ORIGINAL RESEARCH

Enolase-1 serves as a biomarker of diagnosis and prognosis in hepatocellular carcinoma patients

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Background: Hepatocellular carcinoma (HCC) is an aggressive malignancy with high incidence rate and poor prognosis. Enolase-1 (ENO1), a key glycolytic enzyme, has been implicated in the tumorigenesis of various cancers. However, its diagnostic value and clinical significance in HCC are unclear.

Methods: Data of 374 HCC tissues and 50 nontumor tissues were retrieved from The Cancer Genome Atlas database, and the expression level of ENO1 mRNA in HCC was evaluated. In addition, a meta-analysis of 12 HCC cohorts deposited in the Gene Expression Omnibus database was conducted to determine ENO1 expression levels. The diagnostic power of ENO1 in distinguishing HCC tissues from non-HCC tissues was confirmed by receiver operating characteristic (ROC) curve analysis. A tissue microarray comprising 93 HCC specimens and 87 adjacent normal specimens was used to validate ENO1 expression, and its prognostic value in HCC was ascertained by Kaplan–Meier analysis and Cox regression models. In addition, the gene set enrichment analysis was performed to predict the molecular mechanism of ENO1 action in HCC.

Results: ENO1 was overexpressed in HCC tissues and associated with worse outcomes in terms of overall survival (OS) ($P<0.01$) and disease-free survival ($P<0.01$). ENO1 expression ($P<0.01$) was an independent prognostic variable for the OS of HCC patients. Moreover, as per the ROC curve analysis, it had good diagnostic power as well. In addition, elevated expression of ENO1 was significantly correlated with the cell cycle and DNA replication pathway, consistent with its association with pro-proliferative genes such as *MKI67*, *PCNA*, *CDK4*, *CDK2*, and *MELK*.

Conclusion: ENO1 was markedly upregulated and was an oncogene-associated protein in HCC. It is a promising prognostic and diagnostic biomarker for HCC.

Keywords: ENO1, hepatocellular carcinoma, diagnosis, proliferation, cell cycle

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common human malignancy and the third leading cause of cancer-related deaths worldwide.[1–3] Despite the recent advances in therapeutic strategies such as surgical resection, orthotropic liver transplantation, and radio-frequency ablation, the clinical outcomes of HCC patients have not improved due to the asymptomatic nature, late diagnosis, and early metastasis of this cancer.[4–7] Therefore, novel diagnostic and therapeutic strategies are urgently needed to improve the prognosis of HCC patients.

Enolase-1 (ENO1), one of the isoforms of enolase, is a key glycolytic enzyme.[8,9] In addition to glucose metabolism, ENO1 is involved in autoimmune response,

hypoxia endurance, and growth regulation.[10–12] Recent studies have correlated ENO1 with tumorigenesis and cancer progression[13–15] and have also shown its specific involvement in multiple signaling pathways in HCC cells.[2,16,17] However, the clinical significance and diagnostic value of ENO1 in HCC remain to be elucidated.

In this study, we analyzed the HCC expression profile data from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases, as well as HCC tissue microarray (TMA), and found that ENO1 mRNA and protein expression levels were higher in HCC tissues than in the adjacent normal tissues. Furthermore, high expression of ENO1 was associated with poorer overall survival (OS) rates and had reliable diagnostic value in distinguishing HCC tissues from non-HCC tissues. Taken together, ENO1 is a promising prognostic and diagnostic biomarker in HCC patients.

Materials and methods
TCGA data source
The mRNA gene expression data of 374 tumor samples and 50 adjacent normal liver samples were downloaded from TCGA HCC data set (https://tcga-data.nci.nih.gov/tcga/). The follow-up clinical information was available for 327 HCC patients and was utilized to analyze the correlation between ENO1 expression and clinicopathological features. The raw data were processed and analyzed by Biometric Research Branch array tools.

GEO data source
Twelve sets of microarrays – including 1,309 HCC samples and 1,442 nontumor samples – were extracted from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and used for meta-analysis to evaluate the diagnostic power of ENO1. The 12 cohorts consisted of GSE6764, GSE10143, GSE14520, GSE25079, GSE36376, GSE39791, GSE57957, GSE60502, GSE62232, GSE64041, GSE76297, and GSE84005 datasets. Their characteristics including cohort ID, RNA-seq platform, number of samples (nontumor and tumor samples), publication year, and country are summarized in Table S1.

Tissue samples
The TMA of 93 HCC specimens and 87 normal liver specimens (HLiv-HCC180Sur-10) from the regions around cancers was purchased from Outdo Biotech Company (Shanghai, China) and used to further validate ENO1 expression and the prognostic value in HCC. None of the patients whose samples were included in the TMA had received any prior chemotherapy, immunotherapy, or radiotherapy before surgery. The study was approved by the ethics committee of The

First Affiliated Hospital of Zhengzhou University, Zhengzhou, China. Written informed consent of each patient was obtained.

Gene set enrichment analysis (GSEA) and scatter plot analysis
GSEA was used to determine the distribution of the individual genes of the TCGA HCC data sets. If most members of a particular data set were positively related to the expression of ENO1, with significance set at $P<0.05$, the set was termed associated with ENO1. The scatter plot was extracted from the Gene Expression Profiling Interactive Analysis online analysis tool (http://gepia.cancer-pku.cn/).

Statistics for meta-analysis
Stata 12.0 was used to analyze the pooled diagnostic value of ENO1 with the data from GEO data set. Evaluation of heterogeneity was assessed using I^2, with $I^2>50\%$ indicating significant heterogeneity. When heterogeneity was observed between the studies, the random effect model was used and the subgroup analysis was performed. Publication bias was determined by Begg's funnel plot and Egger's test.

Immunohistochemistry (IHC)
IHC was performed as previously described.[18] Briefly, 5-μm-thick TMA sections were dewaxed and treated with hydrogen peroxide to quench endogenous peroxidase activity, followed by overnight incubation with rabbit antihuman ENO1 antibody (1:150; Proteintech, Wuhan, China) at 4°C. The immunoreactive cells were detected by SignalStain® DAB (Cell Signaling Technology, Dancers, MA, USA) and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA, USA). ENO1+ cells containing brown granules were counted independently by two pathologists who were blinded to clinical parameters, and the samples were scored according to the proportion of positive cells as follows: 0, none; 1, <25%; 2, 25%–50%; 3, 51%–75%; and 4, 76%–100%. The staining intensity was scored as follows: 0, none; 1, weak; 2, moderate; and 3, strong. The total staining score (range 0–12) was calculated by multiplying the two subscores, and the samples with scores 0–6 and 7–12 were classified as low expression and high expression, respectively.

Statistical analyses
The statistical analyses were performed using the GraphPad Prism software (Version 6.0; GraphPad Software, Inc., La Jolla, CA, USA) and the SPSS software (Version 23.0; IBM Corporation, Armonk, NY, USA). The Chi-squared test was used to determine the relationship between ENO1 expression levels

and the clinicopathological parameters. Kaplan–Meier method was used for survival analysis. Multivariate Cox proportional hazards method was used to determine the relationship between different variables and survival. Receiver-operating characteristic (ROC) curves were used to analyze the pooled diagnostic value of ENO1 in HCC. Pearson's correlation was used to assess the linear association between two variables. *P*-values less than 0.05 were considered statistically significant.

Results

ENO1 mRNA was overexpressed and correlated with poor prognosis in TCGA HCC cohort

ENO1 was significantly upregulated in the HCC samples (N=374) compared to the nontumor samples (N=50) from the TCGA HCC database (*P*<0.001) (Figure 1A). Furthermore, high ENO1 expression levels were observed in patients with advanced TNM stage and poor differentiation (*P*<0.001) (Figure 1B and C and Table 1). As shown in Table 1, there was no correlation of ENO1 expression with race, age, gender, α-fetoprotein (AFP) levels, and tumor histological type. In addition, high ENO1 expression predicted poorer OS and disease-free survival in HCC patients (Figure 1D and E). Univariate and multivariate Cox regression analyses showed that ENO1 and TNM stage were independent prognostic factors for HCC patients (Table 2). In addition, the GSEA revealed that

overexpression of ENO1 was correlated with gene signatures associated with poor survival (Figure 1F), while low expression of ENO1 was correlated with gene signatures of good survival (Figure 1G). Taken together, high ENO1 expression was related to tumor progression and poor prognosis of HCC patients.

ENO1 expression in HCC was validated by meta-analysis of GEO database HCC microarrays

To further verify the expression of ENO1 in HCC, 12 verified microarrays from the GEO HCC database were analyzed. As shown in the forest plot (Figure 2A), the expression of ENO1 in HCC tissues was higher than that in the nontumor tissues (pooled standard mean difference [SMD]=0.92, 95% CI=0.72–1.13, *P*<0.001) under a random effect model and the results were also obtained using the Chi-squared test (Figure 2B). The sensitivity analysis revealed no significant differences among the included studies (Figure 2C). The funnel plots were basically symmetric (Begg's *P*=0.837, Egger's *P*=0.539), indicating no significant publication bias (Figure 2D). Subgroup analysis suggested that the heterogeneity was partially from different regions and sample sizes of the included cohorts (Figure S1 and Table S2).

ROC curve analysis was used to determine the diagnostic value of ENO1 in distinguishing HCC tissues from non-HCC tissues. The area under the curve (AUC) of the
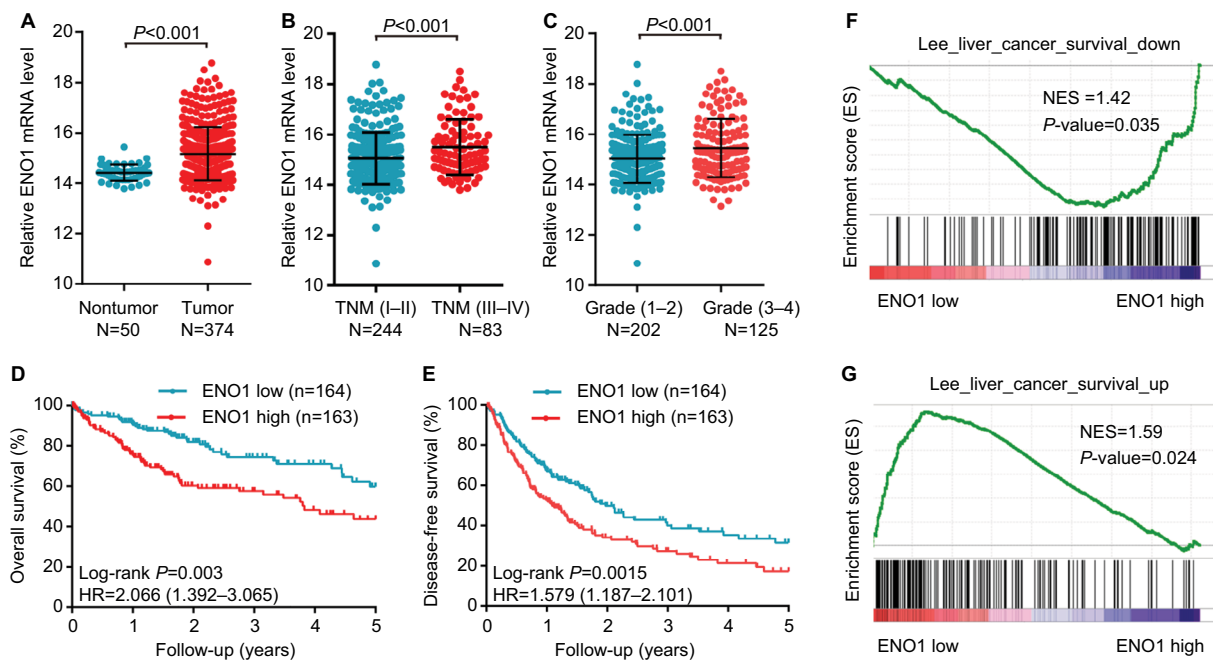
Figure 1 ENO1 mRNA was overexpressed in HCC tissues and negatively correlated with survival in TCGA cohort.
Notes: (**A**) ENO1 mRNA expression in normal tissues and HCC tissues. (**B** and **C**) Expression of ENO1 mRNA in patients with different TNM stages and pathological grades. Kaplan–Meier estimation of OS (**D**) and DFS (**E**) of HCC patients stratified by ENO1 expression. GSEA results showing the correlation between ENO1 expression and the genes associated with poor survival (**F**) and improved survival (**G**) in HCC patients.
Abbreviations: DFS, disease-free survival; ENO1, enolase-1; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; NES, normalized enrichment score; OS, overall survival.

Table 1 The relationship between ENO1 status and clinicopathological features of HCC

Clinicopathological features	Number of cases (n)	ENO1 mRNA expression, n (%)		P-value
		High	**Low**	
Age (years)				
< Median	161	78 (48.4)	83 (51.6)	0.618
> Median	166	85 (51.2)	81 (48.8)	
Race				
Yellow	167	85 (50.9)	82 (49.1)	0.698
White	160	78 (48.7)	82 (51.3)	
Gender				
Male	223	108 (48.4)	115 (51.6)	0.453
Female	104	55 (52.9)	49 (47.1)	
AFP (ng/mL)				
<20	232	109 (47.0)	123 (53.0)	0.105
>20	95	54 (56.8)	41 (43.2)	
TNM stage				
Stage I–II	244	113 (46.3)	131 (53.7)	0.023*
Stage III–IV	83	50 (60.2)	33 (39.8)	
Histological type				
HCC	317	157 (49.5)	160 (50.5)	0.514
Non-HCC	10	6 (60.0)	4 (40.0)	
Differentiation grade				
Grade 1–2	202	89 (44.1)	113 (55.9)	0.008**
Grade 3–4	125	74 (59.2)	51 (40.8)	

Notes: *P<0.05. **P<0.01.
Abbreviations: AFP, α-fetoprotein; ENO1, enolase-1; HCC, hepatocellular carcinoma.

Table 2 Univariate and multivariate analyses of prognostic variables for overall survival in HCC patients

Clinicopathological features	Univariate analysis			Multivariate analysis		
	HR	**95% (CI)**	**P-value**	**HR**	**95% (CI)**	**P-value**
Age (years)						
< Median	1.000		0.389			
> Median	1.189	0.802–1.765				
Race						
Yellow	1.000		0.695			
White	1.082	0.730–1.603				
Gender						
Female	1.000		0.295			
Male	1.243	0.828–1.866				
AFP (ng/mL)						
<20	1.000		0.046*	1.000		0.074
>20	1.519	0.436–0.993		1.458	0.964–2.204	
TNM stage						
Stage I–II	1.000		<0.001**	1.000		0.001**
Stage III–IV	2.631	1.765–3.920		2.466	1.649–3.687	
Histological type						
Non-HCC	1.000		0.454			
HCC	2.126	0.296–15.270				
Differentiation grade						
Grade 1–2	1.000		0.251			
Grade 3–4	1.261	0.849–1.872				
ENO1 expression						
Low	1.000		<0.001**	1.000		0.003**
High	2.073	1.383–3.107		1.861	1.236–2.802	

Notes: *P<0.05. **P<0.01.
Abbreviations: AFP, α-fetoprotein; ENO1, enolase-1; HCC, hepatocellular carcinoma.

A

Study ID	SMD (95% CI)	Weight %
GSE6764	0.11 (−034–0.57)	7.39
GSE10143	1.20 (0.94–1.46)	9.76
GSE14520	0.91 (0.72–1.11)	10.48
GSE25079	0.46 (0.29–0.62)	10.74
GSE36376	0.99 (0.79–1.19)	10.42
GSE39791	0.94 (0.60–1.28)	8.73
GSE57957	1.15 (0.67–1.63)	7.09
GSE60502	0.97 (0.28–1.66)	5.00
GSE62232	0.63 (−0.03–1.30)	5.25
GSE64041	0.99 (0.62–1.36)	8.39
GSE76297	1.26 (1.01–1.51)	9.92
GSE84005	1.41 (0.90–1.91)	6.83
Overall (I^2 =81.1%, P<0.001)	0.92 (0.72–1.13)	100.00

Note: Weights are from random effects analysis

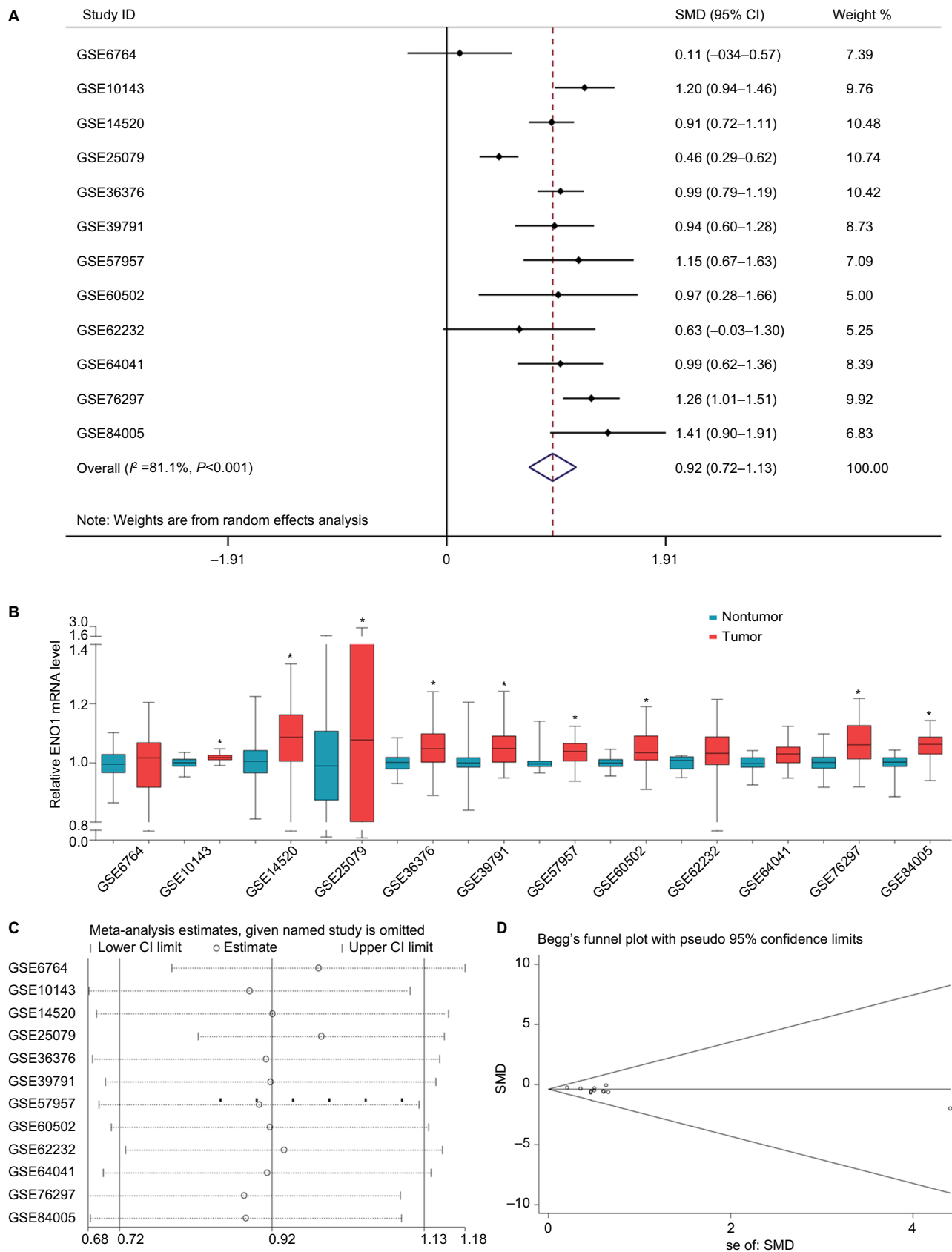−1.91   0   1.91

Figure 2 The expression of ENO1 was markedly increased in HCC tissues and showed high diagnostic value in GEO data set.
Notes: (**A**) Forest plot evaluating differences in ENO1 expression between HCC and normal tissues. The high and low ENO1-expressing tissues were regarded as the experimental and control groups, respectively. (**B**) ENO1 expression in HCC and normal tissues. (**C**) Sensitivity analysis of HRs was calculated by omitting each microarray in turn. (**D**) Funnel plot for the publication bias test of GEO microarrays. Each point represents a single microarray.
Abbreviations: ENO1, enolase-1; GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma; SMD, standard mean difference.
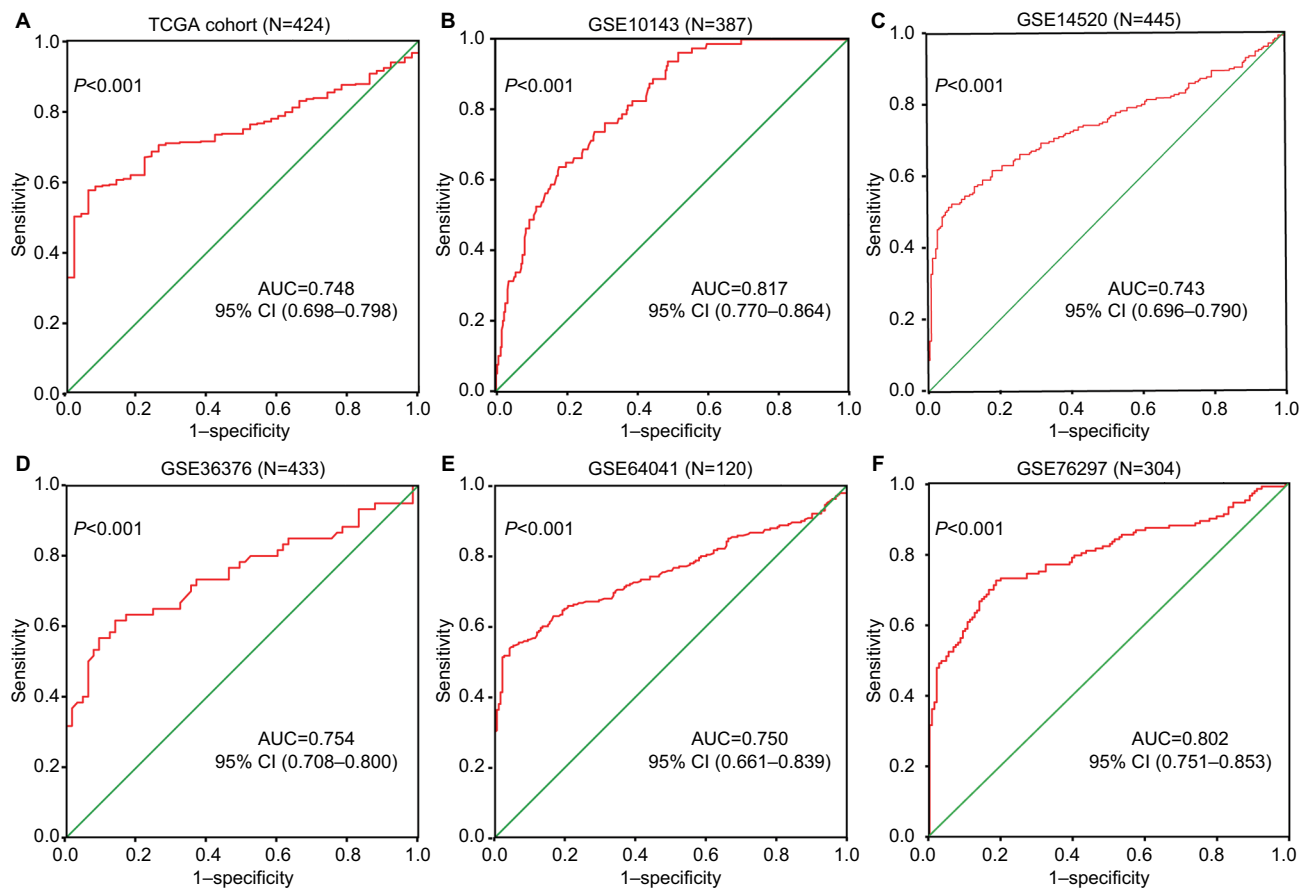
Figure 3 ROC curves for evaluating the diagnostic power of ENO1 in HCC.
Notes: (**A**) TCGA cohort. (**B**) GSE10143. (**C**) GSE14520. (**D**) GSE36376. (**E**) GSE64041. (F) GSE76297.
Abbreviations: AUC, area under the curve; ENO1, enolase-1; HCC, hepatocellular carcinoma; ROC, receiver-operating characteristic; TCGA, The Cancer Genome Atlas.

TCGA HCC cohort was 0.748 (95% CI: 0.698–0.798, *P*<0.001) with a sensitivity of 0.897 and a specificity of 0.744 (Figure 3A). The AUCs of different GEO data sets were as follows: GSE10143, 0.817 (95% CI: 0.770–0.864, *P*<0.001; Figure 3B); GSE14520, 0.743 (95% CI: 0.696–0.790, *P*<0.001; Figure 3C); GSE36376, 0.754 (95% CI: 0.708–0.800, *P*<0.001; Figure 3D); GSE64041, 0.750 (95% CI: 0.661–0.839, *P*<0.001; Figure 3E); and GSE76297, 0.813 (95% CI: 0.710–0.916, *P*<0.001; Figure 3F). The respective specificities of different data sets were 0.821, 0.941, 0.959, 0.862, and 0.897, and the sensitivities were 0.638, 0.516, 0.538, 0.617, and 0.744, respectively. Taken together, ENO1 was a reliable diagnostic marker in HCC.

ENO1 protein was upregulated and related to the poor prognosis in HCC TMA cohort

We also measured the in situ levels of ENO1 protein in the HCC TMA cohort. Consistent with the results obtained with the TCGA and GEO HCC data sets, ENO1 protein was highly expressed in HCC tissues (Figure 4A–C) and was associated with advanced TNM stage (Figure 4D). Furthermore, patients with high levels of ENO1 protein had shorter OS than those with low expression (Figure 4E). Collectively, ENO1 is a potential biomarker for HCC prognosis.

Potential molecular mechanism of ENO1-mediated progression of HCC

Based on the TCGA HCC data, we further predicted the potential mechanism of ENO1 action in HCC using GSEA and found a significant correlation between high ENO1 expression and DNA replication and cell cycle. Scatter plot analysis showed a significant positive correlation between ENO1 expression level and the genes involved in cell cycle and DNA replication (Figure 5A and B), such as MKI67 (*P*<0.001, *R*=0.54; Figure 5C), PCNA (*P*<0.001, *R*=0.46; Figure 5D), CDK4 (*P*<0.001, *R*=0.5; Figure 5E), CDK2 (*P*<0.001, *R*=0.43; Figure 5F), and MELK (*P*<0.001, *R*=0.58; Figure 5G), indicating that ENO1 contributed to the poor prognosis of HCC likely through driving HCC cells' proliferation.
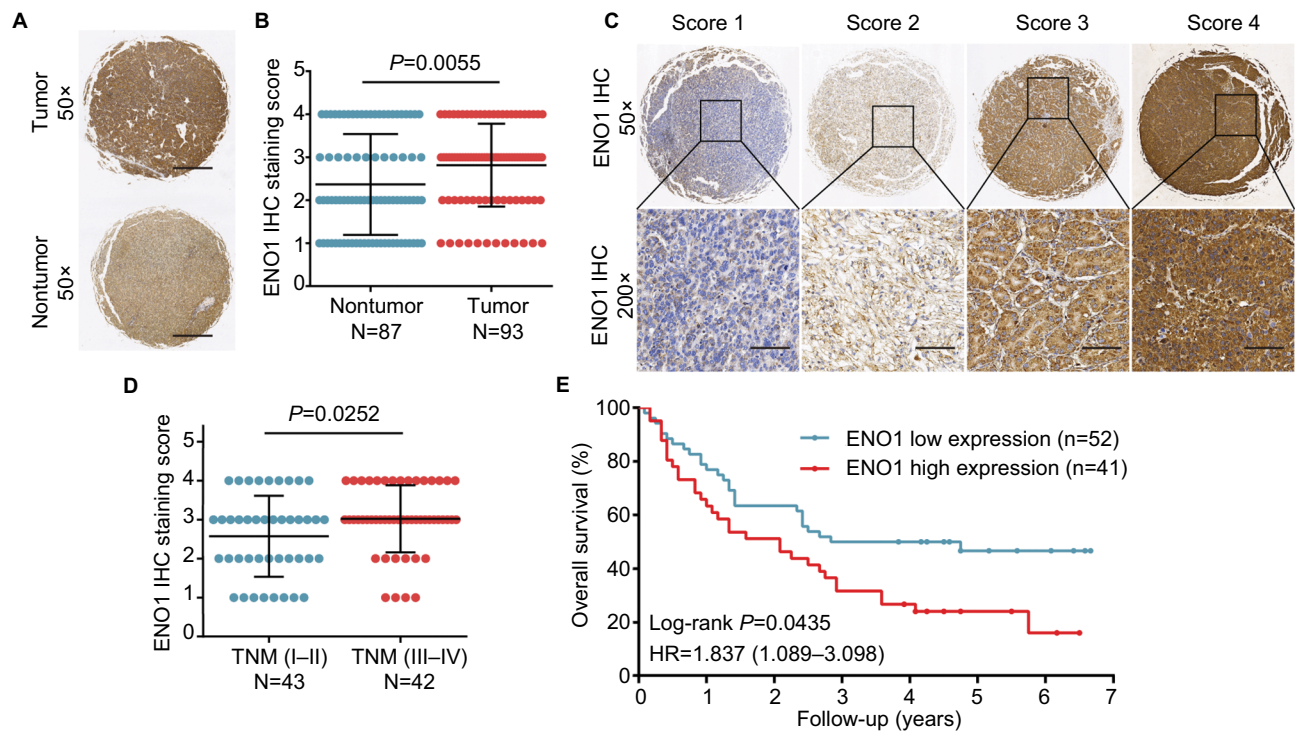
Figure 4 High expression of ENO1 protein was negatively correlated with survival.

Notes: (**A**) Representative ENO1 staining in HCC and normal tissues. (**B**) Increased expression of ENO1 in HCC tissues (*P*=0.0055). (**C**) Representative images of ENO1 staining in HCC tissues. (**D**) Increased expression of ENO1 in advanced TNM stage (*P*=0.0252). (**E**) Kaplan–Meier analysis showing the correlation between ENO1 expression levels and OS of 93 HCC patients.

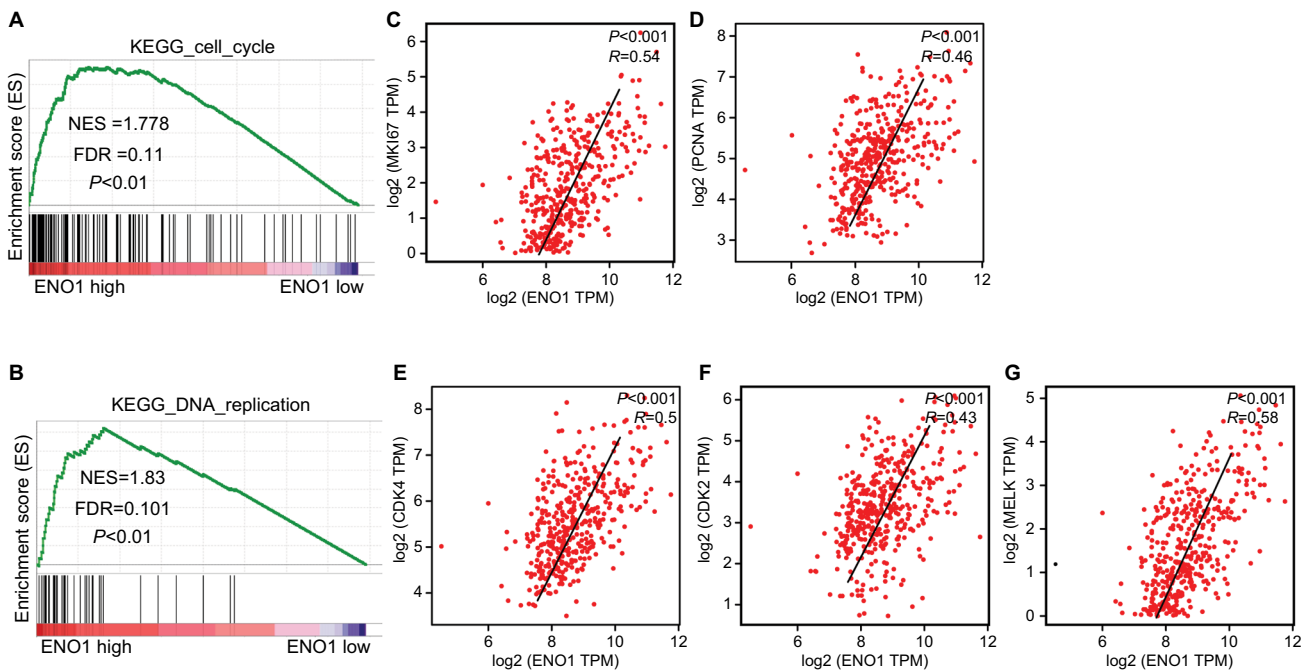**Abbreviations:** ENO1, enolase-1; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; OS, overall survival.

Figure 5 Molecular mechanism of ENO1 action in HCC.

Notes: GSEA of the relationship between high ENO1 expression and genes associated with cell cycle (KEGG_cell_cycle) (**A**), and DNA replication (KEGG_DNA_replication) (**B**). The scatter plot of the linear association between ENO1 expression level and MKI67 (**C**), PCNA (**D**), CDK4 (**E**), CDK2 (**F**), and MELK (**G**).

Abbreviations: ENO1, enolase-1; ES, enrichment score; FDR, false discovery rate; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; NES, normalized enrichment score; TPM, transcripts per millions.

Discussion

HCC represents more than 90% of primary liver cancers and has an overall poor prognosis.[19] Therefore, it is vital to dissect the molecular mechanisms underlying the initiation and progression of HCC. Recent studies showed an oncogenic function of ENO1 in various cancers, with high expression levels observed in cervical squamous cell carcinoma,[13] pancreatic cancer,[11,15] breast cancer,[20] non-small cell lung cancer (NSCLC),[21] nasopharyngeal carcinoma,[22] and HCC.[16,17] We analyzed the expression level of ENO1 on multiple HCC samples using the gene expression data available in TCGA and GEO HCC databases and HCC TMA. We found consistently high ENO1 expression in HCC tissues, indicating its oncogenic role in HCC.

Recent studies have correlated high expression of ENO1 in some primary cancers with tumor progression and poor prognosis. For example, Song et al[23] and Chen et al[24] reported that high expression of ENO1 was significantly correlated with poor prognosis in glioma patients. Similarly, NSCLC patients expressing relatively higher ENO1 levels in the tumors had poorer survival outcomes.[25] Consistent with these findings, we found that high ENO1 expression was positively correlated with the poor prognosis of HCC patients as per the TCGA and HCC TMA data. In addition, univariate and multivariate analyses showed that ENO1 expression was an independent prognostic factor in HCC.

Although previous studies had reported a potential biomarker role of ENO1 in HCC,[16,17] no convincing evidence was available for its diagnostic power in HCC. We therefore conducted a meta-analysis on previous studies retrieved from the GEO HCC data set. The ROC curves showed satisfactory diagnostic performance, thereby substantiating that ENO1 was a reliable diagnostic marker for distinguishing HCC tissues from non-HCC tissues.

We also predicted the potential mechanism of ENO1 action in HCC using GSEA and found a significant correlation between high ENO1 expression and HCC cell cycle and DNA replication. The cell cycle is a complex and strictly controlled process[26] and is frequently dysregulated in tumorigenesis.[27–29] Consistent with our results, several studies had demonstrated an important role of ENO1 in the proliferation and cell-cycle progression of various malignancies, including HCC.[16,21,30] In addition, ENO1 expression was positively correlated with that of MKI67, PCNA, CDK4, CDK2, and MELK, which were involved in cell cycle and DNA replication, and associated with the malignant phenotype of HCC.[31,32]

Conclusion

ENO1 is overexpressed in HCC and associated with cancer progression and poor prognosis. This is the first study to explore the value of ENO1 as a clinical biomarker in HCC and underscore its potential as a potential prognostic and diagnostic biomarker in HCC patients.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Basic characteristics of 12 HCC cohort from GEO

Cohort ID	Platform	Number of samples		Publication year	Country
		Nontumor	**Tumor**		
GSE6764	Affymetrix	40	35	2007	USA
GSE10143	DASL	307	80	2008	USA
GSE14520	Affymetrix	220	225	2010	USA
GSE25079	Affymetrix	289	268	2011	USA
GSE36376	Illumina	193	240	2012	South Korea
GSE39791	Illumina	72	72	2014	USA
GSE57957	Illumina	39	39	2014	Singapore
GSE60502	Affymetrix	18	18	2015	Taiwan, ROC
GSE62232	Affymetrix	10	81	2014	France
GSE64041	Affymetrix	65	60	2016	Switzerland
GSE76297	Affymetrix	151	153	2017	USA
GSE84005	Affymetrix	38	38	2017	China
Total		1,442	1,309		

Abbreviations: GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma.

Table S2 Results of subgroup analysis of the ENO1 expression in nontumor tissues and HCC samples

Subgroup analysis	Number of studies	Number of patients	Pooled HR	*P*-value	Heterogeneity	
					I^2 (%)	*P*-value (χ^2)
Region						
Asian countries	4	623	1.06 (0.89–1.23)	<0.001	0.0	0.486
Western countries	8	2,751	0.83 (0.74–0.92)	<0.001	86.1	<0.001
Sample size						
≤100	5	356	0.83 (0.59–1.07)	<0.001	76.1	0.002
>100	7	2,395	0.89 (0.80–0.97)	<0.001	85.5	<0.001

Abbreviations: ENO1, enolase-1; HCC, hepatocellular carcinoma.

A Study ID   SMD (95% CI)   Weight %

Region (non-Asia)
GSE6764   0.11 (–0.34–0.57)   7.39
GSE10143   1.20 (0.94–1.46)   9.76
GSE14520   0.91 (0.72–1.11)   10.48
GSE25079   0.46 (0.29–0.62)   10.74
GSE39791   0.94 (0.60–1.28)   8.73
GSE62232   0.63 (–0.03–1.30)   5.25
GSE64041   0.99 (0.62–1.36)   8.39
GSE76297   1.26 (1.01–1.51)   9.92
Subtotal (I^2 =86.1%, P=0.000)   0.84 (0.57–1.11)   70.65

Region (Asia)
GSE36376   0.99 (0.79–1.19)   10.42
GSE57957   1.15 (0.67–1.63)   7.09
GSE60502   0.97 (0.28–1.66)   5.00
GSE84005   1.41 (0.90–1.91)   6.83
Subtotal (I^2 =0.0%, P=0.486)   1.06 (0.89–1.23)   29.35

Overall (I^2 =81.1%, P=0.000)   0.92 (0.72–1.13)   100.00
Note: Weights are from random effects
analysis

            –1.91   0   1.91

B Study ID   SMD (95% CI)   Weight %

Sample size (≤ 100)
GSE6764   0.11 (–0.34–0.57)   7.39
GSE57957   1.15 (0.67–1.63)   7.09
GSE60502   0.97 (0.28–1.66)   5.00
GSE62232   0.63 (–0.03–1.30)   5.25
GSE84005   1.41 (0.90–1.91)   6.83
Subtotal (I^2 =76.1%, P=0.002)   0.85 (0.35–1.35)   31.56

Sample size (> 100)
GSE10143   1.20 (0.94–1.46)   9.76
GSE14520   0.91 (0.72–1.11)   10.48
GSE25079   0.46 (0.29–0.62)   10.74
GSE36376   0.99 (0.79–1.19)   10.42
GSE39791   0.94 (0.60–1.28)   8.73
GSE64041   0.99 (0.62–1.36)   8.39
GSE76297   1.26 (1.01–1.51)   9.92
Subtotal (I^2 =85.5%, P=0.000)   0.96 (0.72–1.19)   68.44

Overall (I^2 =81.1%, P=0.000)   0.92 (0.72–1.13)   100.00
Note: Weights are from random effects
analysis
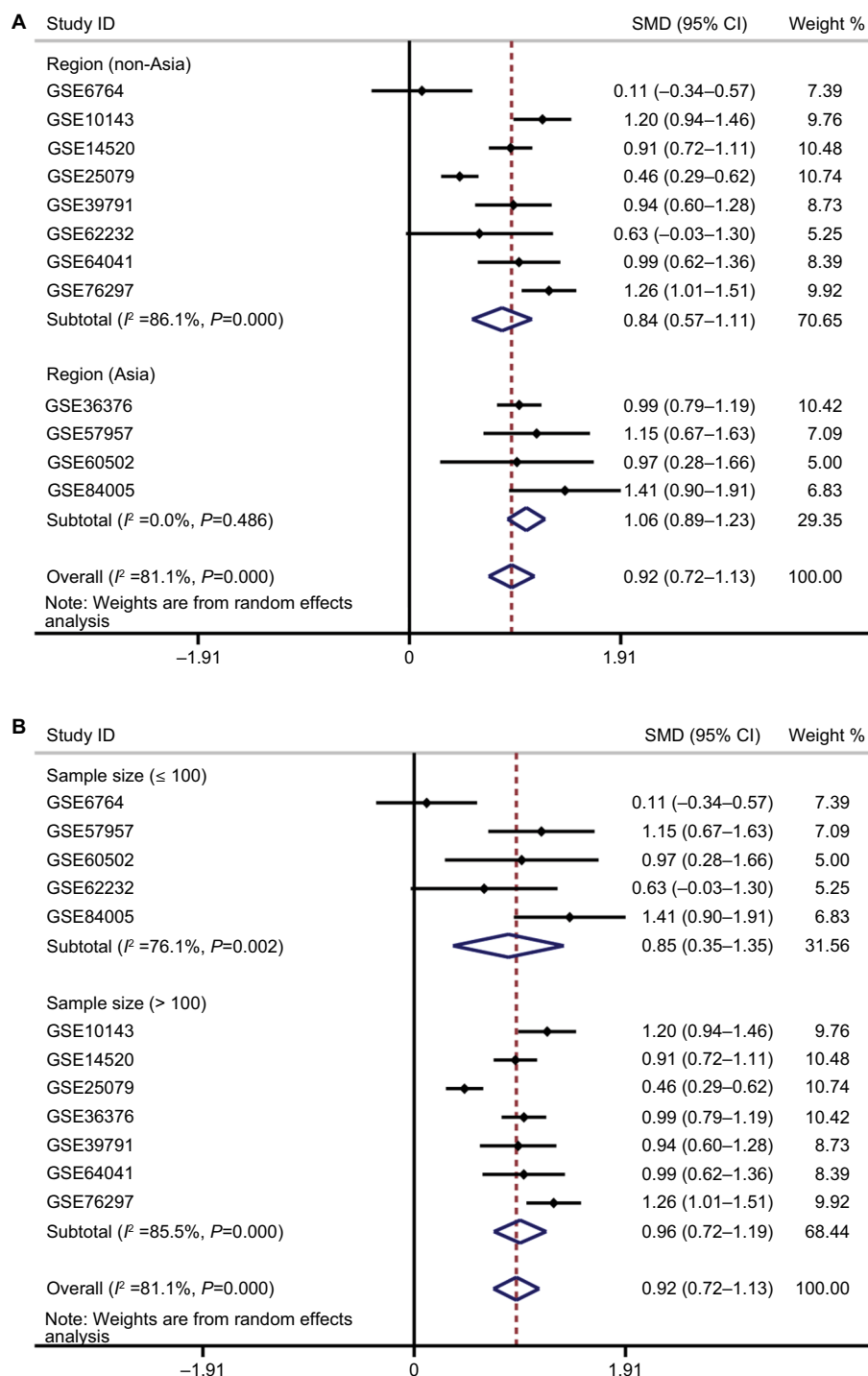
            –1.91   0   1.91

Figure S1 Subgroup analysis for exploring the source of heterogeneity.
Note: Subgroup analysis of the enrolled cohorts based on region (**A**) and sample size (**B**).
Abbreviation: SMD, standard mean difference.