Original Article
Yonsei Med J 2022 Nov;63(11):1007-1015
https://doi.org/10.3349/ymj.2022.0247
Yonsei Medical Journal
YMJ
pISSN: 0513-5796 · eISSN: 1976-2437

Antitumor Effect of Low-Dose of Rapamycin in a Transgenic Mouse Model of Liver Cancer

Hyung Soon Lee[1], Joon Ye Kim[2], Simon Weonsang Ro[3], Myoung Soo Kim[2,4], Haeryoung Kim[5], and Dong Jin Joo[2,4]

[1]Department of Surgery, National Health Insurance Service Ilsan Hospital, Goyang;
[2]The Research Institute for Transplantation, Yonsei University College of Medicine, Seoul;
[3]Department of Genetics and Biotechnology, College of Life Sciences, Kyung Hee University, Yongin;
[4]Department of Surgery, Yonsei University College of Medicine, Seoul;
[5]Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea.

Purpose: We investigate whether low-dose rapamycin is effective in preventing hepatocellular carcinoma (HCC) growth and treating HCC after tumor development in transgenic mice.
Materials and Methods: We established transgenic mice with HCC induced by activated HrasG12V and p53 suppression. Transgenic mice were randomly assigned to five experimental groups: negative control, positive control, tacrolimus only, rapamycin only, and tacrolimus plus rapamycin. The mice were further divided into two groups according to time to commencement of immunosuppressant treatment: de novo treatment and post-tumor development.
Results: In the de novo treatment group, marked suppression of tumor growth was observed in the rapamycin only group. In the post-tumor development group, the rapamycin only group displayed no significant suppression of tumor growth, compared to the positive control group. In T lymphocyte subset analysis, the numbers of $CD4^+$ effector T cells and $CD4^+$ regulatory T cells were significantly lower in the positive control, tacrolimus only, and tacrolimus plus rapamycin groups than the negative control group. Immunohistochemical analysis revealed significantly higher expression of phosphorylated-mTOR, 4E-BP1, and S6K1 in the positive control group than in the rapamycin only group.
Conclusion: Low-dose rapamycin might be effective to prevent HCC growth, but may be ineffective as a treatment option after HCC development.

Key Words: Liver, sirolimus, mice, transgenic, carcinoma, hepatocellular

INTRODUCTION

Post-transplant survival and disease-free survival rates have improved substantially since adoption of the Milan criteria for liver transplantation (LT).[1] Despite this, hepatocellular carcinoma (HCC) recurrence after LT remains a significant problem with limited treatment options.[2] Post-transplant recurrent HCC is less responsive to conventional therapies and is associated with a dismal prognosis.[3] Intense research of the molecular biology of HCC carcinogenesis in recent years has led to the development of effective agents that have been widely tested in preclinical studies using HCC cell lines or xenograft models.[4,5]

Rapamycin, an antibiotic that inhibits mammalian target of rapamycin (mTOR), is used clinically as an immunosuppressive drug to prevent graft rejection after LT.[6] Rapamycin has been shown to inhibit HCC cell growth both in vitro and in vivo and to reduce HCC tumor angiogenesis, both as a single agent and in combination with other chemicals.[4] However, immunosuppression after LT is currently based on the use of calcineurin

Received: June 30, 2022 **Revised:** September 15, 2022
Accepted: September 27, 2022 **Published online:** October 17, 2022
Co-corresponding authors: Haeryoung Kim, MD, PhD, Department of Pathology, Seoul National University Hospital, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea.
E-mail: haeryoung.kim@snu.ac.kr and
Dong Jin Joo, MD, PhD, FACS, Department of Surgery, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
E-mail: djjoo@yuhs.ac

•The authors have no potential conflicts of interest to disclose.

inhibitors (CNI), which have been shown to be associated with an increased risk of HCC recurrence. Despite the known antitumor effects of mTOR inhibitors, there is currently insufficient evidence supporting their application for LT in HCC.[7,8]

To date, the molecular mechanism underlying the antitumor effects of rapamycin remains unclear. Moreover, it is possible that the antitumor effects of rapamycin noted in previous studies may have been caused by the removal or reduction of CNIs.[9] Additionally, the majority of previous studies on the antitumor effects of rapamycin used human HCC xenograft models and a high dose of rapamycin in the chemotherapeutic range of 3–10 mg/kg.[10,11] However, in clinical settings, after LT, rapamycin is widely used in the dose range of low-dose immunosuppressive drugs rather than in the dose range of chemotherapy drugs. Therefore, to reflect the practical use of rapamycin after LT, low-dose rapamycin treatment was applied in our study design to investigate whether low-dose rapamycin is effective in preventing HCC growth and in treating HCC after tumor development in a transgenic HCC mouse model. Additionally, we aimed to evaluate the mechanisms of the antitumor effects of rapamycin.

MATERIALS AND METHODS

Mice and gene transfection

Seven-week-old male C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained according to national and institutional ethical guidelines. The in vivo experimental research proposal was approved by the Committee on Animal Investigation of Yonsei University (IACUC 2016-0066), and in vivo experiments were performed in accordance with the Laboratory Animals Manual and the Laboratory Animal Care and Use Committee, edited by the National Research Council of the National Animal Society.

Non-germline transgenic mouse models were produced using hydrodynamics-based transfection to co-express HrasG12V and a short-hairpin RNA down-regulating p53 (shp53) in combination in the liver. Hydrodynamic injection was performed as reported previously.[12] The plasmids pT2/HrasG12V and pT2/shp53 were prepared using endotoxin-free Maxi Kits (Qiagen, Hilden, Germany). pT2/HrasG12V (oncogene-expressing transposon plasmid, 6.6 kb) was mixed with pT2/C-Luc//PGK-SB13 (transposase-encoding vector, 9.8 kb) at a molar ratio of 2:1 (25 μg and 18.7 μg, respectively). The plasmid pT2/HrasG12V was used as the molar standard for transposons. For the generation of HrasG12V and shp53 transgenic mice, transposons for each transgene were mixed together (12.5 μg pT2/HrasG12V+16 μg pT2/shp53). After mixing transposons with the transposase-encoding plasmids, DNA was suspended in 2 mL of lactated Ringer's solution before injection into the lateral tail veins of male 6–7-week-old C57BL/6 mice (0.1 mL/g body weight) in less than 7 sec.[13]

The mice were randomly assigned to five experimental groups (Table 1): negative control, positive control, rapamycin only, tacrolimus only, and tacrolimus plus rapamycin. The negative control was injected with empty vector into the lateral tail vein. The positive control was injected with HrasG12V and shp53 using hydrodynamics-based transfection. However, neither the negative or positive controls were administered tacrolimus or rapamycin. All control mice received an equal volume of carrier solution by gavage. The mice in the treatment groups were further divided into two groups according to the time to commencement of immunosuppressant treatment (Fig. 1). In the de novo treatment group, rapamycin and/or tacrolimus were administered 1 day after transfection. In the post-tumor development group, rapamycin and/or tacrolimus were given 2 weeks after transfection. The dosages of rapamycin and tacrolimus were based on previous studies in murine models. These studies reported that rapamycin was given orally at a dose of 1.0–3.0 mg/kg/day after solid organ transplantation in mice.[11,14] For tacrolimus, 1.0–3.0 mg/kg/day produced blood trough levels in the human therapeutic range for immunosuppression (5–12 ng/mL).[14,15] Thus, 1.5 mg/kg was chosen as the dose for both rapamycin and tacrolimus, which were administered orally, either singly or in combination, once daily by gavage. The mice were sacrificed 4 weeks after transfection, and body weight and liver weight were examined. All livers, lungs, and spleens were fixed in buffered formalin, and sections were stained with hematoxylin and eosin.

Real-time polymerase chain reaction

To detect expression of alpha-fetoprotein (AFP), total RNA was extracted from tumor tissues using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) and subjected to reverse transcription to synthesize cDNA (AccuPower RT premix kit, Invitrogen Co.). The cDNA was quantified by real-time polymerase chain reaction (PCR) using specific primers of GAPDH; Forward 5'-ACCACAGTCCATGCCATCAC-3', Reverse 5'-TCCACCACCCTGTTGCTGTA-3' and AFP; Forward 5'-AAACCTCCAGGCAACAACCA-3', Reverse 5'-ACTCCAGCGAGTTTCCTTGG-3'. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the ABI PRISM 7500 Current Real-Time PCR machine (Applied Biosystems) according to the manufacturer's protocol. The gene expression levels of AFP were expressed as fold changes

Table 1. Numbers of Mice in Experimental Groups according to the Type of Treatment

	De novo treatment	Post-tumor development treatment
Tacrolimus only	7	5
Rapamycin only	5	5
Tacrolimus+rapamycin	5	5
Negative control	6	
Positive control	6	

https://doi.org/10.3349/ymj.2022.0247

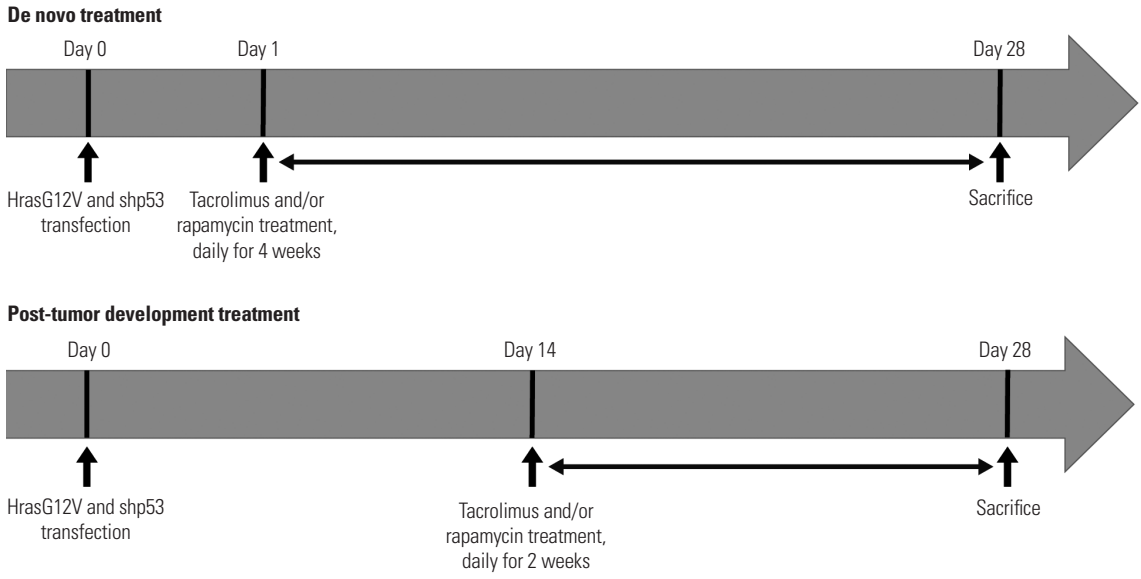

Fig. 1. Schematic representation of experimental design.

relative to GAPDH.

Flow cytometry

To examine the effects of the immunosuppressant treatments on immune cells, splenocytes were collected from mice and subsequently incubated with the appropriately diluted antibodies for 40 min at 4°C. Activated $CD4^+$ effector T cells (Teffs) were stained with APC-Cy7-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD44 antibodies, whereas $CD4^+$ regulatory T cells (Tregs) were fixed/permeabilized after staining with CD4 antibody for intracellular PerCP-Cy5.5-conjugated anti-mouse forkhead box P3 (FOXP3) staining. Flow cytometry was performed using a fluorescence-activated cell sorting (FACS) Verse I or FACS Verse II flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software, v10.0.7 (Tree Star, Inc., San Carlos, CA, USA). All experimental groups were compared to the negative control group. FITC-conjugated anti-mouse CD44 and PerCP-Cy5.5-conjugated anti-mouse FOXP3 antibodies and the Fixation/Permeabilization kit were purchased from eBioscience (San Diego, CA, USA). APC-Cy7-conjugated anti-mouse CD4 antibodies were purchased from BioLegend (San Diego, CA, USA).

Immunohistochemical staining

To measure the expression levels of phosphorylated-mTOR (p mTOR) and its downstream signaling components, eukaryotic translation initiation factor 4E binding protein 1 (4E BP1) and the p70 ribosomal protein S6 kinase 1 (S6K1), in HCC, immunohistochemical staining for p-mTOR, 4E-BP1, and S6K1 was performed on 4 μm-thick tissue sections. In addition, in order to confirm that the tumors were HCC, the tissue sections were stained for AFP, glypican-3, and cytokeratin 19 (CK19). The Ventana BenchMark GX automated platform (Ventana Medical Systems, Tucson, AZ, USA) was used for the immunohistochemical staining. The details of each antibody are summarized in Supplementary Table 1 (only online). Immunostained sections were assessed by an experienced pathologist. The presence of cytoplasmic expression in >5% of tumor cells was regarded as positive for p-mTOR, 4E-BP1, and S6K1 expression. The staining intensity (intensity score 0: no staining, 1: weak, and 2: strong) and the percentage of positive cells (proportion score, 0%–100%) were also assessed.

Statistical analysis

Data are expressed as means±standard deviations and were analyzed using SPSS software, ver. 18.0 (SPSS Inc., Chicago, IL, USA). The significance of intergroup differences was determined using Student's t-test or paired t-test, considering p values<0.05 as statistically significant.

RESULTS

Characteristics of liver tumors in the de novo treatment group

Excluding the rapamycin only group, all mice developed liver tumors after 4 weeks of treatment (Fig. 2). The tumors rapidly grew in the positive control and tacrolimus only groups, resulting in the death of one and three mice, respectively, by 3 weeks. Visual inspection of the livers clearly revealed that there were markedly more tumors in the positive control group than in the rapamycin only group. In both the positive control and tacrolimus only groups, multiple small nodules were evident throughout the liver parenchyma. In contrast, the rapamycin only group displayed significant reductions in the growth of liver tumors and exhibited nearly normal liver. Multiple, sharply demarcated nodules were present in only one mouse (1/5, 20%) of the rapamycin only group. However, the tacrolimus plus

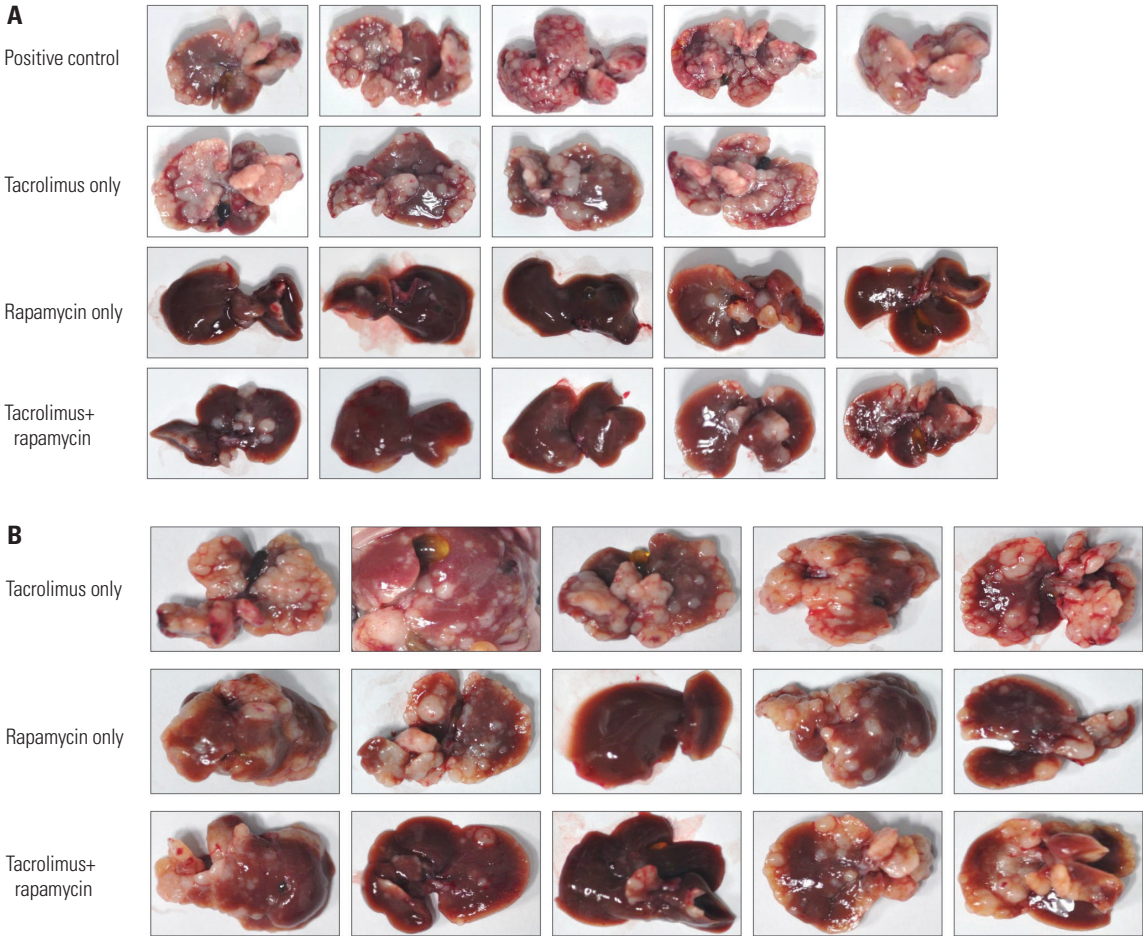

Fig. 2. Gross characteristics of liver tumor development in the de novo treatment group (A) and in the post-tumor development treatment group (B).

rapamycin group developed multiple small nodules throughout the liver parenchyma in three mice (3/5, 60%) and sharply demarcated multiple nodules in two mice. Thus, the evaluation of the gross characteristics revealed that the tacrolimus plus rapamycin group developed significantly more tumors than the rapamycin only group.

Characteristics of liver tumors in the post-tumor development treatment group

In the post-tumor development treatment group, all mice developed liver tumors after 4 weeks of transfection (Fig. 2) with no deaths. Visual inspection of the livers revealed slightly less tumor growth in the rapamycin only group than in the positive control group. Multiple small nodules developed throughout the liver parenchyma in four mice (4/5, 80%) of the rapamycin only group. These findings were significantly different to those of the rapamycin only group in the de novo treatment arm. The tacrolimus plus rapamycin group also developed multiple small nodules throughout the liver parenchyma in three mice (3/5, 60%). Thus, in terms of tumor suppression, the intergroup differences were much less significant in the post-treatment group than in the de novo treatment group. Therefore, subsequent experiments focused on investigating the effect of de novo low-dose rapamycin treatment on the prevention of tumor growth. In the immunohistochemical examination, tumor cells focally expressed AFP and glypican-3, but not CK19, consistent with the characteristics of HCC (Supplementary Fig. 1, only online).

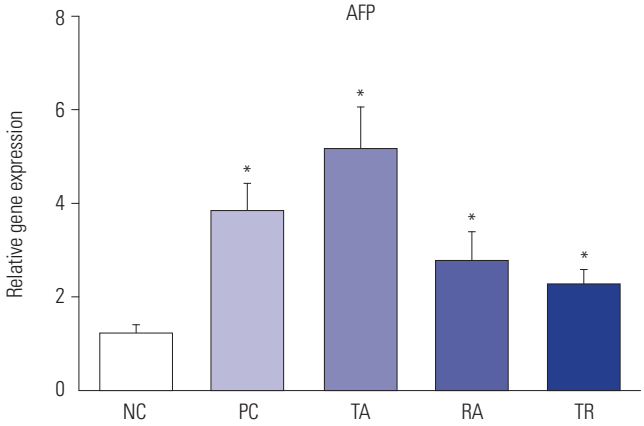

Fig. 3. mRNA expression of AFP in mouse livers following de novo treatment, as measured by real-time polymerase chain reaction. *p<0.05 vs. NC. AFP, alpha-fetoprotein; NC, negative control; PC, positive control; TA, tacrolimus only group; RA, rapamycin only group; TR, tacrolimus plus rapamycin group.

https://doi.org/10.3349/ymj.2022.0247

AFP levels in mouse livers

The AFP mRNA expression levels were significantly higher in all groups than those in the negative control group (Fig. 3). The tacrolimus only group showed significant higher AFP levels than the negative control group (5.2±1.7 vs. 1.3±0.3, p=0.016), followed by the positive control group (3.9±1.5 vs. 1.3±0.3, p=0.017). The rapamycin only and tacrolimus plus rapamycin groups displayed more subtle, but significant higher, AFP levels than the negative control group (2.8±1.3 vs. 1.3±0.3, p=0.047 and 2.3±0.7 vs. 1.3±0.3, p=0.017, respectively).

CD4$^+$ T cell subset changes

FACS analysis of isolated splenocytes revealed that the number of CD4$^+$CD44^{hi} T cells, which represent CD4$^+$ Teffs, was significantly lower in the positive control, tacrolimus only, and tacrolimus plus rapamycin groups, compared to the negative control group (3.1±1.7% vs. 11.2±3.7%, p<0.001; 4.4±2.2% vs. 11.2±3.7%, p=0.007; and 4.4±1.6% vs. 11.2±3.7%, p=0.003, respectively) (Fig. 4). The number of CD4$^+$FOXP3$^+$ T cells, which represent CD4$^+$ Tregs, were also significantly lower in the positive control, tacrolimus only, and tacrolimus plus rapamycin groups than in the negative control group (2.8±1.6% vs. 6.6±

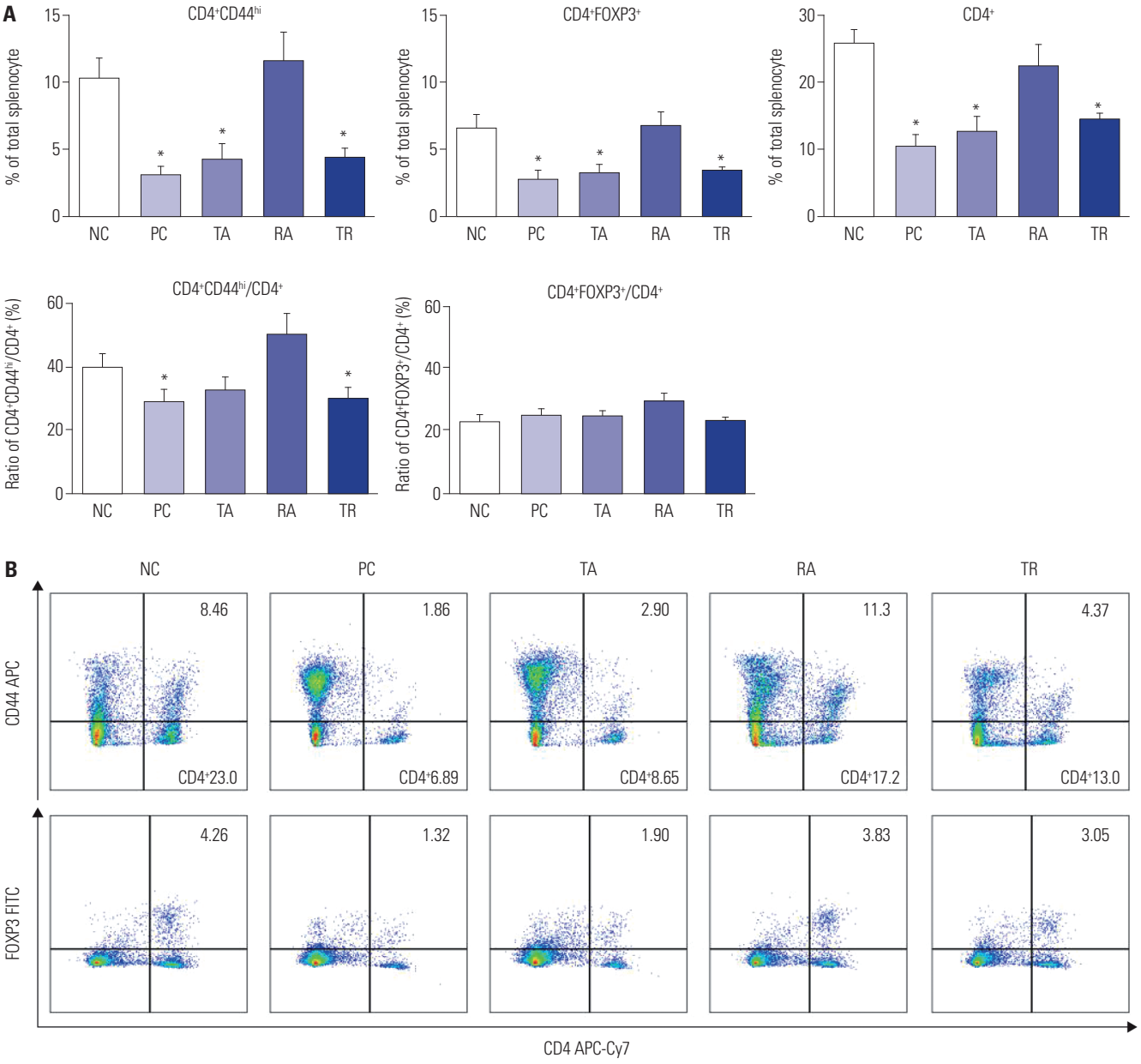

Fig. 4. The CD4$^+$ T cell subset changes in spleens of mice measured by flow cytometry analysis. (A) Dot plots of the negative control, tacrolimus only, rapamycin only, and tacrolimus plus rapamycin treatment groups analysed by flow cytometry. *p<0.05 vs. NC. (B) The number of CD4$^+$ Teffs and CD4$^+$ Tregs were significantly lower in the positive control, tacrolimus only, and tacrolimus plus rapamycin groups compared with that in the negative control group. NC, negative control; PC, positive control; TA, tacrolimus only group; RA, rapamycin only group; TR, tacrolimus plus rapamycin treatment group; FOXP3, forkhead box P3; APC-cy7, allophycocyanin-cyanine7; FITC, fluorescein isothiocyanate.

2.8%, p=0.008; 3.3±1.2% vs. 6.6±2.8%, p=0.018; and 3.4±0.4% vs. 6.6±2.8%, p=0.033, respectively). The total proportion of $CD4^+$ T cells was slightly lower in the rapamycin only group than in the negative control group (22.5±8.7% vs. 25.9 ± 6.0%, p=0.402). Additionally, the ratios of $CD4^+CD44^{hi}/CD4^+$ T cells and $CD4^+FOXP3^+/CD4^+$ T cells in the spleen were analyzed. The ratio of $CD4^+CD44^{hi}/CD4^+$ T cells was significantly lower in the positive control and tacrolimus plus rapamycin groups than in the negative control group (29.0±9.8% vs. 42.7±7.5%, p=0.011 and 29.9±8.6% vs. 42.7±7.5%, p=0.026, respectively), whereas no significant difference was found for the rapamycin only group (50.4±15.7% vs. 42.7±7.5%, p=0.266). The ratio of $CD4^+FOXP3^+/CD4^+$ T cells was slightly higher in the rapamycin only group than in the negative control group (29.8±4.6% vs. 24.5±5.6%, p=0.065). However, the positive control, tacrolimus only, and tacrolimus plus rapamycin groups displayed no significant differences in the ratio of $CD4^+FOXP3^+/CD4^+$ T cells, compared with the negative control group.

Immunohistochemical staining for downstream targets of mTOR

The immunohistochemical staining results for the de novo treatment group are summarized in Fig. 5. p-mTOR staining was predominantly observed in the cytoplasm, whereas 4E-BP1 and S6K1 were observed in both the nucleus and the cytoplasm. The positive control group demonstrated more frequent strong expression of p-mTOR (100% vs. 60%), 4E-BP1 (100% vs. 80%), and S6K1 (100% vs. 40%) than the rapamycin only group. The tacrolimus only group did not display any differences in the staining intensity, compared to the positive control group. The distributions of staining for p-mTOR, 4E-BP1, and S6K1 are described in Table 2. The rapamycin only group displayed significant lower percentages of positive tumor staining for 4E-BP1 and S6K1 than the positive control group (74.0±13.4% vs. 96.0±8.9%, p<0.019 and 28.0±16.4% vs. 64.0±26.1%, p=0.003, respectively). However, the tacrolimus only group and tacrolimus plus rapamycin group did not demonstrate any dif-

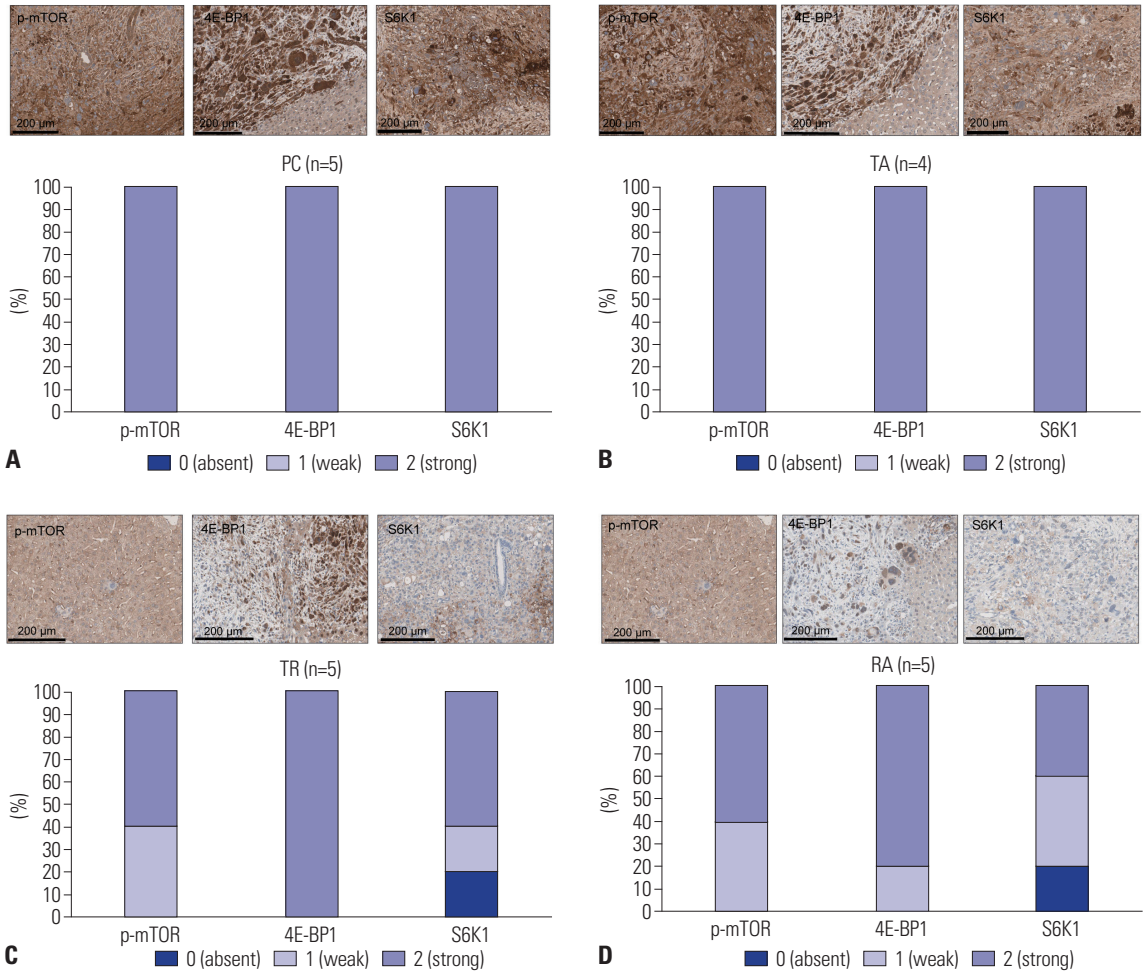

Fig. 5. Immunohistochemical staining results and staining intensity for p-mTOR, 4E-BP1, and S6K1 in the de novo treatment groups. The positive control group (A) demonstrated more frequent strong expression of p-mTOR, 4E-BP1, and S6K1 than the rapamycin only group (D) (100% for all three proteins vs. 60%, 80%, and 40%, respectively). The tacrolimus only group (B) did not display any differences in the staining intensity compared to the positive control group. The tacrolimus plus rapamycin group (C) exhibited more frequent weak expression of p-mTOR and S6K1 than the positive control group. PC, positive control; TA, tacrolimus only group; RA, rapamycin only group; TR, tacrolimus plus rapamycin treatment group; p-mTOR, phosphorylated-mammalian target of rapamycin; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; S6K1, p70 ribosomal protein S6 kinase 1.

https://doi.org/10.3349/ymj.2022.0247

Table 2. Distribution of Staining of p-mTOR, 4E-BP1, and S6K1 in the De Novo Treatment Group

Antibody	Positive control (n=5)	Tacrolimus only (n=4)	*p* value	Tacrolimus+rapamycin (n=5)	*p* value	Rapamycin only (n=5)	*p* value
p-mTOR	74.00±15.17	70.00±23.45	0.803	64.00±13.42	0.302	52.00±25.88	0.149
4E-BP1	96.00±8.94	97.50±4.33	0.761	88.00±17.89	0.406	74.00±13.42	0.019
S6K1	64.00±26.08	52.50±22.17	0.498	46.00±38.47	0.415	28.00±16.43	0.036

p-mTOR, phosphorylated-mammalian target of rapamycin; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; S6K1, p70 ribosomal protein S6 kinase 1.
Values are presented as a mean±standard deviation.

ferences in the distribution of staining, compared to the positive control group.

DISCUSSION

The results of the current study demonstrated successful development of HCC in a transgenic mouse model and revealed an antitumor effect of low-dose rapamycin in de novo treatment. The antitumor effect of low-dose rapamycin was partially observed in combination with tacrolimus, though to a lesser extent than rapamycin only. Moreover, we demonstrated that rapamycin only had a slight antitumor effect after HCC development, indicating that patients are not likely to benefit from rapamycin therapy after HCC development.

To date, CNIs have been the mainstay of maintenance immunosuppression after LT.[16] However, their long-term use is associated with chronic nephrotoxicity, increased risk of infections, de novo malignancies, and recurrence of HCC, which are among the major causes of patient death.[17] The mTOR inhibitor exerts synergistic immunosuppressive efficacy with CNIs, thereby allowing reduction of CNI exposure. Additionally, the antiproliferative effect of mTOR pathway inhibition results in direct antitumor activity. However, there is little evidence supporting the use of mTOR inhibitors for LT in HCC. Moreover, it remains debatable whether rapamycin is effective in preventing HCC growth or treating HCC after recurrence.[6]

A previous international randomized controlled trial (SiLVER-trial) was launched to investigate whether sirolimus-based immunosuppression improves LT outcomes.[8] However, sirolimus did not have a statistically significant effect on recurrence-free survival after 5 years. However, when Schnitzbauer, et al.[7] reported the predictive factors for overall survival in the intention-to-treat cohort of the SiLVER-trial, it was revealed that mTOR-inhibitor treatment for ≥3 months improved outcomes in LT for HCC, especially in patients with AFP-evidence of higher tumor activity. Considering these results, it may be possible that mTOR inhibitors have a preventive effect only in particular cohorts of LT recipients with HCC. Another randomized controlled trial (EVOLVE-1) was conducted among 546 adults with Barcelona Clinic Liver Cancer stage B or C HCC and Child-Pugh A liver function whose disease progressed during or after sorafenib or who were intolerant of sorafenib.[18] However, mTOR inhibitor did not improve overall survival in patients. Consistent with previous reports, the current study demonstrated that rapamycin has an antitumor effect only in the de novo treatment group, giving no significant tumor suppression after tumor development. Thus, low-dose rapamycin may be an ineffective treatment after HCC development.

AFP is the most frequently used serum marker for diagnosis and treatment of HCC,[19] with many previous studies suggesting that it represents a surrogate of tumoral activity and tumor burden.[20] Recently, growing evidence suggests that pretransplant AFP is a useful prognostic marker for selecting LT candidates and assessing risk of recurrence. An increase in the AFP level before LT has been demonstrated to be a predictor of HCC recurrence and to be associated with worse posttransplant survival.[21,22] In the current study, AFP levels were significantly higher in all groups, compared to those in the negative control group, with the tacrolimus only group showing the highest levels. However, the rapamycin only and tacrolimus plus rapamycin groups had slightly higher AFP levels than the negative control group. These results suggest that HCC development was successful in all groups and that HCC was suppressed in the group administered with rapamycin. Thus, low-dose rapamycin may have antitumor activity against HCC.

The dynamics of tumor immunity in terms of T cell function is an important element to consider when selecting novel immunosuppressant options after LT for HCC. The primary mode of action for rapamycin is believed to be through enhancing Treg activity,[23] which is considered to be an important mechanism for immune suppression. However, rapamycin has recently been reported to lower the risk of HCC recurrence in LT recipients, suggesting an impact of mTOR inhibition on antitumor immune responses. Thus, mTOR inhibitors are known to have both immune-stimulating and immune-suppressing effects.[24] In support of this, in murine models of renal cell carcinoma and melanoma, pharmacologic mTOR inhibition has exhibited both immune-stimulating and immune-suppressing effects. Wang, et al.[25] reported that mTOR inhibitor treatment decreased the total number of $CD4^+$ T cells in mice with renal cell carcinoma and melanoma. However, despite an overall decrease in the number of $CD4^+$ lymphocytes, the percent of $CD4^+$ Tregs was higher in groups that received temsirolimus. Consistent with this, the current study demonstrated that rapamycin only slightly lowers the total number of $CD4^+$ T cells, compared with

negative controls. However, the ratio of $CD4^+$ Tregs/$CD4^+$ T cells was higher in the rapamycin only group, compared to that in the negative control group. This result is consistent with previous reports that Tregs are less sensitive to the anti-proliferative effects of mTOR inhibition.[26]

The current study demonstrated a unique result in terms of T cell functions in the dynamics of tumor immunity. The number of Teffs was significantly lower in all study groups except the rapamycin only group, compared to that in the negative control. Previous studies have suggested that $CD4^+$ Teffs are important in the control of tumor progression.[23] Cabrera, et al.[27] reported that patients with HCC have diminished $CD4^+$ T cell function with impaired $CD4^+$ Teff proliferation. Consistent with this, in the current study, HCC markedly developed in all study groups, compared to that in the negative control, with the exception of the rapamycin only group. Moreover, the number of $CD4^+$ Teffs and the ratio of $CD4^+$ Teffs/$CD4^+$ T cells were not significantly different in the rapamycin only group, compared to the negative control group. Thus, the diminished $CD4^+$ Teffs in the positive control, tacrolimus only, and tacrolimus plus rapamycin groups may have resulted from HCC progression. However, further studies are needed to elucidate the impact of rapamycin on $CD4^+$ T cell responses.

The mTOR pathway is a major tumor-initiating pathway in HCC, with upregulation seen in up to 50% of cases.[28] mTOR is present in two different complexes (mTORC1 and mTORC2), and only mTORC1 is sensitive to rapamycin.[29] mTORC1 is involved in the P13K Akt signaling pathway, as p-mTOR phosphorylates the translation initiation factors 4E-BP1 and S6K1, which are necessary for mRNA translation. Alterations in the signaling pathway that leads to mTOR activation results in increased protein synthesis, cell growth, and tumor development.[30] Thus, the mTORC1 signaling pathway controls protein translation and plays an important role in the mechanism of tumor cell growth. Consistent with previous findings, the current study demonstrated that downstream molecules, 4E BP1 and S6K1, were highly expressed in the positive control group than in the rapamycin only group. However, the tacrolimus plus rapamycin group showed no significant difference in 4E BP1 and S6K1 expression, compared to the positive control group. These results suggest that the prevention of HCC growth may require mTOR inhibitor treatment with tacrolimus minimization.

The present study has several limitations. First, it was not possible to measure mean blood trough levels during the immunosuppressant state because of insufficient blood sample size. Second, measurement of tumor volume was not possible due to the diffuse growth pattern of HCC in the transgenic mice. Finally, the current study focused on the direct antitumor effect of low-dose rapamycin. However, in clinical situations, HCC progression and recurrence after LT may be affected by various uncontrollable factors, such as the tumor microenvironment and antitumor immunity of individual patients.

In summary, this study revealed that low-dose rapamycin may be effective to prevent HCC growth, but may be ineffective in reducing tumor growth after HCC development. Mechanistically, low-dose rapamycin treatment may prevent HCC growth through an expansion of $CD4^+$ Tregs and inhibition of downstream target molecules, such as 4E BP1 and S6K1. Additionally, the prevention of HCC growth may require low-dose rapamycin treatment with tacrolimus minimization. Thus, the current study implies that the clinical use of low-dose rapamycin may prevent HCC recurrence after an R0 treatment, such as LT.

ACKNOWLEDGEMENTS

The authors thank Hyejung Lee for her help with immunohistochemical staining. This work was supported by GC Biopharma Corp. Korea.

AUTHOR CONTRIBUTIONS

Conceptualization: Hyung Soon Lee and Myoung Soo Kim. Data curation: Hyung Soon Lee, Joon Ye Kim, and Dong Jin Joo. Formal analysis: Hyung Soon Lee and Joon Ye Kim. Investigation: Hyung Soon Lee and Joon Ye Kim. Methodology: Dong Jin Joo and Haeryoung Kim. Project administration: Dong Jin Joo and Myoung Soo Kim. Resources: Simon Weonsang Ro. Software: Joon Ye Kim. Supervision: Dong Jin Joo and Haeryoung Kim. Validation: Dong Jin Joo and Haeryoung Kim. Visualization: Myoung Soo Kim. Writing—original draft: Hyung Soon Lee and Myoung Soo Kim. Writing—review & editing: Dong Jin Joo and Haeryoung Kim. Approval of final manuscript: all authors.

ORCID iDs

Hyung Soon Lee https://orcid.org/0000-0001-9825-8648
Joon Ye Kim https://orcid.org/0000-0002-1180-2899
Simon Weonsang Ro https://orcid.org/0000-0003-2187-3698
Myoung Soo Kim https://orcid.org/0000-0002-8975-8381
Haeryoung Kim https://orcid.org/0000-0002-4205-9081
Dong Jin Joo https://orcid.org/0000-0001-8405-1531

REFERENCES

1. Mehta N, Bhangui P, Yao FY, Mazzaferro V, Toso C, Akamatsu N, et al. Liver transplantation for hepatocellular carcinoma. Working group report from the ILTS transplant oncology consensus conference. Transplantation 2020;104:1136-42.
2. Lee DD, Sapisochin G, Mehta N, Gorgen A, Musto KR, Hajda H, et al. Surveillance for HCC after liver transplantation: increased monitoring may yield aggressive treatment options and improved postrecurrence survival. Transplantation 2020;104:2105-12.
3. Bodzin AS, Lunsford KE, Markovic D, Harlander-Locke MP, Busuttil RW, Agopian VG. Predicting mortality in patients developing recurrent hepatocellular carcinoma after liver transplantation. Ann Surg 2017;266:118-25.
4. Lu X, Paliogiannis P, Calvisi DF, Chen X. Role of the mammalian target of rapamycin pathway in liver cancer: from molecular genetics to targeted therapies. Hepatology 2021;73 Suppl 1:49-61.

https://doi.org/10.3349/ymj.2022.0247

5. Chew SA, Moscato S, George S, Azimi B, Danti S. Liver cancer: current and future trends using biomaterials. Cancers (Basel) 2019;11:2026.
6. Nashan B. mTOR inhibition and clinical transplantation: liver. Transplantation 2018;102(2S Suppl 1):S19-26.
7. Schnitzbauer AA, Filmann N, Adam R, Bachellier P, Bechstein WO, Becker T, et al. mTOR inhibition is most beneficial after liver transplantation for hepatocellular carcinoma in patients with active tumors. Ann Surg 2020;272:855-62.
8. Geissler EK, Schnitzbauer AA, Zülke C, Lamby PE, Proneth A, Duvoux C, et al. Sirolimus use in liver transplant recipients with hepatocellular carcinoma: a randomized, multicenter, open-label phase 3 trial. Transplantation 2016;100:116-25.
9. De Simone P, Fagiuoli S, Cescon M, De Carlis L, Tisone G, Volpes R, et al. Use of everolimus in liver transplantation: recommendations from a working group. Transplantation 2017;101:239-51.
10. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-35.
11. Selvarani R, Mohammed S, Richardson A. Effect of rapamycin on aging and age-related diseases-past and future. Geroscience 2021;43:1135-58.
12. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther 1999;10:1735-7.
13. Ju HL, Han KH, Lee JD, Ro SW. Transgenic mouse models generated by hydrodynamic transfection for genetic studies of liver cancer and preclinical testing of anti-cancer therapy. Int J Cancer 2016;138:1601-8.
14. Diehl R, Ferrara F, Müller C, Dreyer AY, McLeod DD, Fricke S, et al. Immunosuppression for in vivo research: state-of-the-art protocols and experimental approaches. Cell Mol Immunol 2017;14:146-79.
15. Herbst S, Shah A, Carby M, Chusney G, Kikkeri N, Dorling A, et al. A new and clinically relevant murine model of solid-organ transplant aspergillosis. Dis Model Mech 2013;6:643-51.
16. Kwong AJ, Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, et al. OPTN/SRTR 2019 annual data report: liver. Am J Transplant 2021;21 Suppl 2:208-315.
17. Lee SG, Jeng LB, Saliba F, Singh Soin A, Lee WC, De Simone P, et al. Efficacy and safety of everolimus with reduced tacrolimus in liver transplant recipients: 24-month results from the pooled analysis of 2 randomized controlled trials. Transplantation 2021;105:1564-75.
18. Zhu AX, Kudo M, Assenat E, Cattan S, Kang YK, Lim HY, et al. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. JAMA 2014;312:57-67.
19. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int 2019;39:2214-29.
20. Cillo U, Vitale A, Navaglia F, Basso D, Montin U, Bassanello M, et al. Role of blood AFP mRNA and tumor grade in the preoperative prognostic evaluation of patients with hepatocellular carcinoma. World J Gastroenterol 2005;11:6920-5.
21. Lee JH, Cho Y, Kim HY, Cho EJ, Lee DH, Yu SJ, et al. Serum tumor markers provide refined prognostication in selecting liver transplantation candidate for hepatocellular carcinoma patients beyond the Milan criteria. Ann Surg 2016;263:842-50.
22. Hong G, Suh KS, Suh SW, Yoo T, Kim H, Park MS, et al. Alpha-fetoprotein and (18)F-FDG positron emission tomography predict tumor recurrence better than Milan criteria in living donor liver transplantation. J Hepatol 2016;64:852-9.
23. Li C, Jiang P, Wei S, Xu X, Wang J. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. Mol Cancer 2020;19:116.
24. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. Nat Rev Immunol 2012;12:325-38.
25. Wang Y, Wang XY, Subjeck JR, Shrikant PA, Kim HL. Temsirolimus, an mTOR inhibitor, enhances anti-tumour effects of heat shock protein cancer vaccines. Br J Cancer 2011;104:643-52.
26. Sillito F, Holler A, Stauss HJ. Engineering CD4+ T cells to enhance cancer immunity. Cells 2020;9:1721.
27. Cabrera R, Ararat M, Cao M, Xu Y, Wasserfall C, Atkinson MA, et al. Hepatocellular carcinoma immunopathogenesis: clinical evidence for global T cell defects and an immunomodulatory role for soluble CD25 (sCD25). Dig Dis Sci 2010;55:484-95.
28. Villanueva A, Chiang DY, Newell P, Peix J, Thung S, Alsinet C, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology 2008;135:1972-83.E11.
29. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 2009;10:307-18.
30. Seeliger H, Guba M, Kleespies A, Jauch KW, Bruns CJ. Role of mTOR in solid tumor systems: a therapeutical target against primary tumor growth, metastases, and angiogenesis. Cancer Metastasis Rev 2007;26:611-21.