SPINDLIN1 Promotes Cancer Cell Proliferation through Activation of WNT/TCF-4 Signaling

Jing-Xue Wang, Quan Zeng, Lin Chen, Ji-Chao Du, Xin-Long Yan, Hong-Feng Yuan, Chao Zhai, Jun-Nian Zhou, Ya-Li Jia, Wen Yue, and Xue-Tao Pei

Abstract

SPINDLIN1, a new member of the SPIN/SSTY gene family, was first identified as a gene highly expressed in ovarian cancer cells. We have previously shown that it is involved in the process of spindle organization and chromosomal stability and plays a role in the development of cancer. Nevertheless, the mechanisms underlying its oncogenic role are still largely unknown. Here, we first showed that expression of SPINDLIN1 is upregulated in clinical tumors. Ectopic expression of SPINDLIN1 promoted cancer cell proliferation and activated WNT/T-cell factor (TCF)-4 signaling. The Ser84 and Ser99 amino acids within SPINDLIN1 were further identified as the key functional sites in WNT/TCF-4 signaling activation. Mutation of these two sites of SPINDLIN1 abolished its effects on promoting WNT/TCF-4 signaling and cancer cell proliferation. We further found that Aurora-A could interact with and phosphorylate SPINDLIN1 at its key functional sites, Ser84 and Ser99, suggesting that phosphorylation of SPINDLIN1 is involved in its oncogenic function. Collectively, these results suggest that SPINDLIN1, which may be a novel substrate of the Aurora-A kinase, promotes cancer cell growth through WNT/TCF-4 signaling activation. Mol Cancer Res; 10(3); 326–35. ©2012 AACR.

Introduction

SPINDLIN was first reported as an abundant maternal transcript present in the unfertilized egg and 2-cell, but not the 8-cell, stage embryo during the transition from oocyte to embryo in the mouse (1). Its name is derived from its association with the meiotic spindle, which occurs as a result of phosphorylation modification, in a cell-cycle-dependent fashion. In our earlier report on ovarian cancer cells, we identified human gene SPINDLIN as a new member of the SPIN/SSTY gene family. This family is involved in gametogenesis, and the expression of genes in this family can be detected in early embryos (2). We found that SPINDLIN1 is highly expressed in human early embryonic tissues, including ovary and kidney. However, the expression level of SPINDLIN1 is dramatically decreased and cannot be detected in any embryonic tissues at 8 months. SPINDLIN1 is highly expressed in many kinds of malignant tumor tissues, including ovarian tumors, non–small cell lung cancers, and some hepatic carcinomas (3).

In addition, NIH3T3 cells with ectopic expression of SPINDLIN1 form colonies in culture and tumors in nude mice, suggesting the oncogenic potential of this gene (4). We also reported that SPINDLIN1, a nuclear protein, is relocated during cell mitosis and dynamically distributes along mitotic spindle tubulin, indicating that SPINDLIN1 is a novel spindle protein (5). We also found that ectopic expression of SPINDLIN1 induces cell-cycle delay in metaphase and causes chromosome instability (5, 6).

The WNT signaling pathway regulates cell fate in a large number of developmental processes and differentiation during embryogenesis (7, 8). In addition, the vital role of this pathway in tumorigenesis arouses great research interest (9–11). β-Catenin, stabilized by activation of WNT signaling, is translocated into the nucleus to form nuclear complexes with transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family and subsequently activates several downstream effectors such as C-MYC and cyclin D1. These target genes are known to regulate the cell cycle and contribute to the oncogenic phenotype (12). We believed that uncovering the regulators of WNT/TCF-4 signaling would give rise to a better understanding of the potential mechanisms of carcinogenesis.

Human Aurora-A is commonly amplified in epithelial malignant tumors, and more than 50% of ovarian and breast cancers show enhanced activation of this kinase (13, 14). It has been shown that rodent fibroblasts transfected with Aurora-A form tumors in nude mice, indicating that...
Aurora-A is a cancer susceptibility gene (15). In addition, some studies have shown that Aurora-A plays an essential role in chromosome segregation and cell division (16, 17). Nevertheless, the underlying mechanism by which Aurora-A promotes tumorigenesis remains elusive. Identification of the substrates and further exploration of the signaling pathway linking Aurora-A to other key factors regulating cell growth will provide new insight into understanding tumorigenesis.

Here, we further show that SPINDLIN1 is highly expressed in ovarian cancer tissues and also promotes cancer cell proliferation and tumor growth. Most importantly, SPINDLIN1 is found to function as an activator of WNT/TCF-4 signaling and promotes the expression of the WNT/TCF-4 targets, C-MYC, cyclin D1, and Axin2. Mutation of the 84 and 99 amino acid sites on SPINDLIN1 could largely abolish its effects on WNT/TCF-4 activity and cancer cell proliferation, suggesting that SPINDLIN1 may promote cancer cell growth through the activation of WNT/TCF-4 signaling. Further analysis suggests that the key functional sites of SPINDLIN1, amino acids Ser84 and Ser99, may be candidate phosphorylation sites for Aurora-A. Taken together, our results suggest that SPINDLIN1 promotes cancer cell proliferation by activating the WNT/TCF-4 signaling pathway, and the phosphorylation of Ser84 and Ser99 of SPINDLIN1 may be involved in its effect on WNT/TCF-4 activity.

Materials and Methods

Immunohistochemistry

Immunohistochemistry was conducted on microarray slides containing 53 ovarian cancer tissues and 4 normal tissues obtained from Chaoying Biotechnology. Another 10 cancer-free ovarian tissues were obtained from 307 Hospital center at the Academy of Military Medical Sciences, and 53 ovarian cancer tissues and 4 normal tissues obtained from Chaoying Biotechnology. Immunohistochemistry was conducted on microarray slides containing 53 ovarian cancer tissues and 4 normal tissues obtained from Chaoying Biotechnology. Another 10 cancer-free ovarian tissues were obtained from 307 Hospital center at the Academy of Military Medical Sciences, and 53 ovarian cancer tissues and 4 normal tissues obtained from Chaoying Biotechnology.

Cell culture and transfection

HeLa, A549, H1299, and HEK293T cell lines were purchased from American Type Culture Collection. HeLa and HEK293T cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), and A549 and H1299 cells were grown in RPMI-1640 medium (Sigma). Both media were supplemented with 10% FBS (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin, and all cells were maintained at 37°C in 5% CO₂. Transfections of the cells were conducted using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions.

Antibodies

Mouse monoclonal anti-Aurora-A antibody (Cell Signaling Technology), mouse monoclonal anti–C-MYC (9E10) antibody (Santa Cruz Biotechnology), mouse monoclonal anti-cyclin D1 (A-12) antibody (Santa Cruz Biotechnology), mouse monoclonal anti-GFP antibody (Proteintech Group), rabbit polyclonal anti-glutathione S-transferase (GST) antibody (Proteintech Group), and rabbit polyclonal anti-β-actin antibody (Santa Cruz Biotechnology).

Cell counting kit-8 cell proliferation assay

Viable cells were examined using the CCK-8 Assay (Dojindo). HeLa cells transfected with siRNA-SPINDLIN1 or control oligonucleotide were seeded into 96-well plates at a density of 1 × 10⁵ per well and cultured in DMEM containing 10% FBS. The medium was exchanged for 100 μL DMEM with 10% FBS every 3 days. At the end of the incubation, the cells were stained with crystal violet and colonies were counted. Each sample was repeated in 3 wells.

Tumorigenicity assay

Nude mice were purchased from the laboratory animal center at the Academy of Military Medical Sciences, and
the experiments were carried out in accordance with institutional guidelines for laboratory animals. Four clones of HeLa cells stably overexpressing SPINDLIN1 (SC1, SC2, SC3, and SC4) and 2 clones of control cells (CC1 and CC2) were independently implanted into 4 nude mice. The cells were suspended in PBS, and 3 x 10^6 cells/200 μL were injected subcutaneously into either flank of each mouse. Every 2 or 3 days, tumor diameters were measured, and tumor volumes were calculated using the formula: a x b^2/2 (where a is the largest and b is the smallest tumor diameter). One month after injection, the mice were sacrificed, the tumors were removed, and their weights were recorded.

RNA interference

An siRNA sequence used for depleting SPINDLIN1 was purchased from Sigma-Aldrich. The following target sequences were chosen for RNA interference: 5'-GATT-CAGCATGGGTGAAA-3'. An oligonucleotide sequence was used as a negative control (Sigma-Aldrich).

**In vitro invasion assay**

HeLa or A549 cells were transfected with pcDNA3.1-MYC/HIS(-) or pcDNA3.1-MYC/HIS(-). Matrigel (BD Biosciences), diluted 1:6, was added to each Millicell Hanging Cell Culture Insert (PET membrane, 8.0 μm pore size) and polymerized at 37°C for 4 hours. Cells were added to the upper chamber without FBS, and the lower chamber was filled with the culture medium containing 1.5% FBS. After incubation for 36 hours, the cells on the Matrigel-coated side of the membrane were removed, and cells on the other side were stained with crystal violet. The cells migrating through the membrane were counted in 5 randomly selected fields for each sample, and each sample was repeated in 3 wells.

**Luciferase reporter assay**

A549 and H1299 cells were cotransfected using Lipofectamine 2000 with the TCF/β-catenin–responsive reporter plasmid, pTOPFLASH, and pcDNA3.1-MYC/HIS(-)-SPINDLIN1, or the corresponding empty vector pcDNA3.1-MYC/HIS(-), as well as with the Renilla reference plasmid pRL-null. The control reporter pTOPFLASH transfected with pcDNA3.1-MYC/HIS(-)-SPINDLIN1 was used to exclude any nonspecific effects. After transfection for 36 hours, cells were lysed and then luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega). The procedures followed the manufacturer’s protocol. The results were analyzed using SOFTMAX PRO SOFTWARE (Molecular Devices), and each sample was analyzed in triplicate. Luciferase activity results were normalized to Renilla activity and expressed as the ratio relative to control plasmid activity.

**Immunofluorescence**

HeLa cells plated on coverslips were transfected with pEGFP-C1-SPINDLIN1 for 24 hours. The cells were treated with 1% Triton X-100, followed by incubation with 10% goat serum for 30 minutes. After incubation with the Aurora-A antibody (at a dilution of 1:25) overnight at 4°C, the cells were washed and incubated with TRITC-conjugated goat anti-rabbit IgG diluted 1:50, for 30 minutes at 37°C. After washing, the cells were finally stained with 4,6-diamidino-2-phenylindole (DAPI; 2 μg/mL; Sigma). Microscopic images were acquired using Zeiss LSM 510 META system with a 40× oil objective (Karl Zeiss).

**Immunoprecipitation and immunoblot analysis**

HeLa cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (0.5% sodium deoxycholate, 0.1% SDS, and 1% NP-40) containing protease inhibitor cocktail (Calbiochem). Approximately, 500 μg of total cellular protein was incubated with 10 μL of primary antibody for 1 hour at 4°C. Then, 20 μL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added, and the samples incubated overnight at 4°C. For immunoblot analysis, we followed the previously described methods (2), using anti-Aurora-A and anti-C-MYC antibodies.

**GST pull-down assay**

GST and GST fusion proteins were induced in Escherichia coli strain BL21 (DE3) by addition of 1 mmol/L isopropyl-β-D-1-thiogalactopyranoside (IPTG), and the cultures were maintained at 16°C for 4 hours. The proteins were purified using Glutathione Sepharose 4B (Amersham Bioscience) beads according to the manufacturer’s instructions. HeLa cells transfected with pEGFP-C1-Aurora-A were harvested after 24 hours, and the lysates were incubated with purified GST fusion protein bound to glutathione-agarose beads at 4°C for 4 hours. Then, the mixture was then boiled, and eluted proteins were separated by SDS-PAGE followed by immunoblot analysis using anti-Aurora-A and anti-GST antibodies.

**Reverse transcription PCR**

Total RNA was extracted with TRIzol reagent (Invitrogen) following the recommended protocol. The first strand of cDNA was synthesized using oligo(dT) or a random primer (TaKaRa) and then reverse transcribed by M-MLV (TaKaRa). PCR was carried out in a final volume of 20 μL using rTaq DNA-polymerase (TaKaRa). The PCR cycle conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, finishing with 7 minutes at 72°C. β-Actin was used as an internal standard. The PCR products were visualized in a 1.2% agarose gels by staining with Gel Green (Biotium). The primers for the PCR were as follows:

Forward, 5'-GATT-CAGCATGGGTGAAA-3'.
Reverse, 5'-AACTTCTCCTGGTTCCCT-3' (SPINDLIN1);
Forward, 5'-TACCCTCTCAACGACAGCAG-3'.
Reverse, 5'-TCTTGACATTTCTCCTGCGTG-3' (C-MYC);
Forward, 5'-GCGAGGAACAGAAGTGCG-3'.

**Molecular Cancer Research**

Wang et al.
Reverse, 5'-AGGCCTAGCTAAGCACAGAA-3'
Forward, 5'-GTGAGGCCAGCTCAGAATG-3'
Reverse, 5'-GTCGAGCTCTCGGAAAATG-3' (A52)

In vitro kinase assay

HeLa cells were transfected with the Aurora-A expression vector using Lipofectamine 2000. Cells were collected 36 hours later and lysed. Aurora-A was immunoprecipitated by Protein A/G PLUS-agarose. GST and GST-SPINDLIN1 mutant proteins were expressed in bacteria and purified. The immunoprecipitates were incubated with different GST fusion proteins and labeled with [γ-32P]ATP by the method described previously (18). The proteins were separated by SDS-PAGE, and the gels were analyzed by autoradiography.

Results

SPINDLIN1 is highly expressed in ovarian cancer tissues

To analyze SPINDLIN1 expression in ovarian cancer tissues, we conducted immunohistochemical analysis using the purified SPINDLIN1 antibody. The samples were obtained from a microarray containing 53 different types of ovarian cancer tissues, including serous papillary cystadenocarcinoma, adenocarcinoma, squamous cell carcinoma, and dysgerminoma. Fourteen nontumor ovarian tissues were also included in the analysis for comparison. The results indicated that SPINDLIN1 is mainly expressed in cancer cell nuclei [Fig. 1A (ii–iv)], whereas no expression was found in normal tissues [Fig. 1A(i)]. The statistical data are shown in Fig. 1B. Among grade I cancer tissues, none of the 6 samples were positive for SPINDLIN1. In regard to grade II and grade III/IV tissues, high expression of SPINDLIN1 was observed in 12 of 23 and 11 of 24 samples, respectively (Supplementary Table S1). The results showed that grade II and grade III/IV cancer tissues possessed a higher level of SPINDLIN1 expression than in grade I tissues (Fisher exact test, P < 0.05). These findings corresponded with our first report, which identified SPINDLIN1 as a novel ovarian cancer–related gene and showed that it was overexpressed in ovarian cancer tissues, but not in the normal tissues (3).

SPINDLIN1 promotes cancer cell proliferation and invasion

To study the effects of SPINDLIN1 on cell proliferation, 6 independent clones, including 4 clones of HeLa cells stably expressing SPINDLIN1 (SC1 to SC4) and 2 control clones (CC1 and CC2) were cultured (Fig. 2A). Three SPINDLIN1 clones and 2 control clones were used in colony-forming assays. After 2 to 3 weeks, the number of colonies of HeLa cells increased from 129.33 ± 40.57 (CC1), 111.33 ± 7.64 (CC2) to 218.67 ± 32.47 (SC1, N = 3, P < 0.05), 358.00 ± 44.58 (SC2, N = 3, P < 0.001), and 405.67 ± 45.08 (SC3, N = 3, P < 0.001; Fig. 2B), showing that SPINDLIN1 promoted cell growth in vitro. To conduct the tumorigenicity assay, each of SPINDLIN1 clones (SC1, SC2, SC3, and SC4) were injected into one flank of 4 nude nude mice, respectively. CC1 and CC2 were injected into the other flank of mice. Within 1 month, it became clear that the tumors derived from SPINDLIN1-overexpressing HeLa cells grew faster and were heavier than those produced by control cells (Fig. 2C). The tumor weight increased from 0.18 ± 0.13 g (CC) to 0.43 ± 0.28 g (SC, N = 16, P < 0.01). This result showed that SPINDLIN1 accelerated the growth of HeLa tumors. We used RNA interference to deplete SPINDLIN1 in HeLa cells (Supplementary Fig. S1A, siRNA-SPINDLIN1-1), leading to the slowed growth of cells over 7 days in the CCK-8 assay (Supplementary Fig. S1B). Differences in proliferation appeared beginning on day 2 (N = 6, P < 0.001). In addition, the colony-forming assays also confirmed that the depletion of SPINDLIN1 resulted in reduced colony formation (N = 3, P < 0.01; Supplementary Fig. S1C). These results suggested that SPINDLIN1 depletion slowed the growth of tumor cells.

An in vitro invasion assay showed a significant enhancement of the invasiveness of HeLa and A549 cells transfected with SPINDLIN1 (Fig. 2D). The number of invasive HeLa...
cells increased from 88.27 ± 4.20 to 262.33 ± 13.70 (N = 3, *P < 0.001) and that of A549 cells increased from 114.73 ± 12.63 to 322.67 ± 18.77 (N = 3, **P < 0.001). These results showed that SPINDLIN1 contributed both to the proliferation ability and invasiveness of the tumor cells.

SPINDLIN1 promotes activation of WNT/TCF-4 signaling

Our previous study suggested that SPINDLIN1 might function as a tumor enhancer through activation of the WNT/TCF-4 signaling pathway (19). Here, we confirmed
the direct interaction between SPINDLIN1 and TCF-4 by GST pull-down assay with β-catenin as a positive control and the GST empty vector as negative control (Fig. 3A). Next, we used a luciferase reporter assay to study the effects of SPINDLIN1 on the activity of the TCF/β-catenin-responsive reporter. We found that SPINDLIN1 increased the activity of the pTOPFLASH reporter in both H1299 cells (from 1.07 ± 0.04-fold to 1.72 ± 0.09-fold, N = 3, P < 0.001) and A549 cells (from 1.05 ± 0.02-fold to 3.82 ± 0.14-fold, N = 3, P < 0.001). The activity of the control reporter pTOPFLASH was set as 1.00-fold (Fig. 3B). We then confirmed the reduced activity of the TCF/β-catenin-responsive reporter by RNA interference against SPINDLIN1 (N = 3, P < 0.01; Supplementary Fig. S1D). To test whether SPINDLIN1 can affect the downstream targets of WNT/TCF-4 signaling, the expression of C-MYC, cyclin D1, and Axin2 was analyzed by reverse transcription PCR (RT-PCR) and Western blotting. We found that their expression levels were substantially enhanced in A549 and H1299 cells transfected with SPINDLIN1 (Fig. 3C and D). These results suggested that SPINDLIN1 functions as a WNT/TCF-4 signaling pathway activator.

SPINDLIN1 promotes cancer cell proliferation via WNT/TCF-4 activation

To address the question of whether SPINDLIN1 promotes cancer cell proliferation in a WNT/TCF-4 activation-dependent manner, we set out to analyze the biologic function of SPINDLIN1 mutants that have lost their ability to activate the WNT/TCF-4 signaling pathway.

According to our previous bioinformatics and localization study, amino acid sites 84 and 99 were identified to play significant roles in the cellular localization of SPINDLIN1 and may be involved in its function in the WNT/TCF-4 signaling (19). First, the GST pull-down assay showed that the SPINDLIN1 mutation at sites 84 and 99 (SPINDLIN1-84/99 M) decrease the interaction between SPINDLIN1 and TCF-4 (Fig. 4A). The SPINDLIN1-84/99 M mutant was observed to result in a sharp decrease in the TCF/β-catenin-responsive reporter’s activity in comparison with wild-type SPINDLIN1 in the luciferase reporter assay (from 5.60 ± 0.62-fold to 1.15 ± 0.01-fold, N = 3, P < 0.001). The reporter’s activity was almost as low as that for the empty vector control (Fig. 4B). RT-PCR results showed a substantial decline in C-MYC, cyclin D1 and Axin2 expression levels when sites 84 and 99 of SPINDLIN1 were mutated (Fig. 4C), and Western blotting showed that the mutations significantly decreased C-MYC and cyclin D1 protein levels (Fig. 4D). These data showed that mutation of SPINDLIN1 at these sites abolished its function in the WNT/TCF-4 signaling pathway.

To study whether the mutations of SPINDLIN1 affects its role in cancer cell proliferation, the SPINDLIN1 double mutant was then transfected into cancer cells.
which was then used for colony-forming assays and tumorigenicity assays. As shown in Fig. 4E, the mutations caused a 43% reduction in the number of colonies (from 145.67 ± 6.03 to 82.67 ± 6.43, N = 3, P < 0.001) compared with wild-type, but the numbers were still higher than that observed in cells transfected with the empty vector. The tumorigenicity assay was also conducted for HeLa cells stably expressing the mutated or wild-type SPINDLIN1. The tumor growth rate for the mutant-transfected cells was markedly lower than for the wild-type SPINDLIN1, and the weight of mutants' tumors was reduced by 67% (from 0.35 ± 0.11 g to 0.11 ± 0.14 g, N = 6, P < 0.01; Fig. 4F). The results suggested that SPINDLIN1 promotes tumor cell proliferation by activating the WNT/TCF-4 signaling pathway.

**Figure 4.** Mutation of SPINDLIN1 abolished its effects on WNT/TCF-4 signaling and cancer cell proliferation. A, GST, GST-SPINDLIN1, and GST-SPINDLIN1-84/99 M protein expression was induced in bacteria. Proteins were purified for GST pull-down (top) and incubated with cellular lysates containing C-MYC-TCF-4. After separation by 10% SDS-PAGE, they were immunoblotted with anti-C-MYC antibody (input: 25 μg of protein extracts, bottom). B, pEGFP-C1, pEGFP-C1-SPINDLIN1-84/99 M, and pEGFP-C1-SPINDLIN1-WT were used for luciferase reporter assays. The results were normalized to Renilla activity and are expressed as the ratio relative to the control. Data with error bars indicate the means ± SD. N = 3; ***, P < 0.001 compared with the wild-type (WT) level (Student’s t test). C and D, HeLa cells were transfected with pEGFP-C1, pEGFP-C1-SPINDLIN1-84/99 M, and pEGFP-C1-SPINDLIN1-WT. RT-PCR (C) was carried out to analyze the mRNA levels of C-MYC, cyclin D1, and Axin2, and Western blot analyses (D) were conducted to determine their expression levels, using β-actin as an internal standard. E, HeLa cells transfected with GFP, GFP-SPINDLIN1-84/99 M, and GFP-SPINDLIN1-WT were used for colony-forming assays (top left). Colonies were stained after 2 to 3 weeks (bottom left). The numbers are shown as the means ± SD (right). ***, P < 0.001 compared with wild-type level (Student’s t test). F, HeLa cells stably expressing SPINDLIN1-WT and SPINDLIN1-84/99 M were used for tumorigenicity assays. Each nu/nu mouse was injected with 3 × 10^6 cells in either flank. One month later, the mice were sacrificed and the tumors were removed (left). The tumor weight (middle) and the tumor volume (right) are expressed as the means ± SD. N = 6; ***, P < 0.01 compared with the wild-type level (Student’s t test).
Aurora-A interacts with and phosphorylates SPINDLIN1 at Ser84 and Ser99

Considering the oncogenic characteristic of SPINDLIN1 in cancer cells and its key functional sites, Ser84 and Ser99 were suggested to be candidate phosphorylation sites of Aurora-A by bioinformatics analysis. We hypothesized that the function of SPINDLIN1 may be related to Aurora-A, a Ser/Thr kinase that plays a vital role in the initiation of mitotic and G2-M transition and also takes part in many activities associated with carcinogenesis (20, 21). Aurora-A is found at the centrosomes during interphase and at spindle poles during mitosis (22, 23). We first checked the localization of SPINDLIN1 in GFP-SPINDLIN1–expressing HeLa cells, and endogenous Aurora-A was examined by immunofluorescence with a TRITC-conjugated antibody. The results showed that SPINDLIN1 and Aurora-A localized approximately to the same region: they were both found at the centrosome during interphase, at spindle poles from metaphase to telophase, and at the microtubules of the central body during cytokinesis (Fig. 5A). The results suggested that the biologic functions of SPINDLIN1 and Aurora-A might be related.

To confirm the physical association between Aurora-A and SPINDLIN1 in vitro, we conducted a GST pull-down assay (Fig. 5B). Coomassie-stained gels showed that recombinant GST (used as a negative control) was expressed and purified with the similar efficiency as the GST-SPINDLIN1 fusion protein. Then, cellular lysates containing GFP-Aurora-A were incubated with purified GST-SPINDLIN1 or GST. Western blotting showed that GFP-tagged Aurora-A interacted with GST-SPINDLIN1, whereas band was not detected in the sample incubated with GST alone. These results showed their direct interaction in vitro.

We used co-immunoprecipitation assays to investigate whether Aurora-A interacts with SPINDLIN1 in vivo (Fig. 5C). After cotransfection, HeLa cells with ectopic expression of GFP-Aurora-A and C-MYC-SPINDLIN1 were lysed, and the lysates were immunoprecipitated using mouse anti-C-MYC antibody. The precipitates were analyzed by Western blotting. We observed an Aurora-A band, but this band was absent in the precipitates obtained using mouse IgG (control). These results showed that Aurora-A and SPINDLIN1 interacted in vivo.

Subcellular colocalization results and the observed interaction between Aurora-A and SPINDLIN1 suggested that SPINDLIN1 might serve as a substrate of Aurora-A kinase. To test this assumption, we conducted in vitro kinase assays. Recombinant Aurora-A and various GST-fused fragments of SPINDLIN1 were constructed as described above. The results of autoradiography showed that GST-SPINDLIN1 is phosphorylated by Aurora-A, whereas GST alone was not (Fig. 5D, top). Meanwhile, we found that the kinase phosphorylated fragment 3 (amino acids 51–94) and fragment 6 (amino acids 85–138) of SPINDLIN1. We then narrowed down the phosphorylation locations to amino acids 61–94 and 85–105 (Fig. 5D, middle). To confirm the specific phosphorylation sites, we replaced serines with alanines at S84, S85, S96, and S99 of SPINDLIN1 to generate S-A mutants. The kinase assay showed a lack of phosphorylation of the S84A and S99A mutants, showing that these sites were the phosphorylation sites (Fig. 5D, bottom). The results showed that SPINDLIN1 interacts with Aurora-A, and its functional sites were phosphorylated by this kinase.

Discussion

SPINDLIN1 was first identified as a new member of the SPIN/SSTY gene family and was implicated in ovarian cancers. Its expression is markedly upregulated in ovarian cancer cells and other kinds of cancer cells. We have reported that the constitutive expression of SPINDLIN1 contributes to the malignant transformation of NIH3T3 cells (4). In our previous study, we showed that SPINDLIN1 is involved in metaphase arrest and could affect chromosomal stability. SPINDLIN1 protein is dynamically distributed along mitotic spindle tubulins. We also showed that the overexpression of SPINDLIN1 can induce cell-cycle delay in metaphase and cause chromosomal instability (5). Nevertheless, the molecular mechanisms underlying the oncogenic role of SPINDLIN1 are still largely unknown.

There seems to be a discrepancy in that SPINDLIN1 could cause a delay in mitosis and also cause chromosome instability (2, 5), but our data presented here confirm the fact that SPINDLIN1 stimulates cell growth. In fact, the 2 phenomena are not completely contradictory. Indeed, cell-cycle delay might lead to defects in mitotic spindle organization or DNA separation, ultimately resulting in cell apoptosis. However, it is possible that chromosomal combination takes place in some cells overexpressing SPINDLIN1, which could help those cells escape cell apoptosis and obtain a selective growth advantage. Consequently, those cells undergoing apoptosis overcome the negative influence of SPINDLIN1 and finally acquire an increased growth rate. This possibility may be one explanation for why SPINDLIN1 overexpression induces tumorigenesis.

Accumulating evidence has suggested that the WNT/β-catenin signaling pathway plays an important role in tumorigenesis and development (18, 24–26). Many factors have been identified as cotranscription factors in this signaling pathway. They are known to bind TCF-4 or β-catenin and function as transcription coactivators or inhibitors (27–29). In our previous study, we found that SPINDLIN1 interacts with TCF-4 and activates its activity (19), suggesting that SPINDLIN1 might promote cancer cell proliferation via this signaling pathway. Here, we have confirmed the existence of an interaction between these 2 molecules in cancer cells. Ectopic expression of SPINDLIN1 promotes the activity of a TCF/β-catenin–responsive reporter and enhances the expression levels of C-MYC, cyclin D1, and Axin2, the targets of the WNT/TCF-4 signaling pathway. Amino acid sites 84 and 99 of SPINDLIN1 were shown to be important for the nuclear localization of SPINDLIN1 in our previous study (19). The mutation of these 2 sites results in the abrogation of the nuclear localization of SPINDLIN1, which instead is
localized diffusely throughout the cell. Therefore, we hypothesized that the 2 sites could be important for SPINDLIN1 function. Here, we showed that amino acids 84 and 99 of SPINDLIN1 are indispensable for the promotion of WNT/TCF-4 signaling activity and cancer cell proliferation. The results suggested that SPINDLIN1, with its functional sites at amino acids 84 and 99, functions as a cotranscription factor of the WNT/TCF-4 signaling pathway, promoting cancer cell growth through the upregulation of C-MYC, cyclin D1, and Axin2 expression.

It has been reported that ectopic expression of Aurora-A upregulates telomerase activity and increases C-MYC expression in human ovarian and breast cancer cells (15, 30, 31). Knocking down C-MYC expression by RNA interference suppressed Aurora-A–stimulated telomerase activity (32). Nevertheless, the mechanism by which Aurora-A upregulates C-MYC remains unclear. In this study, we have preliminarily discussed the possible mechanism of Aurora-A. We found that SPINDLIN1 interacts with Aurora-A and is a phosphorylated substrate of the kinase. In addition, the function of the
phosphorylated sites of SPINDLIN1 has been shown here. Therefore, we propose that the effects of Aurora-A on tumorigenesis may be related to WNT/TCF-4 signaling, which is mediated by phosphorylation of the functional sites of SPINDLIN1. However, more research will be needed to investigate this complex process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

References

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Jing-Xue Wang, Quan Zeng, Lin Chen, et al.


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