Marked suppression of tumor growth by FTY720 in a rat liver tumor model: The significance of down-regulation of cell survival Akt pathway

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Abstract. We aim to investigate the anticancer effect of a novel immunomodulator FTY720 on a rat orthotopic liver tumor model. A buffalo rat orthotopic liver tumor model was established by injection of a buffalo hepatoma cell line MH7777 into the right portal vein. FTY720 was administered by intraperitoneal injection starting at 10 days after tumor cell injection at a dosage of 5 mg/kg/day. FTY720 markedly suppressed tumor growth and inhibited tumor progression by selective induction of apoptosis of tumor cells via down-regulation of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3, together with decrease of focal adhesion kinase. Moreover, the proliferation index of tumor cells was significantly reduced to 15.92±5.03% by FTY720 compared with that of 42.92±4.47% in the control group ($p<0.001$). In addition, we confirmed that FTY720 caused no effect on infiltrated lymphocyte in tumor tissue. We conclude that FTY720 is an effective anticancer agent for liver tumor in a rat model without affecting the immune system of the host.

Introduction

Hepatocellular carcinoma (HCC) is the fifth common malignancy worldwide, with 350000 new cases and nearly one million deaths from HCC per year (1). Surgery in terms of hepatic resection or orthotopic liver transplantation may provide potentially curative treatment for HCC patients. However, the high mortality rate is still a problem to confront due to a high recurrence rate following hepatic resection and progression as well as subsequent metastasis of malignant HCC among HCC patients who are on the waiting list for liver transplantation (2-3). Transarterial chemoembolization is widely used as a palliative treatment of adjuvant therapy for unresectable HCC, but a survival benefit might not be always demonstrated especially for patients with tumors of an advanced stage such as those with vascular invasion and metastasis (4). Systemic chemotherapy using interferon, tamoxifen or octreotide has not been confirmed to be an encouraging treatment due to poor response or substantial toxicity (5). It is therefore necessary to develop a novel and effective pharmaceutical therapy for the treatment of HCC. A novel immunomodulator, FTY720, synthesized from a metabolite ISP-1 (myriocin) of the ascomycete fungus *Isaria sinclairii*, was originally developed for prolonging the graft survival rate in assorted animal models of organ transplantation without causing any noticeable side effect (6-8). This drug was subsequently demonstrated to be a noteworthy antitumor agent in the treatment of various cancers including bladder cancer, breast cancer, leukemia and prostate cancer (9-12). Recently, the anticancer ability of FTY720 in HCC cell lines has been demonstrated by inducing tumor cell apoptosis through phosphatidylinositol 3-kinase-mediated Akt dephosphorylation (13). Suppression of liver tumor metastasis was also achieved by FTY720 treatment via down-regulation of the Rac pathway (14). Before FYT720 is tested in clinical trials, it is indispensable to confirm the *in vivo* anticancer effect of FTY720 in an animal liver tumor model with intact immune system.

In the present study, we aimed to study the anticancer property of FTY720 in a rat orthotopic liver tumor model by investigation of the proliferation and progression of liver tumors. We also explored the possible underlying mechanism related to cell survival Akt pathway and FAK expression, as well as the effect of FTY720 on tumor infiltrated lymphocytes.

Materials and methods

Cell line and animals. Buffalo rat hepatoma cell line MH7777 was purchased from the American Type Culture Collection (number CRL1601, ATCC, Manassas, VA, USA). The cell

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Abbreviations: FAK, focal adhesion kinase; HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen; PI, proliferation index; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling

Key words: hepatocellular carcinoma, FTY720, apoptosis, AKT signaling pathway, focal adhesion kinase

line was cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 50 U/ml penicillin G and 50 μg/ml streptomycin (Gibco, BRL) at 37˚C with 5% CO_2 supply. Buffalo rats were housed in a standard animal laboratory with free activity and access to water and chow. They were kept under constant environment conditions with a 12-h light and dark cycle. All operations were performed under clean conditions.

Rat liver tumor model and treatment regimen. Under pentobarbital anesthesia (intraperitoneal injection, 40 mg/kg) and after midline laparotomy, $1.5x10^6$ saline-suspended buffalo hepatoma cell line MH7777 was injected into the right portal vein of each buffalo rat. FTY720 (molecular weight 343.94 Da) was kindly provided by Novartis Pharmaceuticals Ltd (Basel, Switzerland). Rats received FTY720 (1 mg/ml) by intraperitoneal injection starting at 10 days after MH7777 cell line injection at a dose of 5 mg/kg/day for 20, 25 and 30 days. The rats in the control groups were given the same amount of normal saline.

MTT assay. One hundred microliters of MH7777 cell line suspension was seeded in each well of a 96-well plate and incubated at 37˚C in a humidified 5% CO_2 atmosphere. FTY720 was added to each well at various concentrations (0, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μM). After 24-h incubation, the medium was replaced with 100 μl of 5 mg/ml of MTT solution (Amersham, Little Chalfont, UK) and incubated for another 4 h at 37˚C until formation of crystals. The crystals were dissolved by adding 100 μl of dimethylsulfoxide. The absorbance was measured at 570 nm. Each result was obtained at least from four individual experiments.

Western blotting. Whole protein from the cells and tissue samples were extracted using cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with 1 mM of phenylmethyl sulfonyl fluoride and protein concentration was quantified by Bradford method (Bio-Rad, Hercules, CA, USA). Fifty micrograms of proteins were size separated in 12% SDS-PAGE and transferred to nitrocellulose membrane (Amersham). The membrane was blotted with 10% non-fat milk for 1 h at room temperature and probed with primary antibody in proper dilution overnight at 4˚C. Horseradish peroxidase-conjugated secondary antibody (Amersham) was incorporated with primary antibodies for 1 h at room temperature. The immunoreactive signals were visualized by the ECL Plus Western blot system (Amersham). Antibodies used in the experiment included: cleaved blot system caspase-3 (Cell Signaling Technology, Beverly, MA, USA), Phospho-Akt^{ser473} (Cell Signaling Technology) and Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemical staining. Each 4 μM-thick paraffin section was dewaxed in xylene and rinsed in grade alcohol and finally rehydrated in water. The section was placed in citric buffer (pH 6.0) and treated in a microwave oven with high power for 3 min and subsequent low power for 10 min. After peroxidase block for 20 min and 10% goat serum for 30 min, primary antibody CD3-ζ (1:100) (Santa Cruz Biotechnology), cleaved caspase-3 (Cell Signaling Technology),

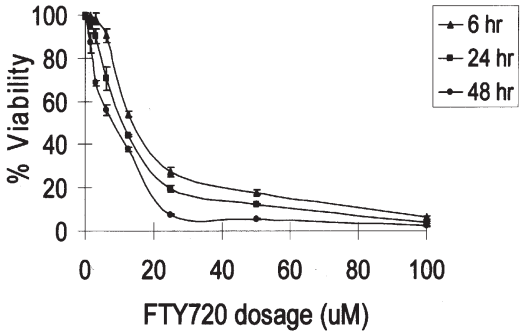

Figure 1. Effect of FTY720 on cell viability in buffalo rat hepatoma cell line MH7777, demonstrated by MTT assay. MH7777 was subjected to FTY720 treatment from 1.56 μM to 100 μM after 6-, 24- and 48-h incubation. Each result represents the mean of at least four individual experiments.

Phospho-Akt^{ser473} (Cell Signaling Technology), or proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology) was applied on the sections and incubated at 4˚C overnight. Secondary antibody from the Envision system (Dako-Cytomation Denmark) was applied and incubated for 30 min at room temperature. The signal was developed by DAB substrate solution. The slide was finally counter-stained by hematoxylin solution.

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay. Each 4 μM-thick paraffin section was detected for apoptotic cells by POD cell death detection kit according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche Biochemicals, Mannheim, Germany).

Quantification of immunohistochemical staining and statistical analysis. TUNEL, PCNA and CD3ζ stained cells were counted in 10 randomly selected fields for each tumor at a x400 magnification. The percentage of positive cells was determined as number of positive cells/total number of cells x100. Continuous variables were expressed as mean and standard deviation. Mann-Whitney U test and χ^2 test were used for statistical comparison. Significance was defined as $P<0.05$. Calculation was made with the help of SPSS computer software (SPSS Inc, Chicago, IL, USA).

Results

In vitro inhibition of tumor growth by FTY720. Results from MTT assay demonstrated that FTY720 triggered a marked loss of cell viability in the MH7777 cell line in time- and dose-dependent manners (Fig. 1). The drug sensitivity for the MH7777 cell line (50% inhibition of growth rate) was determined to be 8, 11 and 14 μM for 6, 24 and 48 h, respectively. Most of the tumor cells (80%) were killed when the concentration of FTY720 was increased to 20 μM after 24-h incubation.

In vivo suppression of liver tumor growth and induction of tumor cell apoptosis by FTY720. Visible tumor nodules were formed in right liver lobes 10 days after tumor cell injection. In the control group, the tumors became bulky and massive

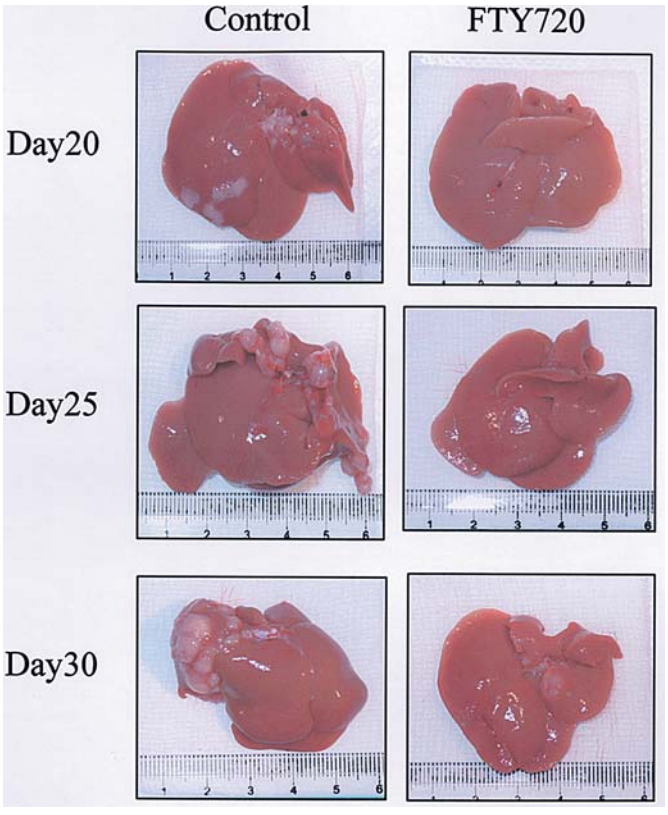

Figure 2. *In situ* appearance of liver tumors in control and FTY720-treated rats.

Table I. Summary of *in vivo* liver tumor model in buffalo rats.

		Control	FTY720	P-value
Day 20	Tumor formed	4/5 (80%)	0/7 (0%)	0.001
	Larger tumor (>3 mm)	0/5 (0%)	0/7 (0%)	
	Venous invasion	0/5 (0%)	0/7 (0%)	
Day 25	Tumor formed	9/9 (100%)	6/6 (100%)	
	Larger tumor (>3 mm)	8/9 (88.9%)	1/6 (16/7%)	0.001
	Venous invasion	8/9 (88.9%)	0/6 (0%)	
Day 30	Tumor formed	8/8 (100%)	4/6 (66.7%)	
	Larger tumor (>3 mm)	7/8 (87.5%)	0/6 (0%)	0.005
	Venous invasion	7/8 (87.5%)	0/6 (0%)	

after 20 days, and almost the whole right robe was occupied by the tumors after 30 days. In contrast, when the buffalo rats were treated with FTY720 at a dosage of 5 mg/kg/day, tumor growth was significantly suppressed in terms of tumor number and size (Fig. 2). The data of liver growth and venous invasion in the two groups are summarized in Table I. Treatment of FTY720 significantly retarded tumor development, which was indicated by the absence of visible liver tumor at day 20 (p=0.001). Furthermore, in the FTY720 treatment group, only one out of six rats developed large tumors (size >3 mm) at day 25 (p=0.001) and no large tumor was observed at day 30 (p=0.005), while 89% and 88% of the rats in the control groups developed large tumors at day 25 and 30, respectively. By

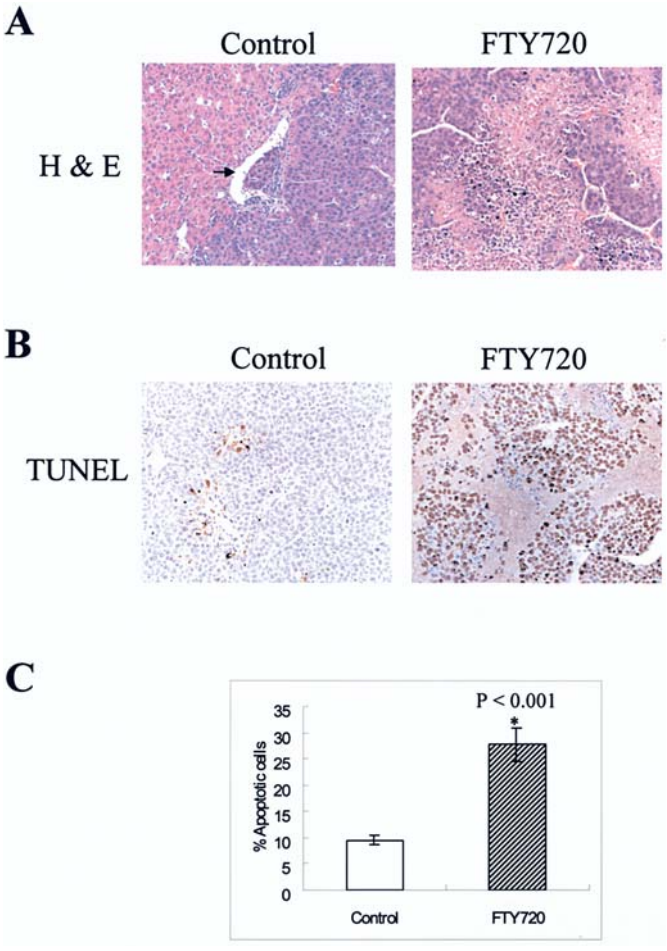

Figure 3. (A) Hematoxylin and eosin (H&E) and (B) TUNEL staining of rat liver tumor tissues in control and FTY720 treatment group at day 30. (C) Quantification and comparison of apoptotic cells in rat liver tumors of control and FTY720 treatment group at day 30.

histological observation (Fig. 3A), intravenous infiltration of tumor emboli was frequently found in the control group counting for 89% at day 25 and 88% at day 30. No intravenous tumor infiltration was found in the FTY720 treatment group.

Morphological examination. Histological finding conducted by hematoxylin and eosin staining, showed that FTY720 treatment induced massive tumor apoptosis and necrosis, but the tumor structure remained intact in the control group (Fig. 3A). TUNEL assay indicated that the number of apoptotic tumor cells was significantly increased after FTY720 treatment (27.75±3.2%) compared to the control group (9.5±0.93%, p<0.001, Fig. 3B and C). The numbers of apoptotic cells in non-tumor liver tissues were similar between the two groups, in which only a few apoptotic cells were detected.

In vitro and in vivo down-regulation of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3 by FTY720. In vitro FTY720 treatment on buffalo rat hepatoma cell line MH7777 resulted in a decrease of Akt phosphorylation and increase of cleaved caspase-3 formation at a low dosage of 11 uM in a time-dependent manner (Fig. 4A). Consistent with the *in vitro* data, FTY720 also reduced phospho-Akt^{ser473} expression and increased cleaved caspase-3 production in tumor tissue

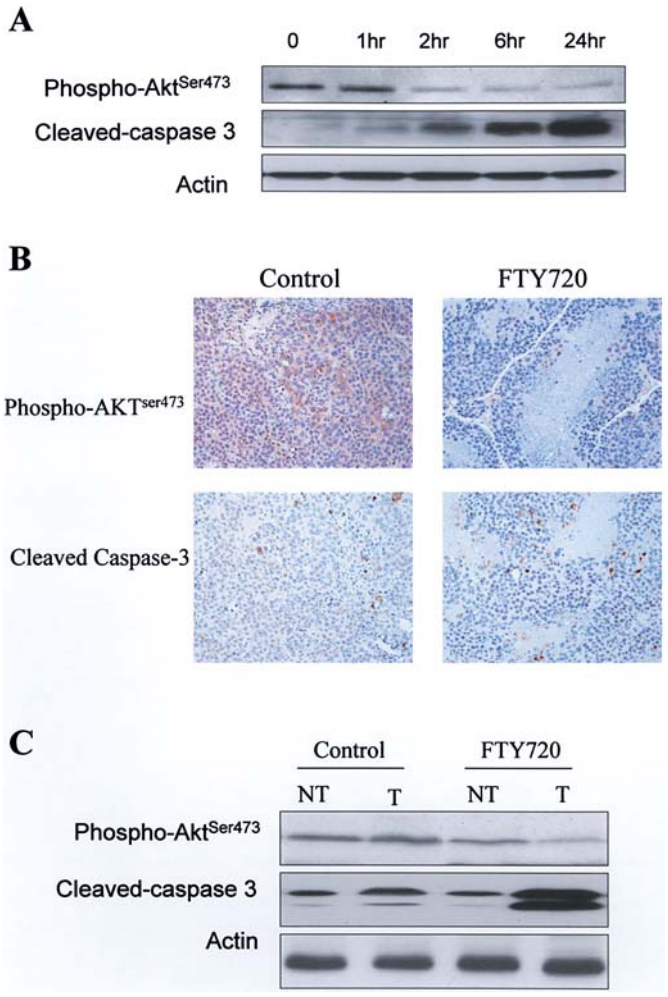

Figure 4. (A) *In vitro* effect of FTY720 on down-regulation of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3 in MH7777. MH7777 was treated with FTY720 at 11 μm (IC50 for 24 h) for 1, 2, 6 and 24 h. Phospho-Akt^{ser473} and cleaved caspase-3 were analyzed by Western blot. Actin expression level was used as normalization control. (B) Immunohistochemical staining of phospho-Akt^{ser473} and cleaved caspase-3 in rat liver tumor tissues of control and FTY720 treatment group at day 30. (C) Western blot analysis of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3 in rat liver tumor tissues of control and FTY720 treatment group at day 30. NT, non-tumor tissue; T, tumor tissue.

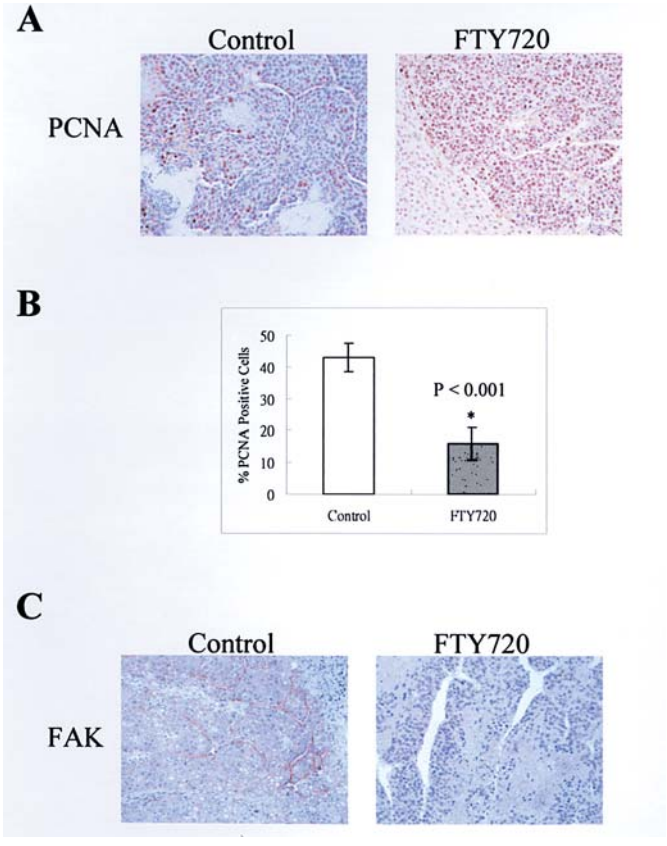

Figure 5. Immunohistochemical staining of (A) proliferating cell nuclear antigen (PCNA) and (C) focal adhesion kinase (FAK) in rat liver tumor tissues of control and FTY720 treatment group at day 30. (B) Quantification and comparison of PCNA positive cells in rat liver tumors of control and FTY720 treatment group at day 30.

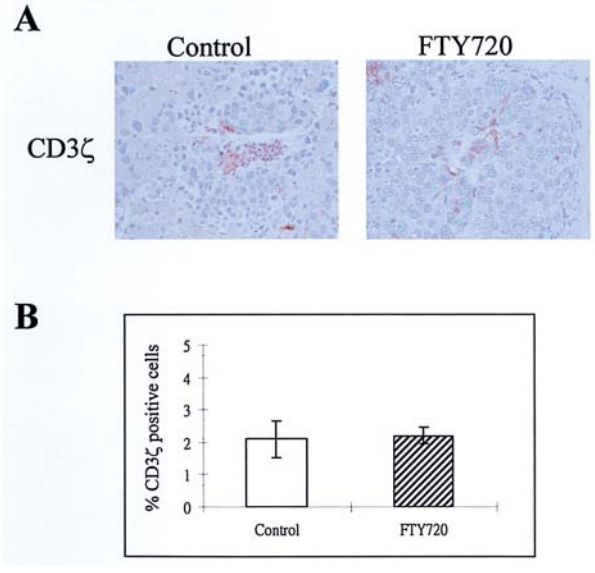

Figure 6. (A) Immunohistochemical staining of CD3ζ in rat liver tumor tissues of control and FTY720 treatment group at day 30. (B) Quantification of CD3ζ positive cells in rat liver tumor of control and FTY720 treatment group at day 30.

comparing with the control group by immunohistochemical staining (Fig. 4B) and Western blotting (Fig. 4C). After FTY720 treatment for 20 days, down-regulation of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3 caused massive tumor cell deaths (Fig. 4B).

In vivo suppression of tumor proliferation and progression by FTY720. FTY720 significantly suppressed rat liver tumor proliferation reflected by less expression of proliferating cell nuclear antigen (PCNA). Results from immunohistochemical staining showed that the number of PCNA positive tumor cells was markedly reduced in the FTY720 treatment group comparing with that in control group (Fig. 5A). The proliferation indexes (PI) calculating as the percentage of PCNA positive tumor cells were determined to be 42.92±4.47% in the control group and 15.92±5.03% in the FTY720 treatment group ($p<0.001$, Fig. 5B).

The protein level of focal adhesion kinase (FAK) in tumor tissues illustrated by immunohistochemical staining was

markedly suppressed by FTY720 treatment compared to the control group in which all tumor tissues were detected with abundant expression of FAK (Fig. 5C).

No side effect on rat immunity by FTY720. Results from the immunohistochemical staining of CD3ζ showed that the number of infiltrated lymphocytes in tumor tissues in the FTY720 treatment group was similar to that in the control group. Only a scattered number of lymphocytes were detected in tumor tissues from both groups (Fig. 6A and B).

Discussion

The high incidence and mortality rate of HCC have urged us to develop effective treatments to eliminate the malignancy of HCC and to improve the survival rate of HCC patients. High recurrence rates after surgical resection and shortage of liver graft donation drive physicians to exploit alternative treatments besides surgical management. Owing to less efficiency of current chemotherapeutic drugs on HCC treatment due to the increase in mutation and possibility of resistance of HCC, there is an urgent need to develop a novel anti-HCC drug to relieve patients who are suffering from HCC. FTY720 is an immunomodulator, which was currently used as a potential anticancer agent for several cancers, for example, bladder cancer, breast cancer, leukemia and prostate cancer. The advantage of the anticancer function of FTY720 was mainly determined by its ability to selectively induce apoptosis of cancer cells but not normal somatic cells (9-12). Our previous data indicated that FTY720 triggers *in vitro* cell death of human hepatoma cell lines and *in vivo* suppresses HCC metastasis in a nude mouse model (13,14). In order to extend the use of FTY720 to clinical application on HCC treatment, it is necessary to test the anticancer effect of FTY720 on HCC using an animal model with intact immune system.

FTY720 with low dosage can induce *in vitro* cell death of different types of cancer cells. The higher sensitivity of cancer cells to FTY720 than normal cells makes FTY720 a reliable anticancer agent (9-12). In this study, we demonstrated that FTY720 exhibited a significant inhibition of cell viability of rat hepatoma cell line MH7777 in time- and dose-dependent manners. The sensitivity of the MH7777 cell line to FTY720 was similar to that in other cancer cell lines.

Akt signaling pathways play important roles in regulating cell survival and protecting tumor cells from apoptosis. FTY720 down-regulates the activation of Akt signaling pathway and subsequently induces cell apoptosis in glioma and leukemia cells (11,15). Our group previously demonstrated the same effect of FTY720 in human hepatoma cell lines (13). The present *in vitro* and *in vivo* studies demonstrated that down-regulation of phospho-Akt^{ser473} and up-regulation of cleaved caspase-3 in tumor cells were caused by FTY720 treatment. The effect of FTY720 on down-regulation of phospho-Akt^{ser473} and activation of cleaved caspase-3 is specific in liver tumor cells but not in normal hepatocytes (16). Indeed, abundant apoptosis and death in liver tumor cells were found in the FTY720 treatment group (Fig. 3B), which may be due to specific down-regulation of Akt signaling pathway in liver tumor cells.

Uncontrolled proliferation of tumor cells is one of the noticeable characteristics in HCC patients (17-19). Suppression of tumor cell proliferation is thus regarded as a critical concern for measuring the efficacy of candidate anticancer agents on suppression of HCC growth. In the present rat orthotopic liver tumor model, FTY720 significantly suppressed liver tumor proliferation to about 16% PI value compared with 43% PI value in the control group, indicating that FTY720 is an effective anti-proliferation agent in liver tumor cells.

FAK was regarded as an important progression marker in many cancers (20-22). FAK plays a crucial role in regulating the early event of integrin-mediated adhesion of tumor cells that facilitates tumor invasion and metastasis (23). Over-expression of FAK in HCC was found to be associated with tumor size and portal venous invasion as well as poorer survival (24,25). Our result showed that the expression of FAK in tumor tissue was significantly suppressed by FTY720 administration (Fig. 5C). Indeed, no venous invasion was detected in buffalo rat liver tissue after FTY720 treatment (Table I). Based on this finding, FTY720 exerts a significant effect on suppressing tumor progression partly due to suppression of FAK expression.

Impairment of immune response and down-regulation of CD3ζ were reported in HCC patients (26). One feature of FTY720 is that it selectively induces apoptosis of infiltrated lymphocytes (27,28). A study on breast cancer found that FTY720 might reduce the number of peripheral lymphocytes in mice (9). Therefore, the major concern of the application of FTY720 on liver cancer is whether it is able to kill tumor-infiltrated lymphocytes. In this study, the result from immunohistochemical staining of CD3ζ showed that the number of infiltrated lymphocytes in tumor tissues was similar between the two groups, suggesting that FTY720 probably does not induce apoptosis of tumor-infiltrated lymphocytes.

In conclusion, we confirmed that FTY720 is an effective anticancer agent for treatment of rat liver tumors by suppressing tumor growth and progression via selectively inducing tumor cell apoptosis and down-regulation of cell survival Akt pathway without affecting the immune system of the host. These may provide important evidence for future clinical application on human HCC patients.

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References

1. Schafer DF and Sorrell MF: Hepatocellular carcinoma. Lancet 353: 1253-1257, 1999.
2. Poon RT, Fan ST, Ng IO and Wong J: Significance of resection margin in hepatectomy for hepatocellular carcinoma: A critical reappraisal. Ann Surg 231: 544-551, 2000.
3. Lo CM and Fan ST: Liver transplantation for hepatocellular carcinoma. Br J Surg 91: 131-133, 2004.
4. Bruix J, Llovet JM, Castells A, *et al*: Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. Hepatology 27: 1578-1583, 1998.
5. Ahn J and Flamm SL: Hepatocellular carcinoma. Dis Mon 50: 556-573, 2004.

6. Muramatsu K, Doi K, Shigetomi M, Kido K and Kawai S: A new immunosuppressant, FTY720, prolongs limb allograft survival in rats. Ann Plast Surg 40: 160-165, 1998.
7. Suzuki S, Enosawa S, Kakefuda T, *et al*: A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. Transplantation 61: 200-205, 1996.
8. Yamasaki T, Inoue K, Hayashi H, *et al*: Effect of a new immunosuppressive agent, FTY720, on survival of islet allografts. Cell Transplant 7: 403-406, 1998.
9. Azuma H, Takahara S, Ichimaru N, *et al*: Marked prevention of tumor growth and metastasis by a novel immunosuppressive agent, FTY720, in mouse breast cancer models. Cancer Res 62: 1410-1419, 2002.
10. Azuma H, Takahara S, Horie S, Muto S, Otsuki Y and Katsuoka Y: Induction of apoptosis in human bladder cancer cells *in vitro* and *in vivo* caused by FTY720 treatment. J Urol 169: 2372-2377, 2003.
11. Matsuoka Y, Nagahara Y, Ikekita M and Shinomiya T: A novel immunosuppressive agent FTY720 induced Akt dephosphorylation in leukemia cells. Br J Pharmacol 138: 1303-1312, 2003.
12. Permpongkosol S, Wang JD, Takahara S, *et al*: Anticarcinogenic effect of FTY720 in human prostate carcinoma DU145 cells: modulation of mitogenic signaling, FAK, cell-cycle entry and apoptosis. Int J Cancer 98: 167-172, 2002.
13. Lee TK, Man K, Ho JW, *et al*: FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation. Carcinogenesis 25: 2397-2405, 2004.
14. Lee TK, Man K, Ho JW, *et al*: Significance of the Rac signaling pathway in HCC cell motility: implications for a new therapeutic target. Carcinogenesis 26: 681-687, 2005.
15. Sonoda Y, Yamamoto D, Sakurai S, Hasegawa M, Aizu-Yokota E, Momoi T and Kasahara T: FTY720, a novel immunosuppressive agent, induces apoptosis in human glioma cells. Biochem Biophys Res Commun 281: 282-288, 2001.
16. Man K, Ng KT, Lee TK, *et al*: FTY720 attenuates hepatic ischemia-reperfusion injury in normal and cirrhotic livers. Am J Transplant 5: 40-49, 2005.
17. Jing Z, Nan KJ and Hu ML: Cell proliferation, apoptosis and the related regulators p27, p53 expression in hepatocellular carcinoma. World J Gastroenterol 11: 1910-1916, 2005.
18. Paiva C, Oshima CT, Lanzoni VP and Forones NM: Apoptosis, PCNA and p53 in hepatocellular carcinoma. Hepatogastroenterology 49: 1058-1061, 2002.
19. Pizem J, Marolt VF, Luzar B and Cor A: Proliferative and apoptotic activity in hepatocellular carcinoma and surrounding non-neoplastic liver tissue. Pflugers Arch 442: R174-R176, 2001.
20. Owens LV, Xu L, Dent GA, Yang X, Sturge GC, Craven RJ and Cance WG: Focal adhesion kinase as a marker of invasive potential in differentiated human thyroid cancer. Ann Surg Oncol 3: 100-105, 1996.
21. Owens LV, Xu L, Craven RJ, *et al*: Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res 55: 2752-2755, 1995.
22. Cance WG, Harris JE, Iacocca MV, *et al*: Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. Clin Cancer Res 6: 2417-2423, 2000.
23. von Sengbusch A, Gassmann P, Fisch KM, Enns A, Nicolson GL and Haier J: Focal adhesion kinase regulates metastatic adhesion of carcinoma cells within liver sinusoids. Am J Pathol 166: 585-596, 2005.
24. Itoh S, Maeda T, Shimada M, Aishima S, Shirabe K, Tanaka S and Maehara Y: Role of expression of focal adhension kinase in progression of hepatocellular carcinoma. Clin Cancer Res 10: 2812-2817, 2004.
25. Fujii T, Koshikawa K, Nomoto S, *et al*: Focal adhesion kinase is overexpressed in hepatocellula carcinoma and can be served as an independent prognostic factor. J Hepatol 41: 104-111, 2004.
26. Maki A, Matsuda M, Asakawa M, Kono H, Fujii H and Matsumoto Y: Decreased expression of CD28 coincides with the down-modulation of CD3ζ and augmentation of caspase-3 activity in T cells from hepatocellular carcinoma-bearing patients and hepatitis C virus-infected patients. J Gastroenterol Hepatol 19: 1348-1356, 2004.
27. Enosawa S, Suzuki S, Kakefuda T, Li XK and Amemiya H: Induction of selective cell death targeting on mature T lymphocytes in rats by a novel immunosuppressant, FTY720. Immunopharmacology 34: 171-179, 1996.
28. Nagahara Y, Enosawa S, Ikekita M, Suzuki S and Shinomiya T: Evidence that FTY720 induces T cell apoptosis *in vivo*. Immunopharmacology 48: 75-85, 2000.