Oncogenic miR-96-5p inhibits apoptosis by targeting the caspase-9 gene in hepatocellular carcinoma

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Abstract. The aberrant expression or alteration of microRNAs (miRNAs/miRs) contributes to the development and progression of cancer. In the present study, the functions of miR-96-5p in hepatocellular carcinoma (HCC) were investigated. It was identified that miR-96-5p expression was significantly upregulated in primary HCC tumors compared with their non-tumorous counterparts. A copy number gain was frequently observed at chromosomal region 7q32.2 in which the *MIR96* locus is located, suggesting that gene amplification may be one of the mechanisms by which miR-96-5p expression is increased in HCC. Transfection of miR-96-5p mimic into HCC cells decreased the expression of *CASP9*, which encodes caspase-9, the essential initiator caspase in the mitochondrial apoptotic pathway, at the mRNA and protein levels. A putative binding site for miR-96-5p was identified in the *CASP9* 3'-untranslated region, and the results of a luciferase assay indicated that *CASP9* is a potential direct target of miR-96-5p. The miR-96-5p mimic increased resistance to doxorubicin- and ultraviolet-induced apoptosis through the decrease in caspase-9 expression in HCC cells. Transfection of miR-96-5p inhibitor enhanced the cytotoxic effect of doxorubicin by increasing caspase-9 expression in the HCC cells, suggesting a synergistic effect between the miR-96-5p inhibitor and doxorubicin. In conclusion, the results of the present study suggest that miR-96-5p, which is frequently upregulated in HCC, inhibits apoptosis by targeting *CASP9*. Therefore, miR-96-5p may be a potential therapeutic target for HCC.

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Introduction

Liver cancer is estimated to lead to ~750,000 mortalities annually worldwide (1). It is more prevalent in men than in women, and is the second leading cause of cancer mortality in men. The most common primary liver cancer is hepatocellular carcinoma (HCC). The risk factors for HCC include infection with hepatitis B or hepatitis C virus, alcohol consumption, and non-alcoholic steatohepatitis (2).

MicroRNAs (miRNAs/miRs) are small non-coding RNAs of between 18 and 25 nucleotides in length that serve a crucial function in gene regulation. miRNAs are known to repress thousands of target genes and co-ordinate normal processes, including cellular proliferation, differentiation and apoptosis (3-5). The aberrant expression or alteration of miRNAs contributes to a range of human pathologies, including cancer (6,7). HCC is no exception, and various HCC-specific miRNA signatures have been reported (8). However, the function of miRNAs in the pathogenesis of HCC remains poorly understood.

One of the hallmarks of human cancer is intrinsic or acquired resistance to apoptosis (9). The evasion of apoptosis contributes to the development and progression of cancer, and to resistance to treatment. Therefore, a better understanding of the molecular mechanisms underlying the resistance of tumors to apoptosis is expected to provide a basis for a rational approach in the development of molecular targeted therapies.

Caspase-9 is the essential initiator caspase required in the mitochondrial apoptotic pathway (10). Upon exposure to intracellular apoptotic stimuli, mitochondria release cytochrome *c*, which binds to an adaptor protein known as apoptotic protease-activating factor 1 (Apaf-1); this binding causes Apaf-1 to oligomerize into a wheel-like heptamer called an apoptosome. Apaf-1 in the apoptosome then recruits caspase-9, which is activated in the apoptosome. The activated caspase-9 is then able to activate downstream executioner caspases, including caspase-3, -6 and -7, and trigger a cascade of events leading to apoptosis. As such, caspase-9 is considered to be a tumor suppressor (11).

To investigate the miRNAs involved in HCC, miRNAs that were upregulated in primary HCC tumors when compared with non-tumor tissues were screened for using a miRNA

microarray. The array analysis revealed that miR-96-5p was among the most significantly upregulated miRNAs in HCC tumors (K. Yasui, unpublished data).

In the present study, the functions of miR-96-5p were investigated in HCC. In addition to the forkhead box O1 (*FOXO1*) gene, which has been identified to be a direct target of miR-96-5p (12-16), the caspase-9 gene (*CASP9*) gene was identified as a potential novel target of miR-96-5p. These results suggest that increased miR-96-5p expression may contribute to resistance to apoptosis via the repression of caspase-9 expression in HCC.

Materials and methods

Reagents and antibodies. Antibodies against caspase-9 (cat. no. 9508), caspase-2 (cat. no. 2224), FOXO1 (cat. no. 2880) and poly(ADP-ribose) polymerase (PARP; cat. no. 9542) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against β-actin (cat. no. A1978) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Doxorubicin was obtained from Toronto Research Chemicals Inc. (Toronto, ON, Canada).

Cell culture and primary tumor samples. Three HCC cell lines (SNU387, SNU449 and HLF) were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Japan Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37˚C. Primary HCC tumor samples and non-tumor samples were obtained from patients who had undergone surgical resection of tumors, as described previously (17). The present study, including the collection and analytical methods, was approved by the Ethics Committees of the Kyoto Prefectural University of Medicine and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient for the use of their tissue samples in the present study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miRNA and mRNA. Total RNA was obtained using TRIzol® (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Residual genomic DNA was removed by incubating the RNA samples with RNase-free DNase I (Takara Bio, Inc., Otsu, Japan) prior to performing RT-qPCR.

Quantification of human (hsa)-miR-96-5p was performed using a fluorescence detection method. Single-stranded cDNA was generated using a Universal cDNA synthesis kit II (Exiqon A/S, Vedbaek, Denmark), according to the manufacturer's protocol. RT-qPCR experiments were performed using the StepOnePlus PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with ExiLENT SYBR-Green master mix (Exiqon A/S). The hsa-miR-96-5p locked nucleic acid (LNA) PCR primer set was purchased from Exiqon A/S. Small nucleolar RNA U49 (Exiqon A/S) was used as an endogenous control for miRNA levels.

The mRNAs were detected by RT-qPCR as described previously (18). The expression level of β-actin was used as an endogenous control for mRNA levels. The primer sequences used for PCR are presented in Table I.

Single-nucleotide polymorphism (SNP) array analysis. Genomic DNA was isolated as described previously (18). Changes in DNA copy number were analyzed in 34 primary HCC tumors using the GeneChip Mapping 250K Sty array (Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and as described previously (18,19).

Immunoblotting. Immunoblotting was performed as described previously (18). The dilutions of the antibodies were 1:1,000 for anti-caspase-9, anti-caspase-2, anti-FOXO1 and anti-PARP; and 1:5,000 for β-actin. For immunodetection, anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology, Inc.) was used as the secondary antibody at a dilution of 1:5,000 or 1:10,000, respectively. Antibody binding was detected using the enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA).

Transfection of miRNA mimics and inhibitors. Hsa-miR-96-5p mimic (#472140-001), cel-miR-39-3p (#479902-001), which was used as a miRNA mimic negative control, hsa-miR-96-5p inhibitor (#4102571-101) and miRNA inhibitor negative control (#199006-101) were obtained from Exiqon A/S. These mimics and inhibitors were transfected into cells using Lipofectamine® RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.), and were used at a final concentration of 12.5 nmol/l, except for SNU449 cells, which were transfected with 12.5, 25 and 50 nmol/l miR-96-5p inhibitor. After 72 h of incubation, cells were harvested for RT-qPCR and immunoblotting analyses.

Identification of the target genes of miR-96-5p. A total of 15 candidate genes were selected (Table II) that were predicted to be target genes of miR-96-5p by miRBase (www.mirbase.org), miRTarBase (mirtarbase.mbc.nctu.edu.tw) and microRNA. org (www.microrna.org). miR-96-5p mimic or negative control mimic was transfected into SNU387 and HLF cells that exhibited low expression levels of miR-96-5p, and the mRNA and protein levels of the 15 genes were determined using RT-qPCR and immunoblotting, respectively, as aforementioned.

Luciferase reporter assay. The 3'-untranslated region (3'-UTR) sequence of *CASP9* mRNA, inclusive of the miR-96-5p target site, was cloned downstream of the firefly luciferase gene in the pMirTarget vector (OriGene Technologies, Inc., Rockville, MD, USA). Site-specific mutations were generated in the *CASP9* 3'-UTR using the KOD Plus Mutagenesis kit (Toyobo Life Science, Osaka, Japan), according to the manufacturer's protocol. The luciferase-*CASP9* 3'-UTR wild-type (WT) vector, the luciferase-*CASP9* 3'-UTR mutant vector or empty vector was transfected into SUN387 cells together with either miR-96-5p mimic or negative control mimic using Lipofectamine® 3000 (Invitrogen). After 48 h of incubation, cells were harvested for the measurement of firefly and *Renilla* luciferase activity using the Dual-Glo Luciferase assay system (Promega Corporation, Madison, WI, USA).

RNA interference. Two small interfering RNAs (siRNAs) targeting *CASP9* (#1 and #2; Ambion; Thermo Fisher Scientific, Inc.) and control (non-silencing) siRNA were delivered into cells using Lipofectamine RNAiMAX (Invitrogen). After 72 h of incubation, cells were harvested for immunoblotting.

Table I. Primer sequences used for PCR.

Gene	Forward primer	Reverse primer
FOXO1	5'-AAGAGCGTGCCCTACTTCAA-3'	5'-CTGTTGTTGTCCATGGATGC-3'
CASP9	5'-GCAAGCAGCAAAGTTGTCGA-3'	5'-GGACTCACGGCAGAAGTTCA-3'
CASP2	5'-TGATGCCTTCTGTGAAGCAC-3'	5'-GCTCAACGGTGGGAGTACAT-3'
CDKN1A	5'-ATGAAATTCACCCCCTTTCC-3'	5'-CCCTAGGCTGTGCTCACTTC-3'
FOXO3	5'-GGCGGACTTTGTGTTTGTTT-3'	5'-AAGCCACCTGAAATCACACC-3'
REV1	5'-TTGTGATGAAGCGCTGGTAG-3'	5'-TTGGTCACTAGCTGGCCTCT-3'
RAD51	5'-CGACTCTCCCTGTCTTCCTG-3'	5'-TTTCCCGGAAGCTTTATCCT-3'
BRMS1L	5'-AGTGAAAACGGAACCACCTG-3'	5'-CCATCAGGCCTCTTAAACCA-3'
EFNA5	5'-AAACGTATTGTGCCCTGGAG-3'	5'-CTGCCACTCCAGAGGAGTTC-3'
AKT1S1	5'-AGGGCTCTTTGTGATGGATG-3'	5'-ACTTGGCGTACTGCTGTGTG-3'
HBP1	5'-CCGTGAAAATGAGGTGGACT-3'	5'-GAAGGCTGGTTCACTCTTCG-3'
PAX6	5'-CCGGCAGAAGATTGTAGAGC-3'	5'-CTCACACATCCGTTGGACAC-3'
IRS1	5'-GCTTTCCACAGCTCACCTTC-3'	5'-CCTCAGTGCCAGTCTCTTCC-3'
STK17A	5'-TTTGTCTGAGTCGGCTGTTG-3'	5'-GTGCCTTTTCCATCCTGAAA-3'
PLAGL1/ZAC	5'-CAAGTGTGTGCAGCCTGACT-3'	5'-ACACTCCTCACACCCAAAGG-3'
ACTB	5'-GTCCACCTTCCAGCAGATGT-3'	5'-TGTTTTCTGCGCAAGTTAGG-3'

Table II. Candidate target genes of miR-96-5p.

Gene	Expression ratio	
	SNU387	HLF
FOXO1	0.30	0.44
CASP9	0.37	0.25
CASP2	0.53	0.44
CDKN1A	0.79	0.64
FOXO3	0.78	0.75
REV1	0.55	0.55
RAD51	0.80	0.65
BRMS1L	0.53	0.50
EFNA5	1.35	1.67
AKT1S1	0.61	0.84
HBP1	0.42	0.38
PAX6	1.40	0.55
IRS1	0.59	0.94
STK17A	0.33	0.24
PLAGL1/ZAC	0.60	0.64

Gene expression levels were determined using RT-qPCR in SNU387 and HLF cells that were transfected with miR-96-5p mimic or control mimic. Values were normalized to levels in cells transfected with control mimic.

Cell viability assay. Cell viability was determined using the water-soluble tetrazolium salt 8 assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol.

Apoptosis assay. Cells were transfected with miR-96-5p mimic or negative control mimic. After 48 h of incubation, the cells were treated with doxorubicin or exposed to ultraviolet (UV) irradiation at 40 J/m^2 (SNU387 cells) or 10 J/m^2 (HLF cells) using a UV HybriLinker HL-2000 (BM Equipment Co., Ltd., Tokyo, Japan). Apoptosis was evaluated from the expression level of cleaved PARP by immunoblotting and the levels of caspase-3/7 activities using the Caspase-Glo 3/7 assay (Promega Corporation).

Statistical analysis. Statistical analyses were performed using SPSS Statistics (version 24.0; IBM Corp., Armonk, NY, USA). Comparisons were made using the Wilcoxon signed-rank test, Mann-Whitney U test, Student's t-test or analysis of variance (ANOVA) followed by post hoc tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-96-5p is upregulated in primary HCC tumors. Using RT-qPCR to analyze 27 paired tumor and non-tumor tissues, it was identified that miR-96-5p expression was significantly upregulated in the HCC tumors compared with their non-tumorous counterparts (Fig. 1A). To investigate the molecular mechanisms by which miR-96-5p is upregulated in the HCC tumors, aberrations in DNA copy number were examined in primary HCC tumors. High-resolution SNP array analysis revealed a frequent copy number gain at chromosomal region 7q32.2, where the *MIR96* locus is located; it was identified in 17/34 (50%) HCC tumors (Fig. 1B). The presence of a copy number gain at the *MIR96* locus was significantly associated with increased expression levels of miR-96-5p in the HCC tumors (Mann-Whitney U test; Fig. 1C).

The potential association between the increased expression levels of miR-96-5p in HCC tumors and various clinicopathological parameters was analyzed. However, no significant association was apparent between the levels of miR-96-5p and any of the parameters, including age, sex, tumor size,
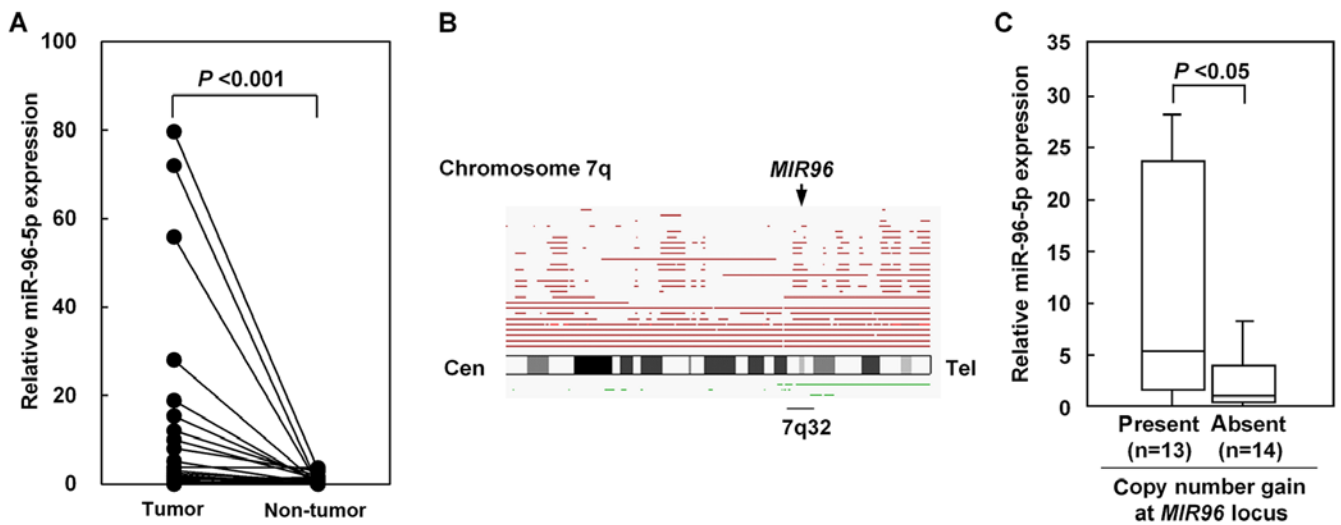
Figure 1. (A) Relative expression levels of miR-96-5p in paired tumor and non-tumor liver tissues from 27 patients with primary HCC were determined using reverse transcription-quantitative polymerase chain reaction. (B) Recurrent copy number gains in the chromosomal region 7q32 were assessed in primary HCC tumors using a GeneChip Mapping 250K Sty array. Copy number gains are indicated by red horizontal lines above the chromosome ideogram. Copy number losses are indicated by green lines under the chromosome ideogram. Each horizontal line represents an aberration detected in a single HCC tumor. The position of the *MIR96* locus is indicated by a black arrow. (C) Association between a copy number gain at the *MIR96* locus and the expression levels of miR-96-5p in primary HCC tumors. The horizontal lines represent the means. The boxes contain the values between the 25th and 75th percentiles. The error bars denote the 5th and 95th percentile values. HCC, hepatocellular carcinoma; *MIR96*, microRNA-96; Cen, centromere; Tel, telomere.

clinical stage, hepatitis B or hepatitis C virus infection and α-fetoprotein level. No association was observed between the levels of miR-96-5p and overall or disease-free survival (data not shown).

CASP9 is identified as a novel direct target of miR-96-5p. To identify the target genes of miR-96-5p, it was investigated whether or not enforced expression of miR-96-5p was able to decrease the expression levels of the 15 candidate genes. These genes were selected because they were predicted to be target genes of miR-96-5p using an *in silico* approach, and they were potential tumor suppressor genes. Results of the RT-qPCR analysis are presented in Table II and Fig. 2A. Representative images of immunoblotting are presented in Fig. 2B. Of the 15 candidate genes, *FOXO1*, *CASP9* and *CASP2* exhibited decreased expression at the mRNA and protein levels following transfection with the miR-96-5p mimic compared with transfection with the control mimic in SNU387 and HLF cells (Fig. 2A and B). To confirm these results, rescue experiments were performed using the miR-96-5p inhibitor in combination with the miR-96-5p mimic. Immunoblot analyses revealed that transfection with the miR-96-5p inhibitor attenuated the suppression of FOXO1, caspase-9 and caspase-2 protein expression by the miR-96-5p mimic (Fig. 2C).

A luciferase assay was performed to investigate whether or not *CASP9* is a direct target of miR-96-5p using a *CASP9* 3'-UTR-luciferase reporter construct. A putative binding site of miR-96-5p was identified in the *CASP9* 3'-UTR (Fig. 2D). SNU387 cells that were co-transfected with the luciferase-*CASP9* 3'-UTR WT vector and miR-96-5p mimic exhibited significantly decreased luciferase activity compared with the cells co-transfected with the luciferase-*CASP9* 3'-UTR WT vector and control mimic (Fig. 2D). In addition, no decrease in luciferase activity was observed when the empty luciferase reporter vector or the luciferase-*CASP9* 3'-UTR mutant vector was co-transfected

with the miR-96-5p mimic (Fig. 2D). Regarding *CASP2*, the luciferase assay was not able to demonstrate that *CASP2* was a direct target of miR-96-5p (data not shown).

Furthermore, the mRNA levels of *FOXO1* and *CASP9* were determined using RT-qPCR in paired tumor and non-tumor liver tissues from 35 patients with primary HCC. The mRNA levels of *FOXO1* and *CASP9* were decreased in the tumors compared with in their non-tumorous counterparts, although the difference was only statistically significant for *FOXO1* (Fig. 2E). However, contrary to expectations, there was no inverse association between the expression levels of miR-96-5p and the mRNA levels of *FOXO1* or *CASP9* in the HCC tumors (data not shown).

miR-96-5p-mediated suppression of caspase-9 inhibits apoptosis. To examine whether miR-96-5p inhibits apoptosis by decreasing caspase-9 expression, caspase-9 expression was knocked down in SNU387 and HLF cells by transfection with two different siRNAs targeting *CASP9*, followed by treatment with doxorubicin, an anticancer agent that causes DNA strand breaks. Caspase-9 knockdown was confirmed by immunoblotting (Fig. 3A). Doxorubicin decreased the cell viability of SNU387 and HLF cells in a dose-dependent manner. However, knockdown of caspase-9 attenuated the doxorubicin-induced decrease in cell viability in SNU387 and HLF cells, when SNU387 cells were treated with doxorubicin at 0.3, 1.2 and 5.0 μmol/l and HLF cells were treated with doxorubicin at 0.15 and 0.3 μmol/l (Fig. 3B).

SNU387 and HLF cells were transfected with the miR-96-5p mimic or control mimic, followed by treatment with doxorubicin. The rate of the doxorubicin-induced decrease in cell viability was significantly lower in cells transfected with the miR-96-5p mimic compared with in cells transfected with the control mimic in a dose-dependent and time-dependent manner (Fig. 3C and D).
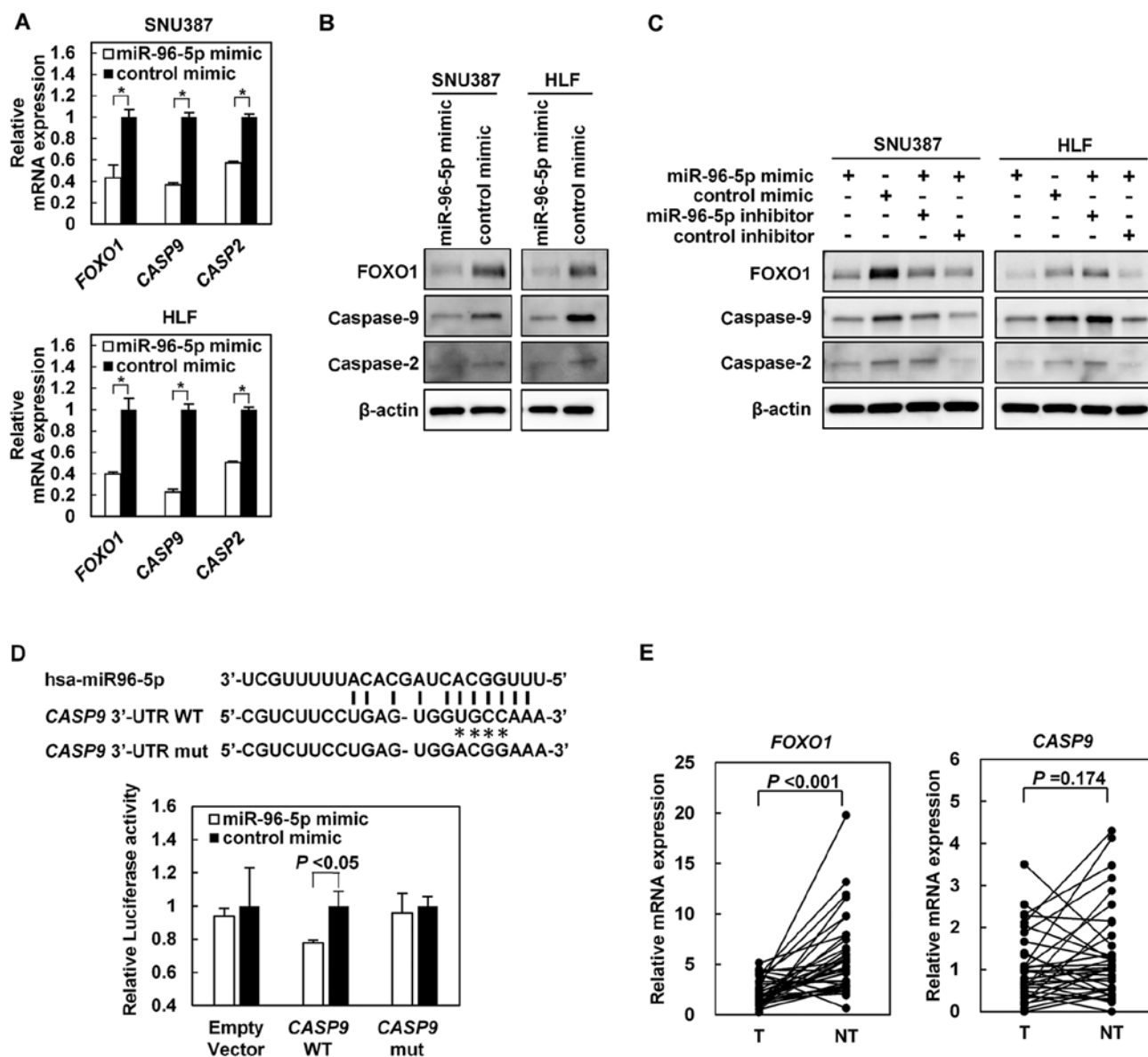
Figure 2. (A) Relative mRNA expression levels of *FOXO1*, *CASP9* and *CASP2* in SNU387 and HLF cells transfected with the miR-96-5p mimic or control mimic. (B) Immunoblot analyses of the indicated proteins in SNU387 and HLF cells transfected with the miR-96-5p mimic or control mimic. β-actin was used as an internal control. (C) Rescue experiments using the miR-96-5p inhibitor in combination with the miR-96-5p mimic. SNU387 and HLF cells were transfected with the miR-96-5p mimic or control mimic in combination with the miR-96-5p inhibitor or control inhibitor. Immunoblot analyses of the FOXO1, caspase-9 and caspase-2 proteins are shown. (D) Upper panel: A putative binding site for miR-96-5p in the *CASP9* 3'-UTR. Point mutations (*) were introduced by site-directed mutagenesis to destroy the base-paring with the miRNA. Lower panel: Luciferase assay. A luciferase reporter plasmid bearing the *CASP9* 3'-UTR WT, CASP9 3'-UTR mut or empty vector was co-transfected with the miR-96-5p mimic or control mimic into SNU387 cells. (E) Relative mRNA expression levels of *FOXO1* and *CASP9* in paired tumor and non-tumor tissues from 35 patients with primary HCC as determined using reverse transcription-quantitative polymerase chain reaction. FOXO1, forkhead box O1; *CASP*, caspase; miR, microRNA; UTR, untranslated region; mut, mutant; WT, wild-type; T, tumor; NT, non-tumor.

Finally, SNU387 and HLF cells were transfected with the miR-96-5p mimic or control mimic, followed by treatment with doxorubicin or UV to induce apoptosis. Immunoblotting of caspase-9 and cleaved PARP revealed that, in SNU387 and HLF cells, transfection with the miR-96-5p mimic decreased caspase-9 expression and attenuated the apoptosis induced by doxorubicin or UV compared with the control mimic (Fig. 3E and F). Results of the caspase-3/7 activity assay indicated that transfection with the miR-96-5p mimic significantly decreased the caspase-3/7 activity in HLF cells treated with doxorubicin compared with similarly treated HLF cells transfected with the control mimic (Fig. 3G).

Apoptosis is induced by the inhibition of miR-96-5p. To evaluate miR-96-5p as a potential therapeutic target for HCC, miR-96-5p inhibitor or control inhibitor was transfected into SNU449 cells, which exhibited increased expression of miR-96-5p. SNU449 cells transfected with a high concentration (i.e. 50 nmol/l) of miR-96-5p inhibitor exhibited decreased cell viability compared with those transfected with the same concentration of the control inhibitor (Fig. 4A and B). Immunoblotting of cleaved PARP identified that the miR-96-5p inhibitor induced apoptosis (Fig. 4C).

Although SNU449 cells transfected with a low concentration (i.e. 10 nmol/l) of miR-96-5p inhibitor did not exhibit
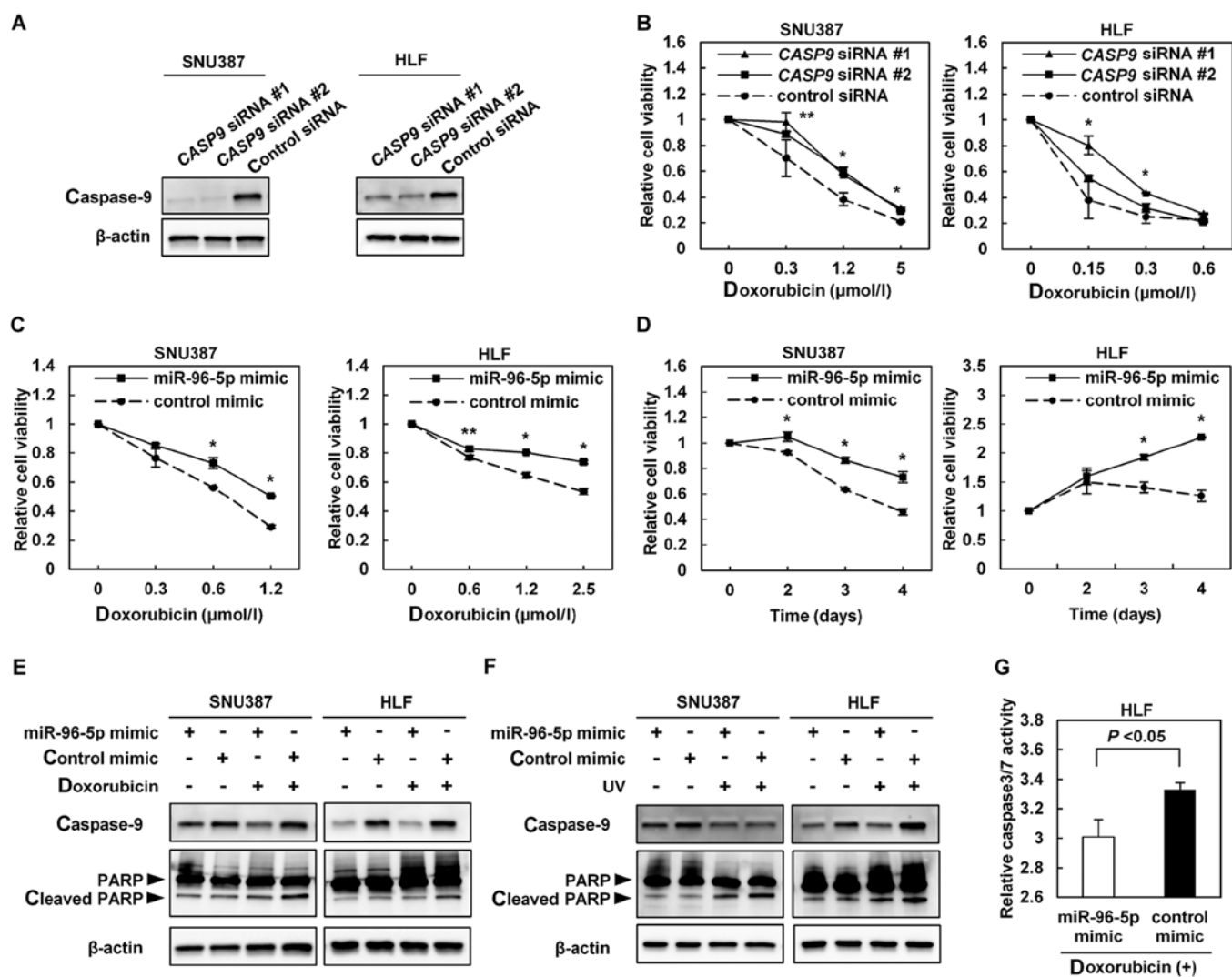
Figure 3. (A) Immunoblot analysis of caspase-9 in SNU387 and HLF cells transfected with *CASP9* siRNA (#1 or #2) or with control siRNA. (B) Effect of knockdown of caspase-9 on the cytotoxicity of doxorubicin. SNU387 and HLF cells were transfected with *CASP9* siRNA (#1 or #2) or control siRNA, then treated with doxorubicin 48 h later at the indicated concentrations. Cell viability was measured 48 h after doxorubicin treatment. *P<0.01 and **P<0.05 vs. control siRNA. SNU387 and HLF cells were transfected with the miR-96-5p mimic or control mimic, then treated with doxorubicin 48 h later. (C) SNU387 and HLF cells were treated with doxorubicin at the indicated concentrations. Cell viability was measured 96 h after doxorubicin treatment. *P<0.01 and **P<0.05 vs. control mimic. (D) SNU387 and HLF cells were treated with doxorubicin at 0.6 and 1.0 μmol/l, respectively. Cell viability was measured at the indicated times. *P<0.01 vs. control mimic. Effect of the miR-96-5p mimic on apoptosis induced by doxorubicin and UV. SNU387 and HLF cells were transfected with the miR-96-5p mimic or control mimic, then treated with doxorubicin or UV 48 h later. (E) SNU387 cells were treated or not treated with 0.6 μmol/l doxorubicin for 6 h. HLF cells were treated or not treated with 2.5 μmol/l doxorubicin for 12 h. (F) SNU387 cells were exposed or not exposed to UV irradiation at 40 J/m². HLF cells were exposed or not exposed to UV irradiation at 10 J/m². Subsequently, the cells were cultured for 18 h. Immunoblot analyses of caspase-9, PARP and cleaved PARP as a marker of apoptosis are shown. (G) Measurement of caspase-3/7 activity. HLF cells were transfected with the miR-96-5p mimic or control mimic, then treated with 2.5 μmol/l doxorubicin 48 h later. Caspase-3/7 activity was measured 12 h after doxorubicin treatment. CASP9, caspase-9; siRNA, small interfering RNA; UV, ultraviolet; miR, microRNA; PARP, poly(ADP-ribose) polymerase.

any changes in cell viability (Fig. 4A and D), they did exhibit increased caspase-9 expression, as revealed by immunoblotting (Fig. 4E). In addition, SNU449 cells transfected with 10 nmol/l miR-96-5p inhibitor prior to treatment with doxorubicin exhibited significantly lower cell viability compared with the cells transfected with the control inhibitor (Fig. 4F).

Discussion

In the present study, it was identified that miR-96-5p was significantly upregulated in the HCC tumors compared with in the non-tumor tissues; this result was consistent with the results of previous studies (13,20). miR-96-5p is a putative oncogenic miRNA that is upregulated in different types of cancer, including breast (14), colorectal (21), prostate (22), bladder (23) and non-small cell lung (24) cancer. However, the underlying molecular mechanism of miR-96-5p upregulation in cancer remains unclear. Although previous studies have identified that transforming growth factor-β1 (25), hypoxia (26) and mitochondrial dysfunction (27) induced the expression of miR-96-5p, these factors did not increase the expression of miR-96-5p in HCC cells in the present study (data not shown). The reason for this difference is unclear, but it may be due to the different types of cell used. The results of the present study suggest that the copy number gain at the *MIR96* locus is one of the molecular mechanisms by which miR-96-5p expression is increased in HCC.
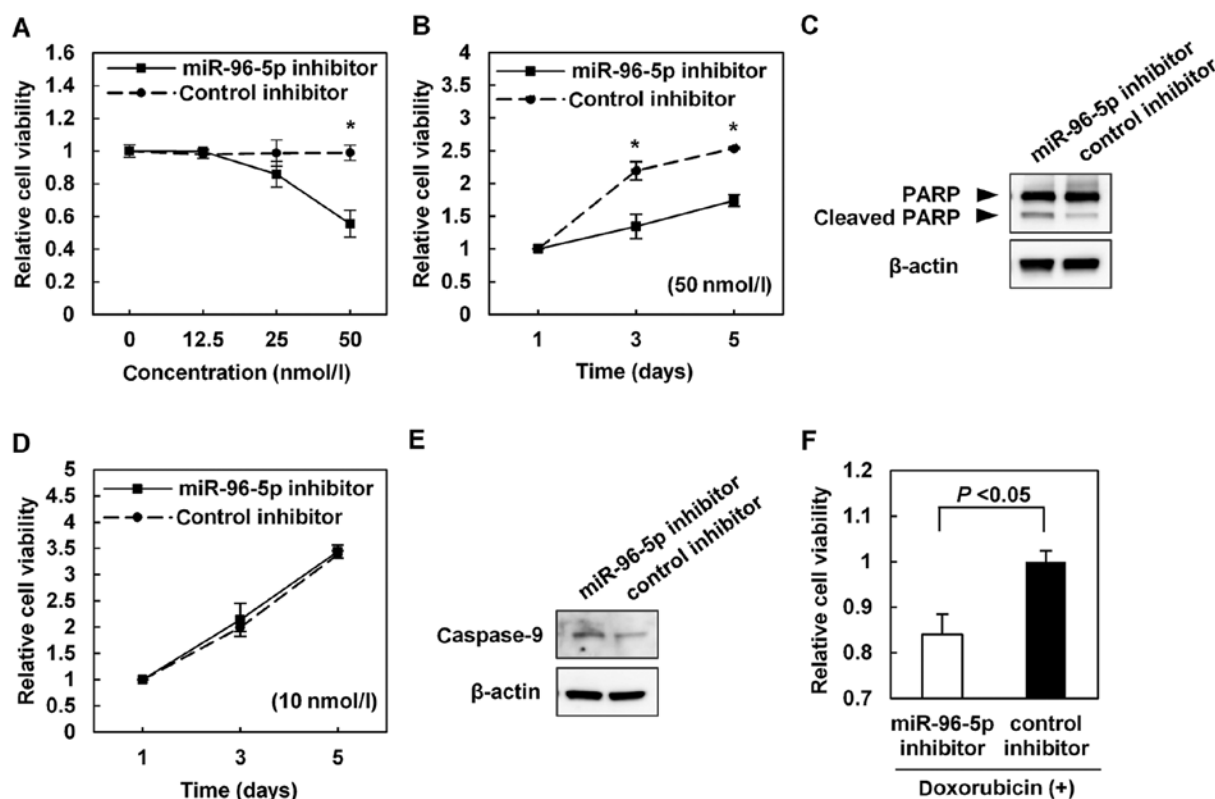
Figure 4. (A) Effect of the miR-96-5p inhibitor on cell viability. SNU449 cells were transfected with the miR-96-5p inhibitor or control inhibitor at the indicated concentrations. Cell viability was assayed 5 days after transfection. *P<0.01 vs. control inhibitor. (B) Effect of a high concentration of miR-96-5p inhibitor on cell viability. SNU449 cells were transfected with 50 nmol/l miR-96-5p inhibitor or control inhibitor. Cell viability was assayed at the indicated times. *P<0.01 vs. control inhibitor. (C) Induction of apoptosis by a high concentration of miR-96-5p inhibitor. SNU449 cells were transfected with 50 nmol/l miR-96-5p inhibitor or control inhibitor, then subjected 48 h later to immunoblot analysis of cleaved PARP as a marker for apoptosis. (D) Cell viability assay of SNU449 cells transfected with 10 nmol/l miR-96-5p inhibitor or control inhibitor. (E) Immunoblot analysis of caspase-9 in SNU449 cells transfected with 10 nmol/l miR-96-5p inhibitor or control inhibitor. (F) Synergistic effect between the miR-96-5p inhibitor and doxorubicin. SNU449 cells were transfected with 10 nmol/l miR-96-5p inhibitor or control inhibitor, then treated with 0.3 μmol/l doxorubicin 48 h later. Cell viability was assayed 48 h after doxorubicin treatment. miR, microRNA; PARP, poly(ADP-ribose) polymerase.

miR-96 belongs to the miR-183/-96/-182 cluster, a conserved polycistronic miRNA cluster. The miR-183/-96/-182 cluster is transcribed into the same primary miRNA. Thus, miR-183, miR-96 and miR-182 are likely to have similar expression levels. However, despite the upregulation of miR-96, unpredictably, the microarray and RT-qPCR analyses revealed that miR-183 and miR-182 were not upregulated in the HCC tumors compared with in the non-tumor tissues. Although the reason for this is unknown, it is possible that there is a difference in stability between the three miRNAs in HCC cells.

Next, target genes of miR-96-5p were identified. Each miRNA is predicted to negatively regulate tens to hundreds of target genes. Several previous studies have identified that *FOXO1* is a direct target of miR-96-5p in HCC (12,13), and in other types of malignancy, including breast (14), prostate (15) and endometrial (16) cancer. FOXO1 orchestrates the regulation of genes involved in the apoptotic response, cell cycle checkpoints and cellular metabolism (14,28). FOXO1 is a putative tumor suppressor (29) and the expression of this gene is downregulated in various types of cancer. In addition, it appears that FOXO1-dependent cell cycle arrest and apoptosis are critical for its tumor-suppressive effect. The results of the present study indicated that the miR-96-5p mimic decreased the expression of *FOXO1* at the mRNA and protein levels in HCC cells, and that the mRNA levels of *FOXO1* were significantly decreased in the primary HCC tumors compared with in their non-tumorous counterparts, confirming that *FOXO1* is a target of miR-96-5p in HCC. Although miR-183 and miR-182 were not upregulated in the HCC tumors in the present study, it has been reported that miR-183, miR-96 and miR-182 concordantly downregulated *FOXO1* in HCC cells (13). In addition to *FOXO1*, previous studies have identified that several genes, including those encoding forkhead box O3 (30), glypican 3 (31), insulin receptor substrate 1 (27), reversion inducing cysteine-rich protein with Kazal motifs (32), metastasis suppressor 1 (33) and protein tyrosine phosphatase non-receptor 9 (34), are potential targets of miR-96-5p in cancers.

The present study attempted to identify novel target genes of miR-96-5p in HCC. Using an *in silico* approach, the putative binding site of miR-96-5p was identified in the *CASP9* 3'-UTR. RT-qPCR and immunoblot analyses revealed that the miR-96-5p mimic decreased *CASP9* expression at the mRNA and protein levels, respectively. Results of the luciferase assay using the *CASP9* 3'-UTR-luciferase reporter construct suggested that *CASP9* is a potential direct target of miR-96-5p. Furthermore, the mRNA levels of *CASP9* were decreased in the primary HCC tumors compared with in their non-tumorous counterparts. Collectively, the results of the present study suggested that *CASP9* is a novel direct target of miR-96-5p,

and that *CASP9* expression is decreased by the upregulation of miR-96-5p in HCC. Since caspase-9 is the essential initiator caspase required for apoptosis signaling and it is considered to be a tumor suppressor, downregulation of caspase-9 by miR-96-5p may be beneficial to cancers.

Resistance to apoptosis contributes to the development and progression of cancer, and to resistance to chemotherapy. Indeed, the present study demonstrated that knockdown of caspase-9 resulted in resistance to doxorubicin-induced apoptosis. Furthermore, it was identified that enforced expression of miR-96-5p led to resistance to doxorubicin- and UV-induced apoptosis through the downregulation of caspase-9. These results suggested that miR-96-5p inhibits apoptosis by decreasing caspase-9 expression. Therefore, upregulation of miR-96-5p may be involved in the development and progression of HCC, and may contribute to resistance to chemotherapy by inhibiting apoptosis through a decrease in caspase-9 expression in HCC. Furthermore, because FOXO1 also induces apoptosis, the miR-96-5p-mediated decrease in FOXO1 and caspase-9 expression may have a synergistic effect on inhibiting apoptosis.

Finally, it was investigated whether miR-96-5p may be a potential therapeutic target in HCC. A high concentration (i.e. 50 nmol/l) of miR-96-5p inhibitor decreased the viability of HCC cells by inducing apoptosis. Although the molecular mechanism underlying this apoptosis is unclear and the high concentration of the inhibitor used may have caused off-target effects, these results suggest that miR-96-5p may be a potential therapeutic target. Interestingly, a low concentration (i.e. 10 nmol/l) of miR-96-5p inhibitor, which did not affect the viability of the HCC cells, enhanced the cytotoxic effect of doxorubicin by increasing caspase-9 expression. These results suggested that HCC cells may be sensitized to doxorubicin by pretreatment with a low concentration of miR-96-5p inhibitor, and that there is a synergistic effect between the miR-96-5p inhibitor and doxorubicin. Of note, in previous studies in which small molecules against miR-96-5p were designed, it was demonstrated that the drugs induced apoptosis in breast cancer cells while leaving healthy cells unaffected (35,36). Taken together, these results and those of the present study indicate that miR-96-5p inhibitor, alone or together with a cytotoxic drug, may provide a promising strategy for the treatment of HCC.

In conclusion, the results of the present study suggest that miR-96-5p, which is frequently upregulated in HCC, inhibits apoptosis by targeting *CASP9*. Therefore, miR-96-5p may be a potential therapeutic target for HCC.

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Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to confidentiality but are available from the corresponding author on reasonable request.

Authors' contributions

NI performed laboratory experiments with the help of AT, YG, KT, TK, TS, NY, OD, YS, AU, TN, KY, MM, HK, YN and YI, and prepared the manuscript. KY designed the present study and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study, including the collection and analytical methods, was approved by the Ethics Committees of the Kyoto Prefectural University of Medicine, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient for the use of their tissue samples in the present study.

Consent for publication

Written informed consent was obtained from each patient.

Competing interests

The authors have no conflicts of interest to disclose.

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