

Prognostic Significance of NBEAL2 in Liver hepatocellular carcinoma

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Abstract

Background: NBEAL2 is a member of the BEACH domain-containing protein (BDCP) family and little is known about the relationship between NBEAL2 and malignancy.

Methods: We downloaded the Gene expression profiles and clinical data of Liver hepatocellular carcinoma (LIHC) from the Cancer Genome Atlas (TCGA) dataset. The expression difference of NBEAL2 in LIHC tissues and adjacent nontumor tissues was analyzed by R software. The relationship between NBEAL2 expression and clinicopathological parameters was evaluated by Chi-square test. The effect of NBEAL2 expression on survival was assessed by Kaplan–Meier survival analysis and Cox proportional hazards regression model. GSEA was used to explore the potential molecular mechanism of NBEAL2 in LIHC.

Results: Up-regulation of NBEAL2 expression was detected in the LIHC tissue compared with adjacent nontumor tissues ($P < 0.001$). The chi-square test showed that no significant correlation between the expression level of NBEAL2 and various clinicopathological parameters (including T, N and M classifications) were detected. The Kaplan–Meier curves suggested that lower NBEAL2 expression was related with poor prognosis. The results of Multivariate analysis revealed that a lower expression of NBEAL2 in LIHC was an independent risk of poor overall survival (HR, 8.873; 95% CI, 1.159-67.936; $P = 0.035$). GSEA suggested that multiple tumor-related metabolic pathways were evidently enriched in samples with the low-NBEAL2 expression phenotype.

Conclusion: NBEAL2 might act as a tumor suppressor gene in the progression of LIHC but the precise role of NBEAL2 in LIHC needs further verification.

Introduction

Hepatic carcinoma is one of the most common low-survival malignancies, which has 42,810 new cases and 30,160 deaths in 2020. Although hepatic carcinoma only causes 5% of all cancer deaths, it is a malignant tumor with a 5-year survival rate of only 18%[1]. Hepatocellular carcinoma (HCC) accounts for the majority of Hepatic carcinoma histology. As HCC symptoms are not obvious in the early stage, most patients with HCC lost the opportunity of surgery when given a precise diagnosis. Hepatic carcinoma is the fastest growing malignant tumor in the past few years, by 2–3% annually despite the speed has slowed recently[1, 2]. Although with the advancements of comprehensive treatments including molecular targeted therapy and immunotherapy, the prognosis of patients with HCC are still poor. It is crucial for exploring the potential biomarker which could serve as a target for HCC treatment and a predictor of prognosis for patients with HCC.

NBEAL2 encodes neurobeachin-like-2, a member of the BEACH domain-containing protein (BDCP) family[3]. BEACH (beige and Chediak-Higashi) has been regarded as a conserved domain consisted of approximately 280 amino acid residues[3]. Neurobeachin-like-2 is believed to play an important role in vesicular trafficking, granule development, apoptosis and receptor signaling[3]. So far, nine members of

BDCP family have been identified as having common features of membrane fission, fusion events and monogenic immunodeficiencies, but the specific role of BDCPs remains still largely unclear[4–7]. Accumulating evidences have suggested that mutations in NBEAL2 is the cause of Gray platelet syndrome(GPS), a rare autosomal recessive disease manifested with bleeding, thrombocytopenia, myelofibrosis and remarkably reduced or disappeared of α -granules[8–10]. Moreover, two other BDCPs, LYST and NBEA, are involved in platelet dense granule defects[7, 11]. However, little is known about the relationship between NBEAL2 and malignancy. A recent study indicated that the expression level of NBEAL2 is capable of influencing the overall survival(OS) of patients with head and neck squamous cell carcinoma(HNSCC). Survival curve proved that decreased expression level of NBEAL2 is correlated with poorer OS. Enrichment analysis revealed the potential link between NBEAL2 and has-miR-137-3p[12], but the effect of NBEAL2 on malignancy is rarely reported in previous research.

In our study, we identified the potential role of NBEAL2 as a prognostic biomarker in LIHC proved by the linkage between NBEAL2 gene expression level and the clinicopathological features as well as prognosis based on The Cancer Genome Atlas (TCGA) dataset.

Materials And Methods

Data Sources

The gene expression data for 294 samples of LIHC tissues, 48 samples of adjacent nontumor tissues and the clinical data of the LIHC patients were downloaded from the TCGA database (<https://portal.gdc.cancer.gov>). The clinical and pathological parameters are shown in Table 1.

Table 1
Characteristics of patients with LIHC

Characteristics	Variable	Patients (190)	Percentages (%)
age	≥ 65 years	73	38.4
	< 65 years	117	61.6
Gender	male	134	70.5
	female	56	29.4
T classification	T1	101	53.2
	T2	51	26.8
	T3	34	17.9
	T4	3	1.6
	Unknow	1	0.5
N classification	N0	140	73.7
	N1	2	1.1
	Nx	48	25.3
M classification	M0	158	83.2
	Mx	32	16.8
Pathological stage	I	101	53.2
	II	50	26.3
	III	39	20.5
Vital status	Alive	169	88.9
	Dead	21	11.1
NBEAL2	High	54	28.4
	Low	136	71.6

Expression And Survival Analysis

Original LIHC gene expression data was merged through Perl programming language while NBEAL2 expression data was extracted from the matrix using R software. The ggplot2 package and coin package were used to visualize the NBEAL2 expression data and draw scatter difference diagrams. R software was used to preprocess clinical data including removing the data with imcomplete clinical information. Subsequently, clinical data and expression data were integrated and matched to obtain the data of 190

patients meeting the requirements. The NBEAL2 expression level was divided into high group and low group in accordance with the C-index and Cut-off value (5-fold crossover verification). Dplyr, caret, survAUC, and survminer packages of R software were used to acquire Kaplan–Meier survival curve.

Univariate And Multivariate Analyses

Univariate and multivariate analyses were performed using Cox proportional hazard regression model with the calculated hazard ratio and 95% confidence interval. Through this model, we assessed the predictive value of various clinicopathological parameters and NBEAL2 expression on survival. Coxph and ggforest commands of R softwares were used to perform and visualize the analyses.

Gene Set Enrichment Analysis(Gsea)

To further understand the molecular mechanism of NBEAL2 in LIHC, the pathways correlated with NBEAL2 in LIHC were explored by GSEA (version 4.1.0). The analysis was performed between datasets with low or high NBEAL2 mRNA expression. The reference gene set (C2.cp.kegg.v7.2.symbols.gmt) was selected. 1000 times permutations of the genome were performed per analysis to identify significantly different pathways. Nominal $P < 0.05$, |normalized enrichment score| > 1 and FDR < 0.25 were regarded as statistically significant.

Statistical analysis

Wilcox.test was used to test the NBEAL2 expression levels in various groups including different tissue types, T stages and pathological stages. The interrelation between NBEAL2 expression and clinicopathological parameters was evaluated by Chi-square (χ^2) test. Kaplan–Meier analysis is created via the survAUC and survminer packages in the R platform. Univariate and multivariate survival analysis were conducted with Cox proportional hazard regression model. IBM SPSS (version 18.0) and R software (version 4.0.2) were used to perform the statistical analyses, and a $P < 0.05$ was regarded statistically significant.

Results

NBEAL2 Expression Analyses

The mRNA level data of NBEAL2 including 294 LIHC tissues and 48 adjacent nontumor tissues were acquired from the TCGA database. In order to ascertain the difference between LIHC and adjacent nontumor tissues, we drawn the scatter plot. As shown in Fig. 1, significant up-regulation of NBEAL2 expression was detected in the LIHC tissue ($P < 0.001$, Fig. 1A), but no differences in the levels of NBEAL2 expression were found between groups classified according to pathological stage ($P = 0.568$, Fig. 1B) and T stage ($P = 0.659$, Fig. 1C).

Kaplan–meier Survival Aanalysis

To investigate the influence of expression level of NBEAL2 on overall survival of patients with LIHC, we performed the Kaplan–Meier risk estimates. As is shown in Figure ($P= 0.0068$, Fig. 2A), a lower NBEAL2 expression level was accompanied by a poorer overall survival in all samples, and a similar result was observed in patients with pathological stage I&II ($P= 0.027$, Fig. 2B), while no statistically differences in stage III&IV($P= 0.15$, Fig. 2C).

The Association Between Nbeal2 Expression And Clinicopathological Parameters

In order to clarify whether NBEAL2 expression is associated with clinicopathological parameters, we made the relevant statistics with the NBEAL2 expression level divided into a high-NBEAL2 expression group and a low-NBEAL2 expression group according to the Cut-off value (5-fold crossover verification). The details about the correlation between NBEAL2 expression and clinicopathological parameters were shown in Table 2. No significant correlation between the expression level of NBEAL2 and various clinicopathological parameters (including T, N and M classifications) were detected.

Table 2
Relationships between NBEAL2 expression and clinicopathological parameters in LIHC

Clinicopathological parameters	NBEAL2 expression		Total	P-value
	High	low		
Age				
≥ 65 years	18(24.7)	55(75.3)	73	0.364
< 65 years	36(30.8)	81(69.2)	117	
Gender				
male	33(24.6)	101(75.4)	134	0.073
female	21(37.5)	35(62.5)	56	
T classification				
I-II	44(29.1)	107(70.9)	151	0.666
III-IV	10(25.6)	29(74.4)	39	
N classification				
Negative	44(31.4)	96(68.6)	140	0.124
Positive	10(20.0)	40(80.0)	50	
M classification				
No	45(28.5)	113(71.5)	158	0.968
Yes	9(28.1)	23(71.9)	32	

NBEAL2 Expression level is a independent predictor of overall survival

As NBEAL2 expression has been discovered to affect survival, Univariate and Multivariate Analysis were performed on 190 LIHC patients with a Cox proportional hazard regression model to verify the impact of NBEAL2 expression and various clinicopathological factors on

survival. As shown in Table 3, Univariate analysis indicated that pathological stage (HR, 5.151; 95% CI, 2.182–12.160; $P < 0.001$), T stage (HR, 4.417; 95% CI, 1.868–10.440; $P < 0.001$), NBEAL2 expression (HR, 9.655; 95% CI, 1.292–72.120; $P = 0.027$) were predictive in terms of survival. We further conducted Multivariate Analysis (including above described clinicopathological factors and NBEAL2 expression),

which identified low NBEAL2 expression level as an crucial independent predictor of poor overall survival(HR, 8.873; 95% CI, 1.159–67.936; $P= 0.035$) (Fig. 3).

Table 3
Univariate analysis and multivariate analysis of correlation of NBEAL2 expression

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
age	1.004	0.970–1.040	0.799	0.992	0.957–1.028	0.673
gender	0.675	0.279–1.629	0.382	0.684	0.270–1.732	0.423
stage	5.151	2.182–12.160	0.000	3.030	0.477–19.271	0.240
T	4.417	1.868–10.440	0.000	1.527	0.254–9.199	0.644
N	2.176	0.8924–5.307	0.087	1.660	0.544–5.068	0.374
NBEAL2	9.655	1.292–72.120	0.027	8.873	1.159–67.936	0.035

Exploration Of Nbeal2-related Signaling Pathways

To further clarify the molecular mechanism of NBEAL2 in LIHC, we analyzed the function of NBEAL2 and its related signal pathway between

high- and low- NBEAL2 expression levels through GSEA. The analysis with c2 as a reference gene set revealed that adherens junction, chronic myeloid leukemia, endocytosis, FC gamma R mediated phagocytosis, GNRH signaling pathway, inositol phosphate metabolism, notch signaling pathway, pancreatic cancer, and pathways in cancer were significantly related with high NBEAL2 expression LIHC(Fig. 3, Table 4). In addition, drug metabolism cytochrome P450, fatty acid metabolism, glycine serine and threonine metabolism, metabolism of xenobiotics by cytochrome P450, peroxisome, PPAR signaling pathway, primary bile acid biosynthesis, retinol metabolism, and tryptophan metabolism were enriched in the low expressed phenotypes of NBEAL2 (Fig. 4, Table 5).

Table 4
Gene sets enriched in the high NBEAL2 expression phenotype

Gene set name	NES	NOM p-value	FDR q-value
KEGG_ADHERENS_JUNCTION	1.78	< 0.001	0.576
KEGG_CHRONIC_MYELOID_LEUKEMIA	1.77	< 0.001	0.415
KEGG_ENDOCYTOSIS	1.86	< 0.001	0.586
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	1.74	0.006	0.234
KEGG_GNRH_SIGNALING_PATHWAY	1.72	0.002	0.181
KEGG_INOSITOL_PHOSPHATE_METABOLISM	1.76	0.002	0.333
KEGG_NOTCH_SIGNALING_PATHWAY	1.73	0.008	0.196
KEGG_PANCREATIC_CANCER	1.74	0.002	0.261
KEGG_PATHWAYS_IN_CANCER	1.73	0.002	0.217

Table 5
Gene sets enriched in the low NBEAL2 expression phenotype

Gene set name	NES	NOM p-value	FDR q-value
KEGG_DRUG_METABOLISM_CYTOCHROME_P450	-2.15	0.002	0.005
KEGG_FATTY_ACID_METABOLISM	-2.06	0.004	0.008
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	-2.14	< 0.001	0.003
KEGG_METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	-2.05	0.006	0.007
KEGG_PEROXISOME	-1.86	0.012	0.026
KEGG_PPAR_SIGNALING_PATHWAY	-1.91	0.004	0.020
KEGG_PRIMARY_BILE_ACID_BIOSYNTHESIS	-2.14	< 0.001	0.002
KEGG_RETINOL_METABOLISM	-2.16	0.002	0.008
KEGG_TRYPTOPHAN_METABOLISM	-1.91	0.004	0.023

Discussion

NBEAL2 encodes a BEACH-domain containing proteins with a crucial role in granule ontogeny of platelets[9, 10, 13]. NBEAL2 encoding protein is expected to have close relationship with CHS1, DLL1 and JAG1, known to play role in hematopoiesis. Mutation of NBEAL2 is a source of a series of disorders of granule ontogeny such as GPS, a severe and inherited bleeding disorders due to the lack of α -granules and their contents. Various NBEAL2 gene defects reported so far cause macrothrombocytopenia and

synthetic disorder of α -granules[14]. However, a previous study demonstrated that deficiency in NBEAL2 and α -granules is capable of affecting the metastasis of melanoma cancer, which is more than their primary function in hemostasis[15]. Vladimir et al. discovered that NBEAL2 was one of biomarkers to detect ovarian cancer with ideal sensitivity and specificity[16]. Previous studies with administration of NBEAL2 knockout mouse demonstrated the failure of α -granules formation in megakaryocytes and platelets. Studies with NBEAL2 knockout mouse highlight emphasized the role of α -granules in promoting immune response, intervention of tissue repair and facilitating malignancy metastasis[17–19]. However, the role of NBEAL2 in malignancy remains unclear and further investigations are required. In this study, we sought to find the evidences that NBEAL2 have a tight relation with LIHC. In addition, potential signaling pathways involved to NBEAL2 in LIHC were explored to further understand the possible mechanism related to the regulation of LIHC progression by NBEAL2.

NBEAL2 expression is proved to play a critical role in LIHC progression in our study, especially as a biomarkers for prognosis in LIHC. The original gene Expression data downloaded from the TCGA database was analyzed and the expression level of NBEAL2 in LIHC and adjacent nontumor tissues was compared. A remarkably higher expression level of NBEAL2 in LIHC compared with adjacent nontumor tissues was observed, which suggested that NBEAL2 was involved in the development of LIHC. The expression levels of NBEAL2 in groups classified by T stage and pathological stage were also detected. NBEAL2 exhibited no significant expression differences between groups classified according to pathological stage and T stage. Considering the close correlation between the expression level of NBEAL2 and survival, these data indicated that NBEAL2 may affect overall survival as a specific and independent impact factor.

Kaplan–Meier survival analysis gave the result that a lower expression of NBEAL2 was accompanied by a worse prognosis. The results of univariate analysis indicated that low NBEAL2 expression was associated with poorer overall survival while pathological stage and T stage were also related with the prognosis of patients with LIHC. Multivariate analysis suggested that the NBEAL2 expression is an independent predictor of the overall survival of LIHC patients. The data set forth in our study supported the potential of NBEAL2 as a biomarker for LIHC. Due to the lack of research on NBEAL2 in malignant tumors, the significance of NBEAL2 in other malignancy remains obscure. Interestingly, a recent study pointed out that high NBEAL2 expression level may be a risk factor for poor survival of patients with IDH1 wild-type Glioblastoma(GBM), which seems contrary with our results[20]. Another research get similar conclusion with us that low NBEAL2 expression level was correlated with poorer overall survival of HNSCC patients[12]. we postulated that NBEAL2 has dissimilar roles in malignancy as it can be a tumor suppressor gene or an oncogene.

To explore the potential molecular mechanism of NBEAL2 in LIHC, GSEA was used to analyze the expression profile data of LIHC tumor tissues grouped by different NBEAL2 expression levels. It was found that adherens junction, chronic myeloid leukemia, endocytosis, FC gamma R (FCGR)-mediated phagocytosis, GNRH signaling pathway, inositol phosphate metabolism, notch signaling pathway, pancreatic cancer, and pathways in cancer were significantly correlated with high NBEAL2 expression

LHC. Since adherens junctions is continuously crucial for maintaining epithelial form and function, and the dysregulation of this pathway damage the integrity of endothelial barrier thus promoting various cancer metastasis[21–23]. Endocytosis is an energy-consumed process, in which cells internalize surrounding substances in the form of vesicles. The imbalance of endocytosis pathways plays an important role in the proliferation, migration, and treatment of cancer cells[24–27]. Fc gamma receptor (FCGR)-mediated phagocytosis is an indispensable part of cellular immunity including antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis activate innate immunity[28, 29]. Since ADCC and phagocytosis participate in tumor cell killing, we speculate that polymorphisms in FCGR-related genes were related with the progression of malignancy. Gonadotropin-releasing hormone (GnRH) is believed to have a tight association with non-small cell lung carcinoma (NSCLC)[30, 31], but the relation between GnRH and LHC remains unclear. Inositol phosphate metabolism pathway affect cell differentiation, migration and regulate phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway[32]. Deregulation of inositol phosphate metabolism are correlated with various types of cancer including hepatocellular cancers, prostate, brain, breast cancers[33–35]. The Notch signaling pathway is evolutionarily conserved and involved in cell proliferation, differentiation and apoptosis throughout the life cycle. Previous research confirmed the positive effect of notch signaling pathway in the amplification of cancer stem cells, which promoting the formation, proliferation and recurrence of the cancer[36–38].

Drug metabolism cytochrome P450, fatty acid metabolism, glycine-serine and threonine metabolism, metabolism of xenobiotics by cytochrome P450, peroxisome, PPAR signaling pathway, primary bile acid biosynthesis, retinol metabolism, and tryptophan metabolism were remarkably associated with low NBEAL2 expression LHC. The suppression of cytochrome P450 (CYP)-mediated drug clearance is observed in the status of systemic inflammation and immunity therapy for cancer[39–41]. The change of CYP enzyme activity may cause either rapidly removal or accumulation of anti-cancer drugs. Reprogramming of fatty acid metabolism has been regarded as a hallmark of cancer, and a recent study pointed out that fatty acid-binding protein 5 (FABP5) upregulation due to the dysregulation of fatty acid metabolism may drive LHC progression[42, 43]. Accumulating evidence highlighted that hyperactivation of the serine and glycine biosynthetic pathway facilitate the formation of tumor[44, 45]. Suppression of xenobiotics metabolism by cytochrome P450 due to either polymorphisms in genes or acquired reason is considered as a risk factor of various malignant tumors[46, 47]. Peroxisome proliferator-activated receptors(PPARs) plays an important role in carcinogenesis and various metabolic disorders[48]. Previous research indicated that alteration of retinol metabolism is significantly correlated with prostate and breast cancer risk[49, 50]. Tryptophan metabolism plays essential roles in both immunity disorder and cancer formation. Recent research supported that tryptophan metabolism could be a potential therapeutic target in cancer[51, 52].

The limitation of our research is the incomplete of clinical information, including lack of tumor size, surgical treatment and surgical details. Finally, the lack of laboratory data makes our research results unconvincing.

In conclusion, the data set forth in our study supported that the expression level of NBEAL2 in LIHC tissues was higher than that in adjacent tissues. On the contrary, lower expression of NBEAL2 in LIHC tissues was correlated with shorter OS. Importantly, univariate and multivariate analyses identified reduced NBEAL2 expression in LIHC as an independent risk factor for shorter OS. Due to the lack of laboratory data, whether it could be a marker for the diagnosis and the prognosis

of LIHC remains obscure.

Declarations

Data availability statement

We analyzed publicly datasets in this research, which can be found in The Cancer Genome Atlas (<https://portal.gdc.cancer.gov/>).

Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

Y.C. designed the research. Y.W., H.S. performed major parts of the research. W.W., F.R., H.J., J.W performed parts of the research. Y.W., J.X. analyzed the data, tested statistics, and coordinated the figures. Y.W. wrote the article. Y.C. revised the article.

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Figures

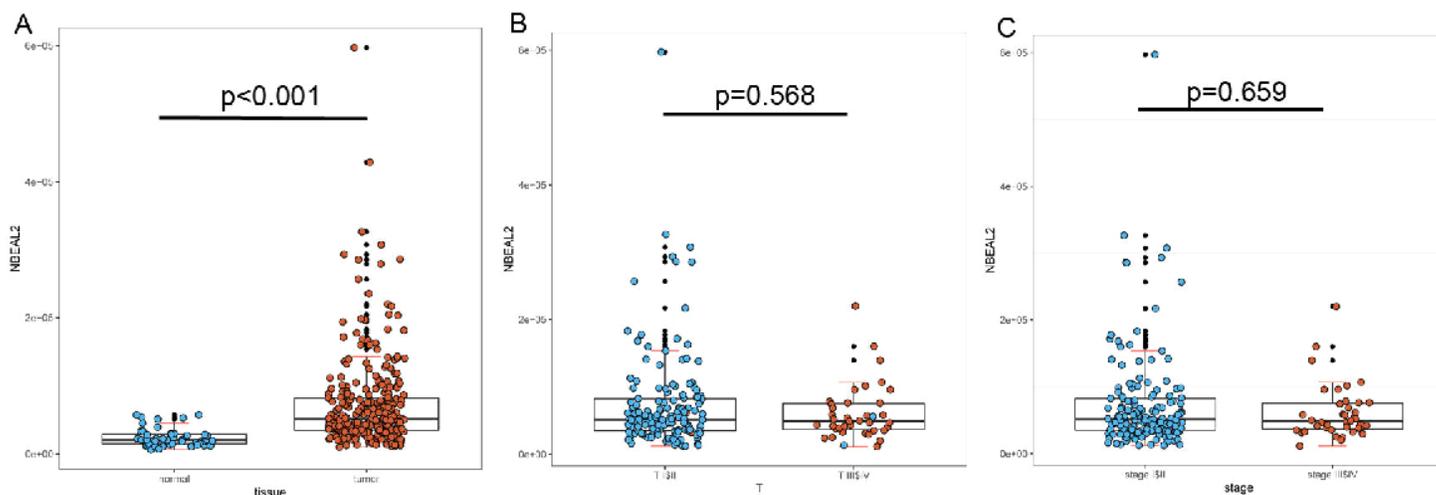


Figure 1

The expression of NBEAL2 and its association with clinicopathological parameters based on TCGA data. (A) Comparison of NBEAL2 expression between liver hepatocellular carcinoma tissues and adjacent nontumor tissues. The expression of NBEAL2 is grouped by T stage (B), pathological stage (C). TCGA, The Cancer Genome Atlas.

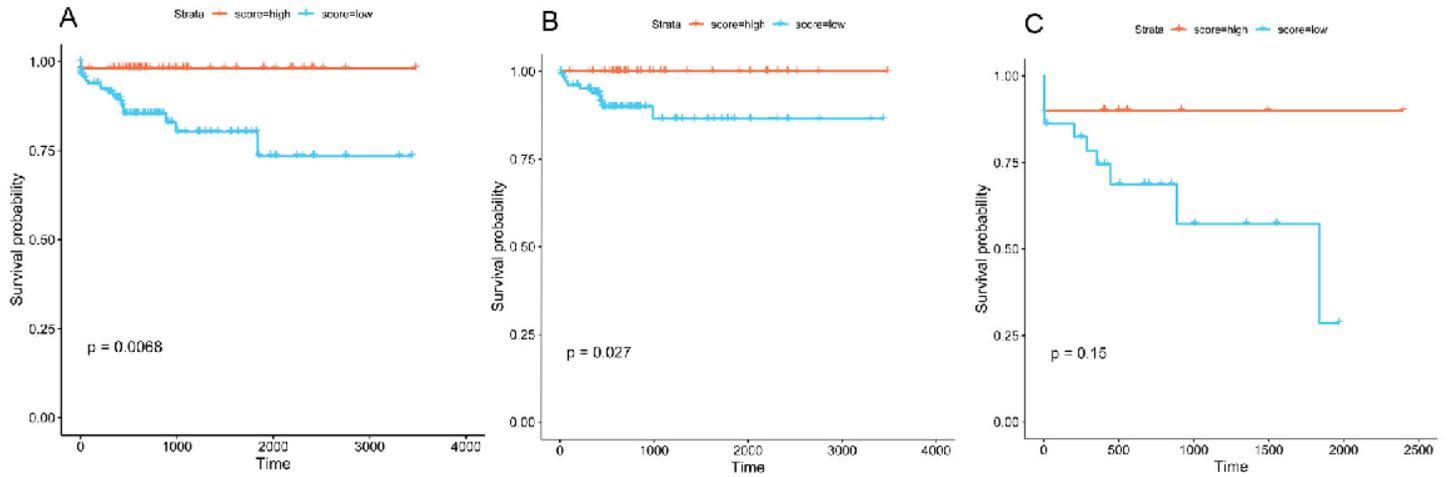


Figure 2

Kaplan–Meier risk estimates Survival analysis in all samples (A), stage I&II (B) and stage III&IV(C).

Hazard ratio

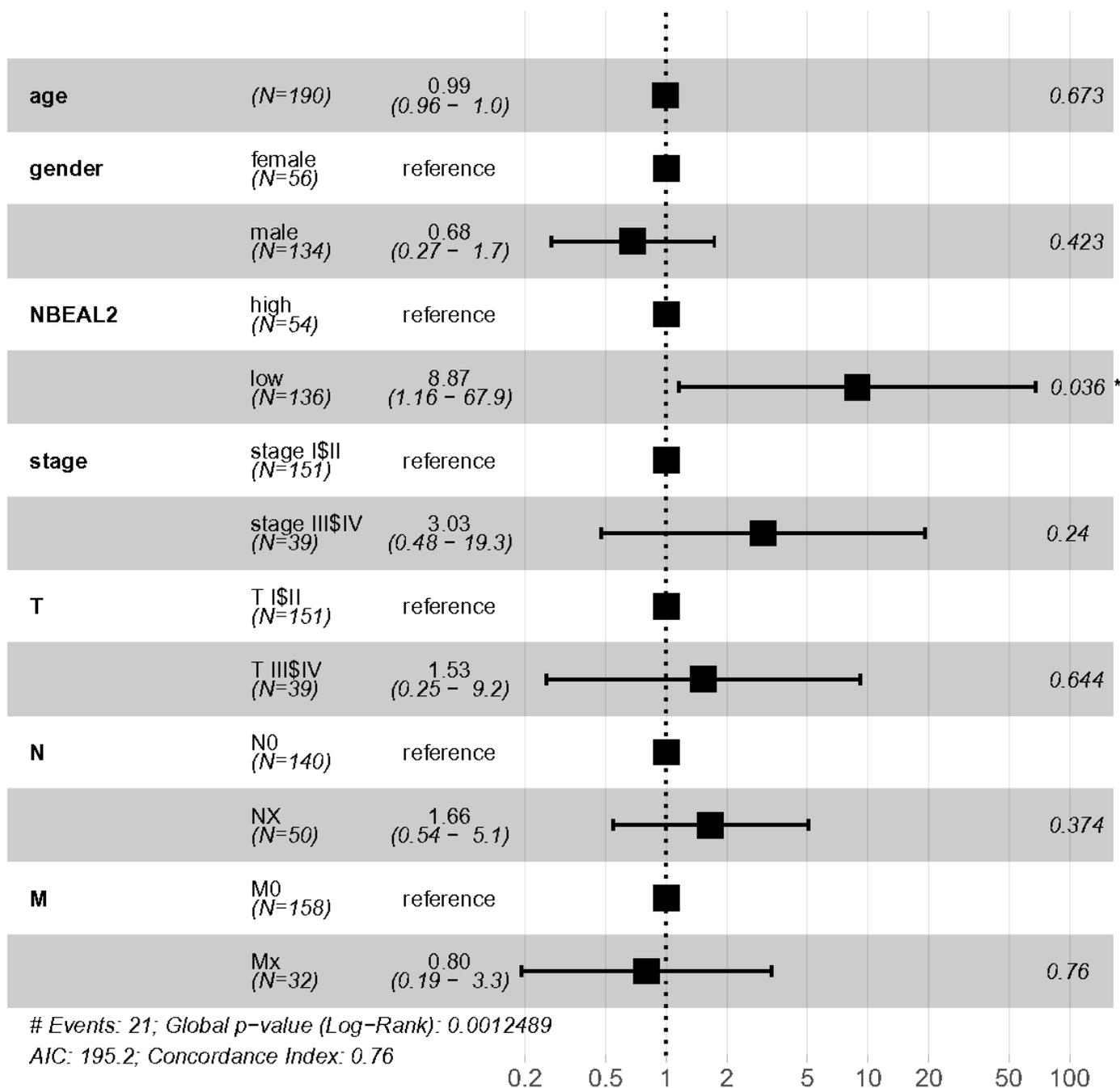


Figure 3

Forest plot for the multivariate Cox proportional hazard regression model. NBEAL2 was an independent predictor of poor survival rate (HR,8.873; 95% CI, 1.159-67.936; P = 0.035). HR, hazard ratio; CI, confidence interval. *P < 0.05, **P < 0.01, ***P < 0.001.

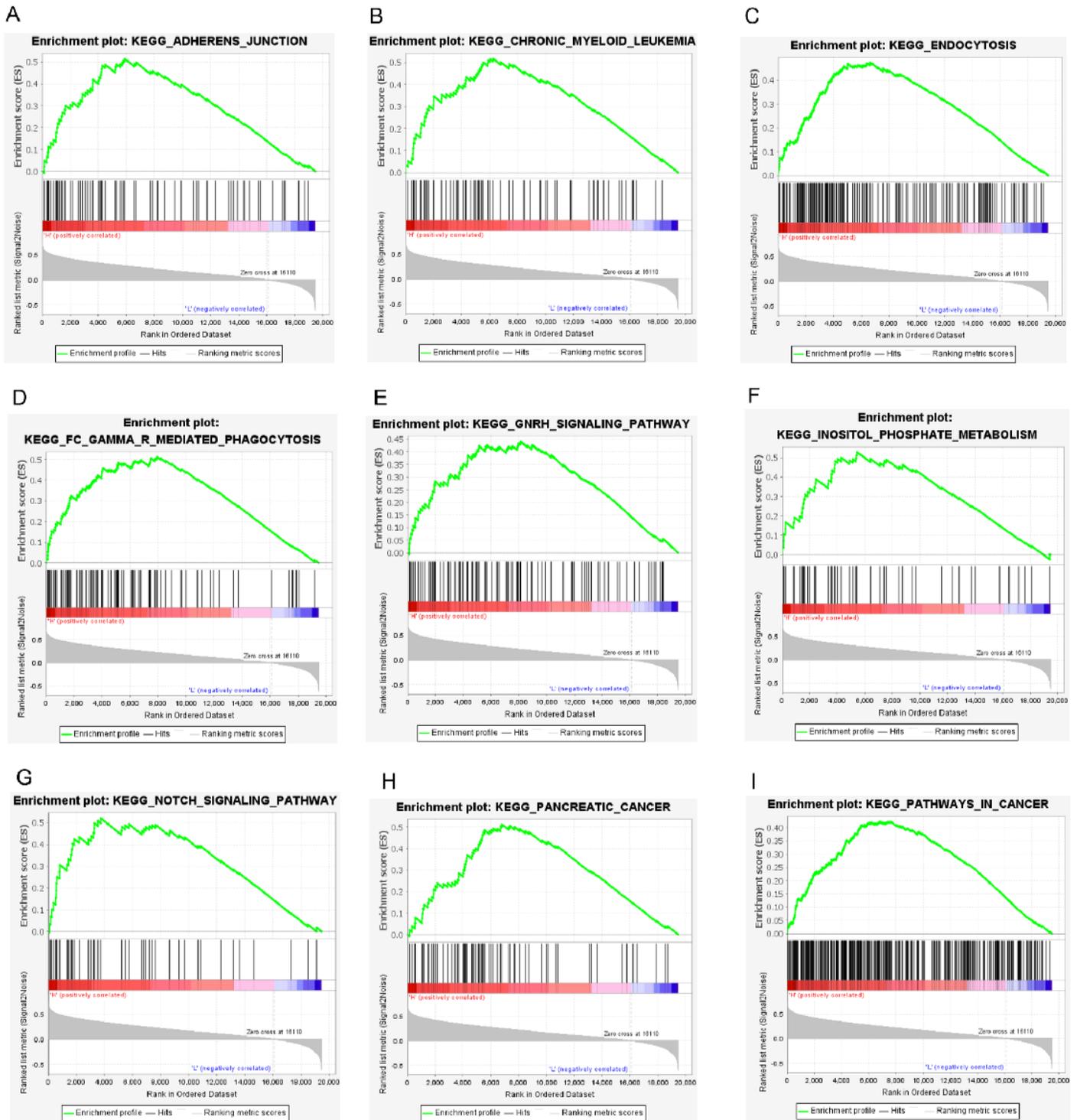


Figure 4

Gene set enrichment analysis results enriched in phenotype of high-NBEAL2 expression group (A) ADHERENS_JUNCTION; (B) CHRONIC_MYELOID_LEUKEMIA; (C) ENDOCYTOSIS; (D) FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS; (E) GNRH_SIGNALING_PATHWAY; (F) INOSITOL_PHOSPHATE_METABOLISM; (G) NOTCH_SIGNALING_PATHWAY; (H) PANCREATIC_CANCER; (I) PATHWAYS_IN_CANCER.

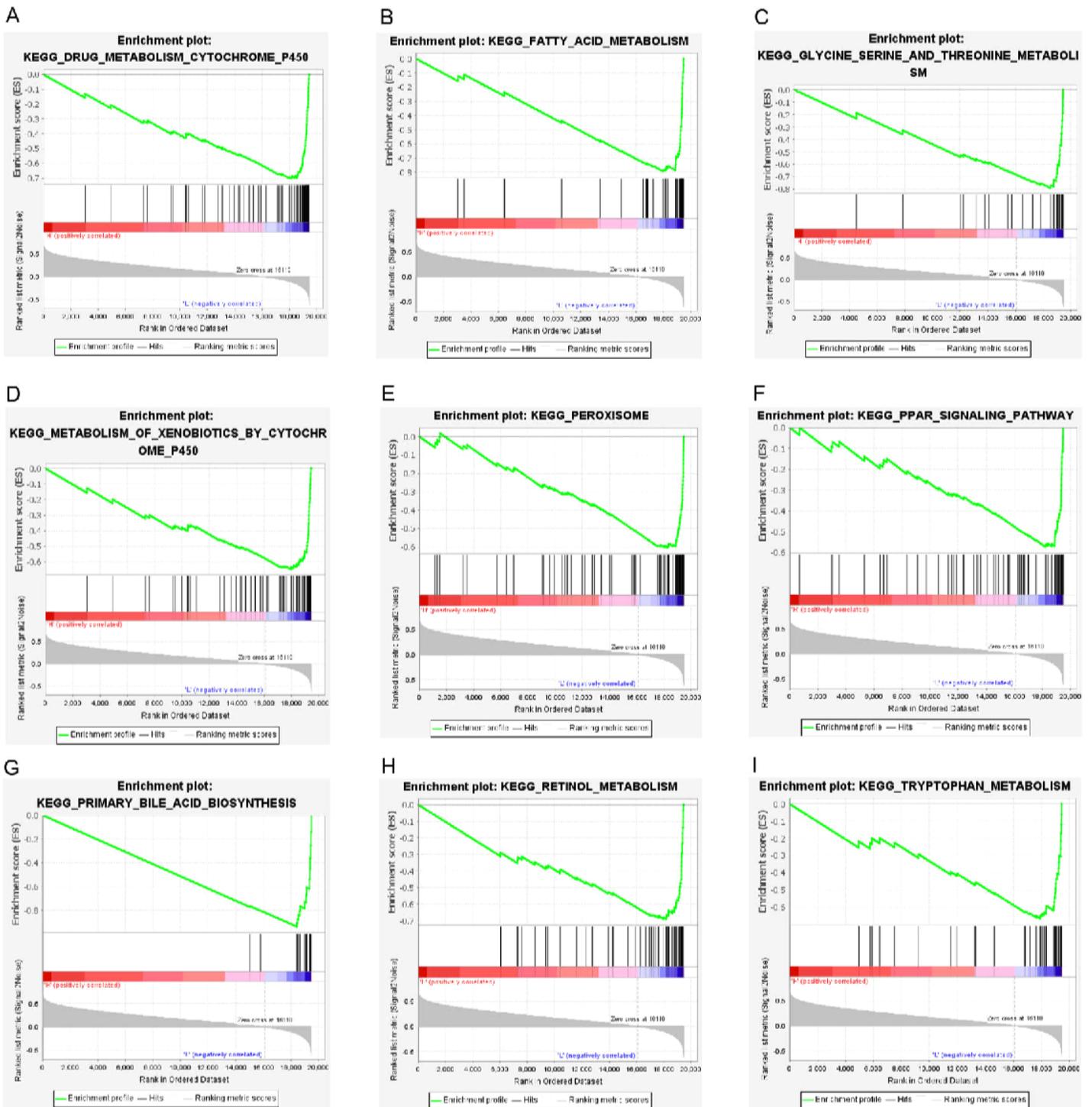


Figure 5

Gene set enrichment analysis results enriched in phenotype of low-NBEAL2 expression group (A) DRUG_METABOLISM_CYTOCHROME_P450; (B) FATTY_ACID_METABOLISM; (C) GLYCINE_SERINE_AND_THREONINE_METABOLISM; (D) METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450; (E) PEROXISOME; (F) PPAR_SIGNALING_PATHWAY; (G) PRIMARY_BILE_ACID_BIOSYNTHESIS; (H) RETINOL_METABOLISM; (I) TRYPTOPHAN_METABOLISM.