined using RT-qPCR analysis. Total RNA was extracted with the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. First strand 5 µl complementary (c)DNA was subjected to real-time PCR reaction using an ABI PRISM 7700 System and TaqMan reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol (18). GAPDH mRNA was used as a reference. The sequence of mRNA was listed as following: AXIN1; forward, 5’AACACATGGTCATGGCAAAGC3’; reverse, 5’TCTTCAGCCTCTGGTCTGG3’ (19); and GAPDH; forward, 5’TCTCTGACACCACCTGCTTATG3’, reverse, 5’AGTGGCAGTGATGGCAGTGGACT3’. The PCR thermocycler conditions included: initial denaturation was performed at 96°C for 15 sec, followed by 30 cycles of denaturation at 97°C for 15 sec, annealing at 62°C for 5 sec, extension at 72°C for 50 sec and the final extension was at 72°C for 10 min. Gene expression was quantified using the 2^ΔΔCt method (20).

Western blotting. A total of 24 h after transfection, protein was extracted from the cells by using a radioimmunoprecipitation lysis extraction kit (Thermo Fisher Scientific, Inc.). The protein concentration was measured using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. A total of 20 µg per lane of protein samples were resolved on a 12% gel using SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The resulting membranes were blocked in 5% milk in 0.1% Tris-buffered saline-Tween for 2 h. Subsequently, the membranes were treated with the following primary monoclonal antibodies overnight at 4°C: Anti-AXIN1 (cat. no. ab131372; dilution, 1:1,000; Abcam, Cambridge, UK), anti-apoptosis regulator Bax (Bax) (cat. no. sc-65,532; dilution, 1:1,000), anti-B-cell lymphoma (Bcl)-2 (cat. no. sc-509; dilution, 1:1,000), anti-phosphorylated (p)-mTOR (cat. no. sc-293132; dilution, 1:1,000), anti-mTOR (cat. no. sc-400140; dilution, 1:1,000), anti-p-AKT (cat. no. sc-7985-R; dilution, 1:1,000), anti-AKT (cat. no. sc-24500; dilution, 1:1,000), anti-P-70S ribosomal protein S6 (S6) (cat. no. sc-8416; dilution, 1:1,000), or anti-S6 (cat. no. sc-9027; dilution, 1:1,000). GAPDH (cat. no. sc-293335; dilution, 1:1,000), GAPDH; forward, 5'AAC ACA TGG TCA TGC CAA GC3' (21); and GAPDH; reverse, 5'TTC TCA GCG TCC TCT GTG G3' (22); and GAPDH; forward, 5'TCC TGC ACCACCAACTGCTTATG3', reverse, 5'AGTGGCAGTGATGGCAGTGGACT3'. The PCR thermocycler conditions included: initial denaturation was performed at 96°C for 15 sec, followed by 30 cycles of denaturation at 97°C for 15 sec, annealing at 62°C for 5 sec, extension at 72°C for 50 sec and the final extension was at 72°C for 10 min. Gene expression was quantified using the 2^ΔΔCt method (20).

Statistical analysis. All experiments were performed ≥2 times. The data are expressed as the mean ± standard deviation. Data analysis was carried out SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by Tukey post hoc test was performed to calculate the P-values. P<0.05 was considered to indicate a statistically significant difference.

Results

Confirming the results of siRNA and construct transfection on AXIN1 expression. To explore the effects of transfection on
AXIN1 expression, an AXIN1 expression vector (pcDNA3.1-AXIN1) and AXIN1-specific siRNA were constructed. As illustrated in Fig. 1A, the mRNA expression of AXIN1 was significantly increased following transfection with pcDNA3.1-AXIN1 compared with the control group (P<0.05). By contrast, the mRNA expression levels of AXIN1 were significantly lower in the siAXIN1 group compared with the siControl group (P<0.05; Fig. 1B). However, no significant difference was observed in AXIN1 mRNA expression following simultaneous transfection with pcDNA3.1-AXIN1+siAXIN1 compared with the control group (Fig. 1C). Western blots for the protein level of AXIN1 revealed similar results (Fig. 1D and E).

Effect of AXIN1 overexpression and silencing on TGCT cell viability. To explore the effects of AXIN1 overexpression and silencing on TGCT cell viability, an MTT assay was performed. A total of 24 h after transfection, cell viability was significantly increased by in the group transfected with siAXIN1 and markedly decreased in the group transfected with pcDNA3.1-AXIN1 compared with the control group of cells simultaneously transfected with scrambled siRNA and empty construct (both P<0.05; Fig. 2), indicating that AXIN1 acts as a tumor suppressor in TGCTs.

Effect of AXIN1 overexpression and silencing on TGCT cell apoptosis. Flow cytometry was performed to explore the effects of AXIN1 overexpression and silencing on TGCT cell apoptosis (Fig. 3). There was no significant difference in the percentage of apoptosis between the siAXIN1 (Fig. 3B) or pcDNA3.1-AXIN1+siAXIN1 (Fig. 3C) groups compared with their respective controls. However, the percentage of apoptosis was significantly increased in the pcDNA3.1-AXIN1 group compared with the control group (Fig. 3A; P<0.05), suggesting that overexpression of AXIN1 induces TGCT cell apoptosis.

Effect of AXIN1 overexpression and silencing on the expression of Bax and Bcl-2 protein in TGCT cells. To explore the potential of AXIN1 overexpression-induced TGCT cell apoptosis, the levels of the apoptosis-associated proteins Bax and Bcl-2 in NTERa2 cells transfected with siAXIN1 and/or pcDNA3.1-AXIN1 were measured. This revealed that the expression of Bax was markedly increased and the expression of Bcl-2 was markedly decreased after transfection with pcDNA3.1-AXIN1 compared with the control group (Fig. 4A). However, there was no notable difference in...
Effect of AXIN1 overexpression and silencing on the PI3K/AKT/mTOR signaling pathway in TGCT cells. To investigate the potential signaling pathway through which the protein levels of Bax or Bcl-2 in the siAXIN1 (Fig. 4B) or pcDNA3.1-AXIN1+siAXIN1 (Fig. 4C) groups compared with their respective controls.
AXIN1 overexpression was inducing TGCT cell apoptosis, the levels of PI3K/AKT/mTOR signaling pathway proteins (p-mTOR, p-AKT, AKT, p-S6 and S6) was investigated (Fig. 5). The results demonstrated that the protein levels of p-AKT, P-mTOR and p-S6 were markedly reduced following transfection with pcDNA3.1-AXIN1, indicating that the PI3K/AKT/mTOR signaling pathway is inhibited by AXIN1 overexpression.

**Discussion**

In the present study, AXIN1 was confirmed to be a candidate tumor suppressor gene in TGCTs. Overexpression of AXIN1 could inhibit TGCT cell viability and induce human EC-derived Ntera2 cell apoptosis through increasing the expression of proapoptotic Bax protein, while decreasing the expression of antiapoptotic Bcl-2 protein. The PI3K/Akt/mTOR signaling pathway was also demonstrated to serve an important role in AXIN1 overexpression-induced TGCT cell apoptosis. These results suggest that AXIN1 is a potential target for gene therapy in TGCTs.

AXIN1 was previously identified to be a product of the mouse *Fused* locus, and is mapped to human chromosome 16p13.3 with 87% similarity to the mouse protein (21). AXIN1 is associated with regulating axis formation during embryonic development (21). Two naturally occurring splicing variants of AXIN1, variant 1 (AXIN1V1) and variant 2 (AXIN1V2) have been reported. AXIN1V1 encodes an 862-amino acid (AA) long polypeptide, whereas AXIN1V2 is a shorter form of AXIN that lacks 36 AAs from exon 8 (8). AXIN1 serves as a scaffold protein through interacting with numerous proteins, enabling it to facilitate the degradation β-catenin, which suggests that it has a tumor suppressor function (22). Several signaling pathways, including that of Wnt, TGF-β, JNK1 and p53, have been reported to be associated with AXIN1 (23-25). A previous study suggested that AXIN1 mutation may be associated with germ cell tumors (17). However, there is little information with respect to the effect of AXIN1 overexpression, rather than mutation, on TGCTs.

One of the mechanisms underlying tumorigenesis is the activation of essential cellular signaling pathways. The essential and well-studied PI3K/AKT/mTOR signaling pathway serves important roles in tumorigenesis (26,27). This signaling pathway contributes to cell proliferation, differentiation, metabolism, cytoskeletal reorganization and apoptosis (28). Activation of the PI3K/AKT/mTOR signaling pathway promotes cancer cell survival and therapy resistance (29-31). Following activation of the PI3K/AKT/mTOR signaling pathway by membrane tyrosine kinase growth factor receptors or G protein-coupled receptors (32,33), functional PI3K is translocated to the plasma membrane where it causes the phosphorylation of phosphatidylinositol 3,4,5-triphosphate (PIP3) (33). Thereafter, p-PIP3 recruits 3'-phosphoinositide-dependent kinase 1 (PDK1) and AKT (34), leading to the phosphorylation and activation of AKT. Phosphorylation of AKT increases cell survival by inactivating proapoptotic factors, including Bax (34,35). Subsequently, p-AKT activates several downstream targets, including those in the mTOR signaling pathway. Activation of mTOR leads to an increase in protein synthesis, including that of certain proteins that are associated with the pathogenesis of a number of tumors, such as cyclin D1 (36). Additionally, activated mTOR can directly phosphorylate PDK1 and activate ribosomal protein S6 kinase b-1 (P70S6K). P70S6K initiates the ribosomal translation of mRNA into proteins that is essential for cell growth, progression and metabolism by phosphorylating S6. Thus, activation of PI3K/AKT/mTOR signaling pathway is a key event in tumorigenesis.

Since AXIN1 may act as a tumor suppressor in TGCTs, the theory that this protective effect may be through inhibition of the PI3K/AKT/mTOR signaling pathway was investigated. To confirm this hypothesis, transfection techniques were utilized to dysregulate the expression of AXIN1 in Ntera2 cells. The effects of transfection on AXIN1 expression were confirmed by RT-qPCR and western blotting. The effects of AXIN1 dysregulation on Ntera2 cell viability and apoptosis were then examined. The results showed that Ntera2 cell viability was significantly increased by knock-down of AXIN1 and markedly decreased by overexpression of AXIN1, further suggesting that AXIN1 functions as a tumor suppressor in TGCTs. In addition, the percentage of apoptotic cells was significantly increased by overexpression of AXIN1. This result was in line with those of previous studies, which also suggested that AXIN1 could significantly induce cancer cell apoptosis (11,37). Next, the potential mechanism of AXIN1 overexpression-induced apoptosis was explored. Overexpression of AXIN1 markedly increased Bax protein expression and markedly decreased Bcl-2 protein expression. One explanation for this effect is that AXIN1 acts of AKT to inhibit the PI3K/AKT/mTOR signaling pathway, decreasing apoptosis-associated protein expression and apoptosis.

The levels of PI3K/AKT/mTOR signaling pathway proteins were assessed in the present study. This demonstrated that the protein levels of p-AKT, p-mTOR and p-S6 were markedly reduced by overexpression of AXIN1, indicating that the PI3K/AKT/mTOR signaling pathway is inhibited by overexpression of AXIN1. Arnold et al (38) suggested that AXIN1 could negatively regulate proto-oncogene c-Myc.
protein expression at a post-translational level, thus acting a tumor suppressor (38). However, in the present study it remains unclear whether AXIN1 regulates or coordinates with oncogene proteins to exert antitumor activity.

In conclusion, the results of the present study confirm that AXIN1 is a candidate tumor suppressor gene in TGCTs and indicate that it exerts this effect through inhibiting the PI3K/AKT/mTOR signaling pathway. This suggests that AXIN1 is a potential target for gene therapy in TGCTs.

References