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Impact of epidermal growth factor single-nucleotide polymorphism on recurrence of hepatocellular carcinoma after hepatectomy in patients with chronic hepatitis C virus infection

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Key words
Hepatocellular carcinoma, hepatectomy, recurrence, epidermal growth factor, single-nucleotide polymorphism

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Epidermal growth factor (EGF) gene single-nucleotide polymorphism (SNP) is associated with an increased risk of hepatic tumors. The study aimed to elucidate the impact of EGF SNP and EGF receptor (EGFR) expression on the recurrence of hepatocellular carcinoma (HCC) after hepatectomy. To examine the impact of EGF SNP and EGFR on recurrent HCC, we retrospectively analyzed 141 HCC patients with chronic hepatitis C virus infection who underwent curative hepatectomy. The EGF *61 GG allele was present in 69 patients (48.9%), AG in 56 (39.7%) and AA in 16 (11.4%). The AA group had a significantly lower rate of intrahepatic metastasis (0% *vs* 16.5%, $P = 0.02$), lower serum EGF concentration (26.3 ± 15.9 pg/mL *vs* 43.4 ± 30.5 pg/mL, $P = 0.02$) and lower proportion of early recurrence (≤2 years; 28.6% *vs* 71.2%, $P = 0.03$) than the AG/GG group. The AA group had significantly higher recurrence-free survival than the AG/GG group ($P = 0.04$), but there was no significant difference in overall survival between these two groups ($P = 0.97$). High versus low EGFR expression analyzed by immunohistochemical staining in cancer cells was not significantly associated with overall survival ($P = 0.37$) or recurrence-free survival ($P = 0.39$). Therefore, EGF *61 AA was associated with a lower risk of recurrence after curative hepatectomy for HCC in patients with hepatitis C virus infection than other genotypes, but EGFR expression in cancer cells was not significantly associated with prognosis.

Hepatocellular carcinoma (HCC) is one of the most common malignant solid tumors, and is generally treated by hepatectomy in patients with well-preserved liver function.[1,2] Even though curative resection improves the prognosis, the 5-year post-hepatectomy overall survival (OS) rate and recurrence-free survival (RFS) rate are 56% and 23%, respectively.[3] The high recurrence rate is thought to result from multicentric carcinogenesis, especially in patients with multiple risk factors.[4–6] As recurrence after hepatectomy is associated with a poorer prognosis, identification of the risk factors for postoperative recurrence may help to improve outcomes.

Epidermal growth factor (EGF) has many biological functions, including stimulation of cell proliferation and differentiation of specific cells.[7,8] Recent studies have reported that the single-nucleotide polymorphism (SNP) A to G mutation at position 61 of the 5′ untranslated region of the EGF gene (rs4444903) is associated with an increased risk of various malignant tumors.[9–11] In patients with HCC, this 61*G polymorphism is associated with an increased risk of hepatocarcinogenesis in patients with chronic hepatitis C virus (HCV) infection and advanced fibrosis.[12] A meta-analysis found that this polymorphism was a risk factor for HCC in a cohort of inhomogeneous patients,[13] whereas another study found that it was not a risk factor for HCC in patients with chronic hepatitis B virus infection.[14] EGF receptor (EGFR) expression is reported to be a predictor of poor prognosis in patients with colon cancer,[15] and inhibition of EGFR expression *in vivo* improved the prognosis of patients with liver cancer.[16,17] These findings indicate that EGFR and its ligand EGF affect hepatocarcinogenesis, but to our knowledge there are no reported studies evaluating the importance of the roles of serum EGF concentration, EGF gene polymorphism and EGFR in recurrence of HCC.

The aim of the present study was to evaluate the impact of SNP *61 in the EGF gene and EGFR expression on recurrence of HCC after hepatectomy.

Materials and Methods

Patients. All patients who underwent curative resection of HCC at Kyushu University Hospital (Fukuoka, Japan) from December 2002 to March 2012 and were seropositive for HCV

antibody were reviewed. Patients who had received preoperative treatment such as hepatectomy, radiofrequency ablation, percutaneous ethanol injection or systemic chemotherapy were excluded from the study. Curative resection was defined as complete macroscopic removal of the tumor. Tumor stage and differentiation and stage of hepatitis activity and liver fibrosis were diagnosed by specialist pathologists according to the TNM stage definitions proposed by the Liver Cancer Study Group of Japan,[18] which are in accordance with the TNM classification system of the International Hepato-Pancreato-Biliary Association[19] and the Metavir score.[20] After discharge, all patients underwent monthly screening for recurrence using ultrasonography and measurement of tumor markers such as alpha-fetoprotein, and 6-monthly computed tomography scanning. If recurrence was suspected, additional investigations such as hepatic angiography were performed. The time of HCC recurrence was defined as the day of diagnosis based on imaging examination findings. All patients provided written informed consent, and the study protocol was approved by the Ethical Committee of Kyushu University.

DNA extraction and epidermal growth factor genotyping. DNA was extracted from the non-cancerous part of resected liver tissues, and genotyping was performed using the Taqman GTXpress Master Mix (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions. The Custom TaqMan SNP Genotyping Assay (Applied Biosystems) was used to identify EGF gene polymorphism (rs4444903).

Enzyme-linked immunosorbent assay. Whole blood samples were collected from all enrolled patients in the operating room before laparotomy. Samples were centrifuged at 3010 *g* for 10 min, and the serum was stored immediately at −80°C. Serum concentrations of EGF were measured using Quantikine enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Immunohistochemical staining and immunoreactivity score. Sections of the resected liver specimens were fixed in 10% buffered formalin, embedded in paraffin, pretreated in a microwave oven for 20 min, and incubated with primary antibodies to EGFR (D38B1, 1:200, Cell Signaling Technology, Danvers, MA, USA). Immunohistochemical staining was detected by an EnVision+ System and DAB kit (DAKO, Glostrup, Denmark). Expression of EGFR was evaluated by two investigators, including a surgical pathologist who was blinded to the clinical details. The immunoreactivity score for EGFR was determined using a modified Allred score[21] by adding a score for the intensity of cell membrane staining (0, none; 1, weak; 2, moderate; 3, strong) to a score for the percentage of positive cells (0, 0%; 1, 1–10%; 2, 11–30%; 3, 31–66%; 4, 67–80%; 5, >80%).

Statistical analysis. All statistical analyses were performed using SAS software (JMP 9.0.1; SAS Institute, Cary, NC, USA). All variables are expressed as the mean ± SD. Categorical variables were compared using the χ^2-test and continuous variables were compared using the non-parametric Wilcoxon test or the parametric *t*-test. OS and RFS were calculated using the Kaplan–Meier method and compared between groups using the log-rank test. A value of $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. This study included 141 consecutive eligible patients with a mean age of 68 ± 7 years. All patients were seropositive for HCV antibody, and 77.3% were male. Ninety-nine patients had Stage I or II tumors. The average tumor size was 3.5 ± 2.5 cm. Forty-eight patients had liver

Table 1. Clinical characteristics of patients carrying AG/GG and AA alleles at rs4444903

rs4444903	All patients (*n* = 141)	AG/GG (*n* = 125)	AA (*n* = 16)	*P*-value
Age (years)	68 ± 7	68 ± 7	70 ± 6	0.36
Gender, male (%)	109 (77.3)	98 (78.4)	11 (68.8)	0.40
Albumin (g/dL)	3.9 ± 0.4	4.0 ± 0.4	3.7 ± 0.5	0.03
Total bilirubin (mg/dL)	0.83 ± 0.32	0.85 ± 0.34	0.72 ± 0.24	0.13
AST (IU/L)	55 ± 30	55 ± 31	55 ± 26	0.93
ALT (IU/L)	56 ± 41	55 ± 40	66 ± 51	0.30
Prothrombin time (%)	86 ± 10	86 ± 11	86 ± 10	0.90
Platelet count (×104/μL)	16.9 ± 17.3	17.3 ± 18.2	13.5 ± 5.6	0.40
ICGR15 (%)	15.4 ± 7.6	15.1 ± 7.5	17.7 ± 7.4	0.20
Child-Pugh Grade A (%)	138 (97.9)	122 (97.6)	16 (100)	0.39
Operation time (min)	344 ± 111	345 ± 112	335 ± 104	0.76
Intraoperative bleeding (mL)	582 ± 496	572 ± 480	663 ± 625	0.51
Maximum tumor size (cm)	3.5 ± 2.5	3.6 ± 2.6	3.0 ± 1.4	0.40
AFP level (log ng/mL)	1.47 ± 1.06	1.49 ± 1.05	1.34 ± 1.11	0.60
DCP level (log mAU/mL)	2.05 ± 0.95	2.09 ± 0.98	1.68 ± 0.63	0.11
Stage (I,II/III,IV)	99/42	86/39	13/3	0.29
Vp, yes (%)	41 (29.1)	37 (29.1)	4 (25.0)	0.70
Im, yes (%)	20 (14.2)	20 (16.0)	0 (0)	0.02
Tumor differentiation (well, moderate/poor)	102/39	90/35	12/4	0.80
Hepatitic activity (0/1/2/3)	4/33/71/33	4/30/61/30	0/3/10/3	0.60
Staging (0/1/2/3/4)	29/31/32/49	25/28/29/43	4/3/3/6	0.94
Achieved SVR, yes (%)	26 (18.4)	24 (19.2)	2 (12.5)	0.50

AFP, alfa-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; $ICGR_{15}$, indocyanine green retention rate at 15 min; Im, microscopic intrahepatic metastasis; SVR, sustained virological response; Vp, microscopic portal vein involvement.

cirrhosis. The clinical characteristics of the enrolled patients are shown in Table 1.

Associations between epidermal growth factor receptor genotype and clinical characteristics. The EGF *61 GG allele was present in 69 patients, AG in 56 patients, and AA in 16 patients. The AA group had a lower rate of intrahepatic metastasis (0% *vs* 16.0%, $P = 0.02$) and lower serum albumin concentration (3.7 ± 0.5 g/dL *vs* 4.0 ± 0.4 g/dL, $P = 0.03$) than the AG/GG group. There were no significant differences between these two groups for other preoperative, intraoperative and pathological factors (Table 1).

There were no significant differences in OS or RFS among patients carrying the AA, AG and GG alleles ($P = 0.99$ and $P = 0.11$, respectively; Fig. 1a,b). There was no significant difference in OS between the AA group ($n = 16$) and the AG/GG group ($n = 125$) ($P = 0.97$; Fig. 1c), but RFS was significantly higher in the AA group than in the AG/GG group ($P = 0.04$; Fig. 1d).

The serum EGF concentration was 47.9 ± 34.6 pg/mL in patients carrying GG, 36.8 ± 21.9 pg/mL in patients carrying AG, and 26.3 ± 15.9 pg/mL in patients carrying AA ($P = 0.01$; Fig. 2a). The AA group had a significantly lower serum EGF concentration than the AG/GG group (26.3 ± 15.9 pg/mL *vs* 43.4 ± 30.5 pg/mL, $P = 0.02$; Fig. 2b). Recurrence was divided into early type (within 2 years after surgery) and late type. The AA group had a significantly lower proportion of early type recurrence than the AG/GG group (28.6% *vs* 71.2%, $P = 0.03$, Table 2).

Associations between epidermal growth factor receptor expression and clinical characteristics. Immunohistochemical analysis showed that EGFR was expressed in the cytoplasm and cell membranes of HCC cells (Fig. 3a), and that the intensity of staining in the cytoplasm correlated with that of the cell membranes. Patients were divided into a high score group (immunoreactivity score >5, $n = 38$) and a low score group (immunoreactivity score ≤5, $n = 103$). Table 3 shows comparisons of clinicopathological factors between these two groups. Univariate analyses showed that the high score group had a significant higher preoperative serum alanine aminotransferase level (67 ± 47 IU/L *vs* 52 ± 38 IU/L, $P = 0.04$), lower des-gamma-carboxy prothrombin level (1.78 ± 0.74 log mAU/mL *vs* 2.14 ± 1.00 log mAU/mL, $P = 0.04$) and smaller maximum tumor size (2.8 ± 1.5 cm *vs* 3.8 ± 2.7 cm, $P = 0.04$) than the low score group. There were no significant differences

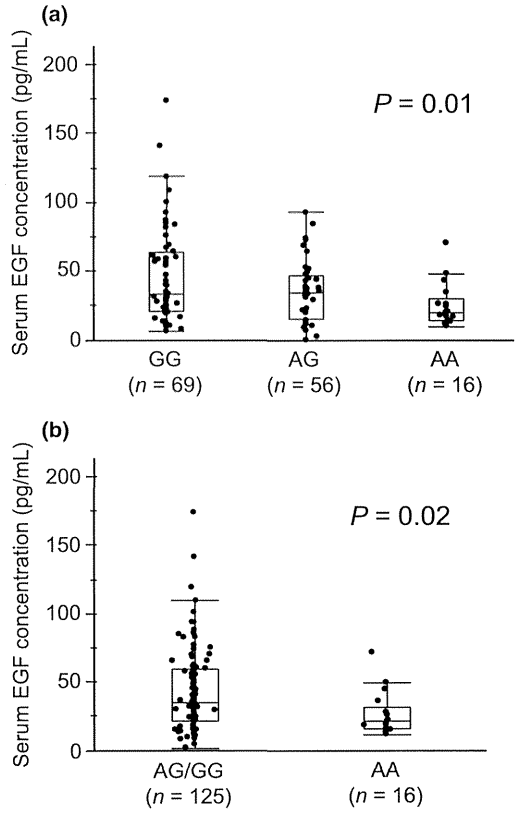

Fig. 2. Comparisons of serum epidermal growth factor (EGF) concentration. (a) There was a significant difference in serum EGF concentration among the three genotypes ($P = 0.01$). (b) The AA group had a significantly lower serum EGF concentration than the AG/GG group ($P = 0.02$).

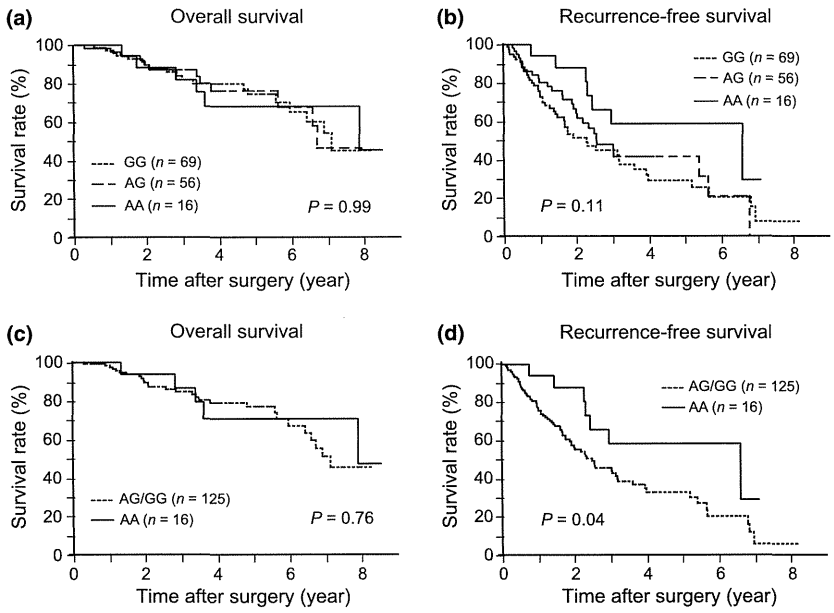

Fig. 1. Comparisons between patients carrying the AA allele and patients carrying other alleles. The AA group had a significantly higher recurrence-free survival rate than the AG/GG group ($P = 0.04$).

Table 2. Proportions of recurrence type in the AG/GG and AA groups

rs4444903	Early type (≦2 years)	Late type (>2 years)	*P*-value
AG/GG (*n* = 66)	47 (71.2%)	19 (28.8%)	0.03
AA (*n* = 7)	2 (28.6%)	5 (71.4%)	

Early indicates recurrence within 2 years after surgery.

in OS or RFS between the high and low score groups ($P = 0.37$ and $P = 0.39$, respectively; Fig. 3c,d).

Discussion

The pathogenesis of HCC involves host genetic factors, environmental factors, and modulation of molecular signaling pathways that contribute to hepatocarcinogenesis and tumor progression.[22] Previous studies report an association between EGF SNP (rs4444903) and an increased risk of hepatocarcinogenesis.[12,23] This may be because EGF gene polymorphism affects serum EGF concentration.[13]

The results of the present study show that patients with HCV infection carrying AA at EGF *61 had a significantly higher RFS after curative hepatectomy for HCC than those carrying other genotypes. A meta-analysis found that the reported proportions of the three genotypes were 41.4% for GG, 43.8% for AG and 14.8% for AA,[13] which are very similar to the proportions in the present study. Abu *et al.*[12] report that the serum EGF concentration was highest in patients carrying GG and lowest in patients carrying AA, and that for each genotype, serum EGF concentration was higher in patients with HCC than without HCC, suggesting an association between higher EGF concentration and increased risk of HCC. Therefore, we divided patients into an AA group and a non-AA group on the basis of serum EGF concentration. In addition, our analysis of recurrence type suggests that a high serum EGF concentration may increase the malignancy of tumor cells and may promote metastatic recurrence rather than multicentric occurrence. Hence, our results indicate that carrying AA at EGF *61 is associated with a lower risk of recurrence of HCC after hepatectomy than other genotypes, because of the lower serum EGF concentration.

Table 3. Comparisons of clinicopathological factors between groups with high and low immunoreactivity scores for EGFR

Factor	High score (*n* = 38)	Low score (*n* = 103)	*P*-value
Age (years)	67 ± 7	69 ± 7	0.29
Gender, male (%)	31 (81.6)	78 (75.7)	0.46
Albumin (g/dL)	3.9 ± 0.4	3.9 ± 0.5	0.92
Total bilirubin (mg/dL)	0.86 ± 0.39	0.83 ± 0.31	0.56
AST (IU/L)	61 ± 29	52 ± 31	0.13
ALT (IU/L)	67 ± 47	52 ± 38	0.04
Prothrombin time (%)	85 ± 9	87 ± 11	0.23
Platelet count (×104/μL)	12.6 ± 4.3	18.4 ± 20	0.08
ICGR15 (%)	17.4 ± 9.0	14.7 ± 6.8	0.06
Operation time (min)	336 ± 88	346 ± 119	0.64
Intraoperative bleeding (mL)	567 ± 368	588 ± 538	0.83
Maximum tumor size (cm)	2.8 ± 1.5	3.8 ± 2.7	0.04
AFP level (log ng/mL)	1.25 ± 0.68	1.56 ± 1.16	0.13
DCP level (log mAU/mL)	1.78 ± 0.74	2.14 ± 1.00	0.04
Stage (I,II/III,IV)	27/11	72/31	0.89
Vp, yes (%)	9 (23.7)	32 (31.1)	0.39
Im, yes (%)	3 (7.9)	17 (16.5)	0.17
Tumor differentiation (well, moderate/poor)	28/10	74/29	0.83
Hepatitic activity (0/1/2/3)	4/4/21/9	3/28/49/23	0.16
Staging (0/1/2/3/4)	26/23/19/35	4/8/13/13	0.11
Achieved SVR, yes (%)	8 (21.1)	18 (17.5)	0.63

AFP, alfa-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EGFR, epidermal growth factor receptor; $ICGR_{15}$, indocyanine green retention rate at 15 min; Im, microscopic intrahepatic metastasis; Vp, microscopic portal vein involvement.

In this study, the EGFR expression of cancer cells was not associated with prognosis. Previous studies report that the intensity of EGFR expression correlates with proliferative activity, stage, intrahepatic metastasis and carcinoma differentiation, but they do not analyze the proportions of cells with EGFR expression.[24] In our samples of resected liver tissue, EGFR expression in cancer cells was heterogeneously distributed even within the same nodule; for that reason, we analyzed EGFR expression in terms of both intensity and proportion, using a modified Allred score. Large tumors were more heterogeneous

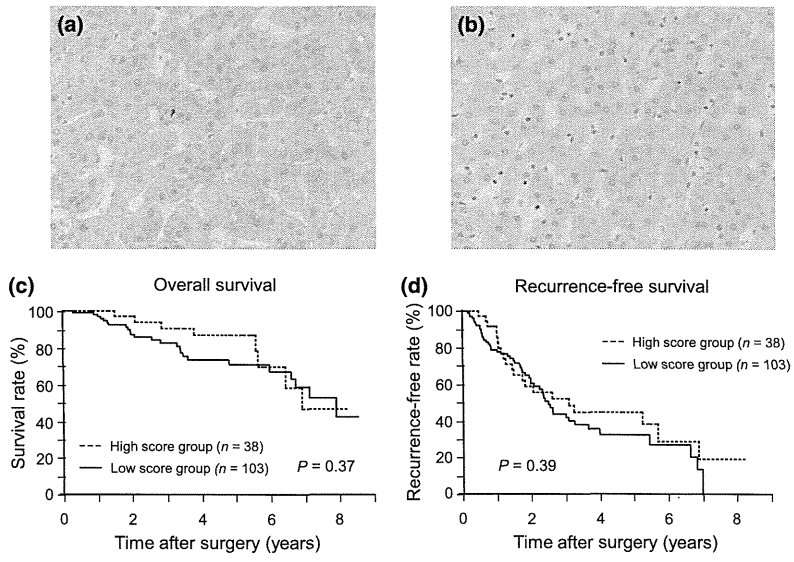

Fig. 3. Immunohistochemical staining of resected liver tissues for epidermal growth factor receptor (EGFR) (original magnification ×400). (a) Cancer cells showing EGFR expression in the cytoplasm and cell membranes. (b) Non-cancerous hepatocytes, showing EGFR expression in the cytoplasm. (c,d) Associations between immunoreactivity scores for EGFR and prognosis after hepatectomy. There were no significant differences in overall survival or recurrence-free survival rates between patients with high and low scores ($P = 0.37$ and $P = 0.39$, respectively).

than small tumors, and the low score group therefore had significantly larger tumor size and higher des-gamma-carboxy prothrombin level than the high score group. In contrast, non-cancerous hepatocytes had homogenous intensity of EGFR expression in their cytoplasm (Fig. 3b). The intensity score of non-cancerous cells was not significantly different between the high score group and the low score group (0.87 ± 0.53 *vs* 0.79 ± 0.55, $P = 0.43$). Recurrence of HCC may be intrahepatic or extrahepatic. Intrahepatic recurrence is mainly caused by multicentric carcinogenesis due to multiple risk factors. We previously reported that hepatitis status, function of the remnant liver and specific gene expression in non-cancerous tissues are associated with the risk of multicentric tumors, and that tumor factors such as tumor size, histological grade and alpha-fetoprotein level are not associated with the risk of multicentric recurrence.[4,5,25] Failure to attenuate hepatic EGF expression in surrounding non-cancerous hepatic tissues is also associated with poor survival in patients with HCC.[26] This and the results of our immunohistochemical analysis suggest that EGFR expression in non-cancerous hepatocytes might have more impact on intrahepatic HCC recurrence after curative hepatectomy than EGFR expression in cancer cells.

Limitations of the present study were the small cohort size and the heterogeneity of our enrolled patients, such as disease free duration after achieved sustained virological response in interferon therapy. In addition, because not all of our recurrent patients received repeat hepatectomy, we could not histologically diagnose all recurrent tumors as intrahepatic metastasis or multicentric occurrence, and we regarded early recurrence ($\leq$2 years) as intrahepatic metastasis and late recurrence (>2 years) as multicenric occurrence in this study. A multicenter study that enables investigation of a large number of homogeneous cases will emphasize our findings.

In conclusion, EGF SNP *61 with the AA genotype was associated with a lower risk of recurrence after curative hepatectomy for HCC in patients with HCV infection than other genotypes, but the EGFR expression of cancer cells was not significantly associated with recurrence after hepatectomy.

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Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Autophagy enhances hepatocellular carcinoma progression by activation of mitochondrial β-oxidation

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Abstract
Background Several types of cancers, including hepatocellular carcinoma (HCC), show resistance to hypoxia and nutrient starvation. Autophagy is a means of providing macromolecules for energy generation under such stressed-conditions. The aim of this study was to clarify the role of autophagy in HCC development under hypoxic conditions.
Methods The expression of microtubule-associated protein 1 light chain 3 (LC3), which is a key gene involved in autophagosome formation, was evaluated in human HCC using immunohistochemistry and western blot. The relationship between LC3 and hypoxia-induced factor 1α (HIF1α) expression was examined using real-time PCR. In addition, human HCC cell line Huh7 was treated with pharmacological autophagy-inhibitor and inactive mutant of Atg4B ($Atg4B^{C74A}$) under hypoxic condition to evaluate the effects of hypoxia-induced autophagy on cell survival, intracellular ATP, and mitochondrial β-oxidation.
Results LC3 was significantly highly expressed in HCC as compared with noncancerous tissues. LC3 expression, correlated with HIF1α expression, was also significantly correlated with tumor size, and only in the context of large tumors, was an independent predictor of HCC recurrence after surgery. In addition, Huh7 treated with autophagy-inhibitor under hypoxia had lower viability, with low levels of intracellular ATP due to impaired mitochondrial β-oxidation.
Conclusions Autophagy in HCC works to promote HIF1α-mediated proliferation through the maintenance of intracellular ATP, depending on the activation of mitochondrial β-oxidation. These findings demonstrated the feasibility of anti-autophagic treatment as a potential curative therapy for HCC, and improved understanding of the factors determining adaptive metabolic responses to hypoxic conditions.

Keywords Autophagy · Cancer progression · Hepatocellular carcinoma

Abbreviations

AFP	Alpha-fetoprotein
Atg	Autophagy-related genes
ATP	Adenosine 5′-triphosphate
DCP	Des-gamma-carboxy prothrombin
HCC	Hepatocellular carcinoma
HIF1α	Hypoxia-induced factor 1α
ICG R15	Indocyanine green retention test at 15 min
LC3	Microtubule-associated protein 1 light chain 3
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
SD	Standard deviation
3MA	3-Methyladenine

Introduction

Hepatocellular carcinoma (HCC) is common and increasing in incidence worldwide [1–4]. HCC grows to a

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relatively large size, sometimes over 10 cm in diameter even when necrosis was observed [5] and it can easily reoccur after therapy [6]. Proliferating cancer cells in tumors growing to a large size require a good supply of nutrients and oxygen. Angiogenesis around tumors is one way of increasing blood flow to provide the required oxygen and energy to the growing tumor [7]. However, recent studies have revealed that nutrition levels, oxygen and glucose are frequently reduced in locally advanced tumors despite tumor vessels having been established [8, 9]. This suggests that the microvasculature around the tumor is structurally and functionally abnormal and not capable of supplying the blood flow needed for cancer cell growth. Furthermore, some aggressive malignant tumors, such as poorly differentiated HCC and pancreatic cancers, are clinically hypovascular [10]. Under these conditions, cancer cells are likely to encounter limited nutrients and oxygen. However, they can exhibit resistance to nutrient deprivation and continue to grow. The mechanisms by which cancer cells obtain energy sources when their external nutrient supply is limited remain is unclear.

Autophagy is a homeostatic mechanism that regulates the turnover of long-lived or damaged proteins and organelles, buffers intracellular constituents and supplies amino acids taken from degradation products of the autolysosome [11]. For the first step, the isolation membrane, a lipid bilayer structure, is developed and sequesters cytoplasmic materials such as organelles, to form autophagosomes. During this step, microtubule-associated protein 1 light chain 3 (LC3), one of the mammalian homologues of yeast autophagy-related gene (Atg) 8, is processed and activated by a ubiquitination-like reaction regulated by Atg7 and Atg3 [12]. First, LC3 proform is cleaved into a soluble form known as LC3-I, which is further modified into a membrane-bound form, LC3-II, and this is followed by recruitment into the autophagosomes. Thus, LC3 is a specific marker of autophagosome formation. Autophagosomes engulf organelles and then fuse with lysosomes to become mature autolysosomes. Accordingly, sequestrated materials are digested into amino acids in the autolysosomes by the lysosomal enzymes [13, 14].

To study the role of autophagy in HCC under hypoxia-induced metabolic stress, we examined LC3 expression as the main marker of autophagosomes, and the other corresponding autophagic genes, Atg5 and Beclin-1, in human tissue samples and HCC cell lines under hypoxic conditions. Our results suggest that high expression of autophagy has the potential to cause malignant tumors to grow in size under hypoxic condition and also promotes poor survival, which can be independently predicted by the autophagic gene LC3 in HCC.

Materials and methods

Human tissue samples

Samples from 102 patients who had undergone liver resection for HCC without preoperative treatment at the Department of Surgery and Science at the Kyushu University Hospital between January 1986 and December 2002 were analyzed using immunohistochemistry [15]. Samples from another 131 patients between January 2004 and March 2009 were analyzed by real-time polymerase chain reaction (PCR). There were no significant differences between the characteristics of HCC patients using immunohistochemistry analysis or real-time PCR analysis (Table S1). Details in Doc. S1.

Reagents and plasmid

3-Methyladenine (3MA) and an inactive mutant of Atg4B ($Atg4B^{C74A}$) were prepared as described previously [15, 16]. Details in Doc. S1.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining and immunofluorescence analysis was performed as previously described [17–20]. Immunoreactivity of cytoplasmic staining in the cancerous region was independently divided into two groups, positive and negative, by two liver pathologists. Positive staining was classified if even a small area of tissue was stained. Details in Doc. S1.

Protein extraction and western blot analysis

Protein extraction and western blot analysis were performed as previously described [21]. Details in Doc. S1.

Real-time PCR

Extraction of total RNA and real-time PCR was performed as previously described [22]. Primers used for real-time PCR are shown in Table S2 and details described in Doc. S1.

Electron microscopy

Analysis of electron microscopy was performed as previously described [20, 23, 24]. For quantification of autophagosome using electron micrographs, high-powered micrographs (×8000–10000) of 10 single cells from multiple distinct low-powered fields were obtained from each specimen [23]. Details in Doc. S1.

Cell culture under hypoxic conditions

For hypoxia treatments, human HCC cell lines were incubated in a humidified hypoxic workstation (MCO-5M, Sanyo, Osaka, Japan) with final oxygen concentrations of 0.1 % O_2 using a Clark-type polarographic electrode (Animas, Frazer, PA, USA). To defect autophagosome formation, cultured cells were treated with autophagy-inhibitor using two methods, 3MA and $Atg4B^{C74A}$, and pre-incubated for 24 h followed by incubation under hypoxia. Details in Doc. S1.

Quantification of intracellular ATP

Total cellular ATP concentration was quantitated using an ATP Detection Reagent kit (Toyo-ink, Tokyo, Japan) as previously described [25]. Details in Doc. S1.

Cytofluorimetric analysis of $\Delta\Psi_m$ and mitochondrial structure

Cytofluorimetric analysis of $\Delta\Psi_m$ and alteration of mitochondrial structure during hypoxia was performed as previously described [26, 27]. Details in Doc. S1.

Measurement of β-hydroxybutyrate

β-hydroxybutyrate concentration was spectrophotometrically assayed as previously described [28]. Details in Doc. S1.

Statistical analysis

All statistical analyses were performed using JMP statistical software version 7.01 (SAS Institute Inc., Cary, NC, USA). All experiments were independently performed three times in triplicate. All variables are expressed as the mean ± standard deviation (SD). Details in Doc. S1.

Results

Upregulation of LC3 expression and autophagy activation in HCC

In the cancer cells of 102 HCC resected tissues, LC3 immunoreactivity was observed in the cytoplasm in 49 of 102 specimens (Fig. 1a). LC3 immunoreactivity was not observed in noncancerous lesions in any specimens. Western blot analysis using a limited set of tissue samples consisting of four matched noncancerous and HCC tissues revealed that expression of LC3-II, a well-known marker of activated autophagy, was detected in the cancerous tissue of case 3 and 4, in which LC3 immunoreactivity was immunohistochemically observed in the cancer cells (Fig. 1b). p62 regulates ubiquitin-positive protein aggregates caused by autophagic deficiency. Considering the results that the expression of p62 were higher in Case 1 and 2 than in Case 3 and 4 both at the cancerous and noncancerous tissues, it also indicated that the autophagic activity were impaired even in the noncancerous tissue and highly impaired in cancerous lesions in the Case 1 and 2.

Relationship between LC3 expression and clinicopathological factors

The positive LC3 expression group had significantly higher serum tumor markers, AFP ($P = 0.0467$) and DCP ($P = 0.0454$), tumor size ($P = 0.0006$) and positive rates of portal vein invasion ($P = 0.0001$) than the negative LC3 expression group (Table 1). Unexpectedly, there were no correlation between the LC3 expression and viral infection. Some patients with HCV or HBV underwent the each pharmacological therapy such as interferon or nucleotide analog, therefore, the activities of hepatitis were difficult to be assessed accurately. That might be the reason why the autophagic activity was necessarily correlated with the presence of the hepatitis clinically.

Association of LC3 with HIF1α expression and HCC recurrence according to HCC tumor size

LC3 (Fig. 1c, $P = 0.0131$) and HIF1α (Fig. 1d, $P = 0.0089$) expression was correlated with tumor size. In the group with a tumor size of ≥3 cm (Fig. 1e), LC3 expression in the high HIF1α expression group was greater than in the low HIF1α expression group ($P = 0.0097$). In any case, LC3 expression in the positive recurrence group was higher than in the negative recurrence group (Fig. 1f, $P = 0.0088$), whereas, in the group with a tumor size of <3 cm, there was no difference in LC3 expression between the high- and low-HIF1α expression groups (Fig. 1g, h). These indicated that only in the case of larger tumors, in which hypoxia and poor nutritional conditions were indicated, the autophagic gene LC3 was highly expressed and that autophagy enhanced tumor growth and promoted HCC malignancy.

Association of high expression of LC3 with tumor size and poor HCC prognosis

For the entire patient group ($n = 131$), the disease-free survival rates of the positive LC3 group (49.0 % at 3 years and 34.7 % at 5 years) was lower than for the negative LC3 group (60.4 % at 3 years and 46.8 % at 5 years) ($P = 0.0056$) (Fig. 2a). In patients with a tumor size of

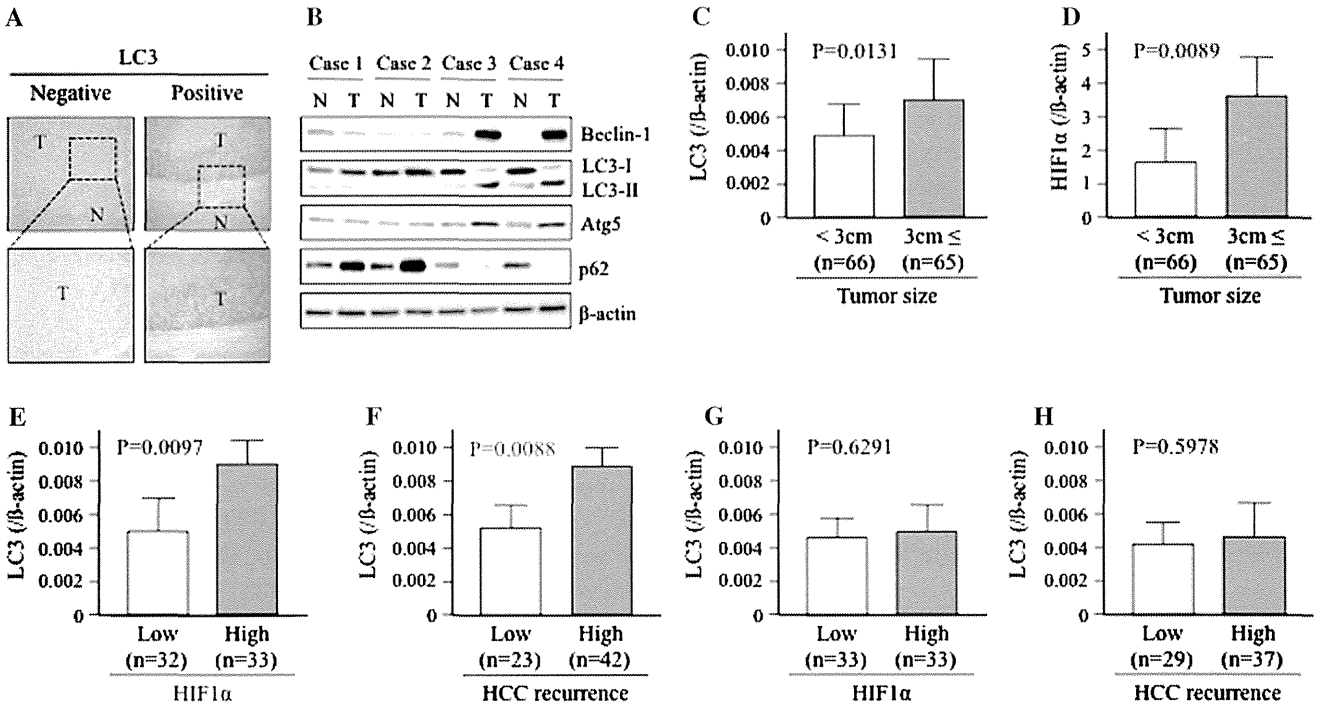

Fig. 1 Upregulation of LC3 protein in HCC human tissue samples (**a**, **b**) and LC3 and HIF1α expression associated with HCC tumor size (**c**–**f**). **a** LC3 immunoreactivity was observed in the cytoplasm of cancer cells, and hepatocytes in non-cancerous tissues were negative for LC3. **b** Western blot analysis of LC3 expression in the four matched HCC tissues and noncancerous tissues. Expression of LC3-II was detected in cancerous tissue in case 3 and 4, but no or only a faint signal was detected in non-cancerous tissue all of the cases. The expression of p62 was higher in case 1 and 2 than in case 3 and 4. **c**–**f** LC3 and HIF1α expression were evaluated by real-time PCR analysis of human HCC samples on the three cohorts: **c**, **d** the total patient population ($n = 131$); **e**, **f** patients with tumor sizes of ≥3 cm ($n = 65$); and **g**, **h** patients with tumor sizes of <3 cm ($n = 66$). They were also divided into two groups consisting of those with high and low expression of HIF1α with a cutoff value with a median of 2.85. LC3 (**c**) and HIF1α (**d**) expression was significantly correlated with tumor size (LC3, $P = 0.0131$; HIF1α, $P = 0.0089$). **e**, **f** In the group with a tumor size of ≥3 cm, LC3 expression in the high HIF1α expression group was significantly greater than that in low HIF1α expression group ($P = 0.0097$) and LC3 expression in the positive recurrence group was significantly higher than in the negative recurrence group ($P = 0.0088$). **g**, **h** In the group with a tumor size of <3 cm, there was no significant difference in LC3 expression between the high and low HIF1α expression groups, and between positive and negative recurrence groups. HCC, hepatocellular carcinoma; HIF1α, hypoxia-induced factor 1α; LC3, microtubule-associated protein 1 light chain 3

≥3 cm ($n = 65$), disease-free survival in the positive LC3 group (48.4 % at 3 years and 32.3 % at 5 years) was lower than in the negative LC3 group (82.6 % at 3 years and 59.8 % at 5 years) ($P = 0.0054$) (Fig. 2b). In contrast, in patients with a tumor size of <3 cm ($n = 66$), there was no difference (Fig. 2c).

Prognostic value of LC3 expression in HCC patients with a tumor size of ≥3 cm

The prognostic factors were evaluated in patients with a tumor size of ≥3 cm using univariate analysis (Table S3), which showed that three parameters namely, ICG R15 ($P = 0.0001$), multiple tumors ($P = 0.0416$) and portal vein invasion ($P = 0.0280$) were predictors of HCC recurrence. Positive LC3 expression was also a predictor of tumor recurrence ($P = 0.0103$). Furthermore, multivariate analysis was conducted with four of the variables (positive LC3 expression, ICG R15 ≥ 14.5 % as median values, multiple tumors and portal vein invasion), and there was no obvious correlation between them (Table 2). Positive LC3 expression was still the independent variable for predicting poor disease free survival ($P = 0.0065$).

Upregulation of LC3 expression in HCC cell lines under hypoxic conditions

Hypoxia markedly increased the number of autophagic vacuoles in Huh7 cells, which appeared as dot-like signals by immunofluorescence analysis (Fig. 3a, b) and electron microscopy (Fig. 3c, d). Expression of LC3-II increased in a time dependent manner (Fig. 3e). These initial observations indicated that HCC cell lines had the potential power to express autophagic activity in response to hypoxic conditions.

Table 1 Immunohistochemical analysis of the correlation between LC3 expression and clinicopathologic characteristics in patients

Variables	LC3 expression ($n = 102$)		P value
	Negative ($n = 53$)	Positive ($n = 49$)	
Age (years)	65 ± 9	62 ± 10	0.1703
Gender (male, %)	77.4	83.7	0.4206
HBs-Ag positive (%)	18.9	22.5	0.6551
HCV-Ab positive (%)	66.0	59.2	0.4744
Serum albumin (g/dL)	4.0 ± 0.4	4.0 ± 0.4	0.3922
Serum T-Bil (mg/dL)	0.9 ± 0.3	0.9 ± 0.3	0.9179
PT (%)	84.1 ± 17.2	87.2 ± 15.5	0.3881
AST (units/L)	49.9 ± 31.0	50.8 ± 24.8	0.8695
ALT (units/L)	51.3 ± 33.7	57.8 ± 51.0	0.4514
ICG R15 (%)	17.0 ± 9.1	15.9 ± 8.7	0.5286
Platelet (10^4/μL)	14.6 ± 7.9	14.9 ± 6.3	0.8053
Child-Pugh A/B, C (%)	86.3/13.7	82.1/17.9	0.5857
Serum AFP (ng/mL)	215 ± 568	14908 ± 63219	0.0468
Serum DCP (mAU/L)	1884 ± 7253	6284 ± 17143	0.0454
Liver cirrhosis (%)	23.5	20.4	0.7062
Tumor size (cm)	3.0 ± 0.4	5.0 ± 0.4	0.0006
Multiple tumors (%)	28.3	38.8	0.2620
Stage I, II/III, IV (%)	66.0/34.0	59.2/40.8	0.4744
Differentiation			
Well, moderate/poor (%)	75.5/24.5	61.2/38.8	0.1207
Portal vein invasion (%)	26.4	69.4	0.0001
Intrahepatic metastasis (%)	13.2	33.3	0.1520

Data are expressed as the mean ± standard deviation

AFP Alpha-fetoprotein, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *DCP* des-gamma-carboxyl prothrombin, *HBs-Ag* hepatitis B surface antigen, *HCC* hepatocellular carcinoma, *HCV-Ab* hepatitis C virus antibody, *ICG R15* indocyanine green retention rate at 15 min, *LC3* microtubule-associated protein 1 light chain 3, *PT* prothrombin time, *T-Bil* total bilirubin

Suppression of autophagy and proliferation of HCC cells under hypoxic conditions

LC3 expression was suppressed using two methods namely, pharmacological inhibition by means of 3MA and transfection of $Atg4B^{C74A}$, which involved type III PI3K inhibition and hampering of the conversion of LC3-I to LC3-II, respectively (Fig. S1). Subsequently, the down-regulation of autophagy under hypoxia inhibited the proliferation of HCC cells (Fig. 4a). In addition, the growth of Huh7 cells transfected with GFP-LC3 was higher than the cells receiving no treatment under hypoxic condition (Fig. 4a). These findings indicated that hypoxia-induced autophagy in HCC cells works to promote cell proliferation through preventing accumulation of damaged protein and organelles.

Suppression of autophagy activity and mitochondrial β-oxidation

The levels of intracellular ATP in autophagy-inhibited HCC cells were lower than in non-treated cells (Fig. 4b). The proportion of HCC cells with low mitochondria membrane permeability treated with autophagy-inhibitor was higher (Fig. 4c). Gene expression levels of MCAD and CPT, L-FABP, and FATP enzymes in autophagy-inhibited cells, which were indicated to have a rate-controlling effect on β-oxidation, the transportation of free fatty acids to mitochondria, and the transport of free fatty acids into hepatocytes, respectively, were significantly lower than those in non-treated cells (Fig. 4d). Consequently, β-hydroxybutyrate levels, final ketone body product, in autophagy-inhibited cells were decreased more than in non-treated cells (Fig. 4e). These findings indicated that HCC cells exposed to hypoxia had the potential power to maintain intracellular ATP through the activation of mitochondrial β-oxidation, which may have been due to the prompt removal of the damaged mitochondria by activated autophagy as a selective degradation system.

Discussion

In the present study, the inalterable role of autophagy in human HCC exposed to hypoxic conditions as tumors grew in size was demonstrated. The LC3 expression was correlated with tumor size, and only in large tumors, was correlated with the expression of HIF1α, hypoxia and the under nutrition marker. Additionally, high expression of LC3 was shown to be an independent predictor of HCC recurrence. Further, analysis of HCC cell lines using autophagy-inhibitor revealed that the hypoxia-induced autophagy in HCC cells worked to promote cell proliferation through maintenance of intracellular ATP. This depended on the activation of mitochondrial β-oxidation with the prompt removal of damaged mitochondria due to activated autophagy. This is the first report demonstrating the mechanism involved in maintaining the intracellular energy sources by means of activated autophagy, as HCC tumors developed in large size under hypoxic stress. Recently some reports demonstrated that the emerging role of autophagy for promoting cell viability in HCC progressions during ischemia-hypoxia condition only in the rodent models [29, 30]. We also demonstrated these results even in human samples clinically and emphasized that 'only in the large tumors' in which the impact of hypoxia and poor conditions were involved, the autophagic activity

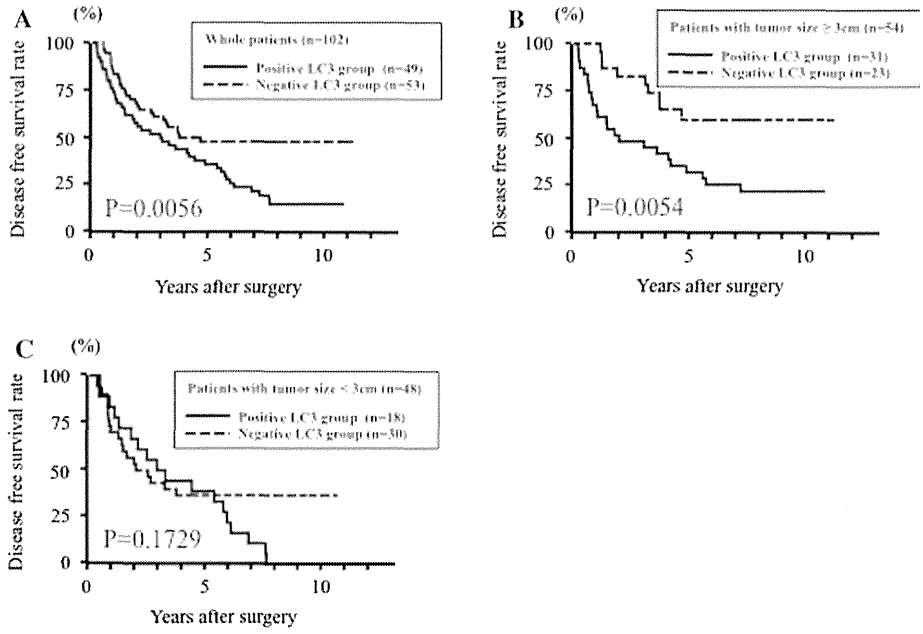

Fig. 2 Association between LC3 expression and patients' prognosis depending on tumor size of HCC. The disease-free survival after surgery were compared between the positive and negative LC3 expression groups by immunohistochemical analysis. The analysis was performed on three cohorts: **a** the total patient group ($n = 102$); **b** patients with tumor sizes of ≥3 cm ($n = 54$); and **c** patients with tumor sizes of <3 cm ($n = 48$). **a** For the entire patient group, the disease-free survival rates of the positive LC3 group (49.0 % at 3 years and 34.7 % at 5 years) was significantly lower than that of the negative LC3 group (60.4 % at 3 years and 46.8 % at 5 years) ($P = 0.0056$). **b** In the patients with a tumor size of ≥3 cm, disease-free survival in the positive LC3 group (48.4 % at 3 years and 32.3 % at 5 years) was significantly lower than in the negative LC3 group (82.6 % at 3 years and 59.8 % at 5 years) ($P = 0.0054$). **c** In patients with a tumor size of <3 cm there was no significant difference between the two groups. *HCC* hepatocellular carcinoma, *LC3* microtubule-associated protein 1 light chain 3

Table 2 Multivariate analysis of risk factors related to postoperative recurrence in patients with HCC tumors of ≥3 cm

Variables ($n = 75$)	Odds ratio	95 % CI	P value
ICG R15 ≥ 14.5 (%)	6.306	2.471–22.704	0.0001
LC3 Positive vs. negative	2.962	1.333–7.841	0.0065
Multiple tumors Positive vs. negative	2.626	1.152–7.210	0.0202
Portal vein invasion Positive vs. negative	2.200	1.012–5.439	0.0465

CI Confidence interval, *HCC* hepatocellular carcinoma, *ICG R15* indocyanine green retention rate at 15 min, *LC3* microtubule-associated protein 1 light chain 3

was highly expressed and its enhancement for the tumor growth and promotion of HCC malignancy, therefore, the newly targeted therapy for autophagy pathway of these adaptive metabolic responses is desired to becomes major challenges to overcome the large sized HCC tumors.

The role of autophagy in cell fate decision remains controversial. Autophagy is claimed to be an indispensable physiological reaction that sustains cell viability under nutrient-starved conditions [31]. Regarding cancer cells, it has been reported that autophagy was highly expressed in many of these cells and that this high expression was a strong factor related to tumor progression [32]. However, autophagy has recently attracted attention in connection with programmed or autophagic cell death. Colell et al. [33] demonstrated that cell death resulting from progressive cellular consumption can be attributed to unrestrained autophagy, which has led to the belief that autophagy is a nonapoptotic form of programmed cell death [34, 35]. However, the role of autophagy, an alternative caspase-independent cell death program, and its underlying molecular mechanism, is still controversial in cancer, especially in tumor progression. About the mTOR pathway, mTOR inhibitors such as RAD001, also an autophagy inducer, does not promote the proliferation of HCC cells and results in cell death [36, 37]. Weiner et al. [38], demonstrated that autophagy is observed in established cancers, but its inhibition during early carcinogenesis actually promotes tumor progression, suggesting that an autophagic switch promotes the transition of a tumor into a state of so-called autophagy addiction in order to maintain viability in hypoxic, nutrient-limited microenvironments. They also discussed that the autophagic function depends on the extent to which cells are capable of enhancing basal levels of autophagy. In fact, the hyperdynamic state of autophagy might be the crisis of cellular life because of its

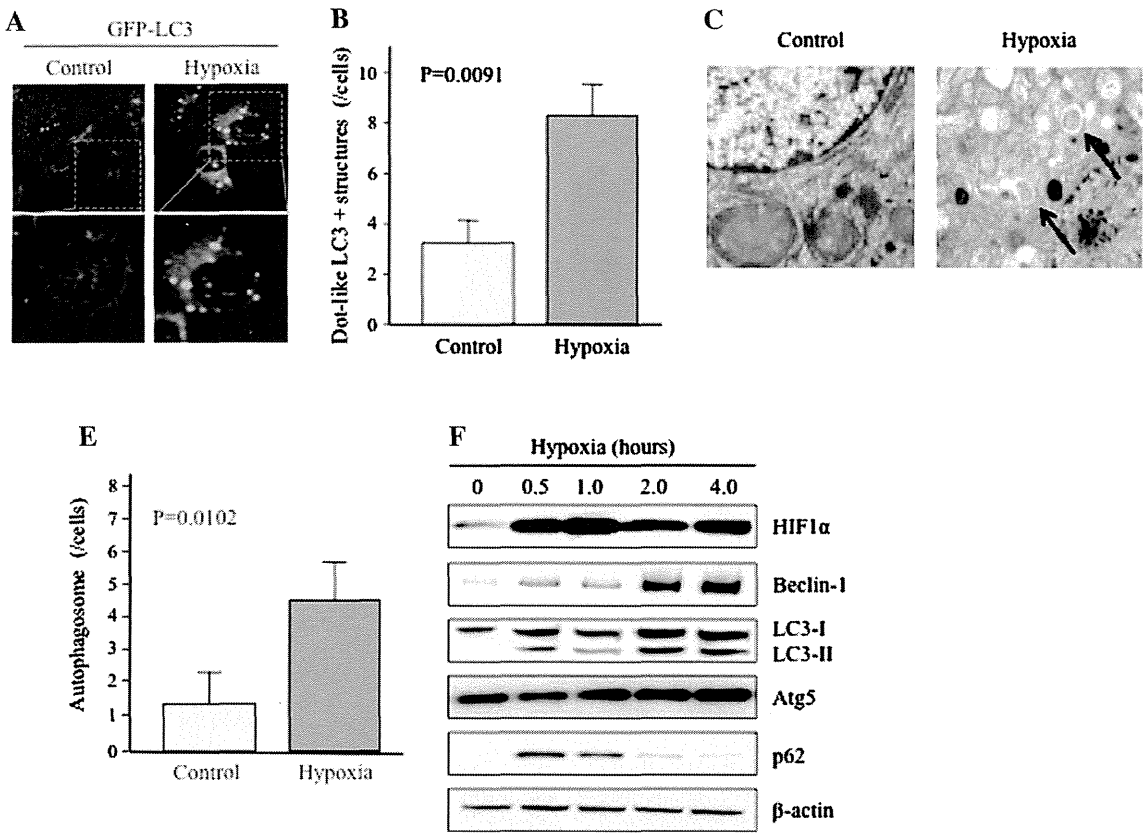

Fig. 3 Upregulation of LC3 protein in HCC cell lines under hypoxic conditions. **a**, **b** Huh7 cells exposed to 2 h hypoxic conditions had markedly increased numbers of autophagic vacuoles, which appeared as dot-like signals by immunofluorescence analysis using antibody against LC3. **c**, **d** Extensive autophagosome formation (*arrowed*) in Huh7 cells exposed to 2 h hypoxic conditions was monitored using electron microscopy. **e** The expression of LC3-II increased in a time-dependent manner when evaluated by western blot analysis using the proteins extracted from Huh7 cells under hypoxic conditions for the indicated times. The expression of the other autophagic genes, Beclin-1 and Atg5, also increased in a time-dependent manner. *Atg* Autophagy-related genes, *HCC* hepatocellular carcinoma, *LC3* microtubule-associated protein 1 light chain 3

consumption of the intracellular even healthy organelle and proteins. Considering the reports of mTOR inhibitors resulted in HCC cell death, the basal levels of autophagy in these setting of HCC cells and whether its inhibitor might excessively induce the autophagic activity or not should be carefully assessed because mTOR is a strong key component in a series of pathways involved in tumor growth and development. We revealed the protective role played by autophagy that involves proliferation of HCC cells due to activation of mitochondrial β-oxidation. Furthermore, we investigated the clinical significance of this finding regarding the link between autophagy and tumor progression. Three HIF1α-dependent molecular mechanisms have been reported by which cells adapt their energy metabolism under hypoxic conditions: inhibition of mitochondrial biogenesis by repression of c-Myc activity [39]; inhibition of acetyl-CoA synthesis by activation of PDK1 [40], and COX4 subunit switching [41]. We demonstrated that protection of mitochondria by autophagy is a fourth component of the HIF1α-mediated metabolic adaptation required to prevent cell death and damaged mitochondria, due to increased ROS levels in HCC cells under low nutrient conditions.

Ding et al. [42] demonstrated that only in an apoptosis compromised background, the expression of the autophagic gene, Beclin 1, and their corresponding autophagic activities were suppressed in HCC. They indicated that the loss of a survival pathway, autophagy, enhanced tumor growth by promoting genome damage and instability in an apoptosis-deficient background, a Bcl-xL positive background. However, their data depended on the assessment of the expression of Beclin-1 and there were multiple molecular machineries involved in the formation of the autophagosome downstream of Beclin-1. Therefore, it was not established whether autophagy was truly activated [39]. Recently, some reports demonstrated Beclin-1 independent autophagy, which acted as a caspase-independent cell death mechanism [43–45]. Additionally, Beclin-1 is the multifunctional gene involved in apoptosis [46, 47]. Thus, Beclin-1 plays a key role in autophagy,

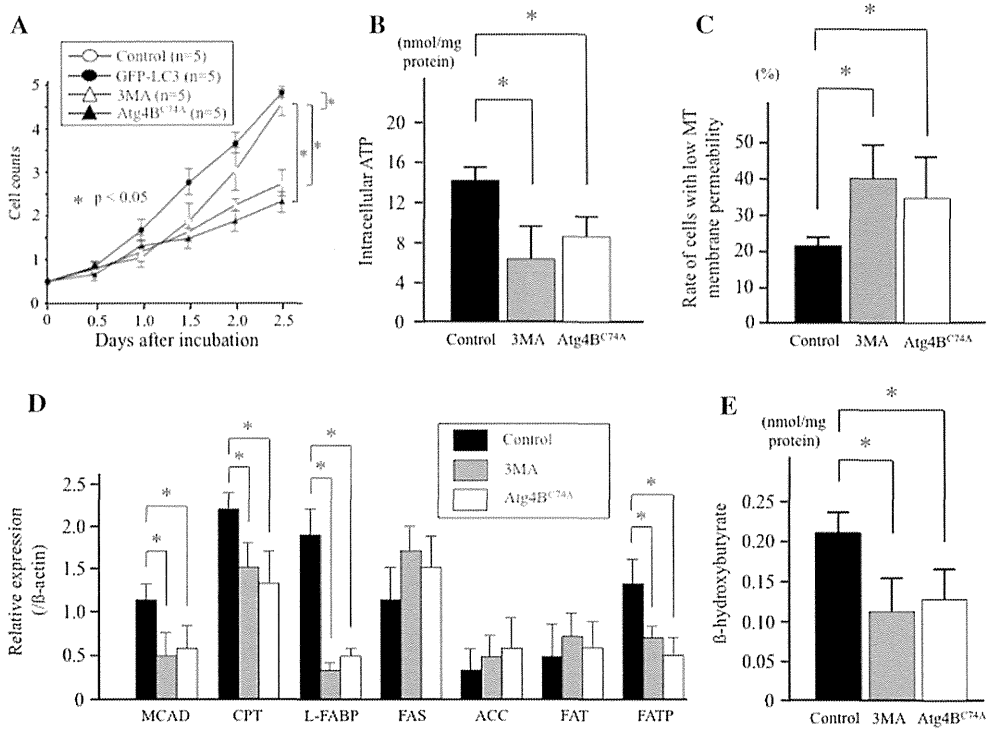

Fig. 4 Impaired proliferation of HCC cell lines treated with autophagy-inhibitor and deterioration of intracellular ATP maintenance through impairment of mitochondrial β-oxidation due to suppressed autophagy activity. Huh7 cells receiving no treatment or treated with autophagy-inhibitor using two methods, pharmacological inhibitor as 3MA and transfection of Atg4B^{C74A}, were incubated under hypoxic conditions of 0.1 % O_2 final concentration for 2 h. **a** Growth of both Huh7 cells treated using the two methods of autophagy-inhibition were significantly lower than that of the cells receiving no treatment under hypoxic conditions. In addition, the growth of Huh7 cells transfected with GFP-LC3 was higher than the cells receiving no treatment under hypoxic condition. **b** The intracellular ATP in the Huh7 cells treated with autophagy-inhibitor under hypoxic conditions was present at significantly lower levels than in cells receiving no treatment. **c** The proportion of Huh7 cells with low levels of mitochondrial membrane permeability treated with autophagy-inhibitor was higher than that of non-treated cells by by FACScan analysis using JC-1 antibody. **d** The gene expression related to mitochondrial β-oxidation in Huh7 cells were examined by real-time PCR. The levels of the enzymes MCAD and CPT, L-FABP, and FATP in Huh7 cells treated with autophagy-inhibitor were significantly lower than that in non-treated cells. **e** The intracellular β-hydroxybutyrate levels in Huh7 cells treated with autophagy-inhibitor under hypoxic conditions were significantly lower than in cells receiving no treatment. *ACC* Acetyl-CoA carboxylase, *ATP* adenosine 5′-triphosphate, *CPT* carnitine palmitoyltransferase, *FAS* fatty acid synthase, *FAT* fatty acid translocase, *FATP* fatty acid transport protein, *L-FABP* fatty acid binding protein, *LC3* microtubule-associated protein 1 light chain 3, *MCAD* medium-chain acyl-CoA dehydrogenase, *PCR* polymerase chain reaction, *3MA* 3-methyladenine

however, Beclin-1 should be carefully analyzed for the assessment of activated autophagy. The standard method for assessing autophagic activity is the demonstration of autophagic vesicles using electron microscopy, [48] and another method is the LC3-based assay. When autophagy occurs, conversion of a fraction of the cytosolic form of LC3-I to the autophagic membrane form of LC3-II can be detected using western blots of LC3 proteins. This change in intracellular localization of LC3 protein is considered to correlate with autophagic activity [49], and LC3 expression detected using immunohistochemistry represents the steady-state LC3 level including both LC3-I and LC3-II. We examined activated autophagy promptly with LC3-II using western blotting and confirmed the high expression of autophagosomes using an electron microscope.

Regarding the energy supply system in surviving cancer cells, several cancer cell lines including pancreatic cancer-derived and colorectal cancer-derived cell lines, are resistant to nutrient-deprived culture conditions [50]. These cells might use some alternative metabolic process to obtain energy for survival. However, untransformed human fibroblasts were completely abolished under the same conditions [51]. These indicated that this phenomenon of starvation-resistance may contribute to survival of cancer cells in nutrient-deficient microenvironments. As an alternative energy source, mammalian cells can use amino acids [52]. If the starvation-resistant cancer cells can raise amino acids from the inside of cells, these amino acids become potential energy sources. Autophagy is an evolutionarily conserved process involving lysosomal degradation of cytoplasmic and cellular organelles

[11, 26]. Autophagy constitutes a stress adaptation that avoids tumor death and has roles in protecting cells against the shortage of nutrients [26, 53]. Furthermore, autophagy is a catabolic process by which cells supply amino acids from self-digested organelles, as an alternative energy source for survival [11, 13, 31]. This function of autophagy seems ideal in fostering the survival of cancer cells in an unfavorable starved microenvironment. We demonstrated that autophagy plays an essential role by maintaining an optimal balance between the competing demands of energy and proliferation under hypoxic conditions. Whereas consideration of cellular energetics favors oxidative metabolism as the most efficient means of producing adequate levels of ATP to maintain cell survival, mitochondrial respiration is also associated with increased amino acid consumption due to autophagy. It was reasonable that the two aspects of HIF1α mediated-autophagic function, namely the protection of mitochondria and the supply amino acids from inside the cell contributed to cell survival in response to hypoxia.

For the recurrence of HCC after surgery, high expression of LC3 is the independent predictive factor in the context of larger sized tumors. Other predictive factors were liver dysfunction defined as ICG R15 < 14.5 %, multiple tumors and portal vein invasion. As HCC tumors grow, it becomes difficult to secure an exogenous energy supply, in particular for the central nest of tumors. However, in small HCC tumors with adequate oxygen supply and enriched nutrient conditions, cells did not struggle to obtain energy from an exogenous nutrient supply. For this reason, in patients with tumors of <3 cm, there was no correlation between LC3 expression, and HIF1α expression and tumor size. However, in patients with tumors of ≥3 cm, LC3 expression was significantly associated with HIF1α expression. To curatively treat HCC, an effective approach may be not only to inhibit the autophagy machinery, but also the HIF1α machinery molecule to prevent energy supply in conditions in which exogenous nutrient supply is extremely limited.

We have provided experimental evidence supporting the conclusion that HIF1α-mediated autophagy is involved in mitochondrial metabolism and is essential to understanding the mechanisms and consequences of the maintenance of intracellular energy in cancer cells under hypoxic conditions. This process requires the HIF1α-dependent induction of autophagy machinery as demonstrated by means of LC3-deficient HCC cell analysis. Understanding the factors determining which of these adaptive metabolic responses to conditions of hypoxia and under nutrition in HCC cells are utilized in vitro and in vivo, and whether these adaptations are successful in preventing cell death, remains a challenge.

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Conflict of interest The authors have no conflicts of interest to declare and have no financial interests linked to this work.

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Surgical Outcomes of Anatomical Resection for Solitary Recurrent Hepatocellular Carcinoma

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Abstract. *Background: For eradicating portal venous tumor extension and intrahepatic metastasis in hepatocellular carcinoma (HCC), anatomical resection is, in theory, preferable. Patients and Methods: We carried-out a retrospective cohort study in 110 patients who underwent curative hepatic resection (anatomical resection; n=20, and limited resection; n=90) for solitary recurrent HCC from 1990-2010. Results: No significant difference was found in short-term surgical results such as mortality, morbidity, and duration of hospital stay between the two groups. Anatomical resection did not influence overall and disease-free survival for all patients with a solitary recurrent HCC. In patients with cancer spread, such as pathological vascular invasion and intrahepatic metastasis (n=61), or with des-γ-carboxy prothrombin (DCP)≥100 mAU/ml (n=73), the disease-free survival rate in the anatomical-resection group was significantly better than that in the limited-resection group (p=0.0452 and p=0.0345, respectively). Conclusion: Anatomical resection should be recommended only for HCC suspected of exhibiting cancer spread as reflected by DCP ≥100 mAU/ml in patients with solitary recurrent HCC.*

Recently, anatomical resection such as subsegmentectomy is the standard treatment for hepatocellular carcinoma (HCC) (1-3). HCCs often invade the portal vein and form intrahepatic metastasis (4). Anatomical resection, in theory, can eradicate intrahepatic metastasis confined to tumor-bearing portal tributaries, and may have prognostic impacts for patients with HCC.

HCC has a high incidence of intrahepatic recurrence in up to 68-98% of patients after initial hepatic resection (5). Repeat hepatectomy has been reported to be safe and to prolong survival after intrahepatic recurrence of HCC(6-8). But anatomical resection cannot always be selected for patients with recurrent HCC because of its technical difficulty and the high recurrent potential of the remnant liver. There is no report on the surgical outcomes of anatomical resection for recurrent HCC in the literature.

We herein presented a retrospective analysis of surgical results including the prognosis of anatomical resection for a solitary recurrent HCC.

Patients and Methods

Patients' characteristics. One hundred and ten patients with solitary recurrent HCC after initial curative hepatic resection who underwent repeat hepatic resections at the Department of Surgery, Hiroshima Red Cross and Atomic Bomb Survivors Hospital, from January 1990 to December 2011 were retrospectively analyzed.

Surgical procedures. Our indications for hepatic resection for recurrent HCC were previously described (9, 10). Essentially, patients with recurrent HCC with disease-free survival (DFS) of one year or less after initial hepatic resection were considered to be contraindicated for repeat hepatic resection (8). The type of operative procedure was principally determined according to the preoperative indocyanine green retention rate at 15 min (ICG 15R); ICGR15 <20% for anatomical resection, and ICGR15 ≥20% for limited resection. Anatomical resection (n=20) includes hemihepatectomy, segmentectomy, and subsegmentectomy or more based on Couinaud's classification (11). The operative procedures conducted in the anatomical resection group are summarized in Table I. In segmentectomy and hemihepatectomy, we divided the liver along the

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Key Words: Recurrent hepatocellular carcinoma, anatomical resection, cancer spread, overall survival, disease-free survival, prognostic factors.

Table I. *Operative procedures of anatomical resection.*

Operative procedure	Subtotal	No
Lobectomy or more	2	
Extendsd or right liver		1
Extended or left liver		0
Caudate		1
Segmentectomy or more	7	
Central bi-segmentectomy		0
Left lateral segmentectomy		3
Medial segmentectomy		2
Anterior segmentectomy		0
Posterior segmentectomy		2
Subsegmentectomy or more	11	
S2		0
S3		2
S5		1
S6		2
S7		1
S8		4
S5+6		1
Total	20	

S: Subsegment defined by Couinaud' s nomenclature (11).

demarcation line appearing after the occlusion of extrahepatic Glisson's pedicle (12). In anatomical subsegmentectomy, we detected intrahepatic Glisson's branches charging the tumor-bearing area under intraoperative ultrasound guidance. We initially divided the liver toward the intrahepatic Glisson's branches, ligated and dissected the Glisson's branch near the pedicle, and finally divided the liver along the demarcation line without using Makuuchi's procedure involving the injection of dye in the portal vein (13). In limited resection (n=90), we divided the liver along a line so as to secure a surgical margin of at least 5 mm, if possible. When securing this margin was impossible, liver parenchymal transection was performed so as not to expose the tumor surface (14, 15).

There was no surgical mortality in any of 110 patients. Complications were evaluated by Clavien's classification of surgical complications, and those with a score of grade II or more were defined as positive (16).

Follow-up and treatment strategy for recurrent HCC. After discharge, all patients were examined for recurrence by ultrasonography and tumor markers such asα-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) every month and by dynamic computed tomography every three months (17). The median follow-up period after repeat hepatic resection was 4.2 years (range=0.7-10.0 years). When second or third recurrence was suspected, we treated by repeat hepatic resection according to the same indications for first recurrent HCC (9,10), ablation therapy, and liodolization (18).

Statistics. The survival curves of the anatomical and limited resection groups were generated by the Kaplan Meier method and compared by the log-rank test. To evaluate the good prognostic factors after repeat hepatic resection for recurrent HCC, we performed multivariate analysis with the Cox proportional hazard model, using

Table II. *Background characteristics, surgical outcomes, tumor-related or other factor.*

Variable	Limited (n=90)	Anatomical (n=20)	*p*-Value
Back ground characteristics			
Age, years	69±10	68±8	0.5630
Male:Female	61:29	15:5	0.5278
Diabetes mellitus (%)	24.4	25.0	0.9584
Albumin (g/dl)	3.9±0.4	4.2±0.4	0.0162
Total bilirubin (mg/dL)	0.8±0.4	0.7±0.2	0.1522
Prothrombin time (%)	87±16	93±12	0.1013
ICGR15(%)	20.2±9.8	17.5±11.0	0.2889
Child-Pugh A:B	85:5	20:0	0.2806
Liver Damage A:B	68:22	19:1	0.0531
Hepatitis B infection (%)	11.2	25.0	0.1064
Hepatitis C infection (%)	74.2	63.2	0.3312
Histological cirrhosis yes:no	61:29	9:11	0.0514
Surgical outcomes			
Surgical time (min)	203±85	281±120	0.0009
Surgical blood loss (g)	583±775	863±1198	0.1928
Transfusion (%)	13.3	25.0	0.1917
Resected volume (g)	36±39	130±81	<0.0001
Surgical margin (mm)	3±4	3±4	0.8107
Complication rate (%)	13	17	0.3910
Duration of hospital stay (days)	19±22	23 ±28	0.4772
Tumor related factors			
Tumor size (cm)	1.9 ±0.8	2.3±1.0	0.0362
Cancer spread: yes:no	47:43	16:4	0.0213
Well or Mod : Poor	74:16	16:4	0.7541
α-fetoprotein (ng/ml)	230±859	136±455	0.6378
DCP (mAU/ml)	76±172	111±311	0.5077
Other factors			
Disease-free interval (years)	3.5±3.1	4.5±3.7	0.2431
Cancer spread of primary HCC yes:no	43:47	10:10	0.9805

ICG R15: Indocyanine green retention rate at 15 min; DCP: des-T-carboxy prothrombin; Mod: moderately-differentiated HCC; Poor: poorly-differentiated HCC.

a variable-selection method by the backward-elimination procedure. A value of $p<0.05$ was set as the cutoff for the elimination using the following 18 clinical, surgical, and tumor-related variables according to the previous reports (2,8,13,19-22): age (< *vs.* ≥65 years); diabetes mellitus (present *vs.* absent);, preoperative serum total bilirubin level (< *vs.* ≥1 mg/dl); ICG R15 (< *vs.* ≥20%); albumin level (> *vs.* ≤3.5 g/dl); Child-Pugh class (A *vs.* B); Liver damage (A *vs.* B) (15); background liver status as assessed histologically (cirrhosis *vs.* non-cirrhosis); preoperative AFP level (< *vs.* ≥100 ng/ml); preoperative DCP level (< *vs.* ≥100 mAU/ml); tumor size (< *vs.* ≥3.0 cm); cancer spread including pathological vascular invasion and intrahepatic metastasis (present *vs.* absent); tumor cell differentiation (well or moderately *vs.* poorly); surgical time (< *vs.* ≥300 min); surgical blood loss (< *vs.* ≥1000 g); operative procedure (anatomical *vs.* limited); intra-operative transfusion (present or absent); surgical margin (> *vs.* ≤5 mm).

Continuous variables were expressed as the mean±S.D. and compared using Student's *t*-test. Categorical variables were compared using the χ^2 test. All analyses were performed with

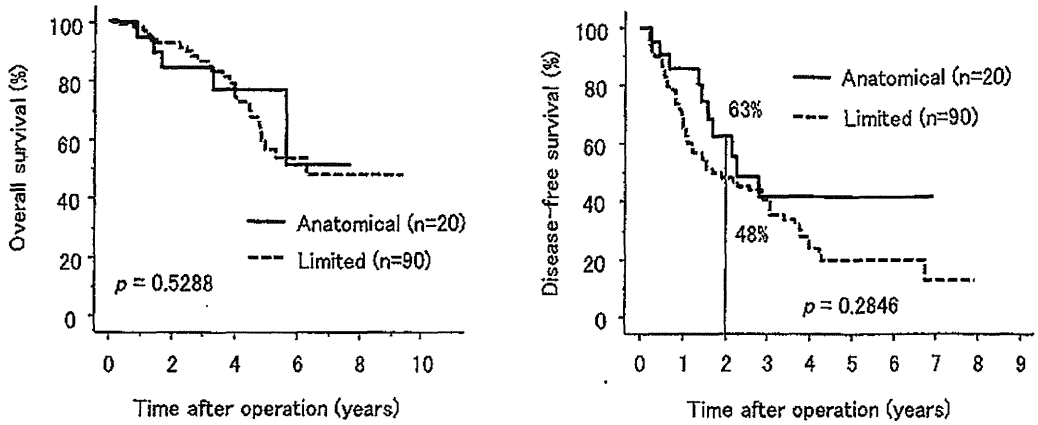

Figure 1. *Overall and disease-free survival curves after anatomical or limited resection for a solitary recurrent HCC (n=110).*

Statview 5.0 software (Abacus Concepts, Berkeley, CA, USA). A *p*-values less than 0.05 were considered to indicate statistical significance.

Results

The background characteristics, surgical outcomes, and tumor-related factors in the anatomical and limited-resection groups are summarized in Table II. In the anatomical resection group, the serum level of albumin was significantly higher than that in the limited-resection group (3.9±0.4 g/dl *vs.* 4.2±0.4 g/dl; p=0.0162). Although the difference was statistically not significant, there were more patients with Liver damage A (75.6% *vs.* 95.0%; p=0.0531) and less histological cirrhosis (67.8% *vs.* 45.0%; p=0.0514) in the anatomical-resection group than in the limited-resection group. As for the surgical outcomes, surgical time (203±85 min *vs.* 281±120 min; p=0.0009) and resected volume (36±39 g *vs.* 130±81 g; p<0.0001) were significantly higher in the anatomical-resection group than in the limited- resection group. But the intraoperative transfusion rate, complication rate, and duration of hospital stay were similar between the two groups. As for tumor-related factors, the tumor size in the anatomical-resection group was significantly larger than in the limited-resection group (1.9±0.8 cm *vs.* 2.3±1.0 cm; p=0.0362). In addition, there were significantly more patients with cancer spread in the anatomical-resection group than in the limited-resection group (52.2% *vs.* 80.0%; p=0.0213). But pathological tumor differentiation, the values of AFP and DCP were similar between the two groups. Briefly summarized, the anatomical-resection group included more patients with good liver function, more surgical stress, and advanced tumor stage.

The overall survival (OS) and disease-free (DFS) curves of the anatomical and limited resection groups are illustrated in Figure 1. The OS was similar in the two groups. The 2-year DFS rates were 48% in the limited resection group and 63% in the anatomical-resection group, but this difference was not statistically significant (p=0.2846). Multivariate analysis identified two factors reflecting good prognosis (Liver damage A, and ICGR15 <20%) influencing OS and three good prognostic factors (Liver damage A, Blood loss<1000 g, and without cancer spread) influencing DFS (Table III). Therefore, in our study, anatomical resection had no prognostic impact beyond that of limited resection for a solitary recurrent HCC.

Table III. *Multivariate analysis for the good prognostic factors after repeat hepatectomy.*

Variable	Hazard ratio	95% Confidence interval	*p*-Value
Overall survival			
Liver damage A	0.35	0.15-0.84	0.0184
ICG15R<2%	0.36	0.14-0.88	0.0265
Absence of cancer spread	0.43	0.18-1.01	0.0543
Tumor size<3.0 cm	0.58	0.34-1.41	0.2330
Disease-free survival			
Liver damage A	0.43	0.25-0.77	0.0043
Blood loss<1000 g	0.45	0.23-0.86	0.0163
Absence of cancer spread	0.51	0.26-0.99	0.0460
Absence of histological cirrhosis	0.74	0.43-1.25	0.2583

ICGR15: Indocyanine green retention rate at 15 min.

The OS and DFS curves of the anatomical (n=16)-and limited-resection (n=45) groups are illustrated in Figure 2 in patients with cancer spread. DFS was significantly better in the anatomical resection group than in the limited resection group (p=0.0452). The two-year DFS rates were 35% in the

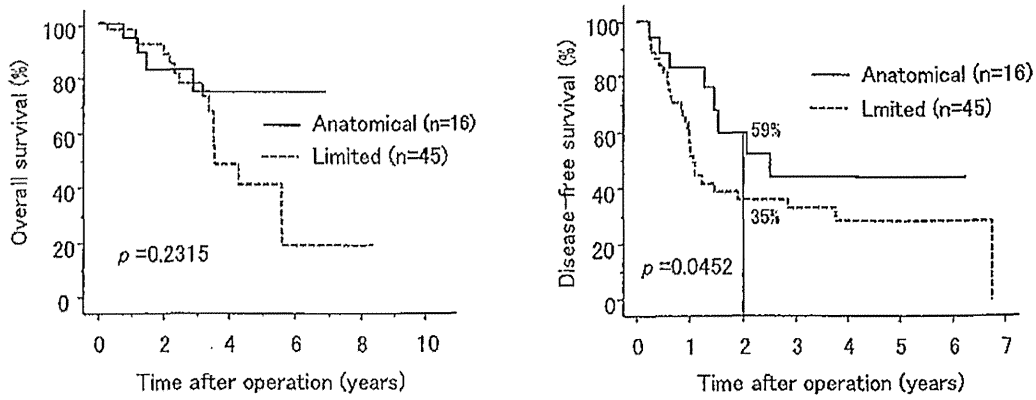

Figure 2. *Overall and disease-free survival curves after anatomical or limited resection for a solitary HCC in patients with cancer spread (n=61).*

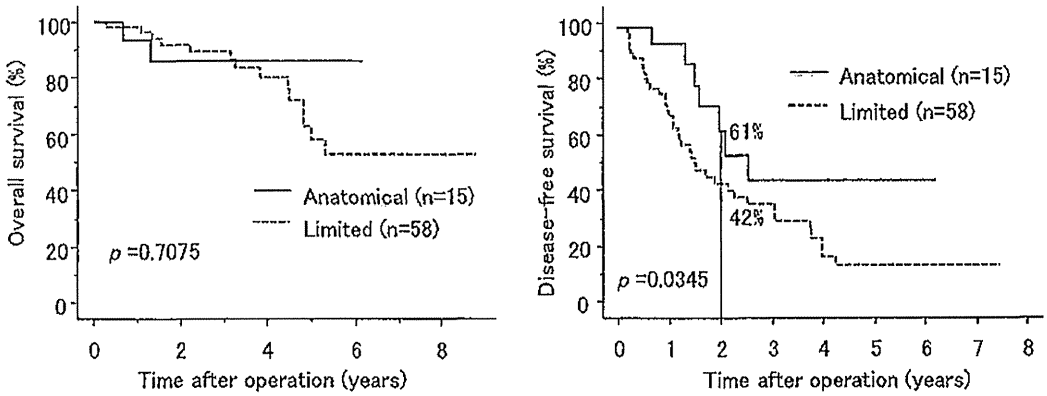

Figure 3. *Overall and disease-free survival curves after anatomical or limited resection for a solitary HCC in patients with DCP≥100 mAU/ml (n=73).*

limited-resection group and 59% in the anatomical-resection group, respectively.

For patients with DCP ≥100 mAU/ml (n=73), the OS and DFS curves of the anatomical (n=15) and limited resection (n=58) groups are illustrated in Figure 3. The DFS was significantly better in the anatomical-resection group than in the limited-resection group (p=0.0345). The two-year DFS rates were 42% in the limited-resection group and 61% in the anatomical-resection group.

Discussion

Repeat hepatic resection for recurrent HCC was first reported to be effective more than two decades ago, and it is still the main option for recurrent HCC treatment (6-8). However, there was no evidence that anatomical resection should be recommended for recurrent HCC in the literature. As far as we are aware of, this is the first report on the surgical outcomes of anatomical resection for recurrent HCC. In our retrospective analysis of 110 patients with a solitary recurrent HCC, anatomical resection was safely performed but the prognostic impacts of anatomical resection were not confirmed.

In patients with recurrent HCC, anatomical resection would be a difficult procedure because of the intra-abdominal adhesions, especially around the hepatic hilum, caused by previous hepatic resection. This was true in our series, in which the surgical time was significantly prolonged in the anatomical-resection group (203±85 min *vs.* 281±120 min; p=0.0009). However, there were no significant differences in short-term surgical results such as complication rate (13% *vs.* 17%; p=0.3910) and the duration of hospital stay (19±22 days *vs.* 23±28 days; p=0.4772) between the two groups. According to the patient backgrounds shown in Table II, patients in the anatomical-resection group showed a better preservation of liver function. We previously reported that liver dysfunction was a strong predictive factor linked to postoperative mortality and

morbidity (23). The maintenance of short-term surgical results in the anatomical-resection group is attributable to the adequate selection of candidates for anatomical resection for recurrent HCC.

In the treatment of HCC, the eradication of intrahepatic metastasis *via* vascular invasion is one of the most important considerations (4). Anatomical resection such as subsegmentectomy taking into consideration both the eradication of intrahepatic metastasis and preservation of liver functional parenchyma would be a theoretically reasonable procedure (1-3). However, in our retrospective analysis, anatomical resection had no prognostic impacts for recurrent HCC, as shown in Figure 1. As shown in Table III, multivariate analysis identified lack of cancer spread as a good prognostic factor influencing DFS. There were significantly more patients with cancer spread in the anatomical resection group than in the limited resection group (52.2% *vs.* 80.0%; p=0.0213). Therefore, there is a possibility that anatomical resection for recurrent HCC leads to favorable DFS in patients with highly recurrent HCC after repeat hepatic resection.

For patients with cancer spread (n=61), DFS was significantly better in the anatomical-resection group than in the limited-resection group (p=0.0452). This result may indicate that anatomical resection also had greater potential for the eradication of intrahepatic metastasis *via* vascular invasion than limited resection also in patients with recurrent HCC. However, anatomical resection was associated with significantly higher surgical stress, including greater surgical time (203±85 min *vs.* 281±120 min; p=0.0009) and resected volume (36±39 g *vs.* 130±81 g; p<0.0001), and the high surgical stress and the decrease of the remnant hepatic reserve resulting from anatomical resection had adverse effects on patient prognosis (15, 24). Anatomical resection, of course, has no beneficial effect against multicentric recurrence. Patients with recurrent HCC after repeat hepatic resection would have high recurrent potential in the remnant liver compared to those with primary HCC (8, 25). Therefore, the better local control of anatomical resection did not lead to better DFS in patients with recurrent HCC.

We have previously reported that a high DCP level could predict cancer spread of HCC (26-28). Koike *et al.* performed a prospective study to clarify the significance of DCP and concluded positivity of DCP to be the strongest predictor for portal vein invasion (29). In patients with DCP ≥100 mAU/ml in our series, DFS was significantly better in the anatomical-resection group than in the limited-resection group (p=0.0452). DCP≥100 mAU/ml should be one of the criteria indicating anatomical resection for recurrent HCC.

In conclusion, anatomical resection should be recommended only for HCC cases suspected of having cancer spread, as reflected by DCP ≥100 mAU/ml in patients with recurrent HCC.

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