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Knockdown of WWP1 inhibits growth and induces apoptosis in hepatoma carcinoma cells through the activation of caspase3 and p53

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ABSTRACT

The activation of oncogenes and the loss of tumor suppressor genes are believed to play critical roles in the pathogenesis of human hepatocellular carcinoma (HCC). The human WW domain containing E3 ubiquitin protein ligase 1 (WWP1) gene is frequently amplified in prostate and breast cancers, however, its role in cancer has not yet been extensively studied. Especially, the role of WWP1 in HCC has not yet been studied. Firstly, we analyzed the expression of WWP1 in HCC samples. We found that protein levels of WWP1 are higher in most HCC cancerous tissues as compared with their matched adjacent non-tumor tissues. Additionally, the WWP1 mRNA was also amplified in all 7 HCC tissues. Knockdown of the endogenous WWP1 using small interfering RNA further showed that deficiency of WWP1 suppressed cell growth and caused apoptosis in HCC cells. Knocking down WWP1 promoted cleaved caspase3 protein and p53 expression in HCC cells, and caspase3 inhibition could prevent cell apoptosis induced by the knockdown of WWP1. All together these results indicate that protein levels of WWP1 in most HCC tissues are higher than non-tumor tissues, and knockdown of WWP1 inhibits growth and induces apoptosis in HCC cells through the activation of caspase3 and p53. Therefore, WWP1 gene might be a potential molecular target of HCC.

1. Introduction

Human hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide and the most common type of primary liver cancer [1]. However, the genetic and biochemical events regulating the development and progression of HCC are still incomplete [2]. The activation of oncogenes and the loss of tumor suppressor genes are believed to play key roles in the pathogenesis of HCC [3]. Thus the discovery of oncogenes associated with HCC and clarifying their mechanism may provide important clues for HCC treatment.

WWP1 (WW domain containing E3 ubiquitin protein ligase1) is an E3 ubiquitin ligase and was first identified by its WW domain [4]. There are many families of E3s, such as the HECT domain ligases, the RING finger ligases, and the U box proteins [5]. WWP1 belongs to the HECT families [6] and includes 922 amino acid residues with the molecular weight of 110 kDa [7]. Similar to other members in the family, WWP1 is consists of three different domains: an N-terminal C2 domain, followed by four protein interacting WW domains, and a C-terminal HECT catalytic domain [6,8].

The WW domain is a short domain of approximately 40 amino acids and it recognizes and binds to Pro-rich polypeptide ligands, such as PPXY or PPLP, as well as phosphorylated serines and threonines or p(S/T) P [9]. The C-terminus of WWP1 possesses a HECT domain and is responsible for its ubiquitin E3 ligase enzyme activity [4]. WWP1 targets a variety of substrate proteins, including p53 [10] and p63 [11], Kruppel-like factors [12], Smad4 [13], p27 [14], TRAF6 [15], and so on. By regulating the proteasome-dependent degradation of specific substrates, WWP1 participates in different biological processes such as cell proliferation and senescence, protein trafficking, and embryonic development [6,14,16].

The human WWP1gene is localized on chromosome 8q21 [17], which is frequently amplified in several human cancers, including

Abbreviations: WWP1, WW domain containing E3 ubiquitin protein ligase 1; HCC, hepatocellular carcinoma; FBS, fetal bovine serum; AFP, α-fetoprotein; 18S rRNA, 18S ribosomal ribonucleic acid; siRNA, small interference RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer saline; PI, propidium iodide.

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prostate and breast cancers [6]. Gain of gene copy number for WWP1 was detected in 31–51% of breast and prostate cancers [18,19]. WWP1 has pro-apoptotic function in Era negative breast cells MCF10 [20]. WWP1 deficiency suppressed cell proliferation and induced apoptosis in Era-positive breast cancer cell lines such as MCF7 and HCC1500 [21].

However, roles of WWP1 in cancer have not yet been extensively studied, and it is still unknown whether WWP1 influences HCC. This study is aimed to investigate the expression of WWP1 in HCC and down-regulation of WWP1 in cell proliferation and apoptosis, to explore the possibility of WWP1 suppression in clinical treatment of HCC.

2. Materials and methods

2.1. Clinical HCC specimens

Human HCC tissues and corresponding surrounding liver samples from 11 Chinese patients were obtained from the Tissue Bank at the Peking University School of Oncology (patients 1–5 and 10–11) and Henan Cancer Hospital (patients 6–9). All samples, collected from surgical resection, were snap-frozen in liquid nitrogen and stored at −80 °C. All human samples were collected in accordance with the Declaration of Helsinki, and use of human tissues was approved by the Institute Research Ethics Committee. The detailed characteristics of these patients are shown in Table 1.

2.2. Cell culture

Human HCC cell lines MHCC97H, were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 [22]. SMMC7721 were cultured in RPMI 1640 media containing 10% FBS at 37 °C in 5% CO_2 [23].

2.3. Real-Time PCR

Real-Time PCR analysis was performed as described [24]. 18S rRNA (18S ribosomal ribonucleic acid) was served as an endogenous control for normalization. The primers were shown as follow: WWP1-F: 5′-ACAGTGGCAATCTCAGCG-3′; WWP1-R: 5′-GCAAAGGT CCATAAGGGT-3′; 18S rRNA-F: 5′-GTAACCCGTTGAACCCCATT-3′; 18S rRNA-R: 5′-CCATCCAATCGGTAGTAGCG-3′.

2.4. Western blotting

The protocol of Western blotting was described as previous study [14]. Antibodies used for Western blotting were: anti-WWP1 (Abnova), anti-caspase3 (Cell Signaling Technology), anti-p53 (Santa Cruz), anti-GAPDH (Cell Signaling Technology) and anti-tubulin (Santa Cruz).

2.5. siRNA-mediated silencing of WWP1

Cells were seeded on 60-mm culture dishes or 6-well micro-culture plates. RNA interference for 48 or 72 h in HCC cells was carried out according to the manufacturer's protocol using Lipofectamine RNAiMAX (Invitrogen). The small interference RNA (siRNA) target sequences for human WWP1 gene are 5′-GAGUUGA UGAUCGUAG-AAG-3′ (siWWP1 #1) and 5′-GAAGTCATCTGT-AACT AAA-3′ (siWWP1 #2), and negative control siRNA was 5′-UUCUC CG-AACGUGUCACGU-3′ (siNC) [14,20].

2.6. MTT assay

Cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. Briefly, MHCC97H or SMMC7721 cells plated in 96-well plates following transfected with siWWP1 #1 or siNC. After transfection for 0, 24, 48, and 72 h, cells were treated with MTT (5 mg/mL, 20 μL). Then cells treatment with dimethylsulfoxide (200 μL), the absorbance was measured at 490 nm. Absorbance for 97H-siNC or 7721-siNC cells was considered to be a control.

2.7. Cell apoptosis assay

MHCC97H and SMMC7721 cells were transfected with siNC and siWWP1 #1 or siWWP1 #2 for 48 or 72 h, and cells were harvested. Cells were washed twice with PBS, then resuspended in cold binding buffer and both Annexin V–FITC and PI were added. Samples were then incubated at room temperature for 15 min in the dark and analyzed by flow cytometry.

2.8. Cell apoptosis in presence of caspase3 inhibitor

Firstly, MHCC97H cells plated in 6-well plates following were transfected with siNC and siWWP1 #1 or siWWP1 #2. After transfection for 6 h, MHCC97H-siWWP1 #1 or MHCC97H-siWWP1 #2 cells were cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h. Cell permeable inhibitor for caspase-3 was purchased from Santa Cruz Biotechnology (sc-3075). Then all cells were harvested, and percentages of cell apoptosis were analyzed by flow cytometry.

2.9. Cell cycle assay

Firstly, MHCC97H cells were transfected with siNC and siWWP1 #1 for 48 or 72 h, and then cells were harvested and fixed in 70% ethanol. Cells were washed twice with phosphate buffer saline (PBS), and then were incubated with 50 mg/mL RNaseA for 30 min in 37 °C, and 2.5 mg/mL propidium iodide (PI) were added. Quantification of the percentage of cell cycle phases was performed by FACS analysis carried out in a FACScan flow cytometer.

Table 1
List of 11 paired HCC tissue samples.

Patient	Sex (F/M)	Age	α-Fetoprotein level (μg/L)	Tumor size (cm^3)	Cancer embolus	Liver cirrhosis
1	M	74	4.21	3.5 × 2.6 × 1.5	No	No
2	F	56	292.7	2.7 × 2.5 × 2.5	No	NO
3	M	62	3.23	3.5 × 3.3 × 4.5	No	No
4	M	36	6.36	3.5 × 3 × 3	Yes	No
5	M	62	4.68	3.5 × 0.6 × 0.6	No	Cirrhosis
6	M	60	1000	15	Yes	Cirrhosis
7	M	57	1210	20 × 20 × 15	Yes	Cirrhosis
8	M	39	1210	4.5 × 4.5 × 4.0	No	Cirrhosis
9	M	60	1189	13 × 12 × 8	No	Cirrhosis
10	F	56	2.25	7.5 × 6.0 × 5.5	No	No
11	M	40	9621	12 × 9 × 5	Yes	No

2.10. Statistical analysis

Data are shown as mean ± SD from at least 3 independently performed experiments. Student's *t* test was used for analysis. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered very significant.

3. Results

3.1. HCC is associated with increased expression of WWP1

To explore the correlation between WWP1 and HCC, we detected WWP1 protein and mRNA expression in HCC tissues and adjacent non-tumor tissues from patients. WWP1 protein levels were increased in 9 of HCC samples compared with adjacent non-tumor tissues and unchanged in 1 of HCC samples among all 11 samples (Fig. 1A). The protein expression of WWP1 was significantly upregulated in HCC as compared with adjacent non-tumor tissue ($P < 0.01$, Fig. 1B), 2.5 times higher in HCC tissue than in adjacent non-tumor tissue (Fig. 1B). WWP1 mRNA levels were also higher in all 7 HCC tissues than adjacent tissues (Fig. 1C). However, WWP1 expression was not associated with α-fetoprotein (AFP) serum levels. Whether the expression of AFP was negative or positive in patients' serum, WWP1 protein levels were higher in most HCC samples than in adjacent non-tumor tissues (Fig. 1A and Tables 1 and 2).

3.2. Knockdown of WWP1 inhibits the proliferation of MHCC97H and SMMC7721 cells

To investigate the biological function of WWP1 in HCC proliferation, we knocked down the expression of WWP1 in MHCC97H or SMMC7721 cells with siWWP1 #1 for 72 h. MHCC97H or SMMC7721 cells treated with control small siRNA (siNC) were used as a negative control. The WWP1 protein was significantly decreased in MHCC97H-siWWP1 #1 and SMMC7721-siWWP1 #1 cells as compared with control cells treated with siNC for 72 h (Fig. 2A and B). The viability of MHCC97H cells treated with siWWP1 #1 for 48 and 72 h strongly decreased as compared with control cells ($P < 0.01$, Fig. 2C). Similar results were obtained from SMMC7721cells ($P < 0.01$, Fig. 2D). Thereby knockdown of WWP1 could suppress the proliferation of HCC cells.

3.3. Deficiency WWP1 causes cell apoptosis of HCC cells

Apoptosis is an important cause of tumor suppression. To clarify how deficiency WWP1 inhibits cell growth, we analyzed the cell apoptosis of MHCC97H cells with siNC and siWWP1 #1 or siWWP1 #2

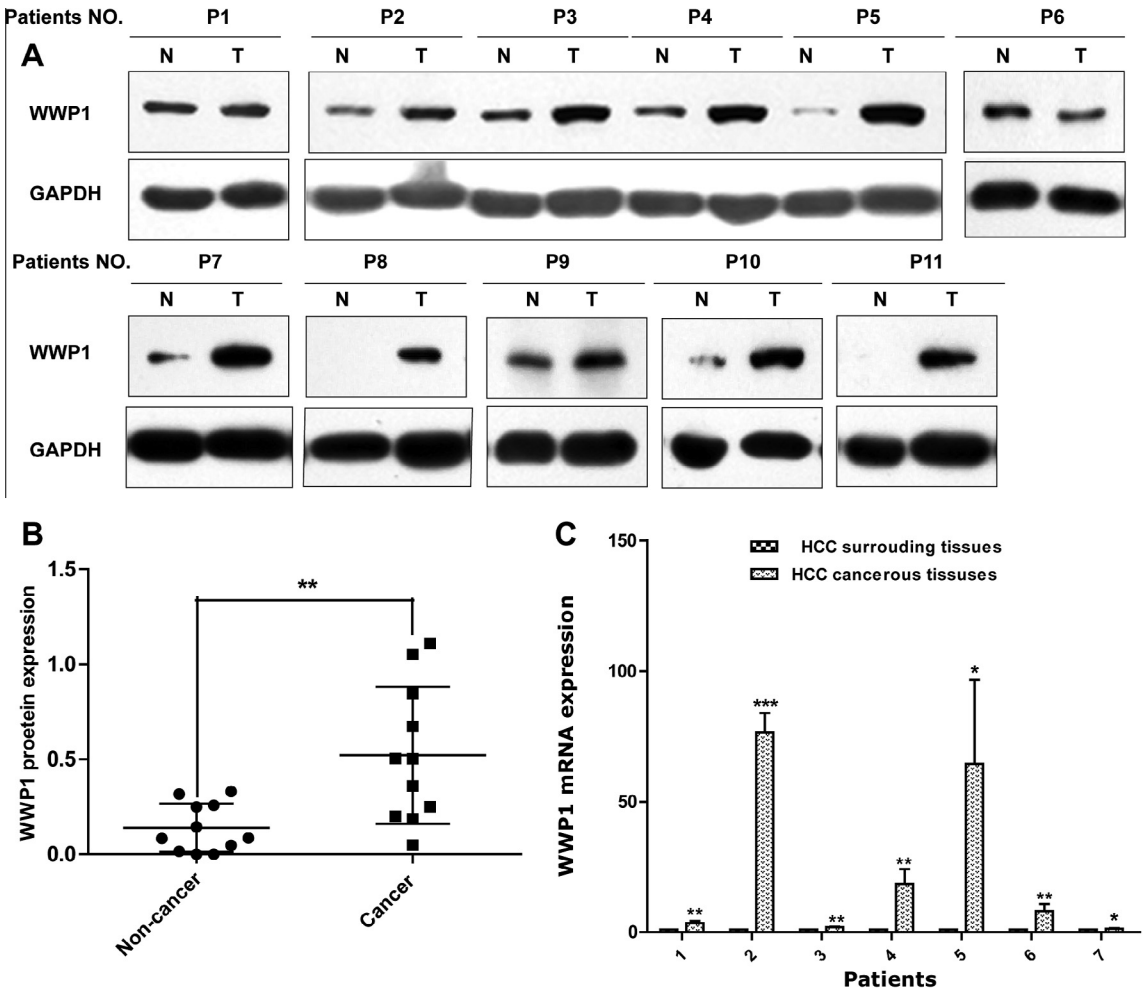

Fig. 1. Characteristics of WWP1 expression in clinical samples. (A) Protein levels of WWP in HCC cancerous tissues and surrounding tissues were examined by Western blotting (N, adjacent non-tumor tissues; T, HCC cancerous tissues). (B) Quantitative analysis of WWP1 protein in HCC cancerous tissues and their matched adjacent non-tumor tissues. (C) Real-Time PCR was performed to detect WWP mRNA in HCC cancerous tissues and their surrounding tissues. Data is showed as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

Table 2
WWP1 and AFP expression in clinical specimens.

WWP1 protein (T/N)	α-Fetoprotein level (μg/L)		Total
	Low (<1000)	High (⩾1000)	
Over-expression	5	4	9
No over-expression	1	1	2
Total	6	5	11

N, adjacent non-tumor tissues; T, HCC cancerous tissues.

transfection for 72 h. Percentages of total and later apoptosis in MHCC97H-siWWP1 #1 or MHCC97H-siWWP1 #2 cells were increased ~15.5% and ~13.5% or ~20.6% and ~10%, respectively, as compared with MHCC97H-siNC cells (Fig. 3A). Similar results were obtained from SMMC7721 cells (Fig. 3B). The discrepancy of early apoptosis between MHCC97H-siWWP1 #1 and MHCC97H-siNC cells is only ~1.1% (Fig. 3A). The discrepancy of early apoptosis between SMMC7721-siWWP1 #1 or SMMC7721-siWWP1 #2 cells and SMMC7721-siNC cells are only 0.6% or 0.14% (Fig. 3B). Therefore, knockdown of WWP1 mainly induced later apoptosis of MHCC97H and SMMC7721 cells.

3.4. Knockdown of WWP1 promotes the protein expression of cleaved caspase3 and p53

Caspase-3 is a crucial executioner of cell apoptosis, because it is responsible for the proteolytic cleavage of many critical proteins, such as poly ADP-ribose polymerase (PARP) [25]. The caspase-3 zymogen actually has no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred [26]. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 or p12 fragments [27]. Since knockdown of WWP1 could induce the apoptosis of HCC cells, we determined the expression of cleaved caspase3 in MHCC97H and SMMC7721 cells with siWWP1 #1 and siWWP1 #2 transfection. Protein levels of cleaved caspase3 were strongly increased in MHCC97H and SMMC7721 treated with siWWP1 #1 and siWWP1 #2 as compared with the control group (Fig. 3C and D).p53 is not only well known for suppressing tumors, but also induce cell apoptosis [10]. WWP1 interacts with the p53 DNA-binding domain and enhances the nuclear export of p53 through promoting p53 ubiquitination, thus inhibits the transcriptional activities of p53 [10]. Our data showed that p53 protein was significantly upregulated in MHCC97H cells treated with siWWP1#1 and siWWP1 #2 as compared with siNC group, 1.3 and 2.5 times higher than in MHCC97H-siNC cells (Fig. 3E). Protein levels of p53 were 2.6 and 3.3 times higher in SMMC7721-siWWP1 #1 and SMMC7721-siWWP1 #2 cells than in SMMC7721-siNC cells (Fig. 3F).

Bcl-2 is an important anti-apoptotic protein, so we also detected the Bcl2 expression in MHCC97H and SMMC7721 with siWWP1#1 transfection. The protein level of Bcl2 was ~0.8 times lower in MHCC97H-siWWP1#1 than in MHCC97H-siNC cells (Fig. S1A). However, the expression of Bcl-2 in SMMC7721-siWWP1 cells was almost unchanged as compared with SMMC7721-siNC group (Fig. S1B).

In conclusion, protein levels of cleaved caspase3 and p53 were obviously increased both in MHCC97H and SMMC7721 cells with siWWP1 transfection (Fig. 3C–F). Thus, silencing WWP1 induced apoptosis depends on promotion of cleaved caspase3 and p53 expression.

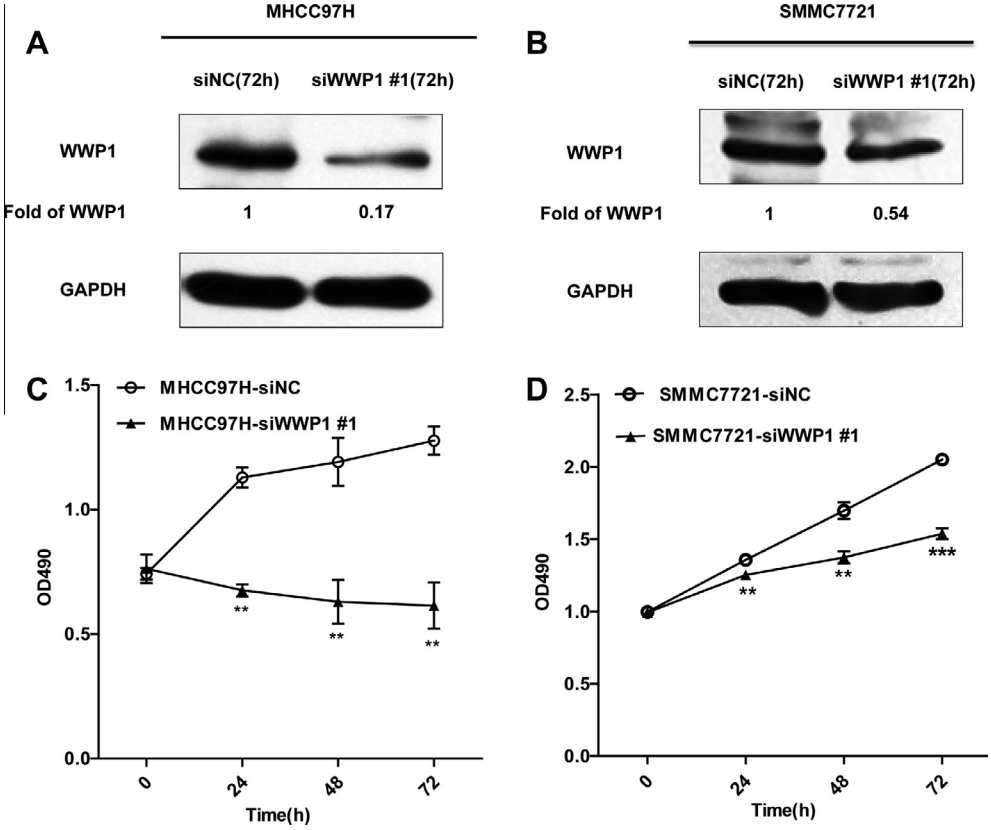

Fig. 2. The impaction of silencing WWP1 on the proliferation of hepatoma carcinoma cells. (A and B) Representative protein blot of WWP1 in MHCC97H or SMMC7721 cells treated with control small siRNA (siNC) or WWP1 siRNA1 (siWWP1 #1) for 72 h. GAPDH was used as a loading control. (A) MHCC97H cells, (B) SMMC7721 cells. (C and D) Cell growth of MHCC97H or SMMC7721 cells treated with siNC or siWWP1 #1 for 0, 24, 48 and 72 h. Data was showed as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$. (C) MHCC97H cells, (D) SMMC7721 cells.

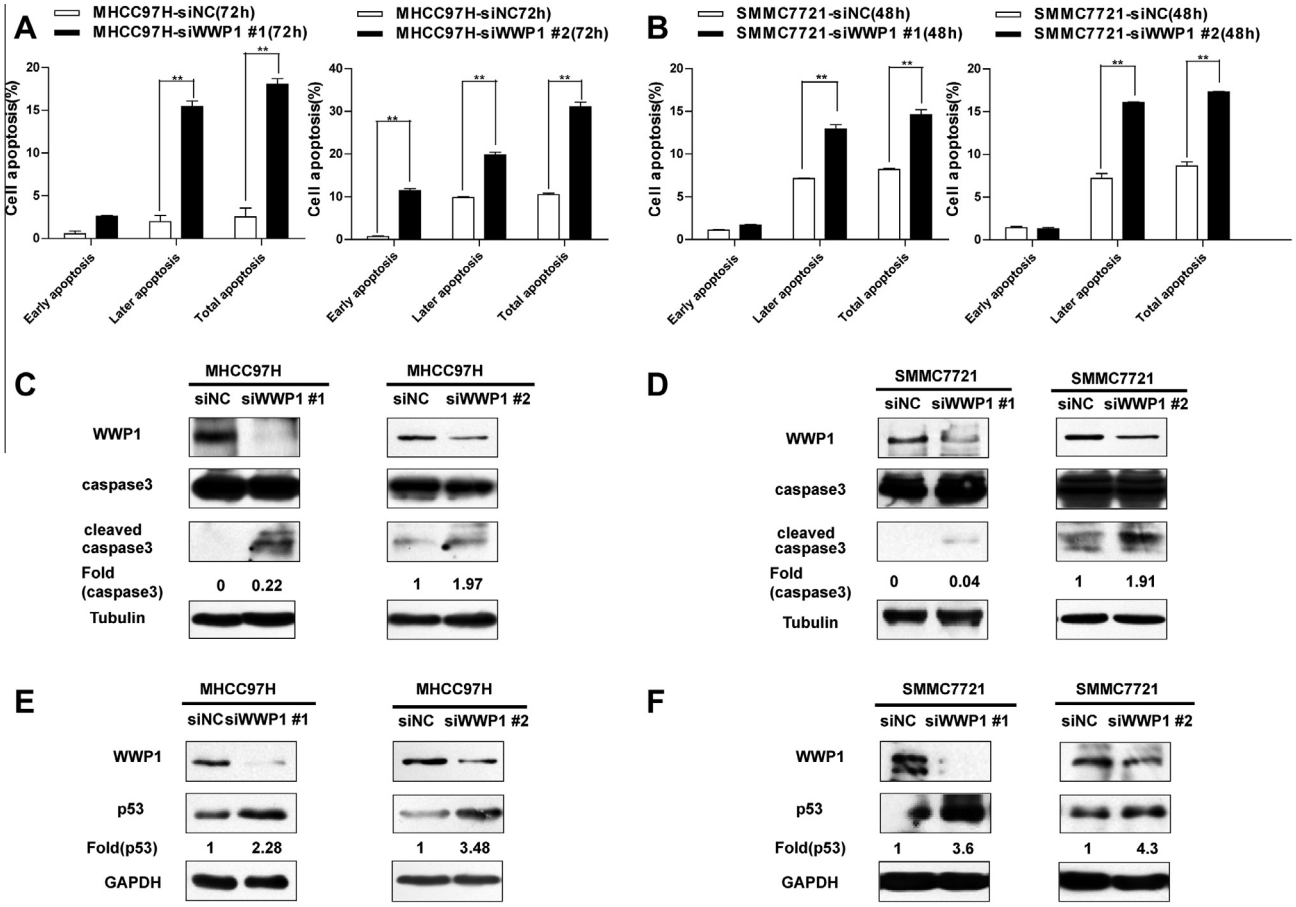

Fig. 3. Knockdown of WWP1 induces cell apoptosis in hepatoma carcinoma cells; down-regulation of WWP1 promotes cleaved caspase3 and p53 expression. (A and B) Knockdown of WWP1 with siWWP1 #1 and siWWP1 #2 induces cell apoptosis of MHCC97H or SMMC7721 cells. (A) MHCC97H cells, (B) SMMC7721 cells. (C and D) Down-regulation of WWP1 with siWWP1 #1 and siWWP1#2 promotes protein levels of cleaved caspase3 in MHCC97H and SMMC7721. (C) MHCC97H cells, (D) SMMC7721 cells. (E and F) Down-regulation of WWP1 promotes p53 expression in MHCC97H and SMMC7721 cells. (E) MHCC97H cells, (F) SMMC7721 cells.

3.5. The caspase3 inhibition could prevent cell apoptosis induced by the knockdown of WWP1

To further clarify that silencing WWP1 induced apoptosis through caspase3 activation, we analyzed the apoptosis of MHCC97H-siWWP1 cells in presence of caspase3 inhibitor. Firstly, MHCC97H cells plated in 6-well plates following were transfected with siNC and siWWP1 #1 or siWWP1 #2. After transfection for 6 h, MHCC97H-siWWP1 #1 or MHCC97H-siWWP1 #2 cells were cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h. Then all cells were harvested, and percentages of cell apoptosis were analyzed by flow cytometry.

Our data showed that percentages of MHCC97H-siWWP1 #1 cells treated with caspase3 inhibitor were decreased about 7% as compared with MHCC97H-siWWP1 #1 in the absence of caspase3 inhibitor, and the percentage of MHCC97H-siWWP1 #1 cells treated with caspase3 inhibitor was almost equal to MHCC97H-siNC cells (Fig. 4A and B). Similar results were obtained in MHCC97H-siWWP1 #2 cells cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h (Fig. 4C). Thereby our study demonstrated that the caspase3 inhibition could prevent cell apoptosis induced by the knockdown of WWP1.

3.6. Down-regulation of WWP1 did not infect cell cycle of HCC cells

We analyzed the cell cycle distribution of MHCC97H cells transfected with siWWP1 #1 for 72 h. Percentages of MHCC97H cells were unchanged at G1 phase (from 61.8% to 60.1%) and G2/M phase (from 10.9% to 13.1%) and S-phase (from 27.3% to 27.0%) with siWWP1 #1 transfection (Fig. S2). So knockdown of WWP1 did not change the phages of cell cycle (G1, S and G2/M) in MHCC97H and SMMC7721 cells (Fig. S2C).

4. Discussion

Hepatocarcinogenesis is a multistep process that involves multiple factors including oncogenes. In this study, we first found that the high expression of WWP1 in HCC; down-regulation of WWP1 inhibited growth and induced apoptosis of HCC cells through activating caspase3 and p53.

The human WWP1 gene is localized on chromosome 8q21, which region is frequently amplified in several human cancers [7,17], thus we examined the WWP1 expression by Western blotting and Real-Time PCR. Our results found that protein levels of WWP1 were very higher in most HCC tissues than their adjacent non-tumor tissues (Fig. 1A and B); the mRNA levels of WWP1 are higher in HCC tissues as compared with their matched surrounding tissues in all 7 paired samples (Fig. 1C). The data is consistent with Chen et al., who found that gain of gene copy number for WWP1was detected in 31–51% of prostate and breast cancers [18,19].

AFP has been widely used in the clinical diagnosis of liver cancer, the diagnostic value of serum AFP is questioned recently because of its low sensitivity [28]. Interestingly, our results showed

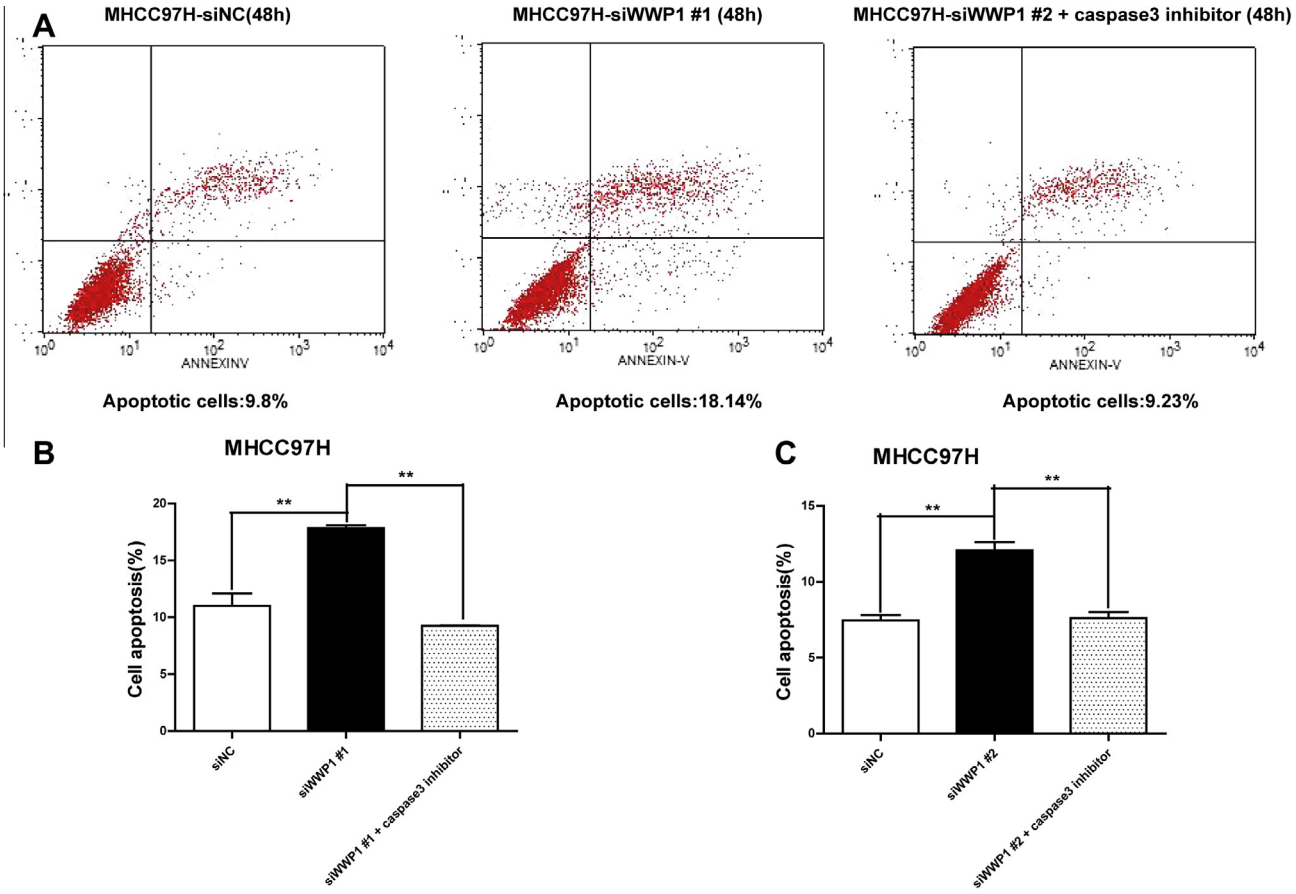

Fig. 4. The caspase3 inhibition could prevent cell apoptosis induced by the knockdown of WWP1. (A) The cell apoptosis distribution of MHCC97H-siNC cells and MHCC97H-siWWP1 #1 cells cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h. FITC-labeled Annexin V-positive cells (upper right and lower right) were considered apoptotic cells. (B) Quantitative analysis of apoptotic percentages of MHCC97H-siNC cells and MHCC97H-siWWP1 #1 cells cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h. (C) Quantitative analysis of apoptotic percentages of MHCC97H-siNC cells and MHCC97H-siWWP1 #2 cells cultured in the presence or absence of caspase3 inhibitor (10 μM) for 48 h.

that the high expression of WWP1 in HCC tissues is not related to AFP levels in serum (Fig. 1A, Table 1 and 2). So it's very significant to study the role of WWP1 in HCC diagnosis in the future. All together, these results demonstrated that WWP1 might play an oncogenic role in HCC and is a potential molecular target of HCC.

In order to clarify whether WWP1 is a potential molecular target of HCC, we determined the role of down-regulation of WWP1 in cell growth by MTT assay. As we supposed, the absorbance of 490 nm both in MHCC97H and SMMC7721 cells was decreased with siWWP1 transfection for 48 and 72 h (Fig. 2C and D).

Cell apoptosis is an important cause of proliferation suppression. To unknown how down-regulation of WWP1 inhibits growth of MHCC97H and SMMC7721 cells, we performed cell apoptosis assay with flow cytometer. The percentage of apoptosis in MHCC97H cells was strongly increased with siWWP1#1 and siWWP1 #2 transfection for 72 h (Fig. 3A). Similar results were obtained in SMMC7721 cells (Fig. 3B). These results were consistent with Chen et al., who demonstrated that down-regulation of WWP1 could induce the apoptosis of breast cancer cells [18]. We also performed cell cycle assay to detect whether deficiency WWP1 could infect cell cycle. Unexpectedly, cell phages of MHCC97H were almost unchanged with siWWP1 transfection for 72 h (Fig. S2).

Caspase3 is a crucial executioner of apoptosis and plays an irreplaceable role in apoptosis [25]. Caspase-3 is activated in apoptotic cells both by intrinsic (mitochondrial) and extrinsic (death ligand) pathways [29,30]. To examine the possible mechanisms underlying siWWP1-mediated cell apoptosis, the expression of cleaved caspase3 in MHCC97H and SMMC7721 cells treated with siWWP1 #1 and siWWP1 #2 was detected by Western blotting. Our data showed that protein levels of cleaved caspase3 were strongly increased both in MHCC97H and SMMC7721 cells with siWWP1 transfection (Fig. 3C and D). Significantly, our results also showed that the caspase3 inhibition could prevent MHCC97H cell apoptosis induced by the knockdown of WWP1 (Fig. 4).The correlation between WWP1 and caspase3 has not been reported, here we demonstrated that knockdown of WWP1 strongly induced the activation of caspase3 protein.p53 is not only well known for suppressing tumors, but also induce cell apoptosis [10]. Therefore, we examined impaction of silencing WWP1 on the expression of p53 in MHCC97H and SMMC7721 cells. As we supposed, protein levels of p53 in these two HCC cells were increased with siWWP transfection (Fig. 3E and F). These results were consistent with Laine et al., who found that WWP promoted the nuclear export of p53 by inducing p53 ubiquitination, thereby reduces the transcriptional activities of p53 [10].

Our results indicated that deficiency WWP1 suppressed cell growth and caused apoptosis in HCC cells through the activation of caspase3 and p53 expression. Protein levels of WWP1 are higher in most HCC cancerous tissues as compared with their matched adjacent non-tumor tissues. This study may provide important clues for HCC treatment, and it's very significant in-depth study the correlation between WWP1 and HCC in the future.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.117.

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