S100A4 mRNA as a prognostic marker and therapeutic target in Wilms tumor (WT)

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Abstract. – OBJECTIVE: Recent studies showed that the S100A4 is candidate prognostic marker or therapeutic targets in cancers. In this study, we first evaluate the expression of S100A4 mRNA in Wilms tumor (WT) and its relationship to the clinicopathological parameters and prognosis. We then tested a hypothesis that the S100A4 gene plays a role in cell proliferation, apoptosis, invasiveness and capillary network formation and regression of established orthotopic tumors of human WT cells.
MATERIALS AND METHODS: Expression of V S100A4 mRNA was examined in 48 surgical specimens of WTs by Quatitive Reverse transcription-PCR analysis (Q-PCR). Correlation between the expression of S100A4 mRNA and clinicopathological parameters was analyzed. We used *in vitro* and *vivo* experiments with RNA interference to evaluate the functional role of S100A4 and its potential as a therapeutic target for WT.
RESULTS: S100A4 mRNA levels were significantly higher in carcinoma specimens than in non neoplastic tissues. S100A4 mRNA expression was significantly correlated with tumor size, vascular invasion, node metastasis and tumor stage. We observed that shRNA-mediated suppression of the S100A4 gene significantly promoted apoptosis and reduced the proliferative and invasive capability, angiogenesis of the WT cells SK-NEP-1 in vitro. S100A4-shRNA-transfected cells exhibited a reduced rate of tumor growth under *in vivo* conditions. Microvascular density (MVD) was reduced by 62% due to S100A4-shRNA treatment (*p* < 0.01).
CONCLUSIONS: Our present results suggest that S100A4 mRNA plays an important role in the development of WT. It might be useful in evaluating the outcome of patients with WT. S100A4 may be a promising therapeutic target for WT.

Key Words:
　Wilms tumor, Prognostic factor, S100A4, Gene treatment.

Introduction

Wilms tumor (WT) is the most common malignant renal tumor in children. In the last few decades there have been major advances in our knowledge of the molecular basis of this tumor and in clinical outcomes[1]. Clearly, a better understanding of the stimuli and signaling pathways involved in Wilms tumor progression would be a substantial advancement. Wilms and other renal tumors are typically angiogenic, invasive and potentially metastatic. Mortality is usually associated with a metastatic phenotype. Essential to the process of metastasis are cellular proliferation, migration and invasion[2].

S100A4 (metastasin)is a ubiquitous small, calcium-binding protein that enables cell migration and invasion to increase cell motility[3]. Consequently, S100A4 expression is up-regulated during wound healing, neurite outgrowth, fibrosis, or neovascularization-all physiological processes that rely on increased cell motility. Recent studies indicate that S100A4 were overexpressed in many malignant neoplasms, and S100A4 overexpression is correlated with tumour invasion, metastasis ,recurrence and poor prognosis[4-9]. The universality of S100A4 expression in a variety of cancers illustrates the potential use of S100A4 as a marker for tumor metastasis and disease progression.

Studies in rodents have provided evidence supporting the direct involvement of S100A4 in tumor progression and metastasis. The role of S100A4 in cancer has been examined most widely in breast cancer models, which have demonstrated that overexpression of S100A4 in non-metastatic mammary tumor cells confers a metastatic phenotype[10-11]. The link between S100A4 and metastasis is further supported by knockdown experiments, as inhibition of

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S100A4 expression by antisense techniques suppresses the metastatic capacity of S100A4-expressing tumor cells in animal models of pancreatic cancer, renal cancer, thyroid cancer, gastric cancer, prostate cancer and osteosarcoma[12-17].

In this study, we first sought to examine the relationship between S100A4 mRNA expression and clinicopathological factors in pediatric WTs. Furthermore, *S100A4* RNA interference was performed in a primary culture model *in vivo* and *in vitro*.

Materials and Methods

Cell and Culture Conditions

SK-NEP-1 Human kidney (Wilms Tumor) cell line obtained from the American Type Culture Collection (ATCC, Shanghai, China) was maintained in the Maccyo'5 (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen Co., NY, USA) in a humidified incubator with 5% CO_2 at 37°C.

Patient Samples

Surgical resection specimens were obtained from 48 patients undergoing surgery for WT at Children's Hospital of Zhengzhou City from Feb 12, 2008 to Jan 20, 2012. The patients had not received chemotherapy or radiation treatment before surgery. After resection of the tumor, the material the nephroblastoma (44 cases) and their normal kidney counterparts (24 cases) was immediately snap-frozen in liquid nitrogen and stored at −80°C. Medical Ethical Committee (Children's Hospital of Zhengzhou City) approved the protocol, and the written informed consent was obtained from every patient's mother and father before surgery. The clinical features of these patients were included in Table I.

Quantitative PCR

Reverse transcriptase reaction was performed in a final volume of 20 µl using 2 µg total RNA and a final concentration of 5 mM $MgCl_2$, 1 X reverse transcription buffer, 1 mM each dNTP, 20 U recombinant RNase inhibitor RNasin, 15 U AMV reverse trancriptase as well as 0.5 µg oligo (dT) primer (Shanghai Shenggong, China). The samples were incubated at 42°C for 1 hour with a final denaturation at 94°C for 15 min.

The amplification of the S100A4 was performed on the LightCycler System (Roche Diagnostics, Mannheim, Germany) using the Platinum SYBR Green qPCR Super Mix-UDG kit (Invitrogen, Karlsruhe, Germany). Therefore, 4 µl of synthesized cDNA was amplified in a total volume of 20 µl containing 10 µl platinum SYBR Green qPCR Super Mix-UDG which included the Taq DNA polymerase, SYBR Green I fluorescent dye, reaction buffer, dNTPs and uracil DNA glycosylase (UDG), 1 µl bovine serum albumin (BSA) and 10 pmol of S100A4 specific primer and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific primers. PCR amplification was then carried out under the following conditions: 95°C for 15 min, followed by 35 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 1 min. The final extension was completed at 72°C for 10 min. In every PCR the hot start was performed to prevent the formation of unspecific products and primer dimers. In addition to the melting curve analysis, specificity of the PCR was controlled by agarose gel electrophoresis and products were sequenced with gene specific oligonucleotides. The amount of S100A4 transcripts was calculated semi-quantitatively in relation to the amount of the amplified housekeeping gene GAPDH. The number of gene copies was calculated by the LightCycler Software Version 3.5.3 according to the second derivative maximum method. The products of Q-PCR were verified on agarose gels.

shRNA Experiments

S100A4 shRNAs were commercially purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Lipofectamine™ (Invitrogen) was used for transfection following the manufacturer's instructions. Before transfection, SK-NEP-1cells cultured at 50% confluence in 6-well plates (10 cm²) were washed two times with OPTIMEM 1 (Invitrogen) without fetal calf serum (FCS) and incubated in 1.5 ml of this medium without FCS for 1 h. Then, cells were transfected with S100A4-RNA duplex formulated into Mirus *Trans*IT-TKO transfection reagent (Mirus Corp., Interchim, France) according to the manufacturer's instructions. Unless otherwise described, transfection used 20 nM RNA duplex in 0.5 ml of transfection medium OPTIMEM 1 without FCS per 5×10^5 cells for 6 h and then the medium volume was adjusted to 1.5 ml per well with Roswell Park Memorial Institute (RPMI) 2% FCS. Silencer™ negative control 1 shRNA was used as negative control under similar conditions (20 nM).Cells were incubated under these conditions

for 48 h and silencing was then confirmed by RT-PCR assays as well as Western blotting. SK-NEP-1/S100A4 shRNA cells were selected with 600 μg/mL G418 for 14 d to acquire the stably transfected SK-NEP-1/S100A4 shRNA cells.

RT-PCR

The total RNA was isolated from cultured cells or tumors *in vivo* using ISOGEN (Nippon gene) according to the manufacturer's method, and a solution containing 5 μg of total RNA was taken for the reverse transcription reaction that was performed with SuperScript 2 Reverse Transcriptase (Invitrogen) and oligo-dT primer. The sequences of the oligonucleotide primers were used as follows: S100A4,5-CAGATCCTGACT-GCTGCCATGGCG-3′,5'-ACGTGTCTGAA GGAGCCATGGTGG-3; the housekeeping gene GAPDH, 5'-CCACCCATGGCAAATTCCATCG CA-3′ and reverse primer, 5'-TCTA GACG-GCAGGTGAGGTCGGCACC-3′. PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide.

Western Blotting

Cells were lysed in TENSV buffer [10 mmol/L HEPES (pH 7.2), 142.5 mmol/L KCl, 5 mmol/L MgCl$_2$, 1 mmol/L EGTA, 0.2% Nonidet P-40, 0.1% aprotinin, and 2 mmol/L phenylmethylsul-

fonyl fluoride] and sonicated at 4°C. The lysates were centrifuged at 15,000 rpm for 15 min, and their protein concentration was adjusted by staining with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA). The 4 × sampling buffer [250 mmol/L Tris (pH 6.8), 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.04% bromophenol blue] was added to each lysate before boiling them for 5 min. The samples were separated by SDS-polyacrylamide electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Minneapolis, MN, USA) and blotted with anti-S100A4 and anti-b-actin (Santa Cruz) antibodies. For detection, enhanced chemiluminescence reagent (Pierce, Waltham, MA, USA) was used with horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad Laboratories).

Cell Viability Assay Using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide (MTT)

Cell viability was measured using MTT (Sigma, St Louis, MO, USA). Briefly, SK-NEP-1cells were cultured in triplicate in 96-well plates at a concentration of 5×10^3 and transfected with 20 ng of the S100A4 shRNA per standard protocol. Untreated cells were maintained under similar conditions to serve as the control (mock). 48 h after transfection, the cells were maintained for different time intervals in serum-supplemented media for 3 days. At 3 days point, 10 μL of freshly prepared MTT reagent (5 mg/mL sterile phosphate buffered saline: PBS) were added to each well and the cells were further incubated for 4 h. The cells were treated with detergent (2 mL Isopropanol supplemented with 6.66 μL concentrated HCl) and mixed thoroughly for color development. A570 was measured using an ELISA plate reader. The OD values obtained were plotted against the respective time intervals. The reduction in cell proliferation following knockdown of S100A4 was quantified as the decrease in the intensity of MTT-formazan (blue color measured calorimetrically at 570 nm) at different time intervals in the SK-NEP-1 cells treated with plasmid vectors compared to the untreated cells (mock).

Apoptosis Assay Using TdT-Mediated Nick end Labelling (TUNEL)

Apoptosis was measured using a commercially available ApopTag Apoptosis Detection Kit (Serological Corporation, Norcross, GA, USA) as per the instructions of the manufacturer. In

Table I. Clinicopatholgic features of patients with WT.

Data	N	*p*-value
Gender		n.s
Female	18	
Male	30	
Age (year)		n.s
≤ 3	37	
> 3	11	
Tumor volume (cm)		n.s
≤ 10	39	
> 10	9	
Clinic stage		n.s
I-II	35	
III-IV	13	
Vascular invasion		n.s
Yes	8	
No	40	
Pathological type		n.s
FH	38	
UH	10	
L metastasis		n.s
Yes	11	
No	37	

brief, SK-NEP-1 cells or the SK-NEP-1 cells transfected with 20 ng of the S100A4 shRNA cells (48 hours after transfection) were grown on Thermanox cover slips (Nalge Nunc, Rochester, NY, USA) in 24-well plates. After 24 h, the cells were washed in PBS and fixed in 1% paraformaldehyde for 15 min at room temperature. After quenching the endogenous peroxidase with 3% hydrogen peroxide, the cells were labelled with the reaction buffer containing TdT-digoxigenin-nucleotide for 1 h at 37°C. The reaction was stopped by adding the stop solution. After washing the cells in PBS, the cells were incubated with anti-digoxigenin peroxidase conjugate at room temperature for 30 min. The cells were washed and incubated with peroxidase substrate and the colour development was monitored under a microscope. The cells were counterstained with light haematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in DePex mounting medium. The cells were viewed under a 40x objective and the cells that fell within the 100 squares of an eye graticule, were counted. The number of apoptotic cells was expressed as a percentage of the total cells counted from five random fields for each cover slip.

Apoptosis Measurement Using Flow Cytometry

Apoptosis was measured using a commercially available annexin-V flouos staining kit. In brief, SK-NEP-1 cells or the SK-NEP-1cells transfected with 20 ng of the S100A4 shRNA cells (48 hours after transfection) were grown in 24-well plates. After 24 h, non-adherent cells were pelleted and added to trypsinized and pelleted adherent cells. The cells were re-suspended in 100 ml of binding buffer containing fluorescein isothiocyanate (FITC) conjugated annexin-V and propidium iodide and incubated at room temperature for 15 min. After the incubation period, 300 ml of binding buffer was added and the cells were analysed in a FACS Calibur (Beckton Dickinson, San Jose, CA, USA). Ten thousand events were recorded from each treatment group.

Matrigel Invasion Assay

SK-NEP-1cells (1×10^6) were transfected with S100A4 shRNA. Cells were incubated for 48 h, trypsinized, counted and 1×10^5 cells were cultured in the upper chamber of a Transwell insert (8 μM pores) coated with matrigel (1 mg/mL) (Collaborative Research Inc., Waltham, MA, USA). The cells were supplemented with 500 μL serum-free media. The lower chamber was filled with 700 μL Dulbecco's Modified Eagle Medium (DMEM) media supplemented with serum that served as a chemoattractant for the migrating cells. The plates were maintained in an incubator at 37°C. Untreated cells were also maintained under similar conditions to serve as the control (mock). 48 h after incubation, the chambers were removed from incubator, non-migrated cells in the upper chamber were scraped carefully, and migrated cells adhering to the lower surface of the Transwell insert were stained with Hema-3. Photographs of the cells were taken at 20 × magnification with a light microscope. The cells were counted and quantification of invasion.

In vitro Angiogenesis Assay

SK-NEP-1 cells (4×10^4) were cultured in 8-well culture slides. After 24 h, the cells were transfected with S100A4 shRNA and allowed to grow for another 24 h. Untreated cells were maintained to serve as the control (mock). 24 h after transfection, serum-containing media was replaced with serum-free DMEM media. After 48 h, conditioned media was collected from transfected cells and untreated cells. Simultaneously, 3×10^4 HMEC (Human Mammary Epitelial Cells) cells were grown for 24 h in 8-well chamber slides. After 24 h, serum-containing media was removed from HMEC cultures and replaced with conditioned media collected from untreated cells and cells transfected with the shRNA. The cells were further grown in conditioned media for 72 h followed by H&E staining. Pictures were taken using a light microscope and observed for capillary-like network formation. The percentage of network formation was quantified as the product of the number of branch points formed by endothelial cells multiplied by the branches from each point and plotted against respective treatments.

Xenotransplantation of SCID Mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Zhengzhou University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Female nu/nu mice, aged 4-6 weeks, obtained from the Shanghai Animal Laboratory Center, were kept in Class 10000 Clean

Room at the Laboratory Animal Center of Zhengzhou University. SK-NEP-1 and the stably transfected SK-NEP-1/S100A4 shRNA cells or control shRNA cells (1×10^9) were subcutaneously injected into 3 nude mice every group. During the next 3 weeks these mice were examined for subcutaneous tumor growth. The tumor volumes were calculated according to the following formula: volume = length × width^2/$_2$.

Immunohistochemistry

Immunohistochemical studies were done on 4 μm-thick sections derived from zinc-fixed, paraffin wax-embedded tumor tissue blocks. These tumors were harvested at the end of the experiments (after 21 days). Sections were subsequently dewaxed, rehydrated, and had endogenous peroxidase activity quenched before S100A4 specific immunohistochemical staining. After specific staining or H&E staining, sections were dehydrated in alcohol and xylene and subsequently mounted.

Microvessel Density

The tumor vasculature was stained with an antibody against CD31, and microvessel density (MVD) was determined by counting CD31-stained vessels of tumor slides by examining "hotspots" according to the manufacture's instruction. Vessel density per 200 field was quan-

tified from 6 to 8 fields per tumor section from the treatment and control groups and expressed as percentage per area (200× field). Percentage per area was assessed using ProImage software.

Statistical Analysis

All values are presented as means ± SEM. Comparisons between groups were analysed using analysis of variance (ANOVA) or Student's t-test, p values less than 0.05 were considered significant. Statistical analyses were performed using SPSS Software for Windows (version 11.0; SPSS, Inc., Chicago, IL, USA). At least three replicates for each experimental condition were performed, and the presented results were representative of these replicates.

Results

S100A4 Show Increased Expression in WT Tissues

Levels of transcripts of S100A4 was increased in tumor samples in comparison to background (expressed as transcript copy number per 50 ng of messenger RNA and standardized with GAPDH; Figure. 1A). Transcript copy numbers for S100A4 were 0.46 ± 0.13 for tumor and 0.12 ± 0.07 for background ($p = 0.025$). The products of Q-PCR were verified on agarose gels (Figure 1B). It is in-
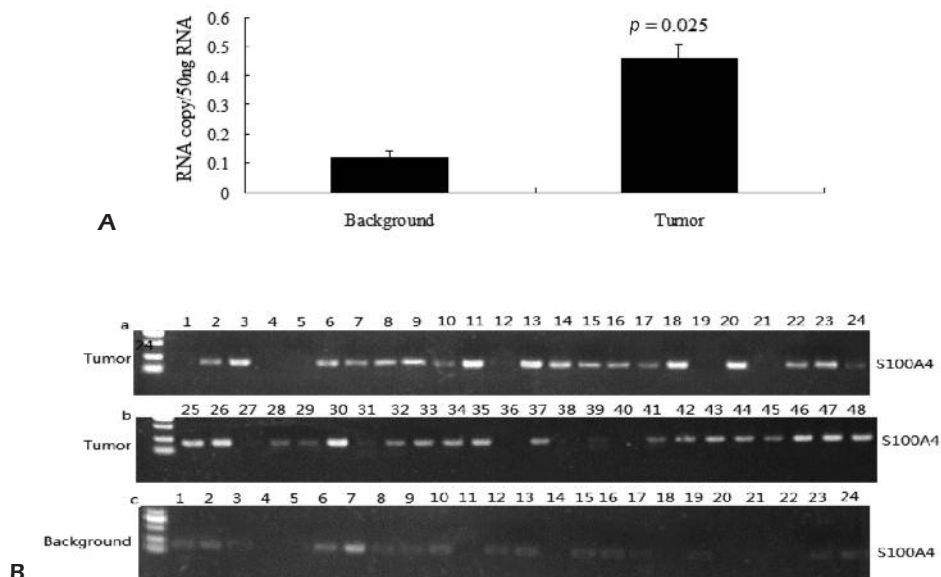
Figure 1. A, Levels of transcripts of S100A4 in tumor samples in comparison to background (expressed as transcript copy number per 50 ug of messenger RNA and standardized with GAPDH). **B,** Represent products of Q-PCR were verified on agarose gels.
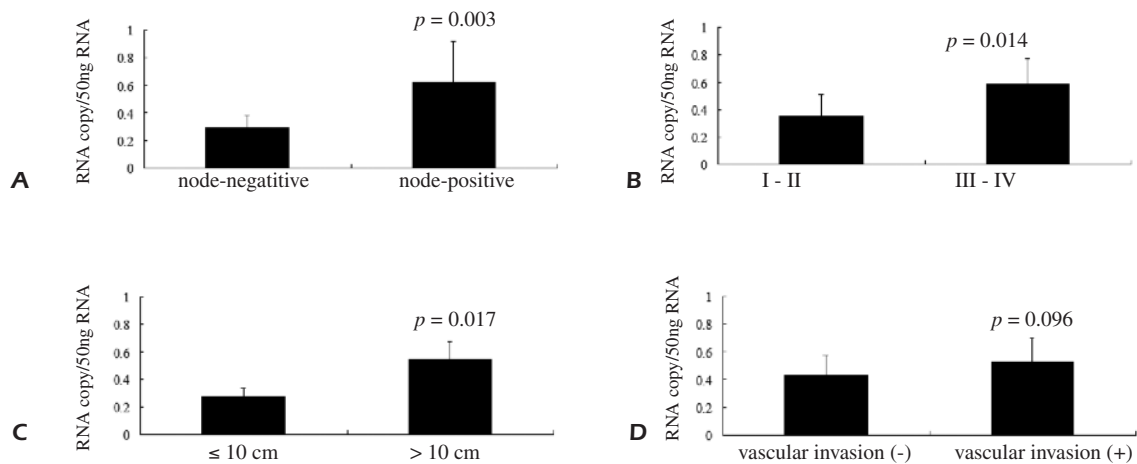
Figure 2. Levels of transcripts of S100A4 in tumor samples in comparison to the clinicopathological factors in pediatric WTs. **A,** Levels of transcripts of S100A4 expression in node-positive tumors compared with node-negative tumors. **B,** Levels of transcripts of S100A4 expression in III-IV stage compared with I-II stage. **C,** Levels of transcripts of S100A4 expression in ≤ 10 cm tumors compared with > 10 cm tumors. **D,** Levels of transcripts of S100A4 expression in patients with vascular invasion compared with patients with without vascular invasion.

teresting to note that S100A4 had increased expression in node-positive tumors compared with node-negative tumors (node positive, 0.62 ± 0.24; node negative, 0.29 ± 0.12; $p = 0.003$) (Figure 2A); Furthermore, S100A4 had increased expression in III-IV compared with I-II tumors (III-IV, 0.58 ± 0.24; I-II, 0.35 ± 0.18; $p = 0.014$) (Figure 2B); S100A4 had increased expression in large tumors compared with little tumors (≤ 10 cm, 0.28 ± 0.14; >10 cm, 0.54 ± 0.23; $p = 0.017$) (Figure 2C). S100A4 also had increased expression in positive vascular invasion compared with negative vascular invasion (positive vascular invasion, 0.53 ± 0.23; negative vascular invasion, 0.43 ± 0.16; $p = 0.096$), although these values were not significantly different (Figure 2D).

shRNA Interference Inhibits S100A4 Expression in SK-NEP-1 Cells

Semi-quantitative RT-PCR analysis of DNA from the S100A4 shRNA transfected SK-NEP-1 cells revealed a significant decrease in mRNA levels of S100A4 as compared to controls and mock-transfected cells (Figure 3A). The S100A4 protein levels were significantly decreased with S100A4 shRNA transfected SK-NEP-1 cells compared to controls and mock-transfected cells (Figure 3B). GAPDH levels and β-actin levels were analyzed at the mRNA levels and protein levels to serve as a loading control and no differences were observed. The mock shRNA also did not have any effect on the mRNA and protein levels.
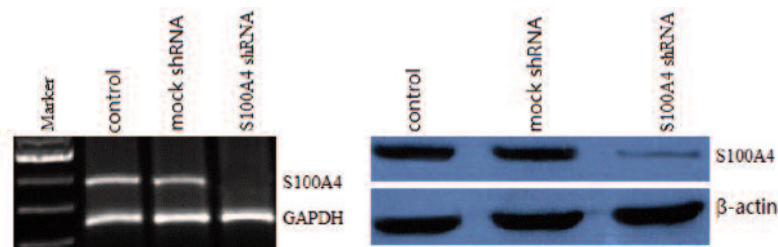

Figure 3. shRNA-mediated knockdown of S100A4 expression in SK-NEP-1 cells. **A,** Semi-quantitative RT-PCR of RNA extracted from SK-NEP-1 parental cells (mock) and cells transfected withS100A4 shRNA. RT-PCR reaction was also performed for GAPDH and served as a loading control. **B,** Following transfection with cell lysates from above cells were probed for S100A4 using a specific antibody for S100A4 by western blotting analysis. β-actin was analyzed as a loading control.

shRNA-Mediated Abrogation of S100A4 Gene Expression Reduce SK-NEP-1 Cell Proliferation

We analyzed the proliferation efficiency of SK-NEP-1 cells upon downregulation of S100A4. shRNA-mediated knockdown of S100A4 decreased proliferation efficiency of cells when compared to parental and mock-transfected cells (Figure 4A).

shRNA-Mediated Abrogation of S100A4 Gene Expression Reduces SK-NEP-1 Cell Apoptosis

Apoptosis measurement by flow cytometry showed that shRNA-mediated knockdown of S100A4 increases apoptosis of SK-NEP-1 cells when compared to parental and mock-transfected cells (Figure 4B). Apoptosis measurement via TUNEL assay has the same results as above (Figure 4C).
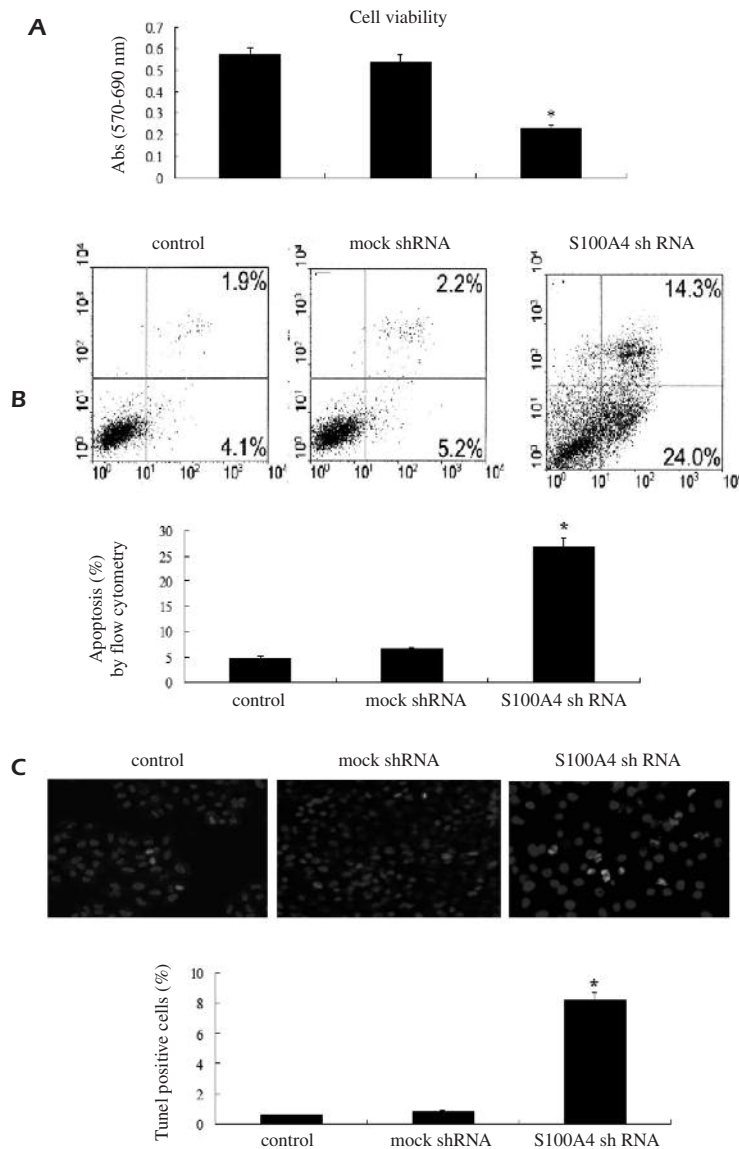
Figure 4. The effect of shRNA-mediated reduction of S100A4 gene expression on proliferation and apoptosis in SK-NEP-1 cells. *A,* SK-NEP-1 cells proliferation decreases following shRNA-mediated reduction of S100A4 gene expression. SK-NEP-1 cells were maintained in triplicate in 96-well plates at a concentration of 5×103 and transfected with S100A4 siRNA as described in Materials and Methods. 72 h after transfection, viable cell mass was measured in both parental and treated cells. Mean ± SD values from 3 different experiments are shown. *B,* SK-NEP-1 cells apoptosis increases following shRNA-mediated reduction of S100A4 gene expression by Annexin V analysis, much more cells showed apoptotic feature compared with control group. These analyses were repeated three times. *C,* SK-NEP-1 cells apoptosis increases following shRNA-mediated reduction of S100A4 gene expression by TUNEL analysis. These analyses were repeated three times. *$p < 0.01$ vs respective untreated groups.
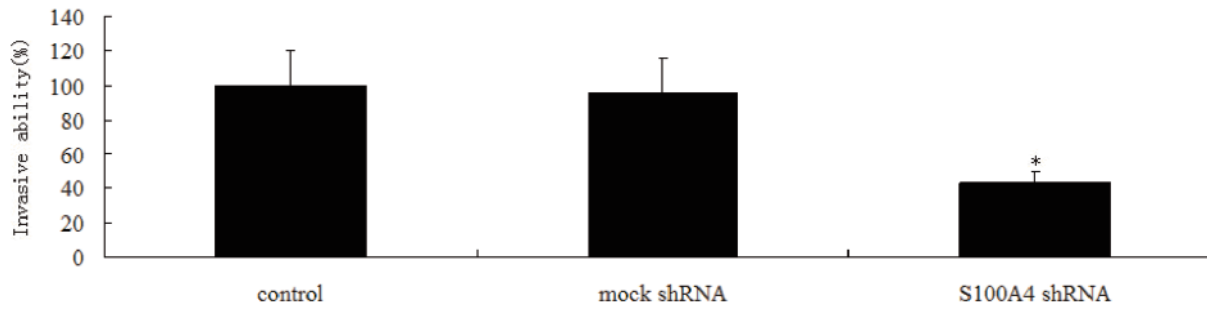
Figure 5. The effect of shRNA-mediated reduction of S100A4 gene expression on invasive capability of SK-NEP-1 cells. Representative histogram showing invasive capability of SK-NEP-1 cells transfected with S100A4 shRNA in a chemoinvasion chamber. (Magnification: ×100). Each bar represents mean ± SE; vs respective untreated groups. *$p < 0.05$. All experiments were repeated three times with similar results.

Knockdown of S100A4 Results in Regression of Tumor Cell Invasion

The S100A4 gene is reported to confer invasive characteristics to various cancer cells; however, its role in the invasion and metastasis of WT is yet unknown. Next, we analyzed the effect of S100A4 gene suppression on the invasive capability of WT cells SK-NEP-1 by employing an *in vitro* matrigel invasion assay. As shown in Figure 5, there was much higher cell migration from SK-NEP-1 cells or mock shRNA transfected SK-NEP-1 cells compared to S100A4 shRNA transfected SK-NEP-1 cells as quantitated by the number of cells migrating out from the well ($p < 0.05$) (Figure 5). These data suggest that the S100A4 gene controls the invasion of cancerous cells during the metastasis of human WT.

Targeting S100A4 Through shRNA Treatment Reduced Angiogenesis Initiated by SK-NEP-1 Cells

The growth of tumors depends on the induction of new capillary blood vessels, which is necessary to support the developing tumor mass. In this study, we used a co-culture system in which microvascular endothelial cells were induced by SK-NEP-1 cells to form capillary-like structures in order to examine the shRNA-mediated suppression of S100A4. Mock and control cells showed well-defined capillary network formation within 72 h. In contrast, SK-NEP-1 cells trans-
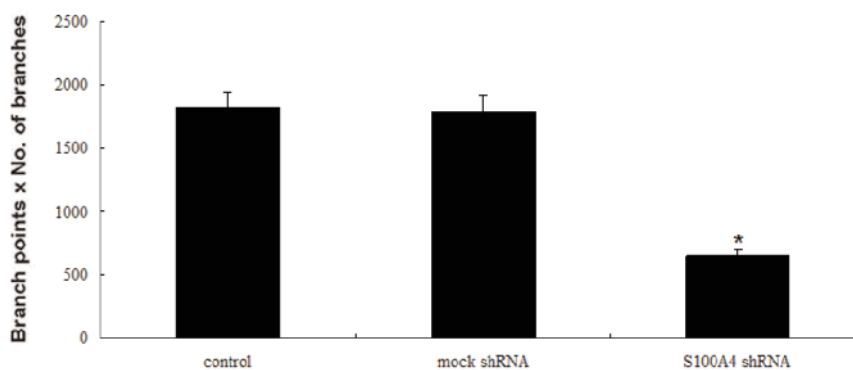
Figure 6. Inhibition of tumor cell-induced capillary network formation by shRNA-mediated reduction of S100A4 gene expression. 4×10^4 SK-NEP-1 cells were cultured in 8-well chamber slides and transfected with S100A4 shRNA and grown for 24 h. At the same time, untreated cells and mock shRNA were maintained for the control. After 24 h, the media was replaced with serum-free media and maintained for another 24 h. Simultaneously, HMEC cells (3×10^4) were maintained in 8-well chamber slides. 24 h following addition of serum-free media to transfected SK-NEP-1 cells, the conditioned media was collected and added to the HMEC cells. The HMEC cells were grown in conditioned media for another 72 h, stained with H&E and capillary network formation was analyzed with light microscopy. The ability of capillary network formation was analyzed as number of branch points and number of branches per branch point and plotted against the respective cells. Each bar represents mean ± SE; vs respective untreated groups. *$p < 0.05$. All experiments were repeated three times with similar results..
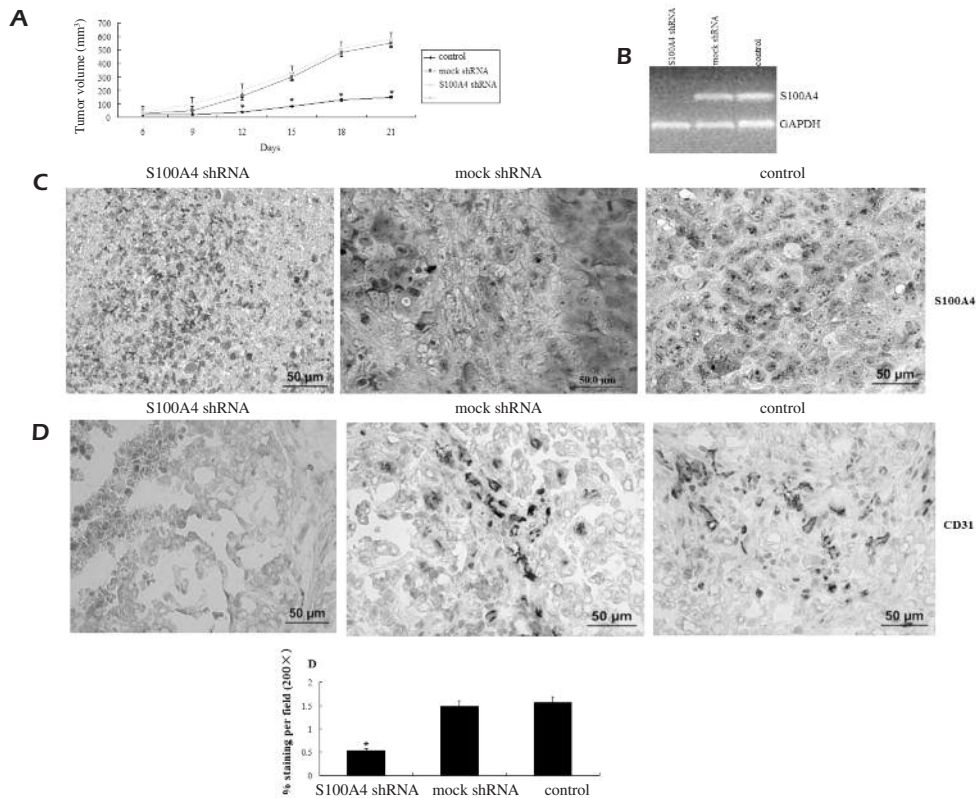
Figure 7. Effect of suppression of S100A4 on subcutaneous tumor growth and MVD. ***A,*** tumor growth curves. Mice received 1×10^8 viable cells (control, mock shRNA and S100A4 shRNA, respectively) by s.c. injection. The curves show tumor growth until day 21. Each bar represents mean ± SE; vs respective untreated groups. *$p < 0.05$. All experiments were repeated three times with similar results. ***B,*** S100A4 mRNA was detected in tumor *in vivo* by RT-PCR assay. ***C,*** S100A4 expression was verified by immunohistochemistry assay. ***D,*** Inhibition of tumor angiogenesis as measured by MVD, quantification of the brown staining revealed that the percentage area occupied by vasculature in S100A4 shRNA-treated tumors was 0.53 ± 0.21% versus 1.56 ± 0.26% and 1.48 ± 0.22% in controls (n = 8). *$p < 0.01$.

fected with S100A4 shRNA completely inhibited tumor cell-induced microvessel morphogenesis.The quantification of branch points and number of branches were undetectable in S100A4 shRNA transfected co-cultures compared to controls and mock (Figure 6).

Effect of Suppression of S100A4 on Subcutaneous Tumor Growth in vivo

We evaluated tumor growth *in vivo* by s.c. implantation of 10^8 tumor cells (control, mock shRNA and S100A4 shRNA, respectively) into nude mice. S100A4 shRNA tumors showed significantly slower growth rates than control cells (control and mock shRNA) at all time points (Figure 7A). There were no significant differences between control and mock shRNA. Suppression of S100A4 expression was verified by immunohistochemistry (Figure 7B) and RT-PCR (Figure 7C) on samples of each tumor.

Effect of Suppression of S100A4 is Associated With Decreased MVD

To determine whether S100A4 inhibition has an effect on SK-NEP-1 tumor vasculature, MVD was analyzed. New blood vessel formation was significantly reduced in S100A4 shRNA transfected tumor in the intervention model. The percentage area occupied by vasculature in S100A4 shRNA-treated tumors was 0.53 ± 0.21% versus 1.56 ± 0.26% and 1.48 ± 0.22% in controls (n = 8; $p < 0.01$; Figure 7D).

Discussion

This is the first report of quantitative analyses of S100A4 mRNA levels in WT tissues. S100A4 mRNA expression levels in tumoral tissues was significantly higher than those in nontumoral tissues. These results suggested that stepwise up-

regulation of S100A4 is related to carcinogenesis of WT .Most S100 proteins are released into the extracellular space. If S100A4 is also released and measurable in the serum, quantitative analysis with serum could also be a promising tool for screening of WT. However, thus far, there have been no reports on S100A4 expression in WT in serum. Though few reports in literature found serum S100A4 mRNA levels could be an important marker for survival predictor and prognosis in breast cancer[18] and clear cell renal cell carcinoma[19].

The biologic functions of several S100 proteins in carcinogenesis have not been fully elucidated to date. However, much interest has focused on S100A4 and some other S100 family members, such as S100A2, S100A6, and S100B, for their potential roles in invasive growth and metastasis of neoplastic diseases. S100A4 or its corresponding mRNA are found at higher levels in metastatic relative to non-metastatic rat[20] and mouse[21] tumor cell lines. Transfection experiments further showed that rodent or human S100A4 can induce a metastatic phenotype in previously non-metastatic rat mammary cells[10]. Conversely, antisense S100A4 RNA or anti-S100A4 ribozyme suppressed the metastatic potential of highly metastatic cell lines[22-23]. The association between S100A4 expression and metastasis observed in animal studies has led to a number of studies examining the utility of S100A4 expression as a prognostic marker in human cancers[4-9].

In the present study, we found S100A4 mRNA were significantly higher in patients with lymph node metastasis and higher tumor stage than in those without lymph node involvement and lower tumor stage. Furthermore, S100A4 mRNA were significantly higher in large tumor size and positive vascular invasion. Our findings suggest that S100A4 mRNA may be as a new and promising prognostic biomarker for WT progression and prognosis.

Although analysis of S100A4 expression has been reported, the role of S100A4 is not well understood. In the present study, we established transiently transfected S100A4 shRNA clones and stably transfected S100A4 shRNA clones, which S100A4 expression was suppressed by shRNA, and investigated the effects of reducing or eliminating S100A4 on the malignant and metastatic potential of the WT cells.

Here we found downregulation of S100A4 reduced *in vitro* proliferation of SK-NEP-1 cells as assessed with the MTT assay. Moreover, cell proliferation decreased significantly in SK-NEP-1 cells treated with S100A4 shRNA as compared to cells treated with Mock shRNA. Furthermore, this reduced proliferation was accompanied by an increase in apoptosis via TUNEL and FCM assay.

Tumor progression occurs through invasion of tumor cells into surrounding normal tissue. Migratory ability of tumor cells is also responsible for metastasis, a key process that aids in tumor propagation. Our study revealed that shRNA-mediated downregulation of S100A4 resulted in a decrease in the migration of SK-NEP-1 cells by Matrigel invasion assay.

Angiostatin is an angiogenesis inhibitor generated by the membranes of platelets. Inhibition of generation of angiostatin and a subsequent favorable effect on angiogenesis was reported by Jurasz et al[24] in HUVEC cells. Our report revealed a remarkable reduction in the invasion and angiogenesis of SK-NEP-1cells following shRNA-mediated targeting of S100A4, compared to controls or mock shRNA-treated groups.

The result from subcutaneous tumor experiment showed remarkable inhibition of tumor growth after injection with S100A4 shRNA-transfected SK-NEP-1 cells compared with tumors established by control or muck shRNA-transfected SK-NEP-1 cells in nude mice. Immunohistochemical staining revealed that S100A4 shRNA transfection reduced MVD. The results indicate that S100A4 shRNA transfection might inhibit tumor by blocking angiogenesis.

Conclusions

The results of our expression analyses suggest that S100A4 mRNA is up-regulated in the WT, and that quantification of S100A6 mRNA may be useful for diagnosis and detection of WT. Our data also indicate that S100A4 is associated with both progression and invasion of WT. The results of this study indicate that inhibition of S100A4 is a very potent inhibitor of cell proliferation, tumor growth, invasion and angiogenesis in a WT model *in vitro* and *vivo*. Our findings suggest that S100A4 may be a promising prognostic marker and therapeutic target for WT.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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