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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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; ICGR₁₅, 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-
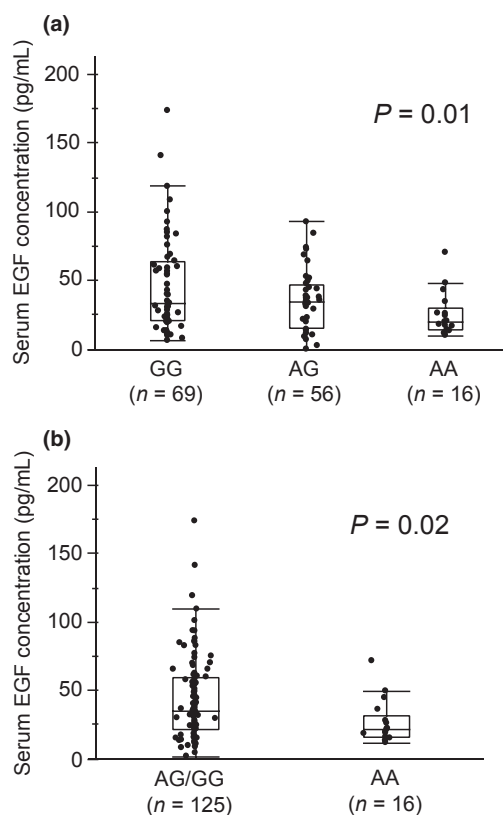
Fig. 2. Comparisons of serum epidermal growth factor (EGF) concentration. (a) There was a significant difference in serum EGF concentration among the three genotypes ($P = 0.01$). (b) The AA group had a significantly lower serum EGF concentration than the AG/GG group ($P = 0.02$).

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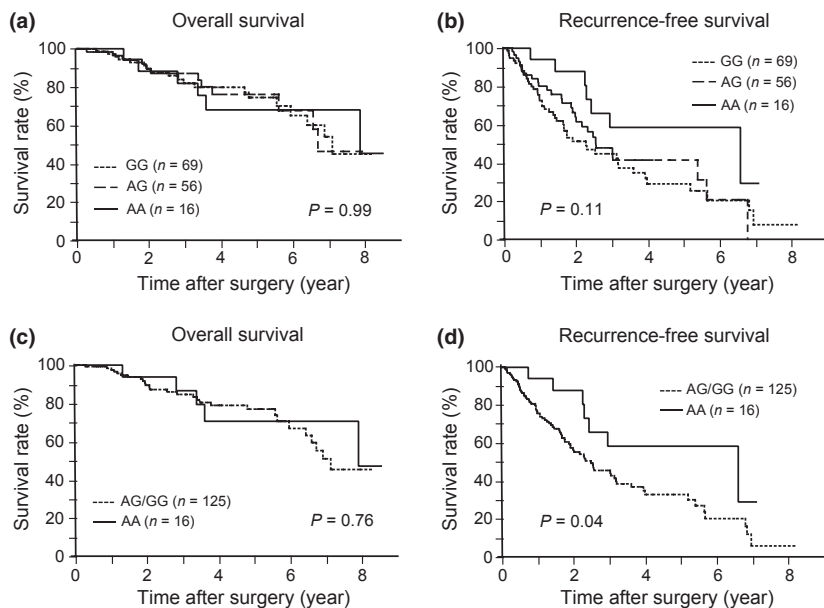
Fig. 1. Comparisons between patients carrying the AA allele and patients carrying other alleles. The AA group had a significantly higher recurrence-free survival rate than the AG/GG group ($P = 0.04$).

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; ICGR₁₅, 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
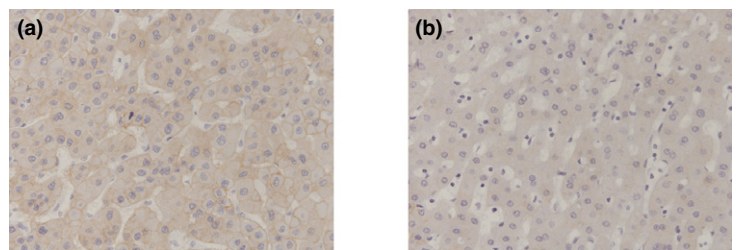

Fig. 3. Immunohistochemical staining of resected liver tissues for epidermal growth factor receptor (EGFR) (original magnification ×400). (a) Cancer cells showing EGFR expression in the cytoplasm and cell membranes. (b) Non-cancerous hepatocytes, showing EGFR expression in the cytoplasm. (c,d) Associations 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).
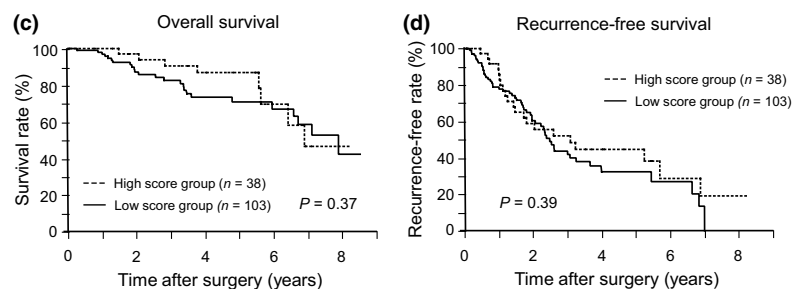
than small tumors, and the low score group therefore had significantly larger tumor size and higher des-gamma-carboxy prothrombin level than the high score group. In contrast, non-cancerous hepatocytes had homogenous intensity of EGFR expression in their cytoplasm (Fig. 3b). The intensity score of non-cancerous 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

Disclosure Statement

The authors have no conflict of interest.

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