学　位　論　文

Angiotensin receptor blocker telmisartan inhibits cell proliferation and tumor growth of cholangiocarcinoma through cell cycle arrest

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Abstract. Cholangiocarcinoma (CCA) is at an advanced stage at the time of its diagnosis, and developing a more effective treatment of CCA would be desirable. Angiotensin II type 1 (AT1) receptor blocker (ARB), telmisartan may inhibit cancer cell proliferation, but the mechanisms by which telmisartan affects various cancers remain unknown. In this study, we evaluated the effects of telmisartan on human CCA cells and to assess the expression of microRNAs (miRNAs). We studied the effects of telmisartan on CCA cells using two cell lines, HuCCT-1 and TFK-1. In our experiments, telmisartan inhibited the proliferation of HuCCT-1 and TFK-1 cells. Additionally, telmisartan induced G_0/G_1 cell cycle arrest via blockade of the G_0 to G_1 cell cycle transition. Notably, telmisartan did not induce apoptosis in HuCCT-1 cells. This blockade was accompanied by a strong decrease in cell cycle-related protein, especially G1 cyclin, cyclin D1, and its catalytic subumits, Cdk4 and Cdk6. Telmisartan reduced the phosphorylation of EGFR (p-EGFR) and TIMP-1 by using p-RTK and angiogenesis array. Furthermore, miRNA expression was markedly altered by telmisartan in HuCCT-1. Telmisartan inhibits tumor growth in CCA xenograft model *in vivo*. In conclusion, telmisartan was shown to inhibit human CCA cell proliferation by inducing cell cycle arrest.

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Abbreviations: CCA, cholangiocarcinoma; ARBs, angiotensin II type 1 receptor blockers; AT1, angiotensin II type 1; AMPK, AMP-activated protein kinase; EGFR, epidermal growth factor receptor; DMSO, dimethyl sulfoxide; CCK-8, Cell Counting Kit-8

Key words: cholangiocarcinoma, telmisartan, cell cycle, angiotensin II type 1 receptor blocker

Introduction

Cholangiocarcinoma (CCA) is a malignant cancer arising from the neoplastic transformation of cholangiocytes, the epithelial cells lining the intrahepatic and extrahepatic bile ducts (1,2). In general, in most CCA cases, the disease is at an advanced stage at the time of its diagnosis. Although surgery including curative liver transplantation is a therapeutic option for selected patients with CCA, 5-year survival rate is very low (3,4). Therefore, developing a more effective treatment of CCA would be desirable.

Angiotensin receptor blocker (ARB), telmisartan is widely prescribed drug for the treatment of hypertension and heart failure. It inhibited the growth of adult T-cell leukemia cell (5), endometrial (6), and gastric cancer cells (7) in several studies. The main mechanism associated with antitumor effect by telmisartan treatment has been shown to inhibit cell proliferation by inducing apoptosis in various cancer cell lines, including prostate (8), renal (9), endometrial (6) and colon (10) cancer lines. More recently, telmisartan induced cell cycle arrest in hematologic and non-hematologic malignancies including our study (5,11).

Cyclin and cyclin dependent kinase (CDKs) complexes are key to the progression of cells through G1/S phase of the cell cycle by inactivating Rb through phosphorylation (12). Over- expression of these molecules are postulated to increase cell turnover and proliferative activity (13). Especially, cyclin D1, and CDKs are essential for driving cells to pass the restriction point (14,15). These mechanisms are associated with the restriction point control the order and timing of cell cycle transition, whereas cell regulatory functions are usually impaired in cancer cells. Overexpression of cyclin D1 has been shown to be a poor prognostic factor in bile duct carcinoma (16). Therefore, repairing cell cycle progression might be an effective strategy for treatment of CCA. In our previous study, telmisartan inhibited human esophageal adenocarci-

noma cell proliferation and tumor growth, inducing cell cycle arrest by regulating cell cycle-related molecules (11). However, the antitumor effect of telmisartan and its mechanism of action in CCA are currently unknown.

In the present study, we report on an investigation of the anti-proliferative effect of telmisartan on CCA cells *in vitro*, and on tumor growth in CCA xenograft model *in vivo*. Cell cycle and cell cycle-related proteins were studied *in vitro*. Furthermore, possible mechanism associated with the anti-tumor effect of telmisartan were also explored, including the activation of receptor tyrosine kinases, angiogenesis, apoptosis and microRNAs (miRNAs).

Materials and methods

Drugs, chemicals and regents. The following were used: Telmisartan (Tokyo Chemical Industry Co. Tokyo, Japan), Trypan Blue (Sigma-Aldrich, St. Louis, MO, USA), RPMI-1640 (Gibco-Invitrogen, Carlsbad, CA, USA), Fetal Bovine serum (FBS, Wako Pure Chemical Industries, Osaka, Japan), penicillin-streptomycin (Invitrogen, Tokyo, Japan), Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan), Cell Cycle Phase Determination kit (Cayman Chemical, Ann Arbor, MI, USA), Annexin V-FITC Early Apoptosis Detection kit (Cell Signaling Technology, Boston, MA, USA), protease inhibitor cocktail (Pro-Prep, complete protease inhibitor mixture; iNtRON Biotechnology, Sungnam, Korea), M30 Apoptosense ELISA kit (PEVIVA AB, Bromma, Sweden), Human Phospho-RTK Array kits and Angiogenesis Antibody Array kits (R&D Systems, Minneapolis, MN, USA). Telmisartan was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). The stock solutions were stored at -20˚C.

Cell lines and culture. The human CCA cell lines HuCCT-1 and TFK-1 were obtained from the Japanese Cancer Research Resources Bank (JCRB). All cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 100 mg/l of penicillin-streptomycin. Cells were incubated at 37˚C in a humidified atmosphere with 5% of CO_2. Passages were made when confluence reached 80-90% by tripsinization with 0.05% (for HuCCT-1) or 0.25% (for TFK-1) trypsin EDTA and gentle mechanical detachment.

Cell proliferation assays. Cell proliferation assays were conducted using CCK-8 according to the manufacturer's instructions, as described in our previous studies (17,18). These assays were conducted in HuCCT-1 and TFK-1 cell lines. Cells ($5.0x10^3$) from each cell line were seeded into the wells of a 96-well plate and cultured in 100 μl of RPMI-1640 supplemented with 10% FBS. After 24 h, the seeded cells were treated with 0, 10, 50, 100 μM telmisartan or vehicle and then cultured for an additional 48 h. CCK-8 reagent (10 μl) was added to each well, and the plates were incubated at 37˚C for 3 h. The absorbance was measured at 450 nm in each well using an automated microplate reader.

Cell cycle and apoptosis analysis. To evaluate the mechanism by which telmisartan inhibits tumor growth, cell cycle and apoptosis analysis were performed separately using a Cell Cycle Phase Determination kit and an Annexin V-FITC Early Apoptosis Detection kit as described in our previous studies (19). HuCCT-1 cells ($1.0x10^6$ cells in a 100-mm dish) were treated with or without 100 μM telmisartan for 24-48 h. The cell cycle distribution was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells. The fixed cells were washed with PBS and 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. A total of 110 μl of PI stain (100 μg/ml) was added to each tube and incubated at 4˚C for at least 30 min prior to analysis. Apoptotic and necrotic cell death was analyzed by double staining with FITC-conjugated Annexin V and PI, which is based on the binding of Annexin V to apoptotic cells with exposed phosphatidylserines and the PI labeling of late apoptotic/necrotic cells with membrane damage. HuCCT-1 cells were treated with or without 100 μM telmisartan for 24 h. Staining was performed according to the manufacturer's instructions. Flow cytometry was conducted using a Cytomics FC 500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA). The percentages of cells were analyzed using Kaluza software (Beckman Coulter).

Gel electrophoresis and western blotting. HuCCT-1 cells ($1.0x10^6$/dish) were seeded in 100-mm culture dishes and cultured for 24 h. Then, 100 μM telmisartan was added, and the cells were further cultured for 24-48 h. The cells were lysed using a protease inhibitor cocktail (Pro-Prep, complete protease inhibitor mixture; iNtRON Biotechnology) on ice for 20 min. Suspensions of lysed cells were centrifuged at 13,000 x g at 4˚C for 5 min; supernatants containing soluble cellular proteins were collected and stored at -80˚C until use. Protein concentrations were measured using a NanoDrop 2000 fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% agarose gels, and the proteins were transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies followed by secondary antibodies. The immunoreactive proteins were visualized on X-ray film using an enhanced chemiluminescence detection system (Perkin-Elmer Co., Waltham, MA, USA). Following primary antibodies were used: anti-β-actin monoclonal antibody (used at 1:10,000; Sigma-Aldrich) and antibodies against cyclin D1 (used at 1:1,000; Thermo Fisher Scientific), cyclin E (used at 1:1,000; Thermo Fisher Scientific), Cdk6 (used at 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Cdk4 (used at 1:1,000; Santa Cruz Biotechnology), Cdk2 (used at 1:2,000; Santa Cruz Biotechnology), and phosphorylated retinoblastoma protein (Rb; used at 1:1,000; BD Pharmingen), and anti-Rb (used at 1:1,000; Santa Cruz Biotechnology). The secondary antibodies included horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit IgG (used at 1:2,000; GE Healthcare Life Sciences, Chalfont, UK).

ELISA for apoptosis. Caspase-cleaved cytokeratin 18 (cCK18) expression was evaluated using an M30 Apoptosense ELISA kit (20). HuCCT-1 cells ($5x10^3$ cells) were seeded in 96-well plates and cultured for 24 or 48 h following the addition of 100 μM telmisartan. The subsequent ELISA procedures were

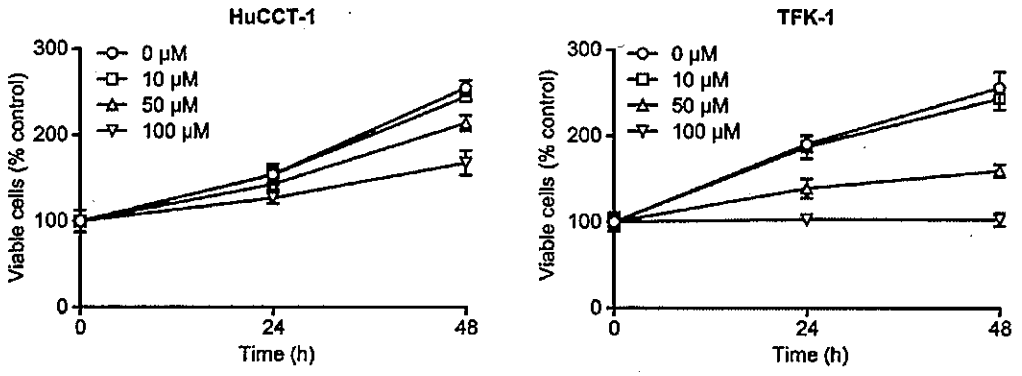

Figure 1. Telmisartan inhibits the proliferation of CCA cells. HuCCT-1 and TFK-1 cells were treated with 0, 10, 50, or 100 μM telmisartan for 24 or 48 h. The data points represent the mean cell number from three independent cultures, and the error bars represent SDs. For each cell line, the conditions at 48 h are significantly different compared with control (0 μM) with $P<0.05$ as assessed by 2-way ANOVA.

performed according to the manufacturer's instructions. The amounts of antigen in the control and treated samples were calculated via interpolation of a standard curve.

Antibody arrays of phosphorylated receptor tyrosine kinases (p-RTKs). Human p-RTKs were assayed using Human Phospho-RTK Array Kits according to the manufacturer's instructions. Briefly, p-RTK array membranes were blocked with 5% BSA/TBS (0.01 M Tris-HCl, pH 7.6) for 1 h and incubated with 2 ml of lysate prepared from the previously mentioned cells after normalization so that the amounts of protein were equal. After 3 washes for 10 min each with TBS plus 0.1% v/v Tween-20 and 2 washes for 10 min with TBS alone to remove unbound materials, the membranes were incubated with an HRP-conjugated anti-phosphotyrosine antibody for 2 h at room temperature. The unbound HRP antibody was washed out with TBS plus 0.1% Tween-20. Finally, each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.). The density of the immunoreactive band obtained 118 on this array was analyzed by densitometric scanning (TIc 119 scanner; Shimizu Co, Ltd., Kyoto, Japan).

Angiogenic profile analysis using an antibody array. A Human Angiogenesis Antibody Array was used according to the manufacturer's protocol. This method is a dot-based assay enabling the detection and comparison of 55 angiogenesis-specific cytokines. Each array membrane was exposed to X-ray film using a chemiluminescence detection system (Perkin-Elmer Co.). The immunoreactive bands were analyzed by densitometric scanning.

miRNA arrays. HuCCT-1 cells were treated with 100 μM telmisartan for 48 h and were stored in RNAprotect Reagent (Qiagen, Venlo, The Netherlands). Total RNA was extracted from cancer cell lines using a miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples typically exhibited $A_{260/280}$ ratios of between 1.9 and 2.1, as determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After RNA measurements were performed with an RNA 6000 Nano kit (Agilent Technologies), the samples were labeled using a miRCURY Hy3/Hy5 Power Labeling kit and were subsequently hybridized to a human miRNA Oligo chip (v. 21.0; Toray Industries, Tokyo, Japan). The chips were scanned with a 3D-Gene Scanner 3000 (Toray Industries), and the results were analyzed using 3D-Gene extraction version 1.2 software (Toray Industries). To determine the difference in miRNA expression between the telmisartan-treated and control samples, the raw data were analyzed using GeneSpring GX 10.0 software (Agilent Technologies). Quantile normalization was performed on the raw data that were above the background level. Differentially expressed miRNAs were determined by the Mann-Whitney U test. Hierarchical clustering was performed using the furthest neighbor method with the absolute uncentered Pearson's correlation coefficient as a metric. A heat map was produced with the relative expression intensity for each miRNA, in which the base-2 logarithm of the intensity was median-centered for each row.

Xenograft model analysis. Animal experiments were performed according to the guidelines of the Committee on Experimental Animals of Kagawa University, Kagawa, Japan. We purchased 21 male athymic mice (BALB/c-nu/nu; 6 weeks old; 20-25 g) from Japan SLC (Shizuoka, Japan). Moreover, the mice were maintained under specific pathogen-free conditions using a laminar airflow rack. The mice had continuous free access to sterilized (γ-irradiated) food (CL-2; CLEA Japan, Inc.) and autoclaved water. Each mouse was subcutaneously inoculated with HuCCT-1 cells ($3x10^6$ cells per animal) in the flank region. Then, when the xenografts were identifiable as masses with a maximal diameter >3 mm, we randomly assigned the animals to 3 groups. These groups were treated with 50 μg per day of telmisartan (n=7), or with 100 μg per day of telmisartan (n=7) or diluent only (n=7). The telmisartan-treated groups were intraperitoneally (i.p.) injected telmisartan (50 or 100 μg) five times per week; the control group was administered 5% DMSO alone. The tumor growth was monitored daily by the same investigators (E. Samukawa and T. Masaki), and the tumor size was measured two times per week. The tumor volume (mm^3) was calculated as the tumor length (mm) x tumor width $(mm)^2/2$ (21). All animals were sacrificed on day 31 after treatment, with all animals remaining alive during this period.

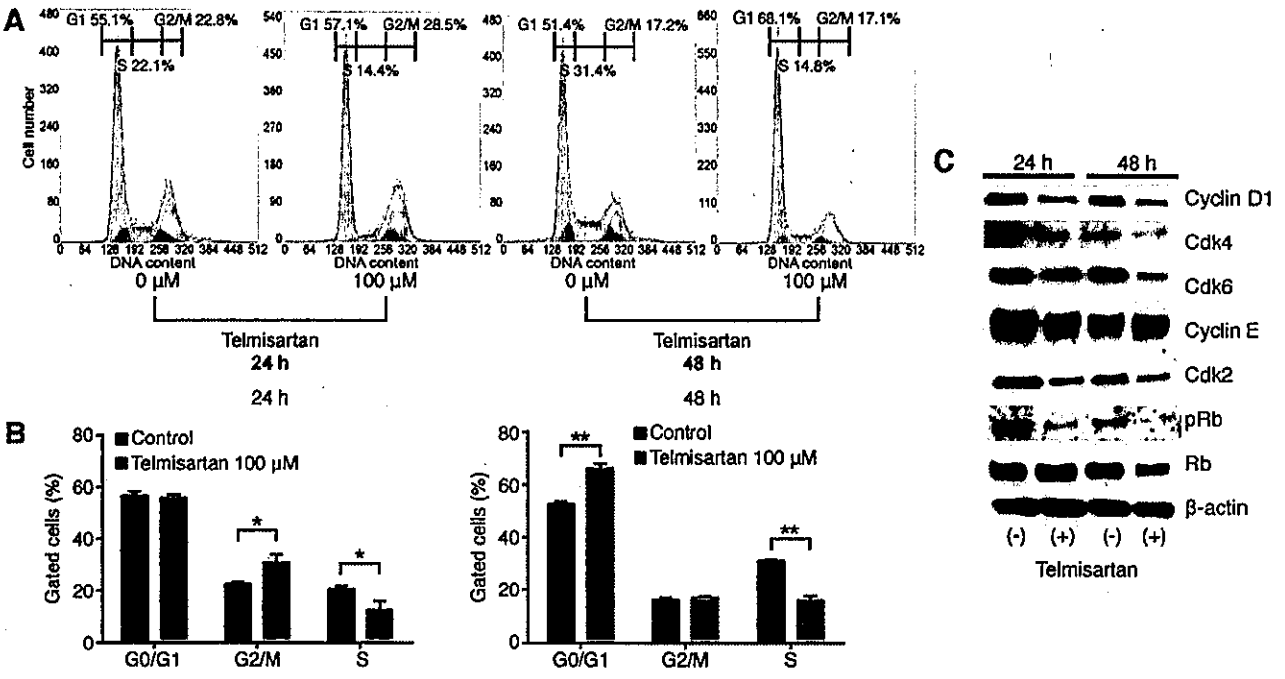

Figure 2. The antiproliferative effects of telmisartan in CCA cells are mediated via cell cycle arrest. (A) HuCCT-1 cells treated with or without 100 μM were analyzed by flow cytometry to estimate the amount of cells in each phase of the cell cycle. (B) The histogram represents the percentage of cells in each cell cycle phase. Telmisartan blocked the cell cycle at G0-G1. (*P<0.05, **P<0.01, vs. control). (C) Expression of cyclin D1, Cdk4, Cdk6, cyclin E, Cdk2, phosphorylated Rb (pRb) and Rb, in HuCCT-1 cells at 24 and 48 h after the addition of 100 μM telmisartan as analyzed by western blotting.

Statistical analyses. All statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA). Comparisons between treatment and control groups were performed using the unpaired t-tests. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Telmisartan inhibits cell proliferation and viability of human CCA cells. To explore the role of telmisartan on the proliferation of CCA cells, HuCCT-1 and TFK1 cells were treated with different concentrations of telmisrtan for 24 to 48 h. HuCCT-1 and TFK-1 cells were grown in 10% FBS and treated with 0, 10, 50, or 100 μM of telmisartan for 48 h. Telmisartan treatment reduced the proliferation of HuCCT-1 and TFK-1 cells (Fig. 1). These results show that telmisartan inhibits cell proliferation in CCA cell lines in a dose-dependent manner.

Telmisartan induces G_0/G_1 phase cell cycle arrest and regulates cell cycle-related proteins in CCA cells. To investigate the effects of telmisartan in the CCA cell lines, we examined cell cycle progression using flow cytometry analysis in HuCCT-1 cells. Flow cytometry was performed on PI stained cells to determine the effects of telmisartan on the cell cycle progression. Treatment with 100 μM of telmisartan increased the population of cells in G_1 phase and reduced the populations of cells in the S phase for 48 h after treatment (Fig. 2A and B).

The effects of telmisartan on the expression of various cell cycle-related proteins in HuCCT-1 cells were evaluated by western blotting. Cells were treated with 0 or 100 μM telmisartan for 24-48 h. The strongest reduction was observed in cyclin D1, key proteins involved in the transition from G_0 to G_1 phase, by telmisartan in a time-dependent manner (Fig. 2C). Additionally, analysis of other proteins associated with the G_0 to G_1 transition indicated that Cdk4 and Cdk6, the catalytic subunits of cyclin D1 were decreased in HuCCT-1 cells 24-48 h after the addition of telmisartan (Fig. 2C). With the treatment of telmisartan, although cyclin E that is the key cyclin for the G1/S transition was unchanged, its catalytic subunit of Cdk2 was decreased (Fig. 2C). Cyclin D1/Cdk4, cyclin D1/Cdk6 and cyclin E/Cdk2 are responsible for the phosphorylation of retinoblastoma (pRB) (22-24). In the present study, pRB was reduced in CCA cells with treatment of telmisartan.

These findings suggest that telmisartan inhibits cell cycle progression from G_0/G_1 to S-phase by decreasing cyclin D1 and CDKs levels, which results in G_1 cell cycle arrest in CCA cells.

No effects of telmisartan on the apoptosis in HuCCT-1 cells. To determine whether telmisartan induced apoptosis, CCA cells were treated with or without 100 μM telmisartan for 24 h and analyzed using Annexin V-FITC/PI staining and flow cytometry. Telmisartan did not change the proportion of apoptotic cancer cells 24 h after treatment in HuCCT-1 cells (Fig. 3A, enclosed areas in bold squares). Additionally, the levels of cCK18 following telmisartan treatment were measured using an M30 ELISA kit. The results showed that telmisartan did not increased the levels of cCK-18 in HuCCT-1 cells (Fig. 3B). These results indicate that telmisartan inhibits CCA cell proliferation without inducing apoptosis.

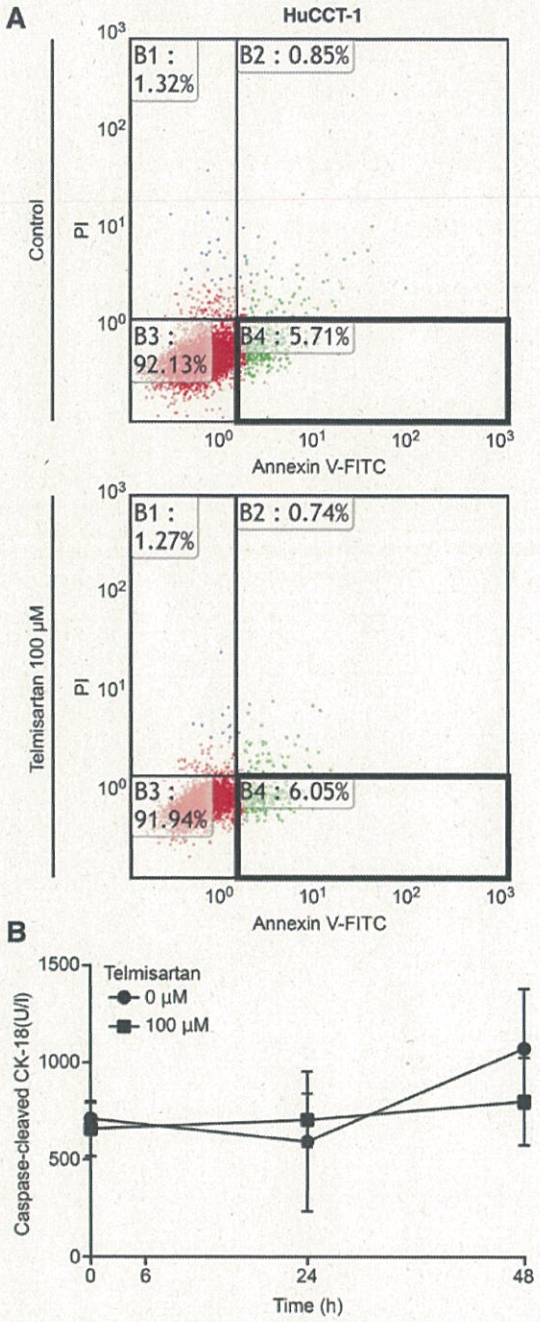

Figure 3. Telmisartan did not induce apoptosis in HuCCT-1 cells. (A) The early apoptotic changes evoked by 100 μM telmisartan at 24-48 h were assessed by flow cytometry. Annexin V-positive and PI-negative cells were regarded as early apoptotic (enclosed areas in bold squares). Telmisartan did not change the proportion of early apoptotic cancer cells among HuCCT-1 cells. (B) The expression of caspase-cleaved keratin 18 (cCK18), which is produced during apoptosis, was determined using an enzyme-linked immunosorbent assay (ELISA). Cells were incubated with or without 100 μM telmisartan.

Effects of p-RTK in HuCCT-1 cells with telmisartan treatment. We used a p-RTK array to identify the key RTKs associated with the antitumor effects of telmisartan. Using an antibody array (Fig. 4A), we simultaneously analyzed the expression of 46 different activated RTKs in HuCCT-1 cells 48 h after telmisartan administration. Telmisartan reduced the expression of phosphorylated EGFR *in vitro* (Fig. 4B). The densitometric analyses of p-EGFR showed decreases of 67.4% (Fig. 4C). Thus, telmisartan may inhibit the activation of EGFR and decreased the cell cycle regulatory molecules partially through the in CCA cells.

Effects of telmisartan on angiogenesis in HuCCT-1 cells. To examine the relationship between angiogenesis and telmisartan, angiogenesis antibody analysis was conducted regarding the antitumor effects of telmisartan (Fig. 5A). Using the antibody array, we simultaneously screened the expression levels of 20 different angiogenesis-related proteins in HuCCT-1 cells with or without telmisartan. The expression levels of TIMP-1 was induced by telmisartan treatment in HuCCT-1 cells as detected by the protein array (Fig. 5B). Densitometric analyses indicated that the ratios of the TIMP-1 spots of the telmisartan-treated cells to those of the untreated cells was 56.0% (Fig. 5C).

Telmisartan affects miRNA expression in HuCCT-1 cells. Using a custom microarray platform, we analyzed the expression levels of 2555 miRNA probes in the cell lines in the presence and absence of telmisartan. Treatment with 100 μM telmisartan for 48 h resulted in the upregulation of 37 miRNAs and the downregulation of 45 miRNAs in HuCCT-1 cells (Table I). Unsupervised hierarchical clustering analysis was conducted using Pearson's correlation, and the results indicated that the telmisartan-treated group clustered separately from the non-treated group (Fig. 6).

Telmisartan inhibits tumor proliferation in vivo. To determine whether telmisartan could affect tumor growth *in vivo*, we subcutaneously injected nude mice with HuCCT-1 cells, followed by i.p. injection of telmisartan. The telmisartan treatment significantly inhibited tumor growth by 55.1% (with 50 μg) and 76.8% (with 100 μg), as determined by integrated tumor growth curves (Fig. 7), compared with that of the untreated control mice ($^{**}P<0.001$). In addition, 100 μg telmisartan treatment did not inhibit tumor growth significantly compared with 50 μg telmisartan-treated mice. Telmisartan had no apparent toxic effects on the mice and no effect on body weight during the study. Furthermore, all animals survived to the end of the experiment.

Discussion

Angiotensin receptor blockers (ARBs) including telmisartan are widely prescribed drugs for the treatment of hypertension. Various cancer cells have recently been reported to express AT1R such as a breast, renal cancer, and hepatocellular carcinoma (25-27). Some epidemiologic studies revealed that the use of ARBs may increase the risk of cancer (28). Meanwhile, several studies indicated that ARB treatment of hypertensive patients was associated with lower cancer incidence and mortality rates (29,30). According to previously controversial clinical studies, the antitumor effect of telmisartan remains inadequately known. Moreover, in previous studies, telmisartan has been shown to inhibit proliferation of various cancer cells (31-33) *in vitro* and tumor growth *in vivo* (5-7). However, there are no any studies on antitumor effects of telmisartan

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

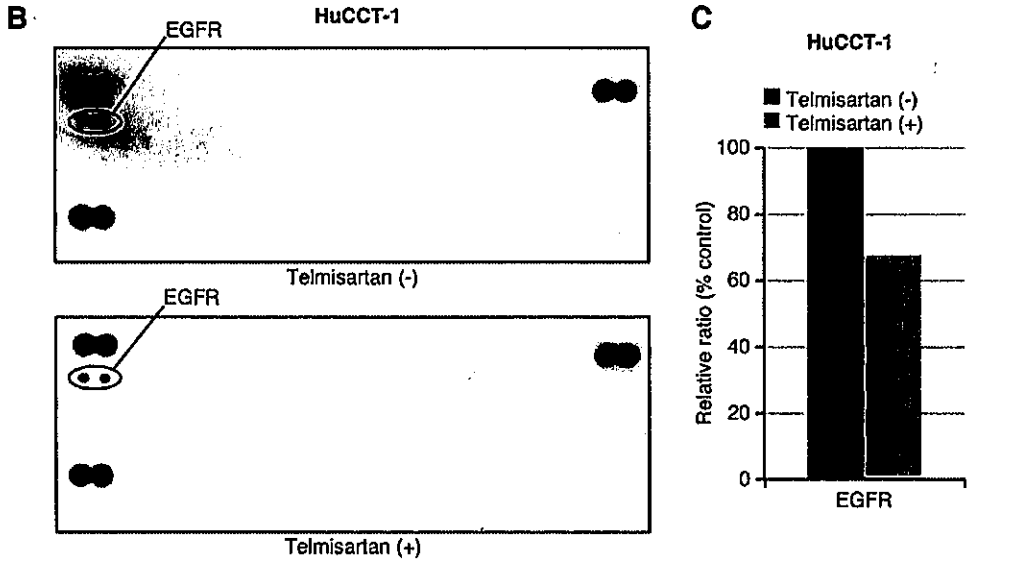

Figure 4. Effects of telmisartan on p-RTK in HuCCT-1 cells. (A) The template indicates the locations of tyrosine kinase antibodies spotted onto a human phospho-RTK array. (B) Representative expression of various phosphorylated tyrosine kinase receptors in HuCCT-1 cells treated with or without 100 μM telmisartan at 48 h. (C) Densitometry indicated that the ratio of p-EGFR spots in telmisartan-treated compared with untreated cells was 67.4%.

A

pos	pos	neg	neg	Angiogenin	EGF	ENA-78	b FGF
GRO	IFN-γ	IGF-1	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
						neg	pos

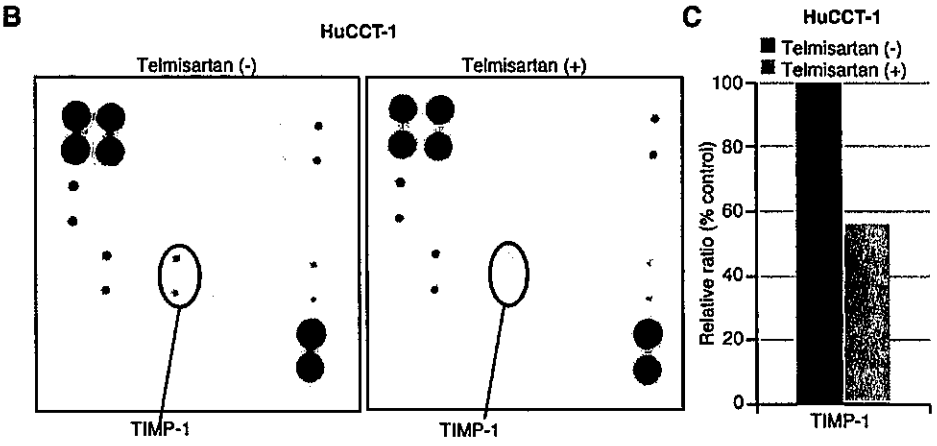

Figure 5. Effects of telmisartan on angiogenesis in HuCCT-1 cells. (A) Template demonstrating the locations of angiogenesis-related proteins spotted onto a human angiogenesis array. (B) Representative expression levels of various angiogenesis-related proteins in HuCCT-1 cells cultured with or without telmisartan. (C) The densitometric ratio of TIMP-1 spots for telmisartan-treated versus untreated cells was 56.0%.

CCA date. Therefore, in the present study, we focused on the antitumor effects of telmisartan in CCA. This report is the first study showing that telmisartan inhibits CCA cells *in vitro* and *in vivo*.

Table I. Statistical analysis results and chromosomal locations of miRNAs in HuCCT-1 cells treated with and without telmisartan.

MicroRNAs	Fold-change[a]	P-value	Chromosomal localization
Upregulated			
hsa-miR-6088	2.41	0.0087	19q13.32
hsa-miR-6131	2.27	0.0050	5p15.2
hsa-miR-4485-3p	2.02	0.0050	11
hsa-miR-3178	1.63	0.0050	16p13.3
hsa-miR-6768-5p	1.54	0.0049	16
hsa-miR-4488	1.51	0.0050	11
hsa-miR-3651	1.48	0.0022	9
hsa-miR-4730	1.48	0.0260	17
hsa-miR-203a-3p	1.48	0.0200	14q32.33
hsa-miR-23b-3p	1.46	0.0114	9q22.32
hsa-miR-29a-3p	1.45	0.0025	7q32.1
hsa-miR-205-5p	1.43	0.0129	1q32.2
hsa-miR-4466	1.43	0.0049	6
hsa-miR-6724-5p	1.42	0.0047	
hsa-miR-4324	1.39	0.0050	19
hsa-miR-138-5p	1.39	0.0049	
hsa-miR-455-3p	1.39	0.0101	9q32
hsa-miR-146a-5p	1.38	0.0103	5q33.3
hsa-miR-6784-5p	1.37	0.0063	17
hsa-miR-1469	1.37	0.0022	15q26.2
hsa-miR-23a-3p	1.34	0.0056	19p13.13
hsa-miR-6869-5p	1.34	0.0129	20
hsa-miR-8059	1.31	0.0159	17
hsa-let-7i-5p	1.31	0.0123	12q14.1
hsa-miR-193a-3p	1.31	0.0050	17q11.2
hsa-miR-6780b-5p	1.3	0.0043	17
hsa-miR-658	1.3	0.0124	22q13.1
hsa-miR-1973	1.29	0.0049	4
hsa-miR-6786-5p	1.29	0.0050	17
hsa-miR-3940-5p	1.29	0.0050	19
hsa-miR-3656	1.27	0.0127	11
hsa-miR-29b-3p	1.23	0.0484	3
hsa-miR-6727-5p	1.23	0.0046	1
hsa-miR-5787	1.23	0.0235	3
hsa-miR-100-5p	1.22	0.0043	11q24.1
hsa-miR-24-3p	1.22	0.0354	8
hsa-miR-638	1.22	0.0043	19p13.2
Downregulated			
hsa-miR-4284	0.43	0.0044	7
hsa-miR-425-5p	0.5	0.0049	3p21.31
hsa-miR-222-5p	0.51	0.0050	Xp11.3
hsa-miR-4521	0.56	0.0049	1
hsa-miR-452-5p	0.57	0.0022	Xq28
hsa-miR-192-5p	0.57	0.0050	11q13.1
hsa-miR-4484	0.58	0.0050	10
hsa-miR-330-3p	0.58	0.0046	19q13.32
hsa-miR-194-5p	0.6	0.0022	
hsa-miR-191-5p	0.61	0.0048	3p21.31
hsa-miR-345-5p	0.61	0.0047	14q32.2

Table I. Continued.

MicroRNAs	Fold-change[a]	P-value	Chromosomal localization
Downregulated			
hsa-miR-4448	0.62	0.0022	3q27.1
hsa-miR-484	0.65	0.0022	16p13.11
hsa-miR-5100	0.66	0.0048	10q11.21
hsa-miR-210-3p	0.66	0.0049	11p15.5
hsa-miR-148b-3p	0.67	0.0064	12q13.13
hsa-miR-584-5p	0.69	0.0050	5q32
hsa-miR-224-5p	0.7	0.0050	Xq28
hsa-miR-1233-5p	0.71	0.0050	15
hsa-miR-301a-3p	0.71	0.0050	17q22
hsa-miR-619-5p	0.71	0.0050	12q24.11
hsa-miR-7977	0.71	0.0023	3
hsa-miR-130a-3p	0.71	0.0035	11q12.1
hsa-miR-7114-5p	0.72	0.0022	9
hsa-miR-130b-3p	0.72	0.0049	22
hsa-miR-106b-5p	0.73	0.0048	7q22.1
hsa-miR-324-5p	0.73	0.0022	17p13.1
hsa-miR-181d-5p	0.74	0.0064	19p13.13
hsa-miR-1913	0.74	0.0087	6
hsa-miR-140-3p	0.75	0.0087	16q22.1
hsa-miR-17-3p	0.75	0.0048	13q31.3
hsa-miR-128-3p	0.76	0.0043	
hsa-miR-141-3p	0.76	0.0100	12p13.31
hsa-miR-331-3p	0.76	0.0129	12q22
hsa-miR-25-3p	0.76	0.0077	7q22.1
hsa-miR-96-5p	0.76	0.0048	7q32.2
hsa-miR-4286	0.77	0.0048	8
hsa-miR-18b-5p	0.77	0.0049	Xq26.2
hsa-miR-181b-5p	0.78	0.0064	
hsa-miR-18a-5p	0.78	0.0063	13q31.1
hsa-miR-3607-5p	0.78	0.0303	5q14.3
hsa-miR-4664-5p	0.79	0.0087	8
hsa-miR-454-3p	0.81	0.0049	17q22
hsa-miR-6840-3p	0.82	0.0079	7
hsa-miR-3135b	0.82	0.0367	6

[a]Fold-change is telmisartan treated/non-treated. Fold change (FC) >1.2, FC <0.83, P-value <0.05.

In the present study, telmisartan induced cell cycle arrest at the G_0/G_1 phase, which was correlated with a remarkable decrease in the expression of cyclinD1 and its catalytic subunits, Cdk2 and Cdk4. Complexes of Cdk4 and Cdk6 with cyclin D1 are required for G_1 phase progression (22), the expression of various cell cycle-related molecules is related to cancer progression and prognosis (22,34). These data indicate that the major cell cycle regulators may be intracellular targets of telmisartan in human CCA cells. In our previous studies, in esophageal adenocarcinoma cells, telmisartan treatment led to a dose-dependent inhibition of proliferation through a decrease in cyclin D1 levels, correlated with blocking cell

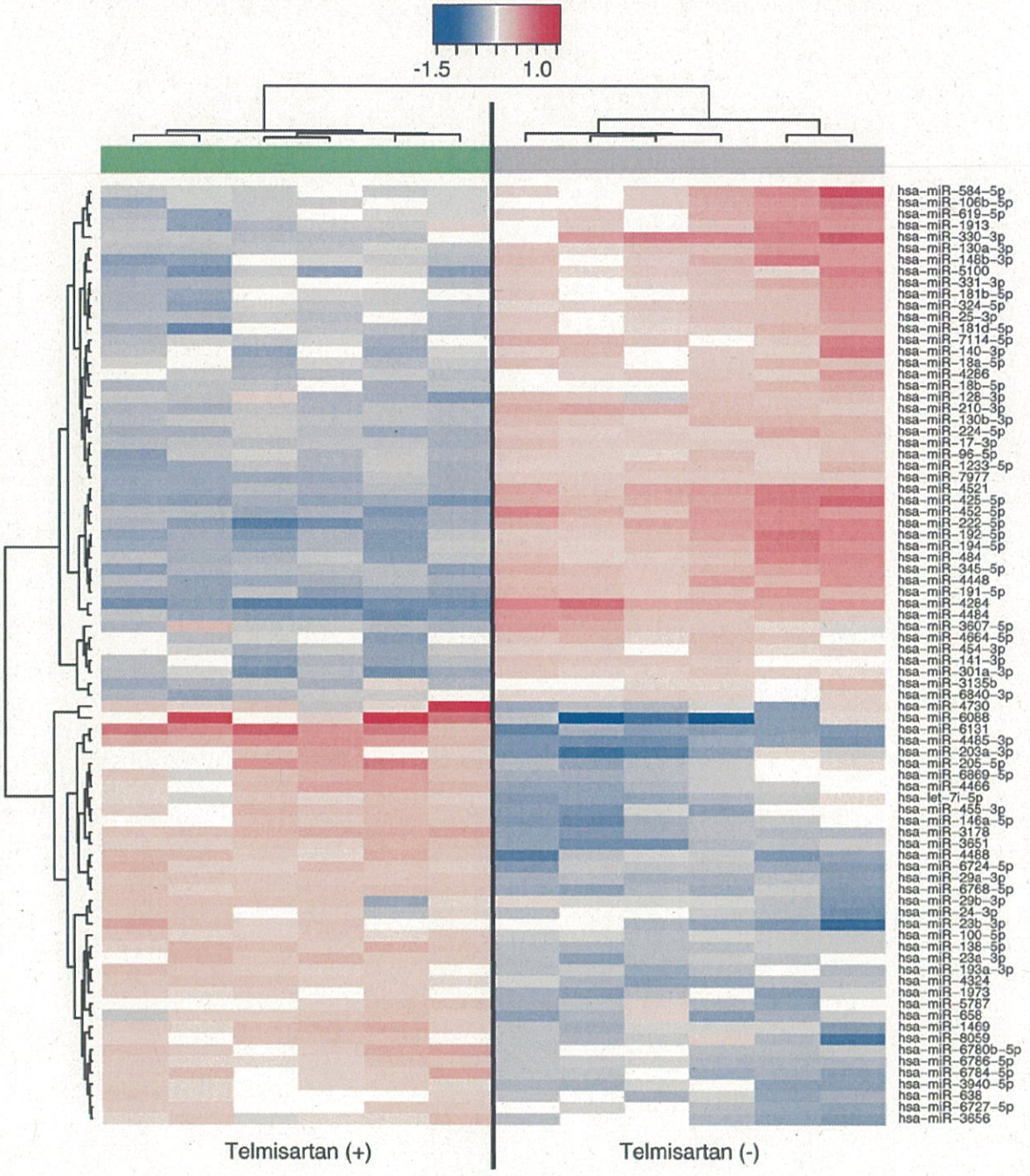

Figure 6. Hierarchical clustering of HuCCT-1 cells cultured with or without telmisartan. The analyzed samples are noted in the columns, and the miRNAs are presented in the rows. The miRNA clustering color scale presented at the top indicates relative miRNA expression levels, with red and blue representing high and low expression levels, respectively.

cycle in the G_0/G_1 phase (11). Our results also demonstrated that telmisartan induce cell cycle arrest in the G_0/G_1 phases by decreasing cyclin D1, Cdk4, Cdk6, Cdk2. Moreover, telmisartan induces antiproliferative effects by phosphorylation of AMPKα in EAC cells, suggesting that the activation of the AMPKα/mTOR pathway inhibits cell cycle regulatory proteins (11). However, in our present study, telmisartan did not induce the phosphorylation of AMPKα in CCA cells (data not shown). Therefore, these results indicate that telmisartan may reduce the expression of cell cycle-related proteins and induced cell cycle arrest without AMPKα/mTOR pathway in CCA cells.

Our *in vitro* study used a higher dose of telmisartan than that used in human treatments (1-10 μM) (35-37). However, telmisartan of higher doses used in the present study has been criticized in similar studies examining other cancer cell types, such as esophagus (11), stomach (7), and prostate cancer cells (8). Our *in vivo* study used a slightly higher dose of telmisartan than that used in human administration. Our results revealed that a slightly higher dose of telmisartan also markedly suppressed

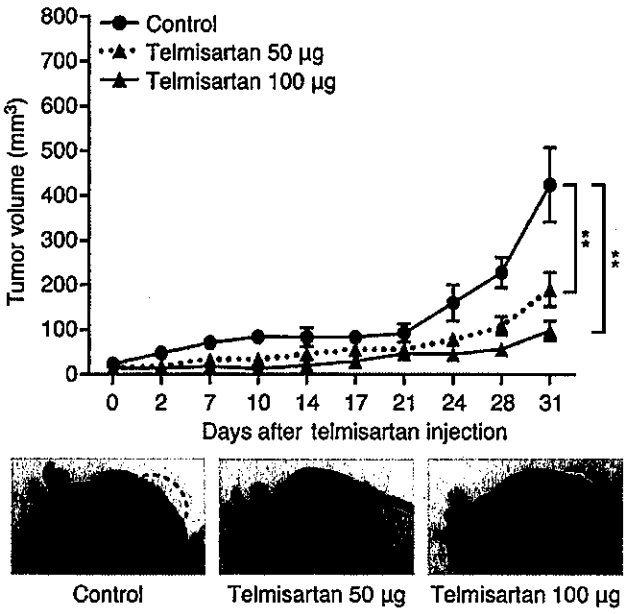

Figure 7. Telmisartan inhibited the growth of HuCCT-1 cell in a xenograft mouse model. HuCCT-1 cells were subcutaneously implanted into the flanks of nude mice. Then, 50 or 100 μg telmisartan was intraperitoneally injected for 31 days, 5 times per week. The tumors were significantly smaller in telmisartan-treated mice compared with vehicle-treated mice. Each point represents the mean ± standard deviation of 7 animals. P≤0.001 by two-way ANOVA (**P<0.001, vs. control).

the growth of subcutaneous CCA tumors in athymic nude mice. These data (*in vivo* study) suggest that the use of telmisartan in CCA treatment may be effective in combination therapy with other anticancer drugs (38).

Telmisartan has been shown to inhibit cell proliferation by inducing apoptosis in various cancer cell lines, namely, prostate (8), renal (9), endometrial (6) and colon (10) cancer lines. To determine whether telmisartan induced apoptosis, HuCCT-1 cells were treated with or without 100 μM telmisartan and analyzed using Annexin V-FITC/PI staining and measuring the levels of cCK18, but it did not induce apoptosis and cell death after telmisartan-treatment in HuCCT-1 cells. These data indicated that telmisartan inhibits CCA cell proliferation without inducing apoptosis. This discrepancy in our data and previous other studies (6,8-10) could due to the differences in the properties of different types of cancers.

Our previous studies evaluated the existence of an association between telmisartan and the inhibition of esophageal adenocarcinoma cell proliferation through p-RTK regulation (11). We demonstrated that telmisartan reduces the phosphorylation of EGFR in CCA cells using p-RTK arrays.

EGFR activation was detected in some human cancers, and EGFR activation could induce cyclin D1, a protein that is important in cell cycle progression (15,39,40,41). In addition, EGFR might act as a hub for transmitting downstream signals to activate some proliferation signals, such as, RAS-MAPK, JAK-STAT, and PI3K-Akt-mTOR pathways (42,43). In short, the EGFR pathway is important in controlling cell cycle. Thus, telmisartan may partially inhibit cell cycle regulatory proteins via decreasing the phosphorylation of EGFR to regulate cell proliferation in CCA cells.

TIMP-1 was overexpressed in the progression of chorangiocarcinoma (44). In our present study, telmisartan also reduced TIMP-1 levels in HuCCT-1 cells. These data suggest that telmisartan may play an important role in angiogenesis and tumor development through reduction of TIMP-1.

MicroRNAs are small, endogenous, noncoding RNA sequences that can modulate protein expression by regulating translational efficiency or the cleavage of target mRNA molecules (45). It has become apparent that miRNAs regulate the development and progression of various cancers (46). To identify the miRNAs associated with the antitumor effects of telmisartan, we used miRNA expression arrays. Several miRNAs were significantly altered following telmisartan treatment *in vitro*. Among these miRNAs, let-7i was significantly upregulated in HuCCT-1 cells treated with telmisartan. The let-7 family contains 13 members and is widely recognized as a class of miRNAs that produce tumor-suppressing effects. Reduction of let-7 family members have been reported in various cancers, including colorectal cancer (47), hepatocellular carcinoma (48), lung cancer (49) and breast cancer (50). The let-7 family members act as tumor suppressor molecules by binding its target oncogenes, such as HMGA2 (51), Ras (52) and c-Myc (53). Moreover, Sun *et al* reported that in breast cancer, let-7 directly inhibits cyclin D1 expression, resulting in low phosphorylation of Akt1, which is critical for the let-7 induced inhibition of mammosphere numbers (54).

In addition, as shown in Table I, miR-3178 was also significantly upregulated in HuCCT-1 cells treated with telmisartan. miR-3178 has lower expression in human pancreatic cancer tissues than in healthy control tissues (55), and hepatocellular carcinoma (HCC) tumor endothelial cells (TECs) than in hepatic sinusoidal endothelial cells (HSECs) (56). miR-3178 acts as a tumor suppressor to inhibit the proliferation, migration, invasion, and angiogenesis and promoted the apoptosis and G1 phase arrest of HCC TECs *in vitro* (57). On the other hand, in the present study, miR-425-5p was remarkably downregulated in HuCCT-1 cells treated with telmisartan. miR-425-5p has recently been reported to be upregulated and to promote tumourigenesis in various cancer types (58-60). PTEN is one of the major target genes for miR-425-5p, and it has been reported as a tumour suppressor (61,62). For this reason, it was suggested that the downregulation of miR-425-5p led to antitumor effect. In the present study, miR-222 was also downregulated in HuCCT-1 cells treated with telmisartan. miR-222 was upregulated in several cancers, and also recognized as oncogenic miRNA (63-67). The cell cycle-dependent kinase inhibitor, p27Kip1 is the target gene of miR-222 (63,64,67). Therefore, the downregulation of miR-222 led to G1 arrest. Thus, telmisartan may regulate cell cycle regulatory molecules through miR-425-5p or miR-222 to regulate cell proliferation in HuCCT-1 cells.

Collectively, our data suggest that telmisartan induced inhibition of human CCA cell proliferation, in part, by the tumor suppressor activities caused by downregulation of above described miRNAs (miR-425-5p and miR-222). Furthermore, in the present study, miR-6088 and miR-6131 were remarkably upregulated in cells treated with telmisartan comparerd with control cells. However, the target gene of these miRNAs remains unknown. Therefore, we need to elucidate further the function of these microRNAs.

Telmisartan is a drug widely used for the treatment of hypertension with limited side effect. Thus, telmisartan of a long-term management of CCA may become effective, and benefits of low costs therapy for the treatment in the patients with hypertension. In conclusion, our results revealed that telmisartan inhibits human CCA cell proliferation by inducing cell cycle arrest and certain miRNAs.

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