Telmisartan inhibits hepatocellular carcinoma cell proliferation *in vitro* by inducing cell cycle arrest

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Abstract. Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third leading cause of cancer-related death. Telmisartan, a widely used antihypertensive drug, is an angiotensin II type 1 (AT1) receptor blocker (ARB) that might inhibit cancer cell proliferation, but the mechanisms through which telmisartan affects various cancers remain unknown. The aim of the present study was to evaluate the effects of telmisartan on human HCC and to assess the expression of microRNAs (miRNAs). We studied the effects of telmisartan on HCC cells using the HLF, HLE, HepG2, HuH-7 and PLC/PRF/5 cell lines. In our experiments, telmisartan inhibited the proliferation of HLF, HLE and HepG2 cells, which represent poorly differentiated types of HCC cells. However, HuH-7 and PLC/PRF/5 cells, which represent well-differentiated types of HCC cells, were not sensitive to telmisartan. Telmisartan induced G_0/G_1 cell cycle arrest of HLF cells by inhibiting the G_0-to-G_1 cell cycle transition. This blockade was accompanied by a marked decrease in the levels of cyclin D1, cyclin E and other cell cycle-related proteins. Notably, the activity of the AMP-activated protein kinase (AMPK) pathway was increased, and the mammalian target of rapamycin (mTOR) pathway was inhibited by telmisartan treatment. Additionally, telmisartan increased the level of caspase-cleaved cytokeratin 18 (cCK18), partially contributed to the induction of apoptosis in HLF cells and reduced the phosphorylation of ErbB3 in HLF cells. Furthermore, miRNA expression was markedly altered by telmisartan *in vitro*. In conclusion, telmisartan inhibits human HCC cell proliferation by inducing cell cycle arrest.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, the sixth most common cancer overall and the third most common cause of cancer-related death worldwide (1). Despite the decreasing incidence of HCC in Asia, western countries are continuously encountering new cases. HCC has one of the worst prognoses of any cancer, with a 5-year survival rate of approximately 15-25% (2,3). Diagnosis at advanced stages and metastasis are associated with a poor prognosis. In clinical practice, HCC is characterized by hypervascularity which is required for tumor growth, invasion and metastasis. Tumor angiogenesis has been reported to be a significant predictor of death and is correlated with the tumor stage (4).

Angiotensin II type 1 (AT1) receptor blocker (ARB) is an antihypertensive drug with established feasibility and safety in clinical use. In addition to HCC cells, various cancer cells have recently been reported to express AT1 receptors (5-7). Several ARBs have been shown to inhibit angiogenesis in various cancer cells expressing AT1 receptor. Angiotensin II promotes cell proliferation during cancer development, and ARBs may suppress proliferation by antagonizing the AT1 receptor (8-10). ARBs inhibit the growth of breast (11), endometrial (12) and gastric cancer cells (13) in several studies. According to some epidemiological studies, the use of ARBs may increase the risk of cancer (14). In contrast, the use of an ARB treatment in hypertensive patients is associated with lower cancer incidence and mortality rates in other studies (15,16).

Among these agents, telmisartan is a widely prescribed drug for the treatment of hypertension, diabetic nephropathy and heart failure. Telmisartan has been shown to inhibit cell proliferation by inducing apoptosis in various cancer cell lines including endometrial (12), prostate 17), renal (18) and

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colon (19) cancer cell lines. Although telmisartan induces cell cycle arrest in hematological malignancies (20), few studies have examined the main antitumor effects of telmisartan, other than apoptosis, on cell cycle arrest in non-hematological malignancies. As shown in our previous study, telmisartan inhibits human esophageal adenocarcinoma cell proliferation and tumor growth by inducing cell cycle arrest through the regulation of cell cycle-related proteins via the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway (21). AMPK is a cellular energy sensor that is expressed in almost all eukaryotic cells (22) and regulates cell growth and proliferation by modulating the mTOR signaling pathway (23,24). In HCC cells, we assumed that telmisartan regulates the proliferation of cancer cells though AMPK activation and focused on AMPK/mTOR signaling.

The present study evaluated the effects of telmisartan on the growth of HCC cell lines and its mechanism of action. Furthermore, possible mechanisms associated with the antitumor effects of telmisartan, including apoptosis, receptor tyrosine kinases (RTKs), angiogenesis and microRNAs (miRNAs), were also explored.

Materials and methods

Chemicals. Telmisartan and valsartan were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Losartan and irbesartan were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). We prepared solutions of telmisartan, valsartan and irbesartan by diluting with dimethyl sulfoxide (DMSO). Losartan was prepared by diluting with H_2O. The stock solutions were stored at -20˚C.

Cell lines and culture. We used five human HCC cell lines, the HLF, HLE, HuH-7 and PLC/PRF/5 cells were obtained from the Japanese Cancer Research Bank (Osaka, Japan), and HepG2 cells were supplied by Riken Cell Bank (Tsukuba, Japan). HLF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2. HLE, HuH-7 and PLC/PRF/5 cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin. HepG2 cells were cultured in MEM supplemented with 10% FBS and penicillin-streptomycin.

Cell proliferation assay. Cell proliferation assays were conducted using the Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Each of the five cell lines was seeded onto 96-well plates (5.0x10³ cells/well) and were cultured in 100 μl of the corresponding medium. After 24 h, the seeded cells were treated with telmisartan, valsartan, losartan or irbesartan (0, 10, 50 or 100 μM) and were cultured for an additional 48 h. At the indicated time-points, the medium in each well was replaced with 100 μl of medium containing the CCK-8 reagent, and the cells were incubated for 3 h. The absorbance was measured at a wavelength of 450 nm using an automated microplate reader.

Flow cytometric analyses of the cell cycle and apoptosis. We conducted a flow cytometric analysis using the Cycle Phase

Determination kit (Cayman Chemical Company, Ann Arbor, MI, USA) to assess the growth inhibition mechanism. HLF cells (1.0x10⁶ cells/100 mm-diameter dish) were treated with 100 μM telmisartan or DMSO control for 12-48 h. Fixed cells were washed with phosphate-buffered saline (PBS) and then stored at -20˚C until analysis by flow cytometry. On the day of analysis, the cells were washed with cold PBS, suspended in 100 μl of PBS plus 10 μl of RNase A (250 μg/ml) and incubated for 30 min. Then, 110 μl of propidium iodide (PI) stain (100 μg/ml) was added to each suspension, and the cells were incubated at 4˚C for at least 30 min prior to analysis.

Apoptosis was analyzed after the telmisartan treatment using flow cytometry and an Annexin V-FITC Early Apoptosis Detection kit (Cell Signaling Technology, Boston, MA, USA). HLF cells (1.0x10⁶ cells/100 mm-dimeter dish) were treated with 100 μM telmisartan or DMSO control for 12 h. Apoptotic and necrotic cell death were analyzed by double staining with FITC-conjugated Annexin V and PI; this staining method is based on the binding of Annexin V to apoptotic cells with exposed phosphatidylserines and the labeling of late apoptotic/necrotic cells with membrane damage by PI. Staining was performed according to the manufacturer's instructions. We repeated the same experiment three times to compare the proportion of apoptotic cells in the telmisartan-treated group and the non-treated group.

Flow cytometry was conducted using a Cytomics FC 500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA) with an argon laser (488 nm). The percentages of cells were analyzed using Kaluza software (Beckman Coulter).

Western blot analysis. Cell lysates were prepared at 4˚C as previously described (25). HLF cells (1.0x10⁶ cells/100 mm-dimeter dish) were seeded and cultured for 24 and 48 h after treatment with 100 μM of telmisartan or DMSO control. The cells were lysed with a protease inhibitor cocktail: PRO-PREP complete protease inhibitor mixture (iNtRON Biotechnology, Seongnam, Korea). Supernatants containing the soluble cellular proteins were collected and stored at -80˚C until use. Protein concentrations were measured using a NanoDrop 2000 spectrofluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Protein aliquots (1-10 μg) were resuspended in sample buffer and separated on 10% Tris-glycine gradient gels via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (26). After blocking, the membranes were incubated with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (27).

The antibodies used for western blot analyses were obtained from the following sources: antibodies against cyclin D1 and cyclin E were obtained from Thermo Fisher Scientific; CDK4, CDK6 and CDK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); AMPKα, p-AMPKα (thr172), mTOR, p-mTOR (thr2448), p70S6K and p-p70S6K (thr389) antibodies were purchased from Cell Signaling Technology; and anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The secondary antibodies included HRP-conjugated anti-mouse and anti-rabbit IgG (Cell Signaling Technology).

Enzyme-linked immunosorbent assay (ELISA) for measuring apoptosis. Caspase-cleaved cytokeratin 18 (cCK18) levels

were measured using the M30 Apoptosense ELISA kit obtained from Peviva Ab (Bromma, Sweden) to evaluate whether telmisartan exerted a pro-apoptotic effect (28). HLF cells (5.0x10³ cells/well) were seeded on a 96-well plate and cultured for 6, 24 and 48 h following treatment with 100 μM of telmisartan. The cells were lysed in polyoxyethylene octyl phenyl ether (NP-40) (Wako Pure Chemical Industries). The subsequent ELISA procedures were performed according to the manufacturer's instructions. The amounts of antigen in the control and unknown samples were calculated using a standard curve.

Antibody arrays of apoptosis-related protein profiles. HLF cells were cultured for 12 h after treatment with 100 μM of telmisartan or DMSO control and then lysed in PRO-PREP. The human apoptosis antibody array kit (R&D Systems, Minneapolis, MN, USA) was used to assess the levels of apoptosis-related proteins. Briefly, proteins were captured by antibodies spotted on a nitrocellulose membrane. Then, the levels of apoptosis-related proteins were assessed using an HRP-conjugated antibody followed by detection via chemiluminescence, and each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer, Waltham, MA, USA). The immunoreactive band density obtained from this array was analyzed by densitometric scanning (TIc scanner; Shimazu Co., Ltd., Kyoto, Japan). We repeated the same experiment three times to compare the telmisartan-treated group with the non-treated group.

Antibody arrays of p-RTKs. HLF cells were cultured for 48 h after treatment with 100 μM of telmisartan or DMSO control and then lysed in PRO-PREP. Human p-RTK array kits (R&D Systems) were used according to the manufacturer's instructions. The levels of phosphoproteins were assessed using an HRP-conjugated antibody. Each array was exposed to X-ray film and analyzed using the same method as described for the other antibody arrays. We repeated the same experiment three times to compare the telmisartan-treated group with the non-treated group.

Antibody arrays of angiogenetic protein profiles. HLF cells were cultured for 48 h after treatment with 100 μM of telmisartan or DMSO control and then lysed in PRO-PREP. The RayBio Human Angiogenesis Antibody array (RayBiotech, Norcross, GA, USA) was used according to the manufacturer's protocol. This method is a dot-based assay that enables the detection and comparison of 20 angiogenesis-specific cytokines. Each array was exposed to X-ray film and analyzed using the same method as described the other antibody arrays. We repeated the same experiment three times to compare the telmisartan-treated group with the non-treated group.

Microarray analysis of miRNAs. After treatment with 100 μM telmisartan or the DMSO control for 48 h, total RNA was extracted from HLF cells using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each RNA sample typically exhibited $A_{260/280}$ ratios between 1.9 and 2.1, which were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara,

CA, USA). After measuring RNA quantity using the RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miRCURY Hy3 Power Labeling kit (Exiqon A/S, Vedbaek, Denmark) and hybridized to a human miRNA Oligo Chip (v.21; Toray Industries, Inc., Tokyo, Japan). Scanning was conducted using the 3D-Gene Scanner 3000 (Toray Industries). The 3D-Gene extraction version 1.2 software (Toray Industries) was used to calculate the raw signal intensity of the images. The raw data were analyzed using the GeneSpring GX 10.0 software (Agilent Technologies) to assess the differences in miRNA expression between the telmisartan-treated and control samples. Global normalization was performed on raw data that were above the background level. Differentially expressed miRNAs were determined using Welch's t-test. The false discovery rate was computed using the Benjamini-Hochberg method (29). Hierarchical clustering was performed using the farthest neighbor method with the absolute uncentered Pearson's correlation coefficient as a metric. A heatmap was produced with the relative expression intensity of each miRNA, in which the base-2 logarithm of intensity was median-centered for each row.

Real-time quantitative polymerase chain reaction (qPCR) analysis of miRNAs. We compared the expression levels obtained from the miRNA arrays with real-time qPCR measurements to validate the data for miR-3651 and miR-7-5p which exhibited significantly greater changes in expression in the microarray analysis. Total RNA was extracted as described above and was diluted to 2.0 ng/μl. TaqMan microRNA assays (Applied Biosystems, Waltham, MA, USA) were adopted to determine the expression levels of miRNAs using U6 small nuclear RNA (RNU6B) as an internal control (Assay ID: 464410mat for miR-3651; 000268 for miR-7-5p; and 001093 for U6). miRNAs were reverse transcribed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems). Reverse transcription was prepared in 15 μl reaction volumes consisting of 5 μl of RNA, 3 μl of 5X RT primer and 12 μl of reverse transcription Master Mix. PCRs were performed in the MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems). Each well contained a 20 μl reaction consisting of 2 μl of cDNA, 1 μl of 20X qPCR assay, 7 μl of nuclease-free water and 10 μl of TaqMan Fast Advanced Master Mix (Applied Biosystems). Using the ViiA7 real-time PCR system (Applied Biosystems), reactions were denatured by an incubation at 95°C for 20 sec followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. The relative expression levels of miR-3651 and miR-7-5p were calculated using the comparative Ct method according to the following formula: 2-ΔCt (ΔCt = miRCt - U6Ct).

Statistical analyses. All statistical analyses were performed using the GraphPad Prism software version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Unpaired t-tests were conducted to compare the data between groups. A P-value of <0.05 was considered significant.

Results

Telmisartan inhibits the proliferation of human HCC cells. We conducted a cell proliferation assay and examined the
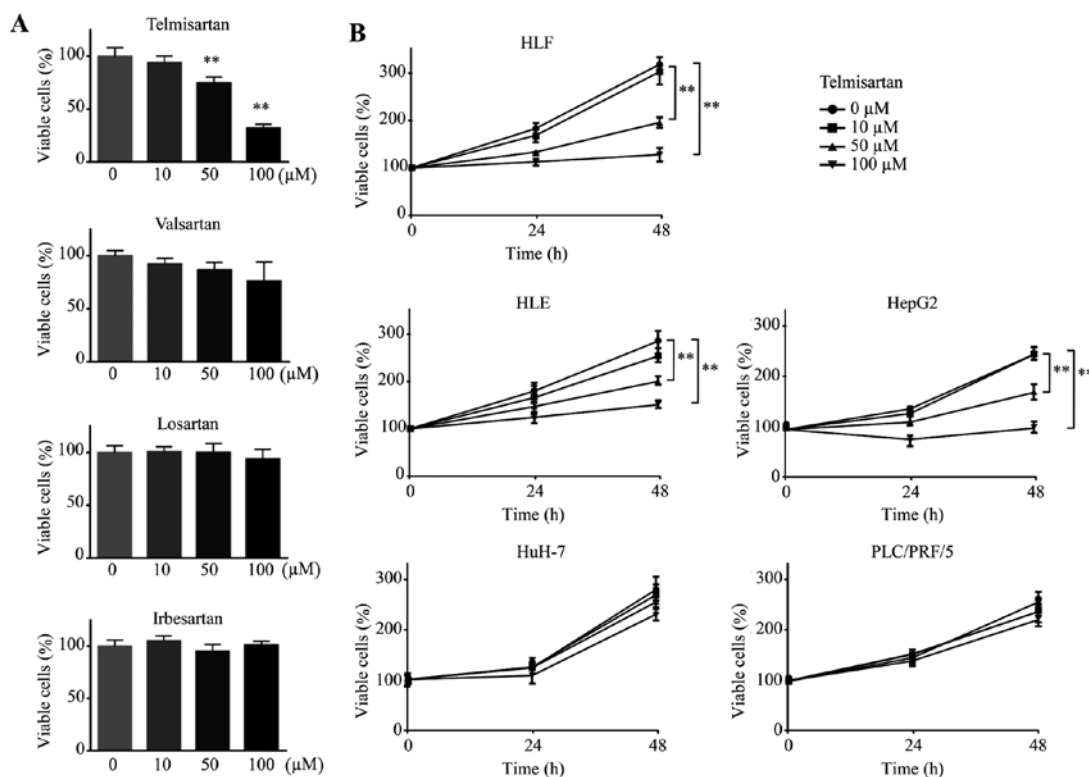
Figure 1. Telmisartan inhibits the proliferation of HLF cells. (A) Effects of the ARBs (telmisartan, valsartan, losartan and irbesartan) on the proliferation of HLF cells. (B) Effects of telmisartan on the proliferation of HCC cells (HLF, HLE, HepG2, HuH-7 and PLC/PRF/5). (**P<0.01).

antitumor effects of four ARBs (telmisartan, valsartan, losartan and irbesartan) on the HLF cell line after treatment for 48 h. Telmisartan reduced the proliferation of HLF cells, whereas none of the other ARBs (valsartan, losartan and irbesartan) affected the proliferation of the HCC cell lines (Fig. 1A). Additionally, five HCC cell lines (HLF, HLE, HepG2, HuH-7 and PLC/PRF/5) were treated with telmisartan, which reduced the proliferation of three HCC cell lines (HLF, HLE and HepG2) but not that of HuH-7 and PLC/PRF/5 cells (Fig. 1B). HLF, HLE and HepG2 cells are classified as poorly differentiated types of HCC cells, and HuH-7 and PLC/PRF/5 cells are classified as well differentiated types of HCC cells (30). Thus, telmisartan inhibits the proliferation of three HCC cell lines in a dose-dependent manner.

Telmisartan induces cell cycle arrest in G_0/G_1 phase and regulates cell cycle-related proteins. We examined cell cycle progression using flow cytometry to further investigate the effects of telmisartan on the HCC cell lines. Treatment with 100 μM telmisartan significantly increased the population of cells in G_1 phase and reduced the population of cells in S phase at 12, 24, 36 and 48 h after treatment (Fig. 2A). The effect peaked in 12 h after treatment and lasted up to 48 h.

The effects of telmisartan on the expression of various cell cycle-related proteins in HLF cells were evaluated by western blotting. Cells were treated with 0 or 100 μM telmisartan for 24 or 48 h. Cyclin D1 and cyclin E, which are key proteins involved in the transition from the G_0 to G_1 phase, exhibited the greatest reduction in expression; telmisartan inhibited cyclin D1 and cyclin E expression in a time-dependent manner (Fig. 2B). Additionally, analysis of other proteins associated with the

G_0 to G_1 transition indicated that the levels of CDK4, CDK6 (the catalytic subunits of cyclin D1) and CDK2 (the catalytic subunit of cyclin E) were decreased in HLF cells after the addition of telmisartan.

Telmisartan induces AMPK phosphorylation and inhibits the mTOR pathway and p70S6K phosphorylation in HLF cells. We focused on AMPK/mTOR signaling to identify the mechanism by which telmisartan induced cell cycle arrest. Telmisartan induced the phosphorylation of AMPK (thr172) in HLF cells, and this effect persisted for at least 48 h (Fig. 3). The levels of the p-mTOR and p-p70S6K proteins decreased in HLF cells following the telmisartan treatment. Thus, telmisartan inhibits cancer cell proliferation by inducing AMPK/mTOR signaling in HCC cells.

Telmisartan partially contributes to the induction of apoptosis in HLF cells. We detected apoptotic cells after telmisartan treatment using flow cytometry. The different quadrants presented in Fig. 4A represent living cells (lower left quadrant), early apoptotic cells (lower right quadrant) and late apoptotic cells (upper right quadrant). A comparison of the percentage of Annexin V positive cells revealed that the average of the triplicate assessment was 4.9% in the telmisartan-treated group and 3.9% in the non-treated group (Fig. 4B). Telmisartan slightly increased the proportion of apoptotic HLF cells 12 h after treatment, but this change was not statistically significant.

HLF cells were subsequently treated with 100 μM telmisartan and the levels of cCK18 following treatment were measured using an M30 ELISA kit to determine whether telmisartan induced apoptosis. Telmisartan significantly
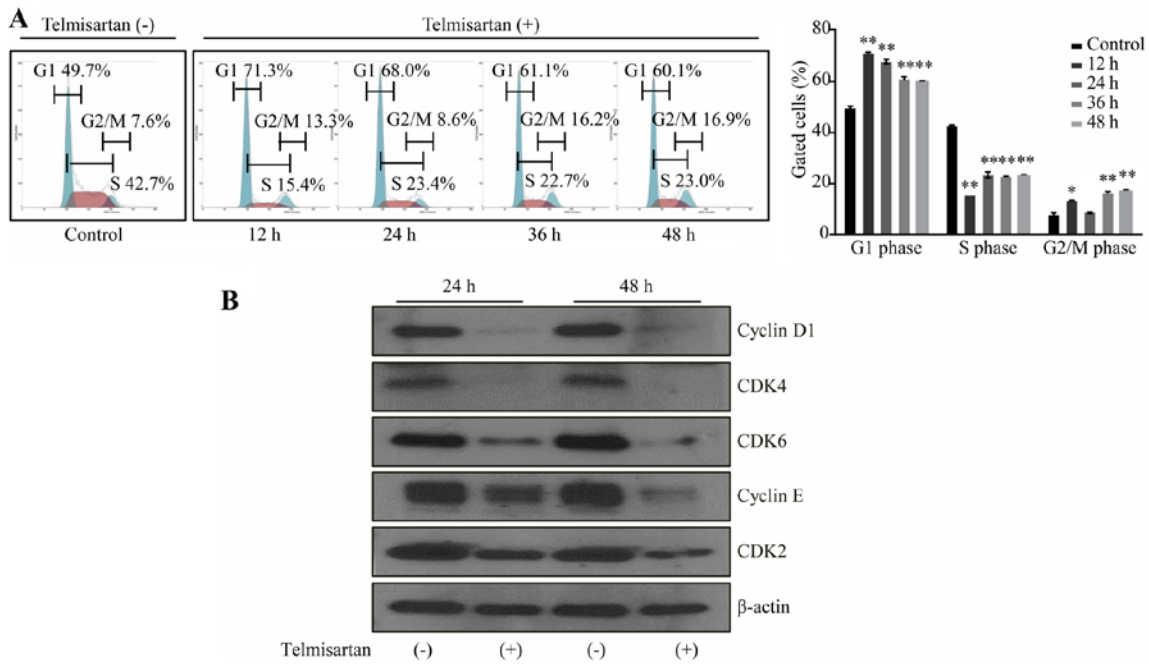
Figure 2. The antiproliferative effects of telmisartan on HCC cells are mediated by the induction of cell cycle arrest. (A) Cell cycle analysis of HLF cells after telmisartan treatment (*P<0.05 and **P<0.01). (B) Western blot analysis of cyclin D1, CDK4, CDK6, cyclin E and CDK2 levels in HLF cells treated with or without telmisartan.
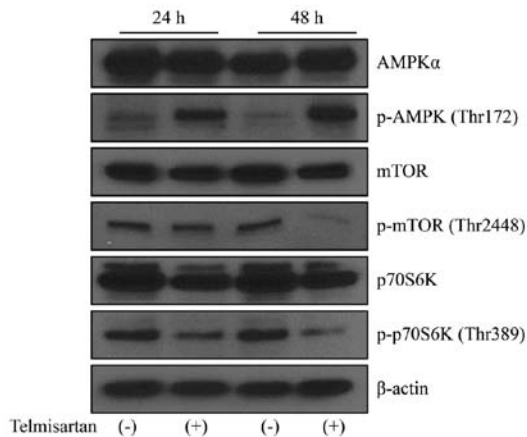

Figure 3. Telmisartan induces the phosphorylation of AMPKα, and the levels of the p-mTOR and p-p70S6K proteins were decreased in HLF cells treated with telmisartan.

increased the cCK18 levels at 6, 24 and 48 h after treatment (Fig. 4C).

Additionally, we used an apoptosis array system to identify the apoptosis-associated proteins that were involved in the antitumor effects of telmisartan. The use of an antibody array enabled us to screen 35 apoptosis-associated proteins in HLF cells treated with or without telmisartan (Fig. 5A). Telmisartan increased the levels of phospho-p53 (ser392) and decreased the levels of survivin in HLF cells (Fig. 5B). Based on densito-metry, the intensities of the phospho-p53 (ser392) and survivin spots from the telmisartan-treated HLF cells were 165.6 and 71.6% of the intensity of the untreated HLF cells, respectively (Fig. 5C). Based on these results, telmisartan may partially inhibit HCC cell proliferation by inducing apoptosis.
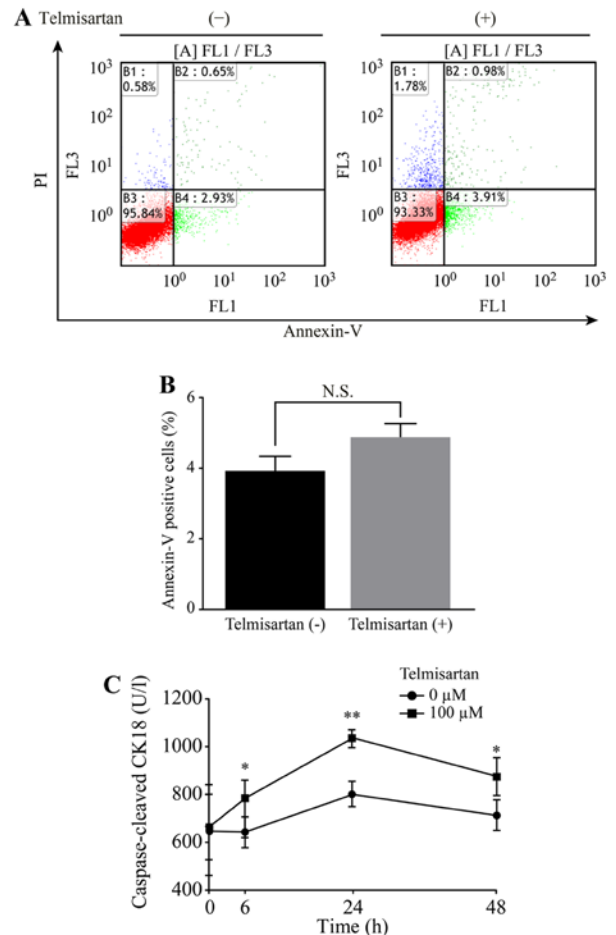

Figure 4. Telmisartan partially contributes to the induction of apoptosis in HLF cells. (A) Flow cytometry assessment of apoptosis in telmisartan-treated HLF cells. (B) Average number of Annexin V positive cells from a triplicate assessment. (C) The levels of cCK18 were measured by ELISA (*P<0.05 and **P<0.01).

A

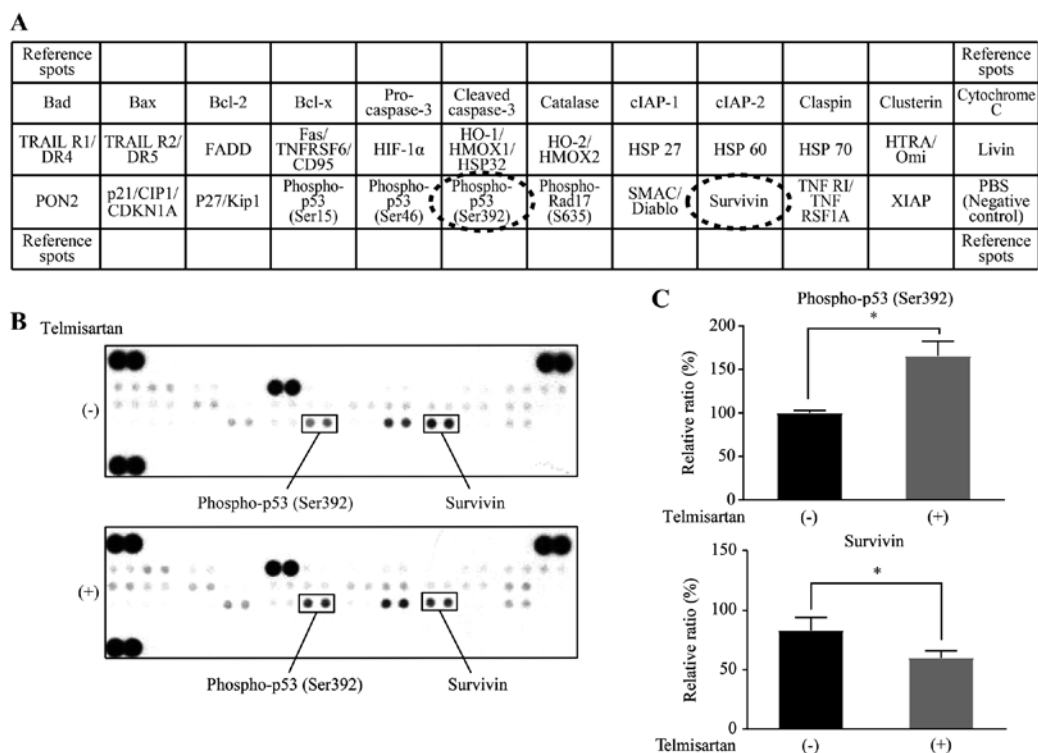| Reference spots											Reference spots
Bad	Bax	Bcl-2	Bcl-x	Pro-caspase-3	Cleaved caspase-3	Catalase	cIAP-1	cIAP-2	Claspin	Clusterin	Cytochrome C
TRAIL R1/ DR4	TRAIL R2/ DR5	FADD	Fas/ TNFRSF6/ CD95	HIF-1α	HO-1/ HMOX1/ HSP32	HO-2/ HMOX2	HSP 27	HSP 60	HSP 70	HTRA/ Omi	Livin
PON2	p21/CIP1/ CDKN1A	P27/Kip1	Phospho-p53 (Ser15)	Phospho-p53 (Ser46)	Phospho-p53 (Ser392)	Phospho-Rad17 (S635)	SMAC/ Diablo	Survivin	TNF RI/ TNF RSF1A	XIAP	PBS (Negative control)
Reference spots											Reference spots

Figure 5. Telmisartan increases the level of phospho-p53 (ser392) and decreases the level of survivin. (A) Template showing the location of apoptosis-related proteins on the human apoptosis antibody array. (B) Representative expression levels of various apoptosis-related proteins in HLF cells treated with or without telmisartan. (C) The densitometric ratio of the telmisartan-treated group to the non-treated group for the phospho-p53 (ser392) and survivin spots (*P<0.05).

A

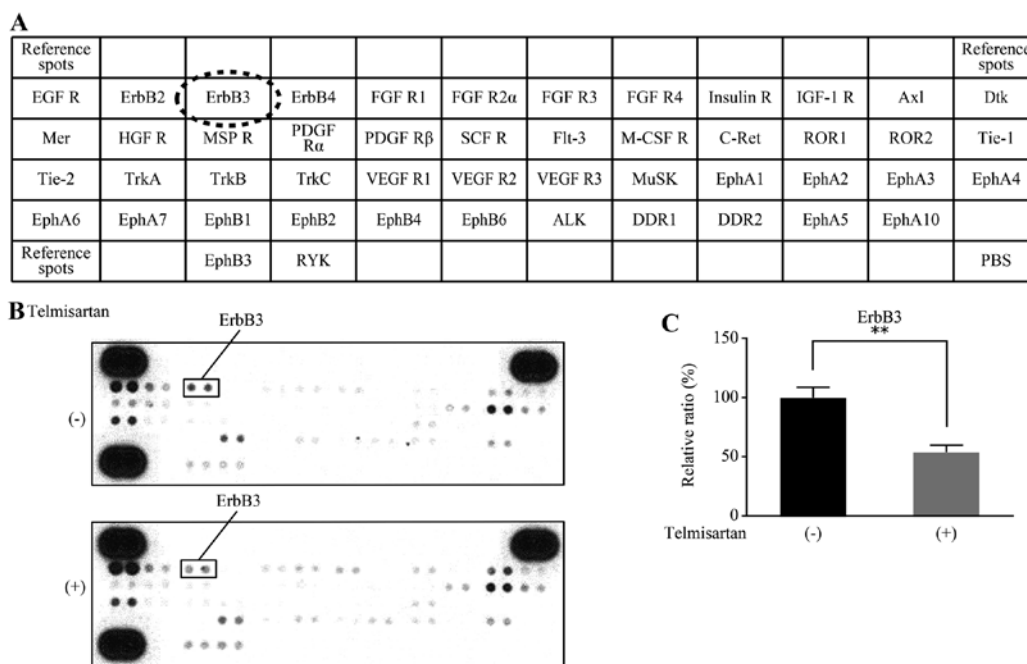| Reference spots											Reference spots
EGF R	ErbB2	ErbB3	ErbB4	FGF R1	FGF R2α	FGF R3	FGF R4	Insulin R	IGF-1 R	Axl	Dtk
Mer	HGF R	MSP R	PDGF Rα	PDGF Rβ	SCF R	Flt-3	M-CSF R	C-Ret	ROR1	ROR2	Tie-1
Tie-2	TrkA	TrkB	TrkC	VEGF R1	VEGF R2	VEGF R3	MuSK	EphA1	EphA2	EphA3	EphA4
EphA6	EphA7	EphB1	EphB2	EphB4	EphB6	ALK	DDR1	DDR2	EphA5	EphA10	
Reference spots		EphB3	RYK								PBS

Figure 6. Telmisartan reduces the levels of p-ErbB3 in HLF cells. (A) Template showing the locations of the tyrosine kinase antibodies on the human p-RTK array. (B) Representative expression of various p-RTKs in HLF cells treated with or without telmisartan. (C) The densitometric ratio of the telmisartan-treated group to the non-treated group for the p-ErbB3 spots (**P<0.01).

Telmisartan reduces the p-ErbB3 levels in HLF cells. We used a p-RTK array to identify the key RTKs associated with the antitumor effects of telmisartan. We simultaneously analyzed the expression of 46 differentially activated RTKs in HLF cells 48 h after telmisartan administration using an antibody array (Fig. 6A). Telmisartan reduced the levels of p-ErbB3 in HLF cells (Fig. 6B). The densitometric analyses of p-ErbB3 showed a decrease of 53.7% (Fig. 6C). Thus, telmisartan may decrease the expression of cell cycle regulatory proteins by inhibiting ErbB3 activation in HCC cells.

A

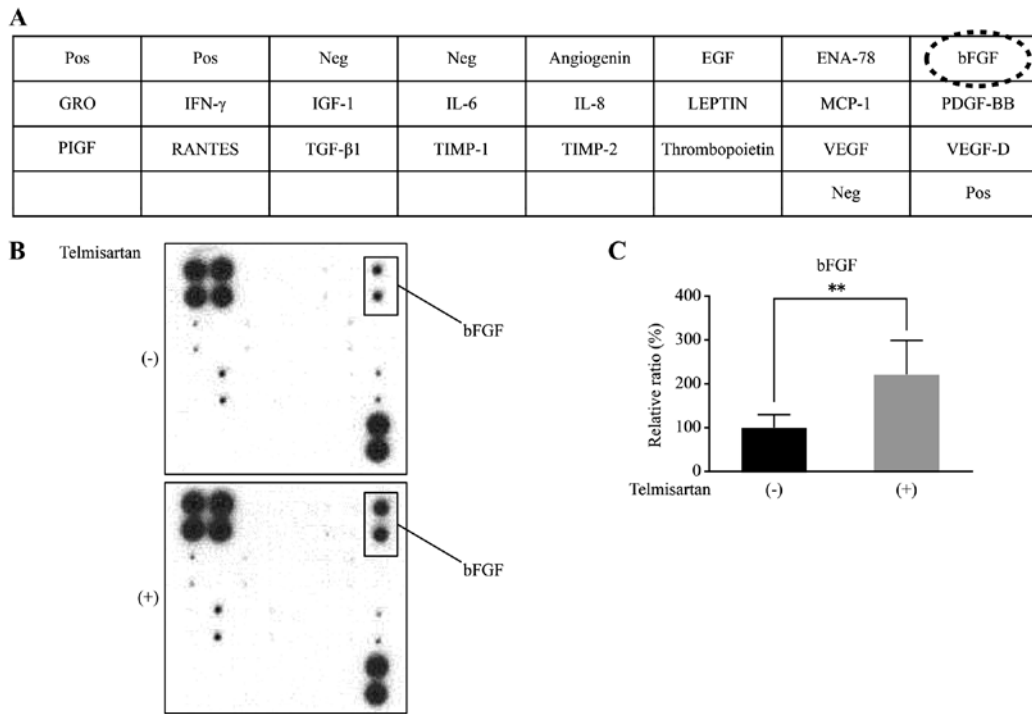| Pos	Pos	Neg	Neg	Angiogenin	EGF	ENA-78	bFGF
GRO	IFN-γ	IGF-1	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PlGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
						Neg	Pos

Figure 7. Telmisartan increases the levels of bFGF in HLF cells. (A) Template showing the locations of angiogenesis-related proteins spotted on the human angiogenesis array. (B) Representative expression levels of various angiogenesis-related proteins in HLF cells treated with or without telmisartan. (C) The densitometric ratio of the telmisartan-treated group to the non-treated group for the bFGF spots (**P<0.01).
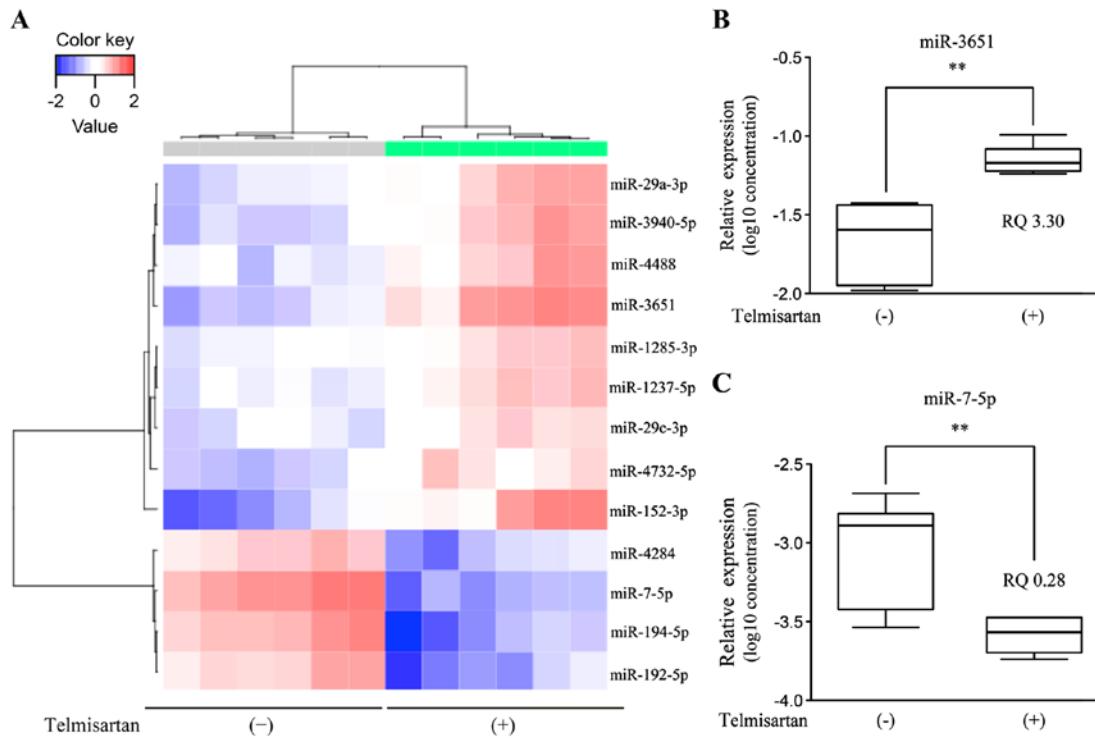

Figure 8. Telmisartan affects miRNA expression in HLF cells. (A) Hierarchical clustering of HLF cells cultured with or without telmisartan according to the expression profiles of numerous differentially expressed miRNAs. The miRNA clustering color scale presented at the top indicates the relative miRNA expression levels, with red and blue representing high and low expression levels, respectively (P<0.001). (B) Real-time qPCR relative quantification (RQ) of miRNAs following telmisartan treatment. miR-3651 expression was significantly upregulated. (C) miR-7-5p expression was significantly downregulated. The $\log_{10}2^{-\Delta Ct}$ value for each miRNA was used to create the figure; the lines represent averages with interquartile ranges (**P<0.01).

Telmisartan affects angiogenesis in HLF cells. An antibody-based analysis of angiogenesis was conducted to investigate the antitumor effects of telmisartan and to examine the relationship between angiogenesis and telmisartan (Fig. 7A). Using

Table I. Statistical results and chromosomal locations of miRNAs evaluated in HLF cells treated with or without telmisartan that exhibited a fold change (FC)>1.5, FC<0.67, or a P-value <0.005.

miRNA	Fold change (Treated/ Untreated)	P-value	Chromosomal location
Upregulated			
miR-4294	3.19	0.0010	10
miR-152-3p	2.65	0.0005	17q21.32
miR-6088	2.62	0.0034	19
miR-3651	2.45	<0.0001	9
miR-3940-5p	2.06	0.0002	19
miR-7150	2.02	0.0040	9
miR-642b-3p	2.00	0.0011	19
miR-6795-5p	1.95	0.0017	19
miR-3191-3p	1.91	0.0031	19
miR-1469	1.89	0.0015	15
miR-29a-3p	1.84	0.0005	7q32.3
miR-4488	1.84	0.0006	11
miR-6887-5p	1.81	0.0048	19
miR-6808-5p	1.77	0.0027	1
miR-6774-5p	1.75	0.0027	16
miR-4732-5p	1.73	0.0003	17
miR-1246	1.72	0.0019	2
miR-4442	1.70	0.0017	3
miR-1237-5p	1.63	0.0004	11
miR-362-5p	1.62	0.0022	X
miR-1343-5p	1.61	0.0039	11
miR-193b-5p	1.57	0.0040	16
miR-29c-3p	1.54	0.0004	1q32.2
miR-6769a-5p	1.53	0.0018	16
miR-8059	1.52	0.0015	17
miR-1285-3p	1.52	0.0008	7
miR-29b-3p	1.51	0.0039	7
miR-8064	1.51	0.0046	3
miR-23c	1.51	0.0030	X
Downregulated			
miR-7-5p	0.29	<0.0001	9
miR-194-5p	0.31	0.0005	1
miR-192-5p	0.37	0.0004	11q13.1
miR-4284	0.46	0.0004	7
miR-1303	0.56	0.0038	5
miR-425-5p	0.58	0.0042	3
miR-6807-5p	0.59	0.0033	19
miR-503-5p	0.60	0.0046	X
miR-15b-3p	0.60	0.0015	3
miR-377-3p	0.60	0.0028	14
miR-542-3p	0.64	0.0026	X
miR-3918	0.65	0.0012	6
miR-200a-3p	0.65	0.0019	1

the antibody array, we simultaneously screened the expression levels of 20 different angiogenesis-related proteins in HLF cells treated with or without telmisartan. The telmisartan treatment increased the b-fibroblast growth factor (bFGF) levels in HLF cells as detected by the protein array (Fig. 7B). According to the densitometric analyses, the ratio of the intensity of the bFGF spots obtained from the telmisartan-treated cells to the spots obtained from the untreated cells was 221.7% (Fig. 7C).

Telmisartan affects miRNA expression. Using a custom micro-array platform, we analyzed the expression levels of 2,555 miRNA probes in the cell lines in the presence or absence of the telmisartan treatment. Treatment with 100 μM telmisartan for 48 h upregulated the expression of 108 miRNAs and downregulated the expression of 55 miRNAs in HLF cells. miR-3651 was significantly upregulated and miR-7-5p was significantly downregulated (Table I). An unsupervised hierarchical clustering analysis was conducted using Pearson's correlation, and the results indicated that HLF cells treated with telmisartan *in vitro* clustered together and were separate from the untreated cell lines (Fig. 8A).

The results of the qPCR assay revealed that the mean miR-3651 level was obviously increased in the telmisartan-treated HLF cells compared with the controls, whereas miR-7-5p expression was significantly decreased in the telmisartan-treated cells compared to the untreated cells (Fig. 8B).

Discussion

ARBs are widely prescribed drugs for the treatment of hypertension. Despite their widespread use, the use of ARBs is associated with an 8% increased risk of cancer (14). However, telmisartan is not associated with an increased risk of cancer (31). Among the ARBs tested in the present study, only telmisartan inhibited HLF cell proliferation. Additionally, telmisartan dose-dependently inhibited the proliferation of three HCC cell lines (HLF, HLE and HepG2) but not the HuH-7 and PLC/PRF/5 cell lines. HLF, HLE and HepG2 cells are classified as poorly differentiated types of HCC cells, and HuH-7 and PLC/PRF/5 cells are classified as well differentiated types of HCC cells (30). Accordingly, poorly differentiated HCC cells were more sensitive to the telmisartan treatment than well to moderately differentiated HCC cells.

Many previous studies have shown that telmisartan exerts an antitumor effect through the induction of apoptosis, as has been demonstrated in endometrial (12), prostate (17), renal (18) and colon cancer (19). However, few reports, including our data in esophageal adenocarcinoma (21), have shown that cell cycle arrest is the major mechanism underlying the antitumor effect of telmisartan. Similarly, the present study showed that telmisartan induced cell cycle arrest at the G_0/G_1 phase by modulating the expression of cell cycle regulatory proteins in HCC cells. According to our flow cytometric analyses, telmisartan induced significant cell cycle arrest in HLF cells. These findings were further corroborated by an analysis of cell cycle-related proteins. The levels of the cell cycle regulatory proteins cyclin D1, cyclin E and CDKs were substantially reduced. Specific cyclin/CDK complexes are activated at different times during cell cycle progression. Complexes of CDK4 and CDK6 with cyclin D1 are required for G_1 phase progression, whereas

complexes of CDK2 with cyclin E are required for the G_1-S transition (32). The expression of various cell cycle-related proteins is related to cancer prognosis (32,33). Therefore, the major cell cycle regulators (cyclin D1 and cyclin E) may be intracellular targets of telmisartan in human HCC cell lines.

More recently, telmisartan was shown to contribute to the activation of AMPK in vascular endothelial cells (34,35). AMPK is a serine/threonine protein kinase that plays many important roles in cellular energy homeostasis (36). Recent studies have documented that the pharmacological activation of AMPK can block cancer cell growth in various human cancers (37). In HCC, we also assumed that telmisartan regulates the proliferation of cancer cells through AMPK activation. In the present study, telmisartan exerted its anti-tumor effects by inducing AMPK phosphorylation in HCC cells, suggesting that activation of the AMPK/mTOR pathway inhibits the expression of cell cycle regulatory proteins. AMPK activation has been shown to inhibit the mTOR pathway and p70S6K phosphorylation, which are involved in protein synthesis, suggesting that this pathway may regulate cell proliferation in various cancer cells (38,39). These reports support our finding that AMPK/mTOR signaling is a pivotal pathway underlying the antiproliferative effect of telmisartan on HCC cells.

As described above, telmisartan inhibits cell proliferation by inducing apoptosis in various cancer cell lines, including urological cancer cell lines (17,18). It also induces apoptosis and DNA fragmentation in human urological cancer (40). The mechanism by which telmisartan induces apoptosis in endometrial cancer was recently elucidated and involves the downregulation of Bcl-2 and Bcl-xL, as well as the upregu-lation of cleaved caspase-3 and PARP (12). HLF cells were treated with or without 100 μM telmisartan for 12 h and analyzed using flow cytometry to determine whether telmis-artan induced apoptosis. Telmisartan did not significantly increase the proportion of apoptotic cells, but it increased the cCK18 levels in HLF cells after 6, 24 and 48 h of treatment. Additionally, telmisartan increased the levels of phospho-p53 (ser392) and decreased survivin expression in HLF cells. The p53 protein is activated by phosphorylation to induce tumor cell apoptosis (41), and survivin is a member of a family of apoptosis inhibitors that play key roles in regulating cell divi-sion and inhibiting apoptosis by blocking caspase activation. However, telmisartan might inhibit HCC cell proliferation partially by inducing apoptosis and regulating the levels of apoptosis-related proteins. Some experimental results, including flow cytometry and western blotting, suggest that the antitumor effect of telmisartan in hepatocellular carcinoma is due to cell cycle arrest rather than apoptosis.

Several signaling pathways, such as the Ras/Raf/mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), insulin-like growth factor receptor, Wnt/β-catenin and Akt/mTOR signaling pathways, have been implicated in hepatic carcinogenesis. In particular, the Ras/Raf/MAPK pathway is typically activated in HCC as a result of increased signaling from upstream growth factors and the inactivation of tumor suppressor genes (42). Telmisartan reduced the phosphorylation of ErbB3 in HCC cells, as measured using p-RTK arrays. In the present study, telmisartan decreased mTOR expression. mTOR, a major

downstream target of the EGFR family, regulates p70S6K. Thus, telmisartan might inhibit cell cycle regulatory proteins by decreasing ErbB3 phosphorylation to regulate HCC cell proliferation.

Candesartan, another ARB, was recently shown to significantly reduce transforming growth factor $\beta1$ (TGF-$\beta1$) expression and suppress tumor proliferation and stromal fibrosis (13). Candesartan also significantly inhibits the growth of tumor xenografts and angiogenesis in mice (13). In the present study, telmisartan increased the bFGF levels in HLF cells. Thus, HLF cells that escape the antitumor effects of telmisartan may express angiogenesis-related proteins.

miRNAs are small non-coding RNA molecules that can regulate the development and progression of various cancers (43). The expression of several miRNAs was signifi-cantly altered *in vitro* following telmisartan treatment. We identified 163 differentially expressed miRNAs (108 upregu-lated and 55 downregulated) in HLF cells in response to telmisartan treatment using a microarray analysis. Several miRNAs that were upregulated upon telmisartan treatment have been reported to be tumor suppressors associated with decreased expression of cyclin/CDK complexes and anti-apoptotic proteins. For instance, the miR-29 family targets Bcl-2 (44), miR-29c-3p modulates cyclin E expression (45), and miR-29b-3p represses CDK2 expression (46). In addi-tion, numerous studies have examined the target molecules of miRNAs associated with cancer progression: miR-126-5p directly regulates a disintegrin and metalloprotease domain 9 (ADAM9) and metalloproteinase 7 (MMP7) expression (47), and miR-152-3p represses DNA methyltransferase 1 (DNMT1) expression (48). Notably, several miRNAs that were down-regulated upon telmisartan treatment have been reported to be oncomiRNAs associated with increased expression of CDK inhibitors: miR-7 inhibits p21-activated kinase 1 (PAC1) (49) and miR-194 directly targets p27kip1 (50). It is possible that these miRNAs interact in a complicated manner and contribute to the antitumor effect of telmisartan, but the suppression of tumor growth via miRNAs has not been fully elucidated. Despite these limitations, our findings have important implications.

In conclusion, telmisartan inhibits human HCC cell prolif-eration by inducing cell cycle arrest via the regulation of cell cycle-related proteins.

Acknowledgements

References

1. Forner A, Llovet JM and Bruix J: Hepatocellular carcinoma. Lancet 379: 1245-1255, 2012.
2. Altekruse SF, Henley SJ, Cucinelli JE and McGlynn KA: Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the United States. Am J Gastroenterol 109: 542-553, 2014.
3. Kim SR, Kudo M, Hino O, Han KH, Chung YH and Lee HS; Organizing Committee of Japan-Korea Liver Symposium: Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. Oncology 75 (Suppl 1): 13-16, 2008.

4. Muto J, Shirabe K, Sugimachi K and Maehara Y: Review of angiogenesis in hepatocellular carcinoma. Hepatol Res 45: 1-9, 2015.

5. Itabashi H, Maesawa C, Oikawa H, Kotani K, Sakurai E, Kato K, Komatsu H, Nitta H, Kawamura H, Wakabayashi G, *et al*: Angiotensin II and epidermal growth factor receptor cross-talk mediated by a disintegrin and metalloprotease accelerates tumor cell proliferation of hepatocellular carcinoma cell lines. Hepatol Res 38: 601-613, 2008.

6. Molina Wolgien MC, Guerreiro da Silva ID, Pinto Nazário AC, Nakaie CR, Correa-Noronha SA, Ribeiro de Noronha SM and Facina G: Genetic association study of angiotensin II receptor types 1 (A168G) and 2 (T1247G and A5235G) polymorphisms in breast carcinoma among Brazilian Women. Breast Care (Basel) 9: 176-181, 2014.

7. Miyajima A, Kosaka T, Asano T, Asano T, Seta K, Kawai T and Hayakawa M: Angiotensin II type I antagonist prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis. Cancer Res 62 (15s): 4176-4179, 2002.

8. Kinoshita J, Fushida S, Harada S, Yagi Y, Fujita H, Kinami S, Ninomiya I, Fujimura T, Kayahara M, Yashiro M, *et al*: Local angiotensin II-generation in human gastric cancer: Correlation with tumor progression through the activation of ERK1/2, NF-kappaB and survivin. Int J Oncol 34: 1573-1582, 2009.

9. Okamoto K, Tajima H, Ohta T, Nakanuma S, Hayashi H, Nakagawara H, Onishi I, Takamura H, Ninomiya I, Kitagawa H, *et al*: Angiotensin II induces tumor progression and fibrosis in intrahepatic cholangiocarcinoma through an interaction with hepatic stellate cells. Int J Oncol 37: 1251-1259, 2010.

10. Du N, Feng J, Hu LJ, Sun X, Sun HB, Zhao Y, Yang YP and Ren H: Angiotensin II receptor type 1 blockers suppress the cell proliferation effects of angiotensin II in breast cancer cells by inhibiting AT1R signaling. Oncol Rep 27: 1893-1903, 2012.

11. Chen X, Meng Q, Zhao Y, Liu M, Li D, Yang Y, Sun L, Sui G, Cai L and Dong X: Angiotensin II type 1 receptor antagonists inhibit cell proliferation and angiogenesis in breast cancer. Cancer Lett 328: 318-324, 2013.

12. Koyama N, Nishida Y, Ishii T, Yoshida T, Furukawa Y and Narahara H: Telmisartan induces growth inhibition, DNA double-strand breaks and apoptosis in human endometrial cancer cells. PLoS One 9: e93050, 2014.

13. Okazaki M, Fushida S, Harada S, Tsukada T, Kinoshita J, Oyama K, Tajima H, Ninomiya I, Fujimura T and Ohta T: The angiotensin II type 1 receptor blocker candesartan suppresses proliferation and fibrosis in gastric cancer. Cancer Lett 355: 46-53, 2014.

14. Sipahi I, Debanne SM, Rowland DY, Simon DI and Fang JC: Angiotensin-receptor blockade and risk of cancer: Meta-analysis of randomised controlled trials. Lancet Oncol 11: 627-636, 2010.

15. Bhaskaran K, Douglas I, Evans S, van Staa T and Smeeth L: Angiotensin receptor blockers and risk of cancer: Cohort study among people receiving antihypertensive drugs in UK General Practice Research Database. BMJ 344: e2697, 2012.

16. Makar GA, Holmes JH and Yang YX: Angiotensin-converting enzyme inhibitor therapy and colorectal cancer risk. J Natl Cancer Inst 106: djt374, 2014.

17. Funao K, Matsuyama M, Kawahito Y, Sano H, Chargui J, Touraine JL, Nakatani T and Yoshimura R: Telmisartan is a potent target for prevention and treatment in human prostate cancer. Oncol Rep 20: 295-300, 2008.

18. Funao K, Matsuyama M, Kawahito Y, Sano H, Chargui J, Touraine JL, Nakatani T and Yoshimura R: Telmisartan as a peroxisome proliferator-activated receptor-γ ligand is a new target in the treatment of human renal cell carcinoma. Mol Med Rep 2: 193-198, 2009.

19. Lee LD, Mafura B, Lauscher JC, Seeliger H, Kreis ME and Gröne J: Antiproliferative and apoptotic effects of telmisartan in human colon cancer cells. Oncol Lett 8: 2681-2686, 2014.

20. Kozako T, Soeda S, Yoshimitsu M, Arima N, Kuroki A, Hirata S, Tanaka H, Imakyure O, Tone N, Honda S, *et al*: Angiotensin II type 1 receptor blocker telmisartan induces apoptosis and autophagy in adult T-cell leukemia cells. FEBS Open Bio 6: 442-460, 2016.

21. Fujihara S, Morishita A, Ogawa K, Tadokoro T, Chiyo T, Kato K, Kobara H, Mori H, Iwama H and Masaki T: The angiotensin II type 1 receptor antagonist telmisartan inhibits cell proliferation and tumor growth of esophageal adenocarcinoma via the AMPKα/mTOR pathway in vitro and in vivo. Oncotarget 8: 8536-8549, 2017.

22. Rehman G, Shehzad A, Khan AL and Hamayun M: Role of AMP-activated protein kinase in cancer therapy. Arch Pharm (Weinheim) 347: 457-468, 2014.

23. Dann SG and Thomas G: The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett 580: 2821-2829, 2006.

24. Fingar DC and Blenis J: Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene 23: 3151-3171, 2004.

25. Masaki T, Tokuda M, Yoshida S, Nakai S, Morishita A, Uchida N, Funaki T, Kita Y, Funakoshi F, Nonomura T, *et al*: Comparison study of the expressions of myristoylated alanine-rich C kinase substrate in hepatocellular carcinoma, liver cirrhosis, chronic hepatitis, and normal liver. Int J Oncol 26: 661-671, 2005.

26. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.

27. Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354, 1979.

28. Schutte B, Henfling M, Kölgen W, Bouman M, Meex S, Leers MP, Nap M, Björklund V, Björklund P, Björklund B, *et al*: Keratin 8/18 breakdown and reorganization during apoptosis. Exp Cell Res 297: 11-26, 2004.

29. Benjamini Y and Hochberg Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 57: 289-300, 1995.

30. Morishita A, Iwama H, Fujihara S, Sakamoto T, Fujita K, Tani J, Miyoshi H, Yoneyama H, Himoto T and Masaki T: MicroRNA profiles in various hepatocellular carcinoma cell lines. Oncol Lett 12: 1687-1692, 2016.

31. Tascilar K, Azoulay L, Dell'Aniello S, Bartels DB and Suissa S: The use of telmisartan and the incidence of cancer. Am J Hypertens 29: 1358-1365, 2016.

32. Masaki T, Shiratori Y, Rengifo W, Igarashi K, Yamagata M, Kurokohchi K, Uchida N, Miyauchi Y, Yoshiji H, Watanabe S, *et al*: Cyclins and cyclin-dependent kinases: Comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 37: 534-543, 2003.

33. Matsuda Y: Molecular mechanism underlying the functional loss of cyclindependent kinase inhibitors p16 and p27 in hepatocellular carcinoma. World J Gastroenterol 14: 1734-1740, 2008.

34. Kurokawa H, Sugiyama S, Nozaki T, Sugamura K, Toyama K, Matsubara J, Fujisue K, Ohba K, Maeda H, Konishi M, *et al*: Telmisartan enhances mitochondrial activity and alters cellular functions in human coronary artery endothelial cells via AMP-activated protein kinase pathway. Atherosclerosis 239: 375-385, 2015.

35. Myojo M, Nagata D, Fujita D, Kiyosue A, Takahashi M, Satonaka H, Morishita Y, Akimoto T, Nagai R, Komuro I, *et al*: Telmisartan activates endothelial nitric oxide synthase via Ser1177 phosphorylation in vascular endothelial cells. PLoS One 9: e96948, 2014.

36. Kahn BB, Alquier T, Carling D and Hardie DG: AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 1: 15-25, 2005.

37. Shackelford DB and Shaw RJ: The LKB1-AMPK pathway: Metabolism and growth control in tumour suppression. Nat Rev Cancer 9: 563-575, 2009.

38. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, *et al*: The antidiabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Mol Cancer Ther 11: 549-560, 2012.

39. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y and Bost F: The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27: 3576-3586, 2008.

40. Matsuyama M, Funao K, Kuratsukuri K, Tanaka T, Kawahito Y, Sano H, Chargui J, Touraine JL, Yoshimura N and Yoshimura R: Telmisartan inhibits human urological cancer cell growth through early apoptosis. Exp Ther Med 1: 301-306, 2010.

41. Hussain SP, Schwank J, Staib F, Wang XW and Harris CC: TP53 mutations and hepatocellular carcinoma: Insights into the etiology and pathogenesis of liver cancer. Oncogene 26: 2166-2176, 2007.

42. Galuppo R, Ramaiah D, Ponte OM and Gedaly R: Molecular therapies in hepatocellular carcinoma: What can we target? Dig Dis Sci 59: 1688-1697, 2014.

43. Morishita A and Masaki T: miRNA in hepatocellular carcinoma. Hepatol Res 45: 128-141, 2015.
44. Xiong Y, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH and Zhuang SM: Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. Hepatology 51: 836-845, 2010.
45. Ding DP, Chen ZL, Zhao XH, Wang JW, Sun J, Wang Z, Tan FW, Tan XG, Li BZ, Zhou F, et al: miR-29c induces cell cycle arrest in esophageal squamous cell carcinoma by modulating cyclin E expression. Carcinogenesis 32: 1025-1032, 2011.
46. Xiao L, Rao JN, Zou T, Liu L, Cao S, Martindale JL, Su W, Chung HK, Gorospe M and Wang JY: miR-29b represses intestinal mucosal growth by inhibiting translation of cyclin-dependent kinase 2. Mol Biol Cell 24: 3038-3046, 2013.
47. Felli N, Felicetti F, Lustri AM, Errico MC, Bottero L, Cannistraci A, De Feo A, Petrini M, Pedini F, Biffoni M, et al: miR-126&126* restored expressions play a tumor suppressor role by directly regulating ADAM9 and MMP7 in melanoma. PLoS One 8: e56824, 2013.
48. Braconi C, Huang N and Patel T: MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. Hepatology 51: 881-890, 2010.
49. Reddy SD, Ohshiro K, Rayala SK and Kumar R: MicroRNA-7, a homeobox D10 target, inhibits p21-activated kinase 1 and regulates its functions. Cancer Res 68: 8195-8200, 2008.
50. Wu X, Liu T, Fang O, Leach LJ, Hu X and Luo Z: miR-194 suppresses metastasis of non-small cell lung cancer through regulating expression of BMP1 and p27^{kip1}. Oncogene 33: 1506-1514, 2014.