의학박사 학위논문

간세포암 절제 후 재발에 대한 Metformin 연관 항종양 효과

Metformin-associated antitumor effect on tumor recurrence after hepatic resection of hepatocellular carcinoma

울산대학교대학원

의 학 과

좌 은 경

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이 논문을 의학박사 학위 논문으로 제출함

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국문 요약

서론: 간세포암 수술적 간 절제후 재발은 여전히 큰 과제로 남아있다. 이 연구는 실험실 연구를 통해 metformin 이 가진 세포독성효과를 조사하고 간세포암으로 간절제술을 시행한 환자에서 metformin 의 항암효과를 조사하고자 한다. 방법: 이 연구는 실험실 연구로써 간세포암으로 간절제술을 시행 받은 885 명 집단의 metformin 투여에 관한 임상적 후향성 연구조사이다. 결과: 실험실에서 이루어진 세포독성 연구에서는 하나의 HepG2.2.15 간 종양과 2 명의 환자로부터 얻은 간세포암 cell lines 을 이용하였다. metformin 투여 후, 세포생사판별시험(cell viability test) 및 세포자멸사 측정(apoptosis assays)에서 metformin 의 세포독성작용은 넥사바(소라페닙,sorafenib) 보다 훨씬 약한 정도로 뚜렷한 효과 보였다. 간세포암으로 간절제술을 시행 후 6 개월 이상 metformin 투여요법을 받은 환자 45 명을 대상으로한 임상결과에서는, metformin 투여 집단과 전체 집단(n=840) 간의 종양 재발율(p=0.61)과 전체 생존률(p=0.52)은 통계적으로 차이가 없었다. 반면, metformin 투여 집단과 성향점수매칭(PSM)을 적용한 집단 (n=225) 사이에서는 종양 재발율(P=0.094)에서는 통계적으로 차이가 없었으나 전체 생존률(p=0.028)에 있어 현저한 차이를 보였다. metformin 투여가 환자 생존에 있어 독립적인 위험인자로 작용하였다.

결론 : 당 실험실 연구 결과는 metformin 의 세포독성효과를 입증하였다. metformin 투여요법은 종양 재발율을 줄이는 효과를 보여주었고, 간세포암으로 간절제술을 시행한 환자 전체 생존률을 현저히 향상 시키는데 도움이 되었다. 간암의 화학적 예방효과와 관련하여 metformin 의 효과를 입증하기 위해서는 다수 센터에서의 다량의 연구가 필요하다.

차 례

그림목차

Abstract

Background: Hepatocellular carcinoma (HR) recurrence following hepatic resection (HR) remains a great concern. This study intended to investigate the metformin-induced cytotoxic effect at in vitro study and to assess the chemopreventive effect of metformin in patients undergoing HR for HCC. **Methods**: This study consisted of a laboratory study and a clinical retrospective study regarding metformin administration in a cohort of 885 patients who underwent HR for HCC. **Results**: In the laboratory study, one HepG2.2.15 liver tumor and 2 patient-derived graft HCC cell lines used for in vitro cytotoxic studies. After metformin treatment, cell viability tests and apoptosis assays revealed noticeable cytotoxic effect of metformin, which was largely weaker than that of sorafenib. In the clinical study including 45 patients with metformin administration for $\geq$ 6 months after HR, there was no statistical difference in tumor recurrence (p=0.61) and overall survival (p=0.52) between the metformin group and all control group (n=840). In contrast, comparison between the metformin group and propensity score-matched control group (n=225), there was no statistical difference in tumor recurrence (p=0.094) but significant difference in overall patient survival (p=0.028). Metformin administration was an independent risk factor for patient survival. **Conclusions**: Our in vitro laboratory study demonstrated presence of cytotoxic effect of metformin. Metformin administration showed a reducing tendency in tumor recurrence rate and helped to induce significant improvement in overall patient survival in patients who underwent HR for HCC. High-volume multicenter studies are necessary to validate the metformin-associated chemopreventive effect on HCC.

Abbreviations:

AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; ADV, AFP-DCP-tumor volume; DM, diabetes mellitus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HR, hepatic resection; NOD-SCID, non-obese diabetic/severe combined immunodeficiency; PDX, patient-derived xenograft; PSM, propensity score-matching; SCID, severe combined immunodeficiency.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and leading causes of cancer-related death [1, 2]. Hepatic resection (HR) is regarded as the first-line treatment in patients with preserved hepatic function, but tumor recurrence is high after curative HR [3, 4]. Many studies have intended to demonstrate the antitumor effect of new agents as postoperative adjuvant therapy after HR, but the clinical impact from these studies was limited [5–8], So far, there is no well-established strategy to lower the risk of HCC recurrence after HR to date.

Metformin is a biguanide agent used in the treatment of type 2 diabetes mellitus (DM). It regulates blood sugar by improving insulin sensitivity and reducing hepatic glucose output by inhibition of gluconeogenesis and glycogenolysis. Recently, metformin is known to be capable of inhibiting cancer cell growth by inducing cell cycle arrest and enhancing apoptosis [9–12]. There are a considerable number of studies presenting that metformin could play a chemopreventive role in other cancers and is associated with reduced risk of HCC [13–15]. Although a few high-volume population-based retrospective studies suggest the possibility of chemopreventive activity of metformin, the effect of metformin on post-resection HCC recurrence remains still unclear.

Therefore this study intended to investigate whether metformin has any cytotoxic effect on in in-vitro liver tumor cell line study and to assess the chemopreventive effect of metformin on HCC recurrence following HR through a propensity score-matched clinical study.

Patients and Methods

Study design

This study consisted of two independent parts as the laboratory research study and the clinical study in order to assess the antitumor effects of metformin.

The laboratory research study was focused to assess whether exposure to metformin has any cytotoxic effect on liver tumor cell lines. In the clinical study, the tumor recurrence rate and overall patient survival period after HR of HCC were investigated to assess whether long-term exposure to metformin has any chemopreventive effect. These 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.

Liver tumor cell lines

We used 3 liver tumor cell lines, one established cell line and two patient-derived xenograft (PDX) tumor cell lines. First, considering that a majority of HCC patients in Korea are associated with hepatitis B virus (HBV) infection, we chose the HepG2.2.15 cell line (Korean Advanced Institute of Science and Technology), which 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, both purchased from Gibco-BRL (Grand Island, NY, USA). Second, we established two PDX tumor cell lines. Small pieces of human HCC tissue were obtained during hepatic resection for HCC in HBV-associated patients who had not undergone preoperative HCC treatment (n = 2). A small tumor fragment of 0.3 g was implanted subcutaneously at the bilateral hind flanks of non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mouse. After confirmation of tumor growth for 3 months, the tumor was harvested and implanted to a MOD-SCID mouse. After confirmation of stable tumor growth, the established first-generation xenograft tumor was serially implanted to severe combined immunodeficiency (SCID) mouse to expand the xenograft tumors. These xenograft tumors were also implanted subcutaneously at the nude mouse for further tumor expansion. These tumors were harvested to establish new PDX tumor cell lines.

In vitro study using liver tumor cell lines

The cytotoxic effect of metformin was evaluated by using the abovementioned 3 liver tumor cell lines. The in vitro drug concentration was determined to be 5-10 mmol/mL for metformin after repeated titration from 5 to 40 mmol/mL for with consideration of the therapeutic ranges in patients with type 2 diabetes [16]. To assess metformin-associated cytotoxicity quantitatively, we used 10 μmol/mL concentration of sorafenib as a reference control [17].

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

Propensity score-matched clinical study using single-institution cohort

The HCC database at our institution was searched to identify patients who underwent primary HR for HCC during the nine years from January 2006 to December 2013. To compare the study groups objectively, patients were narrowly selected according to the following selection criteria: solitary HCC of 2.0–5.0 cm in diameter, curative surgery with anatomical HR, no macroscopic vascular invasion, no extrahepatic metastasis including lymph node metastasis, no preoperative HCC treatment, and Child–Pugh class A. Through this screening process, 939 patients were selected.

These patients were classified according to postoperative administration of metformin. Metformin use was defined as prescription of metformin more than 6 months within the initial 2 years after HR for HCC. In order to assess the long-term outcome related to the defined metformin use, 54 patients who survived for less than 2 years after HR were excluded, leaving 885 patients who survived $\geq$2 years as the whole study cohort. Finally, 45 patients belonged to the metformin group and 840 patients being the control group. The sample number of propensity score-matching (PSM) control group was estimated with a type

I error (α) of 0.10 and a type II error (β) of 0.20 in addition to 10% survival difference; therefore, the sample number of the PSM control group became 225. To overcome possible selection bias, PSM between the metformin study group and control group was applied using multiple logistic regression and a 1:5 matching requirement via the nearest-neighbor matching method [18]. We matched for baseline characteristics (age, sex), background liver disease (viral hepatitis versus others), preoperative level of tumor markers (α-fetoprotein [AFP] and des-γ-carboxy prothrombin [DCP; or proteins induced by vitamin K antagonist or absence-II]), tumor characteristics (size and presence of microvascular invasion), and AFP-DCP-tumor volume (ADV) score [19, 20].

The medical records were reviewed retrospectively after approval by the Institutional Review Board of our institution. Preoperative evaluation, follow-up, and treatment for HCC recurrence have been described previously [4, 21]. Patients were followed up until March 2017 using medical record reviews and through the assistance of the National Health Insurance Service, therefore making the patient follow-up period $\geq$27 months or until death. All patients were followed to identify patient survival status.

Statistical analysis

Numerical data are presented as mean with standard deviation or as median with range. Continuous variables were compared using Student's t test and incidence variables were compared using the chi-square test. Survival curves were estimated by the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazards regression was used for multivariate survival analysis. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS (version 22, IBM, NY).

Results

In vitro cytotoxicity in the cell line study

The MTT assay for cell survival assessment showed a concentration-dependent decrease of cell survival at 20-hour treatment with metformin 5 and 10mmol/mL in HepG2.2.15 cell line (**Fig. 1A**), which was higher than treatment with sorafenib 10 and 20 μmol/mL. In contrast, cell death was lower after metformin treatment than after sorafenib treatment in PDX cell line 1 and 2 (**Fig. 1B and 1C**).

Fluorescence microscopy with DAPI-Hoechst staining showed noticeable apoptosis after metformin exposure in all 3 cell lines, which was comparable with that of sorafenib exposure (**Fig. 2**).

Patient demographics and comparison of post-resection outcomes according to metformin administration

The process for patient selection was depicted in **Fig. 3**. The clinicopathological features of the patients belonged to metformin study group (n=45), non-metformin all control group (n=840), and PSM control group (n=225) are summarized in **Table 1**. The clinicopathological features between the metformin and PSM control groups were very similar each other.

During follow-up with a median period of 62 months (range: 24–135 months), HCC recurrence developed in 351 of 885 patients (39.7%) and all-cause death occurred in 118 of 885 patients (13.3%).

The tumor recurrence rates after HR according to metformin administration were compared, in which the 1-, 3- and 5-year rates were 11.1%, 32.3% and 42.4% in metformin study group; 13.7%, 34.3% and 42.7% in all control group (p=0.61) (**Fig. 4A**); and 17.7%, 42.0% and 54.5% in PSM control group (p=0.094), respectively (**Fig. 5A**).

The overall patient survival rates after HR according to metformin administration were compared, in which the 2-, 3- and 5-year rates were 100%, 97.8% and 83.2% in metformin study group; 100%, 96.4% and 88.6% in all control group (p=0.52) (**Fig. 4B**); and 100%, 89.5% and 67.8% in PSM control group (p=0.028), respectively (**Fig. 5B**).

Risk factor analysis for tumor recurrence and overall survival

The results of the univariate analysis for post-resection prognosis were summarized in **Table 2**, in which significant risk factors were tumor size >3.1 cm and microvascular invasion for tumor recurrence and tumor size >3.1 cm and metformin administration for patient survival.

Multivariate analysis revealed that independent risk factors were tumor size >3.1 cm for tumor recurrence and tumor size >3.1 cm and metformin administration for patient survival (**Table 3**).

Discussion

For patients with HCC, HR is regarded as the first-line treatment in patients with preserved hepatic function, but tumor recurrence is high even after curative HR [3, 4]. Thus there are many attempts to decrease the risk of tumor recurrence in terms of postoperative adjuvant therapy such as interferon Alfa-2b, acyclic retinoid, vitamin K and so on [5–8], but none of them was proven to be effective in prospective controlled trial setting. Vitamin K administration was reported to show antitumor effect in a few patients, but meta-analyses including a randomized controlled study failed to prove its preventive and therapeutic effects [22, 23]. Authors also presented that oral administration of vitamin K2 with or without sorafenib did not show adverse side effects and noticeable antitumor effects occurred in a few patients who had HCC recurrence after HR or liver transplantation [24, 25]. Therefore, there is a great need to find out agents usable for adjuvant chemopreventive setting.

DM is a common chronic disease that is not life threatening in the short-term and is estimated to affect 4-5% of the worldwide population. Along with the increasing prevalence of the Western lifestyle and obesity in the general population, the prevalence of DM is expected to increase rapidly in Asian countries including Korea. DM per se is not life threatening in disease nature, but severe forms of DM might be accompanied by various serious complications leading to deterioration of quality of life and even patient death. Moreover, there is accumulating data showing that patients with DM are also prone to development of cancer including HCC [26-31]. Thus, it is reasonable that a considerable number of patients with HCC are associated with DM in their carcinogenesis.

A Taiwanese nationwide study presented that DM has an adverse effect on patients with HCC regardless of treatment modality, but use of metformin significantly reduces the risk of HCC recurrence and improves the overall outcome of patients after HR if patients survives the initial 2 years [32]. Our present study revealed that there was noticeable reduction in post-resection tumor recurrence rates in the metformin study groups comparing with the PSM control group, which was not statistically significant probably due to small case number. In contrast, we demonstrated that there was a significant improvement in overall patient survival in the metformin group.

There are a few studies which investigated the association between antidiabetic drugs and the risk of developing HCC, and have shown a reduced risk with metformin treatment [22-34]. Metformin has also been demonstrated to inhibit cancer cell growth and proliferation through cell cycle arrest [35]. Although the action mechanisms of anti-tumor effect were not investigated, the presence of certain metformin-associated anti-tumor effect was clearly demonstrated in our present study. It was reported that metformin was capable of attenuating the risk of developing HCC associated with DM in terms of dosage and medication duration, in which metformin inhibited the proliferation of hepatoma cell lines in a dose-dependent manner as well as the risk of developing HCC could also be decreased by increasing the duration of metformin use [13]. Our in vitro cell line study also revealed that anti-tumor effect of metformin appears to be dose-dependent, supporting the suggestion for high-dose long-term administration.

By contrast, so far, the antitumor effect of metformin was demonstrated in only high-volume cohort studies or laboratory researches, implicating that antitumor effect of metformin exists but its prognostic power is not so great enough to be an independent prognostic factor in small or medium-sized volume studies. In our in vitro study, we compare the potency of antitumor effect between metformin and sorafenib, in which the metformin-associated anti-tumor effect was variably comparable to that of sorafenib. Variable treatment response to metformin as well as sorafenib in the different cell lines implicates that a certain proportion of patients may be more benefited chemoprevention with metformin. Thus further laboratory researches should be performed to demonstrate its potency of cytotoxic effect as well as action mechanisms of antitumor effect.

In our clinical study with PSM control group, the independent risk factors were tumor size >3.1 cm for tumor recurrence and tumor size >3.1 cm and metformin administration for patient survival. These results implicate that metformin can be a potential agent for post-resection chemoprevention. Thus, further clinical studies also should be performed to establish the guidelines for patient selection and dosage setting toward wide use of metformin for chemopreventive purpose.

There are some limitations to this study. It was a retrospective single-center study and the study population was not large enough, thus our results may not be generalizable. It is also

necessary to validate the effect of metformin in other geographic regions to extend our results to HCC patients with various background liver diseases other than HBV infection.

In conclusion, our in vitro laboratory study demonstrated presence of cytotoxic effect of metformin. Metformin administration showed a reducing tendency in tumor recurrence rate and helped to induce significant improvement in overall survival in patients who underwent HR for HCC. High-volume multicenter studies and refined laboratory studies are necessary to validate the metformin-associated antitumor effect on HCC.

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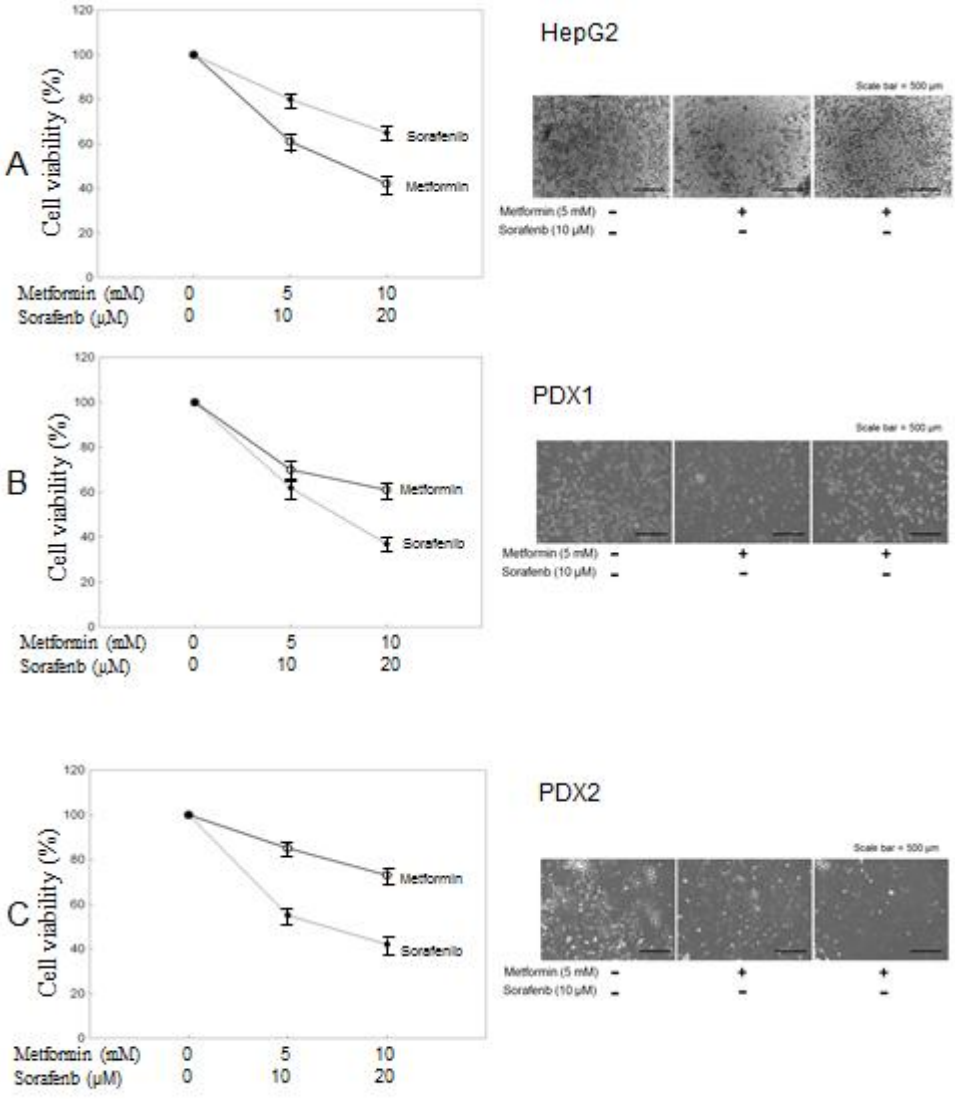

Figure 1. The MTT assay for cell survival assessment. A. HepG2 B. PDX1 C. PDX2

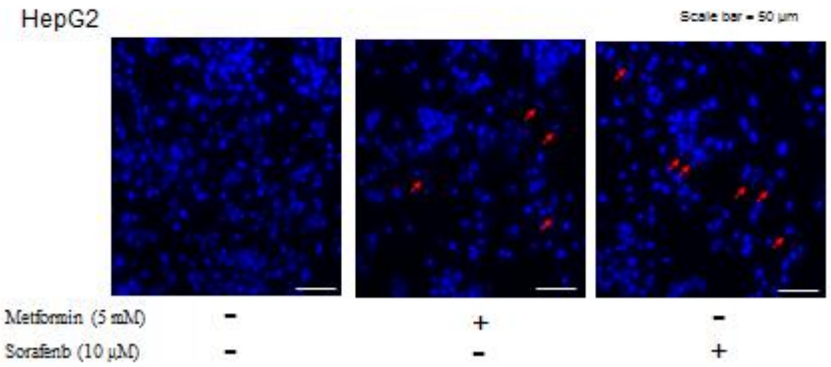

Figure 2. Fluorescence microscopy with DAPI-Hoechst staining.

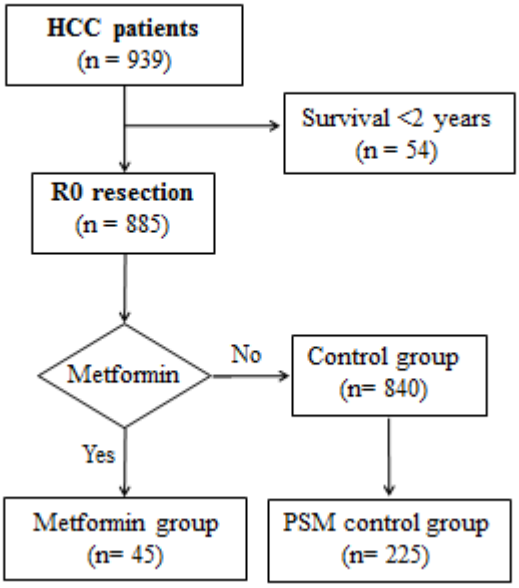

Figure 3. The process for patient selection.

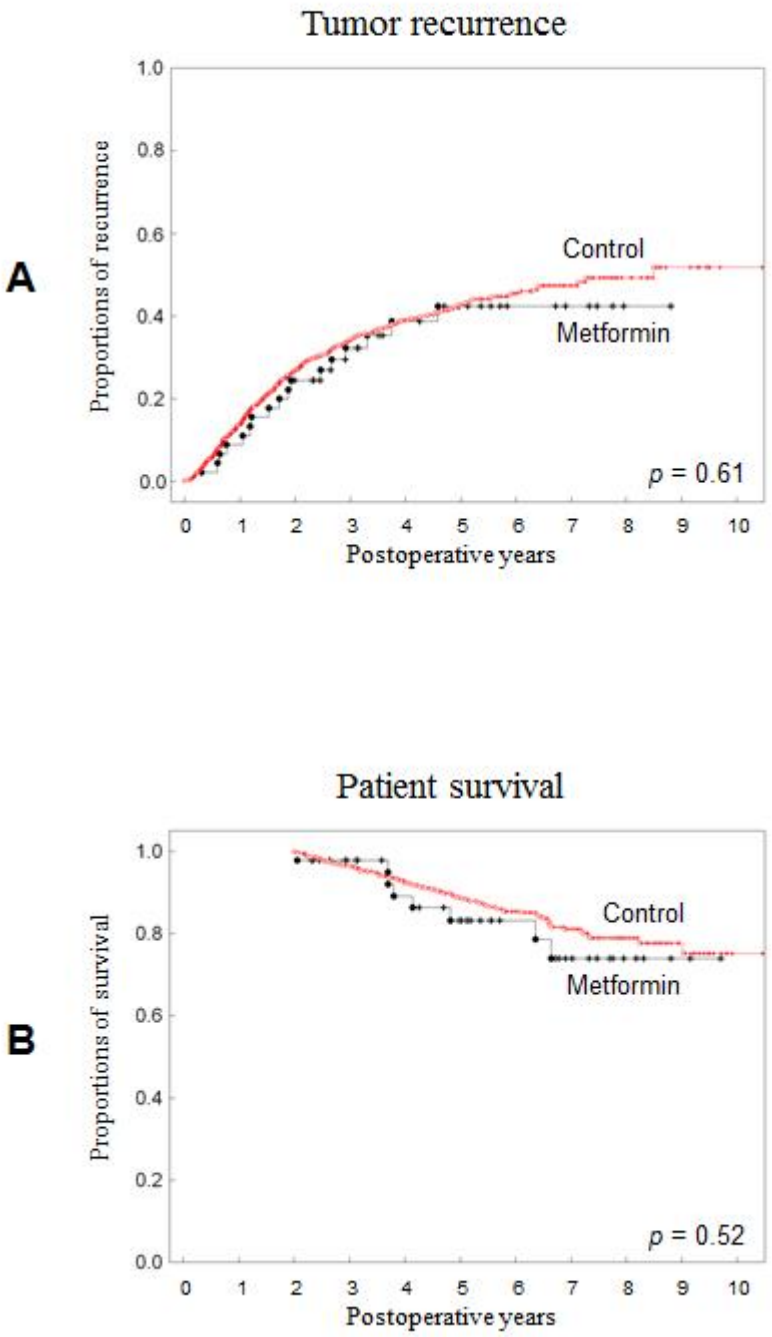

Figure 4. The tumor recurrence rates and the overall patient survival rates after HR according to metformin administration in all control group.

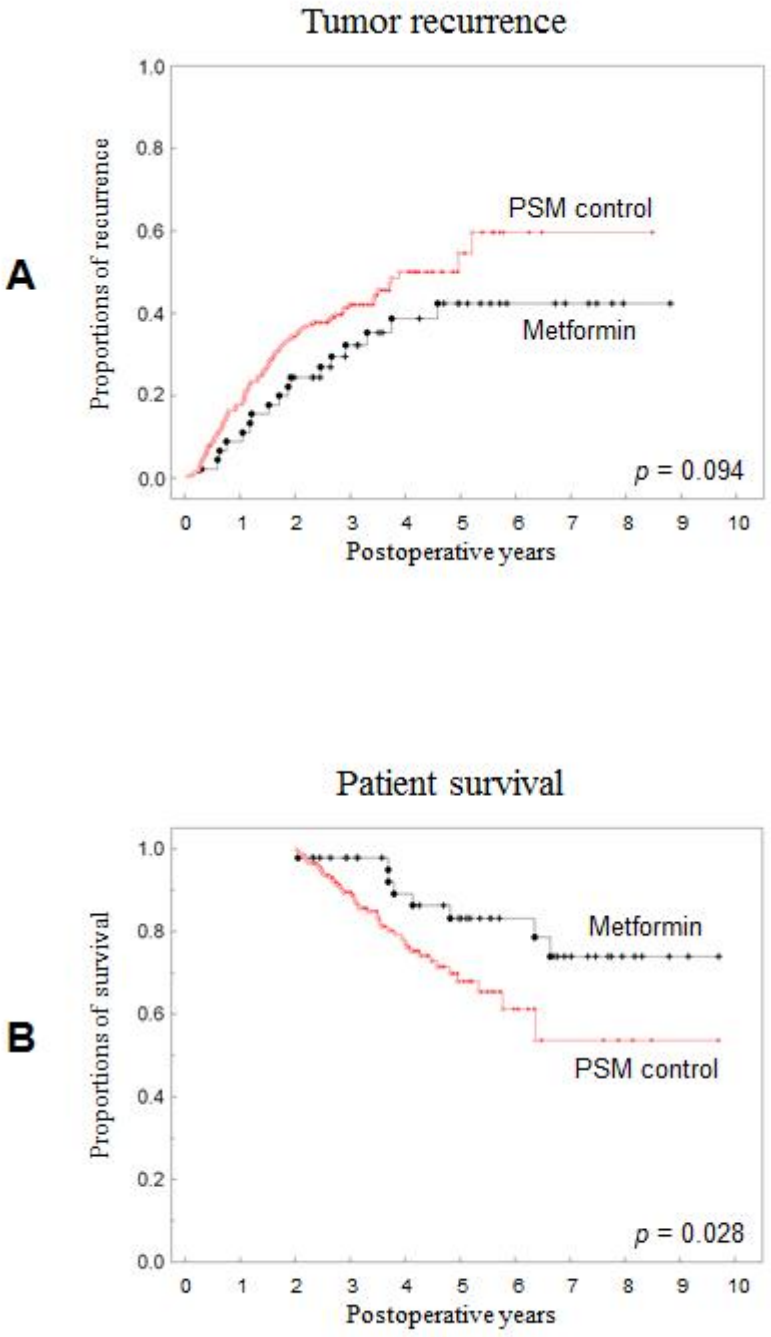

Figure 5. The tumor recurrence rates and the overall patient survival rates after HR according to metformin administration in PSM control group.

Table 1. Comparison of the clinicopathological profiles of the metformin and control group patients.

Parameter	Metformin group (A)	Control group (B)	PSM control group (C)	*p*-value (A vs.B)	*p*-value (A vs.C)
Patient number	45	840	225		
Age (years)	60.8±8.6	57.4±9.5	58.4±8.5	0.021	0.11
Gender (Male / Female) (n)	35 / 10	671 / 169	179 / 46	0.73	0.79
Background liver disease (n)				0.006*	0.060*
HBV	31	710	183		
HCV	4	37	12		
ALD	6	39	15		
Others	4	54	15		
Preoperative blood laboratory profiles (mean±SD)					
Albumin (g/dL)	3.8±0.5	3.8±0.4	3.8±0.4	0.98	0.97
AST (IU/L)	38.2±25.4	38.7±38.5	41.4±33.3	0.93	0.26
ALT (IU/L)	36.2±20.5	39.6±45.2	43.3±38.5	0.62	0.23
Total bilirubin (mg/dL)	0.8±0.4	0.8±0.4	0.8±0.4	0.97	0.98
Platelet count ($10^3/\mu L$)	163.5±46.2	159.3±55.2	156.5±48.3	0.62	0.37
Prothrombin time (INR)	1.02±0.07	1.08±0.09	1.03±0.07	0.021	0.12
AFP (ng/mL) at operation				0.37	0.55
Mean ± SD	176.1±652.9	884.9±3814.2	274.2±2881.2		
Median	8.2	14.5	6.7		
≤7.5 / >7.5 ng/mL (n)	21 / 24	336 / 504	116 / 109		
PIVKA-II (mAU/mL) at operation				0.30	0.83
Mean ± SD	157.8±319.3	464.8±1497.6	234.4±711.3		
Median (range)	47	53	39		
≤40 / >40 mAU/m (n)	22 / 23	345 / 495	114 / 111		
ICG-R_{15} (%)	13.9±8.2	13.0±5.5	12.8±5.7		
MELD score (Mean ± SD)	7.5±1.7	7.7±2.1	7.6±1.9	0.53	0.74
FDG-PET (hypermetabolic / not hypermetabolic) (n)	9/24	345 / 331	101 / 103		
Tumor diameter (Mean ± SD, cm)	3.2±0.9	3.3±0.9	3.1±0.8	0.47	0.45
Tumor volume (Mean ± SD, ml	12.6±11.2	14.3±11.6	12.2±10.7	0.34	0.82
ADV score (Mean ± SD, log)	3.9±1.1	4.5±1.5	3.9±1.2	0.008	0.96
Extent of liver resection (n)				0.75**	1.00**
Trisectionectomy	0	3	1		
Hemihepatectomy	11	161	47		
Bisectionectomy	0	24	7		
Sectionectomy	29	623	149		
Segmentectomy	5	29	21		
Microvascular invasion (present / absent) (n)	7 / 38	155 / 685	42 / 189	0.62	0.67
Most Edmonson-Steiner grade (n)				0.59***	0.41***

WelI-differentiated	12	255	74		
Moderately differentiated	23	443	104		
Poorly differentiated	10	140	47		

HBV, hepatitis B virus; HCV, hepatitis C virus; ALD, alcoholic liver disease; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; ICG-R15, indocyanine green retention test at 15 minutes; MELD, model for end-stage liver disease; FDG-PET, 2-18F-fluoro-2-deoxy-d-glucose positron emission tomography; ADV, AFP-DCP-tumor volume,

*, comparison of HBV vs. non-HBV

**, hemihepatectomy or greater vs. sectionectomy or smaller.

***, WelI-differentiated vs. moderately-to -poorly differentiated.

Table 2. Univariate analyses of factors associated with tumor recurrence and patient survival in 270 patients belonged to the metformin and propensity score matching control groups.

Variables	Patient No.	Median DFS period (mos)	*p*-value	75% OS period (mos)	*p*-value
Background liver disease			0.42		0.88
HBV	214	59		54	
Non-HBV	56	45		51	
Serum AFP			0.13		0.46
≤7.5 ng/mL	137	59		69	
>7.5 ng/mL	133	42		50	
Serum DCP			0.78		0.32
≤40 mAU/m	136	45		59	
>40 mAU/m	134	60		47	
ICG-R15 (%)			0.061		0.12
≤10%	61	67		87	
>10%	140	42		49	
FDG-PET			0.44		0.079
Not hypermetabolic	127	67		64	
Hypermetabolic	110	49		48	
Tumor size			0.001		0.006
≤3.1 cm	154	69		91	
>3.1 cm	116	35		44	
ADV score			0.28		0.97
≤4log	155	60		54	
>4log	114	42		50	
Microvascular invasion			0.044		0.25
Absent		60		55	
Present		22		42	
Tumor differentiation			0.64		0.16
WelI-differentiated	86	69		58	
Moderately-to-poorly differentiated	184	47		49	
Metformin administration			0.12		0.032
No	225	47		49	
Yes	45	70		77	

Median DFS period, disease-free survival period at 50%; 75%OS period, overall survival period at 75%.

Table 3. Multivariate analyses of factors independently associated with tumor recurrence and patient survival in 270 patients belonged to the metformin and propensity score matching control groups.

Variables	Tumor recurrence			Patient survival		
	Hazard ratio	95% CI	*p*-value	Hazard ratio	95% CI	*p*-value
Tumor size (>3.1 cm vs. ≤3.1cm)	1.78	1.23–2.59	0.002	2.07	1.12–3.57	0.009
Microvascular invasion (Present vs. absent)	1.52	0.95–2.46	0.083			
Metformin administration (Yes vs. no)				1.51	1.02–2.22	0.042

CI, confidence interval.