Aberrant Expression of The Esophageal Carcinoma Related Gene 4 As A Prognostic Signature For Hepatocellular Carcinoma

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Abstract

Background. Hepatocellular carcinoma (HCC) is a lethal cancer with increasing incidence, yet the molecular biomarkers that have strong prognostic impact and also hold great therapeutic promise remain elusive.

Methods. Data mining approaches with a set of publicly accessible databases and immunohistochemistry were used to provide a novel insight into the expression pattern and prognostic significance of the esophageal cancer-related gene (ECRG) family members in HCC.

Results. We found that elevated mRNA expression levels of ECRG factors were correlated with better overall survival, relapse-free survival and progression-free survival rates in patients with HCC. Subgroup analyses showed significant associations between ECRG expression and survival outcome in select HCC patients. In addition, immunohistochemical and multivariate analysis confirmed increased ECRG4 expression as an independent prognostic indicator for survival.

Conclusion. Our data suggest that ECRG factors have significant impacts on the survival of HCC patients. The expression of ECRG factors may be involved in HCC progression and could serve as novel biomarkers for predicting more accurate prognosis.

Introduction

Hepatocellular carcinoma (HCC) ranks the third leading cause of cancer death worldwide and the second most common malignancy in China [1, 2]. HCC carcinogenesis is a multistep process with morphological progression involving multiple genetic and epigenetic events. To date, despite new discoveries and technologies dedicated to precision medicine, the molecular mechanisms behind the initiation, progression and pathogenesis of HCC cancer remain unclear. This impedes designing effective and personalized chemo- and bio-therapy strategies. Hence, it is important and intriguing to identify novel molecular factors and to elucidate their biological functions and prognostic implications in HCC.

Through our ongoing endeavors in finding new targets significantly correlated with the outcomes of patients with breast cancer by integrative analysis of existing public online data, we observed that the esophageal cancer-related gene (ECRG) family members may be at the top of the list [3]. The ECRG family was originally cloned and identified from fragments isolated for identifying differentially expressed genes between esophageal cancer samples and normal epithelial controls, and has been described to be fundamental in cancer initiation and progression [4, 5]. Since the identification of ECRGs 20 years ago, increasing biological function data have gradually demonstrated their diverse effects in cancer and the correlation between these proteins and malignant characteristics such as cell cycle and apoptosis, cell proliferation and metastasis, chemo-sensitivity and cellular immunity [4–13]. To date, three ECRG factors have been reported to function in physiology and pathology, including ECRG1 (also called TMPRSS11A), ECRG2 (SPINK7) and ECRG4 (C2orf40). Dysregulated expression of ECRG family members has been observed in different human malignancies [12–18]. Our previous study also

demonstrated promoter methylation-mediated silencing of ECRG4 in nasopharyngeal carcinoma [3]. More recently, our group noticed that attenuated ECRG4 protein expression was significantly correlated with breast cancer metastasis and patient survival [19]. Yet despite such great promise, the specific roles of individual ECRG family members in HCC and their association with patient outcomes have not been well documented. Therefore, in this study we systematically examined the prognostic significance and potential roles of distinct ECRG factors in HCC, based on *in silico* data mining using several online databases. Additionally, immunohistochemical analysis was also performed to confirm the result in conjunction with clinicopathologic parameters and survival data.

Materials And Methods

Oncomine database analysis

To analyze the expression levels of specific ECRGs in a variety of malignancies, Oncomine (http://www.oncomine.org) was used, which is an online cancer microarray database including 715 datasets and 86,733 samples to facilitate and promote discovery from genome-wide expression analyses [20]. Paired Student's t-test was used. A fold-change of 2 with $P<0.01$ was defined as clinically significant.

Gene Set Cancer Analysis (GSCA) database analysis

Gene Set Cancer Analysis (GSCA) is an integrated genomic and immunogenomic web-based platform for gene set cancer research [21]. In this study, gene expression levels of ECRG factors across multiple cancer types were calculated using the GSCA online database (http://bioinfo.life.hust.edu.cn/GSCA/).

Cancer Cell Line Encyclopedia (CCLE) database analysis

The mRNA levels of distinct ECRG factors in multiple types of cancer cell lines were determined using the CCLE database (http://portals.broadinstitute.org/ccle), which is an online encyclopedia of a data collection of gene expression, copy numbers and massively parallel sequences from 1,457 human cancer cell lines [22].

cBioPortal cancer genomics database analysis

The impact of genomic alterations of ECRGs containing gene mutations and copy-number variance on the overall survival (OS) and disease-free survival (DFS) rates of patients with HCC were calculated using the cBioPortal online database (www.cbioportal.org) [23]. cBioPortal for Cancer Genomics allows the visualization, analysis and download of large-scale cancer genomics datasets.

Kaplan-Meier Plotter survival analysis

The prognostic impact of ECRG mRNA expression were analyzed using the Kaplan-Meier Plotter online database (http://kmplot.com/analysis/), which is capable of assessing the effect of 54k genes (mRNA, miRNA, protein) on survival in 21 cancer types including HCC [24, 25]. To investigate the overall survival

(OS), relapse-free survival (RFS) and progression-free survival (PFS) in patients with HCC, the clinical samples were divided into high and low-expression groups by the median value of mRNA expression. After the target probe was separately entered into the database, survival analyses were carried out to generate Kaplan-Meier plots.

Tissue specimens

A tissue microarray chip was purchase from Outdo Biotech Co., Ltd. (Shanghai, China) for immunohistochemistry, providing 100 primary HCC tissues along with 80 paired adjacent noncancerous tissues from patients undergoing curative surgery between February 2008 and June 2010. The median postoperative follow-up period was 41 months (range, 1-88 months). During the follow-up period, 40 (40.8%) patients had died because of disease recurrence and distant metastasis. Tumor grade and stage were classified in accordance with the International Union against Cancer (UICC)/American Joint Committee on Cancer (AJCC) pathologic tumor-node-metastasis (TNM) classification, 7th edition (2010). The clinicopathological parameters are summarized in Table 1. All cases were confirmed by two pathologists. No patients had received neoadjuvant therapy before surgery. Signed informed consents were obtained from patients in accordance with the principles expressed in the Declaration of Helsinki. This study was approved by the Institution Review Board of People's Hospital of Ningxia Hui Autonomous Region.

Table 1
Correlation between ECRG4 expression and clinicopathological factors in HCC patients

Parameters	ECRG4 expression		χ2	*P* value
	Low, *n* (%)	High, *n* (%)		
Age (years)				
<68	20	36	0.332	0.565
≥68	17	24		
Sex				
Female	16	24	0.099	0.753
Male	21	36		
Histological grade				
I/II	21	58	21.644	<0.001
III	16	3		
T classification				
T2	4	0	11.455	0.003
T3	17	45		
T4	16	16		
N classification				
N0	11	40	11.856	0.013
N1/N2	26	21		
M classification				
M0	32	61	1.611	0.204
M1	5	0		
Clinical stage (pTNM)				
I/II	10	40	13.694	<0.001
III/IV	27	21		

Immunohistochemistry and evaluation

Immunohistochemical analysis for ECRG4 was performed using the standard EnVision complex method as described previously [19]. 4-μm sections were cut from specimens that had been fixed in 10% buffered formalin and embedded in paraffin. After undergoing deparaffinization (xylene, 2 times and 10 min each at 37˚C) and rehydration (alcohol gradient, 100, 95, 80 and 70%), endogenous peroxidase blocking (0.3% hydrogen peroxide, 30 min at room temperature) and antigen retrieval (at 121˚C in citrate buffer at 10 mM, pH 6.0 for 10 min), specimens were incubated with a rabbit polyclonal anti-ECRG4 antibody (catalog no. PA5-38791; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a dilution of 1:400 overnight at 4˚C. Immunohistochemical staining was conducted by an EnVision antibody complex (anti-mouse/rabbit) method in conjunction with an EnvisionTM Detection kit (ZSGB-BIO, Beijing, China) with 3,3'-diaminobenzidine as the chromogen substrate. Nuclei were counterstained with 0.5% hematoxylin for 2 min at room temperature. Sections immunostained with normal rabbit IgG (catalog no. ab188776; dilution, 1:50; Abcam, Cambridge, MA, USA) as the primary antibody were used as negative controls. The staining evaluation was performed as follows: ten random 400× microscopic fields per slide were evaluated by two independent observers who were blinded to the clinical information. Global ECRG4 immunostaining was scored using a semi-quantitative integration method combining the percentage of positive cells and the staining intensity. The mean percentage of positively stained cells was scored as follows: 0% (0); 1%-25% (1); 26%-50% (2); 51%-75% (3); and 76%-100% (4). The staining intensity was categorized as follows: absent (0); weak staining exhibited as light yellow (1); moderate staining exhibited as yellow brown (2); and strong staining exhibited as brown (3). The multiplication of the intensity and extent scores was used as the final staining score. For the purpose of statistical evaluation, tumors having a final staining score of ≤ 3 were designated as low expression and those with scores >3 as high expression.

Statistical analyses

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). Associations between different expression levels of ECRG factors and clinicopathological features were analyzed using a χ^2 test. For Kaplan-Meier Plotter and prognostic relevance of ECRG mRNA levels in HCC patients, survival was estimated according to the Kaplan-Meier method and the log-rank test. Significant factors were identified by univariate analysis, and further examined by multivariate regression analysis with a Cox proportional-hazards regression model. $P<0.05$ defined statistical significance.

Results

mRNA expression patterns of ECRG family members in human cancers

Hitherto, three ECRG family members have been identified in various types of human cancer, including hematological malignancies and solid tumors. As shown in Fig. 1A, the Oncomine database contained a total of 138, 255 and 260 unique analyses for ECRG1, ECRG2 and ECRG4, respectively. The mRNA expression levels of all three ECRG factors were significantly higher in cancer tissues than in normal

samples across a wide variety of datasets in different cancer types, except for one study on kidney cancer. Of note, however, the mRNA levels of ECRG factors were not reported in the liver cancer datasets. We further explored the expression levels of ECRG factors across the Cancer Genome Atlas (TCGA) cancer types using the GSCA database. Among three ECRG family members, only ECRG4 exhibited an obvious difference when comparing its expression in liver hepatocellular carcinoma (LIHC) tissues with that in normal tissues (Fig. 1B). In addition, the CCLE database analysis demonstrated that the mRNA expression level of ECRG1, ECRG2 and ECRG4 in liver cancer cells ranked in the 31st, 23rd and 13rd positions, respectively, among all cancer types (Fig. 2).

Correlation between the mRNA levels of ECRG factors and patient survival

The prognostic impact of ECRG factors on the survival of patients with HCC was analyzed using the Kaplan-Meier Plotter survival analysis. High ECRG1 mRNA level appeared to predict a better OS rate for HCC patients (Fig. 3A). Subgroup analyses revealed that high expression of ECRG1 predicted longer OS times in patients with both stage I/II tumors and stage III/IV tumors (Fig. 3B and 3C). High ECRG1 expression also indicated a favorable RFS rate (Fig. 3D). High ECRG1 mRNA level was significantly associated with longer RFS times for male patients (Fig. 3E), but not for the female patients (Fig. 3F). Notably, we observed that elevated ECRG1 was associated with an improved RFS in patients with hepatitis virus infection (Fig. 3G and 3H). In addition, high ECRG1 mRNA level predicted a better PFS rate for HCC patients (Fig. 3I)

Similarly, we observed that high ECRG2 mRNA expression was associated with a better OS rate in HCC patients (Fig. 4A). Subgroup analyses demonstrated that elevated ECRG2 was associated with a favorable OS in both stage I/II patients and stage III/IV patients (Fig. 4B and 4C). High ECRG2 expression implied longer OS times in the subgroups of patients with or without micro vascular invasion (Fig. 4D and 4E). Besides, high ECRG2 levels indicated a improved RFS rate in HCC patients (Fig. 4F). Increased ECRG2 expression was correlated with a longer RFS in patients with hepatitis virus infection (Fig. 4G and 4H). High ECRG2 mRNA level also predicted a better PFS rate for HCC patients (Fig. 4I)

Consistently, up-regulation of ECRG4 was observed to be correlated with a better OS in HCC patients (Fig. 5A). Subgroup analyses revealed that increased ECRG4 mRNA levels illustrated longer OS times in patients with stage I/II tumors and stage III/IV tumors (Fig. 5B and 5C). Increased ECRG4 expression also represented a favorable OS in patients with or without micro vascular invasion (Fig. 5D and 5E). High ECRG4 expression also represented an improved OS in male patients but not in female patients (Fig. 5F and 5G). Furthermore, elevated ECRG4 displayed prolonged RFS and PFS rates for HCC patients (Fig. 5H and 5I).

Correlation between genetic alterations of ECRG factors and patient survival

Subsequently, we assessed gene alterations using the cBioPortal online database. The genetic alteration rates for ECRG1, ECRG2 and ECRG4 were 0.6, 0.3 and 0.2%, respectively (Fig. 6A). However, no significant association was detected between genetic alteration of ECRG factors and OS rates in HCC patients (Fig.

6B, 6D and 6F). Of note, we observed that the genetic alterations of ECRG1 and ECRG4 were correlated with DFS rates, respectively (Fig. 6C and 6G). No significant association was found between genetic alterations of ECRG2 and the DFS rate in HCC patients (Fig. 6E).

ECRG4 expression is an independent prognostic predictor in HCC

In support of the above findings, we further investigated the expression profile of ECRG4 in 100 FFPE specimens using a HCC tissue microarray chip and immunohistochemistry. However, 12 HCC tissue samples in the tissue microarray chip were lost during IHC staining. We observed positive ECRG4 immunostaining in the cytoplasm of tumor cells in 62.2% (61/98) of the HCC samples tested (Fig. 7).Further, low ECRG4 expression was observed to be significantly associated with histological grade, T classification, lymph node metastasis (N classification) and clinical tumor stage (pTNM) (Table 1). Kaplan-Meier survival analyses showed that patients with high ECRG4 expression exhibited a better OS than those with low ECRG4 expression (Fig. 8). Upon univariate analysis, histological grade, clinical stage, and ECRG4 expression, were determined to be responsible for the outcomes of HCC patients (Table 2). However, the multivariate analysis showed that histological grade and ECRG4 expression were independent predictors of prognosis for HCC patients (Table 2).

Table 2
Univariate and multivariate Cox regression analysis for OS in HCC patients

Variables	**Univariate analysis**			**Multivariate analysis**		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Clinical stage	0.412	0.237-0.715	0.002	0.67	0.35-1.284	0.228
Histological grade	4.058	2.381-6.915	<0.001	3.33	1.886-5.879	<0.001
ECRG4 expression	1.836	1.167-2.889	0.009	1.642	1.011-2.667	0.045
HR, hazard ratio; CI, confidence interval						

Discussion

The current study is part of our ongoing research project aimed to identify the correlation between the expression pattern of ECRGs and outcomes of HCC patients in order to identify novel diagnostic and prognostic biomarkers, which may help to guide clinical management of breast cancer in the future. Thus, our present findings using *in silico* analyses provide an insight into the molecular mechanisms underlying this disease. We utilized multiple accessible online databases to perform a systematic and comprehensive analysis of ECRG family members in HCC. Our results indicated that increased expression levels of ECRG1, ECRG2 and ECRG4 levels were significantly associated with better OS, RFS and PFS rates, respectively. These above results suggest their roles as tumor suppressors in cancer development.

In 1998, Su et al discovered how cloning and sequencing expressed ribonucleic acids could be used to implicate genes in the development of esophageal cancer [5]. They compared differences in gene expression profiles between normal esophageal epithelia and esophageal cancer and found four novel esophageal cancer-related genes, i.e. ECRG1, ECRG2, ECRG3 and ECRG4, using a differential displaying technique. The biological functions and expression profiles of ECRG1, ECRG2 and ECRG4 has been reported widely. However, as of yet, the biological role on ECRG3 remains to be elucidated. We also cannot find the relative data about ECRG3 either on Pubmed or the databases used in this study.

It has been reported that single nucleotide polymorphism (SNP) of the ECRG1 gene might be a potential negative prognostic marker in oral squamous cell carcinoma and esophageal cancer [26, 27]. More intensively, another report showed that the ECRG1 290Gln variant allele may act as a genetic susceptibility factor for developing ESCC, especially in the smoking population [28]. To date, studies on ECRG1 have only limited to several cancer types [29–31]. Although ECRG1 has been proposed as a tumor suppressor, its prognostic value on cancers is mostly unknown. We found that high ECRG1 expression was a favorable indicator for predicting the prognosis of HCC patients. Increased ECRG1 levels displayed a significant correlation with better OS rates in a cohort of patients with stage I-II tumors, suggesting that ECRG1 expression may be valuable in predicting the prognosis of patients with early-stage HCC. Increased ECRG1 expression also indicated better RFS rates in male patients and patient with hepatitis virus infection. We inferred that ECRG1 may exert different functions upon diverse conditions due to sex difference and hepatitis virus infection.

To the best of our knowledge, no study has focused on the association between ECRG2 expression and outcomes of patients with cancer thus far. However, it has been reported that short tandem repeat polymorphism in the ECRG2 gene may predict relapse of patients with oral squamous cell carcinoma and esophageal cancer [32, 33]. Previous studies provided evidence that ECRG2 may serve as a tumor suppressor by regulating cell invasion/migration partly through ECM degradation and the urokinase-type plasminogen activator receptor (uPAR)/formyl peptide receptor-like 1 (FPRL1) signaling pathway in several cancer cell lines, including lung, colon and breast cancer [34–36]. The above findings appear to be consistent to our current findings that increased ECRG2 levels were correlated with improved outcomes in patients with HCC. Notably, we found that increased ECRG2 expression was significant correlated with better OS or RFS rates in a cohort of patients with stage I-II tumors or without vascular invasion. These findings suggest ECRG2 as a valuable prognostic predictor for patients with early-stage disease.

Consistent with our present findings, downregulated ECRG4 was observed to be associated with lymph node metastasis, and predicted poor outcomes in a variety of cancer types, including esophageal carcinoma, prostate cancer, gastric cancer, nasopharyngeal carcinoma and renal cell cancer [13, 14, 37, 38]. These above findings have clearly revealed the tumor-suppressor roles of ECRG4. Our recent study also revealed that decreased ECRG4 protein expression was correlated with lymph node metastasis and advanced tumor stage in breast cancer and may serve as an independent high-risk predictor for the prognosis of this malignancy [19]. In this study, we observed that high ECRG4 mRNA expression was a

favorable prognostic factor in patients with breast cancer. Furthermore, it was shown that elevated ECRG4 mRNA levels predicted favorable survival in subsets of patients, which was similar with the result of ECRG1 and ECRG2. Therefore, we hypothesized that ECRG4 expression may play an important role in HCC progression. To support the above observation by data mining approaches, our analysis of immunohistochemistry identified strongly positive correlated tendency between ECRG4 protein overexpression and a favorable OS. As ECRG4 gene product has been identified as a secretary molecule and can be detected in cell culture medium, it is very likely that ECRG4 protein or its derived peptides might potentially be a suitable biotherapeutic reagent for cancer treatment [3, 4]. Thus, we conclude that ECRG4 expression may serve as a prognostic biomarker that also holds great therapeutic promise for HCC.

In summary, different ECRG family members have their impact on the prognosis in HCC patients and could serve as prognostic predictors that hold therapeutic promise for HCC. Future intensive in vitro and *in vivo* research should be conducted to elucidate the exact functions of ECRG factors in the initiation and progression of HCC, which may support the hypothesis that ECRG family members could be prognostic indicators and promising therapeutic targets for precision medicine for HCC treatment.

Declarations

Acknowledgments

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Availability of data and materials

The dataset used and/or analyzed in the current study is available from the corresponding authors upon reasonable request.

Authors' contributions

YY, SH, YD and FH conceived the study, designed experiments, performed the experiments, analyzed the data and drafted the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Signed informed consent was obtained from the patients prior to tissue sample collection. The study protocol conformed to the ethical guidelines outlined in the Declaration of Helsinki and was approved by the Institutional Review Board (approval no. 07-170) of Ningxia Hui Autonomous Region People's Hospital.

Patient consent for publication

Signed informed consent was obtained from the patients prior to tissue sample collection.

Competing interests

The authors declare that they have no competing interests.

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Figures

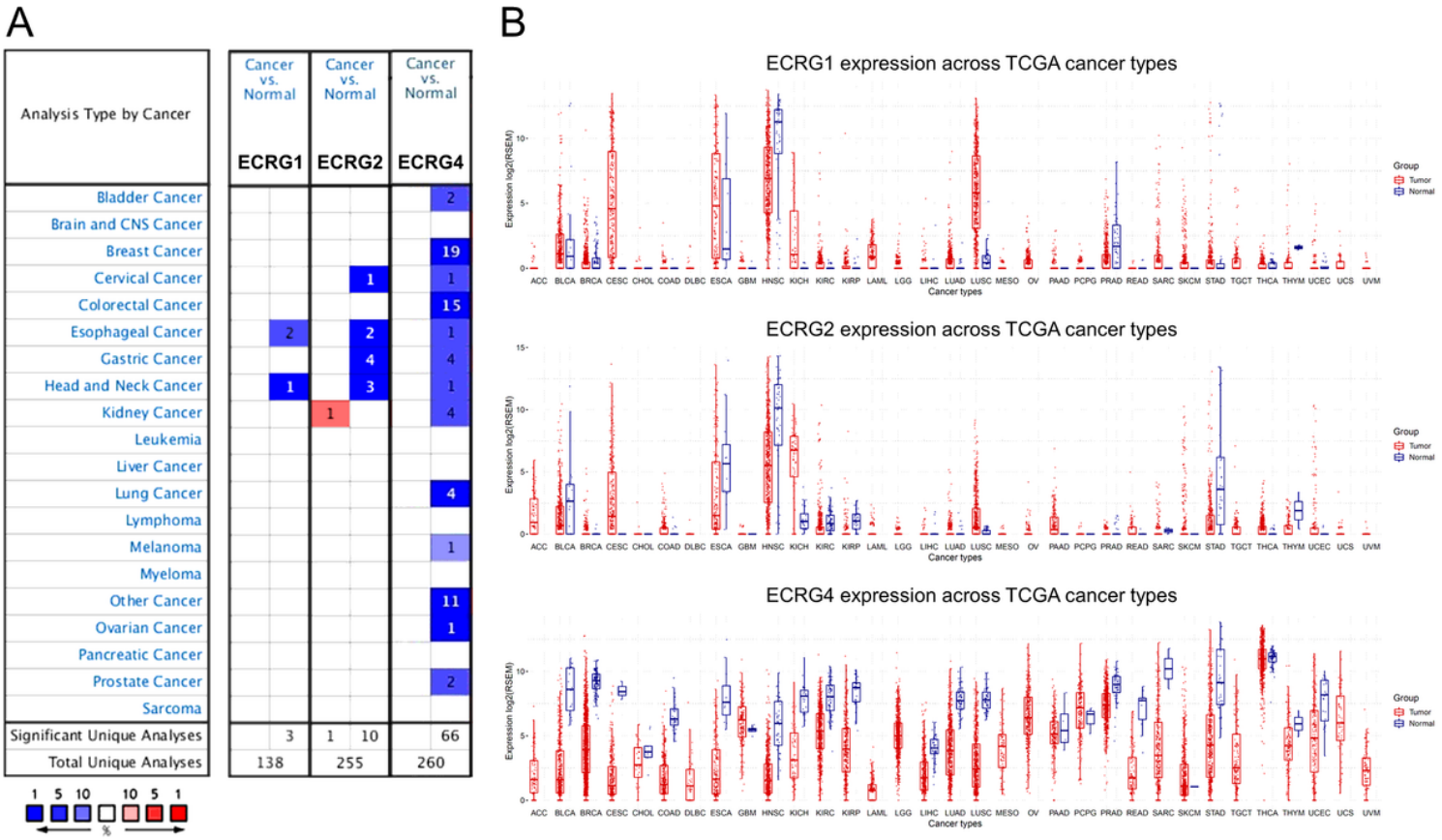

Figure 1

Transcription levels of ECRG family members in different cancer types. (A) A graphic obtained from Oncomine indicates the numbers of datasets with significant overexpression (red) or downregulation (blue) of ECRG factors at the transcriptional level in cancer tissues compared their expression in the corresponding normal tissues. The cell color was determined by the best gene rank percentile for the analyses within the cell, and the gene rank was analyzed by percentile of target genes in the top of all genes measured in each study. The cut-offs for P-values and fold-changes were defined as 0.01 and 2, respectively. ECRG, esophageal cancer-related gene. (B) mRNA expression levels of ECRG factors across TCGA cancer types determined using the GSCA database analysis.

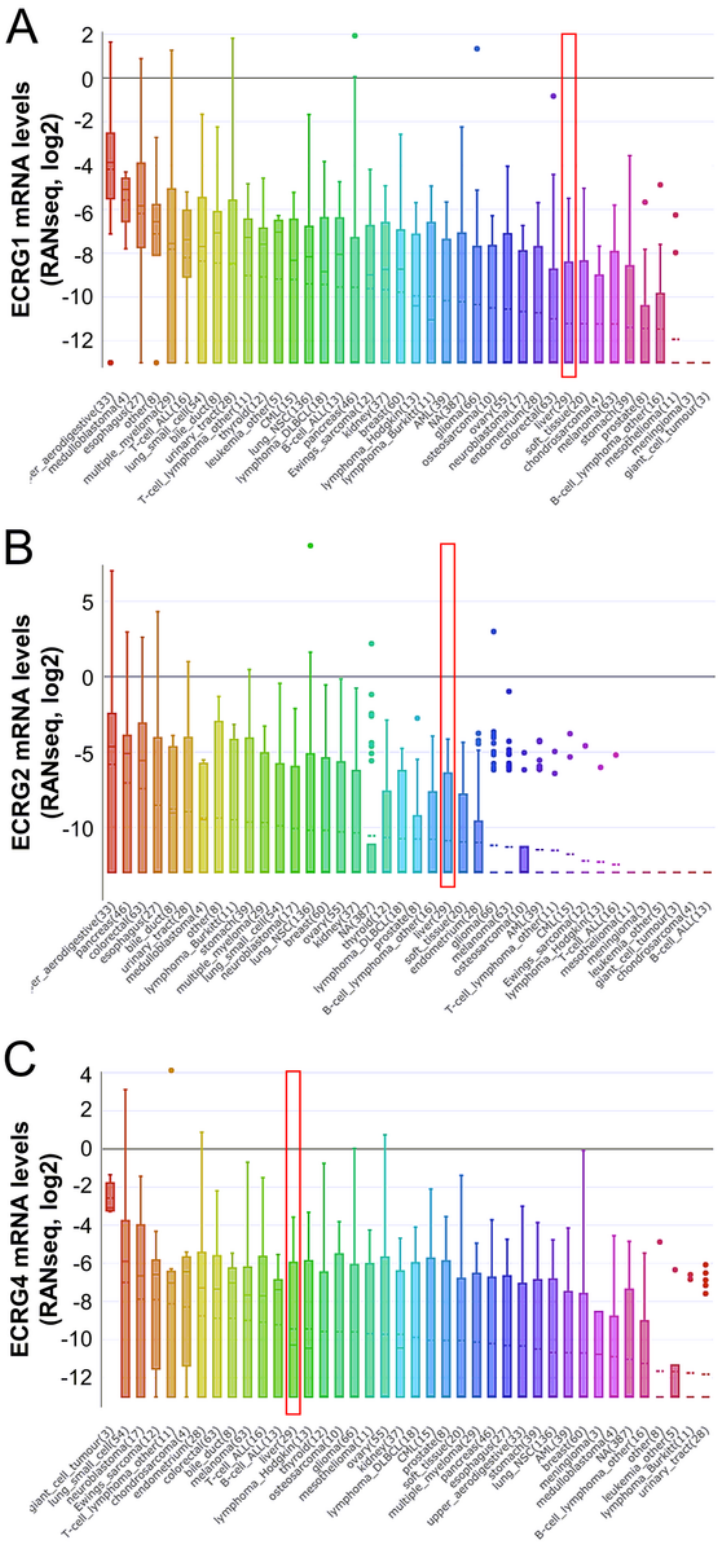

Figure 2

ECRG family members are distinctively highly expressed in liver cancer cell lines according to Cancer Cell Line Encyclopedia (CCLE) analysis. The mRNA expression levels of (A) ECRG1, (B) ECRG2 and (C) ECRG4 ranked the 31st, 23rd and 13rd highest in breast cancer cells among different cancer types (shown in red frame). ECRG, esophageal cancer-related gene.

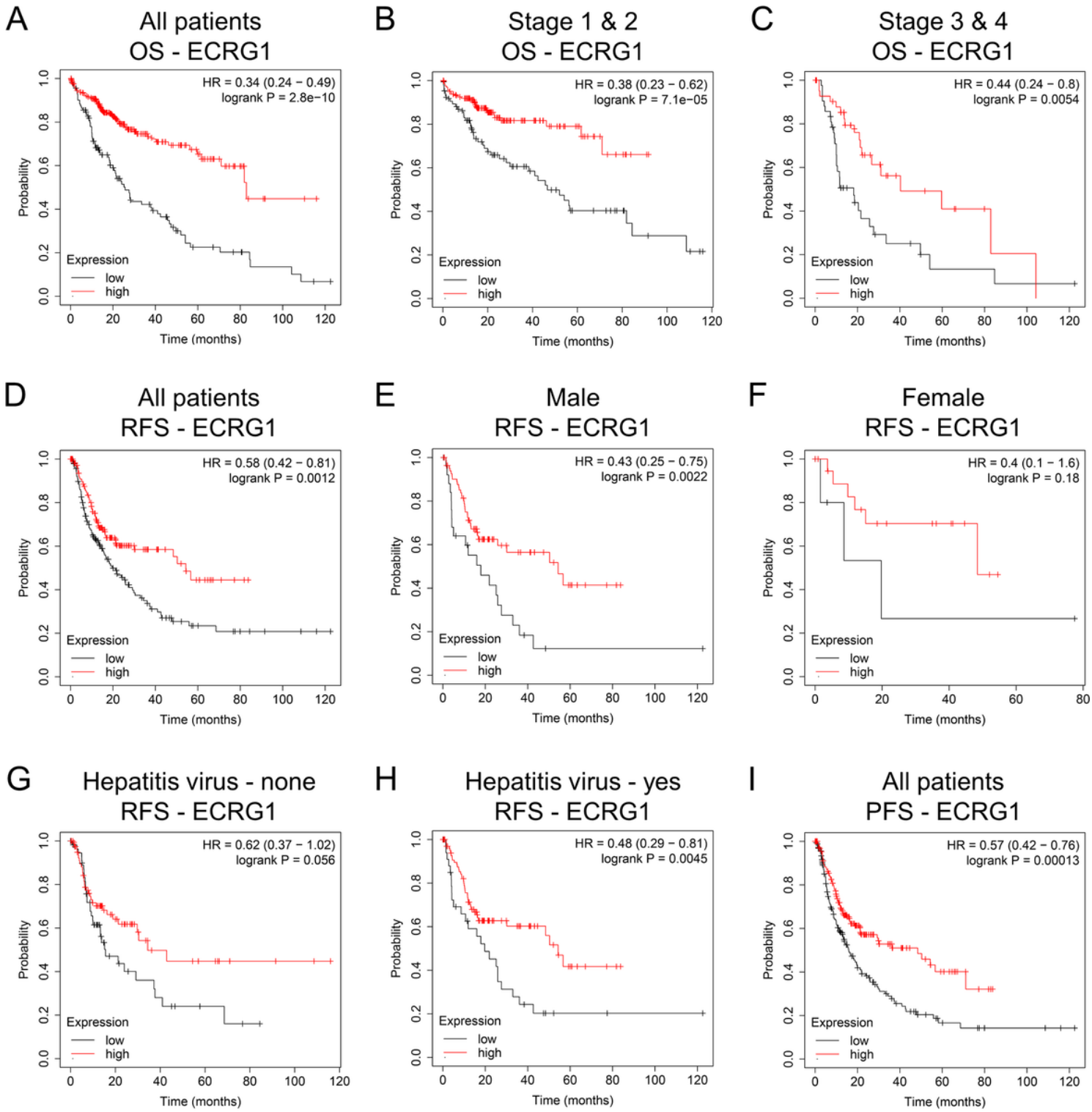

Figure 3

Correlation between ECRG1 mRNA expression and survival of HCC patients using the Kaplan-Meier plotter online database. (A) OS analysis of ECRG1 in all patients. (B and C) OS analysis of ECRG1 in Stage 1-2 and Stage 3-4 patients. (D) RFS analysis of ECRG1 in all patients. (E and F) RFS analysis of ECRG1 in male and female patients. (G and H) RFS analysis of ECRG1 in patients without and with hepatitis virus infection. (I) PFS analysis of ECRG1 in all patients. ECRG, esophageal cancer-related gene; OS, overall survival; RFS, relapse-free survival; PFS, progression-free survival.

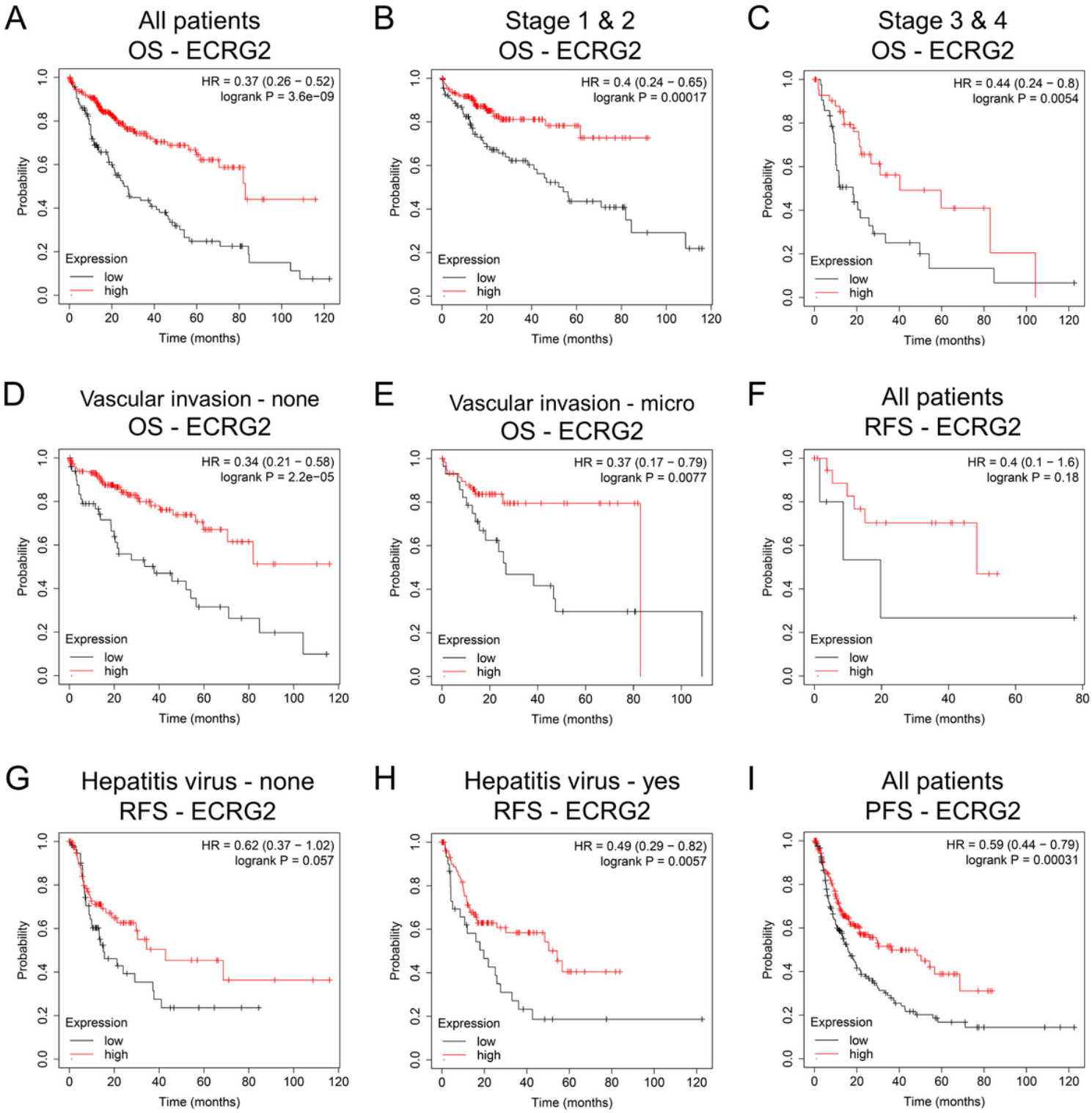

Figure 4

Correlation between ECRG2 mRNA expression and survival of HCC patients using the Kaplan-Meier plotter online database. (A) OS analysis of ECRG2 in all patients. (B and C) OS analysis of ECRG2 in Stage 1-2 and Stage 3-4 patients. (D) OS analysis of ECRG2 in patients without vascular invasion. (E) OS analysis of ECRG2 in patients with micro vascular invasion. (F) RFS analysis of ECRG2 in all patients. (G and H) RFS analysis of ECRG2 in patients without and with hepatitis virus infection. (I) PFS analysis of ECRG2 in

all patients. ECRG, esophageal cancer-related gene; OS, overall survival; RFS, relapse-free survival; PFS, progression-free survival.

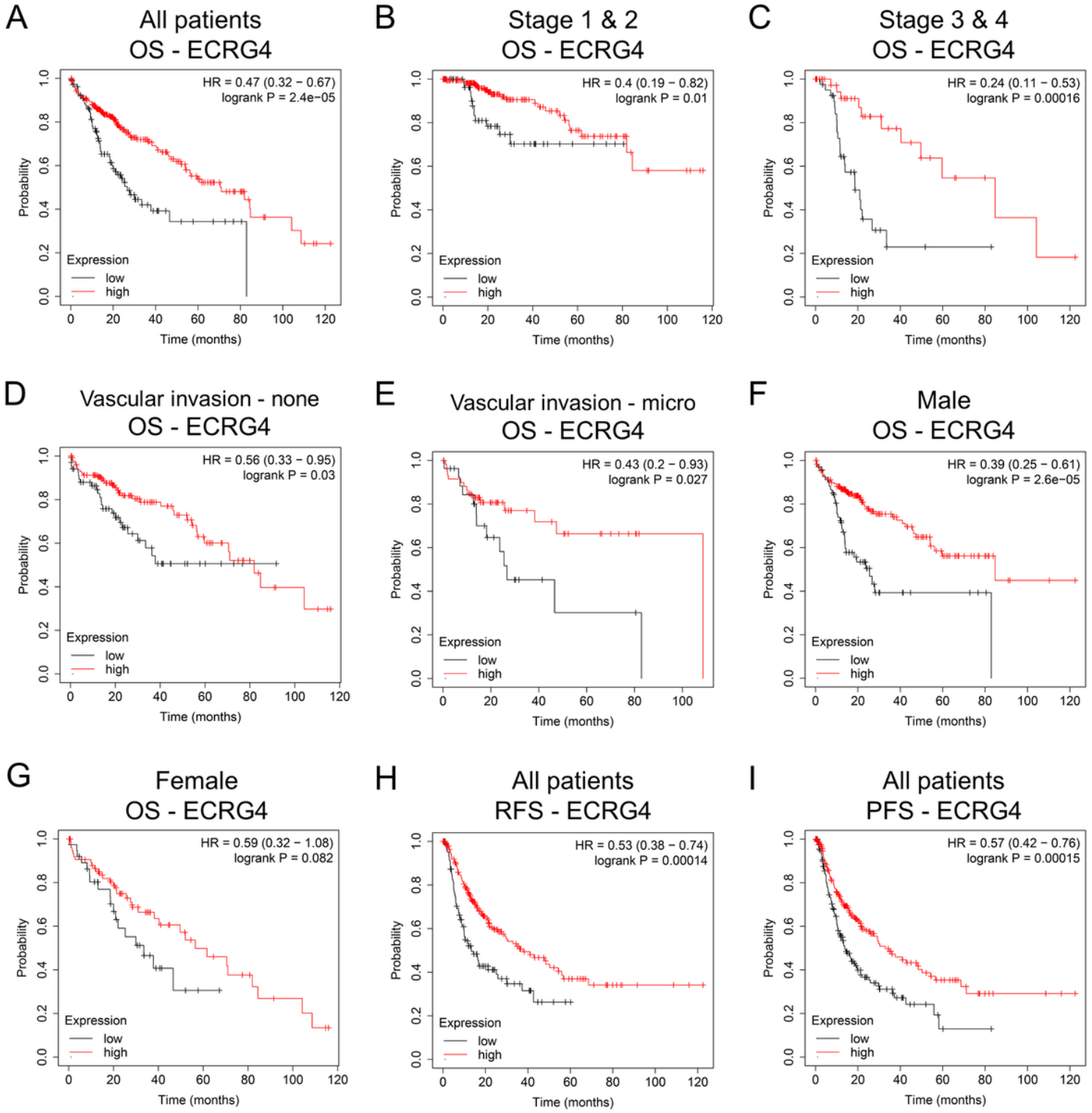

Figure 5

Correlation between ECRG4 mRNA expression and survival of HCC patients using the Kaplan-Meier plotter online database. (A) OS analysis of ECRG4 in all patients. (B and C) OS analysis of ECRG4 in Stage 1-2 and Stage 3-4 patients. (D) OS analysis of ECRG4 in patients without vascular invasion. (E) OS analysis of ECRG4 in patients with micro vascular invasion. (F and G) OS analysis of ECRG4 in male and female

patients. (H) RFS analysis of ECRG4 in all patients. (I) PFS analysis of ECRG4 in all patients. ECRG, esophageal cancer-related gene; OS, overall survival; RFS, relapse-free survival; PFS, progression-free survival.

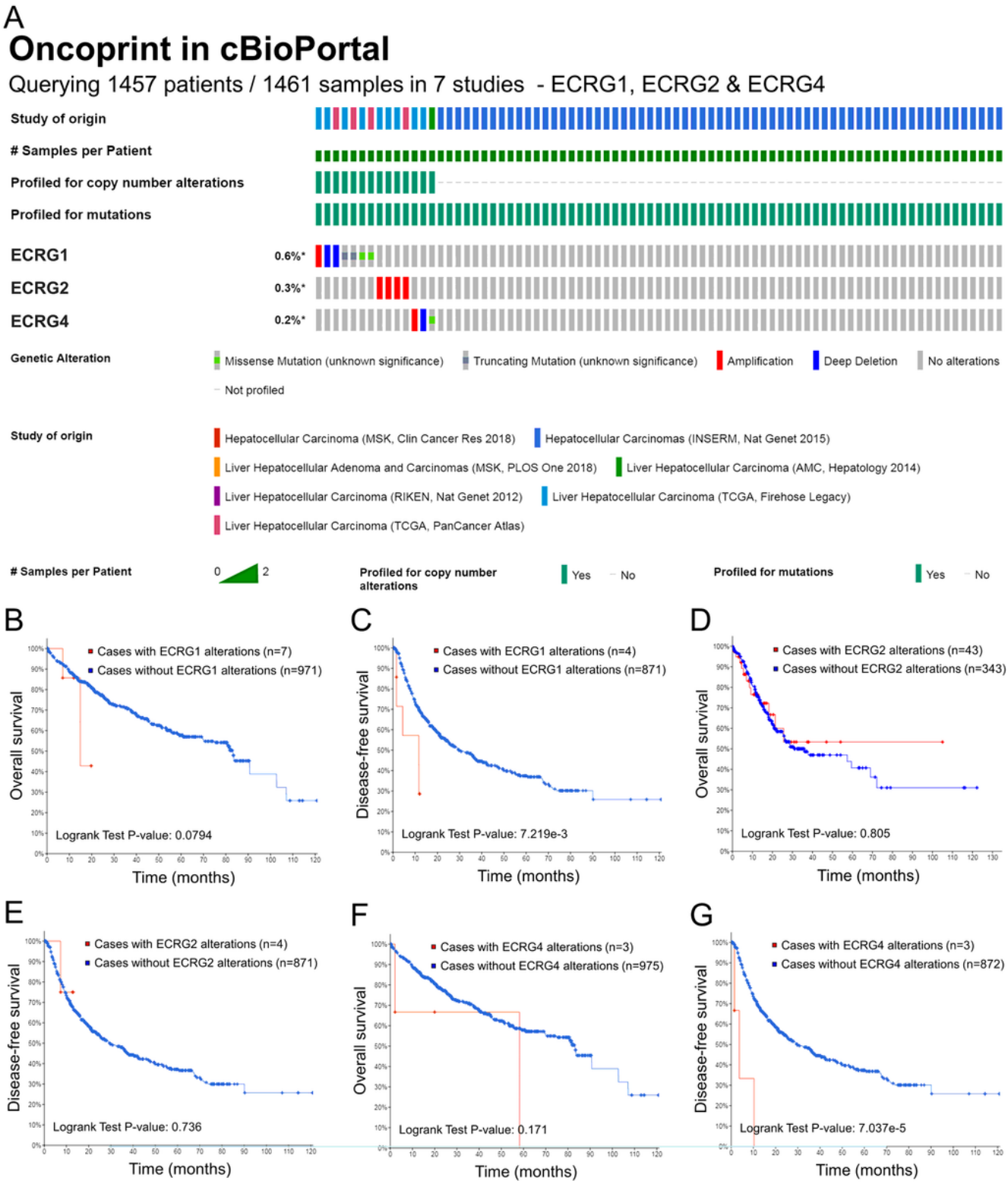

Figure 6

Alterations of ECRG genes and their association with patient survival in HCC via the cBioPortal database analysis. (A) OncoPrint in cBioPortal represented the proportion and distribution of samples with

alterations in ECRG factors. The figure was cropped on the right side to exclude samples without alterations. (B, D, F) The impact of genetic alterations of ECRG1 (B), ECRG2 (D) and ECRG3 (F) on OS rates of HCC patients. (C, E, G) The impact of genetic alterations of ECRG1 (C), ECRG2 (E) and ECRG3 (G) on DFS rates of HCC patients. ECRG, esophageal cancer-related gene; OS, overall survival; DFS, disease-free survival.

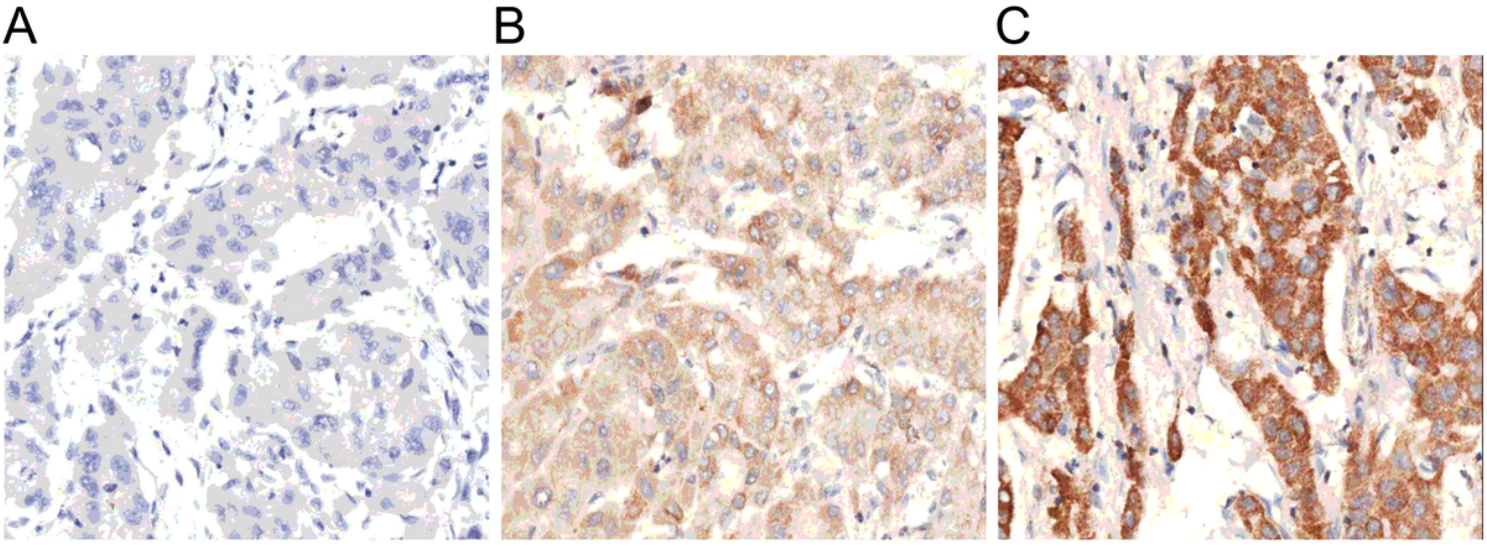

Figure 7

Representative immunohistochemiscal staining for ECRG4 in HCC tissues. (A) negative staining; (B) low staining; and (C) high staining. Original magnification 200×.

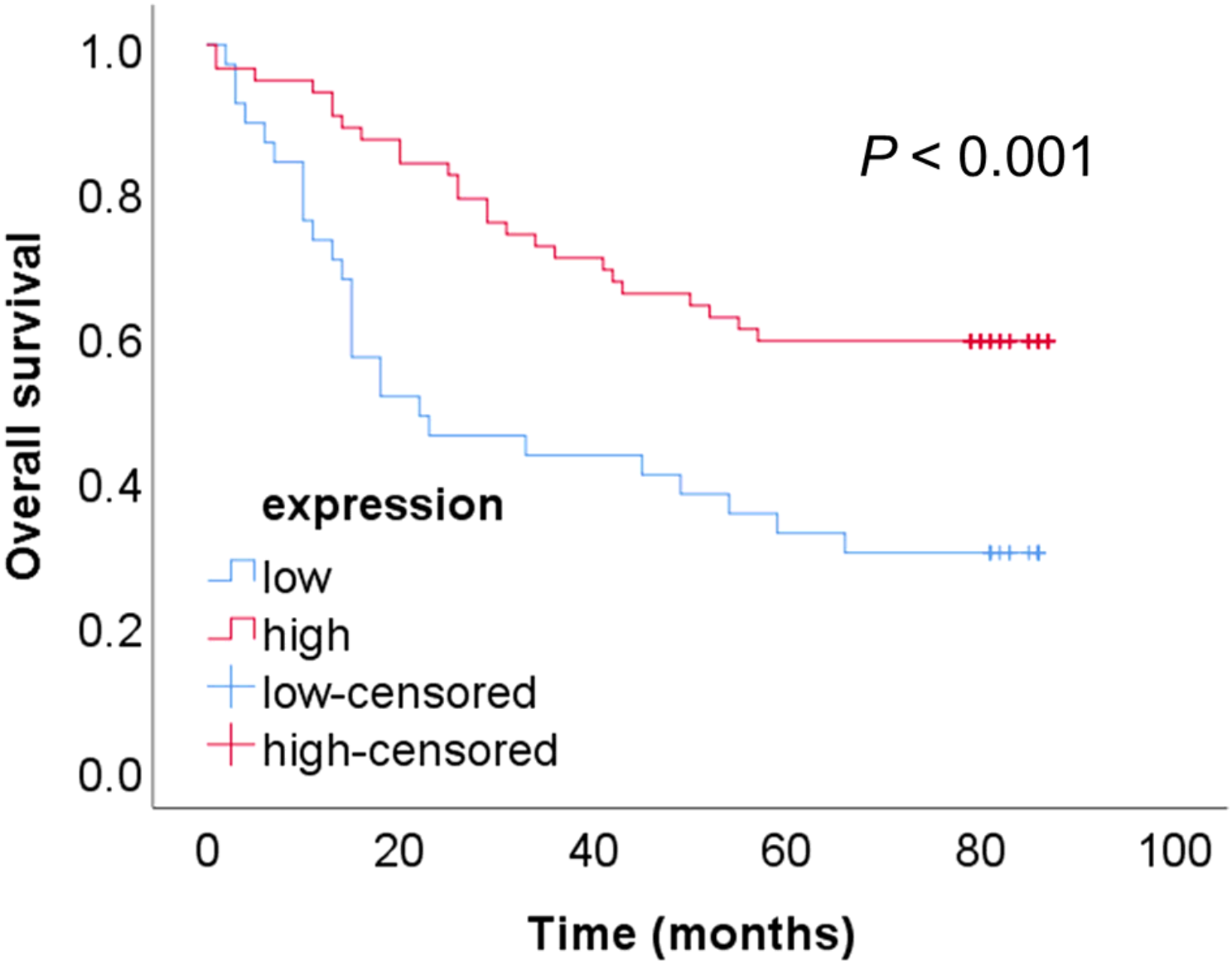

Figure 8

Kaplan-Meier curves comparing the OS rate in HCC patients with high and low protein levels of ECRG4. Low ECRG4 expression was significantly correlated with a shorter OS rate in HCC patients (P < 0.001).