in vivo *38*: 2455-2463 (2024)
doi:10.21873/invivo.13715

Cytoplasmic Expression of the EGFL6 Protein Is an Independent Prognostic Factor for Shortened Patient Survival in Human Hepatocellular Carcinoma

HUI-TING HSU[1,2,3*], YUEH-MIN LIN[4,5*], MING-TAI HSING[6], KUN-TU YEH[4,5], JENG-WEI LU[7,8] and SHUN-FA YANG[1,9]

[1]*Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.;*
[2]*School of Medicine, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[3]*Department of Pathology, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[4]*Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.;*
[5]*School of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.;*
[6]*Department of Neurosurgery, Show Chwan Memorial Hospital, Changhua, Taiwan, R.O.C.;*
[7]*Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark;*
[8]*The Finsen Laboratory, Rigshospitalet/National University Hospital, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark;*
[9]*Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan, R.O.C.*

Abstract. *Background/Aim: Hepatocellular carcinoma (HCC) is the most common primary liver tumor and the second leading cause of cancer-related deaths worldwide. The current study aimed to investigate the clinical relevance of the epidermal growth factor-like domain multiple 6 (EGFL6) expression in HCC and to evaluate whether the expression of EGFL6 in HCC has diagnostic and prognostic significance. Patients and Methods: This study aimed to investigate EGFL6 protein expression levels in 260 HCC tissue specimens using immunohistochemical analyses. The immunohistochemical study demonstrated strong EGFL6 expression in the cytoplasm of non-tumor or normal hepatocytes. Results: The findings revealed that 98 patients exhibited low EGFL6 expression, while 162 patients displayed high EGFL6 expression. We explored the associations between cytoplasmic EGFL6 expression and the clinicopathological features of HCC. Decreased cytoplasmic EGFL6 expression exhibited significant correlations with worse cellular differentiation, higher T classification, vascular invasion, higher stage, and tumor recurrence. Survival analyses, using Kaplan–Meier survival curves for HCC patients, revealed that those with reduced cytoplasmic EGFL6 expression experienced significantly worse disease-free survival (DFS) and disease-specific survival (DSS). Univariate and multivariate analyses identified EGFL6 as an independent predictor for decreased expression, differentiation grade, vascular invasion, stage, or recurrence in cases of DFS or DSS in HCC. Conclusion: This study represents, to the best of our knowledge, the first investigation into the expression of EGFL6 protein in HCC. Taken together, our findings strongly suggest that EGFL6 likely plays a crucial role in the pathogenesis of HCC and indicates that targeting EGFL6 could be a promising therapeutic strategy.*

*These Authors contributed equally to this work.

Correspondence to: Jeng-Wei Lu, Ph.D., Biotech Research and Innovation Centre, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark, Tel: +45 35453799, e-mail: jengweilu@gmail.com; Shun-Fa Yang, Ph.D., Institute of Medicine, Chung Shan Medical University, Taichung, 110, Section 1, Chien-Kuo N. Road, Taichung 402, Taiwan, R.O.C. Tel: +886 424739595 ext. 34253, e-mail: ysf@csmu.edu.tw

Key Words: EGFL6, hepatocellular carcinoma, immunohistochemistry, disease-free survival, disease-specific survival, prognosis.

Liver cancer stands as a highly malignant tumor, ranking sixth in incidence and third in mortality among cancers worldwide (1). Hepatocellular carcinoma (HCC), constituting 90% of liver cancer cases, typically originates from viral hepatitis C or B infections (2). Despite the potential benefit of effective treatments for HCC patients arising from comprehensive studies on the biological and environmental mechanisms driving its occurrence and progression, several challenges

persist. At present, the overall survival (OS) of patients with HCC remains unsatisfactory, with treatment strategies primarily revolving around surgical resection, interventional or radiofrequency ablation, chemotherapy or targeted therapy, and liver transplantation (3-6). The high rates of metastasis and recurrence in HCC, limited effective clinical options, and diminished efficacy of surgical treatment in advanced stages of the disease (7-9). Hence, there is an urgent need to enhance the identification of novel molecular markers and develop effective prognostic signatures to significantly improve the prognosis of patients with HCC (10).

The epidermal growth factor-like (EGFL) domain gene family is named for the protein structures of its members, which contain one or more EGFL domains. Proteins encoded by the EGFL gene family can activate crucial signal transduction pathways, including extracellular signal-regulated kinase (ERK), nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), protein kinase B, and Notch. As a result, this gene family plays a significant role in the occurrence and development of various tumors (11, 12). The epidermal growth factor-like domain multiple 6 (EGFL6, also known as MAEG) was initially discovered in 1999 through high-throughput screening of DNA molecular hybridization and was mapped to the human Xp22 chromosome (13, 14). EGFL6 protein is a secreted factor that plays an important role in promoting endothelial cell migration and angiogenesis (15). High levels of cytoplasmic EGFL6 expression have been found in patients with lung adenocarcinomas. Those with high cytoplasmic EGFL6 expression exhibited a lower 5-year survival rate and shorter median survival time compared to those with low cytoplasmic EGFL6 levels (16). Furthermore, ectopic expression of EGFL6 has been demonstrated to promote cancer cell proliferation, migration, invasion, and tumor progression in breast (17), gastric (18), ovarian, colon (19), and nasopharyngeal (20, 21) cancers. The tumor angiogenic function of EGFL6 was initially implicated in hepatitis C virus (HCV)-associated HCC (22).

In patients with oral cancer, elevated plasma levels of EGFL6 are observed, with higher levels detected in patients with advanced-stage disease compared to those with early-stage disease (23). EGFL6 regulates cell migration and asymmetric division through the SHP2 oncoprotein, leading to the concurrent activation of ERK in ovarian cancer (14). EGFL6-specific antibodies offer promising anti-angiogenic therapies by effectively blocking or knocking down EGFL6, thereby suppressing tumor angiogenesis (15, 17, 24). Similarly, accumulating evidence supports EGFL6 involvement in accelerating and enhancing angiogenesis, carcinogenesis, and tumor progression, suggesting its potential as a putative biomarker for human cancers (16, 17, 19, 20, 23-26).

However, the clinical significance and prognostic implications of EGFL6 expression in HCC have yet to be investigated, encompassing both clinical aspects and the underlying mechanisms. In this study, we investigated the relationship between EGFL6 expression and its clinicopathological characteristics of HCC as well as its prognostic significance. This exploration aimed to elucidate the role of EGFL6 in HCC, potentially refining the treatment effects for HCC patients.

Patients and Methods

Human HCC patients. The study examined samples from 260 patients with HCC treated at Changhua Christian Hospital, Taiwan, between January 1999 and December 2008. Ethical approval for this research was obtained from the Ethics Committees of Changhua Christian Hospital (Changhua, Taiwan, ROC), and all patients provided written, informed consent. The analysis adhered to guidelines approved by the Institutional Review Board (IRB) under the IRB number 151019, approved on January 19, 2016. The HCC patient cohort consisted of 195 males and 65 females. Pathological assessments, including tumor staging and histologic differentiation grading, followed the American Joint Commission on Cancer (AJCC, 7th edition) TNM staging system and the Edmondson and Steiner grading system. Data on age, sex, differentiation grade, T classification, N status, metastasis, vascular invasion, tumor stage, tumor recurrence, Hepatitis B and C infections, cirrhosis, and survival were obtained from histopathological and clinical records.

Tissue microarrays (TMAs). To generate TMAs (5-μm), we selected representative HCC specimens, sectioned them, and stained them using hematoxylin and eosin (H&E). Tissue cylinders (2 mm in diameter) were then extracted from marked regions of paraffin blocks using a semi-automated device. These punched cores included a substantial number of viable tumor cells, showing minimal necrosis in either peripheral or central areas. The tumor specimen punches were organized into new paraffin blocks. After H&E staining of the TMAs, two senior pathologists (Drs. Hui-Ting Hsu and Yueh-Min Lin) confirmed the presence of morphologically representative lesions from the original cancers within these TMAs.

Immunohistochemical staining and scoring. The immunohistochemical staining analysis followed a protocol established in a prior study (10, 27-29). Specifically, we utilized rabbit polyclonal EGFL6 antibody (1:200 dilution; catalog number: ab140079; Abcam, Cambridge, MA, USA) to detect the EGFL6 protein. TMA sections were incubated with anti-EGFL6 antibody overnight at 4˚C. Subsequently, the LASB 2 kit (Dako, Carpinteria, CA, USA) was employed to detect the resulting immune complex, and activity was visualized using aminoethyl carbazole as a substrate. Following this, sections were counterstained using hematoxylin and mounted using Glycergel mounting medium (Dako). To ensure accuracy, appropriate positive and negative controls were integrated into the same immunohistochemical program. Cytoplasmic staining intensity was categorized into four scores: negative staining (0), weak staining (1+), moderate staining (2+), and strong staining (3+). Additionally, the percentage of immunoreactive tumor cells was documented. These scores were blindly evaluated independently by two senior pathologists (Drs Hui-Ting Hsu and Yueh-Min Lin).

Statistical analysis. The relationship between EGFL6 protein expression levels and the clinicopathological parameters of HCC was assessed using the Chi-square test or Fisher exact test. The prognostic significance of EGFL6 protein expression was evaluated using Cox regression models and hazard ratio analysis. Disease-free

survival (DFS) rates were calculated using the Kaplan–Meier method, whereas disease-specific survival (DSS) rates served as the secondary end-point. Survival curve disparities were compared using the log-rank test. Factors independently associated with DFS and DSS were identified through the Cox proportional hazard model, employing univariate and multivariate analyses. Statistical analysis was performed using SPSS statistical software version 17 (SPSS, Inc., Chicago, IL, USA). A *p*-value below 0.05 was considered statistically significant.

Results

Characteristics of patients with HCC. In this study we examined the characteristics of the 260 cases of HCC within the sample population comprising 195 males and 65 females. The ages of patients ranged from 17 to 87 years, with a mean age of 59.47 years. Disease staging was distributed as follows: stage I (121 patients; 46.4%), stage II (70 patients; 26.9%), stage III (61 patients; 23.5%), and stage IV (8 patients; 3.1%). T classification analysis revealed 47.3% of patients were T1, 28.1% T2, 16.9% T3, and 7.7% T4. Additionally, eight patients (3.1%) presented with lymph node metastasis, and six patients (2.3%) initially exhibited signs of metastatic disease. Regarding tumor differentiation, moderately-differentiated tumors were found in 56.9% of patients, while 32.7% had poorly-differentiated tumors, and 10.4% had well-differentiated tumors. Tumor recurrence affected 163 patients (62.7%) during the follow-up period. Among the patients, 154 (59.2%) had a comorbid hepatitis B infection, and 100 (38.5%) had a comorbid hepatitis C infection. Additionally, cirrhosis was clinically diagnosed in 117 (43.8%) patients (Table I).

Table I. *Characteristics of patients with hepatocellular carcinoma.*

Characteristics	Total (%)
Total number	260 (100)
Age (year)	
Mean±S.D.	59.47±13.48
Sex	
Male	195 (75%)
Female	65 (25%)
Stage	
I	121 (46.4%)
II	70 (26.9%)
III	61 (23.5%)
IV	8 (3.1%)
T classification	
T1	123 (47.3%)
T2	73 (28.1%)
T3	44 (16.9%)
T4	20 (7.7%)
Lymph node metastasis	
No	252 (96.9%)
Yes	8 (3.1%)
M classification	
M0	259 (97.7%)
M1	6 (2.3%)
Differentiation	
Well	27 (10.4%)
Moderately	148 (56.9%)
Poorly	85 (32.7%)
Recurrence	
No	97 (37.3%)
Yes	163 (62.7%)
Hepatitis B infection	100 (38.5)
No	106 (40.8%)
Yes	154 (59.2%))
Hepatitis C infection	
No	160 (61.5%)
Yes	100 (38.5%)
Cirrhosis	
No	146 (56.2%)
Yes	114 (43.8%)

Association between cytoplasmic EGFL6 protein expression status and characteristics of patients with HCC. The analysis of immunohistochemical staining revealed strong EGFL6 protein expression in the cytoplasm of non-tumor or normal hepatocytes. The staining intensity observed in these non-tumor hepatocytes served as both an internal positive control and a baseline for scoring EGFL6 staining. The staining patterns of EGFL6 in the cytoplasm of tumor cells appeared relatively homogeneous, without any indications of nuclear staining. Based on the relative intensity of EGFL6 staining in the cytoplasm, we categorized EGFL6 immunostaining results as follows: Negative and weak staining: Scores of 0 and 1+; Moderate and strong staining: Scores of 2+ and 3+; Normal liver control was included for comparison purposes (Figure 1).

The immunostaining results revealed that 98 patients (37.7%) exhibited low EGFL6 protein expression, while 162 patients (62.3%) exhibited high EGFL6 protein expression. In Table II, we employed the Fisher exact test to evaluate the clinical significance of cytoplasmic EGFL6 protein expression levels in HCC tissues. The analysis demonstrated a significant correlation between EGFL6 protein expression and various clinicopathological variables, including differentiation (p=0.007), T classification (p<0.001), vascular invasion (p=0.004), tumor stage (p<0.001), lymph node metastasis (p=0.003), tumor recurrence (p=0.045), two-year survival (p=0.021), and overall survival (p=0.010). There were no significant differences in cytoplasmic EGFL6 protein expression when the results were stratified according to age (p=0.105), sex (p=0.301), distant metastasis (p=0.829), hepatitis B infection (p=0.292), hepatitis C infection (p=0.731), and cirrhosis (p=0.200).

The expression of EGFL6 proteins correlated with shorter survival times. Kaplan–Meier analysis was utilized to assess the correlation between EGFL6 protein expression and disease-

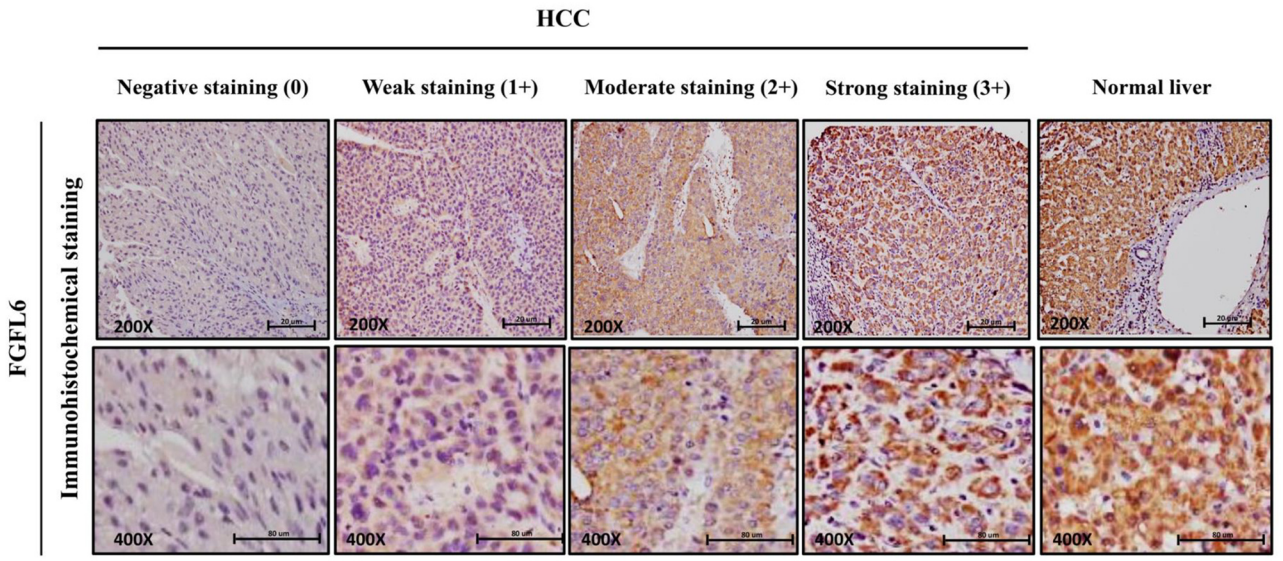

Figure 1. *Immunohistochemical staining and assessment of EGFL6 protein expression in hepatocellular carcinoma (HCC) and normal tissues. The intensity of cytoplasmic staining was graded using the following scores: Negative staining (0); Weak staining (1+); Moderate staining (2+); Strong staining (3+); and a control using normal liver tissue. Images were captured at magnifications of 100× (top panel) and 400× (lower panel). Scale bars indicate 20 um and 80 um, respectively.*

free survival (DFS) and disease-specific survival (DSS) in all 260 patients with HCC. The Kaplan–Meier survival curves for HCC patients indicated that those with low cytoplasmic EGFL6 protein expression experienced significantly worse DSS and DFS compared to those with high cytoplasmic EGFL6 protein expression. This observation was further supported by the results of the log-rank test (p=0.043 and p=0.001, respectively), as depicted in Figure 2.

The prognostic value of clinicopathological characteristics and EGFL6 in HCC patients assessed through univariate and multivariate analysis. The prognostic value of EGFL6 in HCC patients was assessed through univariate and multivariate analysis using the Cox regression model and hazard ratios. Our goal was to explore the correlations among DFS, DSS, and various clinicopathological variables in patients with HCC. These variables included EGFL6 protein expression (Low, High), differentiation grade (G1-G2, G3), vascular invasion (No, Yes), tumor stage (I-II, III-IV), and tumor recurrence (No, Yes).

The DFS rates among patients with HCC indicated a significant association with EGFL6 protein expression [95% confidence interval (CI)=1.14-2.12, p=0.006; 95%CI=1.08-2.02, p=0.014], differentiation grade (95%CI=1.14-1.57, p<0.001; 95%CI=1.27-2.42, p=0.001), vascular invasion (95%CI=1.31-2.44, p<0.001) and tumor stage (95%CI=1.32-1.85, p<0.001; 95%CI=1.30-2.99, p=0.001), as shown in Table III. Meanwhile, the DSS rates among patients with HCC demonstrated significant associations with EGFL6 protein expression (95%CI=1.22-2.29, p=0.001; 95%CI=0.97-2.08, p=0.007), differentiation grade (95%CI=1.28-1.86, p<0.001; 95%CI=1.36-2.90, p=0.001), vascular invasion (95%CI=1.80-3.91, p<0.001; 95%CI=1.09-2.75, p=0.021), tumor stage (95%CI=1.52-2.23, p<0.001; 95%CI=1.44-3.59, p<0.001), and tumor recurrence (95%CI=1.59-4.02, p<0.001; 95%CI=1.33-3.45, p=0.002), as presented in Table IV.

Discussion

The cause of HCC has been linked to genetic alterations (30, 31) and a number of environmental risk factors (32). However, the high heterogeneity of tumors and the complexity of underlying mechanisms mean that these biomarkers lack sufficient sensitivity and specificity to assess the prognosis of patients with HCC, despite the current clinical use of a variety of HCC tumor markers for diagnosis (10, 27, 28, 33). Previous literature has found that aberrant expression of multiple oncogenes and tumor suppressor genes could have antitumor or cancer-promoting effects (34). Various biomarkers have been associated with the incidence and disease progression of HCC, indicating their critical role in tumorigenesis (10, 27, 28, 33). Serum alpha-fetoprotein (AFP) levels are frequently utilized for the identification and monitoring of HCC patients; nevertheless, many individuals with advanced HCC exhibit normal AFP levels in clinical diagnosis (35, 36). So far, gene expression and genome-

Table II. *Patient characteristics and the status of cytoplasmic EGFL6 expression assayed using immunohistochemistry.*

Variables	Total, n (%)	EGFL6, n (%)		*p*-Value
		EGFL6 (–)	EGFL6 (+)	
Total number	260 (100)	98 (37.7)	162 (62.3)	
Age	59.47±13.48	58.7±14.5	60.0±12.8	0.105
Sex				
Female	65 (25)	28 (28.6)	37 (22.8)	0.301
Male	195 (75)	70 (71.4)	125 (77.2)	
Differentiation				
Well	27 (10.4)	3 (3.1)	24 (14.8)	0.007*
Moderately	148 (56.9)	57 (58.2)	91 (56.2)	
Poorly	85 (32.7)	38 (38.8)	47 (29.0)	
T classification				
T1-T2	196 (75.4)	61 (62.2)	135 (83.3)	<0.001**
T3-T4	64 (24.6)	37 (37.8)	27 (16.7)	
Vascular invasion				
No	123 (47.3)	35 (35.7)	88 (54.3)	0.004*
Yes	137 (52.7)	63 (64.3)	71 (45.7)	
Stage				
I-II	191 (73.6)	59 (60.2)	132 (81.5)	<0.001**
III-IV	69 (26.4)	39 (39.8)	30 (18.5)	
Lymph node metastasis				
No	252 (96.9)	91 (92.9)	161 (99.4)	0.003*
Yes	8 (3.1)	7 (7.1)	1 (0.6)	
Recurrence				
No	97 (37.3)	29 (29.6)	68 (42.0)	0.045*
Yes	163 (62.7)	69 (70.4)	94 (58.0)	
Distant metastasis				
No	259 (97.7)	141(100)	118 (96.3)	0.829
Yes	6 (2.3)	3 (0.0)	3 (3.7)	
Hepatitis B infection				
No	106 (40.8)	44 (44.9)	62 (38.3)	0.292
Yes	154 (59.2)	54 (55.1)	100 (62.3)	
Hepatitis C infection				
No	160 (61.5)	59 (60.2)	101 (62.3)	0.731
Yes	100 (38.5)	39 (39.8)	61 (37.7)	
Cirrhosis				
No	146 (56.2)	60 (61.2)	86 (53.1)	0.200
Yes	114 (43.8)	38 (38.8)	76 (46.9)	
Two-year survival				
Dead	74 (28.5)	36 (36.7)	38 (23.5)	0.021*
Alive	186 (71.5)	62 (63.3)	124 (76.5)	
Overall survival				
Dead	157 (60.4)	72 (73.5)	85 (52.5)	0.010*
Alive	103 (39.6)	26 (26.5)	77 (47.5)	

EGFL6 (–): low-expression; EGFL6 (+): high-expression. *p*-Value was measured using Fisher Extract Test. *$p<0.05$; **$p<0.01$.

based candidate markers for patients with HCC are under evaluation (37, 38).

In this study, we investigated the protein expression level of EGFL6 in HCC and examined its correlation with patient prognosis. Immunohistochemical results confirmed the expression pattern of EGFL6 in HCC specimens. We also found that cytoplasmic protein levels of EGFL6 were significantly decreased in HCC tissues. Interestingly, strong cytoplasmic expression of EGFL6 protein was observed in positively stained cells, particularly in the cytoplasm of non-tumor or normal hepatocytes (Figure 1). The biological role of EGFL6 in HCC remains unclear. While EGFL6 functions as an oncogene in breast, colorectal, gastric, ovarian cancers (14, 17-19), oral squamous cell carcinoma (23), lung adenocarcinoma (16), and nasopharyngeal carcinoma (20), our results suggest that a high level of EGFL6 is insufficient to promote cancer development in HCC. Since the protein levels of EGFL6 are key determinants of EGFL6 activity in the cell (39), the decreased activity of EGFL6 in liver cells may correlate with the tumorigenesis of HCC.

Morbidity and mortality in patients with HCC who undergo surgical treatment have decreased in recent years. However, the prognosis of patients with HCC remains unsatisfactory, with a 5-year postoperative survival rate ranging from 25% to 49%. The primary factors influencing the survival rate of patients with HCC are chemotherapy resistance, metastasis, and recurrence. The expression of EGFL6 and its correlation with prognosis have been intensively studied in many cancers. Over-expression of EGFL6 has been shown to be associated with a poor prognosis in breast, colorectal, ovarian, gastric cancers (14, 17-19), and lung adenocarcinoma (16). In our study, patients with HCC who expressed lower levels of cytoplasmic EGFL6 protein had a shorter DFS and DSS compared to those expressing higher levels of cytoplasmic EGFL6 protein (Figure 2). These observations suggest that the expression profile, levels, and prognostic significance of EGFL6 may differ among various types of cancers.

Previous research shows that a high level of plasma EGFL6 was correlated with TNM stage, advanced T status, and distant metastasis in patients with oral squamous cell carcinoma (23). In colorectal cancer, higher EGFL6 expression was associated with advanced T stage, an increased risk of lymph node metastasis, a higher risk of distant metastasis, and poorer histological differentiation (19). In the statistical analysis of 260 real-world HCC samples, we observed a correlation between lower levels of cytoplasmic EGFL6 protein expression and clinicopathological variables typical of patients with HCC. These variables included differentiation, T classification, vascular invasion, tumor stage, lymph node metastasis, tumor recurrence, two-year survival, and overall survival. Lower cytoplasmic EGFL6 protein expression was associated with the development and progression of HCC. It is noteworthy that HCC shows no significant correlation with age, sex, distant metastasis, hepatitis B infection, hepatitis C infection, or cirrhosis (Table II). Our results are consistent with previous studies indicating that EGFL6 expression levels are closely related to histological differentiation, T classification, tumor stage, and lymph node metastasis.

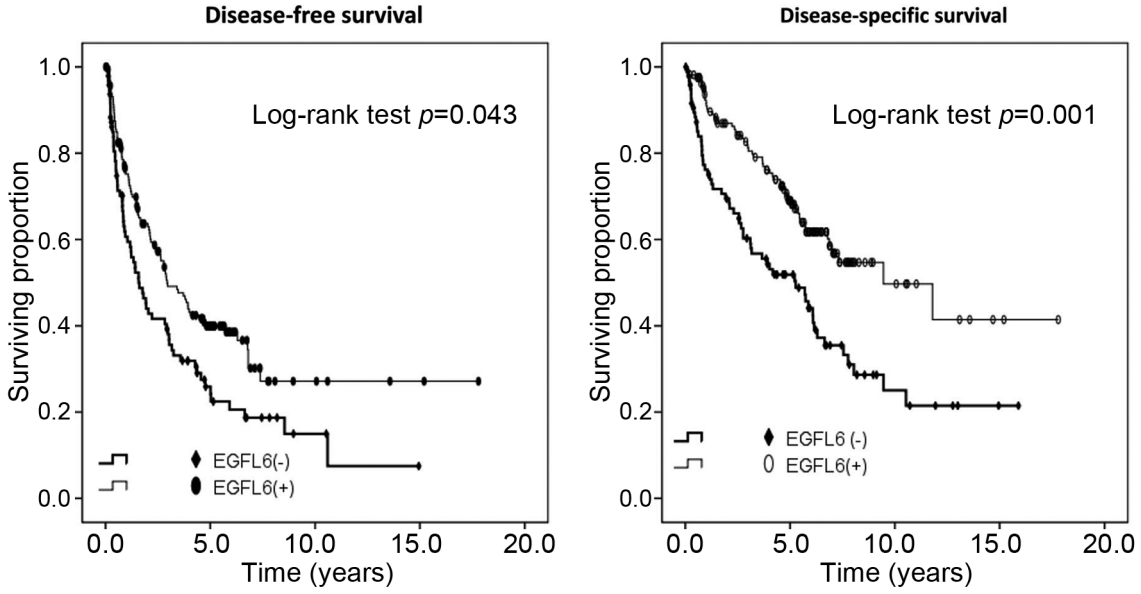

Figure 2. *Kaplan–Meier survival curves depicting disease-free survival (DFS) and disease-specific survival (DSS) stratified by EGFL6 protein expression in hepatocellular carcinoma (HCC). The Kaplan–Meier survival analysis examined DFS and DSS among patients with HCC based on varying EGFL6 protein expression levels. Distinct differences were observed in the high and low EGFL6 protein expression groups, as determined using the log-rank test.*

Table III. *Univariate and multivariate analysis of disease-free survival rate in hepatocellular carcinoma.*

	Univariate			Multivariate		
	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
EGFL6 expression						
High	1.0	1.14-2.12	0.006*	1.0	1.08-2.02	0.014*
Low	1.55			1.48		
Differentiation grade						
G1-G2	1.0	1.14-1.57	<0.001**	1.0	1.27-2.42	0.001*
G3	1.34			1.75		
Vascular invasion						
No	1.0	1.31-2.44	<0.001**	1.0	0.88-1.88	0.188
Yes	1.79			1.29		
Stage						
I-II	1.0	1.32-1.85	<0.001**	1.0	1.30-2.99	0.001*
III-IV	1.56			1.97		

*$p<0.05$; **$p<0.01$; CI: confidence interval.

To predict clinical prognosis and enhance subsequent postoperative HCC management, it is important to identify a new biomarker that can be used in combination with common clinicopathological risk factors. Therefore, in this study, we investigated and characterized the ability of EGFL6 as an independent prognostic factor in predicting HCC outcomes. Indeed, univariate or multivariate Cox regression analyses of DFS revealed that EGFL6 expression, differentiation grade, vascular invasion, and stage have significant prognostic value for patients with HCC (Table III). Furthermore, univariate or multivariate Cox regression analyses of DSS revealed that EGFL6 expression, differentiation grade, vascular invasion, stage, and recurrence also have significant prognostic value for patients with HCC (Table IV). According to this evidence, our results suggest that EGFL6 may have a tumor-suppressor function in liver

Table IV. *Univariate and multivariate analysis of disease-specific survival rate in hepatocellular carcinoma.*

	Univariate			Multivariate		
	Hazard ratio	95%CI	*p*-Value	Hazard ratio	95%CI	*p*-Value
EGFL6 expression						
High	1.0	1.22-2.29	0.001*	1.0	0.97-2.08	0.007*
Low	1.67			1.42		
Differentiation						
G1-G2	1.0	1.28-1.86	<0.001**	1.0	1.36-2.90	<0.001*
G3	1.55			1.98		
Vascular invasion						
No	1.0	1.80-3.91	<0.001**	1.0	1.09-2.75	0.021*
Yes	2.66			1.73		
Stage						
I-II	1.0	1.52-2.23	<0.001**	1.0	1.44-3.59	<0.001**
III-IV	1.84			2.78		
Recurrence						
No	1.0	1.59-4.02	<0.001**	1.0	1.33-3.45	0.002*
Yes	2.52			2.14		

*$p<0.05$; **$p<0.01$; CI: confidence interval.

cancer cells. Further functional studies on the effects of reduced EGFL6 expression may provide additional evidence supporting its tumor-suppressive role in HCC.

Conclusion

In summary, our findings highlight two key points: first, the presence of EGFL6 protein in the cytoplasm emerges as a critical biomarker linked to poor survival in HCC, and second, a decrease in cytoplasmic EGFL6 protein expression may signal an unfavorable prognosis for HCC. However, it remains premature to assert EGFL6 protein as an entirely independent prognostic factor for HCC. Nevertheless, the evidence strongly indicates EGFL6 protein as an independent prognostic indicator for DFS and DSS in patients with HCC. Furthermore, the robust correlation between EGFL6 protein expression and HCC not only positions EGFL6 protein expression as a potential prognostic marker but also underscores its potential utility in developing enhanced therapeutic strategies for patients with HCC.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

HTH, YML, MTH, KTY, and JWL conducted the analysis and drafted the article. They also contributed to data interpretation. HTH, YML, JWL, and SFY contributed to writing the manuscript. All Authors critically reviewed and approved the final version.

Acknowledgements

This research received no external funding.

References

1 Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F: Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71(3): 209-249, 2021. DOI: 10.3322/caac.21660

2 Zhou J, Sun H, Wang Z, Cong W, Wang J, Zeng M, Zhou W, Bie P, Liu L, Wen T, Han G, Wang M, Liu R, Lu L, Ren Z, Chen M, Zeng Z, Liang P, Liang C, Chen M, Yan F, Wang W, Ji Y, Yun J, Cai D, Chen Y, Cheng W, Cheng S, Dai C, Guo W, Hua B, Huang X, Jia W, Li Y, Li Y, Liang J, Liu T, Lv G, Mao Y, Peng T, Ren W, Shi H, Shi G, Tao K, Wang W, Wang X, Wang Z, Xiang B, Xing B, Xu J, Yang J, Yang J, Yang Y, Yang Y, Ye S, Yin Z, Zhang B, Zhang B, Zhang L, Zhang S, Zhang T, Zhao Y, Zheng H, Zhu J, Zhu K, Liu R, Shi Y, Xiao Y, Dai Z, Teng G, Cai J, Wang W, Cai X, Li Q, Shen F, Qin S, Dong J, Fan J: Guidelines for the Diagnosis and Treatment of Hepatocellular Carcinoma (2019 Edition). Liver Cancer 9(6): 682-720, 2020. DOI: 10.1159/000509424

3 Siegel RL, Miller KD, Jemal A: Cancer statistics, 2018. CA Cancer J Clin 68(1): 7-30, 2018. DOI: 10.3322/caac.21442

4 Qi XS, Guo XZ, Han GH, Li HY, Chen J: MET inhibitors for treatment of advanced hepatocellular carcinoma: A review. World J Gastroenterol 21(18): 5445-5453, 2015. DOI: 10.3748/wjg.v21.i18.5445

5 Yin JM, Sun LB, Zheng JS, Wang XX, Chen DX, Li N: Copper chelation by trientine dihydrochloride inhibits liver RFA-induced inflammatory responses in vivo. Inflamm Res 65(12): 1009-1020, 2016. DOI: 10.1007/s00011-016-0986-2

6 Magistri P, Tarantino G, Ballarin R, Berretta M, Pecchi A, Ramacciato G, DI Benedetto F: The evolving role of local

treatments for HCC in the third millennium. Anticancer Res 37(2): 389-402, 2017. DOI: 10.21873/anticanres.11331

7 Donne R, Lujambio A: The liver cancer immune microenvironment: Therapeutic implications for hepatocellular carcinoma. Hepatology 77(5): 1773-1796, 2023. DOI: 10.1002/hep.32740

8 Brown ZJ, Tsilimigras DI, Ruff SM, Mohseni A, Kamel IR, Cloyd JM, Pawlik TM: Management of hepatocellular carcinoma. JAMA Surg 158(4): 410, 2023. DOI: 10.1001/jamasurg.2022.7989

9 Foerster F, Gairing SJ, Müller L, Galle PR: NAFLD-driven HCC: Safety and efficacy of current and emerging treatment options. J Hepatol 76(2): 446-457, 2022. DOI: 10.1016/j.jhep.2021.09.007

10 Lu JW, Ho YJ, Lin LI, Huang YC, Yeh KT, Lin YH, Lin YM, Tzeng TY: JMJD2B as a potential diagnostic immunohistochemical marker for hepatocellular carcinoma: A tissue microarray-based study. Acta Histochem 117(1): 14-19, 2015. DOI: 10.1016/j.acthis.2014.10.002

11 Andersen M, Trapani D, Ravn J, Sorensen JB, Andersen CB, Grauslund M, Santoni-Rugiu E: Methylation-associated silencing of microrna-126 and its host gene egfl7 in malignant pleural mesothelioma. Anticancer Res 35(11): 6223-6229, 2015.

12 Wu F, Shirahata A, Sakuraba K, Kitamura Y, Goto T, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K: Down-regulation of egfl8: A novel prognostic biomarker for patients with colorectal cancer. Anticancer Res 31(6): 2249-2254, 2011.

13 Yeung G, Mulero JJ, Berntsen RP, Loeb DB, Drmanac R, Ford JE: Cloning of a novel epidermal growth factor repeat containing gene EGFL6: Expressed in tumor and fetal tissues. Genomics 62(2): 304-307, 1999. DOI: 10.1006/geno.1999.6011

14 Bai S, Ingram P, Chen YC, Deng N, Pearson A, Niknafs YS, O'Hayer P, Wang Y, Zhang ZY, Boscolo E, Bischoff J, Yoon E, Buckanovich RJ: EGFL6 regulates the asymmetric division, maintenance, and metastasis of ALDH+ ovarian cancer cells. Cancer Res 76(21): 6396-6409, 2016. DOI: 10.1158/0008-5472.CAN-16-0225

15 Chim SM, Qin A, Tickner J, Pavlos N, Davey T, Wang H, Guo Y, Zheng MH, Xu J: EGFL6 promotes endothelial cell migration and angiogenesis through the activation of extracellular signal-regulated kinase. J Biol Chem 286(25): 22035-22046, 2011. DOI: 10.1074/jbc.M110.187633

16 Chang CC, Sung WW, Hsu HT, Yeh CM, Lee CH, Chen YL, Liu TC, Yeh KT: Validation of EGFL6 expression as a prognostic marker in patients with lung adenocarcinoma in Taiwan: a retrospective study. BMJ Open 8(6): e021385, 2018. DOI: 10.1136/bmjopen-2017-021385

17 An J, Du Y, Fan X, Wang Y, Ivan C, Zhang XG, Sood AK, An Z, Zhang N: EGFL6 promotes breast cancer by simultaneously enhancing cancer cell metastasis and stimulating tumor angiogenesis. Oncogene 38(12): 2123-2134, 2019. DOI: 10.1038/s41388-018-0565-9

18 Huo FC, Zhu WT, Liu X, Zhou Y, Zhang LS, Mou J: Epidermal growth factor-like domain multiple 6 (EGFL6) promotes the migration and invasion of gastric cancer cells by inducing epithelial-mesenchymal transition. Invest New Drugs 39(2): 304-316, 2021. DOI: 10.1007/s10637-020-01004-2

19 Zhang QW, Zhang XT, Tang CT, Lin XL, Ge ZZ, Li XB: EGFL6 promotes cell proliferation in colorectal cancer via regulation of the WNT/β-catenin pathway. Mol Carcinog 58(6): 967-979, 2019. DOI: 10.1002/mc.22985

20 Zhu Z, Ni H, You B, Shi S, Shan Y, Bao L, Duan B, You Y: Elevated EGFL6 modulates cell metastasis and growth via AKT pathway in nasopharyngeal carcinoma. Cancer Med 7(12): 6281-6289, 2018. DOI: 10.1002/cam4.1883

21 Kang J, Wang J, Tian J, Shi R, Jia H, Wang Y: The emerging role of EGFL6 in angiogenesis and tumor progression. Int J Med Sci 17(10): 1320-1326, 2020. DOI: 10.7150/ijms.45129

22 Mas VR, Maluf DG, Archer KJ, Yanek KC, Fisher RA: Angiogenesis soluble factors as hepatocellular carcinoma noninvasive markers for monitoring Hepatitis C virus cirrhotic patients awaiting liver transplantation. Transplantation 84(10): 1262-1271, 2007. DOI: 10.1097/01.tp.0000287596.91520.1a

23 Chuang CY, Chen MK, Hsieh MJ, Yeh CM, Lin CW, Yang WE, Yang SF, Chou YE: High level of plasma EGFL6 is associated with clinicopathological characteristics in patients with oral squamous cell carcinoma. Int J Med Sci 14(5): 419-424, 2017. DOI: 10.7150/ijms.18555

24 Noh K, Mangala LS, Han HD, Zhang N, Pradeep S, Wu SY, Ma S, Mora E, Rupaimoole R, Jiang D, Wen Y, Shahzad MMK, Lyons Y, Cho M, Hu W, Nagaraja AS, Haemmerle M, Mak CSL, Chen X, Gharpure KM, Deng H, Xiong W, Kingsley CV, Liu J, Jennings N, Birrer MJ, Bouchard RR, Lopez-Berestein G, Coleman RL, An Z, Sood AK: Differential effects of EGFL6 on tumor versus wound angiogenesis. Cell Rep 21(10): 2785-2795, 2017. DOI: 10.1016/j.celrep.2017.11.020

25 Oberauer R, Rist W, Lenter MC, Hamilton BS, Neubauer H: EGFL6 is increasingly expressed in human obesity and promotes proliferation of adipose tissue-derived stromal vascular cells. Mol Cell Biochem 343(1-2): 257-269, 2010. DOI: 10.1007/s11010-010-0521-7

26 Wang X, Gong Y, Wang D, Xie Q, Zheng M, Zhou Y, Li Q, Yang Z, Tang H, Li Y, Hu R, Chen X, Mao Y: Analysis of gene expression profiling in meningioma: deregulated signaling pathways associated with meningioma and EGFL6 overexpression in benign meningioma tissue and serum. PLoS One 7(12): e52707, 2012. DOI: 10.1371/journal.pone.0052707

27 Lu JW, Chang JG, Yeh KT, Chen RM, Tsai JJ, Hu RM: Overexpression of Thy1/CD90 in human hepatocellular carcinoma is associated with HBV infection and poor prognosis. Acta Histochem 113(8): 833-838, 2011. DOI: 10.1016/j.acthis.2011.01.001

28 Lu JW, Chang JG, Yeh KT, Chen RM, Tsai JJ, Hu RM: Decreased expression of p39 is associated with a poor prognosis in human hepatocellular carcinoma. Med Oncol 28 Suppl 28(S1): 239-245, 2011. DOI: 10.1007/s12032-010-9707-9

29 Hsu HT, Lin YM, Hsing MT, Yeh KT, Lu JW, Yang SF: Correlation between low cytoplasmic expression of XBP1 and the likelihood of surviving hepatocellular carcinoma. In Vivo 38(3): 1316-1324, 2024. DOI: 10.21873/invivo.13571

30 Yang TH, Chan C, Yang PJ, Huang YH, Lee MH: Genetic susceptibility to hepatocellular carcinoma in patients with chronic hepatitis virus infection. Viruses 15(2): 559, 2023. DOI: 10.3390/v15020559

31 Lu JW, Ho YJ, Yang YJ, Liao HA, Ciou SC, Lin LI, Ou DL: Zebrafish as a disease model for studying human hepatocellular carcinoma. World J Gastroenterol 21(42): 12042-12058, 2015. DOI: 10.3748/wjg.v21.i42.12042

32 Gomez-Quiroz LE, Roman S: Influence of genetic and environmental risk factors in the development of hepatocellular carcinoma in Mexico. Ann Hepatol 27 Suppl 27: 100649, 2022. DOI: 10.1016/j.aohep.2021.100649

33 Ho YJ, Chang J, Yeh KT, Gong Z, Lin YM, Lu JW: Prognostic and clinical implications of WNK lysine deficient protein kinase 1

expression in patients with hepatocellular carcinoma. In Vivo 34(5): 2631-2640, 2020. DOI: 10.21873/invivo.12081

34 Lee EY, Muller WJ: Oncogenes and tumor suppressor genes. Cold Spring Harb Perspect Biol 2(10): a003236, 2010. DOI: 10.1101/cshperspect.a003236

35 Zhang J, Chen G, Zhang P, Zhang J, Li X, Gan D, Cao X, Han M, Du H, Ye Y: The threshold of alpha-fetoprotein (AFP) for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis. PLoS One 15(2): e0228857, 2020. DOI: 10.1371/journal.pone.0228857

36 Yamao T, Yamashita YI, Imai K, Umezaki N, Tsukamoto M, Kitano Y, Arima K, Miyata T, Nakagawa S, Okabe H, Hayashi H, Chikamoto A, Ishiko T, Baba H: Clinical significance of preoperative hepatocellular carcinoma with high *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein, but low alpha-fetoprotein. Anticancer Res 39(2): 883-889, 2019. DOI: 10.21873/anticanres.13189

37 Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP: Prognostic molecular markers in hepatocellular carcinoma: A systematic review. Eur J Cancer 43(6): 979-992, 2007. DOI: 10.1016/j.ejca.2007.01.004

38 Yim SH, Chung YJ: An overview of biomarkers and molecular signatures in HCC. Cancers (Basel) 2(2): 809-823, 2010. DOI: 10.3390/cancers2020809

39 Su G, Wang W, Xu L, Li G: Progress of EGFL6 in angiogenesis and tumor development. Int J Clin Exp Pathol 15(11): 436-443, 2022.

Received June 17, 2024
Revised July 11, 2024
Accepted July 12, 2024