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The JNK inhibitor SP600129 enhances apoptosis of HCC cells induced by the tumor suppressor WWOX

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Abstract

Background/Aims—The FRA16D fragile site gene WWOX is a tumor suppressor that participates in p53-mediated apoptosis. The c-jun N-terminal kinase JNK1 interacts with WWOX and inhibits apoptosis. We investigated the function of WWOX in human hepatocellular carcinoma (HCC) and the effect of JNK inhibition on WWOX-mediated apoptosis.

Methods—Allelic imbalance on chromosome 16 was analyzed in 73 HCCs using 53 microsatellite markers. WWOX mRNA in HCC cell lines and primary HCCs was measured by real-time RT-PCR. Effects of WWOX on proliferation and apoptosis and the interaction between WWOX and JNK inhibition were examined.

Results—Loss on chromosome 16 occurred in 34 of 73 HCCs. Of 11 HCC cell lines, 2 had low, 7 intermediate, and 2 had high WWOX mRNA. Of 51 primary tumors, 23 had low WWOX mRNA. Forced expression of WWOX in SNU387 cells decreased FGF2-mediated proliferation and enhanced apoptosis induced by staurosporine and the JNK inhibitor SP600129. Conversely, knockdown of WWOX in SNU449 cells using shRNA targeting WWOX increased proliferation and resistance to SP600129 induced apoptosis.

Conclusions—WWOX induces apoptosis and inhibits human HCC cell growth through a mechanism enhanced by JNK inhibition.

Keywords

WWOX; hepatocellular carcinoma; JNK; apoptosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death worldwide, with approximately 600,000 deaths in 2002(1). Risk factors for HCC include chronic hepatitis B and C virus infections, alcohol, non-alcoholic steatohepatitis, and dietary aflatoxins(2). The multi-kinase inhibitor sorafenib is currently the only approved agent for advanced HCC(3). Understanding the pathogenesis of HCC will allow the development of novel therapies.

Several genes within common fragile sites function as tumor suppressors in HCC, including FHIT (FRA3B), Parkin (FRA6E) and M6P/IGF2R (FRA6E) (4–9). Chromosome 16q is often altered in HCC, with loss of part or the entire arm (10,11) (12,13). The WW-domain containing oxidoreductase (WWOX) gene spans 1.5 Mb within the FRA16D fragile site at 16q23 (14–17). WWOX is highly expressed in secretory epithelial cells, endocrine and exocrine organs, and urinary epithelial cells (18). WWOX has tumor suppressor properties (19)(59) and has been implicated in breast (14,20–26), ovarian (27–29), prostate (30–32), oral (33,34), gastric (35,36), pancreatic (37), esophageal (38), cutaneous (39) (40–42), thyroid (43) and lung carcinomas (25,44–48) and meningiomas(49).

Homozygous deletions in the WWOX gene have been detected in cancer (12,29,50,51), but point mutations of WWOX are rare (21,38). Multiple WWOX splice variants, some of which have a dominant negative function, are expressed in cancer (52). WWOX is downregulated by promoter methylation in hematopoietic, pancreatic, breast, bladder and lung cancers (25,50). In knock-out mice, haploinsufficiency of WWOX is tumorigenic (47,53–55). These alterations are similar to those of the FRA3B gene, FHIT, in cancer (5,25,56).

Functionally, WWOX is essential for TNF, UV, staurosporine and p53 mediated cell death (20). WWOX is localized in the mitochondria, nucleus, and Golgi (57,58). WWOX is released from mitochondria during the mitochondrial membrane permeability transition, translocates to the nucleus, and cooperates with p53 in mediating apoptosis (20,57,58). c-jun N-terminal kinase (JNK) interacts with WWOX, phosphorylating WWOX at Tyr33 and Tyr61 residues and inhibiting WWOX-mediated apoptosis (59,60). Consequently, it appears that WWOX is important in the p53 and JNK1 pathways. Both p53 and c-jun are important growth regulators in HCC, therefore it is likely that WWOX plays an important role in the pathogenesis of HCC. Prior evaluation of WWOX mRNA expression in human HCC cell lines showed that 13 of the 18 cell lines examined expressed low levels of WWOX mRNA (61).

We report here an analysis of WWOX expression in primary HCCs and the functional effects of suppression or restoration of WWOX expression in HCC cells. To determine the role of WWOX in hepatocarcinogenesis, we investigated 1) WWOX expression in HCCs, 2) allelic imbalance (LOH) in the WWOX gene region, 3) homozygous deletions in the WWOX gene in HCC cell lines, 4) the effect of WWOX on FGF2 mediated cell proliferation and 5) the effect of WWOX on HCC cell apoptosis induced by the non-specific kinase inhibitor, staurosporine, and the JNK inhibitor, SP600129.

MATERIALS AND METHODS

HCC tissues and cell lines

Paired tumor and benign liver tissues were obtained from 109 patients undergoing resections for HCC at Mayo Clinic between 1987 and 2003, snap-frozen in liquid nitrogen and stored at −80C. The Mayo Clinic Institutional Review Board approved the study.

Clinical characteristics were ascertained from the medical record in February 2007 (Table 1). HBV and HCV status was confirmed by PCR. Of the 109 patients, 75 were Caucasians

(68.80%), 4 Asians (3.66%), 1 Hispanic (0.91%), 1 Black (0.91%), 6 Pacific Islanders (5.50%) and 23 unknown race (21.10%).

DNA from 73 patients was used for LOH analysis. WWOX mRNA expression and splice variant analyses were performed on a subset of samples that yielded high-quality RNA: real time PCR, 51; Northern blotting, 11; and splice variant analysis, 33 (variants from 11 were sequenced).

Eleven human HCC cell lines (Hep3B, HepG2, HUH7, PLC/PRF5, SKHep1, SNU182, SNU387, SNU398, SNU423, SNU449, and SNU475) obtained from the American Type Culture Collection were maintained in RPMI1640 with 10% fetal bovine serum (FBS) at 37C and 5% CO_2.

Real-time PCR

RNA was purified using Trizol (Invitrogen) followed by RNAeasy column clean up (Qiagen Inc.)(62). Quantitative real-time PCR was performed using cDNA from 51 pairs of HCC tumor and benign tissue and 11 HCC cell lines on an ABI 7300 Sequence Detection System using 2X TaqMan Universal Master Mix and ABI Assay on Demand gene expression kits for WWOX and 18S ribosomal RNA (Hs_00249590_m1, Hs_00251319_m1 and 4333760). Primers were from both the 5'- and 3'-regions of the WWOX mRNA. A standard curve was prepared using WWOX cDNA cloned into the Topo-TA vector. Standards were measured in triplicate; test samples in duplicate.

WWOX splice variants

Analysis of splice variants was performed for 11 HCC cell lines and 33 benign and primary HCC tumor pairs. cDNA was prepared from 4 ug of total RNA using the Superscript III First Strand Synthesis System (Invitrogen Life Technologies Inc., Carlsbad, CA). 4 ug of commercial human liver RNA (Clontech Lab. Inc.) was used to prepare control cDNA for all the experiments performed. First and second nested PCR reactions were performed as previously described (29). PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed. For 11 of the primary HCCs, the 1.2 kb WWOX transcript and other smaller non-full length transcripts were subcloned into the TOPO TA plasmid vector (Invitrogen Life Technologies) and sequenced with appropriate primers on an ABI 377 DNA sequencing system.

LOH analysis

LOH analysis of DNA from 73 HCC and benign tissue pairs used 53 microsatellite markers on chromosome 16. Nine markers were within FRA16D, with four of the nine within the WWOX gene. LOH was performed as described previously(63).

Exon PCR and analysis for homozygous deletions

Exon screening using DNA extracted from 11 HCC cell lines was performed using primers for each of the 9 exons and standard PCR conditions as previously described (14). PCR products were separated on 2% agarose gels and examined for homozygous deletions.

Methylation specific PCR

DNA from 11 HCC cell lines was treated with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange CA), and eluted in 40 ul of elution buffer. One ul of bisulfite-modified DNA was amplified in a final reaction volume of 25 ul, using 1 X PCR buffer, 1.5 mM $MgCl_2$ (Applied Biosystems), 200 uM each dNTP's (Roche Diagnostics, Indianapolis, IN), 50 uM each of the methylation specific primers and 1.25 units of AmpliTaq Gold (Applied

Biosystems). CpGenome Universal Methylated Bisulfite-treated human genomic DNA (Chemicon, Temecula, CA) was used as a positive control for methylation specific PCR. The amplification was performed using an initial hot-start step at 95C for 12 min, followed by 32 cycles of 95C for 45 s, annealing temperatures of either 65C or 58C for 45 s, extension at 72C for 45 s, and a final extension step at 72C for 10 min. The primers used for the methylation specific PCR were WWOX-M-F1 (5' – TTTATTGTTCGTGTCGTTTAGTTTCGC – 3') (+144 – +170) and WWOX-M-R1 (5' – GCAATACGCAAACGTAAACGATCGA – 3') (nucleotides +1 – +25 from the transcription start site) giving a 170 bp product and WWOX-M-F2 (5' – GCCCCTACTCCGGCGAAACGGGAGCGGC -3') (+488 – +516) and WWOX-M-R2 (5' – GCGCACTCCAACGCAACGCGTACGA – 3') (+310 – +334) giving a 207 bp product.

cDNA cloning

A 1,251 bp WWOX cDNA (bases 61 to 1311 of GenBank NM_016373, missing 13 amino acids at the C-terminal) was cloned into the Creator™ System plasmid, pDNR-1 (Clontech). Cre recombinase was used to place the WWOX sequence into the pLP-EGFP-C1 acceptor to produce plasmid pLP-WWOX-EGFP-C1, which expresses a WWOX-EGFP fusion protein.

shRNA targeting WWOX mRNA

An shRNA construct targeting WWOX mRNA was made by inserting the sequence 5'–AAGACTCAGTGGGAACATC–3' into plasmid pSS-H1p, provided by Dr. Daniel D. Billadeau.

Transient and stable transfections of the SNU387 and SNU449 cell lines

HCC cell line SNU449, which has a high level of WWOX expression, and SNU387, which has a low level of WWOX expression, were selected for transfection. SNU449 carries a G to A substitution at nucleotide 481 of the p53 gene, leading to an alanine to threonine mutation at codon 161 which is associated with nuclear p53 accumulation, while SNU387 has a truncated p53 gene due to an A to T substitution at nucleotide 490 which leads to a stop codon at position 164.

SNU387 cells were transfected with control vector or the pLP-WWOX-EGFP-C1 plasmid. Cells were seeded at 100,000 cells per ml in 6 well plates, in RPMI1640 with 10% FBS. After overnight incubation, cells were transfected using 2 μg DNA and 3 ul Lipofectamine 2000 reagent (Invitrogen). 48 hours later selection was started using G418 (Invitrogen)(58c). Established clones (SNU387 Vector, SNU387 WWOX1 and SNU387 WWOX3) were grown in 150 μg/ml G418.

SNU449 cells were transfected with the WWOX-shRNA-pSS-H1p construct as above; established clones were grown in 350 μg/ml G418.

WWOX antibodies

Anti-WWOX monoclonal antibody H2267 was provided by Dr. Akira Watanabe (64).

Western immunoblotting

Twenty micrograms of total cellular protein from SNU449 stably transfected with shRNA targeting WWOX was resolved by SDS-PAGE (61). Blots were probed with WWOX monoclonal antibody H2267 and anti-phospho-ERK antibody (Cell Signaling Technologies Inc., MA). Anti-Actin and anti-total ERK were used to control for loading.

Cell proliferation and apoptosis assays

SNU449 cells and clones stably transfected with the pSSH1p vector or the WWOX-shRNA-pSS-H1p plasmid, were plated at 5,000 cells per well. SNU387 cells and clones stably-transfected with vector or pLP-WWOX- EGFP-C1 were plated at 10,000 cells per well in RPMI1640 with 10% FBS. Cells were treated with staurosporine, FGF2(65), or the JNK inhibitor SP600129(66). Cell proliferation was assessed by cell counting, MTT assay, or BrdU incorporation(67). Apoptosis was assessed by DAPI staining and fluorescence microscopy (68).

RESULTS

WWOX mRNA expression is decreased in 45% of HCCs

WWOX mRNA expression in 11 HCC cell lines and 51 tumor and benign tissue pairs was determined by real time RT-PCR using 18S ribosomal RNA as an internal control (Figure 1A and B). We found moderately high WWOX mRNA expression in SNU449 cells and essentially undetectable expression in SNU387 cells as previously reported (48). Of the 51 primary HCCs, WWOX was downregulated in 23 (45%) as compared to the adjacent benign tissue.

45% of HCCs show allelic imbalance (LOH) on chromosome 16

The 73 tumors were designated as non-informative (homozygous) or informative (heterozygous). For informative samples, a ratio was determined between tumor and benign tissue based on relative peak fluorescence values. Samples were scored as negative (no LOH) or positive (LOH)(Figure 2). 39 (53%) showed no LOH, 13 (18%) showed partial LOH on chromosome 16q, 6 (8%) showed loss of the entire q arm, and 15 (21%) showed loss of both the p and q arms. There was no significant correlation between partial or complete loss of chromosome 16 and patient survival.

WWOX splice variants occur more frequently in tumor than benign tissues

Of the 11 HCC cell lines, 4 expressed only the full-length WWOX mRNA transcript; the remaining 7 expressed only splice variants without the full-length transcript. Of the 33 pairs of benign and primary HCC tumors, the full-length 1.25 kb transcript was expressed by every benign and tumor tissue. In addition, several tumor and benign tissues expressed shorter splice variants. Seven of the 33 primary HCCs expressed only the full-length transcript, 10 expressed alternative splice variants in tumor but not in associated benign tissue, 15 expressed variants in both tumor and benign tissue, and one HCC expressed a smaller variant in benign tissue only. Therefore, 25 (76%) of the 33 tumors expressed splice variants, whereas only 14 (42%) of the 33 benign tissues expressed splice variants ($p < 0.01$). All the splice variants were previously described in other tumor types (29). The functional consequences of these splice variants are not completely elucidated, however some may have dominant negative effects on WWOX function and others may be resistant to degradation. In particular, the WWOXDelta5-8 variant missing exons 5–8 (encoding the oxidoreductase domain) lacks a phosphorylation site for the tyrosine kinase activated Cdc42-associated kinase (Ack1), which is critical for polyubiquitination and degradation of WWOX(69). Hence, it fails to undergo Ack1-induced degradation and may also have a dominant negative effect on WWOX function.

Analysis for homozygous deletions

None of the cell lines had homozygous deletions of the WWOX gene. As previously reported, the PLC/PRF/5 (Alexander) cell line showed an extra PCR fragment representing an additional exon (61).

No evidence of WWOX promoter methylation

None of the 11 HCC cell lines showed evidence of promoter methylation.

Downregulation of WWOX in SNU449 increases proliferation and decreases staurosporine-induced apoptosis

SNU449 cells were transiently-transfected with a WWOX shRNA plasmid construct or the control vector pSS-H1p. In control DMSO treated cells, suppression of WWOX expression significantly increased proliferation. Staurosporine significantly reduced cell viability to approximately 25% of control values in both vector-transfected and WWOX suppressed cells (Figure 3A). Suppression of WWOX expression resulted in a decrease in both baseline and staurosporine-induced apoptosis (Figure 3B and 3C).

In SNU449 cells stably-transfected with the WWOX shRNA plasmid, real-time PCR showed 40% lower WWOX mRNA than in vector-transfected controls; Western immunoblotting also showed a 30% decrease in WWOX protein (Figure 3D).

The proliferation rates of the Vector and WWOX shRNA-transfected cells were measured by cell counting and BrdU incorporation. Downregulation of WWOX increased SNU449 shRNA cells compared to SNU449 Vector cells by 2 days after cell plating, persisting to day 5 (Figure 3E). Downregulation of WWOX also significantly enhanced unstimulated and FGF2-stimulated cell proliferation (Figure 3F).

Restoration of WWOX expression markedly stimulates apoptosis and inhibits proliferation of SNU387 cells

SNU387 cells, which express low levels of WWOX, were transfected with pLP-WWOX-EGFP-C1 or the control pLP-EGFP-C1. After transient transfection, there was a profound change in cell morphology (Figure 4A), with development of nuclear condensation, cytoplasmic vacuolization and markedly increased cell death in WWOX-transfected cells, with up to 95% cell death at 72 hours (Figure 4B and C). With this profound degree of cell killing it was impossible to select SNU387 clones expressing WWOX at high levels.

Twelve to sixteen weeks after beginning drug selection, we were able to isolate two WWOX-transfected clones resistant to G418. Both clones, SNU387 WWOX1 and SNU387 WWOX3, showed substantial inhibition of proliferation compared to vector-transfected cells (Figure 4D). The WWOX-transfected clones showed significantly increased staurosporine-induced apoptosis (Figure 4E). Finally, basal and FGF-stimulated proliferation were also decreased by WWOX transfection (Figure 4F).

The JNK inhibitor SP 600129 enhances WWOX-induced apoptosis and growth inhibition

Phosphorylation by JNK1 kinase appears to regulate WWOX function. To assess the interaction of JNK kinase with WWOX in HCC, we examined the effects of JNK kinase inhibition on apoptosis and cell proliferation in cells expressing WWOX at low or high levels. SNU387 Vector, SNU387 WWOX1 and SNU387 WWOX3 cells treated with SP600129 for 6 hours were examined by phase contrast and fluorescence microscopy after DAPI staining (Figure 5A). SNU387 WWOX1 and SNU387 WWOX3 cells showed increased apoptosis when compared to vector-transfected cells. Apoptosis was significantly enhanced by SP600129 (Figure 5B). SP600129 also enhanced the WWOX-induced decrease in cell proliferation (Figure 5C). Next, we examined the effect of WWOX and SP600129 on phosphorylation of ERK1/2 kinase, which is downstream of JNK kinase. As expected, treatment of SNU387 Vector cells with SP600129 substantially inhibited ERK phosphorylation. In addition, concurrent expression of WWOX further inhibited ERK phosphorylation (Figure 5D).

Finally, we examined the effect of treatment with SP600129 on apoptosis and proliferation in cells with shRNA-mediated downregulation of WWOX. After downregulation of WWOX, SP600129-induced apoptosis was significantly decreased (Figure 5E). Further, while down-regulation of WWOX increased cell proliferation, SP600129 significantly reduced the proliferation of SNU449 cells to about 25% of control (Figure 5F).

DISCUSSION

The WWOX gene spans the FRA16D fragile site at chromosome 16q23. WWOX has been implicated in p53-mediated apoptosis and functions as a tumor suppressor in several cancers. Mutations and deletions in the coding region of WWOX rarely occur in cancer (11). Suppression of WWOX expression by promoter hypermethylation has been demonstrated in squamous cell lung carcinomas, invasive breast carcinomas and transitional cell bladder carcinomas, with methylation frequencies of 62.5%, 53%, and 29%, respectively (24). In contrast, WWOX does not appear to be regulated by methylation in breast cancer cell lines (19).

Eleven (61%) of 18 human HCC cell lines showed downregulation of WWOX mRNA expression, and 75% showed low or undetectable levels of WWOX protein, suggesting the involvement of the gene in hepatocarcinogenesis (61). Therefore, we 1) determined WWOX expression in primary HCCs; 2) investigated the regulation of WWOX expression in HCC, 3) determined whether WWOX functions as a tumor suppressor in HCC cells, and 4) investigated the interaction of WWOX with JNK pathway inhibition.

We found that modulation of WWOX in HCC cells has a dramatic effect on cell morphology, proliferation, and apoptosis. Forced expression of WWOX produced significant morphologic changes, induced spontaneous apoptosis, and profoundly inhibited cell proliferation. Conversely, downregulation of WWOX led to increased cell proliferation, supporting the tumor suppressor role of WWOX in HCC.

To elucidate the mechanisms regulating inhibition of WWOX function in HCC, we analyzed mRNA expression, LOH, mRNA splice variants, and promoter methylation of the WWOX gene.

WWOX mRNA expression was decreased in 45% of HCCs when compared to surrounding benign tissue. LOH analysis showed that chromosome 16 is frequently lost in its entirety in HCC. Since the 16q arm also contains the E cadherin tumor suppressor at 16q22, there may be functional cooperativity between loss of WWOX and loss of E cadherin and other tumor suppressors on chromosome 16. E cadherin loss has been shown to predict aggressive clinical behavior of HCCs, including recurrence of disease after liver transplantation(70–74).

WWOX promoter region methylation was not found in any of the 11 HCC cell lines tested by methylation-specific PCR. Treatment of HCC cell lines with the demethylating agent 5-aza-2’-deoxycytidine did not result in enhanced WWOX mRNA expression, confirming that WWOX is not regulated by methylation. It is possible that, similar to the observation in breast cancer, the WWOX promoter is regulated by methylation in primary HCCs, but not in cell lines.

Multiple splice variants of the WWOX gene have been described, some of which may have a dominant negative function, suggesting that WWOX function is regulated by alternative splicing. Tumors were significantly more likely than benign tissues to have splice variants, but no statistically significant correlation was found between the presence or the absence of splice variants and any clinical variables. This may have been due to the relatively small number of tumors examined.

WWOX profoundly enhanced both spontaneous and staurosporine-induced apoptosis and inhibited HCC cell proliferation. JNK1 kinase phosphorylates WWOX and antagonizes its p53-mediated effects on apoptosis and the cell cycle. To elucidate the potential role of JNK kinase in WWOX-mediated effects in HCC and the potential for inhibition of JNK as a therapeutic modality, we treated HCC cells with the JNK inhibitor SP600129. SP600129 enhanced WWOX-induced apoptosis and also cooperated with WWOX in suppressing HCC cell proliferation. This is consistent with the reported effect of JNK in driving cyclin D1 expression and cell proliferation during liver regeneration and during stimulation of liver cell growth by the HCV non-structural protein 3(75), but contrasts with evidence that phosphorylation and activation of JNK in both non-transformed and transformed hepatocytes promotes apoptosis induced by oxidative stress, death receptor signaling, acetaminophen and troglitazone(76–79). Thus, JNK appears to mediate either pro-apoptotic or pro-survival effects depending on the cell type and system studied.

In summary, forced expression of WWOX in HCC cell lines by transient or stable transfection induces profound changes in cell morphology, a high rate of spontaneous apoptosis, and increased sensitivity to apoptosis induced by the JNK inhibitor SP600129, supporting the tumor suppressor function of WWOX in HCC. Measures to enhance WWOX expression or WWOX dependent pathways in HCCs with low WWOX expression may be of therapeutic importance. The available data suggests that WWOX is a critical regulator of the p53 and JNK1 kinase stress response pathways. Additional studies to explore the role of these pathways in WWOX-function are anticipated.

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Abbreviations

HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; WWOX, WW domain containing oxidoreductase gene; FHIT, fragile histidine triad gene; FBS, fetal bovine serum.

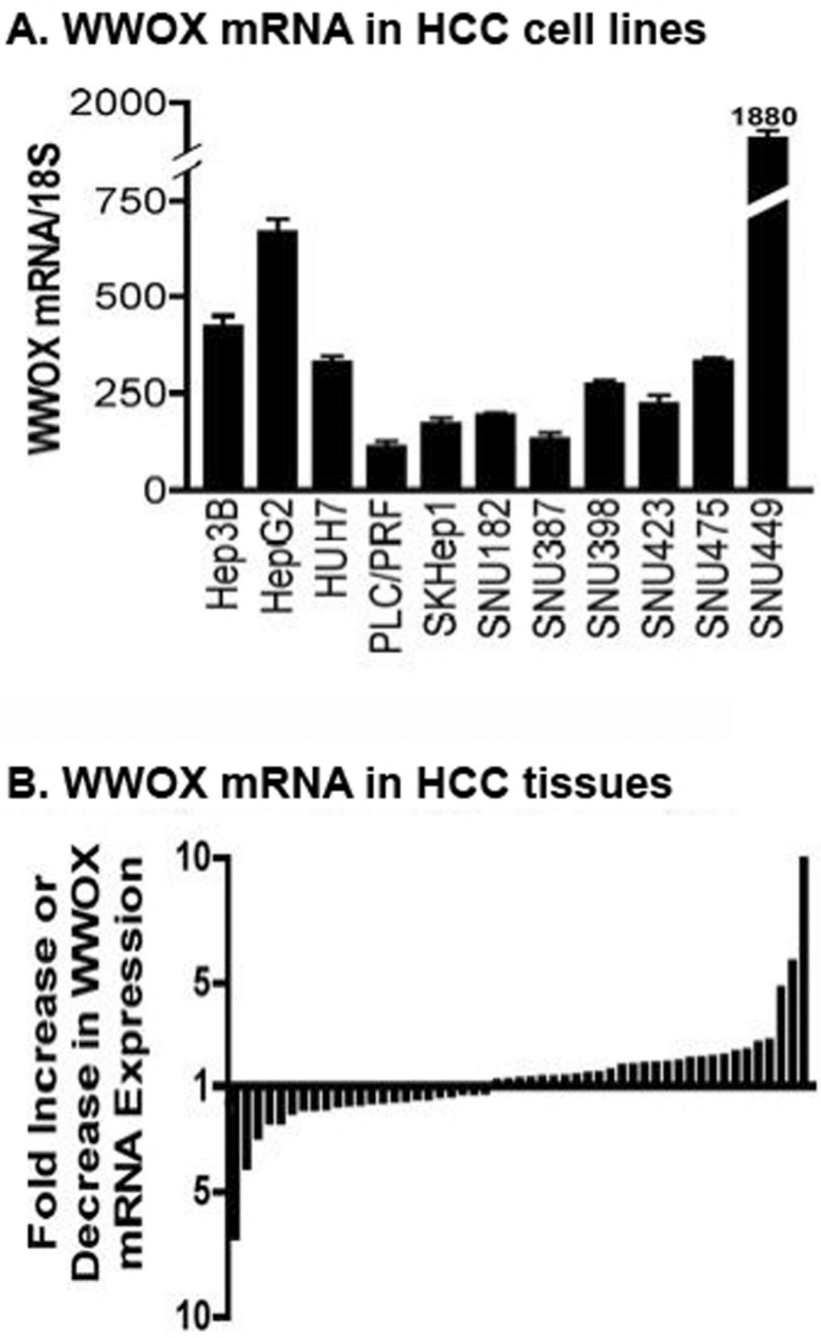

Figure 1. WWOX mRNA expression in HCC cell lines and primary tumors. WWOX mRNA expression of HCC cell lines and benign and tumor tissue pairs were evaluated by real-time PCR
(A) Of 11 HCC cell lines analyzed, 2 showed very low expression, 7 intermediate and 2 higher expression levels of WWOX mRNA. (B) WWOX mRNA expression was decreased in 45% of HCCs compared to adjacent benign tissue.

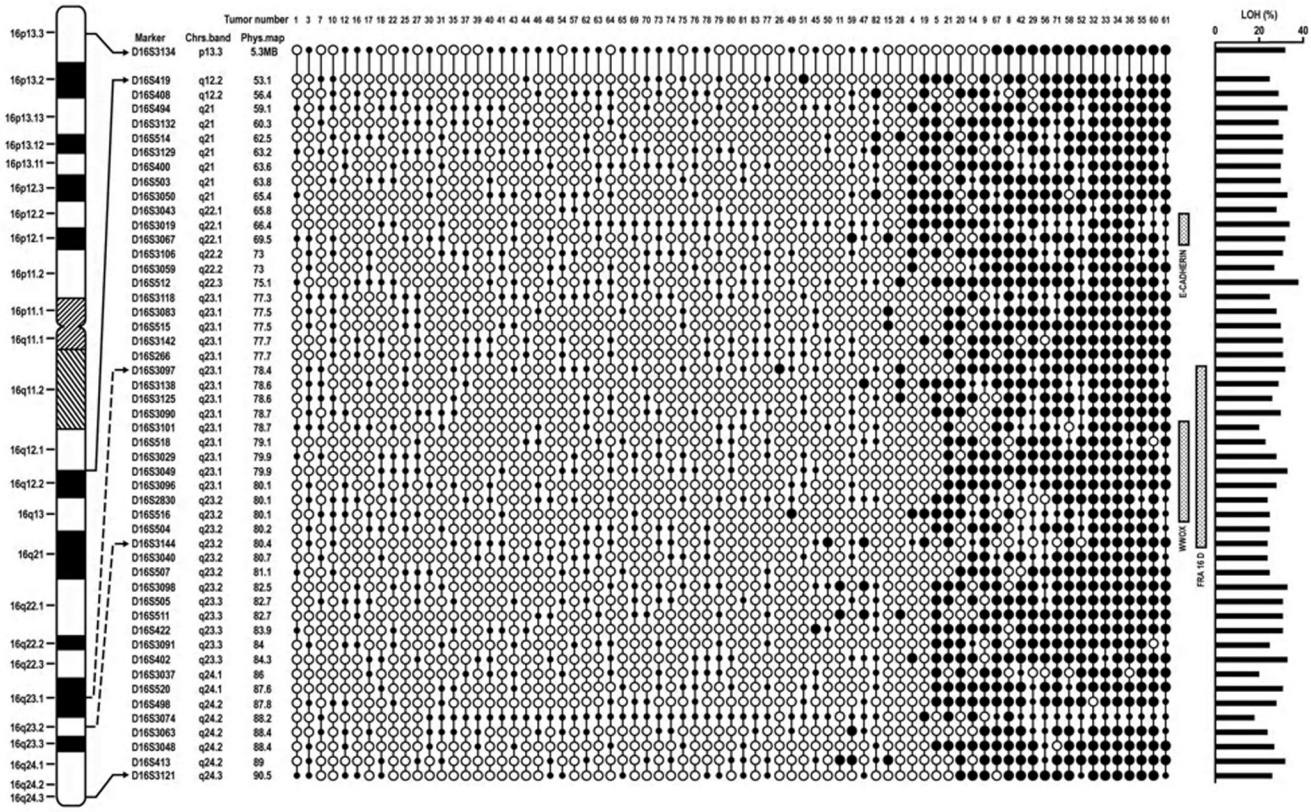

Figure 2.

LOH analysis of chromosome 16 in 73 primary HCCs. Five of 73 HCCs analyzed showed loss of chromosome 16q only, an additional 15 showed loss of the entire chromosome; the peak LOH at the WWOX locus was 40%. LOH analysis of 73 HCCs chromosome 16 using 53 markers showed no LOH in 39 of the samples, partial LOH of a few markers in 13, loss of the entire q arm in 6, and total loss of both the p and q arms in 15. Informative samples were scored as LOH based on a ratio of benign and its corresponding tumor value obtained from the relative fluorescence of the peaks. Legend: white circles – no LOH, small black dots – non-informative (homozygous), large black dots – LOH.

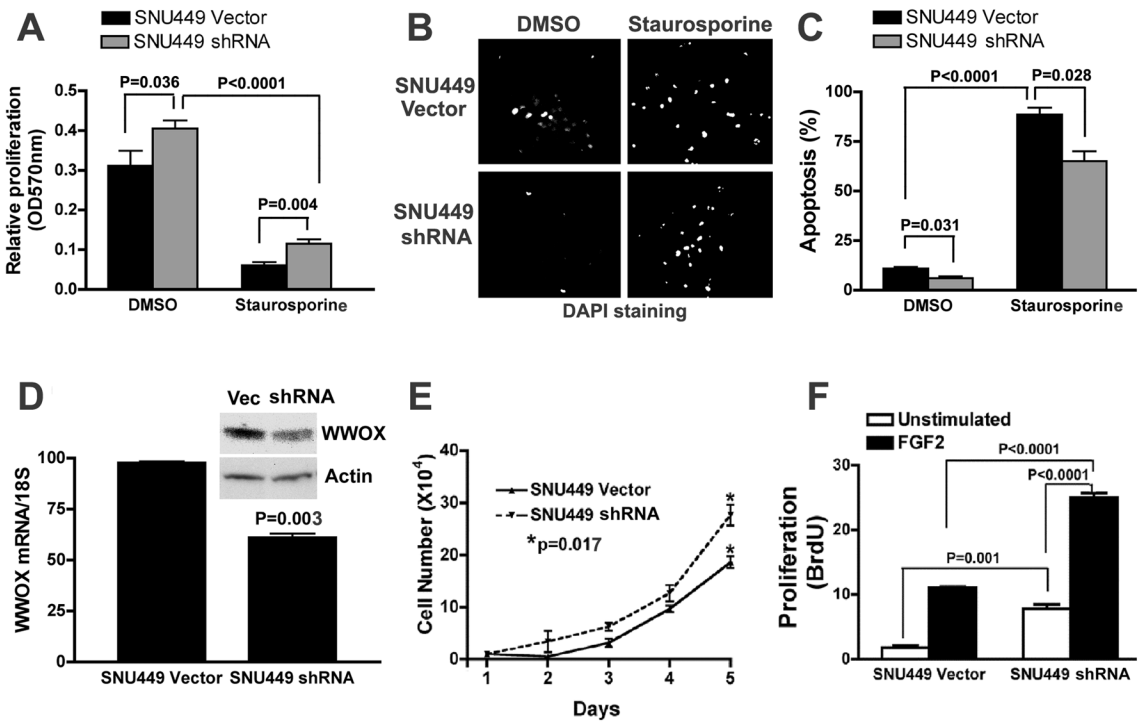

Figure 3. shRNA-mediated knockdown of WWOX in SNU449 cells inhibits staurosporine-induced apoptosis and enhances FGF2-induced cell proliferation

(A) Relative proliferation of vector and WWOX shRNA transfected SNU449 cells assessed by MTT assay. Transient transfection of WWOX shRNA increased proliferation of SNU449 cells. Staurosoporine substantially inhibited proliferation of both vector and shRNA transfected cells. (B and C) WWOX shRNA decreased the sensitivity of SNU449 cells to staurosporine-induced apoptosis, as measured by DAPI staining and fluorescence microscopy. (D) SNU449 cells stably transfected with WWOX shRNA have approximately 40% lower WWOX mRNA expression than pSSH1p Vector control cells by real-time PCR; this result was confirmed by Western immunoblotting (inset). (E) Stable knockdown of WWOX increased proliferation of SNU449 cells. (F) Suppression of WWOX expression significantly enhanced FGF-mediated proliferation of SNU449 cells, as measured by BrdU incorporation.

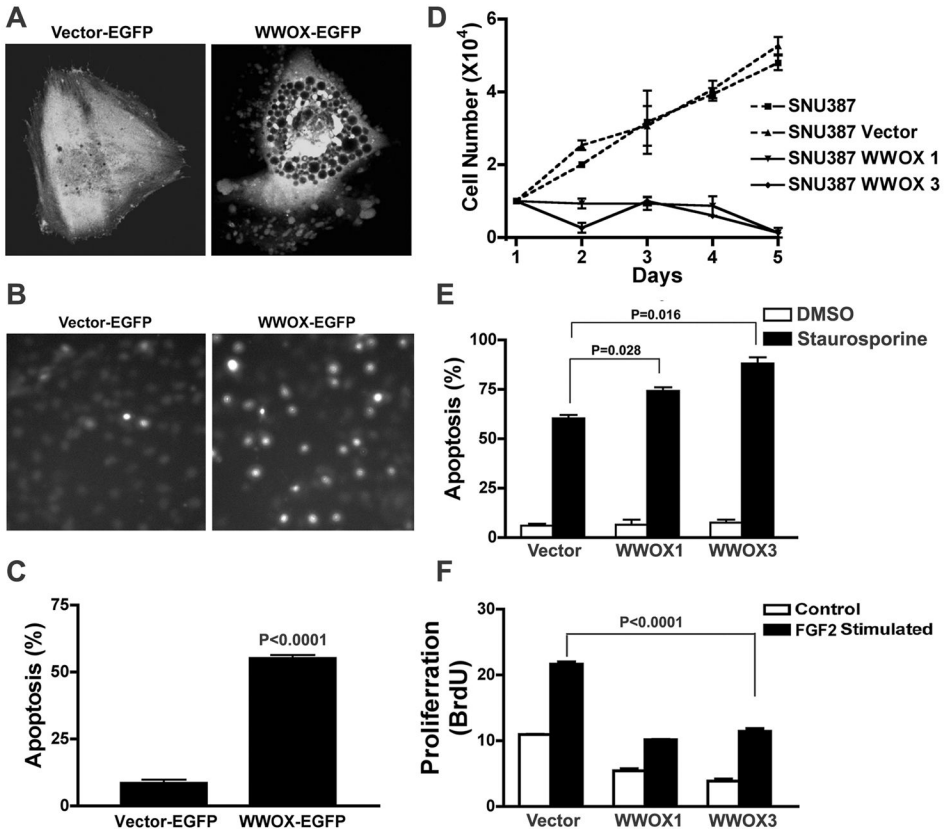

Figure 4. Transient or stable forced expression of WWOX in SNU387 cells induced marked morphologic changes, increased staurosporine-induced apoptosis and inhibited FGF2-mediated cell proliferation

(A and B) High and low-power fluorescence images of SNU387 cells transiently transfected with Vector or a WWOX-pLP-EGFP-C1 construct. The WWOX-expressing cells exhibit profound vacuolation and increased apoptosis. (C) Quantitation showing substantially increased apoptosis in SNU387 cells transiently transfected with WWOX, as compared to vector-transfected controls. (D) Cell counting assay showing markedly decreased proliferation rate of two clones of SNU387 cells stably transfected with WWOX (WWOX1 and WWOX3), when compared to parental (SNU387) or vector-transfected control cells (SNU387 Vector). (E) WWOX-expressing SNU387 WWOX1 and WWOX3 clones have increased sensitivity to staurosporine-induced apoptosis. (F) WWOX-expressing SNU387 WWOX1 and WWOX3 clones show decreased baseline and FGF-stimulated BrdU incorporation as shown here at 96 h after plating.

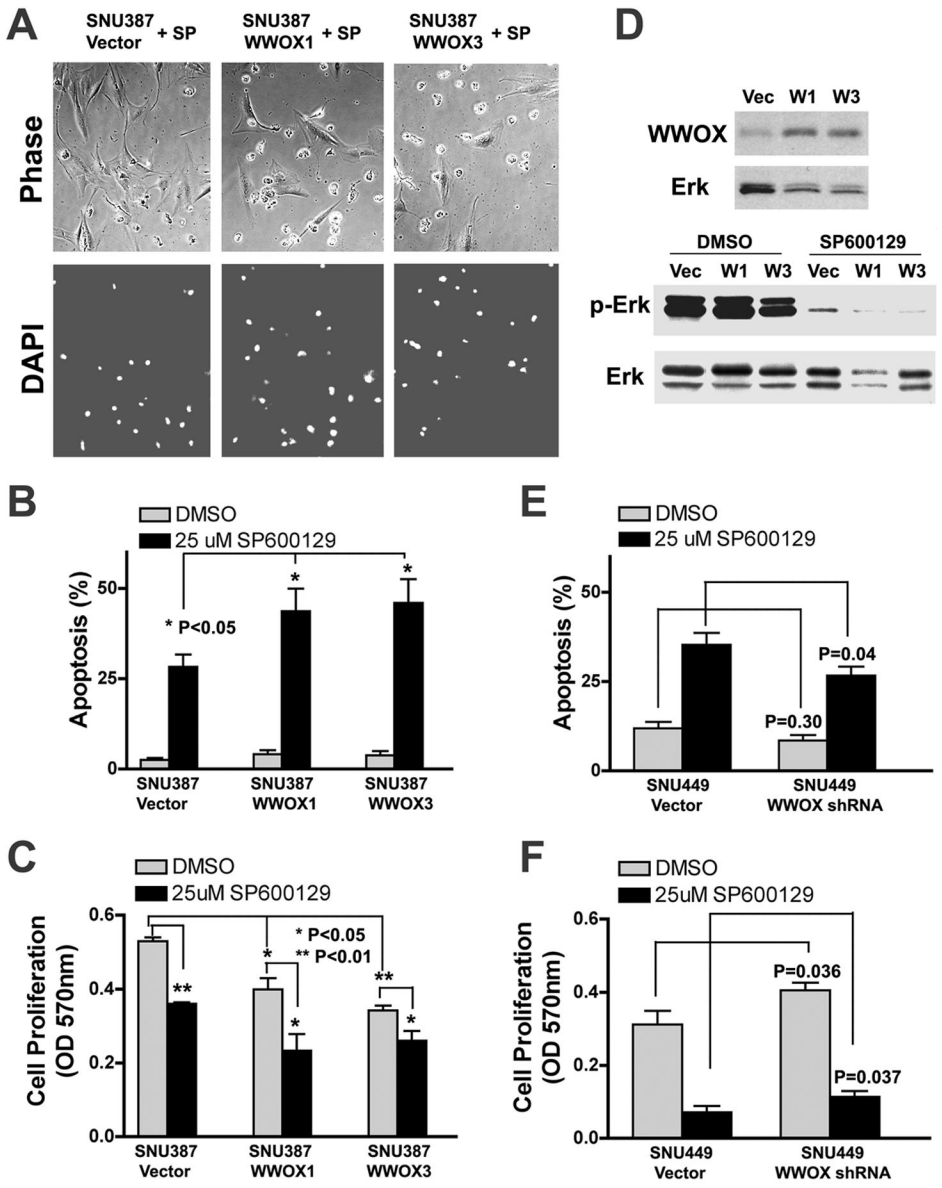

Figure 5. Effects of the JNK inhibitor, SP 600129, on apoptosis and proliferation in SNU387 and SNU449 HCC cell lines with forced expression or downregulation of WWOX expression

(A) SNU387 clones (Vector, WWOX1 and WWOX3) were treated with 25 μM SP600129 for 6 hours and morphology and apoptosis were observed by phase contrast (A upper panels) and fluorescence microscopy following DAPI staining (A lower panels). (B) SP600129 enhances the sensitivity of SNU387 cells to apoptosis induced by WWOX expression. (C) WWOX expression decreases proliferation of SNU387 cells. SP600129 further inhibits proliferation of both vector and WWOX-expressing cells. (D) Western immunoblotting confirms the increased expression of WWOX after stable transfection of SNU387 cells (upper panels). Immunoblotting against phosphoERK showed no significant change after transfection with WWOX, however, expression of WWOX enhances SP600129 inhibition of ERK phosphorylation. Total ERK is used as a loading control (lower panels). (E) Downregulation of WWOX in SNU449 cells leads to decreased sensitivity to SP600129-induced apoptosis. (F) Downregulation of WWOX in SNU449 cells significantly enhanced cell proliferation. This effect was not completely abrogated by treatment with SP600129.

Table 1

Clinical Characteristics of 109 Study Participants

Variable	Participants n (%)
Sex (Male)	65 (59.6)
Age (median, range), years	68 (30 – 91)
Survival (median, range), months	25.7 (0.16 – 206)
Tumor Grade*	
1	12 (11.0)
2	49 (45.0)
3	38 (34.9)
4	7 (6.4)
Etiology#	
HBV	14 (14.1)
HCV	10 (10.1)
HBV & HCV	4 (4.0)
Alcohol	15 (15.2)
Hemochromatosis	4 (4.0)
No known risk factors	52 (52.5)

*Grade was unknown for 3 participants;

#Etiology was unknown for 10 participants