의학박사 학위논문

간세포암이 재발한 간이식 수혜자에서의 sorafenib 과 mTOR 억제제의 항암효과

Antitumor effect of sorafenib and mTOR inhibitor in liver transplantation recipients with hepatocellular carcinoma recurrence

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권 재 현

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이 논문을 의학박사 학위 논문으로 제출함

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Abstract

This study aimed to evaluate the antitumor effect of sorafenib, mTOR inhibitor (mTORi) , and their combination therapy in liver transplantation (LT) recipients with hepatocellular carcinoma (HCC) recurrence via laboratory-based research study followed by subsequent validation in the clinical LT setting. In the laboratory study, HepG2.2.15 liver tumor cell line and two patient-derived graft HCC cell lines were used for in vitro cytotoxic studies. HepG2.2.15 cells were also implanted to nude mice followed by oral administration of sorafenib and everolimus for in vivo study. After treatment with everolimus and sorafenib, cell viability and apoptosis assays revealed noticeable cytotoxic effects with individual agents and augmented effects by combination therapy. In vivo mouse study demonstrated similar cytotoxic outcomes, with maximal decrease of tumor weight and the greatest inhibition of α-fetoprotein (AFP) and hepatitis B virus X (HBx) expression by combination treatment. In the clinical study including 232 LT recipients, post-recurrence survival rates were not different following sorafenib administration (p=0.168), but were significantly improved following mTORi administration (p<0.001). Combination therapy demonstrated no noticeable synergistic antitumor effect. Although there was a prognostic difference according to the time of post-transplant tumor recurrence (p<0.001), mTORi-associated antitumor effect was present regardless of the time of tumor recurrence (p ≤0.098). Our laboratory study demonstrated synergistic antitumor effects of sorafenib and mTORi, but this was not reproduced in our clinical LT study. Because mTORi administration was well tolerated and beneficial for post-recurrence survival, administering mTORi in LT recipients with HCC recurrence appears worthwhile.

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Abbreviations

AFP, α-fetoprotein; ALD, alcoholic liver disease; AMC, Asan Medical Center; cccDNA, covalently closed circular DNA; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, hepatitis B virus X; HCV, hepatitis C virus; LT, liver transplantation; MELD, model for end-stage liver disease; mTOR, mammalian target of rapamycin; NOD-SCID mouse, non-obese diabetic/severe combined immunodeficiency mouse; PDX, patient-derived xenograft, PIVKA-II, proteins induced by vitamin K antagonist or absence-II; UCSF, University of California, San Francisco;

Introduction

Hepatocellular carcinoma (HCC) is an established indication for liver transplantation (LT).[1] Although LT candidates are prudently selected according to selection criteria for HCC, tumor recurrence develops in a considerable proportion of LT recipients.[2-5] Post-transplant HCC recurrence is associated with poor outcomes, probably because of inevitable immunosuppression and a high incidence of extrahepatic metastasis.[5,6]

HCC recurrence after LT is initially treated with various locoregional treatment modalities, but frequently, such treatments are no longer applicable because of ongoing tumor progression, and sorafenib is finally administered.[7-9] The therapeutic effects of sorafenib in LT recipients have been investigated in a few studies,[8,9] but are believed to be suboptimal. Mammalian target of rapamycin inhibitor (mTORi) is frequently reported to be beneficial to reduce the risk of HCC recurrence after LT,[6,10] but its effect on established post-transplant HCC recurrence is still unclear.[11,12]

Combination therapy with sorafenib and mTORi has been attempted due to their mechanisms of action in HCC pathogenesis despite the risk of augmented adverse side-effects.[11,12] Such combination therapy has been reported to have a synergistic antitumor effect in experimental animal studies,[13,14] but its clinical effects are still debated because of small number of cases and oncological heterogeneity in the study patients.[11,12] Thus, the present study aimed to assess the antitumor effects of sorafenib, mTORi, and their combination therapy in a laboratory-based research study and to subsequently validate them in the clinical LT setting.

Materials and methods

Study design

This study comprised two independent investigations: a laboratory research study and a clinical LT study. To assess the antitumor effects of sorafenib, mTORi, and their combination therapy, the study candidates were divided into four study groups as sorafenib, mTORi, their combination, and control groups.

The laboratory research study was focused on assessment of the synergistic antitumor effects with combination therapy and included in vitro and in vivo studies on tumor growth using an established HepG2 cell line and patient-derived xenograft (PDX) tumor cell lines.[15] In the clinical LT study, the survival periods after HCC recurrence were compared among the abovementioned four groups to validate the antitumor effects. These study designs are depicted in Fig. 1. The study protocols were approved by the Ethical Committee of Animal Study in the Asan Institute of Life Sciences and Institutional Review Board of the Asan Medical Center.

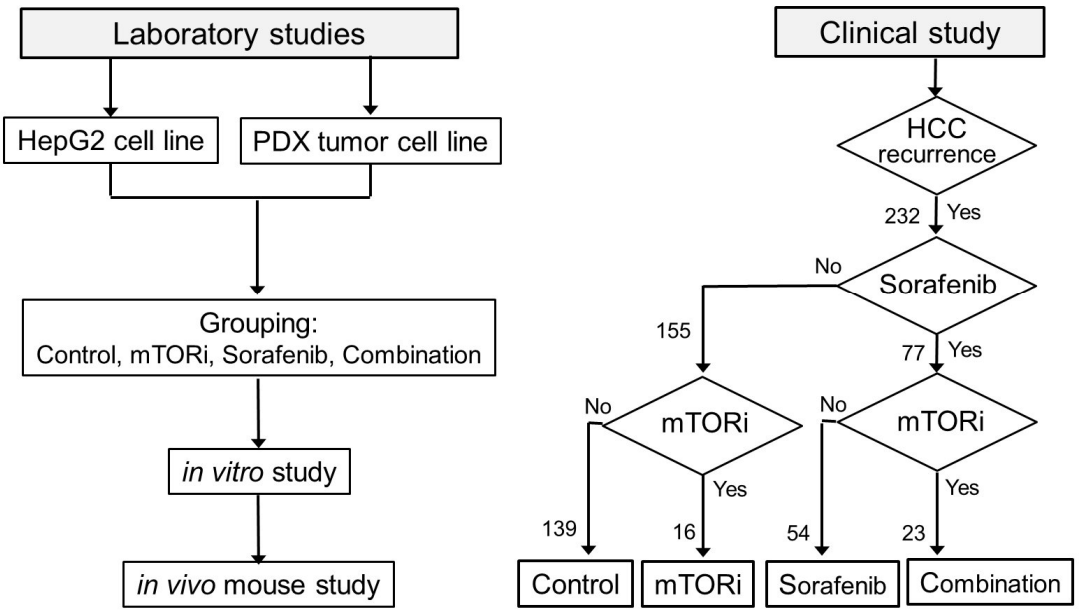

Fig. 1. Schematic illustration of study design for the laboratory and clinical studies.

In vitro study using HepG2 cell line

Because majority of HCC patients in Korea have associated hepatitis B virus (HBV) infection, we chose the HepG2.2.15 cell line (Korean Advanced Institute of Science and Technology) that is derived from the human hepatoblastoma cell line HepG2 with HBV transfection. This liver tumor cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA).

The effects of sorafenib and everolimus were evaluated using this liver tumor cell line. To reproduce the therapeutic ranges in HCC patients and LT recipients, the in vitro drug concentration was determined to be 10 μmol/mL for sorafenib and 10 nmol/mL for everolimus after repeated titration studies (5 to 20 μmol/mL for sorafenib and 5 to 20 nmol/mL for everolimus).[16-20] Duration for drug exposure was set to be 20 hours.

To assess cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Duchefa, Haarlem, the Netherlands) assays were performed using 12-well plates. The optical density was assessed at 550 nm using a microplate reader (Bio-Rad). Cell survival was expressed as the percentage of absorbance in drug-treated cells relative to that in untreated cells. The cells were also observed under a fluorescence microscope after 4′,6-diamidino-2-phenylindole (DAPI)–Hoechst staining (Sigma-Aldrich, Poole, Dorset, UK).

Western blot assays were performed to assess HBV X (HBx) protein expression and apoptosis. Cell extracts were separated by polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with a primary antibody against HBx, poly(ADP-ribose) polymerase (PARP)-1, cleaved PARP-1, cleaved caspase-3, and actin, followed by incubation with a secondary antibody. Antibodies against HBx (ALX-804-278-C100) were purchased from Enzo Life Sciences (Farmingdale, NY, USA); antibodies against PARP (B-10, sc-74470) and cleaved PARP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against actin (AC-15, A3854)

were purchased from Sigma-Aldrich (Poole, Dorset, UK); and antibodies against cleaved caspase-3 (Asp175, cat no. 9664) were obtained from Cell Signaling (Danvers, MA, USA). Proteins in samples were detected using a Supersignal pico-enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Unless specified, cell lysates containing 20 μg protein were analyzed.

In vitro study with PDX tumor cell lines

Small pieces of human HCC tissue were obtained during hepatic resection for HCC in HBV-associated patients who did not undergo any preoperative HCC treatment (n = 2). A small tumor fragment of 0.3 g was implanted subcutaneously at the bilateral hind flanks of a non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mouse. After the confirmation of tumor growth for 3 months, the tumor was harvested and implanted to a NOD-SCID mouse. After the confirmation of stable tumor growth, the established first-generation xenograft tumor was serially implanted into SCID mice to expand the xenograft tumors. These xenograft tumors were also implanted subcutaneously into nude mice for further tumor expansion. The tumors were then harvested to establish new PDX tumor cell lines that were used to perform in vitro study for assessment of cell viability with MTT assay.

In vivo mouse study using the HepG2 cell line

A tumor mass of HepG2.2.15 cells (0.3 g) was implanted subcutaneously at the hind flank of 6- to 8-week-old nude mice, and the mice were fed for 2 weeks for tumor growth. Thereafter, 5 mg/kg sorafenib and 5 μg/kg everolimus were administered orally for 3 weeks, consistent with the oral dosage used in HCC patients and LT recipients after consideration of their plasma clearance rates in the mouse models.[17-20] Gross tumor morphology and three-dimensional volume of the tumors were assessed every day, and the mice were sacrificed on

day 21 of drug administration.

Western blot assays were performed to assess α-fetoprotein (AFP) expression at the harvest tumors. Cell extracts were separated by polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated with primary antibody against AFP (C-19: sc-8108, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin followed by incubation with a secondary antibody.

In addition, reverse transcription-polymerase chain reaction (RT-PCR) was also performed to assess expression of HBx and covalently closed circular DNA (cccDNA) of HBV at the harvest tumors. The following primer sets were used: HBx forward, 5‘-TGCCAACTGGATCCTTCGCGGGACGTCCTT-3‘; and HBx reverse, 5‘-GTTCACGGTGGTCTCCATG-3‘; cccDNA forward, 5‘-CTCCCCGTCTGTGCCTTCT-3‘ (nucleotides 1548–1566); and cccDNA reverse, 5‘-GCCCCAAAGCCACCCAAG-3‘ (nucleotides 1903–1886). The data were expressed as the fold change in the HBx and cccDNA levels from the harvest tumors to the normal human liver control tissues. Details of these PCR methods are described elsewhere.[21]

In vivo mouse study using the PDX tumor cell lines were not performed because the outcomes from in vitro studies between the HepG2 cell line and PDX tumor cell lines were very similar, thus their in vivo study outcomes were anticipated to be similar to those with HepG2 cell line.

Clinical LT study on 232 patients who had post-transplant HCC recurrence

The LT database at our institution was searched to identify patients who underwent LT for HCC over a 14-year period from January 2000 to December 2013, and 1486 of 3850 LT recipients were selected. Of these, 232 recipients (15.6%) had post-transplant HCC recurrence until December 2015. These 232 patients were divided into four groups according to administration with sorafenib and mTORi (sirolimus or everolimus) (Fig. 1). Patients who

had not continued drug treatment for 3 months were not included in the corresponding drug group. Patients were followed up until February 2017 with a medical record review and assistance from the National Health Insurance Service, thereby making the overall patient follow-up period $\geq$ 38 months and the post-recurrence follow-up period $\geq$ 14 months, or until patient death. All patients were completely followed up to identify patient survival status. All LT recipients who were diagnosed with HCC before LT or at the explant liver underwent follow-up examinations every month during the first year and every 3 months thereafter. A detailed follow-up protocol based on the relative risk of HCC recurrence has been previously described.[5] The general principles of treatment for recurrent HCC lesions were applied to LT recipients with HCC recurrence.[4,5,22] Locoregional treatment including surgical resection was initially attempted if indicated, but a considerable proportion of patients with advanced HCC recurrence used sorafenib as the final treatment modality. A wide range of heterogeneity was noted in the time of treatment initiation and in the duration of sorafenib administration; thus, we decided to measure the overall post-recurrence survival period instead of the survival periods after sorafenib administration. Patients with poor general condition or impaired graft liver function beyond Child–Pugh class A were not indicated for sorafenib in principle. Sorafenib administration was stopped if disease progression developed according to the modified Response Evaluation Criteria in Solid Tumors.[23] In Korea, sorafenib has been covered by social health insurance since late 2009.

Because mTORi was not included as the primary immunosuppressant at our institution, it was not commonly used before development of HCC recurrence or de novo malignancy. In contrast, it was preferentially administered to LT recipients with HCC recurrence after introducing sirolimus in 2008 and social health insurance coverage for everolimus in early 2016 in Korea.

Statistical analysis

Continuous variables are reported as means with standard deviation or medians with ranges and were compared using Student's *t*-test. Categorical variables were compared using chi-square or Fisher's exact test, as appropriate. Survival curves were generated using the Kaplan–Meier method and compared using log-rank test. A p-value <0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad, La Jolla, CA, USA) and SPSS version 22 (IBM, New York, NY, USA).

Results

In vitro cytotoxicity in the HepG2 cell line study

The MTT assay for cell survival assessment demonstrated a concentration-dependent decrease in cell survival after 20-hour treatment with 5 to 20 μmol/mL sorafenib and 5 to 20 nmol/mL everolimus. The greatest decrease was observed after combined treatment with sorafenib and everolimus. Noticeable differences were observed between treatment with 5 μmol/mL sorafenib + 5 nmol/mL everolimus and that with 10 μmol/mL sorafenib + 10 μmol/mL everolimus, but not between treatment with 10 μmol/mL sorafenib + 10 μmol/mL everolimus and that with 20 μmol/mL sorafenib + 20 μmol/mL everolimus (Fig. 2A). Thus, the drug concentrations of 10 μmol/mL sorafenib + 10 μmol/mL everolimus were used for further in vitro studies.

A microscopic assessment of the cell morphology revealed abundant apoptosis after treatment with sorafenib, everolimus, and their combination (Fig. 2B). Fluorescence microscopy with DAPI–Hoechst staining revealed apoptosis after treatment with sorafenib or everolimus, and the highest apoptosis levels were observed after combination therapy (Fig. 2C).

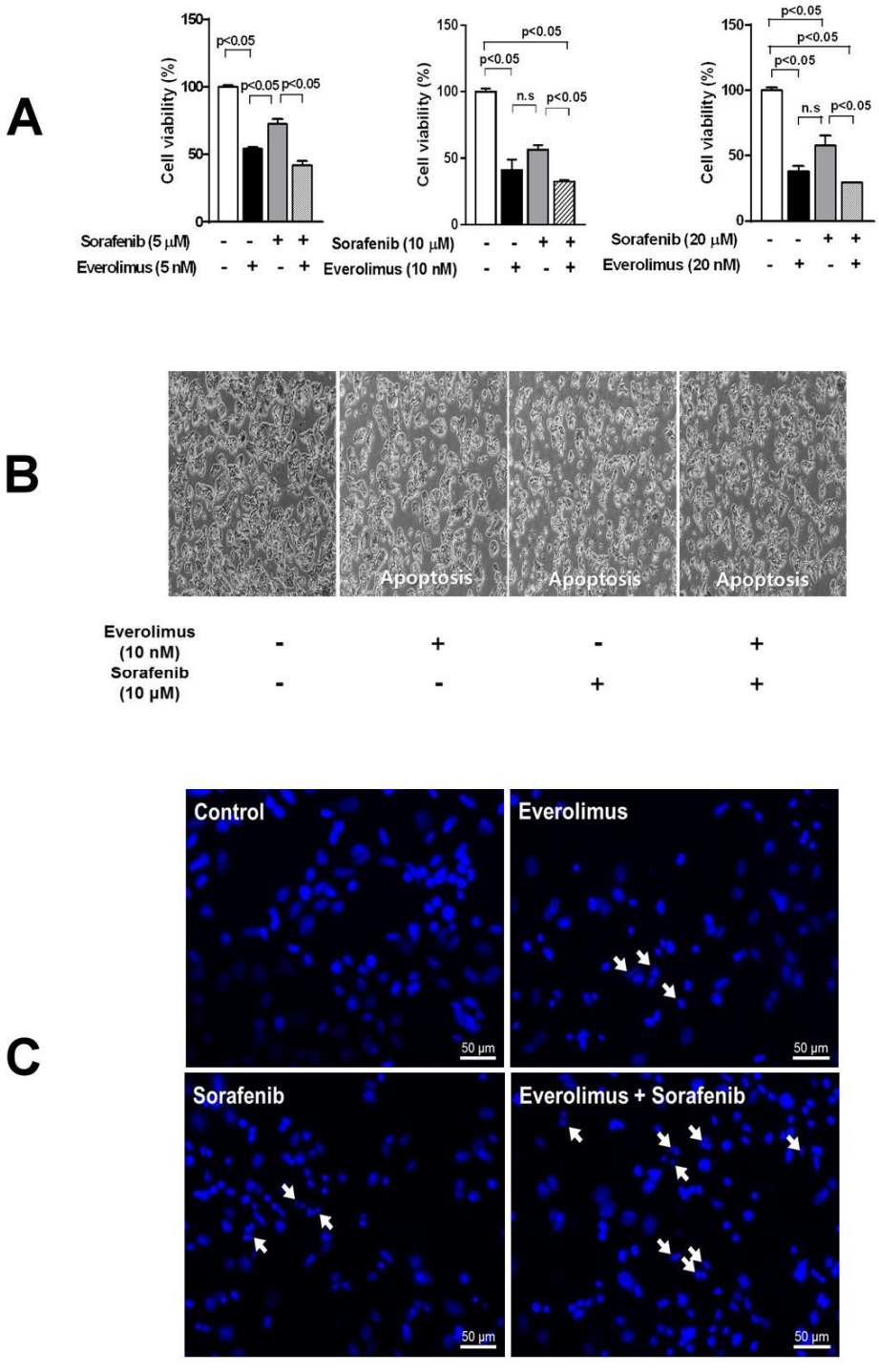

Fig. 2. In vitro cytotoxicity in the HepG2 cell line studies with sorafenib and everolimus showing MTT assay for cell survival assessment (**A**), microscopic assessment of the cell morphology (**B**), and fluorescence microscopy with DAPI–Hoechst staining (arrows indicate apoptosis) (**C**).

Treatment with sorafenib or everolimus led to a noticeable downregulation of HBx expression, and the lowest expression was observed after combination therapy (Fig. 3A). Apoptosis was assessed by staining for PARP-1, cleaved PARP-1, and cleaved caspase-3 after treatment with sorafenib or everolimus, and the highest staining was observed after combination therapy (Fig. 3B).

In vitro cytotoxicity in PDX tumor cell line study

MTT assay showed that the decrease of cell viability was observed after either sorafenib or everolimus treatment and the highest decrease was observed after combination therapy, which were similar to those of the HepG2 cell line studies (Fig. 3C and 3D).

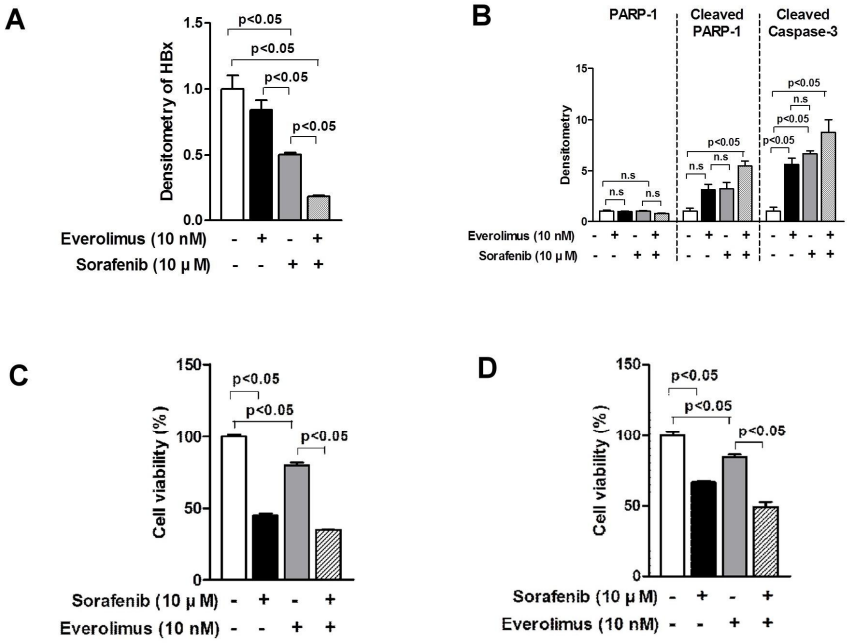

Fig. 3. In vitro cytotoxicity studies with sorafenib and everolimus. With HepG2 cell line, Western blot and densitometry for hepatitis B virus X protein (HBx) expression (**A**) and for apoptosis markers PARP-1, cleaved PARP-1, and cleaved caspase-3 (**B**). MTT assay for cell survival assessment using two patient-derived xenograft hepatocellular carcinoma cell lines were also performed (**C** and **D**).

In vivo inhibition of tumor growth in the HepG2 cell line study

Daily observations of tumor volume over 21 days revealed noticeable tumor growth after administration of sorafenib or everolimus, and marked inhibition of tumor growth after combination therapy (Fig. 4A). Gross tumor morphology at day 21 was compared (Fig. 4B). Tumor weight at day 21 was compared among the control, sorafenib, everolimus and combination groups, and was greatly decreased after combination therapy (Fig. 4C).

Expression of AFP at the tumor tissues was inhibited after administration of either sorafenib or everolimus, and maximal inhibition occurred after combination treatment (Fig. 4D). Expression of HBx (Fig. 4E) and HBV cccDNA (Fig. 4F) at the tumor tissues was also down-regulated after administration of either sorafenib or everolimus, and maximal decrease occurred after combination therapy.

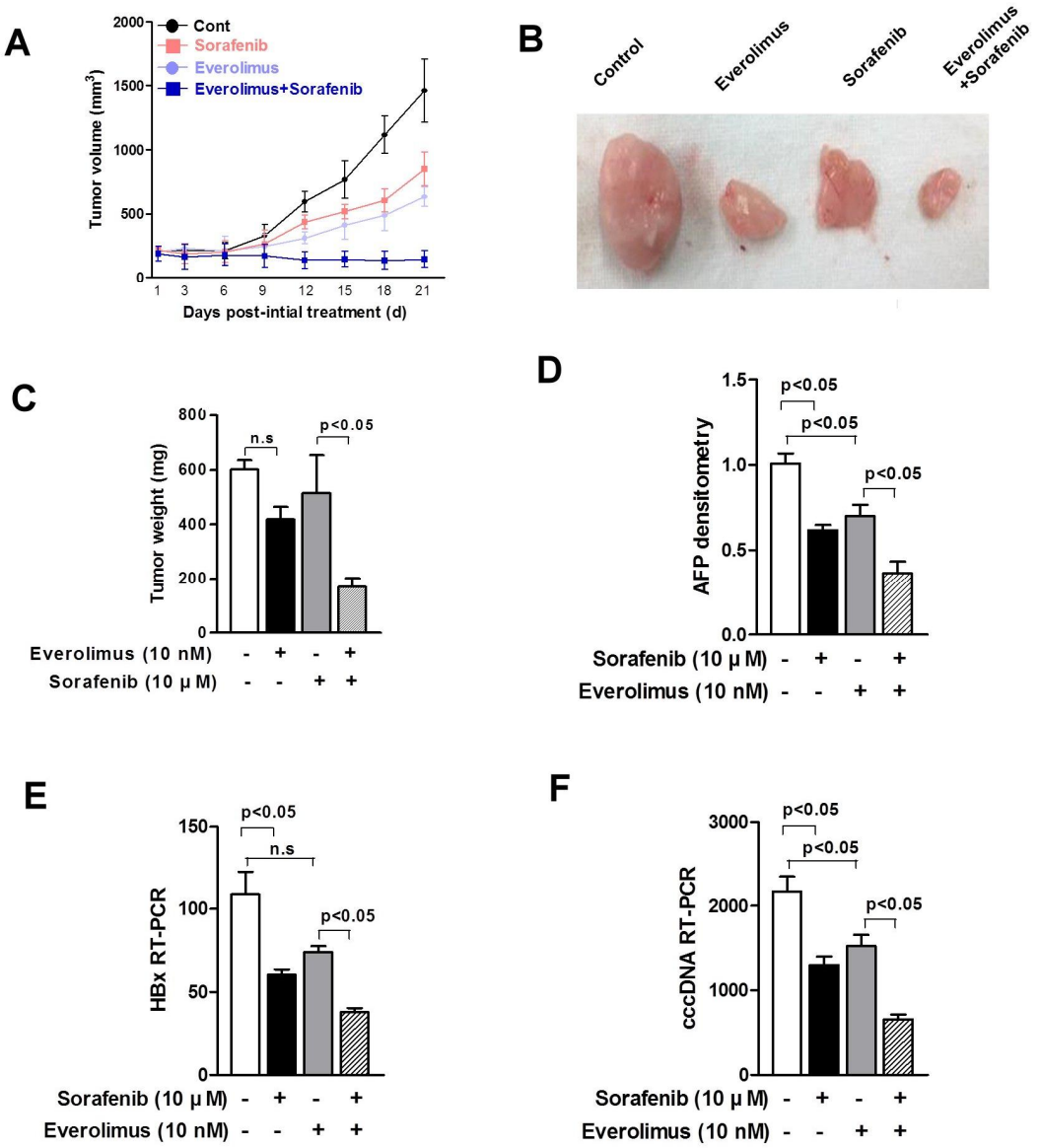

Fig. 4. In vivo inhibition of tumor growth in the nude mouse study with HepG2 cell line according to administration of sorafenib and everolimus. **A**) Daily changes in tumor volume; **B**) Comparison of tumor gross morphology at day 21 after medication; **C**) Comparison of tumor weight at day 21 after medication; **D**) Expression of α-fetoprotein (AFP) at the tumor tissues; **E**) Expression of hepatitis B virus X protein (HBx) at the tumor tissues; and **F**) Expression of hepatitis B virus covalently closed circular DNA (cccDNA) at the tumor tissues.

Clinical profiles and medication status of 232 LT recipients with HCC recurrence

The number of patients in the control, sorafenib, mTORi, and combination groups were 139, 16, 54, and 23, respectively (Fig. 1). The demographics of these four groups are summarized in Table 1, and their preoperative parameters were not statistically different. None of them had administered mTORi before HCC recurrence.

Initially, sorafenib was administered to 118 patients, 42 (35.6%) of them dropped out within 3 months because of adverse side effects, poor general condition, or rapid tumor progression. In 21 patients (17.8%) of them, sorafenib administration had to be stopped within the first month primarily because of the intolerable adverse side effects even after dosage reduction. In the remaining 77 patients, sorafenib administration was maintained over a median period of 6 months (range: 3–71).

mTORi was initially administered to 51 patients, 12 (23.5%) of them dropped out within 3 months because of adverse side effects and other causes. mTORi administration had to be stopped in only four (7.8%) of these patients because of severe adverse side effects despite dosage reduction of mTORi. In the remaining 39 patients, mTORi administration was maintained over a median period of 12 months (range: 3–60). Only sirolimus was available in Korea before 2016, which was subsequently replaced with everolimus because only everolimus was financially covered by the social health insurance in LT recipients. After insurance coverage for everolimus, the drop-out rate of mTORi was lowered to 6.7% (2 of 30). Thus, 23 patients had used sirolimus only, six switched from sirolimus to everolimus, and 22 used everolimus only. None of these patients experienced overt acute rejection requiring high-dose steroid therapy.

Table 1. Comparison of the clinical features of liver transplantation recipients with hepatocellular carcinoma recurrence treated with sorafenib and mTOR inhibitor

Parameters / Groups	Sorafenib (−) mTORi (−)	Sorafenib (−) mTORi (+)	Sorafenib (+) mTORi (−)	Sorafenib (+) mTORi (+)	p-value
Patient number	139	16	54	23	
Age (years)	53.7 ± 6.5	51.4 ± 5.2	52.8 ± 5.6	53.5 ± 5.5	0.54
Male gender (n)	127 (91.4%)	15 (93.8%)	48 (88.9%)	21 (91.3%)	0.97
Background liver disease					0.67
HBV	112 (87.8%)	13 (81.3%)	48 (88.9%)	21 (91.3%)	
HCV	11 (7.9%)	2 (12.5%)	2 (3.7%)	0	
ALD	4 (2.9%)	1 (6.3%)	1 (1.9%)	1 (4.3%)	
Others	2 (1.4%)	0	3 (5.6%)	1 (4.3%)	
MELD score	14.0 ± 7.3	11.3 ± 4.8	11.4 ± 4.4	14.4 ± 8.7	0.061
AFP (ng/mL)	49.9	14.8	20.5	19.3	0.69
PIVKA-II (AU/L)	54	24	35	34	0.66
LT type (n)					0.28
Living-donor	128 (92.1%)	16 (100%)	53 (98.1%)	22 (95.7%)	
Deceased-donor	11 (7.9%)	0	1 (1.9%)	1 (4.3%)	
Milan criteria (n)					0.18
Within	70 (50.4%)	12 (75%)	24 (44.4%)	10 (43.5%)	
Beyond	69 (49.6%)	4 (25%)	30 (55.6%)	13 (56.5%)	
UCSF criteria (n)					0.33
Within	81 (58.3%)	13 (81.3%)	32 (59.3%)	15 (65.2%)	
Beyond	58 (41.7%)	3 (18.8%)	22 (40.7%)	8 (34.8%)	
AMC criteria (n)					0.11
Within	95 (68.3%)	15 (93.8%)	37 (68.5%)	19 (82.6%)	
Beyond	44 931.7%)	1 (6.3%)	17 (31.5%)	4 (17.4%)	

Salvage LT (n)	13 (9.4%)	3 (18.8%)	13 (24.1%)	4 (17.4%)	0.059
Disease-free survival after LT (months)	22.6 ± 26.1	33.6 ± 29.6	18.9 ± 26.2	27.3 ± 31.8	0.23

Long-term outcomes in LT recipients with HCC recurrence treated with sorafenib and mTORi

The overall and post-recurrence patient survival rates were 84.5% and 54.0% at 1 year, 49.6% and 28.0% at 3 years, 33.6% and 19.9% at 5 years, and 19.0% and 4.5% at 10 years, respectively (Fig. 5A and 5B).

The post-recurrence survival rate differed significantly according to the recurrence time after LT (p <0.001, Fig. 5C); thus, patients with late recurrence 1 or 3 years post-transplant demonstrated significantly prolonged overall survival outcomes than those with earlier recurrence (p <0.001, Fig. 5D).

The post-recurrence survival rate after sorafenib treatment demonstrated no significant difference (p=0.168, Fig. 6A). In contrast, the post-recurrence survival rate after mTORi treatment demonstrated a significant difference (p<0.001, Fig. 6B). The post-recurrence survival after combined therapy demonstrated a significant difference (p=0.011, Fig. 6C). Compared with the sorafenib group, the combination therapy group demonstrated a significant prolongation in post-recurrence patient survival period (p=0.006, Fig. 6D). These findings indicated that the antitumor effect was demonstrated after mTORi administration but not after sorafenib administration.

Because there was a prognostic difference in the time of tumor recurrence, patients in the mTORi group were divided accordingly. mTORi treatment demonstrated a significant prognostic difference in patients with tumor recurrence within the first year (p=0.006, Fig. 7A), during the second and third years (p=0.021, Fig. 7B), and after 3 years (p=0.098, Fig. 7C). Considering the small patient numbers in these subgroups, these findings indicated that

the beneficial antitumor effect was present after mTORi administration regardless of the time of tumor recurrence.

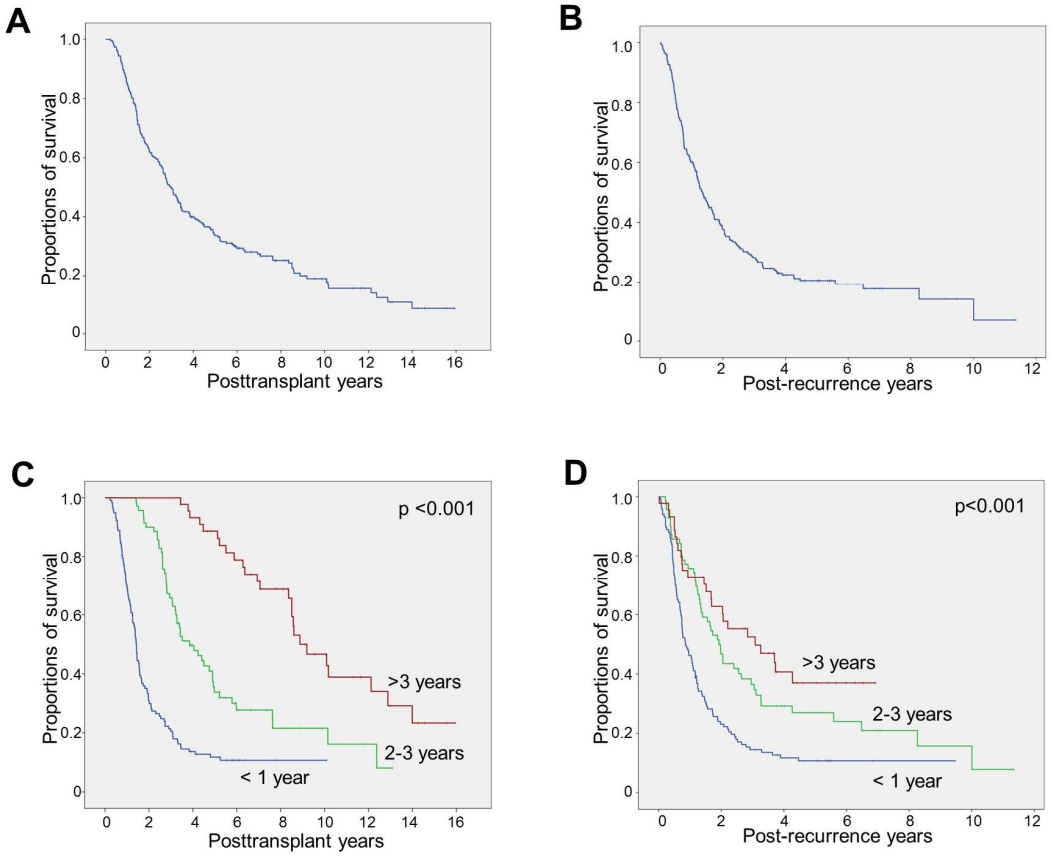

Fig. 5. Overall patient survival (**A**) and post-recurrence patient survival curves (**B**). The overall (**C**) and post-recurrence (**D**) patient survival curves according to the time of hepatocellular carcinoma recurrence after liver transplantation operation are also depicted.

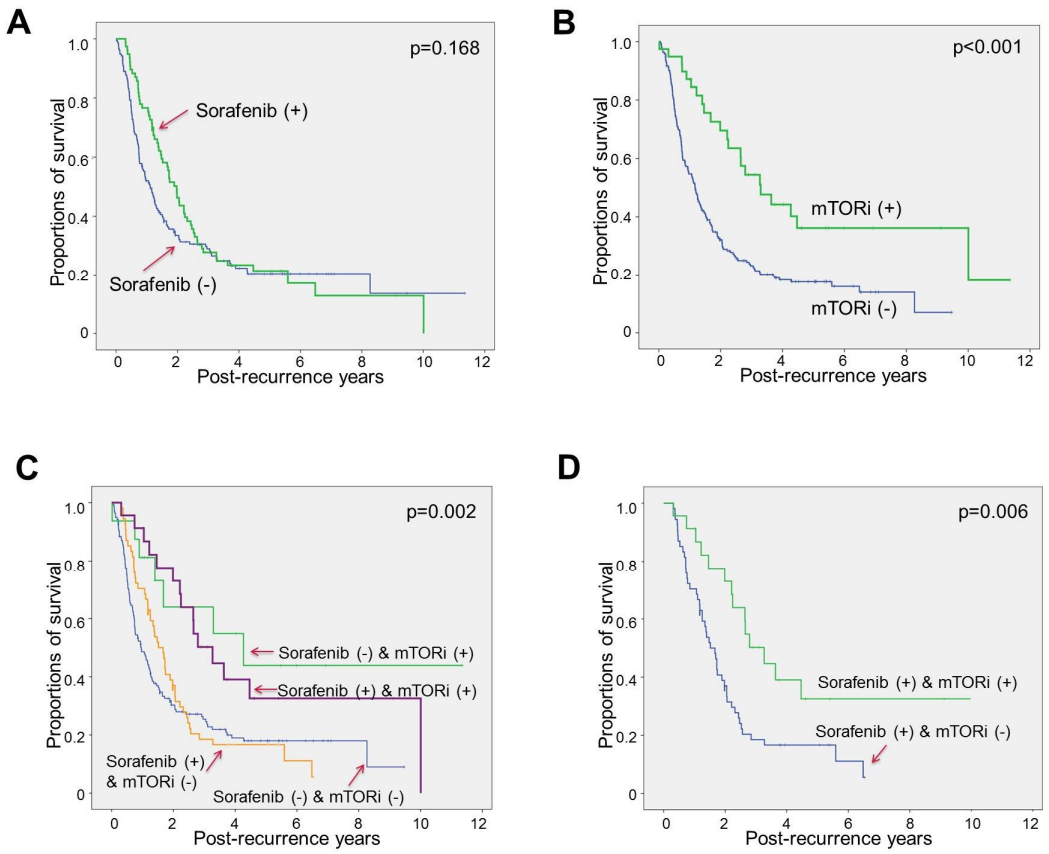

Fig. 6. Comparison of the post-recurrence patient survival curves for treatment with sorafenib (**A**), mTOR inhibitor (mTORi) (**B**), and their combination (**C**). In patients receiving sorafenib, the post-recurrence survival curves according to mTORi administration were compared (**D**).

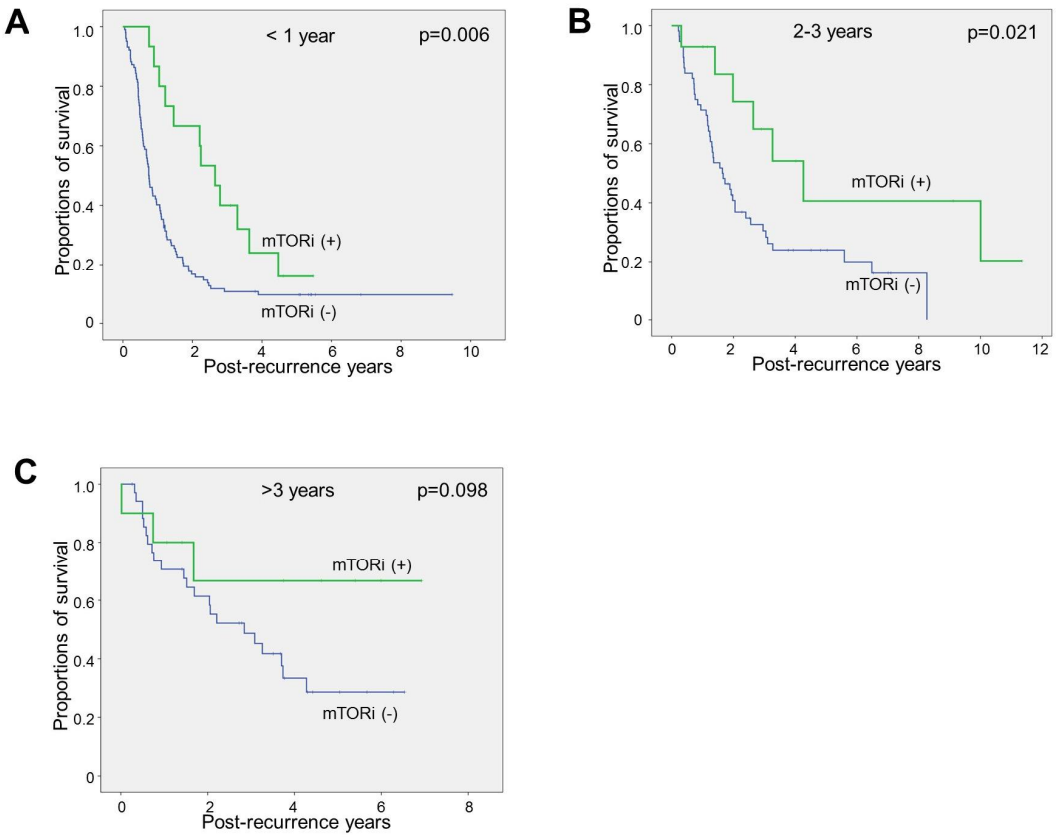

Fig. 7. Comparison of the post-recurrence patient survival curves for treatment with mTORi. The post-recurrence survival curves according to mTORi administration were compared in patients with hepatocellular carcinoma recurrence within the first year after liver transplantation operation (**A**), in patients with recurrence during the second and third years (**B**), and in patients with recurrence after 3 years (**C**).

Discussion

Sorafenib is a multikinase inhibitor with therapeutic efficacy against HCC.[24,25] It blocks tumor cell proliferation by targeting the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway; exerts an anti-angiogenic effect by targeting the tyrosine kinases vascular endothelial growth factor (VEGF) receptor-2, VEGF receptor-3, platelet-derived growth factor receptor-β, Ret, and c-Kit; and inhibits phospho-eIF4E and

Mcl-1 protein.[24-26] Although sorafenib noticeable improves the survival of patients with advanced HCC,[27,28] its potential mechanisms of action that induce clinical benefits are not yet well known. Furthermore, its therapeutic effect on post-transplant HCC recurrence is still largely unknown.[8,9]

mTORi has dual properties of immunosuppressive and antitumor effects. Its immunosuppressive effects are induced by the inhibition of interleukin (IL)-2-mediated T-cell proliferation,[29] unlike those of calcineurin inhibitors that prevent calcineurin from activating IL-2 production. Its antitumor effects are induced by inhibiting cell growth and angiogenesis, and it is clinically used for several malignancies.[30] An analysis of the high-volume data from the Scientific Registry of Transplant Recipients has revealed that mTORi-based immunosuppressive therapy is associated with improved survival after LT for HCC, but has also revealed a trend toward lower survival rates in non-HCC recipients, thus confirming the specificity of its beneficial impact on cancer.[6] Everolimus is effective and is associated with a low rate of HCC recurrence in LT recipients.[31-33] However, so far, majority of clinical LT studies have focused on prevention of HCC recurrence but not on the treatment of established HCC recurrence. Although we preferentially switched the primary immunosuppressant toward mTORi after HCC recurrence and de novo malignancy, there is still a lack of strong evidence supporting immunosuppressive regimen using mTORi.[31-34]

It is difficult to quantify the antitumor effect of sorafenib and mTORi in the clinical setting of LT recipients with HCC recurrence because of small number of cases and oncological heterogeneity in the study patients.[11,12] Therefore, we needed to confirm the antitumor effects of these agents in a controlled in vivo animal study. Our present study clearly demonstrated that the antitumor effects of sorafenib or everolimus alone were noticeably present in both in vitro and in vivo mouse studies and that their antitumor effects were significantly augmented by their combination, confirming the results of previous animal studies.[13,14]

However, contrary to our expectation, any synergistic effect of combination therapy was

not clearly demonstrated in LT recipients. Furthermore, the antitumor effect of sorafenib alone was not identified in LT recipients with HCC recurrence.[9] According to the Korean clinical practice guidelines, sorafenib is the last therapeutic option for HCC because the social health insurance policy permits its use only after logoregional treatment is no longer applicable. Thus, the time of starting sorafenib administration was usually delayed until definite local tumor progression or multiple metastases occurred. Through our clinical experience on sorafenib treatment (unpublished data), we recognized that the therapeutic efficacy of sorafenib is lower in non-LT patients with far advanced HCC compared with that in non-LT patients with less advanced HCC. In addition, sorafenib administration had to be stopped in patients showing disease progression during sorafenib treatment, and they were usually provided only the best supportive care. We did not include patients with sorafenib treatment for <3 months in the sorafenib group. We believe that such a late sorafenib administration was one of the main causes of its reduced therapeutic efficacy.

In contrast, the present study demonstrated that the antitumor effect of mTORi alone was evident in LT recipients, which was quite comparable with that of the in vivo mouse study. This finding on the antitumor effect of mTORi on established HCC recurrence was not reported before, and is consistent with that of previous clinical studies on its preventive effect on HCC recurrence.[31-33] The nude mouse is athymic; thus, the depleted T-lymphocyte function facilitates tumor cell growth. In contrast, mTORi selectively inhibits only IL-2-mediated T-cell proliferation in normal human individuals; thus, tumor growth might be more suppressed in LT recipients than in the nude mouse model because their innate immunity against tumor cells may be well preserved. The mTOR signaling pathways are associated with the carcinogenesis of human HCC; thus, its blockage by mTORi may also significantly contribute to tumor growth inhibition.[35]

A Korean single-institution study found that 12 patients who received combination therapy with sorafenib and sirolimus demonstrated better post-recurrence survival outcomes than 27

patients with best supportive care for post-transplant HCC recurrence.[12] In a Spanish multi-center study on 31 LT recipients with HCC recurrence, combination therapy resulted in partial response in one patient and stable disease in 13; thus, the overall clinical benefit rate was 53.8%.[36] However, considering the results of our present study, we presumed that such prognostic benefit might have been greatly influenced by mTORi rather than the combination therapy. To our knowledge, our study has the largest number of post-transplant HCC recurrence cases from a single institution and, to date, is the only clinical study to evaluate the individual antitumor effects of sorafenib and mTORi. Considering the poor outcomes of post-transplant HCC recurrence, it would be neither practical nor ethical to perform a controlled clinical study on this topic. Instead, to further validate our findings, it is necessary to perform multi-center studies.

When administering combination therapy with sorafenib and mTORi, a high probability of adverse side effects is anticipated. The 3-month drop-out rate of sorafenib was 35.6% (42 of 118 patients) in our present study; sorafenib administration had to be stopped in 21 of these 42 patients within the first month primarily because of severe adverse side effects even after dosage reduction. In contrast, mTORi administration had to be stopped in only four of 51 patients (7.8%) due to serious adverse side effects because dosage reduction combined with other immunosuppressive agents could effectively alleviate the adverse side effects. It was noticeable that the drop-out rate with everolimus treatment was noticeably decreased after it was covered by the social health insurance. Therefore, if LT recipients can tolerate sorafenib, we believe that it is not difficult to add mTORi because the adverse side effects can be readily managed with dosage reduction. In contrast, for LT recipients who administer mTORi, add-on of sorafenib may be not simple because of high probability of adverse side effects.

This study has some limitations. This was a retrospective single-center study in an HBV-endemic area, and majority of our patients underwent living-donor LT. Another major

limitation is that the Korean health insurance policy permits the use sorafenib only in patients who cannot receive locoregional treatment; thus, its administration was usually started at very advanced stages of HCC recurrence. A unique strong point of this study is that the survival status of all patients was completely followed up.

In conclusion, our laboratory study demonstrated a synergistic antitumor effect of sorafenib and mTORi, but this was not reproduced in the clinical LT setting probably because of late sorafenib administration at advanced stages of HCC recurrence. Considering that mTORi alone was beneficial for the post-recurrence patient survival and was also well tolerated, administering mTORi alone or in combination with sorafenib after HCC recurrence appears worthwhile.

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국문 요약

본 연구에서는 간세포암이 재발한 간이식 수혜자에서의 sorafenib와 mTOR 억제제 (mTORi) 의 항암효과를 규명하고자 실험실 연구와 임상 연구를 시행하였다. In vitro 실험에서는 HepG2.2.15 간세포암 세포주와 환자의 간세포암에서 추출한 세포주 (PDX) 를 사용하여 sorafenib과 everolimus를 각각 주입하였을 때와 병합하여 주입하였을 때의 세포독성 효과를 평가하는 실험을 진행하였다. In vivo에서는 생쥐실험모델을 통하여 HepG2.2.15 간세포암 세포주를 주사하여 종양 증식을 시킨 뒤에, sorafenib과 everolimus를 복용하였을 때와 병합하여 복용시켰을 때 종양의 증식 정도를 비교하였다. HepG2.2.15 간세포암 세포주와 PDX 세포주 모델에서 세포 생존도와 세포자멸은 대조군에 비해서 everolimus와 sorafenib 각각 모두 유의하게 세포독성 효과를 보였고, 이 두 가지를 병합요법으롤 사용했을 때 그 효과는 각각을 개별적으로 사용했을 때 보다 유의하게 세포독성 효과가 증강되어 나타났다. In vivo 생쥐실험모델에서도, everolimus와 sorafenib을 사용했을 때보다 병합요법을 하였을 때 종양 조직의 무게가 유의하게 더 감소하였고, 알파태아단백 (AFP) 및 hepatitis B virus X (HBx) 발현의 유의한 감소를 보였다. 232명의 간이식 수혜자를 대상으로 한 임상 연구에서는, 대조군에 비해서 sorafenib을 사용한 환자에서는 재발 후 생존율에 유의한 차이가 없었지만 (p=0.168), everolimus를 사용한 환자에서는 재발 후 생존율이 유의하게 증가하였다 (p<0.001). everolimus와 sorafenib 병합 요법은 유의한 상승효과를 보이지는 않았다. 이식 후 재발한 시기에 따라 생존율에 유의한 차이를 보였지만 (p<0.001), everolimus의 항암 효과는 재발한 시기에 관계 없이 나타났다. 본 연구에서는 실험실 모델에서는 everolimus와 sorafenib의 병합요법이 상승효과를 나타내었으나, 간이식 수혜자를 대상으로 한 임상연구에는 그러한 효과를 보이지 않았다. 간세포암이 재발한 이식 환자에

게 everolimus를 사용하는 것은 부작용이 적고 재발 이후 생존을 향상시킬 수 있기 때문에 임상적으로 효용성이 있을 것으로 보인다.