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Original Articles

Angiotensin receptor blockade attenuates cholangiocarcinoma cell growth by inhibiting the oncogenic activity of Yes-associated protein

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ABSTRACT

Cholangiocarcinoma (CCA) is a destructive malignancy with limited responsiveness to conventional chemotherapy. Although angiotensin receptor blockers (ARBs) have gained attention for their potential anticancer activity, little is known about their effects on CCA. The transcriptional co-activator, Yes-associated protein (YAP) is a critical oncogene in several cancers, including CCA. Following recent evidence showing that YAP is regulated by angiotensin II (AT-II), we investigated the effects of an ARB, losartan, on two human CCA cell lines (KKU-M213 and HuCCT-1) with regards to YAP oncogenic regulation. Losartan suppressed AT-II-induced CCA cell proliferation in a dose-dependent manner, induced apoptosis, decreased YAP (Ser127), and downregulated the YAP target genes CTGF, CYR61, ANKRD1, and MFAP5. However, losartan did not affect epithelial–mesenchymal transition, differentiation, or stemness in the CCA cells. Xenograft tumor growth assay showed that oral administration of a low clinical dose of losartan considerably reduced subcutaneous tumor burden and attenuated intratumor vascularization in CCA cell-derived xenograft tumors in BALB/c nude mice. These results indicate that ARB therapy could serve as a potential novel strategy for CCA treatment.

1. Introduction

Cholangiocarcinoma (CCA) is a hepatobiliary cancer with a steadily increasing annual incidence [1,2]; it is associated with a poor 5-year survival rate and limited therapeutic options [1,2]. Although surgical resection is the first-line treatment of choice for intrahepatic CCA, 20%–40% of patients are not eligible for surgery as their tumors are locally advanced, have metastasized, involve blood vessels, or have extended into both hepatic lobes [3]. This results in a median survival of approximately 24 months for most patients diagnosed with CCA. According to a phase III randomized controlled trial for biliary tract cancers performed in the UK, the treatment of choice for unresectable CCA comprises a cisplatin/gemcitabine combination [4]. However, the success of these treatment agents remains limited, and their long-term usage is often associated with severe side effects. Alternative approaches are therefore needed. Further, identifying a clinically available compound, which exerts antitumor activity against CCA through a novel molecular target and with proven safety for long-term administration, would be extremely important.

Yes-associated protein (YAP) is a primary effector of the Hippo tumor-suppressor pathway and has been identified as a transcriptional co-activator that interacts with p73, Runx2, Tbx5, SMAD, ErbB4, Pax3, and TEAD family proteins [5–7]. Hippo/YAP signaling is an evolutionarily conserved pathway that regulates tissue growth and organ size by modulating cell proliferation, apoptosis, and self-renewal of progenitor cells [8,9]. Central to the Hippo pathway is a kinase cascade, wherein MST1/2 kinases (ortholog of Drosophila Hippo) and SAV1 form a complex to phosphorylate LATS1/2 [5–7]. Phosphorylated LATS1/2 kinases sequentially phosphorylate and inhibit two major downstream effectors of the Hippo pathway: the transcription co-activators YAP and TAZ [5–9]. Recent studies have indicated that YAP is a critical oncogene whose dysregulation can lead to tumorigenesis [10–12]. When the Hippo enzymatic cascade collapses, YAP is dislodged from its cytoplasmic anchorage and shuttles into the nuclei,

Abbreviations: CCA, cholangiocarcinoma; ARB, angiotensin receptor blocker; YAP, Yes-associated protein; AT-II, angiotensin II; GPCR, G protein-coupled receptor; AT1R, angiotensin II type 1 receptor; RAAS, the renin-angiotensin-aldosterone system; HUVEC, human umbilical vascular endothelial cell; EMT, epithelial-to-mesenchymal transition

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where it acts as a transcription co-activator stimulating target downstream genes and acquiring oncogenicity by binding with TEAD family transcription factors [10–12]. Oncogenic YAP has been shown to occur both in human CCA cell lines and patient specimens; further, nuclear YAP can be potentially used as an independent prognostic marker for overall survival in CCA [13]. Thus, pharmacological YAP inhibitors may comprise novel therapeutic targets.

G protein-coupled receptors (GPCRs) are thought to modulate the Hippo/YAP pathway [14]. Such is the case of angiotensin II type 1 receptor (AT1R), a central regulator of the renin-angiotensin-aldosterone system (RAAS). Its ligand, angiotensin II (AT-II), plays a key role in many pathophysiological activities, including vascular hormone secretion, tissue growth, and cancer [15,16]. Currently, AT1R blockers (ARBs) are clinically used to treat hypertension and heart failure, because of their effects on the cardiovascular system, and we previously reported that a highly hydrophilic ARB, losartan, showed suppressive effects against the growth of several experimental tumors including hepatocellular carcinoma and pancreatic cancer [17,18]. Recent bench research has also revealed that AT-II binding to the AT1R can activate YAP by inhibiting the Hippo pathway in podocytes [19]. However, it remains unknown whether this process is associated with CCA development and whether losartan can inhibit CCA growth in conjunction with YAP/TEAD regulation.

The present study investigated the impact of AT-II on CCA cell growth, considering Hippo/YAP regulation and the anticancer properties of the ARB losartan against human CCA cells. We show that losartan suppresses AT-II-stimulated CCA cell proliferation via YAP inactivation and inhibits intratumor angiogenesis in CCA-derived murine xenograft models. On the basis of these results, we propose that losartan should be evaluated as a novel treatment modality for CCA.

2. Materials and methods

2.1. Compounds and cell culture

Human AT-II acetate salt was obtained from BACHEM (Bubendorf, Switzerland), and losartan potassium was supplied by Merck & Co., Inc. (New Jersey, USA). Verteporfin (Merck KGaA., Darmstadt, Germany) was used as a YAP inhibitor. Two human CCA cell lines, KKU-M213 and HuCCT1, and human umbilical vascular endothelial cells (HUVECs) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). HepG2 were obtained from the RIKEN BRC CELL BANK (Ibaraki, Japan). KKU-M213, HUVEC, and HepG2 were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific K.K., Kanagawa, Japan) supplemented with 10% fetal bovine serum and 1% ampicillin/streptomycin. HuCCT1 was cultured in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum and 1% ampicillin/streptomycin. The cells were grown in the media recommended by the respective suppliers.

2.2. Human CCA xenografts

Six-week-old male athymic nude mice (BALB/cSlc-nu/nu) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed in stainless steel mesh cages under controlled conditions (temperature: 23C ± 3C, relative humidity: 50% ± 20%, 10–15 air changes/hour, illumination: 12 h/day). The animals were provided tap water *ad libitum* throughout the experimental period.

For tumor inoculation, 1×10^6 cells were suspended in 200 μL of medium with Matrigel High Concentration (Corning, Tewksbury, MA, USA; 1:1) and injected subcutaneously into the mice' bilateral flanks. Tumors were measured with a caliper, and their volume was calculated using the following formula:

$$\frac{1}{2}[(Width)^2 \times Length]$$

Seven days after inoculation, the intervention mice were orally administered losartan at a dose of 30 mg/kg and the mice in the vehicle group were administered an equivalent volume of saline solution ($n = 5$). All mice were sacrificed 35 days after administration. The tumors were then collected, and their sizes were recorded. Serum biological markers were measured using routine laboratory methods.

All animal procedures were performed in compliance with the recommendations of the Guide for Care and Use of Laboratory Animals (National Research Council), and the study was approved by the animal facility committee of Nara Medical University (authorization number: 11693).

2.3. Statistical analysis

Data were analyzed with either Student's t-tests or one-way analysis of variance followed by Bonferroni's test for multiple comparisons, as appropriate. All tests were two-tailed, and a level of $p < 0.05$ was considered statistically significant.

Additional methods can be found online in the Supplementary materials and methods.

3. Results

3.1. Losartan suppressed human CCA cell growth in vitro

To investigate the antitumor effect of losartan on human CCA, we first evaluated mRNA expression level of AT-II type1 receptor (AGTR1) on two human CCA lines (KKU-M213, HuCCT-1). As shown in Fig. 1A, both CCA lines exhibited higher mRNA levels of *AGTR1* than HepG2, a human liver cancer line, and lower levels than HUVECs, which are recognized models of AGTR1 expression [20,21]. These findings demonstrate that both CCA lines express AGTR1.

Given the high AGTR1 expression levels, we next assessed the proliferative activity of AT-II on these CCA lines. In both cell lines, AT-II induced cell proliferation in a dose-dependent manner (10^{-10}–10^{-5} M) (Fig. 1B). We then investigated whether AT-II blockade suppressed CCA cell proliferation by treating CCA cells with different concentrations of losartan (0.1–10 μM) plus AT-II (10^{-6} M). Results showed that AT-II-stimulated CCA cell proliferation was markedly suppressed by losartan at lower doses than the equivalent plasma concentration level of patients receiving a clinical dose (i.e., 10 μM) (Fig. 1C) [22]. A time-course analysis showed a significant losartan-induced cytostatic effect at 1 μM, 24 h after starting the reaction (Fig. 1C). Losartan-treated KKU-M213 and HuCCT-1 cells also exhibited an increase in TUNEL-positive apoptosis compared with controls (Fig. 1D). As shown in Fig. 1E, losartan did not affect the proapoptotic factors, BAK and BAX. Regarding the antiapoptotic factors, the levels of Mcl-1 were decreased in both losartan-treated CCA lines, whereas no changes were not found in the levels of Bcl-xL. These results suggest that losartan inhibits Mcl-1-regulated antiapoptotic activity in CCA cells, and that losartan-treated CCA cells show enhanced caspase-3 cleavage (Fig. 1E and F).

3.2. Losartan interfered with the Hippo-YAP pathway in human CCA

We investigated the impact of AT-II and losartan on Hippo-YAP signaling in KKU-M213 and HuCCT-1 cells. Since YAP is usually activated by its dephosphorylation in cancer cells, we first evaluated the effect of changing YAP phosphorylation through AT-II and/or losartan treatment. Although AT-II did not affect YAP expression, it exerted a profound effect on phosphorylated-YAP (p-YAP(Ser127)), indicating that AT-II induces YAP dephosphorylation in both cell lines (Fig. 2A and B). Losartan effectively suppressed p-YAP downregulation (Fig. 2A and B). We next assessed the phosphorylation status of LATS1, a negative

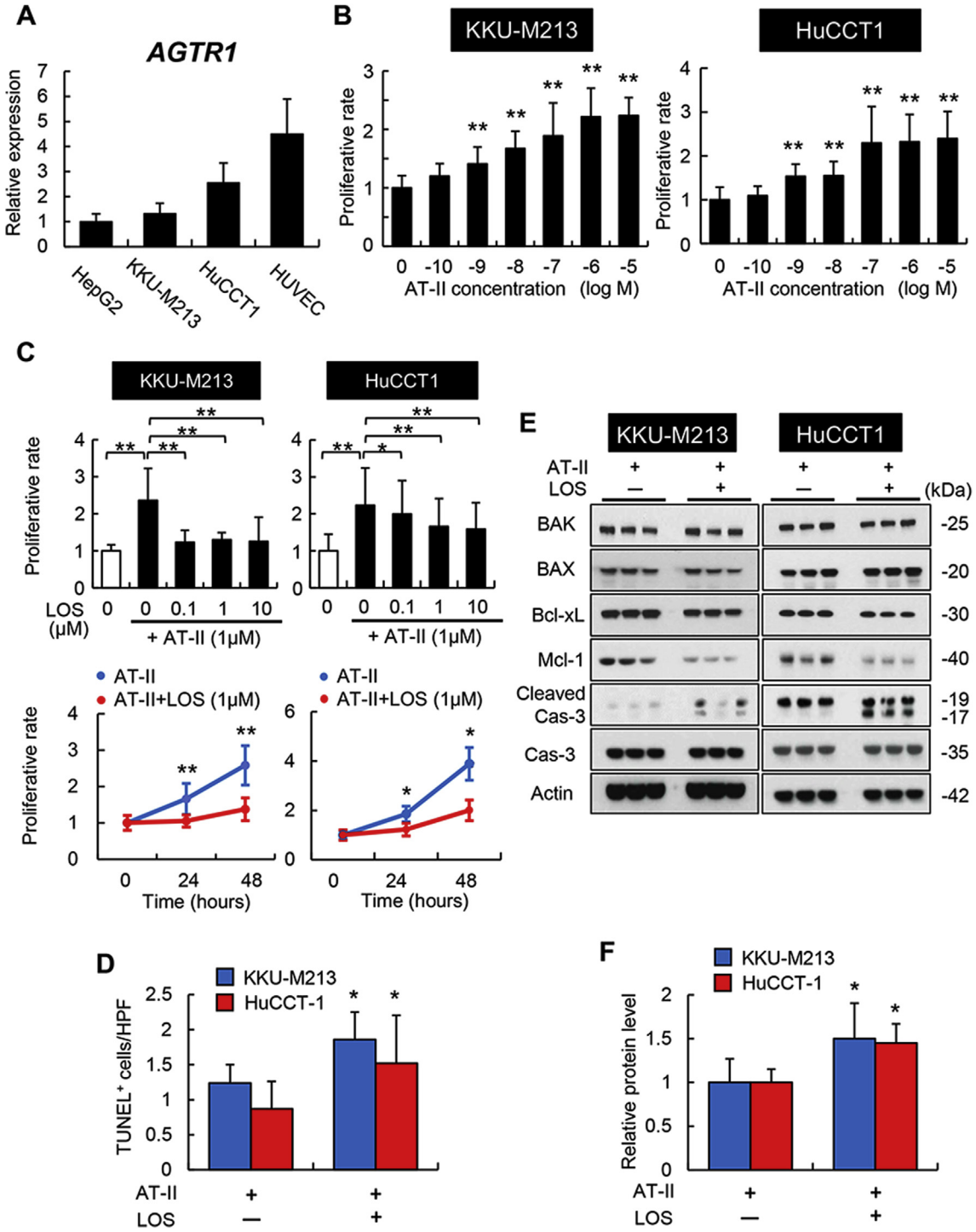

Fig. 1. Losartan inhibits *in vitro* cell proliferation and antiapoptotic activity of human cholangiocarcinoma. (A) Relative mRNA levels of *AGTR1* in cholangiocarcinoma cell line, KKU-M213 and HuCCT1. HepG2 and HUVEC cells were used as controls. (B) Angiotensin-II induced the proliferation of KKU-M213 (Left panel) and HuCCT-1 (Right panel) in a dose-dependent manner. (C) LOS inhibited the AT-II-stimulated proliferation of KKU-M213 (Left panels) and HuCCT-1 (Right panels) in a dose (Upper panels)- and time (Lower panels)- dependent manner. (D) Quantification of TUNEL$^+$ cells in cultured KKU-M213 and HuCCT-1. The number of TUNEL$^+$ cells in high-power field (HPF) were counted for quantification. (E) Western blots of whole cell lysates for apoptotic markers in cultured KKU-M213 and HuCCT-1. (F) The quantification of Cleaved Cas3/Cas-3 is shown. The expression levels were quantified by using NIH image J software and were normalized to actin. AT-II; angiotensin-II, LOS; losartan. Relative mRNA expression levels were measured by quantitative RT–PCR (qRT–PCR). *GAPDH* was used as internal control for qRT–PCR (A). Actin was used as the loading control for WB (E). For analysis, the cells were treated with angiotensin-II (10^{-6} M) and/or losartan (10^{-6} M) for 24 h (D, E). Quantitative values are indicated as ratios to the values of untreated cells (B, C), and of LOS-untreated cells (F). Data are mean ± SEM (n = 8; A, B, and C. n = 6; D. n = 3; F). *,P ≤ 0.05; **,P ≤ 0.01 (B, C, D, and F).

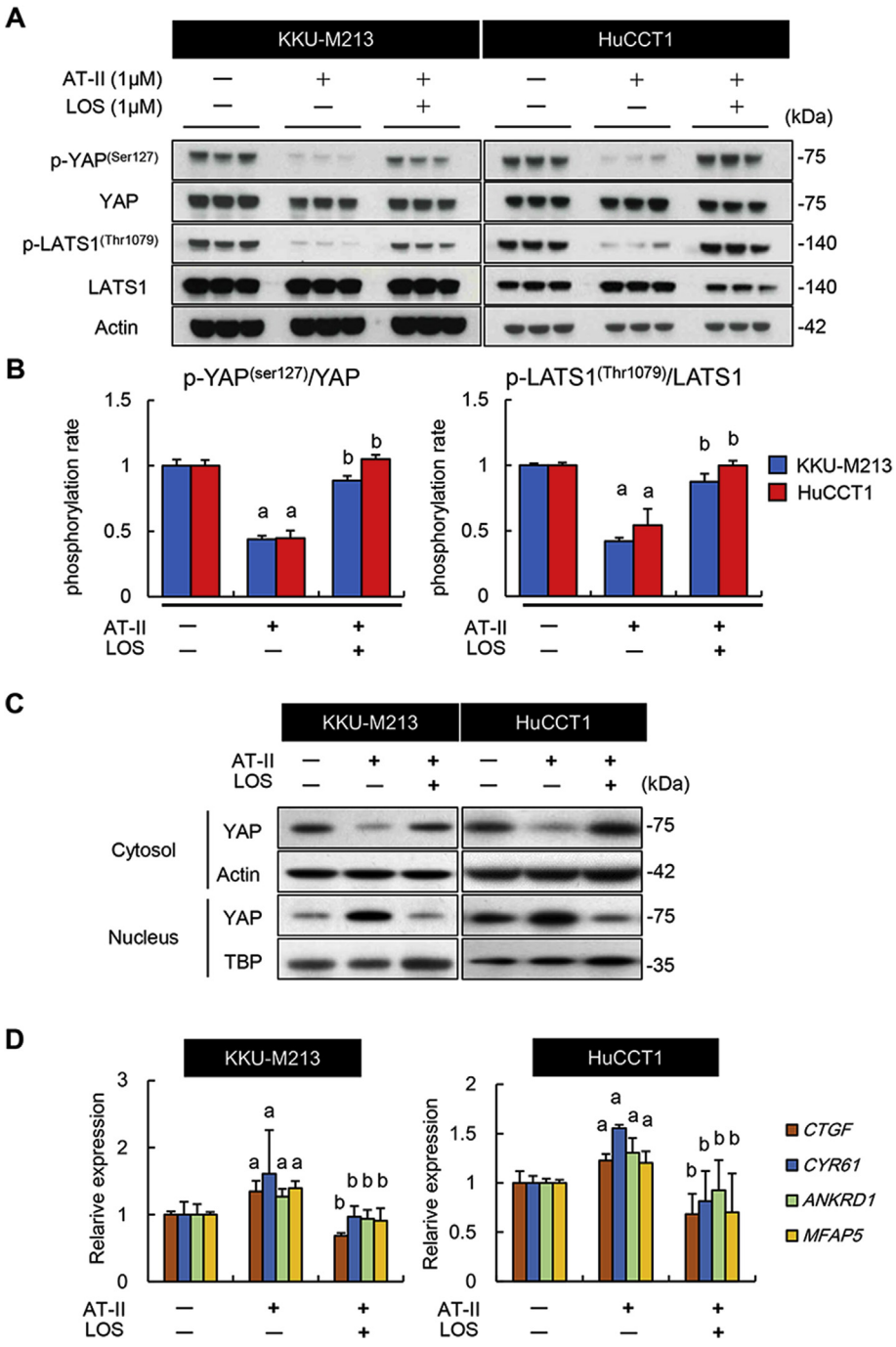

Fig. 2. Losartan inhibits the YAP oncogenic activation in human cholangiocarcinoma cells *in vitro*. (A) Western blots of whole cell lysates for YAP (Ser127) and LATS1 (Thr1079) phosphorylation. (B) Relative phosphorylation rate of phosphorylated YAP(Ser127)/YAP (Left panel), phosphorylated LATS1(Thr1079)/LATS1 (Right panel). The expression levels were quantified by using NIH image J software and were normalized to actin. (C) Western blots of cytosol and nuclear fractions for YAP. (D) Relative mRNA levels of YAP/TEAD target genes, *CTGF*, *CYR61*, *ANKRD1* and *MFAP5* in cultured cholangiocarcinoma cell lines. AT-II; angiotensin-II, LOS; losartan. Actin or TBP was used as the loading control for WB of whole cell lysate and cytosol fraction (A and C) or nuclear fraction (C), respectively. The mRNA expression levels were measured by quantitative RT-PCR (qRT-PCR). *GAPDH* was used as internal control for qRT-PCR (D). For analysis, the cells were treated with angiotensin-II (1 μM) and/or losartan (1 μM) for 24 h (A-D). Quantitative values are indicated as ratios to the values of untreated cells (B, D). Data are mean ± SEM (n = 3; B. n = 8; D). a, $P \leq 0.01$ vs untreated group [AT-II(-)/LOS(−)]; b, $P \leq 0.01$ vs angiotensin-II-treated and losartan-untreated group [AT-II(+)/LOS(−)] (B, D).

regulator of YAP. Similar to the molecular action on YAP, neither AT-II nor losartan stimulated LATS1 expression, although losartan inhibited AT-II-induced LATS1 dephosphorylation in both lines (Fig. 2A and B). YAP acquires oncogenic activation by translocating into the nucleus following its dephosphorylation. We therefore evaluated YAP levels both in the cytosolic and nuclear fractions of CCA cells. Upon AT-II stimulation, nuclear YAP levels increased, whereas cytosolic YAP levels decreased in both CCA cultures, compared with controls. Those AT-II-induced alterations were recovered by losartan treatment (Fig. 2C), demonstrating that losartan inhibits AT-II-mediated nuclear translocation of YAP in CCA cells. Consistent with YAP activation, AT-II treatment increased the mRNA levels of selected YAP/TEAD target genes including CTGF, CYR61, ANKRD1, and MFAP5. Losartan inhibited this upregulation (Fig. 2D), which shows its ability to interfere with AT-II-stimulated YAP oncogenic activation in human CCA cells.

To validate the relationship between the antiproliferative effect of losartan and YAP inactivation in CCA cells, we evaluated its effect in CCA proliferation under verteporfin-induced YAP silencing. Verteporfin, a YAP inhibitor, significantly attenuated cell proliferation and reduced mRNA levels of TEAD target genes in both CCA cell lines, which shows that it efficiently inhibits YAP activation (Supplementary Figs. S1A and S1B). It is noteworthy that the antiproliferative effects of losartan were not found under the presence of verteporfin (Supplementary Fig. S1C). These results support the mechanistic insight that losartan exerts an inhibitory effect on CCA growth via interference with AT-II-stimulated YAP activation.

Additionally, we analyzed whether losartan could regulate certain cancer properties in these CCA lines, including epithelial-to-mesenchymal transition (EMT), differentiation, and stemness. We found no effect of losartan on the mRNA levels of selected markers for EMT (CDH1

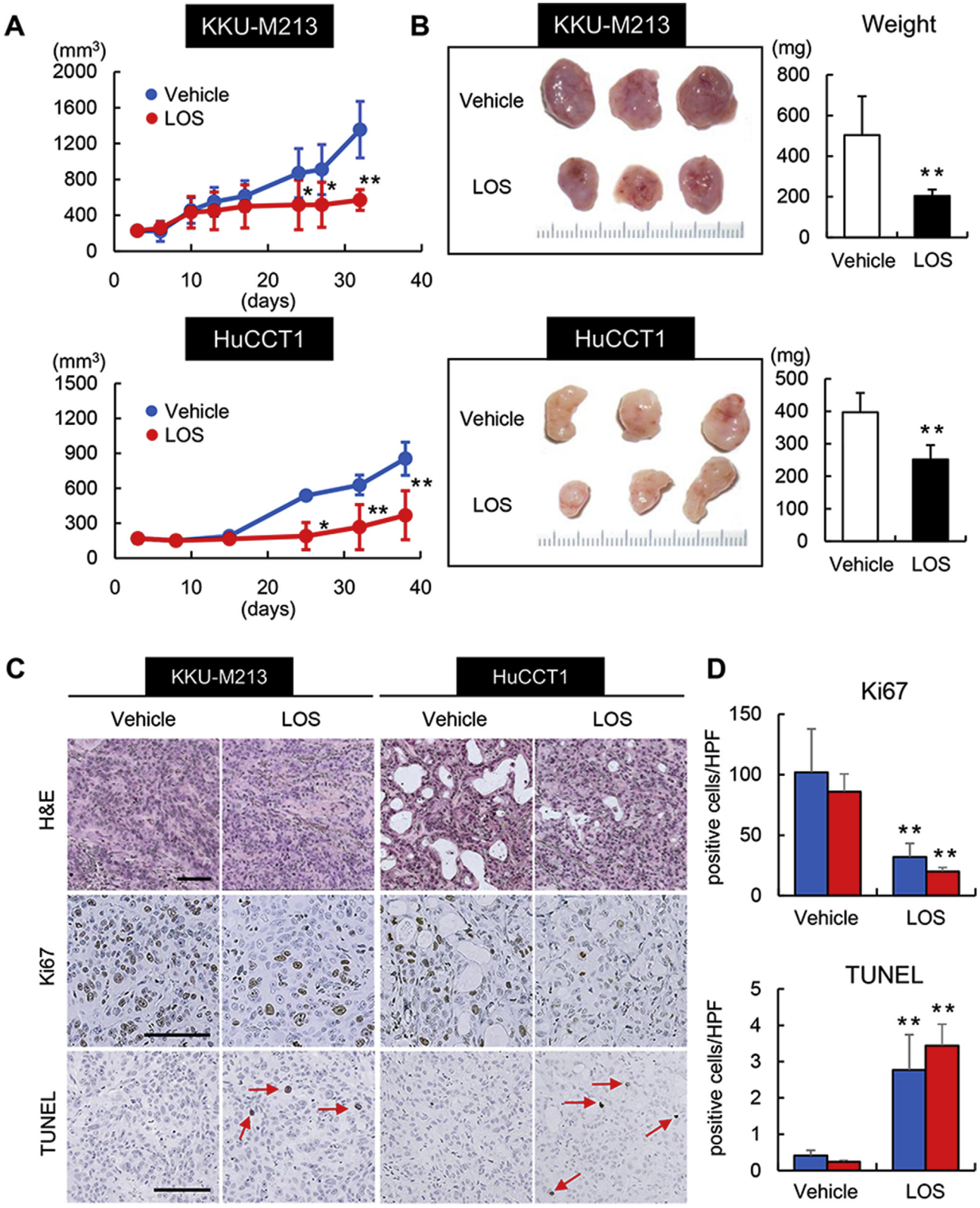

Fig. 3. Losartan suppresses cholangiocarcinoma growth in tumor-bearing nude mice. (A) Daily oral administration of losartan (30 mg/kg) for 5 weeks resulted in almost 50% reduction in tumor volume of KKU-M213 and HuCCT-1 grafted on nude mice. (B) Left panels: representative photographs of untreated or losartan-treated tumor-bearing nude mice just before sacrifice and resected subcutaneous tumors. Right panels: Losartan administration significantly decreased tumor weight of KKU-M213 and HuCCT-1 grafted on nude mice. (C) Representative pictures of KKU-M213- and HuCCT-1-grafted subcutaneous tumors stained with H&E (Upper panels), Ki67 (Middle panels), and TUNEL (Lower panels). Red arrows indicate TUNEL positive CCA cells. Scale bars; 50 μm. (D) Quantification of Ki67- (Upper panel) and TUNEL- (Lower panel) positive cells. The number of immunopositive cells in high-power field (HPF) were counted for quantification. LOS; losartan-treated group. Data are mean ± SD (n = 10; A, B, D). *,P ≤ 0.05; **,P ≤ 0.01 vs Vehicle-treated group (A, C, D).

and VIM; Supplementary Fig. S2A), differentiation (HNF4a, ALB, KRT7, and KRT19; Supplementary Fig. S2B), and stemness (EPCAM and CD44; Supplementary Fig. S2C).

3.3. Losartan reduced human xenograft tumor burden by inactivating YAP in the CCA

Given its in vitro inhibition of human CCA cell growth, we evaluated the effect of losartan on the growth of xenograft CCA tumors in athymic nude mice. Both KKU-M213 and HuCCT-1 cell-derived xenograft tumors grew progressively in vehicle-treated control mice, whereas losartan-treated mice exhibited a slower tumor growth at a clinically comparable dosage of 30 mg/kg/day (Fig. 3A and B). After the experiments, the mean tumor weights were significantly lower in losartan-treated than in control mice (Fig. 3B). We further confirmed that losartan administration did not damage intrahepatic biliary epithelial cells, using histological and serological assessments (Supplementary Figs. S3A and S3B). Intratumor cell viability was evaluated by immunohistochemistry, which showed that losartan potently abrogated Ki67-positive cell proliferation while simultaneously inducing TUNEL-positive cell apoptosis in tumors derived from both KKU-M213 and HuCCT-1 cells (Fig. 3C and D). Notably, in accordance with the results from cultured CCA cells, intratumor Mcl-1 levels were reduced in losartan-treated mice as compared with vehicle-treated mice (Supplementary Fig. S4A).

As described above, YAP is activated by LATS-mediated dephosphorylation, translocating into the nucleus, where it binds to transcriptional factors such as TEAD [5–12]. We therefore performed immunohistochemical analyzes to investigate the intracellular localization of YAP in subcutaneous tumors, aiming to determine whether the losartan's antitumor effects were involved in YAP regulation. Fig. 4A shows that YAP localized both in the cytoplasm and nuclei in the tumors of vehicle-treated mice, but only in the cytoplasm of losartan-treated mice tumors. Quantitative analysis revealed that the tumors of losartan-treated mice exhibited a lower ratio of cells with nuclear YAP than those of vehicle-treated mice. This suggests that in xenograft CCA tumors, the nuclear translocation of YAP is inhibited by losartan-induced AT-II blockade. Consistent with the results in cultured cells, losartan-treated mice exhibited subcutaneous tumors attenuated p-LATS1 and p-YAP downregulation, thus indicating that losartan influences deactivation of LATS1 and YAP (Fig. 4B). Intratumor expression of YAP/TEAD target genes (i.e., CTGF, CYR61, ANKRD1, and MFAP5) was significantly suppressed in losartan-treated mice (Fig. 4C).

3.4. Losartan attenuated intratumor angiogenesis in human CCA

Since AT1R blockade is known to cause an antiangiogenic effect, we assessed its effect on tumor angiogenesis using xenograft models [16,18]. Quantitative evaluation of CD34-positive microvessels showed that tumor neovascularization induced by both KKU-M213 and HuCCT-1 cells was attenuated by losartan (Fig. 5A). Consistent with this result, the intratumor mRNA levels of Vegfa were decreased in the losartan-treated mice (Fig. 5B).

To explore the functional mechanism underlying these antiangiogenic effects, we investigated the impact of losartan on vascular endothelial cells. Our in vitro experiments revealed that neither AT-II nor losartan affected HUVEC proliferation (Fig. 5C). In contrast, an in vitro endothelial tubular formation assay showed that tubular formation was strongly promoted by AT-II, and that losartan significantly suppressed the observed tubular formation (Fig. 5D). These findings indicate that losartan-induced tumor angiogenesis could be attributed to the direct inhibition of endothelial tubular formation. Given the basic evidence that YAP plays a role in endothelial tubular network formation, we next examined the mRNA levels of YAP/TEAD target genes in cultured HUVECs to determine whether losartan could regulate YAP transcriptional activity in the endothelial cells [23,24]. Interestingly, AT-II stimulation increased the mRNA levels of YAP/TEAD target genes, whereas losartan inhibited this upregulation (Fig. 5E). These results indicate that losartan inhibits AT-II-mediated YAP/TEAD transcription in HUVECs. We then assessed the effect of losartan on tumor interstitial matrix indirectly affecting intratumor vascular perfusion [25] and found that losartan did not alter the intratumor expression levels of the stromal collagen-related factors, TGFB1, COL1A1 and EDN1 (Supplementary Fig. S4B).

4. Discussion

ARBs are widely used to manage cardiovascular diseases and chronic kidney disease, and available epidemiologic evidence suggests that they affect cancer incidence. An increasing number of studies have also elucidated the involvement of RAAS signaling, especially the AT1R/AT-II axis, in certain cancers, including breast, lung, prostate, pancreas, and liver cancers [18,26–29]. It is noteworthy that some preclinical studies have showed an impact of AT-II on CCA development and progression of CCA. For example, Okamoto et al. demonstrated that the AT1R/AT-II axis was strongly associated with an interaction between CCA and hepatic stellate cells, during tumor progression and fibrogenesis [30]. They also reported that AT-II plays a key role in CCA EMT, through its effect on the SDF1/CXCL4 axis [31]. However, the mechanism underlying the inhibitory effects of ARBs on CCA cell proliferation remains to be clarified.

The present study focused on the communication between the AT1R/AT-II and Hippo/YAP pathways during CCA progression, aiming to explore potential molecular interactions. Among the many regulatory factors in Hippo/YAP signaling, GPCRs are one of the most powerful inducers of the YAP oncogenic pathway [14,32], which recognize extracellular ligands and transduce them to heterotrimeric G proteins, which in turn transduce the intracellular signals to appropriate downstream effectors, thus affecting several signaling pathways [33]. In the Hippo/YAP pathway, GPCRs inhibit the activity of LATS1/2 via Gα12/13, effectively releasing YAP from LATS1/2-mediated inhibition [14]. Thus, pharmacological blockade of GPCRs produce an anticancer effect through inhibition of YAP oncogenic activity. Among the known GPCRs, AT1R is pharmacologically relevant, given the clinical availability of antagonists [34]. We therefore hypothesized that these agents could have anticancer effect via regulation of the Hippo/YAP signaling pathway.

Our in vitro analysis revealed that AT1R stimulation by AT-II induced cell proliferation in cultured CCA cells with decreased LATS1(Thr1079) phosphorylation, which was accompanied by a decreased phosphorylation of its target, YAP(Ser127). In line with this finding, the YAP and TEAD target genes (e.g., CTGF, CYR61, ANKRD1, and MFAP5) were also upregulated by AT-II stimulation. Remarkably, blockade of the AT1R/AT-II axis by losartan abrogated the resulting CCA cell proliferation, thus demonstrating that decreases in LATS and YAP phosphorylation were suppressed by the inhibited upregulation of the YAP/TEAD target genes. These results are supported by the findings from the xenograft model, which showed that losartan suppressed tumor growth through attenuated Ki67-positive cell proliferation and YAP-nuclear shuttling. Several molecular studies have shown that interaction between YAP and the TEAD family regulates tumor growth by controlling cell cycle at the G1 phase [13,35]. It has also been reported that ARB arrests cell cycle at the G0/G1 phase, which leads to a marked decrease in cyclinD1 expression [36]. Our results are in line with those findings, suggesting that YAP signaling inactivation is strongly associated with the observed antiproliferative effect of losartan. Intriguingly, losartan also inhibited cell proliferation and reduced TEAD target gene expression in both CCA cell lines even in AT-II-free cultures (data not shown). Recently, Samukawa et al. have shown that telmisartan, another ARB, induces inhibition of human CCA cell proliferation, partly through tumor suppressing activity mediated by downregulation of miRNAs, including miR-222 [36]. Notably, Li et al. have shown that

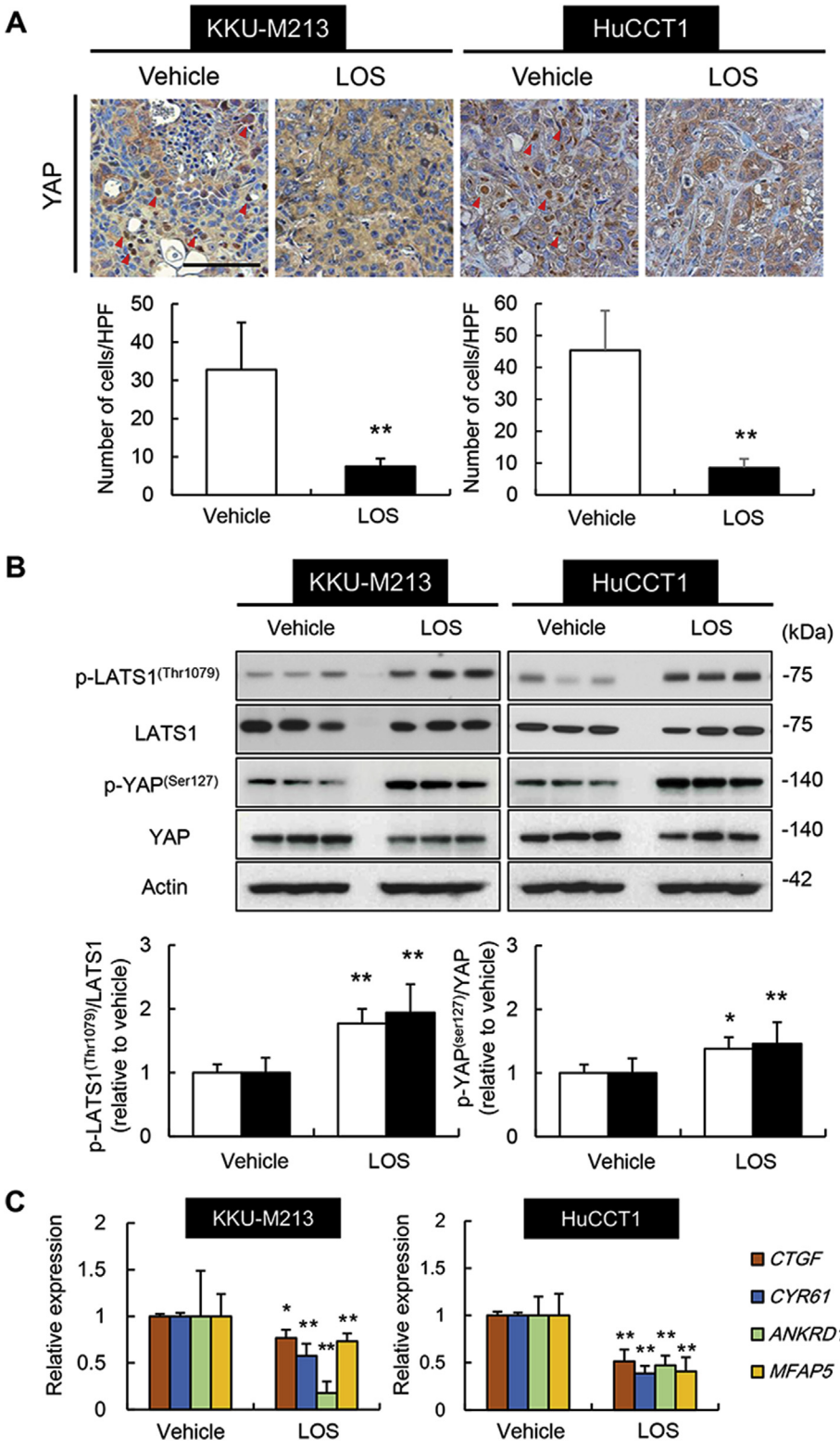

Fig. 4. Losartan interferes with YAP nuclear shuttling in subcutaneous cholangiocarcinoma tumors. (A) Representative pictures of KKU-M213- and HuCCT-1-grafted subcutaneous tumors stained with YAP (Upper panels). Red triangles indicate intranuclear YAP localization. Scale bars; 50 μm. Quantification of nuclear YAP-positive cells (Lower panels). The number of immunopositive cells in high-power field (HPF) were counted for quantification. (B) Western blots of whole cell lysates from tumor tissues for YAP (Ser127) and LATS1 (Thr1079) phosphorylation (Upper panel). Relative phosphorylation rate of phosphorylated LATS1(Thr1079)/LATS1 (Lower left panel), and phosphorylated YAP (Ser127)/YAP (Lower Right panel). The expression levels were quantified by using NIH image J software and were normalized to actin. (C) Relative mRNA levels of YAP/TEAD target genes, *CTGF*, *CYR61*, *ANKRD1* and *MFAP5* in KKU-M213 (Left panel)- and HuCCT-1 (Right panel)-grafted subcutaneous tumor tissues. LOS; losartan-treated group. Actin was used as the loading control for WB (B). The mRNA expression levels were measured by quantitative RT-PCR (qRT-PCR). *GAPDH* was used as internal control for qRT-PCR (C). Quantitative values are indicated as ratios to the values of vehicle-treated group (B, C). Data are mean ± SD (n = 10; A) and ± SEM (n = 3; B. n = 10; C). *, P ≤ 0.05; **, P ≤ 0.01 vs vehicle-treated group (A–C).

miR-222 can regulate the YAP/TEAD signaling pathway [37], suggesting that miR-222/YAP/TEAD signaling may also be involved in the antiproliferative effects on human CCA cells as an additional pathway, although further investigation is required to clearly elucidate this mechanism.

Losartan also inhibited antiapoptotic activity downregulating Mcl-1 in CCA cells. After Rizvi et al. demonstrated that the YAP and Hippo signaling pathways culminate in an Mcl-1-regulated tumor survival, it has been proposed that the effect of losartan on anti-apoptosis was associated with YAP inactivation [38]. However, the role of YAP in apoptosis is controversial and there is evidence that YAP acts as a pro-apoptotic effector by potentiating p73-mediated apoptosis upon DNA damage [39–42]. Given that such opposing functions of YAP on apoptosis are dictated by the cellular context, further studies are required to clarify the mechanism by which apoptosis is induced by losartan in terms of YAP inactivation. Besides its role in cell survival, YAP regulates

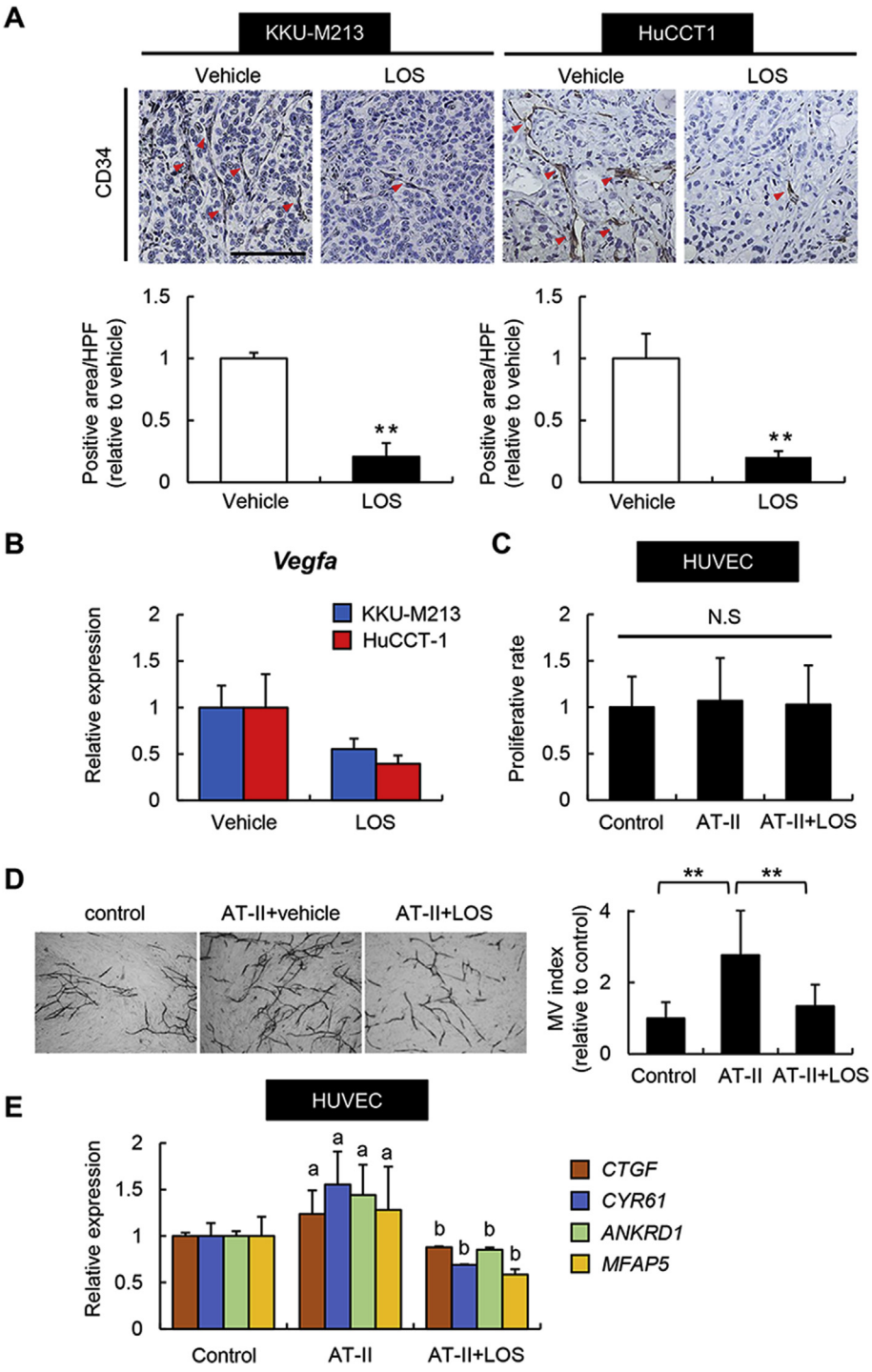

Fig. 5. Losartan attenuates intratumor angiogenesis in cholangiocarcinoma. (A) Representative pictures of KKU-M213- and HuCCT-1-grafted subcutaneous tumors stained with CD34 (Upper panels). Red triangles indicate $CD34^+$ immunopositive new vessels. $CD34^+$ immunopositive new vessels in high-power field (HPF) were quantified by NIH imageJ software (Lower panels; Left; KKU-M213, Right; HuCCT-1). Scale bar; 50 μm. (B) Relative mRNA levels of *Vegfa* in KKU-M213- and HuCCT-1-grafted subcutaneous tumor tissues. (C) Neither angiotensin-II (10^{-6} M) nor losartan (10^{-6} M) affect endothelial cell proliferation. (D) Characteristics (Left panels) and index (Right panel) of in vitro endothelial tubular formation. Losartan (10^{-6} M) attenuated angiotensin-II (10^{-6} M)-stimulated tubular formation. Microvessel (MV) index was quantified by NIH imageJ software. (E) Relative mRNA levels of YAP/TEAD target genes, *CTGF*, *CYR61*, *ANKRD1* and *MFAP5* in cultured HUVECs. AT-II; angiotensin-II, LOS; losartan. Relative mRNA expression levels were measured by quantitative RT–PCR (qRT–PCR). *GAPDH* was used as internal control for qRT–PCR (B and E). Quantitative values are indicated as ratios to the values of vehicle group (A, B) and untreated cells (C–E). Data are mean ± SD (n = 10; A), and ± SEM (n = 10; B and D. n = 8; C and E). **,$P \leq 0.01$ vs vehicle-treated group (A, B) and untreated cells (D). a, $P \leq 0.01$ vs untreated group [AT-II(-)/LOS(−)]; b, $P \leq 0.01$ vs angiotensin-II-treated and losartan-untreated group [AT-II(+)/LOS(−)] (E).

EMT, differentiation, and stemness [6–13,43]. We therefore evaluated the effects of losartan on these properties of cancer and found that it did not alter the mRNA levels of selected markers associated with EMT, differentiation, and stemness.

The results from the present study show that losartan was likely to improve subcutaneous CCA tumor growth more potently than proliferation in both lines. Following such discrepant findings, we assessed the impact of losartan on tumor microenvironment by hypothesizing that, since the AT1R/AT-II axis is strongly associated with tumor angiogenesis, it might also affect intratumor neovascularization. We have previously reported that AT-II could play an important role in the angiogenic activity in certain cancers, such as hepatocellular or pancreatic cancer. Therefore, we investigated the losartan-related changes in CCA angiogenic status, as a measure of cancer microenvironmental regulation.

We found that losartan attenuated the intratumor microvasculature of xenograft CCA and proposed two possible mechanisms for this antiangiogenic effect: first, losartan may directly inhibit vascular endothelial growth via the AT1R/AT-II axis, as supported by our finding of a losartan-induced inhibitory effect on endothelial tubular formation in vitro. Moreover, we identified a regulatory effect of AT1R/AT-II on the activation of YAP/TEAD signaling in HUVECs. Choi et al. have shown that YAP is a critical angiogenesis regulator, playing a role in endothelial cell migration, sprouting and tubular network formation [23]. Kim et al. also revealed multifaceted roles of YAP in endothelial cell behaviors, including junction assembly, metabolism in sprouting

angiogenesis, and barrier formation and maturation [24]. Taken together, these findings support our hypothesis that losartan's antiangiogenic effect is partially associated with YAP inactivation in endothelial cells.

Second, YAP signaling inactivation may lead to a suppression of tumor-derived proangiogenic activity in CCA. Among the YAP/TEAD target genes, MFAP5 is secreted by cancer cells facilitating angiogenesis and survival [44,45]. In ovarian cancer, high levels of MFAP5 expression correlate with poor survival and increased microvasculature density [46]. MFAP5 has been suggested to function as a direct transcriptional YAP target in human CCA, and its secretion may be promoted by oncogenic YAP activation, thus leading to increase in tumor angiogenesis both in vitro and in vivo [44]. Consistent with these findings, we showed that, in both CCA lines, AT-II stimulates MFAP5 upregulation via YAP activation, while losartan attenuates this upregulation and suppresses neovascularization. Therefore, losartan's antiangionenic effects may derive from inhibition of YAP's oncogenic activity, as well as from a direct impact on vascular endothelial cells.

In conclusion, the results from the present study indicate that losartan has an inhibitory effect on CCA growth through attenuating of YAP oncogenic activity. Although we approached the functional relationship between the AT1R/AT-II and Hippo/YAP pathways in CCA growth, it should be considered that YAP is regulated by other pathways, independently of Hippo signaling (e.g., Wnt/β-catenin and AMP-activated protein kinase) [47,48]. Therefore, further studies are needed to evaluate the functional interaction between these pathways and the AT1R/AT-II pathway in the oncogenic activation of YAP in human CCA. Additionally, ARB monotherapy seems to exert a limited curative effect against CCAs. Thus, we propose that it may make a significant contribution by combining anticancer drugs including gemcitabine in the clinical practice. Given that ARBs are clinically used without serious side effects, these agents may eventually emerge as a novel treatment class for patients with nonresectable CCA.

Author contribution

S.S performed most of the in vitro and in vivo experiments, analyzed and interpreted all data, and drafted the manuscript. K.K designed the study, orchestrated all the experimental data, and drafted the manuscript. N.N, K.S, S.S, K.N, K.K, H.K, M.K, K.M, T.N, and A.M offered technical/material support.

H.Y supervised the study, revised the article, and finally approved its submission.

Disclosure of conflicts of interest

No potential conflicts of interest were disclosed by all authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.07.021.

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