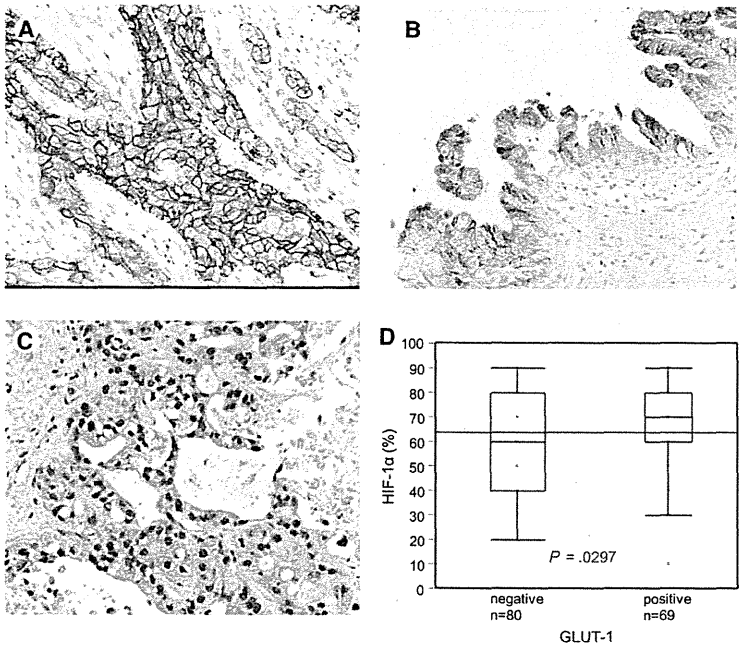

Fig. 1 GLUT-1 (A), GLUT-2 (B) and HIF-1α (C) expression in cholangiocarcinoma (×200). HIF-1α expression in perinecrotic area (C). D, Correlation between GLUT-1 and HIF-1α expression. The GLUT-1–positive group has a significantly higher percentage of HIF-1α–positive cells than the GLUT-1–negative group ($P = .0297$).

2.6. Statistical analysis

Statistical analysis of group differences was performed by the χ^2 test, Fisher exact test, and the Mann-Whitney U test. Patient survival was defined as the period of survival between surgery and the date of the last follow-up or until death due to the disease. The Kaplan-Meier method was used for the survival analysis, and comparisons were made based on the log-rank test. For multivariate analysis, the Cox proportional hazards model was used. $P < .05$ was considered statistically significant.

3. Results

3.1. Comparison of GLUT-1 and GLUT-2 expression and clinicopathological findings

In intrahepatic cholangiocarcinoma, GLUT-1 membranous expression was frequently observed near the necrotic areas (Fig. 1A), and GLUT-2 membranous and/or cytoplasmic expression was detected in the adenocarcinoma of perihilar location (Fig. 1B), in particular, non-invasive lesions. Red blood cells were strongly positive for GLUT-1, and hepatocytes were positive for GLUT-2. The percentage of the GLUT-1 positive group was 46% (69/149), and that of the GLUT-2 positive group was 21% (31/149). Compared with the GLUT-1–negative group, the GLUT-1–positive group showed larger tumor size ($P = .0031$), more frequent poor differentiation ($P < .0001$), extensive necrosis ($P < .0001$), more frequent lymphatic invasion ($P = .0031$), perineural invasion ($P = .0293$), and lymph node metastasis ($P < .0001$). Compared to the GLUT-2–negative group, the GLUT-2–positive group showed smaller tumor size ($P = .0326$), more frequent well differentiation ($P = .0015$), perihilar location ($P < .0001$), non–mass-forming type ($P < .0001$), perineural invasion ($P = .0049$), and lymph node metastasis ($P = .0248$) (Table 2).

The GLUT-1–positive group had a significantly higher percentage of HIF-1α–positive cells than the GLUT-1–negative group (GLUT-1–positive group, 68.0 ± 2.4%;

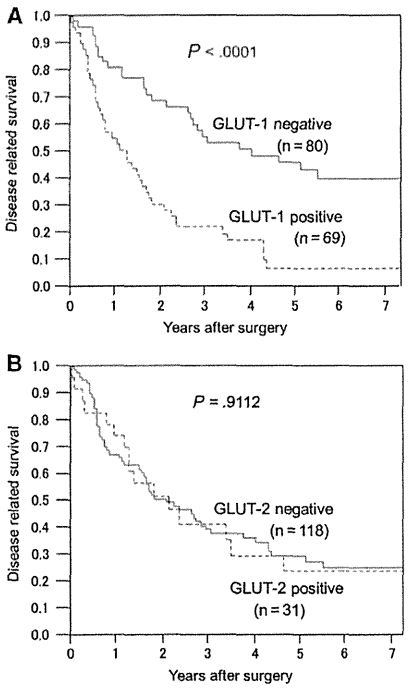

Fig. 2 A, The survival rates of patients with GLUT-1–positive expression were significantly worse than those of patients with GLUT-1–negative expression ($P < .0001$). B, There were no statistical differences between the survival rates of GLUT-2–positive expression and patients with GLUT-2–negative expression ($P = .9112$).

GLUT-1–negative group, 60.8 ± 2.2%; $P = .0297$) (Fig. 1D), but there is no statistical difference between GLUT-2 expression and the percentage of HIF-1α–positive cells.

3.2. Post-operative survival analysis

Patients in the GLUT-1–positive group showed significantly poorer survival compared with the negative group ($P < .0001$) (Fig. 2A), whereas there were no differences between survival rates for the two groups in terms of GLUT-2 expression ($P = .9112$) (Fig. 2B). Univariate analysis indicated that tumor size, tumor differentiation, tumor necrosis, lymphatic invasion, perineural invasion, and lymph node metastasis were significant prognostic factors (Table 3). Multivariate analysis revealed that lymphatic invasion and lymph node metastasis were independent prognostic factors (Table 3).

3.3. The effects of GLUT-1 inhibition on cell migration and invasion

Quantitative real-time RT-PCR analysis revealed down-regulation of GLUT-1 mRNA in RBE cells transfected with GLUT-1 siRNA compared with those transfected with control siRNA (Fig. 3A). The number of migrating and invading cells was significantly lower in GLUT-1 siRNA transfectants than in their control counterparts (Fig. 3B, migration assay; control siRNA 117.8 ± 11.3, GLUT-1 siRNA 63.5 ± 11.3, $P = .0276$) (Fig. 3C, invasion assay; control siRNA 110.5 ± 7.1, GLUT-1 siRNA 24.9 ± 7.1, $P = .001$).

3.4. Expression of GLUT-1 and GLUT-2 in BilIN

Normal biliary epithelium is negative for GLUT-1 and GLUT-2 expression. GLUT-1 was expressed in 30% (6/20) of BilIN-1, 45% (5/11) of BilIN-2, and 25% (2/8) of BilIN-3. GLUT-2 was expressed in none (0/20) of BilIN-1, 45% (5/11) of BilIN-2, and 50% (4/8) of BilIN-3. GLUT-2 expression is significantly higher in BilIN-2 and BilIN-3 than in BilIN-1 ($P = .0004$), whereas there was no statistical difference in the expression of GLUT-1 in BilINs (BilIN1 vs. BilIN2 and BilIN3, $P = .9046$) (Fig. 4).

4. Discussion

GLUT-1 expression correlates with poor outcome in several digestive cancers, such as hepatocellular carcinoma [14], pancreatic carcinoma [15], gallbladder cancer [16], esophageal cancer [17], gastric cancer [18], colon cancer [19], and rectal cancer [20]. A previous study showed that GLUT-1 is expressed especially in moderately to poorly differentiated cholangiocarcinoma [12]. However, no study has evaluated the relationship between GLUT-1 expression and outcomes in cholangiocarcinoma. In the current study, we demonstrated that GLUT-1 expression was correlated with higher malignant potential and, only in the univariate analysis, was a poor prognostic factor in cholangiocarcinoma.

In ovarian cancers, HIF-1α expression is associated with GLUT-1 expression, but GLUT-1–positive areas are narrower, more heterogeneous, and more localized than areas that extensively express HIF-1α [21]. Similarly, our results show that GLUT-1 expression is correlated with HIF-1α expression and that GLUT-1–positive areas, such as perinecrotic areas, are more limited than HIF-1α–positive areas. GLUT-1 expression is also reported to be regulated in a HIF-1α–independent manner, such as c-Myc, Akt, *KRAS/BRAF* mutation, and miR-1291 [22-25].

These days, GLUT inhibitors are being tested in Phase 1 and Phase 2 trials, such as in osteosarcoma, non–small cell lung cancer and prostate cancer [6,26]. In previous studies, Amann et al proposed GLUT-1 as a therapeutic target for

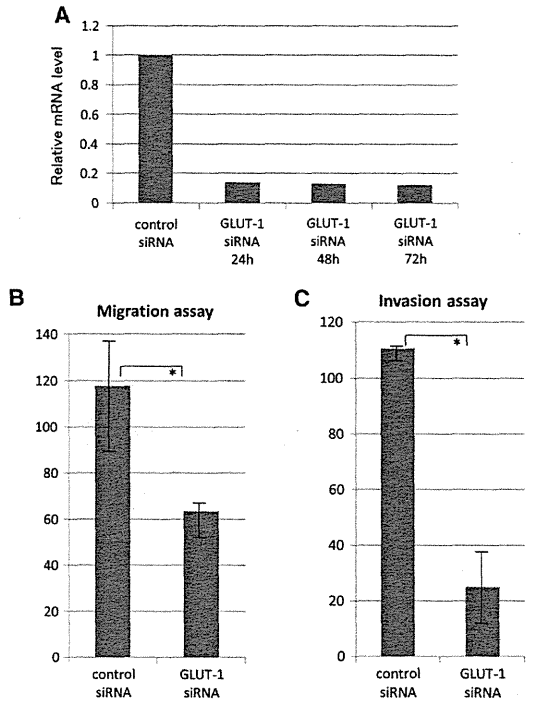

Fig. 3 Effects of GLUT-1 inhibition using GLUT-1 siRNA on migration and invasion of cholangiocarcinoma cells. A, GLUT-1 mRNA level of RBE cells transfected with GLUT-1 siRNA relative to transfected control siRNA. Analysis of migration (B) and invasion (C) potential of these cells. Down-regulation of GLUT-1 significantly inhibited migration and invasion in RBE cells. Vertical axis showed the migration (B) and invasion (C) cells/field. $^*P < .05$.

hepatocellular carcinoma [6,27]. GLUT-1 not only affects glucose uptake and utilization but also influences tumorigenic features such as metastasis, chemoresistance, and escape from immune surveillance [6]. More recent reports suggest that GLUT-1 is a potential new therapeutic target for laryngeal squamous cell carcinoma [28]. In our study, the inhibition of GLUT-1 using GLUT-1 siRNA showed decreased cell migration and invasion potential in cholangiocarcinoma cells. Our results indicate that GLUT-1 may play an important role in aggressive behavior and tumor invasiveness in cholangiocarcinoma cells. Furthermore, we expect GLUT-1 to be a novel therapeutic target in cholangiocarcinoma.

GLUT-2 expression is high in perihilar, non-mass–forming types (periductal infiltrating type and intraductal growth type) of cholangiocarcinoma, as well as in high-grade BilINs. BilIN is a premalignant or noninvasive lesion of perihilar-type cholangiocarcinoma [29]. Therefore, GLUT-2 may be associated with carcinogenesis of large bile ducts. It is often difficult to arrive at a differential diagnosis of a BilIN lesion solely through morphology. A previous study showed that S100P may be a useful differential marker of precursor lesions of cholangiocarcinoma [30]. Another study documented that pancreatic intraepithelial neoplasia (PanIN)-1B and higher grade PanINs showed extensive GLUT-2 expression, and that GLUT-2 expression may be useful for detecting precursor lesions of pancreatic carcinoma [31]. In our study, all the BilIN-1 lesions and normal bile ducts were negative for GLUT-2 expression, whereas half of high-grade BilIN lesions were strongly positive. GLUT-2 is not fully sensitive but rather a highly specific stain for high-grade precursor lesions of invasive cholangiocarcinoma, and it may be a helpful diagnostic marker of atypical biliary lesions. Although 21% of cholangiocarcinomas were positive for GLUT-2, there were few positive cells in the invasive areas of the GLUT-2–positive cases described above. Therefore, GLUT-2 may be associated with an early stage of carcinogenesis from high-grade neoplasia to invasive cholangiocarcinoma. However, the total number of BilIN cases is small in our study; thus, a further large-series study is needed.

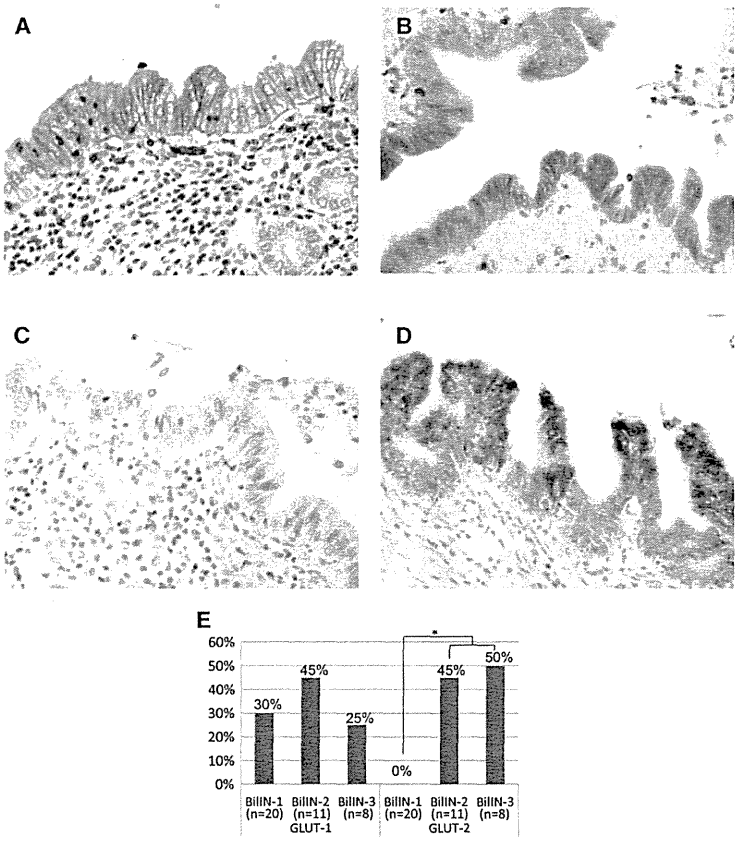

Fig. 4 GLUT-1 and GLUT-2 expression in BilIN. GLUT-1 expression in BilIN-1 (A) and BilIN-2 (B) (×200). GLUT-2 expression in BilIN-1 (C) and BilIN-2 (D) (×200). E, The positive rate of GLUT-1 and GLUT-2 expression in BilIN. All cases of BilIN-1 are negative for GLUT-2 expression. *$P < .05$ (BilIN-1 vs. BilIN2 and BilIN-3).

In summary, we suggest that GLUT-1 expression correlates with higher malignant potential and poor outcome and that the inhibition of GLUT-1 might be a beneficial therapeutic strategy for cholangiocarcinoma. GLUT-2 expression is associated with cholangiocarcinogenesis of the large bile duct and may help to identify high-grade BilINs from atypical bile ducts.

Acknowledgments

The authors appreciate the technical support from Ms. Tateishi and the Research Support Center, Graduate School of Medical Sciences, Kyushu University. English usage in this paper was reviewed by KN International.

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Cancer Science
Japanese Cancer Association JCA
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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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Funding Information
Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 24390320).

Received February 23, 2014; Revised March 24, 2014; Accepted April 3, 2014

Cancer Sci 105 (2014) 646–650

doi: 10.1111/cas.12415

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

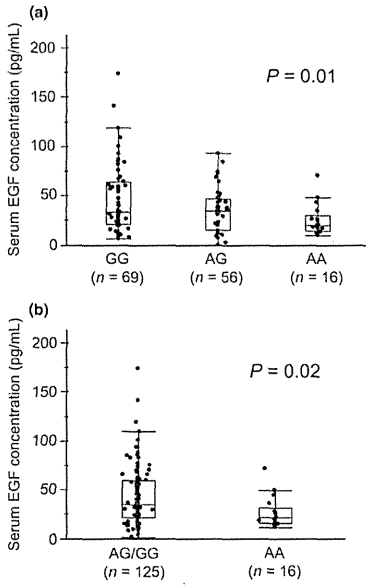

Fig. 2. Comparisons of serum epidermal growth factor (EGF) concentration. (a) There was a significant difference in serum EGF concentration among the three genotypes ($P = 0.01$). (b) The AA group had a significantly lower serum EGF concentration than the AG/GG group ($P = 0.02$).

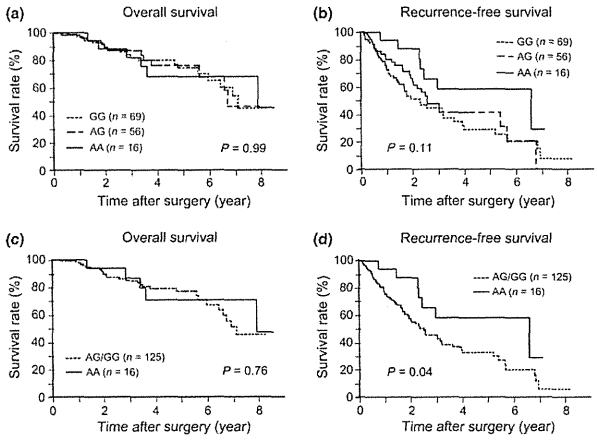

Fig. 1. Comparisons between patients carrying the AA allele and patients carrying other alleles. The AA group had a significantly higher recurrence-free survival rate than the AG/GG group ($P = 0.04$).

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

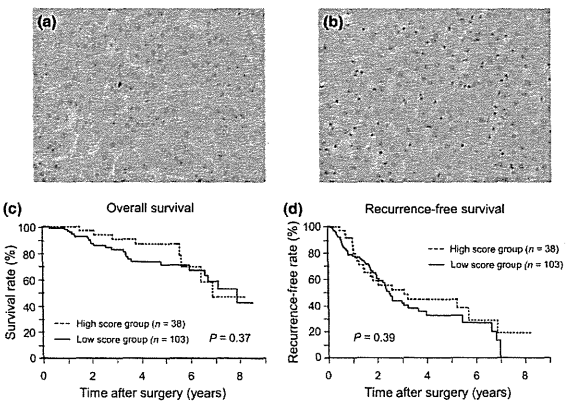

Fig. 3. Immunohistochemical staining of resected liver tissues for epidermal growth factor receptor (EGFR) (original magnification ×400). (a) Cancer cells showing EGFR expression in the cytoplasm and cell membranes. (b) Non-cancerous hepatocytes, showing EGFR expression in the cytoplasm. (c,d) Associations 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 (≤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.

Acknowledgements

This study was supported in part by a Grant-in Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 24390320).

Disclosure Statement

The authors have no conflict of interest.

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