Int J Clin Exp Med 2015;8(9):14962-14968
www.ijcem.com /ISSN:1940-5901/IJCEM0009687

Original Article

Role of WNT1-inducible-signaling pathway protein 1 in etoposide resistance in lung adenocarcinoma A549 cells

Yunhua Xu, Shun Lu

Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai 200030, China

Received April 28, 2015; Accepted June 23, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Object: The aim of this study was to explore the role of WNT1-inducible-signaling Pathway Protein 1 (WISP-1) in etoposide resistance in lung adenocarcinoma A549 cells. Methods: WISP-1 overexpression A549 lung adenocarcinoma cell was established. After exposure to ultraviolet (UV) and etoposide, cell viability and apoptosis were evaluated. Moreover, western-blot was employed to examine the expression of apoptotic pathway proteins. In addition, a nude mice tumor model was established to examine the effect of WISP-1 overexpression in vivo and TUNEL staining was used to assess cell apoptosis of tumor tissue. Results: WISP-1 overexpression significantly increased cell viability and decreased cell apoptosis after treatment with UV and etoposide. Decreased expression of Bad and Bax and increased expression of Bcl-2 was found after etoposide treatment in WISP-1 overexpressed cells. A significantly increasing of tumor volume in WISP-1 overexpressed group was found and TUNEL staining revealed that decreased cell apoptosis in WISP-1 overexpressed group. Conclusion: Our results demonstrated that WISP-1 may have a facilitating role in etoposide resistance through increasing cell viability and decreasing cell apoptosis.

Keywords: WNT1-inducible-signaling pathway protein 1, lung adenocarcinoma, A549, etoposide, apoptosis

Introduction

Lung cancer is the second most common cancer in both men and women, accounting for approximately 15% of all newly diagnosed cancers [1, 2]. The statistical data shows that the overall 5-year survival rate of lung cancer is only 15.8%, making it one of the most deadly and difficult to diagnose cancers in humans. Despite extensive effects have been paid into developing new therapy for lung cancer, it remains the most common cause of cancer deaths worldwide, which is more than breast, colon, and prostate cancer combined [3, 4].

As a downstream mediator of Wnt signaling, Wnt-inducible signaling protein-1 (WISP-1/CCN4) is upregulated in a number of chronic fibrotic disorders effecting the lung, liver and kidney [5-7]. Functionally, WISP-1 has shown its ability to induce proliferation and drive epithelial to mesenchymal transition in alveolar epithelial cells [8]. Despite the emerging evidence for a role for WISP-1 in lung, the biology of the protein remains poorly described.

As a semi-synthetic derivative of podophyllotoxin originating from podophyllum peltatum or podophyllum emodi [9, 10], E*toposi*de acts on topoisomerase II, an enzyme involved in DNA processing during its replication, transcription and recombination and the mode of its action is known to be related to the major chemotherapeutic effect of the agent, i.e. cell death induction [11, 12]. However, the role of WISP-1 in etoposide induced cell death has not yet explored.

Here, we conducted this study to explore the role of WISP-1 in etoposide resistance in A549 lung adenocarcinoma cells. Our results demonstrated that WISP-1 may have a facilitating role in etoposide resistance through increasing cell viability and decreasing cell apoptosis in vitro and in vivo.

Materials and methods

Cell culture and drug treatment

The human lung adenocarcinoma A549 cell lines were purchased from Institute of Shanghai

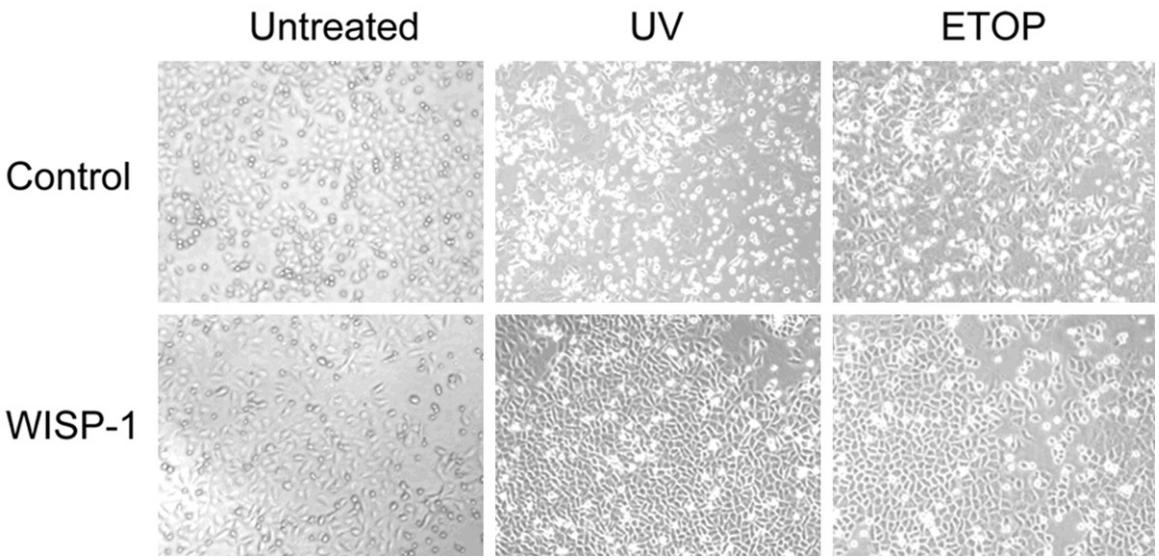

Figure 1. WISP-1 overexpression obviously improved cell viability. Cell morphology of WISP-1overexpressed lung adenocarcinoma A549 cells after treatment with ultraviolet (UV) radiation or etoposide. A549 cells were treated with UV light at 50 J/m^2 for 24 hrs or with etoposide at 50 μM for 24 hrs.

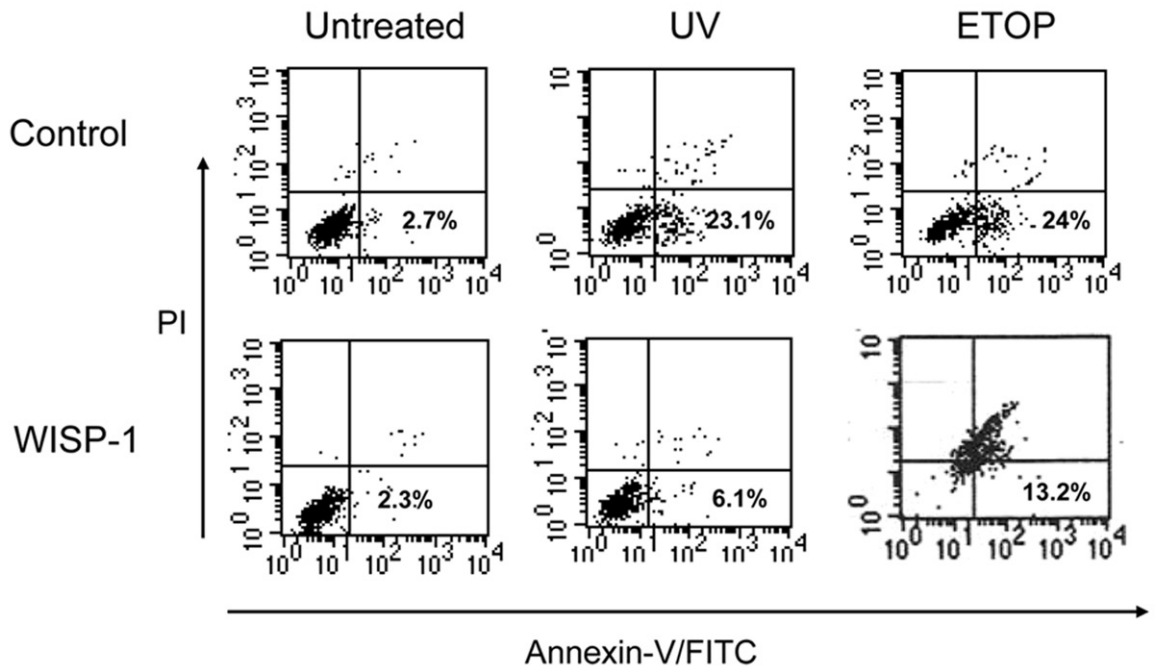

Figure 2. WISP-1 overexpression significantly decreased lung adenocarcinoma A549 cell apoptosis induced by ultraviolet radiation or etoposide.

Biochemistry and Cell biology, Chinese Academy of Science (Shanghai, China). The A549 cells were maintained at 37°C and 5% CO_2 incubator in DMEM media with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged when 90% confluence was reached and the culture media was replaced with DMEM media containing 1% fetal bovine serum before exposed to treatment.

Establishment of WISP overexpressed cell line

The overexpressing WISP-1 cDNA (Genebank No. NM_003882) clone was supplied from

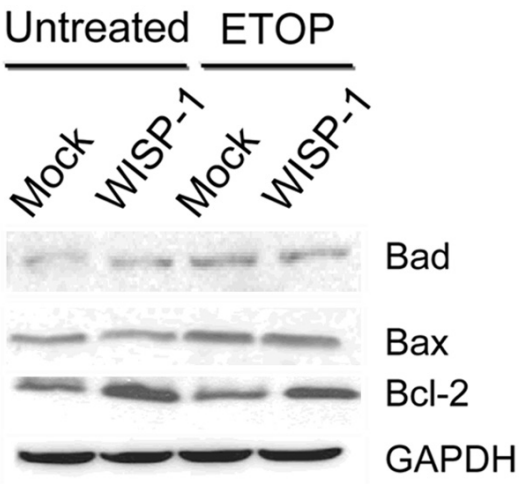

Figure 3. The expression of apoptosis pathway protein in WISP-1 overexpressed lung adenocarcinoma A549 cells. Etoposide can decrease the expression of Bad, Bax expression, and increase the expression of Bcl-2.

GeneCopoeia Inc. (Maryland, USA) and the schematic diagram of the overexpressing WISP-1 vector pReceiver-Lv105 was shown in supplemental data (Figure S1). WISP-1-expressing A549 cell line was established by lentiviral infections. Recombinant lentiviruses were generated by transfecting the lentiviral constructs into 293FT packaging cells with lipofectamine 2000 (Life Technology, CA, USA) according to the manufacturer's instructions. Forty-eight hours post-transfection, lentivirus-containing supernatant was collected. Target cells were infected at about 40% confluence by adding the lentivirus-containing medium supplemented with 8 μg/mL polybrene. Infection medium was replaced with DMEM and 10% FBS 124 h later. Cells were split into DMEM that contains 2.5 μg/mL puromycin at 48 h post-infection. Drug-resistant clones were picked and expanded. Total protein was extracted for Western analysis to assess exogenous WISP-1 expression (Figure S2).

Cell viability assay

After preparing the single cell suspension, 4 × 10^3 cells in 100 μL culture media were seeded in 96-well plate in quadruplicate overnight and exposed to UV light at 50 J/m^2 for 24 hrs or etoposide at 50 μM for 24 hrs. Cell morphology was observed under a light microscope. All the experiments were performed for three times.

Assay for cell apoptosis

Cells (2 × 10^6 cells/well) within a 6 well plate were exposed to UV light at 50 J/m^2 for 24 hrs or etoposide at 50 μM for 24 hrs. After that, Cell were stained with FITC-conjugated Annexin V and PI and analyzed by FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

In vivo model

Athymic nude (nu/nu) mice at 5 weeks old were purchased from SLAC Animal Inc (Shanghai, China). The nude mice were randomized to four treatment groups: (1) Control vector, (2) Overexpressing WISP-1, (3) Ovrexpressing WISP-1 + etoposide, (4) Control vector + etoposide; 2 × 10^6 WISP-1 overexpressing A549 cells and control A549 cells were injected subcutaneously into the right drawleg of the nude mice. When the tumors were 100 mm^3 in size, the mice were treated with etoposide administered intraperitoneally (I.P.). Mice were then assessed for tumor size every 7 days by measuring tumor length (L), width (W) and height (H), and tumor volume was calculated by using following equation: $V = (\frac{\pi}{6} \times L \times W \times H)$. At the day 28 post-treatment, all the mice were sacrificed and the tumors were excised, weighted and photographed. For histological analysis, tumors from the treated groups and the control group were fixed in 10% formalin and then conducted with paraffin embedded sections for TUNEL staining.

Ethical approval of the study protocol

All the mouse experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Chest Hospital, Shanghai Jiao Tong University and adhered to accept international guidelines generally for animal experimentation.

TUNEL staining

The tumor tissue sections were processed with TUNEL staining according to the manufacturer's instructions (Roche, Switzerland). The samples were analyzed under light microscope with a magnification of 400 ×. Five fields were randomly picked and the cells with red particles presented in the nucleus were recognized as positive.

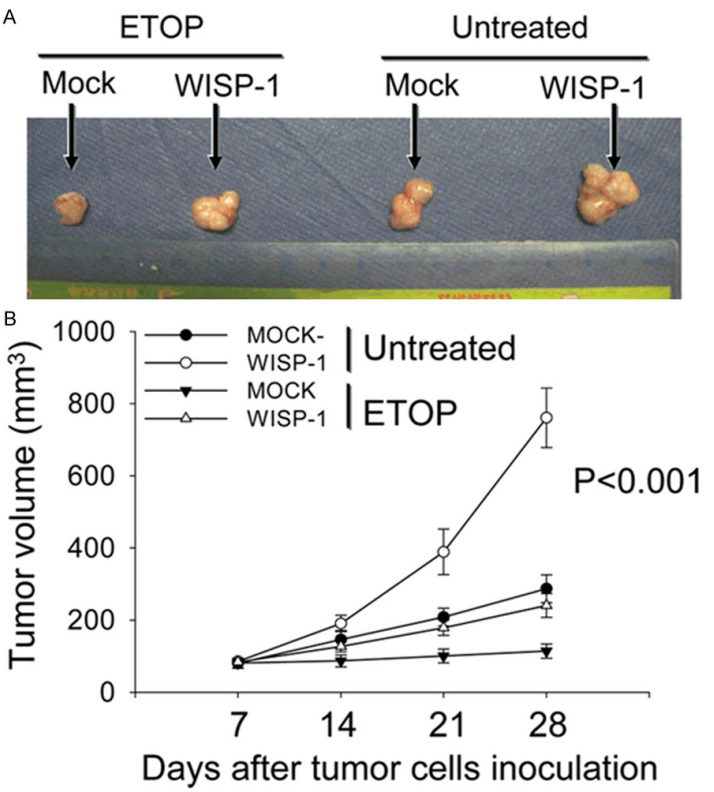

Figure 4. WISP-1 overexpression on tumor growth inhibition by etoposide in a nude mice model. A. Etoposide treatment could significantly suppress the A549 cell growth in a nude mice while WISP overexpression partially restored the cancer cell growth. B. Time course of tumor volume in different treatment group. A549 cells (2×10^6 cells) were transplanted subcutaneously and tumor volume was measured.

Western-blot analysis

The cell protein was prepared according to previous description. Cellular or cytosol protein was separated by electrophoresis on SDS-PAGE gel and then transferred onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking, the blots were incubated with the antibodies to Bad (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (Cell signaling Technology, USA), Bcl-2 (Cell signaling Technology, USA). GAPDH and β-actin (Bioworld, China) were used as loading control. The appropriate HPR conjugated secondary antibodies (Jackson ImmunoResearch, USA) were used at 1:5000. The protein bands detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce, Rockford, IL USA) on X-ray films (Koda, Lexington, MA, USA). Relative quantification of the protein was determined by Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

All the statistical analyses were performed by SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The data was presented as Mean ± SD. One way-ANOVA was used to examine the difference in three or more groups. $P < 0.05$ was recognized as significant difference.

Results

WISP-1 overexpression increased cell viability

We firstly examined the effect of WISP-1 overexpression on the cell death of A549 cells induced by UV and etoposide. The results showed that obviously decreased floating cells in WISP-1 overexpression group, suggesting WISP-1 overexpression could significantly increase the A549 cell viability after the treatment with UV and etoposide (**Figure 1**).

WISP-1 overexpression decreased cell apoptosis

Furthermore, we examined the effect of WISP-1 overexpression on UV or etoposide induced cell apoptosis. Compared to the control group, WISP-1 overexpression significantly decreased cell after UV (23.1% vs. 6.1) or etoposide treatment (24.0% vs. 13.2) (**Figure 2**).

Effect of WISP-1 overexpression on apoptotic pathway

After etoposide treatment, the A549 cells were lysed and the related apoptotic pathway was examined. Etoposide treatment could significantly increase Bad and Bax expression and decrease Bcl-2 expression in mock cells. In WISP-1 overexpressed cells, decreased expression of Bad and Bax and increased expression of Bcl-2 was found after etoposide treatment, indicating apoptosis inhibition effect of WISP-1 in etoposide induced cell apoptosis (**Figure 3**).

Effect of WISP-1 overexpression on tumor growth

We established a nude mouse model to examine the role of WISP-1 overexpression on the

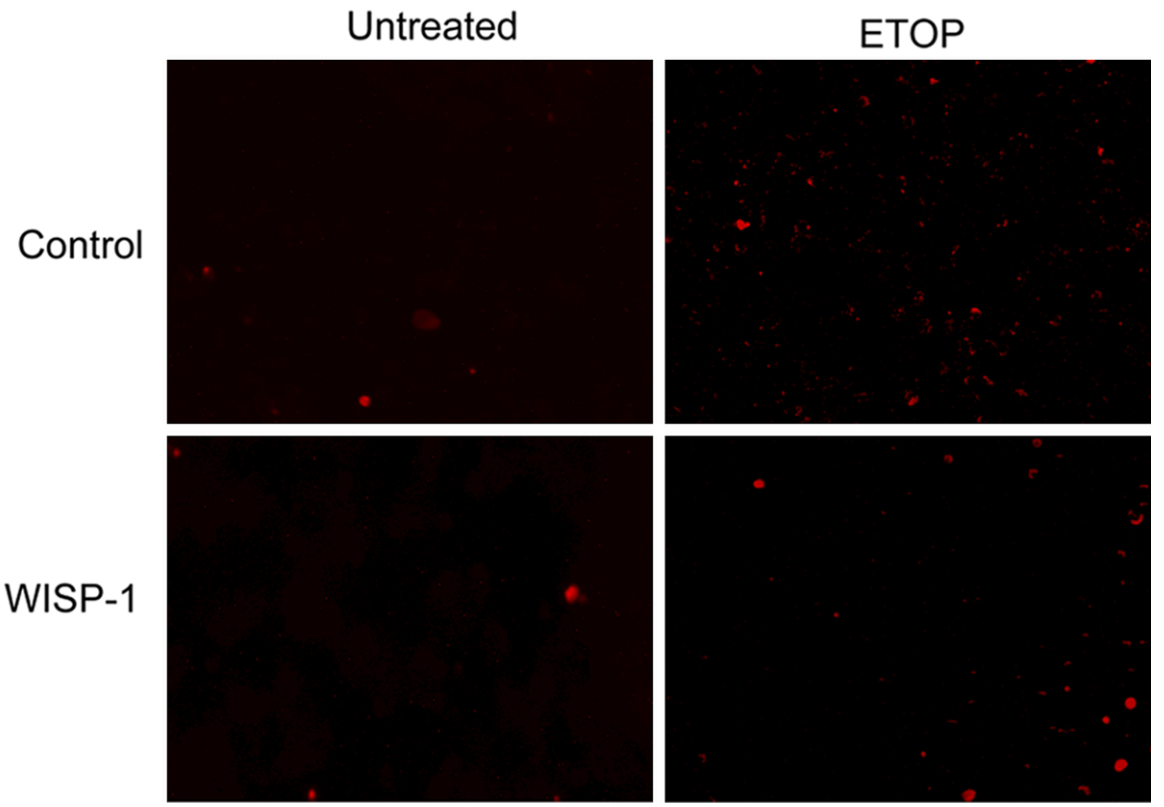

Figure 5. Cell apoptosis of tumor tissue derived from a lung adenocarcinoma A549 subcutaneous transplantation nude mice model. TUNEL staining was used to determine the cell apoptosis.

tumor growth. WISP-1 overexpression significantly suppressed the tumor growth inhibition by etoposide (**Figure 4A**). We also performed the tumor volume examination. A significantly increasing of tumor volume in WISP-1 overexpressed group was found at Day 21 and Day 28 while the tumor volume in etoposide treated WISP-1 overexpressed cells was comparable to mock cells without treatment (**Figure 4B**). In addition, we performed the TUNEL staining on the tumor tissue and the results showed that WISP-1 overexpression suppressed the cell apoptosis induced by etoposide (**Figure 5**).

Discussion

In present study, our results demonstrated WISP-1 overexpression significantly increased cell viability and decreased cell apoptosis after treatment with UV and etoposide. Decreased expression of Bad and Bax and increased expression of Bcl-2 was found after etoposide treatment in WISP-1 overexpressed cells. A significantly increasing of tumor volume in WISP-1 overexpressed group was found and TUNEL staining revealed that decreased cell apoptosis in WISP-1 overexpressed group. To the best of our knowledge, this is the first study to evaluate the role of WISP-1 in A549 lung cancer cells.

Konigshoff et al. previously showed that WISP-1 neutralization with antibodies exerted a survival benefit and improved lung function in the bleomycin model of pulmonary fibrosis [13]. Furthermore, Structural variants of WISP-1 generated by differential splicing or proteolysis have been suggested in a number of pathological settings [14, 15]. WISP-1 has been supposed to exert pleiotropic, cell-specific functions with potential distinct paracrine and autocrine functions which may be attributed to the use of differing cell-surface receptors in different cell types. In addition, whilst WISP-1 has been suggested to interact with the small leucine rich proteoglycans biglycan and decorin [16] through activating Akt signaling pathway [17]. Putative integrin recognition sites in the vWC, TSP1 and CT domains were found in WISP-1 proteins and two recent reports showed that an interaction between full length WISP-1 and

the $\alpha_V\beta_5$ integrin [18-20] although the domains responsible for these interactions were not identified. Here, our showed that WISP-1 overexpression could increase cell viability and decrease cell apoptosis after the treatment with etoposide or UV. Furthermore, through a nude mice model, we observed the similar phenomenon. A significantly increasing of tumor volume in WISP-1 overexpressed group was found at Day 21 and Day 28 while the tumor volume in etoposide treated WISP-1 overexpressed cells was comparable to mock cells without treatment.

Etoposide (VP-16), a semi-synthetic derivative of podophyllotoxin, acts as a topoisomerase II inhibitor by forming a ternary complex. As one of the most widely used cancer chemotherapy agents [21], etoposide has been used clinically both as a single agent and a constituent of combination chemotherapy regimens and has been applied to improve the treatment of various human cancers [22]. Several studies have demonstrated that etoposide induces apoptotic cell death. However, the exact molecular mechanism leading to apoptotic cell death by etoposide remains to be elucidated. As critical components of apoptotic pathway, Bcl-2 family members contain at least one of the four conserved α-helical motifs named Bcl-2 homology (BH) domains (BH1-BH4) [23]. Until now, three groups of Bcl-2 family members were found and they were Bcl-2, Bcl-xL and Mcl-1; the multidomain proapoptotic members, such as Bax and Bak; and the BH3 domain only proteins, such as Bim, Bid, Bad and Bik [24]. According to previous studies, mitochondrial membrane act as the principal site in which the action of apoptosis is regulated by Bcl-2 family proteins. The anti-apoptotic multidomain proteins (Bcl-2, Bcl-xL, Bcl-w and Mcl-1) are mainly located in the mitochondria and they can prevent the translocation and/or activation of Bax-like proteins in the mitochondria [25], inhibiting cytochrome c release from the mitochondria or mitochondrial membrane depolarization. Bcl-2 family members could also present on the endoplasmic reticulum (ER) and participate in regulating ER-mediated apoptosis [26]. The chemotherapeutic drugs resistance of tumor cells is a challenging problem in cancer treatment. Upre gulation of the anti-apoptotic molecules in tumors impairs remission and cure with chemotherapy, protecting the tumor cells from the apoptotic effects of various antineoplastic agents. Elevated expression of the anti-apoptotic protein Bcl-2 is found in numerous human tumors [27]. Anti-apoptotic Bcl-2 proteins inhibit apoptosis induced by various stimuli including chemotherapeutics [28]. The functional blockade of Bcl-2 or other anti-apoptotic proteins, such as Bcl-xL, could either induce apoptosis in cancer cells or sensitize these cells to chemotherapy [29]. Etoposide treatment could significantly increase Bad and Bax expression and decrease Bcl-2 expression in mock cells. In WISP-1 overexpressed cells, decreased expression of Bad and Bax and increased expression of Bcl-2 was found after etoposide treatment, indicating apoptosis inhibition effect of WISP-1 in etoposide induced cell apoptosis.

In conclusion, our results demonstrated that WISP-1 may have a facilitating role in etoposide resistance through increasing cell viability and decreasing cell apoptosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shun Lu, Shanghai Lung Cancer Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, No. 241 Huaihai West Road, Shanghai 200030, China. Tel: +86-021-22200000; Fax: +86-021-62804970; E-mail: lussh007@163.com

References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.

[2] Edwards BK, Ward E, Kohler BA, Eheman C, Zauber AG, Anderson RN, Jemal A, Schymura MJ, Lansdorp-Vogelaar I, Seeff LC, van Ballegooijen M, Goede SL, Ries LA. Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. Cancer 2010; 116: 544-573.

[3] Pacheco-Pinedo EC, Durham AC, Stewart KM, Goss AM, Lu MM, Demayo FJ, Morrisey EE. Wnt/beta-catenin signaling accelerates mouse lung tumorigenesis by imposing an embryonic distal progenitor phenotype on lung epithelium. J Clin Invest 2011; 121: 1935-1945.

[4] Dela Cruz CS, Tanoue LT, Matthay RA. Lung cancer: epidemiology, etiology and prevention. Clin Chest Med 2011; 32: 605-644.

[5] Jiang F, Parsons CJ, Stefanovic B. Gene expression profile of quiescent and activated rat he-

patic stellate cells implicates Wnt signaling pathway in activation. J Hepatol 2006; 45: 401-409.
[6] Wang D, Dai C, Li Y, Liu Y. Canonical Wnt/beta-catenin signaling mediates transforming growth factor-beta1-driven podocyte injury and proteinuria. Kidney Int 2011; 80: 1159-1169.
[7] Zulato E, Favaretto F, Veronese C, Campanaro S, Marshall JD, Romano S, Cabrelle A, Collin GB, Zavan B, Belloni AS, Rampazzo E, Naggert JK, Abatangelo G, Sicolo N, Maffei P, Milan G, Vettor R. ALMS1-deficient fibroblasts over-express extra-cellular matrix components, display cell cycle delay and are resistant to apoptosis. PLoS One 2011; 6: e19081.
[8] Stephens S, Palmer J, Konstantinova I, Pearce A, Jarai G, Day E. A functional analysis of Wnt inducible signalling pathway protein -1 (WISP-1/CCN4). J Cell Commun Signal 2015; 9: 63-72.
[9] Canel C, Moraes RM, Dayan FE, Ferreira D. Podophyllotoxin. Phytochemistry 2000; 54: 115-120.
[10] Guerrero E, Abad A, Montenegro G, Del Olmo E, Lopez-Perez JL, San Feliciano A. Analgesic and anti-inflammatory activity of podophyllotoxin derivatives. Pharm Biol 2013; 51: 566-572.
[11] Cortes F, Mateos S, Pastor N, Dominguez I. Toward a comprehensive model for induced endoreduplication. Life Sci 2004; 76: 121-135.
[12] Marchetti F, Pearson FS, Bishop JB, Wyrobek AJ. Etoposide induces chromosomal abnormalities in mouse spermatocytes and stem cell spermatogonia. Hum Reprod 2006; 21: 888-895.
[13] Konigshoff M, Kramer M, Balsara N, Wilhelm J, Amarie OV, Jahn A, Rose F, Fink L, Seeger W, Schaefer L, Gunther A, Eickelberg O. WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. J Clin Invest 2009; 119: 772-787.
[14] Cervello M, Giannitrapani L, Labbozzetta M, Notarbartolo M, D'Alessandro N, Lampiasi N, Azzolina A, Montalto G. Expression of WISPs and of their novel alternative variants in human hepatocellular carcinoma cells. Ann N Y Acad Sci 2004; 1028: 432-439.
[15] Yanagita T, Kubota S, Kawaki H, Kawata K, Kondo S, Takano-Yamamoto T, Tanaka S, Takigawa M. Expression and physiological role of CCN4/Wnt-induced secreted protein 1 mRNA splicing variants in chondrocytes. FEBS J 2007; 274: 1655-1665.
[16] Desnoyers L, Arnott D, Pennica D. WISP-1 binds to decorin and biglycan. J Biol Chem 2001; 276: 47599-47607.
[17] Su F, Overholtzer M, Besser D, Levine AJ. WISP-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase. Genes Dev 2002; 16: 46-57.
[18] Ono M, Inkson CA, Kilts TM, Young MF. WISP-1/CCN4 regulates osteogenesis by enhancing BMP-2 activity. J Bone Miner Res 2011; 26: 193-208.
[19] Hou CH, Tang CH, Hsu CJ, Hou SM, Liu JF. CCN4 induces IL-6 production through alphavbeta5 receptor, PI3K, Akt, and NF-kappaB singling pathway in human synovial fibroblasts. Arthritis Res Ther 2013; 15: R19.
[20] Liu JF, Hou SM, Tsai CH, Huang CY, Hsu CJ, Tang CH. CCN4 induces vascular cell adhesion molecule-1 expression in human synovial fibroblasts and promotes monocyte adhesion. Biochim Biophys Acta 2013; 1833: 966-975.
[21] Chen AY, Liu LF. DNA topoisomerases: essential enzymes and lethal targets. Ann Rev Pharm Toxicol 1994; 34: 191-218.
[22] Dubrez L, Goldwasser F, Genne P, Pommier Y, Solary E. The role of cell cycle regulation and apoptosis triggering in determining the sensitivity of leukemic cells to topoisomerase I and II inhibitors. Leukemia 1995; 9: 1013-1024.
[23] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993; 74: 609-619.
[24] Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; 28: 1322-1326.
[25] Murphy KM, Streips UN, Lock RB. Bcl-2 inhibits a Fas-induced conformational change in the Bax N terminus and Bax mitochondrial translocation. J Biol Chem 2000; 275: 17225-17228.
[26] Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, Krause KH. Bcl-2 decreases the free Ca2+ concentration within the endoplasmic reticulum. Proc Natil Acad Sci U S A 2000; 97: 5723-5728.
[27] Reed JC, Miyashita T, Takayama S, Wang HG, Sato T, Krajewski S, Aime-Sempe C, Bodrug S, Kitada S, Hanada M. BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem 1996; 60: 23-32.
[28] Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nat Rev Cancer 2008; 8: 121-132.
[29] Real PJ, Cao Y, Wang R, Nikolovska-Coleska Z, Sanz-Ortiz J, Wang S, Fernandez-Luna JL. Breast cancer cells can evade apoptosis-mediated selective killing by a novel small molecule inhibitor of Bcl-2. Cancer Res 2004; 64: 7947-7953.

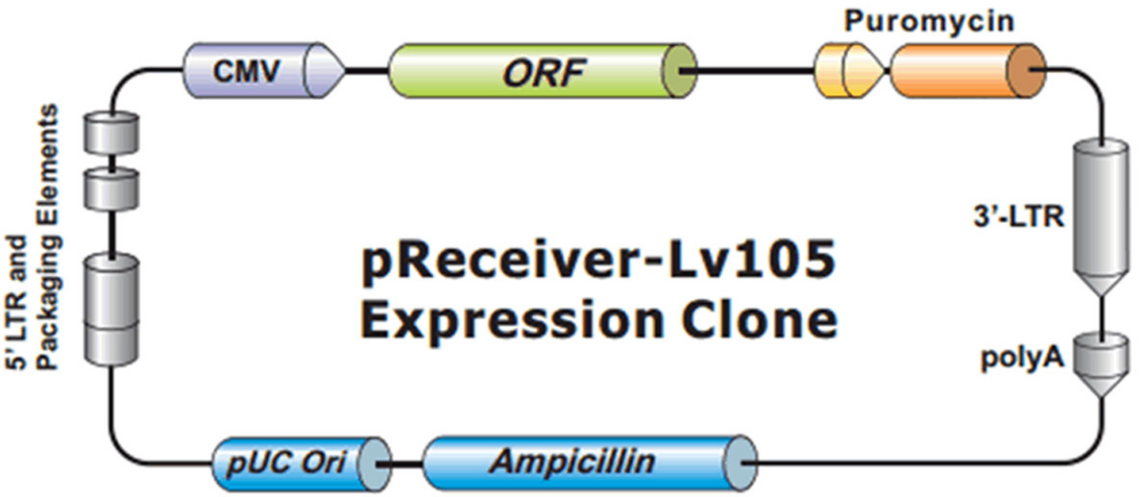

Figure S1. The schematic diagram of the overexpressing WISP-1 lentiviral vector OmicsLink™ Expression vector carrying the CMV (cytomegalovirus) promoter and anti-Puromycin gene and with WISP-1 cDNA insertion.

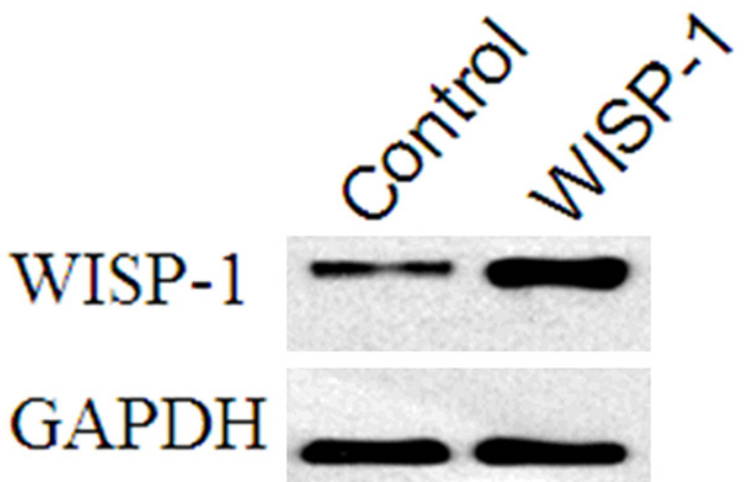

Figure S2. Western blot analysis of protein level of WISP-1 from A549 cells transfected with WISP-1 overexpressing vector (left lane) and A549 cells transfected with control vector (right lane). GAPDH was loaded for inner control.