WT1 Promotes Invasion of NSCLC via Suppression of *CDH1*

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Introduction: The Wilms' tumor gene (*WT1*) has been identified as an oncogene in many malignant diseases, and aberrant *WT1* expression has been linked to development, progression, and prognosis of non–small-cell lung cancer (NSCLC). We sought to investigate the underlying mechanism of *WT1* and metastasis in NSCLC.

Methods: Real-time polymerase chain reaction was applied to detect *WT1* and *CDH1* mRNA in 159 NSCLC samples and corresponding adjacent tissues. Stable clones with overexpression and knockdown of *WT1* were generated with plasmid and shRNA via lentivirus technology in H1568 and H1650 NSCLC cell lines. Wound-healing assay, transwell assays, and polymerase chain reaction array were carried out for invasion evaluation. Dual luciferase reporter assay was performed to validate the effect of *WT1* on *CDH1*.

Results: The level of the *WT1* mRNA was negatively correlated with that of E-cadherin (*CDH1*) and associated with pathological stage, metastasis, and survival rate of 159 NSCLC patients. A series of genes were regulated by *WT1*, and *WT1* could suppress *CDH1* transcription via direct binding to its promoter and may enhance the invasive ability of H1568 and H1650 NSCLC cell lines.

Conclusions: *WT1* expression was correlated with clinical stage, metastasis, and survival rate in 159 NSCLC patients. Via direct binding to the promoter, *WT1* could suppress *CDH1* and promote NSCLC invasion.

Key Words: *WT1*, *CDH1*, Non–small-cell lung cancer, Invasion, Metastasis.

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Lung cancer (including non–small-cell lung cancer [NSCLC] and small-cell lung cancer) remains the leading cause of cancer-related deaths in the world.[1] Although great efforts have been made toward improving early diagnosis and effective treatment, including findings of new biomarkers,[2–4] establishment of modified operation, and development of specific drugs,[5–9] the prognosis is still poor.

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The most common features of malignancy are invasion and metastasis, which are characterized by the ability of cancer cells to invade into adjacent area, intravasate into blood or lymphatic vessels, and extravasate into a distant environment. Metastasis, which is primarily responsible for the low 5-year survival rates (approximately 10%–15%),[10,11] is especially required for aggressive NSCLC, and once cancer cells lose contact with the neighboring cells, they become motile, invade the surrounding area, migrate, enter the circulation, extravasate into the target organ, proliferate, and metastasis. Therefore, the investigation of this area is important for patients with advanced NSCLC, but far from satisfaction to date.

The Wilms' tumor gene 1 (*WT1*) encodes a 49 to 52 kDa protein with an N-terminal domain involved in RNA/protein interactions[12] and is an important regulator of cell growth and development in the embryo kidney, adult urogenital system, and central nervous system.[13] The C-terminal domain harbors four zinc fingers, which permit binding to target DNA sequences for its transcriptional regulatory function.[14] Through two alternative splicing motifs of exon 5 (17 amino acid) and exon 9 (3 amino acid, KTS region), four main protein isoforms of WT1 are designated as A (-/-), B (+/-), C (-/+), and D (+/+).[15] A large number of genes coding for growth factors (e.g., *TGF-β*, *CSF-1*), growth factor receptors (e.g., *insulin R*, *IGF-IR*, *EGFR*), transcription factors (e.g., *EGR*, *WT1*, *cMyc*, *Pax2*, *Dax-1*, and *Sry*), and other proteins (e.g., ODC, MDR1, Hsp70, p21, Bcl-2) have been identified as *WT1* target genes.[16] Although first recognized as a tumor suppressor in Wlims' tumor (nephroblastoma),[17] based on accumulating evidence, *WT1* has been demonstrated to act as an oncogene in other sorts of malignancies including leukaemia,[18–20] breast cancer,[21,22] and lung cancer[23,24] et al., which suggested it acts in a dichotomous manner. The reason why *WT1*, the chameleon gene,[25] plays the very opposite role still remains unclear.

Studies have identified Sox9, Snail, and *CDH1* as being deregulated by *WT1* in *WT1* conditional knockout mice, although available evidence for *WT1* in direct transcriptional regulation is only available for *Snail* and *CDH1,* which was only reported in cardiovascular progenitor cells.[26] The present study was designed to investigate the relationship between *WT1* and its downstream molecule E-cadherin in NSCLC. We demonstrated that the expression of *WT1* and E-cadherin (*CDH1*) were correlated and also associated with metastasis in NSCLC samples. We further showed that in vitro, *WT1-D* enhanced the invasiveness of H1568 and H1650 NSCLC cells via negative modulation of E-cadherin by directly bind to its promoter.

PATIENTS AND METHODS

Patients and Tissue Samples

The study was performed on a total of randomly selected 159 NSCLC patients, of whom 102 had adenocarcinomas and the remaining 57 had squamous cell carcinomas. The patients were treated at People's Hospital of Jiangsu Province between September 2007 and August 2009, and all the specimens were collected under a protocol approved by the Human Ethics Committee of Nanjing Medical University. Each patient participated after providing informed consent. The median age of the patients was 64 years (range 49–85 years) and most of them were men (73%).

A total of 318 tissue specimens were included in the study with one tumor sample and one corresponding adjacent sample for each patient. All specimens were histologically classified by a professional pathologist according to the national NCCN guidelines for NSCLC version 3.2011[27] using blind method. Survival rate was calculated using 36 months as cutoff point.

DNA and RNA Preparation

Total RNA was extracted from tissue specimens using the TRIzol method (Invitrogen, Shanghai, China) and cDNA was synthesized using reverse transcriptase kit (TAKARA, Tokyo, Japan) according to the manufacturers' protocol.

Real-Time Polymerase Chain Reaction for *WT1* and *CDH1* RNA Expression

WT1 and *E-cadherin* (*CDH1*) mRNA levels were measured by real-time polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq (TAKARA). *WT1* and *CDH1* transcription values were normalized against the expression of *β-actin*. Amplification conditions, primers, and probes sequences for the *WT1* and *β-actin* were from the work by Sitaram et al.[28] and for *CDH1* were the same as those in the work by Martínez-Estrada et al.[26]

Cell Culture

H1568 (CRL-5876), H1650 (CRL-5883), and 293T (CRL-11268) cell lines from ATCC (Manassas, VA) were used for the present study. H1568 and H1650 were cultured in RPMI 1640 medium whereas 293T was cultured in Dulbecco's Modified Eagle Medium (DMEM) high-glucose medium, both supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). All cells were maintained in a humidified 37°C incubator with 5% CO_2.

Lentivirus Production and Transduction

WT1 gene was synthesized (purchased from GenScript, Nanjing, China) with restrictive digestion site of MluI in both ends, subcloned into pLV-GFP plasmid (gifted by Prof. Beicheng Sun, University of Nanjing Medical University, China), and named pLV-GFP-*WT1*. To generate plasmid-expressing *WT1*-shRNA, double-stranded oligonucleotides were cloned into pLL3.7 vector (gifted by D. Yun Chen, University of Nanjing Medical University, China) and named pLL3.7-*WT1*-shRNA. The sequences of WT1-shRNA used are aac TCAGGGTTACAGCACGGTC ttcaagaga GACCGTGCTGTAACCCTGA ttttt c. The uppercase letters represent *WT1*-specific sequence, and lowercase letters represent hairpin sequences. Recombinant lentivirus was generated from 293T cells using calcium phosphate precipitation. H1568 and H1650 were transfected with lentivirus using polybrene (8 μg/ml).

Western Blotting Assay

Proteins were extracted from cultured cells, quantitated using a protein assay (bicinchoninic acid [BCA] method; Beyotime, Shanghai, China). Proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membrane, blocked in 4% dry milk at room temperature for 1 hour, and immunostained with primary antibodies at 4°C overnight using anti-*WT1* (1:1000, 6F-H2; Millipore, Billerica, MA), anti-E-cadherin (1:1000; Abcam, Cambridge, United Kingdom), and *β-actin* (1: 20,000; Dako, Glostrup, Denmark). The results were visualized via a chemiluminescent detection system (Pierce ECL Substrate Western blot detection system; Thermo, Rockford, IL) and exposed in Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA).

Cell-Proliferation Assay (Cell-Counting Kit-8)

Cells were seeded into 96-well plates (6.0 × 103 cells per well). Cell viability was assessed by cell-counting kit-8 assay (Beyotime Institute of Biotechnology, Shanghai, China). The absorbance of each well was read on a spectrophotometer (Thermo) at 450 nm (A450). Three independent experiments were performed in quintuplicate.

Wound-Healing Assay

Cells were seeded in six-well plates and cultured to confluence. Wounds of 2-mm width were created with a plastic scriber and the floating cells were washed away thrice with phosphate buffered saline (PBS). After incubation in a serum-free medium for 48 hours, cultures were observed and photos were taken under a microscope. A minimum of five randomly chosen areas was measured.

Transwell Invasion Assay

The invasive ability of the cells was investigated using Transwells (8-μm pore size; Corning Costar Corp, Bedford, MA) put into the 24-well plates. First, 50 μl Matrigel (50 μg/ml; BD Biosciences, San Jose, CA) was added onto each surface of the chamber, incubated for 2 hours for solidification, then the supernatant was washed away with warm PBS. H1568 and H1650 were suspended in RPMI 1640 containing 2% fetal bovine serum. A total of 100 μl of the cell suspension (5 × 104 cells) was added to the upper chamber coated with Matrigel, and 400 μl of RPMI 1640 containing 10% fetal bovine serum was added to the lower compartment. After incubation for 48 hours at 37°C in a 5% CO_2 humidified incubator, the Matrigel and cells on the upper surface of the filter were removed with cotton swabs and the cells that invaded into the lower surface were fixed with 2% paraformaldehyde, stained with crystal violet. Then the filters were removed from