Original Research

A functional polymorphism in the epidermal growth factor gene predicts hepatocellular carcinoma risk in Japanese hepatitis C patients

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Running header: EGF SNP in a Japanese HCC population

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Abstract

Background: A single nucleotide polymorphism (SNP) in the epidermal growth factor (EGF) gene (rs4444903) has been associated with increased risk of cancer including hepatocellular carcinoma (HCC). The aim of this study was to examine the relationship between EGF SNP genotype and the development and prognosis of HCC in a Japanese population.

Methods: Restriction fragment-length polymorphism was used to determine EGF SNP genotype in 498 patients including 208 patients with HCC. The level of EGF mRNA expression in cancerous tissues was measured by quantitative reverse transcription polymerase chain reaction. The correlation between EGF SNP genotype and prognosis was statistically analyzed in patients with HCC.

Results: The proportion of A/A, A/G and G/G genotype were 5.3%, 42.8%, and 51.9% in patients with HCC, whereas, in those without HCC they were 8.6%, 35.9%, and 55.5%, respectively, which revealed that the odds ratios (OR) of developing HCC was higher in patients with a G allele (OR = 1.94, $P = 0.080$ for A/G patients, OR = 1.52, $P = 0.261$ for G/G patients as compared with A/A patients). In particular, when the analysis was limited to the 363 patients with hepatitis C, the OR for developing HCC was 3.54 ($P = 0.014$) for A/G patients, and 2.85 ($P = 0.042$) for G/G patients as

compared with A/A patients. Tumoral EGF mRNA expression in G/G patients was significantly higher than that in A/A patients ($P = 0.033$). No statistically significant differences were observed between EGF SNP genotype and disease-free and overall survival.

Conclusion: The EGF SNP genotype might be associated with risk for development of HCC in Japanese patients but not with prognosis. Of note, the association is significantly stronger in patients with hepatitis C which is the main risk factor for HCC in Japan.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, and the third most common cause of death from cancer worldwide.[1] The regions of high incidence for this disease are generally Eastern and South Eastern Asia and Middle and Western Africa. Because most patients developing HCC are affected with viral hepatitis and followed-up for decades, it is necessary to analyze new molecular markers that can be used to identify high risk populations that may be suitable for more intensive screening or prevention strategies.[2]

Currently, molecular alterations in tumors are being scrutinized at a genome-wide scale, covering different dimensions such as gene expression, epigenetic changes, chromosomal aberrations, and more recently, next generation sequencing.[3] As for HCC, there have been many reports regarding molecular markers associated with developing HCC.[2,4-6] These molecular markers could be useful in the clinic, however; racial differences have been reported and need to be examined more thoroughly.[5-7]

Epidermal growth factor (EGF) was isolated in 1962 and has been shown to stimulate the proliferation and differentiation of epidermal and epithelial tissues via binding to the EGF receptor (EGFR).[8-10] EGF is well known to be associated with various malignant tumors such as melanoma and esophageal carcinoma via autocrine or

paracrine pathways.[11,12] Shahbazi et al[13] identified a single nucleotide polymorphism (SNP) involving A to G transition at position 61 in the 5' untranslated region of the EGF gene (rs4444903) and demonstrated that the G/G genotype was associated with an increased risk of developing malignant melanoma compared to the A/A genotype.

Recently, Tanabe et al[2] showed that the G/G genotype was correlated with an increased risk of developing HCC in both a mixed North American population and a Caucasian French population, and that serum and liver tissue EGF levels were higher in A/G and G/G patients as compared to A/A patients. Furthermore, Abu Dayyeh et al[7] expanded this study in large cohort size, and demonstrated the differences in the distribution of polymorphisms by race and the incidence of HCC. However, conflicting results have been reported from China with two studies showing an association between the G allele and HCC risk,[14,15] while another report found no association between the EGF polymorphism and HCC risk.[16]

Thus far, there has been no report examining the EGF genotype and its association with HCC in a Japanese population. Even though the allelic distribution is expected to be similar between Chinese and Japanese patients, hepatitis C virus (HCV) is the major risk factor for HCC in Japan whereas hepatitis B virus (HBV) is the major risk factor in China and other Eastern countries. The aim of this study was to investigate

the relationship between the EGF SNP and risk of HCC. In addition, to our knowledge, this is the first report to analyze the EGF SNP as a prognostic factor for HCC patients.

Materials and methods

Study population and specimens

A total of 498 patients with liver disease were single ethnic Japanese and retrospectively enrolled in this study during the period from 1994 to 2010 at Nagoya University Hospital with exclusion of autoimmune liver disease, hereditary liver diseases like Wilson's disease, drug-related hepatitis and obstructive jaundice. 208 patients were diagnosed histologically as having HCC and underwent liver resection, and the other 290 patients were followed up for either hepatitis or liver cirrhosis.

In 208 patients who underwent liver resection, the collected samples from the resected specimen were stored immediately in liquid nitrogen at −80˚C until analysis. Genomic DNA was obtained by digestion with proteinase K followed by phenol/chloroform extraction. Total RNA was isolated from each of the frozen samples with the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For the 290 patients without liver resections, genomic DNA was extracted from 150 μL of whole blood using a commercial kit (QIAamp DNA Blood mini Kit, Qiagen).

All 498 patients were single ethnic Japanese, and written informed consent, as required by the Institutional Review Board of Nagoya University, was obtained from all patients.

EGF genotyping

The EGF SNP rs4444903 was analyzed by using restriction fragment-length polymorphism (RFLP) as described previously.[13] In brief, genomic DNA was subjected to polymerase chain reaction (PCR) amplification by using GeneAmp® PCR System 9700 (Life technologies, Carlsbad, CA, USA). It was performed under the following conditions: initial denaturation at 95˚C for 5 minutes, followed by 35 cycles at 95˚C for 30 seconds, 51˚C for 30 seconds, and 72˚C for 1 minute with a final extension step of 7 minutes at 72˚C to amplify nucleotide positions −78 to +164 of the EGF gene. The following primers were used, forward: 5’-TGTCACTAAAGGAAAGGAGGT-3’ and reverse: 5’-TTCACAGAGTTTAACAGCCC-3’. 20 μL of PCR product was digested overnight with 5 units of AluI (Roche, Basel, Switzerland) at 37˚C, separated by electrophoresis in a 3% agarose gel and visualized by staining with ethidium bromide. AluI digestion of the 242 base pair (bp) PCR product containing the 61*G allele produced 15, 34, and 193 bp fragments, while digestion of the 61*A allele produced 15,

34, 91, and 102 bp fragments. The experiment was repeated twice for each sample to ensure accuracy.

Determining EGF mRNA expression

EGF mRNA in HCC tissues was measured by quantitative reverse transcription-PCR. Total RNA from each sample was used to synthesize complementary DNA by single-strand reverse transcription (M-MLV Reverse Transcriptase, Life technologies). The level of EGF mRNA was expressed as the ratio of EGF mRNA PCR product to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample for standardization. The following primer sequences were used, EGF forward: 5'-CTTGTCATGCTGCTCCTCCT -3', reverse: 5'- GAGGGCATATGAAAGCTTCG-3'. Expression of GAPDH was quantified using the following primer, forward: 5'-AACGGCTCCGGCATGTGCAA-3', reverse: 5'-GGCTCCTGTGCAGAGAAAGC-3'. Real-time detection of the emission intensity of SYBR Green was performed with an ABI prism 7000 Sequence Detection System (Life technologies). All PCR reactions were performed under the following conditions: initial denaturation at 95˚C for 30 minutes, followed by 40 cycles at 95˚C for 15 seconds and 60˚C for 40 seconds. Experiments were performed in triplicate to ensure accuracy.

Statistical analysis

Chi-square test was used to determine whether observed genotype frequencies were consistent with Hardy-Weinberg equilibrium. Statistical differences between groups were evaluated using χ^2 test for qualitative variables, and unpaired t-test or ANOVA for quantitative variables. Comparisons of groups with regard to odds of HCC were made using a logistic regression model, and age and gender were included as covariates in addition to genotype in adjusted analysis. Overall survival and disease-free survival rates were calculated using the Kaplan-Meier method, and the difference was analyzed using the log-rank test. Independent prognostic factors were analyzed with the Cox proportional hazards regression model in a stepwise manner. Statistical analysis was performed using JMP® 9 software (SAS Institute Inc, Cary, NC, USA). The presence of a statistically significant difference was denoted by $P < 0.05$.

Results

EGF genotyping of patients

RFLP was used to determine EGF SNP genotype in 208 patients with HCC and 290 patients with either hepatitis or liver cirrhosis (Figure 1A and B). The ratio of A/A, A/G and G/G genotypes were 5.3%, 42.8%, and 51.9% in 208 patients with HCC, whereas in 290 patients without HCC, the ratios were 8.6%, 35.9%, and 55.5%, respectively. The frequencies of the EGF polymorphism in this study population were consistent with Hardy-Weinberg equilibrium ($P = 0.864$).

Patient characteristics

Our Japanese population consisted of 208 patients with HCC and 290 patients without HCC, and their characteristics are shown in Table 1. There was a significant difference in the distribution of age and gender between the HCC group and those patients without HCC ($P < 0.001$, $P < 0.001$, respectively). There was also a significantly higher incidence rate of HCC in HBV patients than in HCV patients ($P < 0.001$).

Patient characteristics were also stratified by EGF genotype and are shown in Table 2. There were no significant differences in age and gender with respect to the EGF SNP. A total of 391 patients had HCV and 64 patients had HBV, and there were no significant differences between these etiologies and EGF genotype ($P = 0.127$).

EGF mRNA expression level

Presence of the G allele has been reported to increase the expression of EGF and may be one mechanism by which the EGF SNP increases the risk of HCC.[2] To examine the correlation between EGF genotype and its expression, the mRNA level of EGF in cancerous tissues were measured by real-time PCR. The relative ratio of A/G and G/G patients were significantly higher than that of A/A patients (P = 0.069, P = 0.033, respectively; Figure 2).

Relative risk of developing HCC based on EGF genotype

In order to explore the association between EGF genotype and the susceptibility of HCC development, the odds ratio (OR) for the risk of HCC was statistically analyzed, using the A/A genotype as a reference (Table 3). The proportion of A/A, A/G and G/G genotypes were 5.3%, 42.8%, and 51.9% in patients with HCC, whereas in patients without HCC, the ratios were 8.6%, 35.9%, and 55.5%, respectively, which revealed that the OR of developing HCC were 1.93 (95% confidence interval [CI] 0.92-4.27, P = 0.081) in A/G patients and 1.51 (95% CI 0.73-3.30, P = 0.268) in G/G patients compared with A/A patients. Importantly, after adjusting for age and gender, the OR for the A/G and G/G genotypes were 2.57 and 1.90 (P = 0.030, P = 0.133, respectively).

Next, we restricted the analysis to patients with hepatitis C, which is the most

common risk factor for HCC in Japan (Table 4). This analysis demonstrated a significant association between the EGF genotype and risk of HCC. The OR were 3.54 (P = 0.014) in A/G patients and 2.85 (P = 0.042) in G/G patients in the crude analysis, and 7.58 (P < 0.001) in A/G patients and 4.61 (P = 0.008) in G/G patients in the adjusted analysis.

We also analyzed the risk limited to hepatitis B, which is the second most frequent cause of HCC in Japan and accounts for about 15% of cases. The proportion of A/A, A/G and G/G genotypes were 11.4%, 40.9%, and 47.7% in patients with both HBV and HCC, while the proportion were 3.1%, 43.1%, and 53.9% in patients with both HCV and HCC. Even though the A allele tended to be more frequent in HBV patients with HCC, there was no significant difference compared to the proportion in patients with both HCV and HCC (P = 0.136). In addition, EGF genotype was not a significant risk factor for HCC in HBV patients (data not shown).

Univariate and multivariate analysis of clinicopathological factors for overall survival

In univariate analysis for overall survival on various clinicopathological parameters, tumor size (≥3 cm), vascular invasion, pathological stage III or IV and alpha-fetoprotein (AFP) level (≥20 ng/ml) were all significant risk factors for poor overall survival (P =

0.009, $P < 0.001$, $P = 0.048$, $P = 0.015$, respectively; Table 5). In multivariate analysis, tumor size, vascular invasion and AFP level were all independent risk factors for poor survival. No statistically significant differences were observed between EGF SNP genotype or EGF mRNA expression and overall survival, and the disease-free and overall survival stratified by EGF SNP in resected HCC patients are shown in Figure 3.

Discussion

Hepatocarcinogenesis is thought to be deeply associated with chronic HBV or HCV infection, and in fact, a certain proportion of chronically infected HBV and HCV individuals will develop HCC. Thus, multiple genetic and epigenetic factors may affect HCC development in this background.[17] Among these genetic alterations, dysregulation of EGF/EGFR signaling pathway is thought to be one of the most important factors in early hepatocarcinogenesis.[18,19]

Previously, it has been reported in several studies that the distribution of the EGF polymorphism varies by race.[2,7,14,16,20] In our Japanese population, the allelic frequencies of the G allele and A allele were 0.73 and 0.27 which is very similar to what has been reported for Chinese and African Americans. This distribution is strikingly different from Caucasians though where the allelic frequencies of the G and A alleles are roughly 0.40 and 0.60. It is interesting to speculate that these differences in distribution might explain the high incidence of HCC in East Asia and Africa, but this requires further study.

A meta-analysis of five studies examining the EGF genotype and HCC risk concluded that the G allele was a risk factor for HCC independent of ethnicity and etiology.[21] This is in contrast to previous studies examining the association between the

EGF genotype and risk of HCC in Chinese patients with HBV.[14-16] However, a more recent meta-analysis examined these three Chinese studies together and revealed a significant association between the G allele and HCC risk, and concluded that the original discrepancy was probably due to the fact that these were single case-control studies with small sample sizes.[22] In addition, when they analyzed all eight published studies on the EGF polymorphism and HCC risk, they found that the 61G allele was a risk factor for HCC while the 61A allele is protective. Not surprisingly, the A/A genotype occurs in less than 10% of Asians and African Americans but is present in roughly 30% of Caucasians.

It is difficult to explain why the risk of developing HCC is higher in patients with the A/G genotype and not the G/G genotype. One would expect that risk of HCC would increase with the number of copies of the G allele, however; it has been observed occasionally in other studies that the OR were higher for A/G patients.[2,14,20] We suspect that this might be due to relatively small sample sizes. Regardless, A/G and G/G patients had higher risk of developing HCC than patients with the A/A genotype in our Japanese population. In addition, the OR that we report here are higher than what has been observed in studies examining other polymorphisms,[23-25] and this observation might suggest that EGF SNP strongly contributes to the development of HCC. Also,

given that Japan is an insular country and therefore a genetically isolated area,[26] the high-risk identified by the EGF SNP in this Japanese population might suggest for more disease specificity. Thus, our observations overall strengthen the previous assertion that the EGF SNP is associated with the development of HCC.

Of note, when the analysis was limited to patients with hepatitis C and adjusted for age and gender, the risk of developing HCC increased dramatically in A/G and G/G patients. This finding is of particular importance as HCV is the main risk factor for HCC in Japan. In addition, several recent mechanistic studies support this finding. First, EGFR was recently shown to be a host factor for HCV cellular entry as EGFR signaling leads to assembly of the host tetraspanin receptor complex.[27,28] Ligands that activate EGFR, especially EGF, therefore increase HCV cellular entry,[27] and the EGF SNP is known to increase EGF liver levels.[2]

The association between EGF genotype and prognosis of HCC patients has never been described so far and remains controversial in other malignancies such as glioma and esophageal carcinoma.[29-31] In this study, no statistical association was found between EGF genotype and both disease-free and overall survival in HCC patients. This result could be explained by the fact that the prognosis of HCC, as opposed to other malignancies, is subject to various individual conditions such as cirrhosis leading to

multicentric carcinogenesis and residual liver function.[32] Interestingly, EGF is part of a gene expression signature associated with poor overall survival in HCC patients who have had a resection and in cirrhosis patients.[33,34] This signature was derived from the surrounding non-tumoral liver tissue and might help explain why the increased tumoral EGF expression seen in our A/G and G/G patients does not associate with survival. It might be that EGF expression alone is not powerful enough to predict prognosis or it could be that EGF expression in the surrounding, non-tumoral tissue will have more prognostic value. Consistent with this, Falletti et al[35] have reported that the EGF genotype associates with advanced fibrosis at a young age in HCV patients. They hypothesize that the EGF polymorphism is responsible for a more rapid disease progression early during the course of HCV infection which would be consistent with its role in cellular entry.

In conclusion, allelic frequencies of the EGF polymorphism in Japanese patients were similar to what has been reported previously for other high risk HCC groups including Chinese and African Americans. The A/G and G/G genotypes were associated with increased risk of HCC, especially in HCV patients where increased EGF levels might lead to a more rapid disease progression. We suggest that more intensive

follow-up in HCV patients with G allele might lead to earlier diagnosis and better outcomes for HCC.

Disclosure

The authors report no conflicts of interest in this work.

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Figure legends

Figure 1 EGF SNP was analyzed by using restriction fragment-length polymorphism and the representative results are shown: (**A**) HCC patients and (**B**) Not HCC patients including hepatitis or cirrhosis patients.

Abbreviations: bp, base pair; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; SNP, single nucleotide polymorphism.

Figure 2 EGF mRNA expression level based on the genotypes in HCC patients.

Abbreviations: EGF, epidermal growth factor; HCC, hepatocellular carcinoma; mRNA, messenger ribonucleic acid.

Figure 3 (**A**) DFS based on EGF SNP in HCC patients and (**B**) OS based on EGF SNP in HCC patients.

Abbreviations: DFS, disease-free survival; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; N.S., not significant; OS, overall survival; SNP, single nucleotide polymorphism.

Table 1 Patient characteristics with or without HCC

	HCC (n = 208)	**Not HCC (n = 290)**	***P* value**
Age (years), mean ± SD	63.1 (± 9.9)	52.7 (± 13.2)	< 0.001
Sex (male/female)	176/32	169/121	< 0.001
Child-Pugh score (A/B/C)	193/15/0	265/18/7	0.674[b]
Etiology, n (%)			
HBV	44 (68.8%)	20 (31.3%)	< 0.001[c]
HCV	130 (33.3%)	261 (66.8%)	
Alcohol	0 (0.0%)	3 (100.0%)	
Multiple factors[a]	3 (60.0%)	2 (40.0%)	
Non-B, non-C	31 (88.6%)	4 (11.4%)	

Note: Statistical differences between the HCC and not HCC groups were evaluated using the χ^2 test for qualitative variables, and unpaired *t*-test for quantitative variables. [a]Multiple factors in HBV, HCV, and alcohol; [b]comparison between Child-Pugh score A and B or C; [c]comparison between HBV and HCV.

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; SD, standard deviation.

Table 2 Patient characteristics, stratified by EGF genotype

	A/A (n = 36)	**A/G (n = 193)**	**G/G (n = 269)**	***P* value**
Age (years), mean ± SD	56.4 (± 14.1)	56.2 (± 13.2)	57.7 (± 12.6)	0.487
Sex (male/female)	26/10	143/50	176/93	0.125
Child-Pugh score (A/B/C)	33/1/2	176/13/4	249/19/1	0.865[b]
Etiology, n(%)				
HBV	8 (22.2%)	28 (14.5%)	28 (10.4%)	0.127[c]
HCV	26 (72.2%)	148 (76.7%)	217 (80.7%)	
Alcohol	0 (0.0%)	0 (0.0%)	3 (1.1%)	
Multiple factors[a]	0 (0.0%)	0 (0.0%)	5 (1.9%)	
Non-B, non-C	2 (5.6%)	17 (8.8%)	16 (6.0%)	

Note: The statistical differences between each group were evaluated using the χ^2 test for qualitative variables, and ANOVA for quantitative variables. [a]Multiple factors in HBV, HCV, and alcohol; [b]comparison between the Child-Pugh score A and B or C; [c]comparison between HBV and HCV.

Abbreviations: ANOVA, analysis of variance; EGF, epidermal growth factor; HBV, hepatitis B virus; HCV, hepatitis C virus; SD, standard deviation.

Table 3 Analysis of EGF genotype in a Japanese population

	HCC (n = 208) n (%)	**Not HCC (n = 290) n (%)**	**Crude analysis**		**Adjusted analysis[b]**	
			OR (95% CI)	***P* value[a]**	**OR (95% CI)**	***P* value[a]**
Genotype						
A/A	11 (5.3%)	25 (8.6%)	1 [Reference]		1 [Reference]	
A/G	89 (42.8%)	104 (35.9%)	1.94 (0.93-4.32)	0.080	2.68 (1.10-6.32)	0.024
G/G	108 (51.9%)	161 (55.5%)	1.52 (0.74-3.35)	0.261	1.93 (0.84-4.65)	0.122
Allele						
A	111 (26.7%)	154 (26.6%)	1 [Reference]			
G	305 (73.3%)	426 (73.4%)	0.99 (0.75-1.32)	0.963		

Note: Comparisons of the groups with regard to the odds of HCC were made using a logistic regression model, and age and sex were included as covariates, in addition to genotype, in adjusted analysis. [a]Logistic regression model; [b]age and sex were included as covariates in adjusted analysis.

Abbreviations: CI, confidence interval; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; OR, odds ratio.

Table 4 Analysis of EGF genotype in Japanese patients with HCV

	HCC (n = 130) n (%)	HCV (n = 233) n (%)	Crude analysis		Adjusted analysis[b]	
			OR (95% CI)	*P* value[a]	OR (95% CI)	*P* value[a]
Genotype						
A/A	4 (3.1%)	21 (9.0%)	1 [Reference]		1 [Reference]	
A/G	56 (43.1%)	83 (35.6%)	3.54 (1.27-12.63)	0.014	7.58 (2.30-30.43)	< 0.001
G/G	70 (53.9%)	129 (55.3%)	2.85 (1.03-10.0)	0.042	4.61 (1.46-17.87)	0.008
Allele						
A	64 (24.6%)	125 (26.8%)	1 [Reference]			
G	196 (75.4%)	341 (73.2%)	1.12 (0.79-1.59)	0.516		

Note: The analysis was limited to the 363 patients with HCV. Comparisons of the groups with regard to the odds of HCC were made using a logistic regression model, and age and sex were included as covariates, in addition to genotype, in adjusted analysis. [a]Logistic regression model; [b]age and sex were included as covariates in adjusted analysis.

Abbreviations: EGF, epidermal growth factor; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

Table 5 Univariate and multivariate analysis of clinicopathological factors for overall survival

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Sex (male vs female)	1.23 (0.62-2.80)	0.567		
Age (≥65 vs <65)	1.30 (0.80-2.14)	0.287		
Virus (HCV vs HBV)	1.28 (0.70-2.54)	0.432		
Histological differentiation (well vs others)	0.72 (0.37-1.31)	0.298		
Tumor size (≥3 cm vs <3 cm)	2.12 (1.20-4.01)	0.009	1.92 (1.03-3.80)	0.040
Tumor multiplicity (multiple vs solitary)	1.68 (0.98-2.79)	0.060		
Pattern of fibrous growth (infiltrative vs expansive)	1.89 (0.98-3.38)	0.057		
Formation of fibrous capsule (present vs absent)	0.81 (0.18-1.40)	0.431		
Capsular infiltration (present vs absent)	0.81 (0.50-1.34)	0.410		
Septal formation (present vs absent)	0.72 (0.54-1.56)	0.716		
Vascular invasion (present vs absent)	2.61 (1.53-4.35)	< 0.001	2.48 (1.17-5.36)	0.018
Pathological Stage[a] (I or II vs III or IV)	0.60 (0.37-0.99)	0.048	0.73 (0.34-1.49)	0.401
AFP level (≥20 ng/ml vs <20 ng/ml)	1.86 (1.13-3.15)	0.015	1.81 (1.08-3.10)	0.024
EGF genotype (A/G vs A/A)	1.52 (0.54-6.35)	0.468		
EGF genotype (G/G vs A/A)	1.32 (0.47-5.52)	0.633		
EGF mRNA expression (≥median vs <median)	0.83 (0.50-1.37)	0.469		

Note: Independent prognostic factors were analyzed with the Cox proportional hazards regression model. [a]According to TNM staging by the Liver Cancer Study Group of Japan.

Abbreviations: AFP, alpha-fetoprotein; CI, confidence interval; EGF, epidermal growth factor; HBV, hepatitis B virus; HCV, hepatitis C virus; OR, odds ratio; mRNA, messenger ribonucleic acid; TNM, tumor, node, metastasis.

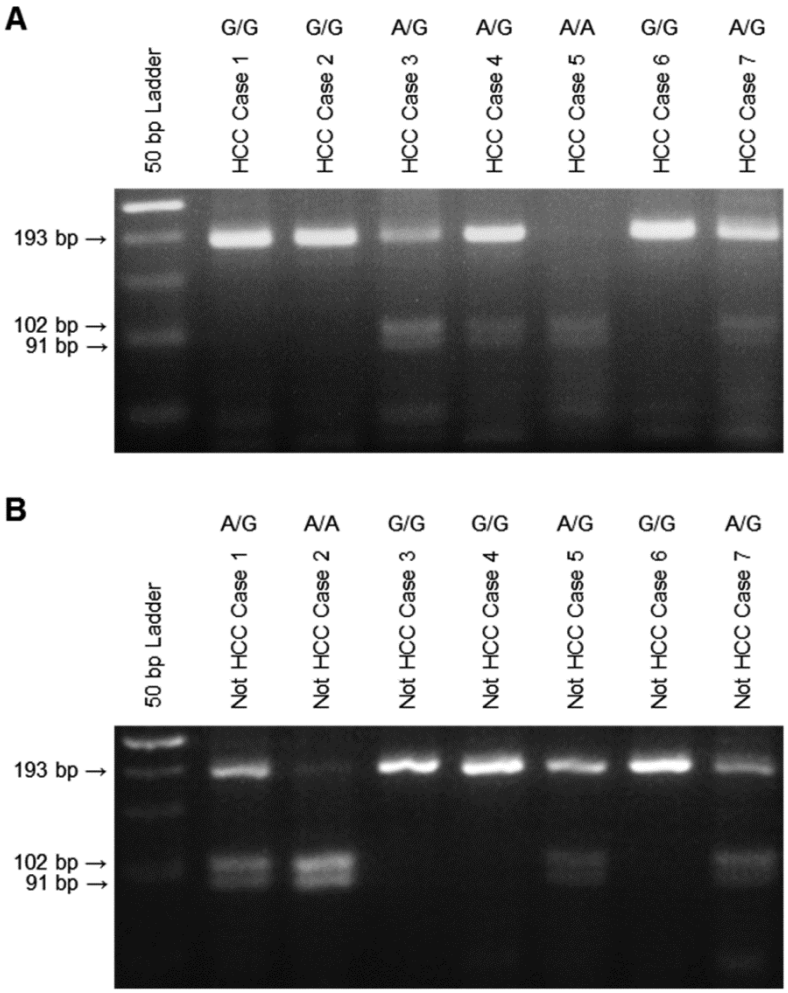

Figure 1 EGF SNP was analyzed by using restriction fragment-length polymorphism, and the representative results are shown: (A) HCC patients and (B) Not HCC patients including hepatitis or cirrhosis patients.

Abbreviations: bp, base pair; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; SNP, single nucleotide polymorphism.

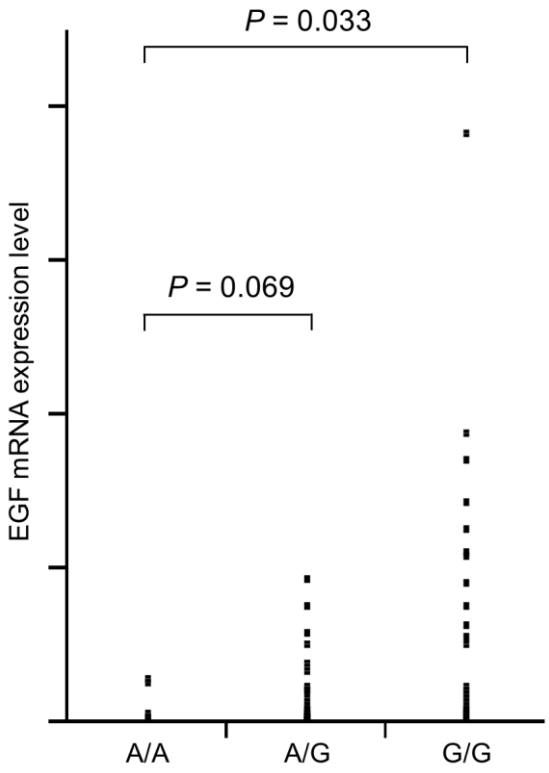

Figure 2 EGF mRNA expression level based on the genotypes in HCC patients. Abbreviations: EGF, epidermal growth factor; HCC, hepatocellular carcinoma; mRNA, messenger ribonucleic acid.

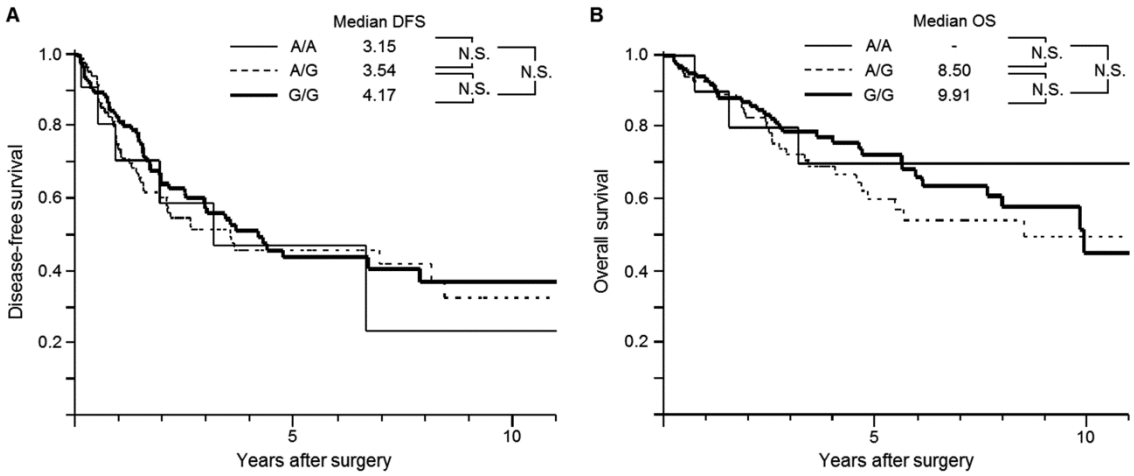

Figure 3 (A) DFS based on EGF SNP in HCC patients and (B) OS based on EGF SNP in HCC patients.

Abbreviations: DFS, disease-free survival; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; N.S., not significant; OS, overall survival; SNP, single nucleotide polymorphism.