**WWOX** induces apoptosis and inhibits proliferation of human hepatoma cell line SMMC-7721

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**Abstract**

**AIM:** To investigate the effects of the **WWOX** gene on the human hepatic carcinoma cell line SMMC-7721.

**METHODS:** Full-length **WWOX** cDNA was amplified from normal human liver tissues. Full-length cDNA was subcloned into pEGFP-N1, a eukaryotic expression vector. After introduction of the **WWOX** gene into cancer cells using liposomes, the **WWOX** protein level in the cells was detected through Western blotting. Cell growth rates were assessed by methyl thiazolyl tetrazolium (MTT) and colony formation assays. Cell cycle progression and cell apoptosis were measured by flow cytometry. The phosphorylated protein kinase B (AKT) and activated fragments of caspase-9 and caspase-3 were examined by Western blotting analysis.

**RESULTS:** **WWOX** significantly inhibited cell proliferation, as evaluated by the MTT and colony formation ass-
says. Cells transfected with **WWOX** showed significantly higher apoptosis ratios when compared with cells transfected with a mock plasmid, and overexpression of **WWOX** delayed cell cycle progression from G1 to S phase, as measured by flow cytometry. An increase in apoptosis was also indicated by a remarkable activation of caspase-9 and caspase-3 and a dephosphorylation of AKT (Thr308 and Ser473) measured with Western blotting analysis.

**CONCLUSION:** Overexpression of **WWOX** induces apoptosis and inhibits proliferation of the human hepatic carcinoma cell line SMMC-7721.

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**Key words:** WWOX; SMMC-7721; Apoptosis; Proliferation; Hepatic carcinoma

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**INTRODUCTION**

The tumor suppressor gene **WW**OX is localized in a common fragile site FRA16D (locus 16q23.3-24.1). Protein encoded by **WW**OX is an oxidoreductase containing two WW protein interaction domains. The biological role of the protein is not yet defined, although there are hypotheses that it may play a part in steroid hormones me-
tabolism and ErbB4 receptor signaling pathway. Low expression level of the WWOX gene has been observed in many types of cancers, possibly due to the loss of heterozygosity or epigenetic changes, such as methylation of CpG islands in promoter region. Several researches have revealed loss of heterozygosity of WWOX locus in gastric, pancreatic, esophageal and lung cancers. The role of WWOX in hepatic carcinoma is not well understood, and few studies have reported the effects of WWOX on hepatic carcinoma. In this study, we investigated the apoptotic effects of the WWOX gene on the human hepatic carcinoma cell line SMMC-7721.

MATERIALS AND METHODS

Materials

The eukaryotic expression vector pEGFP-N1 and Escherichia coli DH5α competent cells are routinely maintained by the central laboratory at our hospital. The hepatoma cell line SMMC-7721 was obtained from the Chinese Academy of Sciences (Shanghai, China). Dulbecco’s modification of Eagle’s medium Dulbecco (DMEM) culture medium was purchased from Gibco BRL (Gaithersburg, United States). Fetal bovine serum was obtained from Sijiqing Biological Engineering Material (Hangzhou, China). The following materials were used: RNasey Protect Mini-kit (QIAGen Co., Germany), SMARTTM PCR cDNA synthesis kit (Clontech Co., United States), DNA gel extraction kit (Dalian TaKaRa Co., China), plasmid mini-preparation kit (Shanghai HuaSun Biotechnology Co., China), KOD-Plus DNA polymerase (TOYOBO Co., United States), T4 DNA ligase and the HindⅢ and Kpn I restriction enzymes (New England Biolabs, United States), DNA polymerase (Roche, New York, United States), pEGFP-N1 and pCMV6-eGFP plasmid (OriGene Technologies, Inc, United States), G418 (Life Technology, Paisley, Scotland) and PBST (pH 7.4). The restriction enzymes were purchased from Promega, UK.

Cell lines and culture conditions

SMMC-7721 cells were cultured in DMEM medium (HyClone Inc, United States) supplemented with 10% calf bovine serum in a 37℃ and 5% CO2 incubator.

Construction of pEGFP-N1-WWOX vector and establishment of cell line SMMC-7721 that stably expresses WWOX

The WWOX open reading frame was amplified from a cDNA clone using the forward primer 5’GGAGCGGGTGAG-3’ and the reverse primer 5’GGATCCCGAGTTGAGTAC-T3’, which introduced Kpn I and HinDⅢ restriction endonuclease sites. WWOX cDNA digested with Kpn I and HinDⅢ was cloned into a pEGFP-N1 eukaryotic expression vector. The resulting vector was transfected into SMMC-7721 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). An empty vector construct was used as a negative control. After 24-48 h, the transient transfection efficiency was determined under an Olympus fluorescence microscope. The cells were then passaged at appropriate ratios to six-well plates. The next day, the cells were cultured in the presence of 1000-2000 g/mL G418 (Life Technology, Paisley, Scotland), which was increased in concentration in a stepwise manner over 14 d. Cells highly expressing green fluorescent protein (GFP) were selected.

Western blotting

Protein was extracted from cultured cells using lysis buffer. After a 30-min incubation on ice, the lysates were heated at 100℃ for 15 min and centrifuged at 12 000 × g for 15 min at 4℃. Lysates containing an equal amount of protein (25 μg) were dissolved in sodium dodecyl sulfate (SDS) sample buffer, separated on 12% SDS slab gels, and transferred electrophoretically onto polyvinylidene difluoride membranes. Equal protein loading and transfer were confirmed by Ponceau S staining. After being blocked with 5% non-fat dry milk in Tris-Buffered Saline and Tween 20 (10 mmol Tris-HCl, pH 8.0, 100 mmol/L NaCl and 0.05% Tween), the membrane was incubated at 4℃ overnight with the appropriate primary antibodies. Following washing, horseradish peroxidase conjugated secondary antibody was applied to the membrane. Proteins bound by the secondary antibody were visualized by enhanced chemiluminescence (Amersham Bioscience) according to the manufacturer’s instructions. The expression of GAPDH was measured as a control, and each experiment was performed in triplicate.

Cell growth assays

Cell growth was determined by the methyl thiazolyl tetrazolium (MTT) assay (Sigma, United States). Briefly, 1 × 104 cells were seeded onto 96-well plates with four replicates for each condition. Approximately 72 h later, MTT reagent was added to each well at 5 mg/mL in a 20 μL volume, and the reaction was incubated for another 4 h. The formazan crystals formed by viable cells were subsequently solubilized in dimethyl sulfoxide, and the absorbance (A) at 490 nm was measured.

Plate colony formation assay

Approximately 100 cells were added to each well of a six-well culture plate. After incubation at 37℃ for 15 d, cells were washed twice with phosphate-buffered saline (PBS) and stained with Giemsa solution. The number of colonies containing ≥ 50 cells was counted under microscope [plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%]. Each experiment was performed in triplicate.

Cell cycle analysis

Fifty-eight hours after treatment, logarithmically grow-
ing cells were collected and washed with PBS three times and fixed with 75% ethanol at -20°C for at least 1 h. After extensive washing with PBS, the cells were suspended in Hank's balanced salt solution containing 50 mg/mL RNase A (Boehringer Mannheim) and 50 mg/mL propidium iodide (PI) (Sigma-Aldrich), incubated for 1 h at room temperature, and were analyzed by FACScan (Becton Dickinson).

**Apoptosis assays**

Apoptosis was analyzed 48 h after treatment using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions.

**Statistical analysis**

Data were presented as the mean ± SD. Comparisons of experimental values between cisplatin-treated cells and untreated controls were conducted using analysis of variance or the Kruskal-Wallis rank test. Statistical significance was defined as P < 0.05.

**RESULTS**

**Overexpression of WWOX in the cell line SMMC-7721**

To study the biological functions of WWOX, we introduced WWOX into SMMC-7721 cells using a pEGFP-N1 eukaryotic expression vector containing the WWOX gene. Seven stably transfected cell clones were obtained. Western blotting analysis with anti-GFP antibodies showed that WWOX-pGFP fusion protein in the SMMC-7721 cell clones was highly expressed compared with control cells and control-vector cells (Figure 1).

**WWOX inhibits cell growth in vitro**

To analyze the function of WWOX, we studied the rate of cell growth in the WWOX-expressing SMMC-7721 cells. The results from the colony formation assay indicated that SMMC-7721 cells overexpressing WWOX formed significantly fewer colonies than did the control clone cells and the control-vector cells (P < 0.05) (Figure 2). Cells transfected with WWOX also showed significantly decreased cell proliferation compared with control cells and control-vector cells when examined by the MTT assay (Table 1, Figure 3).

**Overexpression of WWOX arrests the cell cycle in G1 and induces apoptosis of SMMC-7721 cells**

To detect the effect of WWOX overexpression on the cell cycle, we measured the cell cycle distribution in WWOX-expressing SMMC-7721 cells. In these lines, there was a marked decrease in the S-phase population, while the G1 population was significantly increased compared with the control vector and wild type SMMC-7721 cells (P < 0.05). Neither cell lines showed significant changes in the G2 population (Figure 4, Table 2). Cells transfected with pEGFP-N1-WWOX demonstrated more apoptosis than did cells transfected with the mock
Hu BS et al. WWOX induces apoptosis and growth inhibition in carcinoma cells

Caspase-9 and caspase-3 activation by WWOX
Expression of cleaved caspase-9 and caspase-3 was upregulated, as measured by Western blotting in cells that were transfected with pEGFP-N1, compared with either the cells transfected with a control vector or parental wild-type cells (Figure 6).

Phosphorylation of Akt decreased by WWOX
To evaluate the effect of WWOX on Akt/PKB activity, the phosphorylation level at Akt Thr308 and Ser473 was examined with specific phospho-Akt antibodies. Western blot analysis showed that WWOX significantly reduced the level of Akt/PKB phosphorylation (Figure 7).

DISCUSSION
Hepatic carcinoma is a highly invasive and clinically challenging tumor, and its molecular basis remains poorly understood. We used a gain-of-function approach by plasmid or the parent cells ($P < 0.05$) (Figure 5).

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DISCUSSION
Hepatic carcinoma is a highly invasive and clinically challenging tumor, and its molecular basis remains poorly understood. We used a gain-of-function approach by
introducing WWOX into wild-type cells to investigate the effect of the WWOX gene on SMMC-7721, the human hepatic carcinoma cell line. Our data suggest that WWOX can significantly inhibit cell proliferation and induce cell apoptosis of the hepatic carcinoma cell line SMMC-7721. Overexpression of WWOX delayed the cell cycle progression from G1 into S phase, as demonstrated by flow cytometry.

Apoptosis plays a central role in tumor development, and a lack or failure of apoptosis leads to the development of many tumors, including hepatocarcinoma[16-17]. This suggests that induction of apoptosis in tumor cells might be an effective approach for delaying tumor progression. In this study, we found that overexpression of WWOX induces apoptosis in the hepatic carcinoma cell line SMMC-7721.

There are, at least, two broad extrinsic and intrinsic pathways that lead to apoptosis[18-20]. The extrinsic pathway begins with the binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) and results in the
recruitment of Fas-associated protein with death domain and pro-caspase-8 to the Fas complex. This increase in the local concentration of pro-caspase-8 leads to its autocatalysis and activation. Activated caspase-8 cleaves pro-caspase-3, which then undergoes autocatalysis to form active caspase-3, a principle effector caspase of apoptosis. The intrinsic apoptosis pathway always begins with mitochondrial damage, which results in the release of cytochrome C from the damaged mitochondria. In the cytosol or on the surface of the mitochondria, cytochrome C is bound to the protein Apaf-1 (apoptotic protease activating factor), which activates the initiating caspase, caspase-9, which then activates caspase-3. Both caspase-8 and caspase-9 can activate the effector caspase, caspase-3, by proteolytic cleavage, and the subsequent processes result in nuclear DNA fragmentation and the formation of apoptotic bodies. This indicates that activation of caspase-3 is a central event for the process of apoptosis. Based on these results, we speculate that WWOX allows the release of cytochrome C from mitochondria, resulting in the activation of caspase-9 and caspase-3 in sequence and finally induces apoptosis of HCC cells. Consistent with this hypothesis, the results of Western blotting showed that WWOX overexpression induced the activation of caspase-9 and caspase-3.

Our work also shows that WWOX downregulates the phosphorylation of Akt/PKB at Thr308. Akt/PKB is phosphorylated at two regulatory sites, Thr308 and Ser473, which are essential for its activation. Activated Akt/PKB can phosphorylate BAD, a pro-apoptotic Bcl-2 family member, and the forkhead transcription factors (27,28), leading to their inactivation and cell survival. It has been reported that the phosphorylation of caspase-9 can regulate its activity. Akt/PKB is a regulator of cell survival and apoptosis, and its activation in a variety of cells can protect against apoptosis. Akt/PKB is phosphorylated at serine 473, which is essential for its activity.

WWOX blocks the activation of Akt, thereby attenuating the activity of a major anti-apoptotic pathway and inducing cell apoptosis. It remains unclear how WWOX affects Akt phosphorylation, as it does not affect PI3-kinase activity directly (29). Other potential consequences of WWOX inhibition, such as the modulation of the RAS-signaling pathway, the expression of p53 and other members of the B-cell lymphoma 2 family, such as myeloid cell leukemia-1, the activation of the sphingomyelin-ceramide pathway, and interference with nuclear factor-KB (NF-κB) merit further investigation in the future.

In conclusion, WWOX may play a key role in tumor cell proliferation and carcinogenesis. Overexpression of WWOX can suppress the growth of HCC cells by inhibiting cell growth and inducing cell apoptosis. Apoptosis is induced by WWOX through the activation of the caspase cascade, which is correlated with the phosphorylation of Akt/PKB. These results suggest a potential role for WWOX as an effective chemotherapeutic and chemopreventive strategy against human liver cancer.

REFERENCES
1. Aqeilan RI, Donati V, Gaudio E, Nicoloso MS, Sundvall M, Korhonen A, Lundin J, Isola J, Sudol M, Joensuu H, Croce CM, Elenius K. Association of Wwox with ErbB4 in breast cancer. Cancer Res 2007; 67: 9330-9336
2. Aqeilan RI, Hagan JP, de Bruin A, Rawahneh M, Salah Z, Gaudio E, Siddiqui H, Volinia S, Alder H, Lian JB, Stein GS, Croce CM. Targeted ablation of the WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. Endocrinology 2009; 150: 1530-1535
3. Kuroki T, Trapasso F, Shiraishi T, Alder H, Mimori K, Mori M, Croce CM. Genetic alterations of the tumor suppressor gene WWOX in esophageal squamous cell carcinoma. Cancer Res 2002; 62: 2258-2260
4. Yendamuri S, Kuroki T, Trapasso F, Henry AC, Dumon KR, Huebner K, Williams NN, Kaiser LR, Croce CM. WW domain-containing oxidoreductase tumor suppressor leads to impaired steroidogenesis. Endocrinology 2009; 150: 1530-1535
5. Ishii H, Veccheione A, Furukawa Y, Sutheesophon K, Han SY, Druck T, Kuroki T, Trapasso F, Nishimura M, Saito Y, Ozawa K, Croce CM, Huebner K, Furukawa Y. Expression of FRA16D/WWOX and FRA3B/FHIT genes in hematopoietic malignancies. Mol Cancer Res 2003; 1: 940-947
6. Kuroki T, Yendamuri S, Trapasso F, Matsuyama A, Aqeilan RI, Alder H, Rattan S, Cesari R, Nolli ML, Williams NN, Mori M, Kanematsu T, Croce CM. The tumor suppressor...
gene WWOX at FRA16D is involved in pancreatic carcinogenesis. *Clin Cancer Res* 2004; **10**: 2459-2465

7 Agellan RI, Kuroki T, Pekarsky Y, Albagha O, Trapasso F, Bafra R, Huebner K, Edmonds P, Croce CM. Loss of WWOX expression in gastric carcinoma. *Clin Cancer Res* 2004; **10**: 3053-3058

8 Driouch K, Prydz H, Monese R, Johansen H, Lidereau R, Frangen E. Alternative transcripts of the candidate tumor suppressor gene, WWOX, are expressed at high levels in human breast tumors. *Oncogene* 2002; **21**: 1832-1840

9 Gourley C, Paige AJ, Taylor KJ, Scott D, Francis NJ, Rush R, Aldaz CM, Smyth JF, Gabra H. WWOX mRNA expression profile in epithelial ovarian cancer supports the role of WWOX variant 1 as a tumour suppressor, although the role of variant 4 remains unclear. *Int J Oncol* 2005; **26**: 1681-1689

10 Guler C, Uner A, Guler N, Han SY, Iliopoulos D, Hauck WW, McCue P, Huebner K. The fragile genes FHIT and WWOX are inactivated coordinately in invasive breast carcinoma. *Cancer* 2004; **100**: 1605-1614

11 Ishii H, Furukawa Y. Alterations of common chromosome fragile sites in hematopoietic malignancies. *Int J Hematol* 2004; **79**: 238-242

12 Paige AJ, Taylor KJ, Taylor C, Hillier SG, Farrington S, Scott D, Porteous DJ, Smyth JF, Gabra H, Watson JE. WWOX: a candidate tumor suppressor gene involved in multiple tumor types. *Proc Natl Acad Sci USA* 2001; **98**: 11417-11422

13 Sbrana I, Veroni F, Nieri M, Puliti A, Barale R. Chromosomal fragile sites FRA3B and FRA16D show correlated expression in initial and recurrent glioblastomas: modulation by radiochemotherapy. *J Neurol Neurosurg Psychiatry* 2001; **72**: 16568-16575

14 Shi J, Deininger M, Streffer J, Grote E, Wickboldt J, Dichtl W, Weller M, Meyermann R. BCL-2 family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy. *J Neurol Neurosurg Psychiatry* 1999; **67**: 763-768

15 Wellers M, Malipiero U, Aguzzi A, Reed JC, Fontana A. Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. *J Clin Invest* 1995; **95**: 2633-2643

16 Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239-257

17 Evan G, Littlewood T. A matter of life and cell death. *Science* 1998; **281**: 1317-1322

18 Yang J, Liu X, Bhalla K, Kim CN, Ibredo AM, Cai J, Peng TL, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997; **275**: 1129-1132

19 Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479-489

20 Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998; **17**: 37-49

21 Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996; **86**: 147-157

22 Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; **90**: 405-413

23 Sakai T, Liu L, Teng X, Mukai-Sakai R, Shimada H, Kaji R, Mitani T, Matsumoto M, Toida K, Ishimura K, Shishido Y, Mak TW, Fukui K. Nucluc recruiting Apaf-1/pro-caspase-9 complex for the induction of stress-induced apoptosis. *J Biol Chem* 2004; **279**: 41131-41140

24 Arnould D, Gaume B, Karbowski M, Sharpe JC, Cecconi F, Youle RJ. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J* 2003; **22**: 4385-4399

25 Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 1997; **275**: 661-665

26 Franke TF, Kaplan DR, Cantley LC. PI3K downstream AKT blocks apoptosis. *Cell* 1997; **88**: 435-437

27 Fang X, Yu S, Eder A, Mao M, Bast RC, Boyd D, Mills GB. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncoogene* 1999; **18**: 6635-6640

28 Rena G, Prescott AR, Guo S, Cohen P, Unterman TG. Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targeting. *Biochem* 2001; **354**: 605-612

29 Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death caspase-9 by phosphorylation. *Science* 1998; **282**: 1318-1321

30 Zhou H, Summers SA, Birnbaumu MJ, Pittman RN. Inhibition of Akt kinase by cell-permeable ceramide and its implications for ceramide-induced apoptosis. *J Biol Chem* 1998; **273**: 16568-16575

31 Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* 2000; **275**: 11397-11403

32 Lin MT, Lee RC, Yang PC, Ho FM, Kuo ML. Cyclooxygenase-2 inducing Mcl-1-dependent survival mechanism in human lung adenocarcinoma CL1.0 cells. Involvement of phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* 2001; **276**: 48997-49002

33 Lee YJ, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. *J Biol Chem* 2003; **278**: 37041-37051