WASF2 Overexpression and Promoter Hypomethylation Are Associated With Poor Clinical Outcomes in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most common and lethal cancers worldwide. Wiskott-Aldrich syndrome protein family member 2 (WASF2) is an integral member of the actin cytoskeleton pathway that plays a crucial role in cell motility. In this study, we aimed to explore the role of WASF2 in HCC carcinogenesis and its regulatory mechanism.

Methods: *WASF2* expression in HCC was analyzed using six public RNA-seq datasets and 66 paired tissues from patients with HCC. Role of *WASF2* in HCC cell phenotypes was evaluated using small interfering RNA (siRNA) *in vitro* and *in vivo*. Epigenetic regulatory mechanism of *WASF2* was assessed in the Cancer Genome Atlas liver hepatocellular carcinoma project (TCGA_LIHC) dataset and also validated in 38 paired HCC tissues.

Results: *WASF2* is overexpressed in HCC and is clinically correlated with prognosis. *WASF2* inactivation decreased the viability, growth, proliferation, migration, and invasion of Huh-7 and SNU475 HCC cells by restoring G2/M checkpoint function, inducing cell death, and inhibiting epithelial-mesenchymal transition, and hindering actin polymerization. In addition, *WASF2* knockdown using siWASF2 in a xenograft mouse model exerted tumor suppressive effect. Furthermore, we observed a negative correlation between *WASF2* methylation status and mRNA expression. The cg24162579 CpG island in the *WASF2* 5' promoter region was hypomethylated in HCC compared to matched non-tumor samples. Patients with high *WASF2* methylation and low *WASF2* expression displayed the highest overall survival.

Conclusions: *WASF2* is overexpressed and hypomethylated in HCC and correlates with patient prognosis. Moreover, *WASF2* inactivation exerts anti-tumorigenic effects on HCC cells *in vitro* and *in vivo*, suggesting that *WASF2* could be a potential therapeutic target for HCC.

Background

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer, comprising around 70–85% of all cases [1]. Despite the recent development of targeted therapies and immunotherapies, HCC-related mortality has increased over the last three decades [2]. Moreover, there is a lack of standard molecular biomarkers for predicting treatment responses due to the phenotypic and molecular complexity and heterogeneity of HCCs [3]; therefore, it is important to understand the pathogenic mechanisms underlying HCC development and progression.

Wiskott-Aldrich syndrome protein family member 2 (WASF2), also known as WASP family verprolin-homologous protein 2, is an integral member of the actin cytoskeleton pathway that is activated by Ras-related C3 botulinum toxin substrate 1 (Rac1). The WASF2-actin-related protein 2/3 (Arp2/3) complex causes nucleation during actin assembly, leading to the formation of lamellipodia [4, 5]. In cancer, studies have shown that WASF2 is involved in the invasion and metastasis of malignant B16F10 mouse melanoma cells [6] and the motility and invasion of pancreatic cancer cells (S2-013 and PANC-1) [7]. Although WASF2 has been reported to be overexpressed in human HCC tissues compared to surrounding

non-tumor tissue and positively correlate with poor overall survival (OS) [8], the role of WASF2 in HCC cell phenotypes remains unclear. In addition, few studies have investigated the regulatory mechanism of WASF2, apart from one recent study in human umbilical vein endothelial cells and human aortic endothelial cells which suggested microRNA-1253 as a novel regulator of WASF2 [9].

In this study, we confirmed that WASF2 overexpression in HCC tumor tissues is associated with poor clinical outcomes in patients with HCC and found that WASF2 suppression interrupts HCC cell growth, proliferation, migration, and invasion *in vitro* and *in vivo* by regulating the cell cycle, apoptosis, and epithelial-mesenchymal transition (EMT). Moreover, we suggest that cg24162579 CpG island methylation in the *WASF2* promoter is an important mechanism for controlling WASF2 expression and that patients with cg24162579 hypomethylation display more aggressive tumor behavior and a poorer prognosis than those with cg24162579 hypermethylation.

Methods

Patients and samples

Sixty-six pairs of frozen HCC tissues and corresponding non-cancerous liver tissues were obtained from the Ajou University Hospital (Suwon, South Korea). Patient demographics and clinical characteristics are presented in Additional file 1: Table S1. All experiments were performed according to the Declaration of Helsinki and the study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-KSP-16-365 and AJIRB-BMR-SMP-17-189). Anonymous serum samples and clinical data were provided by the Ajou Human Bio-Resource Bank, and the need for informed consent was waived.

22K human protein microarray

Protein microarray experiments were performed using a HuProt human proteome microarray v3.0 (CDI Laboratories Inc., Mayaguez, PR, USA) with 20 human plasma samples [five patients with liver cirrhosis, five patients with HCC (at diagnosis, 6 months before diagnosis, and 1 year before diagnosis)]. Briefly, each protein chip was equilibrated using microarray buffer (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.8 mM KH₂PO₄ pH 7.4; 0.05% Triton X-100) for 5 min at 22–25°C and then incubated with 5% skimmed milk (BD Biosciences, San Jose, CA, USA) in microarray buffer for 1 h at 22–25°C. To identify autoantibodies in HCC samples, a blocked protein chip was washed three times with microarray buffer for 10 min, incubated with 20 µg/mL of serum in reaction buffer (50 mM Tris-Cl pH 7.5; 2 mM DTT; 2.5 mM MgCl₂) for 8 h at 4°C, and washed with microarray buffer for 10 min. The washed chip was then incubated with Alexa Fluor goat-anti rabbit 647-conjugated secondary antibodies (1:5 000) diluted in microarray buffer containing 1% skimmed milk for 30 min at 22–25°C. The chip was then washed three times with microarray buffer, dried by centrifugation in a 50-mL conical tube (200 × *g* for 2 min), and scanned using a GenePix 4000B instrument (Molecular Devices, San Jose, CA, USA). All spotted proteins were probed using glutathione S-transferase antibodies with Alexa Fluor goat-anti rabbit 546-conjugated secondary antibodies. The signal intensity of each spot was recorded as the ratio of foreground to

background signal and normalized to glutathione S-transferase. The mean signal intensity of all proteins on the chip was calculated.

Acquisition and analysis of the expression and methylation data in public omics databases

To analyze *WASF2* mRNA expression in HCC, we obtained expression datasets from The Cancer Genome Atlas liver hepatocellular carcinoma project (TCGA_LIHC), the International Cancer Genome Consortium liver cancer RIKEN Japan (ICGC_LIRI), and gene expression omnibus databases from the National Center for Biotechnology Information [GSE6764, GSE12443, GSE77314, GSE89377 (Catholic University of Korea's liver hepatocellular carcinoma project, Catholic_LIHC), GSE93392, and GSE102418].

To analyze *WASF2* methylation status, we detected genome-wide DNA methylation in TCGA_LIHC. Level 3 data sets obtained using an Infinium HumanMethylation450 BeadChip were analyzed using R software (<http://www.R-project.org/>). The correlation between *WASF2* methylation and expression was analyzed and visualized using MEXPRESS (<https://mexpress.be/>).

Cell culture, treatments, and transfection

Human HCC cell lines (Huh-7, Hep3B, PLC/PRF/5, SNU368, SNU398, SNU423, SNU449, and SNU475) were acquired from the Korean Cell Line Bank (Seoul, South Korea). Immortalized normal hepatocytes (MIHA) were provided by Dr Roy-Chowdhury (Albert Einstein College of Medicine, Bronx, NY, USA). HCC and MIHA cells were cultured in RPMI 1640 or Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Invitrogen, Waltham, MA, USA) and 100 units/mL penicillin-streptomycin (GenDEPOT, Barker, TX, USA). THLE-2 immortalized normal hepatocytes were obtained from the AmericanType Culture Collection (ATCC, Bethesda, MD, USA) and cultured in bronchial epithelial cell growth medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen), 5 ng/mL epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA), 70 ng/mL phosphoethanolamine (Sigma-Aldrich), and antibiotics. Cells were grown in a humidified incubator with 5% CO₂ at 37°C.

Cells were starved in serum-free medium for 4 h prior to treatment with human recombinant transforming growth factor-β1 (TGF-β1; 20 ng/mL; R&D Systems, Minneapolis, MN, USA) dissolved in sterile 4 mM HCl supplemented with 0.1% bovine serum albumin (BSA; R&D Systems) for 24 h. Cells were also transfected with 100 nM small interfering RNAs (siRNA) purchased from Bioneer (Daejeon, South Korea) and negative-control RNA duplexes synthesized by Genolution (Seoul, South Korea) for 24–48 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA isolation and quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from frozen tissues and cell lines using QIAzol reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA in a 10 µL final volume using 5X PrimeScriptTM RT Master Mix (Takara Bio, Shiga, Japan). Quantitative real-time

polymerase chain reaction (qRT-PCR) was performed using amfiSure qGreen Q-PCR Master Mix (GenDEPOT) and monitored in real time using a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primer sequences used are listed in Additional file 1: Table S2. All assays were performed three times.

Western blot analysis

Proteins were extracted from tissues and cell lysates using radio immunoprecipitation buffer containing Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Equal amounts of total proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffer saline and 0.1% Tween-20 for 1 h at 22–25°C, incubated overnight with primary antibodies at 4°C, and then incubated with HRP-conjugated secondary antibodies at 22–25°C for 1 h. Chemiluminescence signals were detected using Clarity™ Western ECL Substrate (Bio-Rad Laboratories) and visualized using ChemiDoc™ (Bio-Rad Laboratories). Antibodies are listed in Additional file 1: Table S3 and S4.

Cell viability and growth assays

To analyze cell viability, two HCC cell lines transfected for 48 h in 60 mm dishes were harvested by trypsinization, stained using 0.4% trypan blue solution (Invitrogen), and counted using a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany).

To analyze cell growth, cells were seeded in 12-well plates, transfected with WASF2-targeting siRNA (siWASF2) or negative control siRNA (siNC), and incubated for 90 min at 37°C with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biosesang, Seongnam, South Korea) solution. Dark blue formazan products were dissolved in dimethyl sulfoxide (Biosesang) and absorbance was detected using a TECAN SUNRISE Microplate Reader (TECAN, Zürich, Switzerland) at a wavelength of 570 nm.

Clonogenic proliferation assay

Cells were transfected with siWASF2 or siNC in 60 mm dishes for 48 h, reseeded in 6-well plates (2, 4, or 6 × 10³ cells per well), and incubated at 37°C in a CO₂ incubator for 10 days. Cell colonies were washed with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde for 30 min, stained with 0.5% crystal violet overnight at 22–25°C, and counted using ImageJ software (Laboratory for Optical and Computational Instrumentation, Madison, WI, USA).

Wound healing assay

Cells were transfected with siWASF2 or siNC in 60 mm dishes for 48 h, reseeded in 6-well plates (1.5 × 10⁶ cells per well), and incubated at 37°C in a CO₂ incubator for 24 h. The cell monolayer was scratched manually using a sterile micropipette tip. Initial (0 h after scratching) and residual (48 h after scratching)

gap widths were imaged using an Olympus CKX53 (Olympus, Tokyo, Japan) photomicrograph. Each area was measured three times and expressed as a percentage.

Apoptosis and cell cycle assays

Apoptosis was detected using an Annexin V-FITC Apoptosis Detection Kit (Koma Biotech, Seoul, South Korea). Briefly, cells transfected for 48 h were harvested by trypsinization, rinsed in PBS, and resuspended in 1× binding buffer before being incubated with 1.25 µL of Annexin V-FITC solution at 22–25°C for 20 min in the dark, stained with 10 µL propidium iodide (PI; Sigma-Aldrich), and analyzed using a FACS Aria III flow cytometer (BD Biosciences).

Also, apoptotic cells were evaluated by Hoechst 33342 and PI staining. The transfected cells were fixed in 4% paraformaldehyde (Biosesang) for 10 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 20 min. Then treated with Hoechst 33342 (10 µg/mL; Invitrogen) for 10 min, stained with PI (20 µg/mL; Sigma-Aldrich) for 20 min, and observed on Olympus IX71 (Olympus).

To analyze the cell cycle, transfected cells were collected, fixed using 70% ethanol for 3 h at 4°C, washed in PBS, and stained using 200 µL PBS containing 10 µg/mL RNase A, 1% Triton X-100, and 30 µg/mL PI (Sigma-Aldrich) for 30 min in the dark. The proportion of cells at each stage of the cell cycle was determined using a FACS Aria III flow cytometer (BD Biosciences). Experiments were performed in triplicate.

Migration and invasion assays

For the *in vitro* cell migration and invasion assays, we used 24-well plates and cell culture inserts (BD Biosciences). Prior to the invasion assay, the upper compartment of the cell culture insert was coated with 100 µL of Matrigel (BD Biosciences) diluted to 0.3 mg/mL in serum-free media. After Huh-7 and SNU475 cells had been transfected with siWASF2 or siNC for 24 h, they were seeded onto the cell culture insert with serum-free media and 5–20% fetal bovine serum as a chemoattractant for 24–72 h at 37°C in a 5% CO₂ humidified incubator. Migratory or invasive cells on the lower side of the membrane were stained using a Diff-Quik staining kit (Sysmex Corporation, Chuo-ku, Japan) and imaged using a CKX53 inverted microscope (Olympus) at 200× magnification. Cells were counted in three random fields of view.

Immunofluorescence

Cells were seeded in 24-well plates and fixed in 4% paraformaldehyde (Biosesang) for 10 min at 22–25°C and permeabilized in PBS with 0.2% Triton X-100 (PBST; Sigma-Aldrich) for 10 min. After the cells were washed in PBST, the plates were blocked with 2.5% BSA in PBST for 30 min and incubated with the following primary antibodies: F-actin (1:200; Abcam, Waltham, MA, USA). The plates were washed and then incubated with AlexaFluor-488-conjugated secondary antibodies for 2 h and stained with 4', 6-diamidino-2-phenylindole. The stained plates were analyzed by Olympus IX71 (Olympus).

Animal experiments

Five-week-old BALB/c female nude mice were purchased from ORIENT BIO (Seongnam, South Korea) and six-week-old athymic female nude mice were purchased from Koatech (Pyeongtaek, South Korea), placed in individually ventilated cages under a pathogen-free environment, and allowed to acclimate for two weeks before being used in experiments.

For *in vivo* subcutaneous xenograft assay, Huh-7 cells transfected with siWASF2 or siNC (5×10^5 cells) were diluted in 0.1 mL Matrigel/serum-free Dulbecco's Modified Eagle's Medium and injected subcutaneously into the flanks of BALB/c nude mice. Tumor diameter was measured three times a week for 18 days in the tumor-bearing mice using a digital caliper. Tumor volume was calculated as follows: $Tumor\ volume = 0.52 \times length \times width^2$.

At the end of the experimental period, tumors were removed for weighing and histological analysis. Tumor tissue samples were immediately flash-frozen in liquid nitrogen for RNA and protein extraction and placed in 10% neutral buffered formalin for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC).

For *in vivo* lung metastasis assay, *ras*-transformed NIH-3T3 cells transfected with siWASF2 or siNC (3×10^5 cells) were mixed with 0.2 mL serum-free Dulbecco's Modified Eagle's Medium and injected into the athymic nude mice through tail veins. The mice were sacrificed 14 days after cell inoculation, and the number of the peritoneal nodules were assessed.

All animals were maintained according to the Guide for the Care and Use of Laboratory Animals and experiments were approved by the Ethics Committee for Laboratory Animal Research Center of Ajou University Medical Center (IACUC-2020-0007).

IHC

Tissue samples from patients with HCC were fixed in formalin, embedded in paraffin, and cut into 5- μ m sections that were deparaffinized in xylene, hydrated in graded alcohol, and incubated with primary antibodies (listed in Additional file 1: Table S3) overnight at 4°C. After being washed three times, the sections were incubated with secondary antibodies for 1 h and then a peroxidase substrate until the desired stain intensity had developed.

In the BALB/c nude mouse model, tumors were harvested, fixed in 10% neutral buffered formalin, and embedded in paraffin blocks before being cut into 5- μ m sections and deparaffinized. One section was stained with H&E and another was evaluated by IHC. Both sections were incubated with the antibodies listed in Additional file 1: Table S3.

Bisulfite sequencing polymerase chain reaction (BSP)

Genomic DNA was isolated from human liver tissues and cell lines using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. The DNA quality and concentration of each sample was checked using a NanoPhotometer® N60 (Implen, Munich, Germany) and 1 μ g of DNA was

modified using an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Briefly, DNA was heat-denatured and bisulfite-converted using CT-conversion reagent in a thermocycler. The DNA was bound to a Zymo-spin IC column and desulfonated using M-desulfonation buffer. After being eluted from the column using 10 μ L of M-elution buffer, 3 μ L of bisulfite-converted DNA was used for BSP to amplify the specific *WASF2* promoter region (cg24162579) using TaKaRa EpiTaq HS (Takara Bio) with a T100 Thermal Cycler (Bio-Rad Laboratories). The primers used to detect *WASF2* CpG island methylation were designed using MethPrimer 2.0 (<https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>; Additional file 1: Table S2). PCR was conducted under the following cycling conditions: 40 cycles of 98°C for 10 s, 63°C for 60 s, and 72°C for 30 s. The PCR products were cloned into a pTOP vector and transformed into DH5 α -competent *E. coli* cells using a TOPcloner TA kit (Enzymomics, Daejeon, Korea). Five clones per HCC cell line were expanded overnight and plasmid DNA was extracted using a QIAGEN Plasmid Mini kit (Qiagen). Purified plasmid DNA was sequenced using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) with an M13 primer to analyze the methylation status of specific CpG sites.

Methylation-specific polymerase chain reaction (MSP) and quantitative MSP (qMSP)

MSP and qMSP were performed using primers for methylated or unmethylated DNA designed using MethPrimer. Briefly, 2 μ L of bisulfite-treated genomic DNA was amplified using TaKaRa EpiTaq HS (Takara Bio) under the following cycling conditions: 40 cycles of 98°C for 10 s, 60°C for 40 s, and 72°C for 30 s. The PCR products were analyzed using 2% agarose gel electrophoresis. qMSP was performed using an amfiSure qGreen Q-PCR Master Mix (GenDEPOT) and was monitored in real-time using an ABI 7300 Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 34 s, and 72°C for 30 s, followed by a single cycle of 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s to generate dissociation curves. Relative DNA methylation was calculated using the difference between the Ct values of the methylated and unmethylated PCR products. All measurements were performed in triplicate. The primer sequences used for MSP and qMSP are listed in Additional file 1: Table S2.

Hydroxyurea (HU), 5-aza-2'-deoxycytidine (5-aza) treatment

Cells were treated with 0.5 mM HU (Sigma-Aldrich) or 5 μ M 5-aza (Sigma-Aldrich) for 48–72 h at 37°C in a CO₂ incubator, with the culture medium replaced daily. The treated cells were harvested and used to detect *WASF2* methylation and expression.

Statistical analyses

Receiver operating characteristic (ROC) curves were produced using SPSS version 22 (SPSS, Chicago, IL, USA). OS and disease-free survival (DFS) curves were plotted using Graphpad™ 8.0 software (GraphPad Software, San Diego, CA, USA) using the Kaplan-Meier product limit method. Significant differences between survival curves were determined using the log-rank test. All experiments were performed at least three times. All samples were analyzed in triplicate. Results are presented as the mean $\pm$ standard error of

the mean (SEM). Significant differences between experimental groups were assessed using paired or unpaired Welch's *t*-tests and one-way analysis of variance (ANOVA) in Graphpad™ 8.0 software. *P* values of < 0.05 were considered statistically significant.

Results

Identification of potential driver markers of HCC development using proteomic and transcriptomic databases

To identify potential early diagnostic markers in hepatocarcinogenesis, we analyzed protein and gene expression using the following systemic strategies. Plasma samples from five patients with liver cirrhosis and five patients with early HCC (at diagnosis, 6 months before diagnosis, and 1 year before diagnosis) were analyzed using a 22K protein chip. All subjects were male with a liver cirrhosis or HCC etiology involving hepatitis B virus. Serial clustering analysis revealed the positive expression of 47 autoantibodies from liver cirrhosis to HCC. Although most autoantibodies were not detected in the liver cirrhosis group, all were differentially expressed in the 1 y ago group (1 year before HCC diagnosis), 6 m ago group (6 months before HCC diagnosis), and Dx group (at HCC diagnosis; Fig. 1A).

To select more precise diagnostic biomarkers for HCC, we performed ROC curve comparative analysis (Fig. 1B). Out of the 47 autoantibodies, 20 were significantly specific and sensitive as biomarkers, with an area under the curve (AUC) of > 0.8 (Additional file 1: Table S5). Venn diagram analysis performed with this ROC result and the OS analysis of TCGA_LIHC identified one putative candidate as early diagnostic marker for HCC: WASF2 (Fig. 1C).

WASF2 overexpression is closely related to the clinical outcome of HCC

First, we examined *WASF2* expression in large cohorts of HCC patients from TCGA_LIHC, ICGC_LIRI, and National Center for Biotechnology Information gene expression omnibus databases. Notably, *WASF2* was significantly overexpressed in HCC compared to non-tumor samples in three large cohort datasets (TCGA_LIHC, ICGC_LIRI, and GSE77314; Additional file 2: Fig. S1A) and was remarkably overexpressed in early HCC compared to non-cancerous samples in multistage datasets of hepatocarcinogenesis (GSE6764, GSE12443, and GSE114564; Fig. 1D). Next, we validated *WASF2* expression using qRT-PCR with a set of 66 randomly selected matched pairs of HCC tissues, of which 40 (60.6%) showed *WASF2* overexpression (Fig. 1E). Increased *WASF2* protein expression was confirmed by western blotting in 22 randomly selected human HCC tissues with corresponding non-cancerous liver tissues, revealing that *WASF2* was overexpressed in 16 pairs (72.7%; Fig. 1F). Therefore, *WASF2* overexpression was confirmed in HCC in both public omics databases and our cohort. To select HCC cell lines overexpressing *WASF2*, we measured endogenous *WASF2* levels using qRT-PCR and western blotting in eight different liver cell lines, including the MIHA immortalized normal hepatic cell line. Six of the eight HCC cell lines exhibited high

WASF2 expression compared with the MIHA cells; in particular, SNU475 and Huh-7 had the highest WASF2 expression and were selected for further experiments (Fig. 1G). Next, we performed IHC analysis on non-tumor and tumor tissues from patient with available archived tissue to confirm the expression of the putative marker, WASF2, with brown spots indicating positive antibody staining. Subsequently, WASF2 were positively expressed in tumor tissue compared to non-tumor tissue (Fig. 1H). To further validate the results mentioned before, the expression level of WASF2 protein in HCC tissues from Human Protein Atlas (HPA; <https://www.proteinatlas.org/>) was evaluated and 75% of HCC tissues showed positive expression of WASF2 (Additional file 2: Fig. S1B, C). Since *WASF2* expression was upregulated in all HCC data sets, we assessed the association between *WASF2* expression and the prognosis of HCC patients in the TCGA_LIHC datasets. Kaplan-Meier survival analysis indicated that patients with high *WASF2* expression displayed significantly lower 5-year OS, disease specific survival and progression-free survival rates than those with low *WASF2* expression (Fig. 1I).

WASF2 deactivation results in tumor suppression in vitro

Consequently, this molecule may function as potential oncogene in HCC. Thus, we analyzed WASF2 as a novel driver molecule in hepatocarcinogenesis.

To investigate the biological functions of WASF2 in HCC, its expression was reduced using RNA interference. First, we assessed the efficiency of three siRNAs-targeting *WASF2* in Huh-7 and SNU475 cells, and selected the siRNA with the highest efficiency for further analyses (Additional file 2: Fig. S2A, B). The growth rates of the two HCC cell lines transfected with negative control siRNA and siWASF2 for 96 h were assessed using cell viability and MTT assays, which revealed that *WASF2* knockdown significantly reduced the viability and growth of Huh-7 and SNU475 cells (Fig. 2A). Clonogenic and scratch wound healing assays were also performed to assess the rate of proliferation and the wound healing ability of the two HCC cell lines. These assays indicated that individual *WASF2* knockdown significantly reduced the proliferation and serum-stimulated wound-healing efficacy of Huh-7 and SNU475 cells (Fig. 2B, C).

Gene signatures correlated with WASF2 expression are associated with poor prognosis in patients with HCC

Having confirmed WASF2 overexpression at both the mRNA and protein levels in HCC, we assessed the prognostic relevance of *WASF2* expression in TCGA_LIHC dataset. Gene signatures with expression patterns that correlated highly with *WASF2* expression ($n = 1\ 354$) were selected for cluster analysis ($P < 0.001$; $r > 0.3$ or $r < -0.3$) and HCC patients were divided into *WASF2* high (*WASF2*_high) and *WASF2* low (*WASF2*_low) groups (Fig. 2D). Since the heat map showed two distinct clusters depending on the group, we performed survival analyses with the two groups. Kaplan-Meier survival analysis indicated that patients with HCC who displayed high *WASF2* expression had significantly lower 5-year OS and DFS rates than those with low *WASF2* expression (OS, log-rank $p < 0.0001$, hazard ratio [HR] = 2.31; DFS, log-rank $p < 0.0001$, HR = 1.97; Fig. 2E).

To elucidate the functional role of the gene signatures that correlated with *WASF2* expression, we performed gene set enrichment analysis (GSEA) using The Molecular Signatures Database (MSigDB). *WASF2*-related gene signatures were enriched in the majority of the cancer-associated pathways, of which G2M_CHECKPOINT was identified as a highly significant enriched gene set (Fig. 2F). The four representative enrichment plots further revealed that these *WASF2*-related genes were significantly associated with the cell cycle, liver cancer survival, liver cancer proliferation, and HCC metastasis (Fig. 2G).

Targeted *WASF2* suppression elicits cell arrest, death, and reduced metastatic potential in HCC

Since the G2M checkpoint was the most significant function associated with the *WASF2*-related gene signature in The Molecular Signatures Database (MSigDB), we performed flow cytometry cell-cycle analysis. *WASF2* knockdown significantly increased the proportion of cells in the G2M phase and altered the expression of cell-cycle regulatory proteins (Fig. 3A). Therefore, we analyzed cell death by staining with Annexin V-FITC/PI after *WASF2* knockdown, with subsequent FACS analysis revealing that *WASF2* knockdown significantly induced apoptosis in Huh-7 and SNU475 cells. In addition, *WASF2* knockdown elevated the expression of the cell death regulators cleaved poly ADP-ribose polymerase (PARP), cleaved caspase-3, and cleaved caspase-9 in HCC cells (Fig. 3B). Additionally, Hoechst 33342 and PI were stained to assess morphological changes. When *WASF2* knockdown, it is observed that the apoptotic and necrotic cells increased in Huh-7 and SNU475 cells (Additional file 2: Fig. S3A).

GSEA showed that the *WASF2*-related gene signatures were also enriched in the malignant features of HCC cells; therefore, we conducted *in vitro* migration and invasion assays to identify the role of *WASF2* in the progression of HCC. Modified Boyden chamber assays revealed that *WASF2* knockdown significantly suppressed the migratory and invasive responses of both Huh-7 and SNU475 cells (Fig. 3C). To elucidate the regulatory effect of *WASF2* on EMT, we detected the expression of EMT regulatory proteins in HCC cells stimulated with TGF- β 1 (20 ng/mL) using western blot analysis. *WASF2* knockdown increased zonula occludens-1 (ZO-1) and E-cadherin expression in Huh-7 and SNU475 cells but decreased fibronectin and Snail expression (Fig. 3D). Interestingly, a positive correlation was observed between *SNAI1* with *WASF2* in the tumor group of the TCGA_LIHC dataset (Pearson's correlation coefficient $r = 0.26$, $P < 0.0001$), but not in the non-tumor group (Pearson's correlation coefficient $r = 0.08$, $P = 0.55$; Fig. 3E).

According to previous studies, *WASF2* is the key molecule related to the actin cytoskeleton and reconstruction of the action affects migration and invasion of cancer cells [10]. To identify the *WASF2* is associated with the actin signaling pathway, we performed the analysis of the actin cytoskeleton signaling pathway related to *WASF2* using Ingenuity Pathway Analysis software (IPA; Qiagen, www.qiagen.com/ingenuity). *WASF2* has a positive correlation with Arp2/3 and F-actin and subsequently causes actin polymerization (Additional file 2: Fig. S3B). Next, cell motility characteristics and expression of F-actin were visualized by immunofluorescence staining, and protein levels of several molecules

related to the actin cytoskeleton were confirmed through western blot analysis. In immunofluorescence staining, *WASF2* knockdown caused less formation of filopodia around the cells and down-regulated F-actin (Fig. 3F, G). Rac and cell division control protein 42 homolog (Cdc42) are Ras-related GTP-binding proteins that control the actin cytoskeleton, and Arp2 and Arp3 are involved in the initiation of actin polymerization [11]. The actin network is a dynamic structure with continuous assembly and disassembly, and F-actin, which plays an important role in actin polymerization, is a polymer of actin [12]. The results showed that Arp2, Arp3, and F-actin were notably decreased in the *WASF2* knockdown group compared with the negative control group. An interesting observation was that the protein expression level of Rac/Cdc42 was consistent after the *WASF2* knockdown and even the result of the phosphorylated form (p-Rac/Cdc42) was the same as Rac/Cdc42 (Fig. 3H). Therefore, the dynamic reorganization of the actin is a prerequisite for the migration of cancer cells, and these results reveal that *WASF2* is involved in the construction of the complex actin cytoskeleton.

***WASF2* suppression attenuates HCC tumorigenesis in vivo**

To gain further insights into the regulatory effect of *WASF2*, we analyzed *WASF2* expression in mouse and rat genomic datasets and assessed the tumor-suppressive effect of *WASF2* knockdown *in vivo*. Consistently, *WASF2* expression was increased in early and advanced HCC samples from both diethylnitrosamine (DEN)-induced mouse and rat HCC models from the gene expression omnibus database compared to corresponding control liver tissues (Fig. 4A). To validate the tumor suppressive effects of *WASF2* knockdown *in vivo*, we subcutaneously injected si*WASF2*-transfected Huh-7 cells into BALB/c female nude mice. We observed that the overall rate of tumor growth was significantly lower in the *WASF2*-knockdown (Huh-7_si*WASF2*) group than in the negative control (Huh-7_NC) group (Fig. 4B), as were tumor size at sacrifice (Fig. 4C). Also the average volume of tumor and *WASF2* expression in tumor tissues were significantly lower in the Huh-7_si*WASF2* group than the Huh-7_NC group (Fig. 4D). Therefore, we performed IHC analysis for anti-*WASF2*, anti-Ki67, anti-SNAIL, and anti-cleaved caspase-3 in xenograft tumor sections from the Huh-7_si*WASF2* and Huh-7_NC groups. To avoid quantifying any non-tumoral areas, the xenograft sections were stained using H&E and *WASF2*. As expected, the results showed that the expression levels of the cytosol of *WASF2*, the nucleus of Ki67, and the cytosol and nucleus section of SNAIL were decreased in the Huh-7_si*WASF2* group. Whereas, the expression levels of the cytosol and nucleus of cleaved caspase-3 increased (Fig. 4E). To investigate the effects of *WASF2* on HCC cells metastasis, *in vivo* metastasis assays were performed. First, we confirmed the efficiency of siRNAs-targeting *WASF2* in *ras*-NIH-3T3 cells (Fig. 4F), and then injected si*WASF2*-transfected *ras*-NIH-3T3 cells into athymic female nude mice through tail veins. There were no distinct changes in the body weight between the two groups (Fig. 4G) during 14 days of cell inoculation. However, lung metastases were clearly observed in the *ras*-NIH-3T3_NC group but not in the *ras*-NIH-3T3_si*WASF2* group the time of sacrifice (Fig. 4H). Also, we observed the number of lung nodules in the *ras*-NIH-3T3_si*WASF2* group were significantly decreased than that in the *ras*-NIH-3T3_NC group (Fig. 4I). Together, these *in vivo* analyses indicate that *WASF2* suppression attenuates the *in vivo* tumorigenic and metastatic potential of HCC.

WASF2 promoter hypomethylation causes overexpression in hepatocarcinogenesis and is associated with poor prognosis in patients with HCC

Since WASF2 was overexpressed at the mRNA and protein levels in HCC, we attempted to identify the mechanism of WASF2 activation. In TCGA_LIHC dataset, *WASF2* methylation correlated significantly and negatively with *WASF2* expression in the tumor group (Pearson's correlation coefficient $r = -0.33$, $P < 0.0001$; Fig. 5A). In addition, *WASF2* hypomethylation was concentrated in the tumor group compared to the normal group in 44 matched pairs of HCC patients from TCGA_LIHC data set (Fig. 5B). Therefore, we analyzed the correlation between *WASF2* expression and the DNA methylation of CpG islands in the 5' promoter region using MEXPRESS. Regression analysis demonstrated a significant correlation between *WASF2* expression and the methylation of five CpG sites (cg20745431, cg16406658, cg03436453, cg00307483, and cg24162579; Fig. 5C), among which cg24162579 was only significantly hypomethylated in the tumor samples of 44 matched pairs of patients with HCC from TCGA_LIHC (Additional file 2: Fig. S4A).

Consequently, we assessed the clinical relevance of *WASF2* methylation status in patients with HCC using the cg24162579 site. First, we performed BSP and MSP analysis on the *WASF2* promoter in immortalized normal liver hepatocytes (THLE-2 and MIHA) and HCC (Hep3B and Huh-7) cell lines targeting the region including the cg24162579 site (-833 to +534 bp relative to the transcriptional start site). Two CpG sites (site 1 and 2) in the *WASF2* promoter region were clearly methylated in Huh-7 cells (Fig. 5D), but only one was methylated in other cell lines (Additional file 2: Fig. S4B). We also analyzed *WASF2* promoter methylation levels in paired human HCC tissues using qMSP, finding that 60 and 56% of the tumor tissues in the test ($n = 20$) and validation ($n = 18$) cohorts, respectively, were hypomethylated compared to non-tumor tissues (Fig. 5E, F). We decided to assess the clinical relevance of *WASF2* methylation status in these 38 patients with HCC. Interestingly, *WASF2* methylation was lower in HCC tissues with vascular invasiveness than without it (Fig. 5G, left) and the DFS of HCC patients with cg24162579 hypomethylation was lower than that of patients with hypermethylation (Fig. 5G, right). We also observed that the *WASF2* methylation status significantly and negatively correlated with *WASF2* expression in these 66 HCC tissues (Pearson's correlation coefficient $r = -0.32$, $P = 0.048$; Fig. 5H).

To determine whether *WASF2* is regulated by DNA methylation-dependent epigenetic mechanisms, we treated Hep3B and Huh-7 cells with the hyper-methylating agent HU or DNA methylation inhibitor 5-aza and then measured *WASF2* promoter DNA methylation using MSP and *WASF2* expression using qRT-PCR and western blotting. HU treatment increased DNA methylation and decreased *WASF2* expression at the RNA and protein levels in Huh-7 cells compared to untreated cells but did not affect Hep3B cells (Fig. 5I, J). However, after treatment with 5-aza on Hep3B cells, the expression of *WASF2* RNA and protein was increased (Fig. 5K). These results suggest that *WASF2* expression is regulated via a DNA methylation-mediated epigenetic mechanism. To investigate the effect of DNA methyltransferases (DNMT)-dependent methylation on *WASF2* in HCC cells, first, after knockdown of each DNMTs in Huh-7 and Hep3B, the

expression levels of DNMT1, DNMT3a, and DNMT3b were measured. The expression level of each *DNMTs* mRNA in the Huh-7 and Hep3B was significantly decreased compared to the negative control (Additional file 2: Fig. S4C, top), and the expression of *WASF2* was upregulated (Additional file 2: Fig. S4C, bottom).

Finally, we observed the clinical association between *WASF2* methylation and overexpression in TCGA_LIHC dataset. Although cg24162579 CpG methylation in the *WASF2* promoter was significantly downregulated according to HCC tumor stage (Fig. 6A, left), *WASF2* expression was significantly upregulated (Fig. 6A, right). Since *WASF2* methylation and expression were significantly correlated with HCC tumor differentiation, we assessed their prognostic effects in patients with HCC according to *WASF2* methylation and expression level using the median beta value or median *WASF2* expression as a threshold. Patients in the low methylation group had a shorter OS than those in the high methylation group (log-rank $p = 0.038$, HR = 1.65; Fig. 6B), and when analyzed further, patients with high-methylation (Hyper) and low-expression (Low) showed the most favorable OS, progress-free survival (PFS), DFS, and disease-specific survival (DSS; $P = 0.001$, $P = 0.027$, $P = 0.045$, and $P = 0.039$, respectively; Fig. 6C, D). Also, the multivariate analysis demonstrated that *WASF2* with hypomethylation and with high expression was an independent poor prognostic factor for OS in HCC (Additional file 1: Table S6–9). These results suggest that *WASF2* methylation and expression appear to have a significant correlation with prognosis of patients with HCC.

Discussion

Through an in-depth analysis of public omics databases and validation experiments with HCC cell lines, xenograft mouse model and human samples, our study demonstrates the comprehensive functional characterization of *WASF2* in HCC and suggests that cg24162579 CpG site methylation in the promoter may be an important regulatory mechanism of *WASF2*.

We attempted to detect autoantibodies for tumor-associated antigens and identify hepatocarcinogenesis biomarkers using 22K protein chip analysis in patients with or without HCC [13, 14]. *WASF2* autoantibodies were detected in plasma at the time of diagnosis in all five patients with early HCC, as well as the plasma of four patients 1 year before diagnosis. In this study, we found that patients with high *WASF2* expression in TCGA_LIHC dataset had a lower OS rate than those with low *WASF2* expression. Consequently, we decided to characterize the role of *WASF2* in HCC and its regulatory mechanism.

WASF2 plays a key role in cell motility by relaying Rac activation to induce Arp2/3-mediated actin polymerization [15], while playing critical roles in cell movement and angiogenesis during embryogenesis [5, 16]. In addition, *WASF2* overexpression and/or clinical relevance have been reported in cancers such as melanoma, pancreatic cancer, and breast cancer as well as in relation to liver metastasis in colorectal cancer [6, 7, 17–19]. In HCC, *WASF2* mRNA and protein levels are higher than in paired surrounding non-tumor tissues ($n = 31$) and are significantly associated with aggressive tumor behavior. Furthermore, patients with high *WASF2* expression in HCC tissue ($\geq 26\%$ cancer cells) have a lower OS than those with

low expression ($\leq 25\%$ cancer cells) [8]. Since the role of WASF2 in the phenotype of HCC remains largely unclear, our study provides a comprehensive understanding of WASF2 expression in HCC tissues as well as its correlation with clinical outcomes, and demonstrates the effects of WASF2 suppression in HCC *in vitro* and *in vivo*. Notably, we found that *WASF2* expression was increased in HCC tissues from six public omics databases and increased significantly according to the progression of liver disease, while patients with high *WASF2* expression had a lower OS, DSS and PFS than those with low *WASF2* expression. Furthermore, we validated WASF2 mRNA and protein overexpression in 60% (40/66) and 72.7% (16/22) of the patients with HCC from our own cohort, respectively, before examining the biological functions of WASF2 in HCC using *in vitro* and *in vivo* mouse models. Interestingly, WASF2 inactivation decreased the viability, growth, proliferation, migration, and invasion of Huh-7 and SNU475 HCC cells by restoring G2/M checkpoint function, inducing HCC cell death, inhibiting EMT, and involving actin polymerization. Moreover, WASF2 inactivation caused tumor suppressive effects in the xenograft mouse model. Therefore, our study demonstrates that WASF2 suppression affects the cell cycle, cell death, EMT regulation, and actin cytoskeleton, which are all critical steps in tumorigenesis [20–22].

A recent study suggested microRNA-1253 as a novel epigenetic regulator of WASF2 in human umbilical vein endothelial cells and human aortic endothelial cells [9]; however, no studies have yet reported epigenetic mechanisms that regulate WASF2 expression in cancer and studies on WASF2 regulation in cancer cells are limited. We found a negative correlation between *WASF2* methylation status and mRNA expression in TCGA_LIHC dataset and revealed that cg24162579 was only significantly hypomethylated CpG island in the *WASF2* 5' promoter region in HCC samples from TCGA_LIHC dataset. Moreover, cg24162579 methylation decreased and *WASF2* expression increased according to HCC tumor differentiation grade, while patients with low cg24162579 methylation showed a significantly lower OS than those with high methylation. Together, these findings suggest that patients with hypermethylation and low *WASF2* expression displayed the most favorable OS.

Conclusions

In conclusion, we found that *WASF2* is overexpressed in HCC and correlates with poor patient prognosis. Furthermore, *WASF2* suppression decreased HCC cell growth, migration, and invasion by reducing G2/M checkpoint function, inducing HCC cell death, inhibiting EMT, and hindering actin polymerization in HCC. Therefore, we suggest that *WASF2* plays a pivotal role in HCC development and progression, and that cg24162579 methylation is a key epigenetic mechanism that regulates *WASF2* expression and predicts prognosis.

Abbreviations

Arp2/3: actin-related protein 2/3

ANOVA: analysis of variance

AUC: area under the curve

BSP: bisulfite sequencing polymerase chain reaction

Catholic_LIHC: the Catholic University of Korea's liver hepatocellular carcinoma project

Cdc42: cell division control protein 42 homolog

DEN: diethylnitrosamine

DFS: disease-free survival

DNMT: DNA methyltransferase

DSS: disease-specific survival

EMT: epithelial-mesenchymal transition

ES: enrichment score

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GSEA: gene set enrichment analysis

H&E: hematoxylin and eosin

HCC: hepatocellular carcinoma

HU: hydroxyurea

HPA: human protein atlas

ICGC_LIRI: International Cancer Genome Consortium liver cancer RIKEN Japan

IHC: immunohistochemistry

IPA: ingenuity pathway analysis

MSigDB: the Molecular Signatures Database

MSP: methylation-specific polymerase chain reaction

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OS: overall survival

PARP: poly ADP-ribose polymerase

PBS: phosphate-buffered saline

PFS: progress-free survival

PI: propidium iodide

qMSP: quantitative methylation-specific polymerase chain reaction

qRT-PCR: quantitative real-time polymerase chain reaction

Rac1: Ras-related C3 botulinum toxin substrate 1

ROC: receiver operating characteristic

SEM: standard error of mean

siNC: negative control small interfering RNA

siRNA: small interfering RNA

siWASF2: WASF2-targeting small interfering RNA

TCGA_LIHC: The Cancer Genome Atlas liver hepatocellular carcinoma project

TGF- β 1: transforming growth factor- β 1

WASF2: Wiskott-Aldrich Syndrome Protein Family Member 2

ZO-1: zonula occludens-1

5-aza: 5-aza-2'-deoxycytidine

Declarations

Ethics approval and consent to participate

All experiments were performed according to the Declaration of Helsinki and the study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-KSP-16-365 and AJIRB-BMR-SMP-17-189). Anonymous serum samples and clinical data were provided by the Ajou Human Bio-Resource Bank, and the need for informed consent was waived. All animals were maintained according to the Guide for the Care and Use of Laboratory Animals and experiments were approved by the Ethics Committee for Laboratory Animal Research Center of Ajou University Medical Center (IACUC-2020-0007).

Consent for publication

Not applicable.

Availability of data and materials

All data that support the findings of this study are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JWE, SSK and HRA made substantial contributions to the conception and design of the present study. HRA, GOB, JAS and MGY performed the in vitro experiments. SSK and JWE acquired and analyzed the data. SSK, HCK and JHY interpreted all the datasets in the present study. JWE, SSK and HJC drafted the initial manuscript and critically revised it for important intellectual content. JWE, SSK and JYC confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

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Figures

Fig. 1

Figure 1

Identification of potential HCC driver markers using a 22K human protein microarray system and clinical relevance of WASF2 overexpression. A Heatmap of 47 differentially expressed autoantibodies from liver cirrhosis to HCC (LC, liver cirrhosis; 1 y ago, 1 year ago; 6 m ago, 6 months ago; Dx, HCC diagnosis). B ROC analysis of 47 autoantibodies. C Venn diagram analysis of the ROC result for 47 autoantibodies and OS from TCGA_LIHC. D Differential expression of WASF2 in human hepatocarcinogenesis in GSE6764,

GSE12443, and GSE114564 datasets (one-way ANOVA, post hoc comparisons, Tukey's test). E Bar chart shows the fold change in tumor (T) versus non-tumor (NT) WASF2 expression in 66 matched pairs of human HCC tissues and corresponding noncancerous adjacent liver tissues F Western blotting analysis of WASF2 in 22 matched pairs of human HCC tissues with corresponding noncancerous adjacent liver tissues. G WASF2 mRNA and protein expression in one immortalized non-transformed hepatocyte cell (MIHA) and eight HCC cell lines analyzed using qRT-PCR (top) and western blot analysis (bottom). H Representative IHC photomicrographs of WASF2 in HCC (T, tumor tissues; NT, non-tumor tissues). Scale bar = 300 μ m. I Kaplan-Meier survival analyses of WASF2 expression in TCGA_LIHC datasets for overall survival (left), disease-specific survival (middle), and progress-free survival (right; log-rank test *P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 2

Figure 2

The anti-tumorigenic effects of WASF2 knockdown in HCC cells and Identification of the functional characterization of WASF2 inactivation according to the gene signature correlated with WASF2 expression. A Growth analyses. Left: morphology and number of Huh-7 and SNU475 cells transfected with NC or siWASF2. Scale bar = 100 μ m. Cell number was counted using trypan blue (mean $\pm$ SEM, n = 3, unpaired t-test). Right: MTT assay (mean $\pm$ SEM, n = 3, unpaired t-test). B Clonogenic and C scratch

wound healing assay in Huh-7 and SNU475 cells. Clonogenic assay: left, representative colony images; right, graphical representation of colony number from three random images (mean $\pm$ SEM; n = 3). Scale bar = 10 μ m. Wound healing assay: left, representative images; right, graphical representation of the percentage of migrated cells from three random images (mean $\pm$ SEM; n = 3, unpaired t-test). *P < 0.05; **P < 0.01; ***P < 0.001. D Cluster analysis was performed on 1,354 gene signatures that correlated highly with WASF2 expression (P < 0.001, r > 0.3 or r < -0.3) in TCGA_LIHC dataset. Patients were divided into WASF2 high or WASF2 low groups. E Kaplan-Meier analysis of overall survival (top) and disease-free survival (bottom; ***P < 0.0001; log-rank test). F Bar chart of the top 20 gene set lists enriched in the WASF2-associated gene signature reported by The Molecular Signatures Database (MSigDB) (FDR q < 0.01). G GSEA plots for the HCC-related gene sets derived from the WASF2 signature. y-axis represents ES, x-axis represents genes (vertical black lines) in the gene sets. Significance was determined from the nominal P values ($\leq$ 0.05) and false discovery rate ($\leq$ 0.25) from GSEA.

Fig. 3

Figure 3

Regulatory effect of WASF2 on EMT. A Cell cycle analysis in Huh-7 and SNU475 cells after siWASF2 transfection using flow cytometry. The percentage of cells in G2/M phase was analyzed using a FACSaria III flow cytometer. Western blot analyses of cell-cycle modulators in two HCC cell lines (mean $\pm$ SEM; n = 3, unpaired t-test). B Apoptosis analysis using flow cytometry. Dot plots indicate apoptosis ratios using PI and FITC-annexin V (left). Bar graph shows average percentage of apoptotic cells (right).

Western blot analysis of PARP, cleaved PARP, caspase-3, cleaved caspase-3, caspase-9, and cleaved caspase-9. GAPDH was used as a loading control. C Representative cell images and bar charts showing the number of migrating and invading cells transfected with siWASF2 captured in three random fields following modified Boyden chamber motility assay (top) and Transwell invasion assay (bottom; mean $\pm$ SEM; n = 3, unpaired t-test). Scale bar = 50 μ m. D Western blot analysis of EMT molecules in the presence or absence of TGF- β 1 (20 ng/mL) in Huh-7 and SNU475 cells. E Correlation analysis between WASF2 and SNAIL mRNA expression in non-tumor liver tissues (n = 50, Pearson's correlation coefficient, r = 0.08, P = 0.55, top) and HCC tissues (n = 371, Pearson's correlation coefficient, r = 0.26, P < 0.0001, bottom) from TCGA_LIHC dataset. F IF staining of F-actin and G observation of filopodia formation (white arrows) in SNU475 cells transfected with negative-control siRNA (NC) or siWASF2. Representative fluorescence images are shown. Scale bar = 100 μ m. H Western blot analysis of molecules related to the actin cytoskeleton in Huh-7 and SNU475.

Fig. 4

Figure 4

WASF2 suppression attenuates HCC tumorigenesis and metastatic in vivo. A Differential expression of WASF2 in a mouse model of DEN-induced hepatocarcinogenesis (left: GSE93392) and a rat model (right: GSE102418) (one-way ANOVA, post hoc comparisons, Tukey's test). B Tumor growth in mice injected subcutaneously with Huh-7 cells inactivating WASF2 (n = 6 per group; unpaired t-test). C Mouse images and mass images of xenograft mouse model established with Huh-7 cells transfected with negative control siRNA and siWASF2. The arrows indicate tumor mass. D Box plot of tumor weight in two groups (left) and box plot of WASF2 expression in xenograft tumor tissues assayed using qRT-PCR (right, n = 6 per group; unpaired t-test). E Representative images of H&E and IHC staining for WASF2, Ki-67, SNAIL, and cleaved caspase-3 in xenograft tumors derived from negative-control siRNA (NC) or siWASF2 transfected Huh-7 cells (right). Scale bar = 100 μ m. F The efficiency of siWASF2 in ras-NIH-3T3 cells and G body weight among the two groups of mice (n = 5 per group; unpaired t-test). H Representative in vivo images of nodules (arrow) 18 days after injection of transfected ras-transformed NIH-3T3 cells with negative control siRNA (n = 5) or siWASF2 (n = 5) using a lung metastasis mouse model. I The number of nodules on the surface of mouse lungs were counted. *P < 0.05; **P < 0.01; ***P < 0.001. Data were shown as mean $\pm$ SEM.

Fig. 5

Figure 5

Oncogenic WASF2 activation mechanism by methylation in HCC. A Correlation analysis between WASF2 mRNA expression and methylation in TCGA_LIHC dataset (n = 360, Pearson's correlation coefficient, $r = 0.22$, *** $P < 0.0001$). B Density plot of the methylation status in the non-tumor (NT) and tumor (T) groups. Methylation distribution across all sites. x-axis represents methylation level as mean β -values. y-axis represents relative density. C DNA methylation of CpG islands in the 5' promoter region corresponding to

WASF2 expression. Right: Pearson's correlation coefficient r and p values for Wilcoxon rank-sum test between each parameter and WASF2 expression. Red = significant difference. D The genomic locale of WASF2 and the CpG island in its promoter region. Arrow indicates transcriptional start site. The CpG island region was predicted using MethPrimer and the regions analyzed by BSP are indicated (top). Sequence detail of the BSP regions (-833 to -534 bp) in the WASF2 gene promoter. CpG dinucleotides (CG) in this region are indicated in red capitals and numbered 1–4. Methylation status of CpGs in the WASF2 promoter region in HCC cell lines (bottom). E Bar chart of CpG methylation in the WASF2 promoter in the test cohort (top; $n = 20$) and validation cohort (bottom; $n = 18$) determined by MSP in paired tumor (T) and non-tumor (NT) tissues. F Representative gel images of MSP results. Primers are specific for unmethylated (U) or methylated (M) DNA. G Violin plot (left) of WASF2 methylation based on the vascular invasion status of the test and validation cohorts [without invasiveness, No ($n = 15$); with invasiveness, Yes ($n = 23$); * $P < 0.05$]. Disease-free survival of HCC patients with low or high WASF2 DNA methylation (right). P values determined by the log-rank test. H Correlation of WASF2 methylation status and its expression in 66 HCC tissues. I Methylation status (left) and levels (right) of WASF2 in Huh-7 and Hep3B cells with/without HU treatment. J WASF2 mRNA (left) and protein (right) expression in Huh-7 and Hep3B cells with/without HU treatment. K WASF2 mRNA and protein expression in Hep3B cells with/without 5-aza treatment.

Fig. 6

Figure 6

Clinical association between WASF2 methylation and overexpression. A Differences in cg24162579 CpG site methylation in the WASF2 promoter region according to HCC tumor grade (left). Differences in WASF2 expression according to HCC tumor grade (right). B Overall survival curves according to cg24162579 CpG site methylation in TCGA_LIHC dataset. C Overall survival, Progression free survival, Disease free survival, and Disease specific survival curves according to the level of WASF2 methylation and expression in TCGA_LIHC dataset. D Forest plot of univariate Cox regression analyses of clinical parameters on overall survival, progress-free survival, disease-free survival, and disease-specific survival *P < 0.05; **P < 0.01; ***P < 0.001. HCC differentiation was defined using the Edmondson grade (grade 1, G1; grade 2, G2; grade 3, G3; and grade 4, G4).

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