Original Article

ACTB may serve as a predictive marker for the efficacy of lenvatinib in patients with HBV-related early-stage hepatocellular carcinoma following partial hepatectomy: a retrospective cohort study

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Background: The lack of effective biomarkers for the treatment of postoperative recurrence in hepatocellular carcinoma (HCC) persists despite lenvatinib therapy. This study aims to identify beta-actin (*ACTB*) as a predictive biomarker for lenvatinib that can facilitate individualized treatment for HCC.

Methods: This retrospective study included a subset of patients with HCC who underwent partial hepatectomy, with some receiving postoperative lenvatinib treatment and others not receiving lenvatinib treatment. A propensity score matching (PSM) analysis of patients who underwent treatment with or without lenvatinib following HCC partial hepatectomy was performed. Immunohistochemistry was employed to determine the levels of *ACTB* expression in HCC samples obtained from matched patients (n=225) enrolled in this study. The X-Tile was employed to determine the optimal cut-off point of *ACTB* levels for predicting time to recurrence (TTR). To assess the correlation between *ACTB* levels and lenvatinib efficacy, a subgroup analysis of TTR was conducted. A Cox regression model with an interaction term was utilized to assess the predictive significance of the model. Subsequently, a nomogram was developed and its discriminative ability

and predictive accuracy were assessed using the concordance index (C-index) and calibration curve. For the investigation of the *ACTB* expression, HCC and para-tumoral normal tissues were employed. The patient-derived xenograft (PDX) model was utilized to validate the correlation between *ACTB* levels and lenvatinib responsiveness.

Results: After PSM, a total of 76 patients who underwent postoperative lenvatinib treatment were included in the analysis, with a median TTR of 24.35 months. Early-stage HCC patients with lower levels of *ACTB* exhibited a more favorable response to lenvatinib therapy compared to those with higher levels. The reduced expression of *ACTB* was indicative of the benefits of lenvatinib, as opposed to higher levels {hazard ratio (HR) =0.243 [95% confidence interval (CI): 0.096–0.619], P<0.001, P value for interaction =0.014}. In approximately 81.8% of cases involving HCC patients, there was an observed increase in the expression of *ACTB*. Multivariate analysis of the lenvatinib cohort revealed Child-Pugh [HR =5.416 (95% CI: 1.390–21.104), P=0.015], Barcelona Clinic Liver Cancer (BCLC) stage [HR =2.508 (95% CI: 1.116–5.639), P=0.026], and *ACTB* [HR =5.879 (95% CI: 2.424–14.259), P<0.001] score as independent factors for TTR, and all were included in the nomogram. The survival probability based on the calibration curve showed that the prediction of the nomogram was in good agreement with the actual observation. The C-index of the nomogram for predicting survival was 0.76 (95% CI: 0.71–0.84). Moreover, the PDXs derived from tumors exhibiting low levels of *ACTB* expression demonstrated a heightened sensitivity to lenvatinib treatment.

Conclusions: In patients with tumors treated with lenvatinib, low *ACTB* expression can predict a lower risk of recurrence. The validation of this potential biomarker in independent cohorts is necessary prior to its implementation for precision treatment stratification in patients undergoing partial hepatectomy for early-stage HCC.

Keywords: Hepatocellular carcinoma (HCC); lenvatinib; hepatitis B virus (HBV); personalized therapy; partial hepatectomy

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Introduction

Hepatocellular carcinoma (HCC) is a prevalent malignancy worldwide, ranking fourth in cancer-related mortality globally (1,2). Liver resection remains the primary therapeutic option, despite the fact that less than 30% of patients meet the criteria for potentially curative treatment. However, due to high recurrence and death, survival after resection is unsatisfactory, with the 5-year overall survival (OS) of approximately 50%. The majority of patients experience recurrence following resection, with a postoperative recurrence rate ranging from 60% to 70% within a 5-year period (3). Factors contributing to recurrence include the presence of microscopic vascular invasion (MVI), elevated serum α-fetoprotein (AFP) levels, multiple tumors, higher grades of hepatitis activity and cirrhosis, as well as satellite nodules (4-7). Several randomized trials have investigated various adjuvant therapeutic interventions, such as transcatheter arterial chemoembolization (TACE), targeted therapy, interferon (IFN), internal radiation, and chemotherapy, with the aim of providing long-term recurrence or OS. However, as far as we are aware, there are currently no standardized treatment options available (3,8,9).

Lenvatinib is an orally administered multikinase inhibitor that selectively targets PDGF receptor α, fibroblast growth factor (FGF) receptors 1–4 (FGFR1–4), vascular endothelial growth factor (VEGF) receptors 1–3, KIT and RET. It has been authorized as a first-line systemic treatment for advanced HCC patients (10-12). This was the result of a randomized phase-III clinical trial in patients with advanced HCC, which demonstrated that lenvatinib had comparable OS to sorafenib while exhibiting superior progression-free survival (PFS) (13). Furthermore, lenvatinib was indicated with the efficacy of preventing the recurrence of HCC for patients after surgery (14,15). Despite numerous studies, no predictive factors for lenvatinib response have been identified. Given the limitations of the currently

available clinicopathological biomarkers, a study suggests that molecular biomarker should be used in addition to clinicopathological marker (16). At present, most of the studies on biomarkers related to the prediction of HCC targeted therapy are still in the preliminary stage, and more large samples and prospective clinical studies are needed to verify them. Currently, some studies have successively reported various substances or cells as biomarkers for predicting the prognosis of patients with HCC after liver resection (16,17), but there are few biomarkers that predict the efficacy of lenvatinib. In a random phase III non-inferiority trial (REFLECT), there was a trend that lower VEGF, angiogenin-2 (Ang-2), and FGF21 serum levels at baseline were associated with better outcomes with lenvatinib; early changes in rehabilitation and treatment of contaminated Soil in the treatment of HCC, Ang-2 levels, AFP response, baseline albumin bilirubin (ALBI) score, change in the ALBI score, neutrophil to lymphocyte and platelet to lymphocyte ratios were early predictors of objective response (OR) in patients with HCC undergoing lenvatinib treatment (17,18). However, to date, none of the biomarkers have been validated for clinical use. Worse still, the status of lenvatinib biomarker expression in HCC and its correlation with lenvatinib response remain elusive. Therefore, it is imperative to investigate the correlation between biomarkers and its efficacy in treating HCC, as well as to develop innovative indicators for patient selection to optimize adjuvant treatment outcomes (19,20).

Given the widely held assumption that the beta-actin (*ACTB*) gene is constitutive and ubiquitous, performing housekeeping activities, recent research suggests a broader range of functional roles for this protein (21). Biological research suggests that a significant deficiency in *ACTB* protein levels can lead to detrimental effects on cell shape, migration, proliferation, and gene expression. These effects may hinder the proper development of vital organs such as the kidney, heart, and brain. Nevertheless, mounting evidence indicates that *ACTB* is expressed abnormally in various malignancies, disrupting the cytoskeleton and thereby impeding tumor invasion and metastasis (22-28). In the current research field of HCC, the expression of *ACTB* was downregulated in HCCs with varying degrees of invasiveness and Tumor-Nodes-Metastasis (TNM) stages, and the 3'-untranslated region (UTR) of *ACTB* played a critical role in HCC progression (29).

The current study investigated the correlation between *ACTB* and patient TTR in lenvatinib-treated individuals with high risk of recurrence, suggesting that *ACTB* may serve as a potential biomarker for predicting lenvatinib efficacy in HCC patients at high risk of recurrence. To the best of our knowledge, this is the first attempt to identify a predictive biomarker and build a development model in accordance with the CONSORT guidelines for patients with hepatitis B virus (HBV)-related HCC who have undergone partial hepatectomy and received lenvatinib treatment. We present this article in accordance with the TRIPOD reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-942/rc).

Highlight box

Key findings

- The expression of beta-actin (*ACTB*) influences the efficacy of lenvatinib for patients with hepatitis B virus-related early-stage hepatocellular carcinoma (HCC) after surgery.

What is known and what is new?

- Lenvatinib effectively improves the prognosis of patients with HCC after surgery.
- HCC with low expression of *ACTB* is sensitive to lenvatinib, indicating low recurrence of patients after surgery.

What is the implication, and what should change now?

- It is recommended to detect the expression of *ACTB* in HCC tissue after surgery. The patients with low-level *ACTB* HCC should prioritize using lenvatinib to prevent recurrence.

Methods

Patients and treatment

In conducting this retrospective clinical cohort study, we employed a comprehensive research design and retrospectively enrolled a total of 256 HCC patients who had not received preoperative therapy at the Eastern Hepatobiliary Surgery Hospital (EHBH) to identify parameters associated with the benefits of lenvatinib in the adjuvant setting between 2009 and 2020. Between 2018 and 2020, a total of 76 patients received adjuvant lenvatinib treatment following surgical intervention and were prospectively monitored until the conclusion of this study (the lenvatinib group). Incorporating insights from previous research and a review of pertinent literature, we have included baseline characteristics of patients in our study. These characteristics have been determined based on the professional assessment of pathologists, outlining tissue and tumor features such as the presence of liver

cirrhosis. Additionally, for specific data of particular interest, a separate and detailed examination has been conducted, as exemplified by our focused scrutiny on data related to *ACTB*. The control group comprised patients who underwent surgery between 2009 and 2011 but did not receive adjuvant therapy. During the first-year post-surgery, patients underwent follow-up visits every 2–3 months. After 1 year, they underwent follow-up assessments every 3–6 months to evaluate liver function and serum tumor markers. The duration between surgical resection and the day of recurrence (or the last follow-up) was utilized to ascertain the time to recurrence (TTR). Table S1 presents a comprehensive overview of their clinicopathological features. After propensity score matching (PSM), a cohort (n=225) was formed by conducting a propensity matching analysis on the patients who met the criteria, as shown in Table S2. The following selection criteria were employed: (I) patients with Child-Pugh A/B liver function and unilateral HCC, who exhibited at least two risk factors for recurrence, including the presence of MVI, high levels of AFP, multiple tumors and satellite nodules; (II) lenvatinib therapy was initiated within one-month post-surgery and continued for a duration of at least 1 year or until disease recurrence. The subjects were orally administered lenvatinib once daily in 28-day cycles, with a dosage of 12 mg/day (for body weight ≥60 kg) or 8 mg/day (for body weight <60 kg). Dose interruptions, followed by reductions to 8 mg/day or 4 mg/day (or 4 mg every other day), were permitted for lenvatinib-related toxicities; (III) hepatitis B surface antigen (HBsAg) and/or hepatitis B core antibody (HBcAb) were detected as positive, while hepatitis C antibody was identified as negative; (IV) refraining from preoperative therapies such as radiofrequency ablation, high-intensity focused ultrasound, or chemoembolization was the approach taken for HCC patients; (V) all patients underwent surgical treatment, and postoperative pathology confirmed that the tumor was R0 removed To conduct immunohistochemistry (IHC) staining, tissue slices were obtained from primary HCC patients. The procedural flow of HCC patients is illustrated in *Figure 1A*.

The TTR, which was the primary endpoint of this study, was defined as the duration from surgery to recurrence. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the institutional ethics board of Eastern Hepatobiliary Surgery Hospital (No. EHBHKY2023-K036-P001) and informed consent was obtained from all individual participants.

Surgical procedure

The surgical team used conventional approaches to execute all surgical procedures. The surgical procedures were performed through a bilateral subcostal incision. The abdominal cavity was thoroughly examined to assess the extent of local disease and identify any extrahepatic metastases. Intraoperative ultrasonography was performed to determine the number, size, and vascular connection of the lesions. Pringle's maneuver was employed to interrupt hepatic blood flow, utilizing cycles of 15 minutes occlusion followed by 5 minutes of reperfusion. The liver resection was performed using the clamp-crushing technique (30).

IHC analysis

The primary HCC and corresponding para-tumoral fresh specimens were promptly obtained post-surgery, fixed in neutral buffered formalin, and embedded in paraffin for subsequent analysis via hematoxylin and eosin (HE) or IHC staining as previously reported (31). For *ACTB* staining, the slides were incubated with a monoclonal antibody against human β-actin (D6A8, Cell Signaling Technology, Boston, USA) at a dilution of 1:100. The secondary antibody used was an horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins/HRP (P0448, DAKO, Copenhagen, Denmark). The final steps involved the utilization of a colorimetric reagent solution containing diaminobenzidine (Liquid DAB + Substrate Chromogen System, K3468, DAKO), followed by counterstaining with hematoxylin. The specificity of *ACTB* antibody was assessed through western blotting. The slides stained in different batches exhibited minimal experimental variability, indicating excellent reproducibility. Simultaneous experimental controls were implemented. To observe the stained sections, we utilized an imaging system comprising of a Leica DFC420 CCD camera attached to a Leica DM IRE2 microscope procured from Leica Microsystems Imaging Solutions in Cambridge, UK. The images were captured using Leica QW in Plus v3 software at 200× magnification for each individual core (32). The degree of hepatic inflammation and fibrosis was evaluated utilizing the Glasgow University Score for Liver Pathology (GS) staging system. The quality of immunostaining was evaluated independently by two pathologists who were blinded to the clinical information and patient outcomes. The pathologists utilized the positive pixel count method provided by Image-

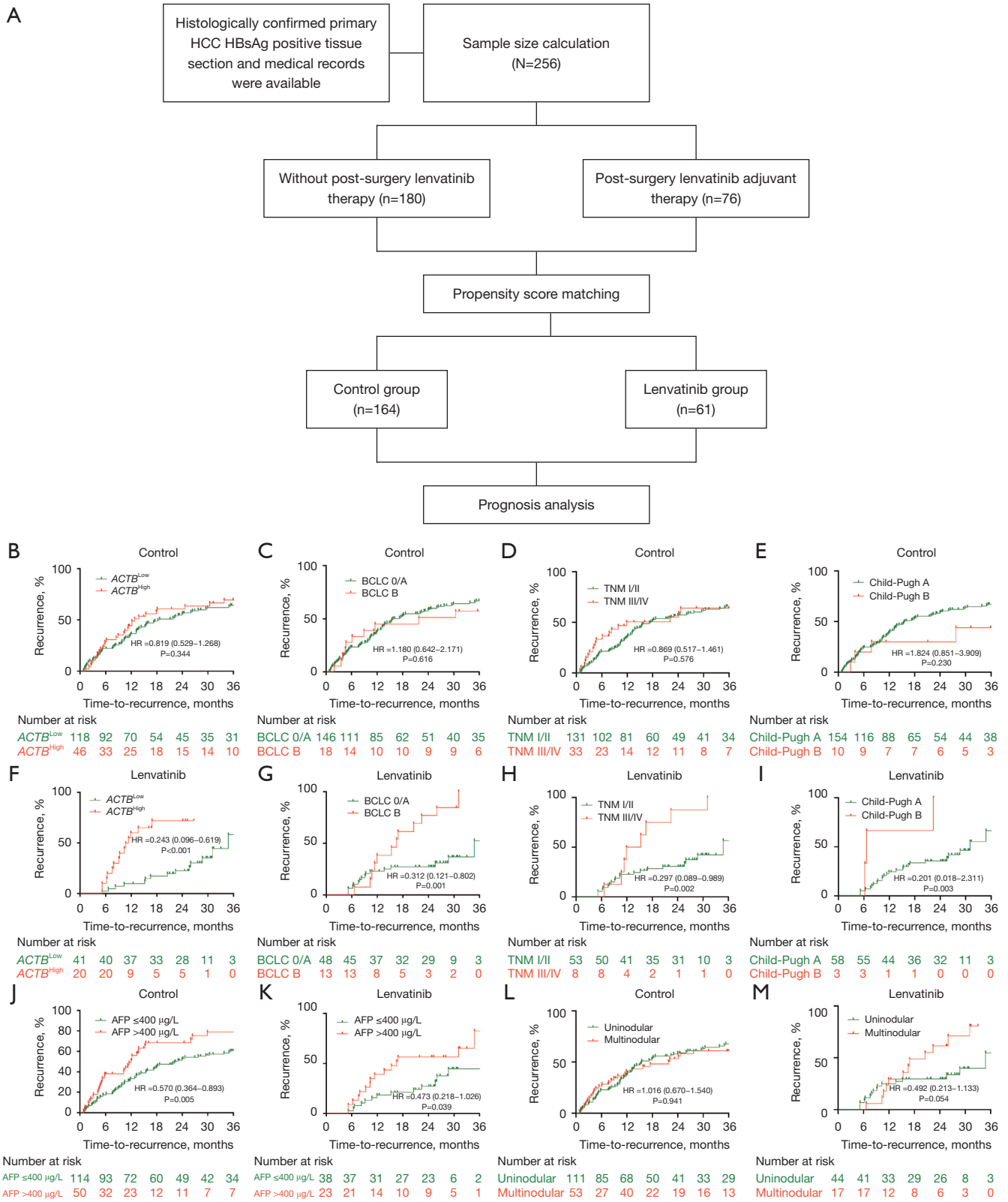


Figure 1 Factors of stratification influencing the efficacy of adjuvant lenvatinib therapy. (A) The flow chart diagrams depicting HCC patients; (B) the TTR post-surgery was compared between patients treated without adjuvant lenvatinib therapy, stratified by '*ACTB*^{Low}' and

'*ACTB*^{High}' groups; (C) the TTR post-surgery was compared between patients treated without adjuvant lenvatinib therapy in BCLC 0/A and BCLC B groups; (D) the TTR post-surgery was compared between TNM I/II and BCLC III/IV groups, without adjuvant lenvatinib therapy administered to the patients; (E) the TTR post-surgery was compared between Child-Pugh A and B groups of patients treated without adjuvant lenvatinib therapy; (F) the TTR post-surgery was compared between patients treated with adjuvant lenvatinib therapy, stratified by '*ACTB*^{Low}' and '*ACTB*^{High}' groups; (G) the TTR post-surgery was compared between patients treated with adjuvant lenvatinib therapy in BCLC 0/A and BCLC B groups; (H) the TTR post-surgery was compared between TNM I/II and BCLC III/IV groups, with adjuvant lenvatinib therapy administered to the patients; (I) the TTR post-surgery was compared between Child-Pugh A and B groups of patients treated with adjuvant lenvatinib therapy; (J,K) the TTR post-surgery was compared between patients who received adjuvant lenvatinib therapy and those who did not, in AFP ≤400 μg/L and AFP >400 μg/L groups; (L,M) the TTR post-surgery was compared between uninodular and multinodular groups of patients treated with or without adjuvant lenvatinib therapy. HCC, hepatocellular carcinoma; HBsAg, Hepatitis B surface antigen; *ACTB*, beta-actin; HR, hazard ratio; BCLC, Barcelona Clinic Liver Cancer Staging; TNM, Tumor-Nodes-Metastasis; AFP, α-fetoprotein; TTR, time to recurrence.

Pro plus V6.0 (Media Cybernetics, Bethesda, MD, USA) to quantify and assess high-resolution digital images they gathered. The software automatically calculates the total intensity of positive (brown staining) pixels and measures the overall area of the image. The staining score for each sample was determined by calculating the sum of positive pixel intensities divided by the total area. To stratify patients in the adjuvant lenvatinib group based on their expression levels, we selected the upper tertile of IHC staining scores for lenvatinib target in HCC tissues. The X-Tile software was utilized to determine the cut-off point for *ACTB* protein and mRNA levels, which enabled the categorization of patients into two groups based on their high or low *ACTB* expression levels.

mRNA analysis

Real-time polymerase chain reaction (RT-PCR) analysis was performed using a SYBR Green PCR Kit (Roche, Switzerland, Basel) and a Light Cycler 480 System (Roche). Fifty pairs of HCC and corresponding peritumoral normal tissues were used for the analysis of β-actin mRNA. GAPDH was used as internal reference. The sequences of primers used are listed below:

- β-actin forward primer (5'-3'): AATCGTGCGTGACATTAAGGAG; reverse primer (5'-3'): ACTGTGTTGGCGTACAGGTCTT.
- GAPDH forward primer (5'-3'): ACCACAGTCCATGCCATCAC; reverse primer (5'-3'): TCCACCACCCTGTTGCTGTA.

Patient-derived xenograft (PDX) model

To establish the PDX model, primary HCC specimens were obtained from six independent patients and utilized for xenograft formation as previously reported (33). For this study, the primary tumor specimens were obtained post-surgery and subsequently washed with DMEM containing antibiotics. The specimens were then sliced into 20–30 mm^3 sections and subcutaneously implanted bilaterally in male BALB/c nude mice. Once the original xenografts reached a volume of 500–800 mm^3, they were retrieved and sliced into 30 mm^3 cubes before being subcutaneously implanted into male BALB/c nude mice. Following size-matching of the xenografts (150–200 mm^3), the mice were randomly assigned to two treatment groups (lenvatinib or vehicle). Mice bearing xenografts were orally administered daily doses of lenvatinib (10 mg/kg body weight) (34) or vehicle for 21 days, and the tumor volume was normalized at day 21. Each model was generated using the following number of mice: Patient #1, n=5 for both lenvatinib and vehicle groups; Patient #2, n=5 for both lenvatinib and vehicle groups; Patient #3, n=5 for vehicle group and n=4 for lenvatinib group; Patient #4, n=5 for both lenvatinib and vehicle groups; Patient #5, n=5 for both lenvatinib and vehicle groups; Patient #6, n=4 for vehicle group and n=5 for lenvatinib group. The tumor volumes were quantified as described, and Table S3 provides a comprehensive overview of *ACTB* expression in the six distinct primary HCCs used to develop the PDX model, along with relevant clinical features of the patients. IHC was conducted to evaluate the expression of *ACTB* in primary HCCs used for developing PDX models. The cutoff threshold for IHC staining score to distinguish between high and low HCCs was 88.4. In addition to obtaining written informed consent from each participant, the Institutional Review Board at EHBH authorized all the protocols and procedures of this study. The growth rate of tumors was calculated using the following equation:

$$\text{Tumor growth rate} = \frac{\text{Normalized tumor volume at day 21} - \text{normalized tumor volume at day 0}}{\text{Normalized tumor volume at day 0}} \div 21 \quad [1]$$

Statistical analysis

Statistical analysis utilizing the SPSS V22.0 program (IBM, Chicago, IL, USA) was applied to identify risk variables. The baseline clinicopathological features of the patients were evaluated using the χ^2 test. The categorical variables were categorized based on clinical outcomes, and the selection of groups was made prior to the modeling process. The Mann-Whitney test for abnormal distributed variables was utilized to compare the continuous variables. PASS 17 program was employed to compute the sample size, the median TTR time of lenvatinib and control group were used as basis, tests were two-sided and α is equal to 0.05. Propensity score matching analysis was used to match HCC patients who received treatment with or without lenvatinib employing psmatch2 tools, the hypothesis test for the balance of variables before and after matching employing pstest tools in STATA 17 version MP (https://www.stata.com). With the assistance of X-Tile statistical software (version 3.6.1, Yale University, New Haven, CT, USA), the optimal threshold points for TTR were determined. The presence of substantial HCC subpopulations was shown by an X-Tile plot, and a two-dimensional projection of each conceivable subpopulation was utilized to demonstrate the strength of the link between a biomarker and an outcome (34). X-Tile plots were constructed to determine the degree of quantitative elements including *ACTB* were present. For subgroup analysis, we employed a Cox proportional hazards regression model and the Kaplan-Meier technique to determine hazard ratios (HRs) and median recurrence time. An interaction term was included in the Cox proportional hazards regression model to explore interactions between therapy, biomarkers, or clinical factors. Based on R packages "rms", Cox regression coefficients were also utilized to construct a nomogram, while calibration curves were employed to evaluate the concordance between observed and predicted outcomes derived from the nomogram (35). Multivariate analysis of TTR in matched patients was conducted using regression models (Table S4). All statistical evaluations were two-tailed, and a significance level of P<0.05 was considered statistically significant.

Results

Relationships between the expression of ACTB and patient outcomes were investigated in participants who underwent adjuvant lenvatinib therapy following surgery

The HBV infection status and utilization of antiviral therapy were succinctly summarized in Table S5. To investigate the correlation between *ACTB* expression and lenvatinib response as well as patient outcome in HCC samples, IHC staining was performed to measure and score *ACTB* expression in 76 post-surgical patients who received adjuvant lenvatinib treatment. According to the stratified factors affecting the efficacy of lenvatinib adjuvant therapy, the analysis was made (*Figure 1*). The patients were subsequently stratified into cohorts based on their *ACTB* expression levels. The control group did not exhibit a comparable level of performance (*Figure 1B-1E*) by Kaplan-Meier estimates. However, univariate analysis revealed that patients with high levels of *ACTB* (HR =5.014, P<0.001), BCLC stage B (HR =3.318, P=0.002), TNM stage III/IV (HR =3.485, P=0.004), Child-Pugh stage B (HR =5.265, P=0.008), AFP >400 μg/L (HR =2.160, P=0.045) and multinodular tumors (HR =2.427, P=0.023) were at an elevated risk for reduced TTR in the lenvatinib group (*Table 1*). Furthermore, patients in the lenvatinib group with low *ACTB* levels (HR =0.243, P<0.001) (*Figure 1F*), BCLC stage 0/A (HR =0.312, P=0.001) (*Figure 1G*), TNM stage I/II (HR =0.297, P=0.002) (*Figure 1H*), or Child-Pugh A (HR =0.201, P=0.003) (*Figure 1I*) exhibited a significantly longer TTR. Furthermore, upon subjecting these variables to multivariate analysis, the results indicated that elevated levels of *ACTB* (HR =5.879, P<0.001), BCLC stage B (HR =2.508, P=0.026) or Child-Pugh stage B (HR =5.416, P=0.015) were independent risk factors for TTR and associated with shorter TTR (*Table 1*). Simultaneously, we compared the baseline characteristics of patients in the lenvatinib group and control group based on the high and low expression of *ACTB*, and the results revealed no significant differences between the groups (Table S6). This suggests their potential in predicting recurrence among patients treated with adjuvant lenvatinib.

Table 1 Univariate and multivariate analysis of TTR of patients receiving adjuvant lenvatinib

Variables	Subgroup	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
Gender	Female *vs.* male	1.931 (0.889–4.196)	0.096	–	–
Age (years)	>50 *vs.* ≤50	1.176 (0.549–2.519)	0.677	–	–
Tumor size (cm)	>3.5 *vs.* ≤3.5	2.709 (0.812–9.034)	0.105	–	–
Tumor number	Multinodular *vs.* uninodular	2.427 (1.129–5.217)	0.023	NA	NA
MVI	No *vs.* yes	0.522 (0.244–1.115)	0.093	–	–
AFP (μg/L)	>400 *vs.* ≤400	2.160 (1.019–4.578)	0.045	NA	NA
Cirrhosis	Yes *vs.* no	1.093 (0.513–2.330)	0.818	–	–
HBV-DNA (IU/mL)	Low *vs.* high	0.975 (0.463–2.054)	0.946	–	–
Child-Pugh	B *vs.* A	5.265 (1.548–17.907)	0.008	5.416 (1.390–21.104)	0.015
TNM	III/IV *vs.* I/II	3.485 (1.507–8.058)	0.004	NA	NA
BCLC	B/C *vs.* 0/A	3.318 (1.544–7.128)	0.002	2.508 (1.116–5.639)	0.026
ACTB	High *vs.* low	5.014 (2.199–11.430)	<0.001	5.879 (2.424–14.259)	<0.001

TTR, time to recurrence; MVI, macroscopic vascular invasion; AFP, α-fetoprotein; HBV, hepatitis B virus; TNM, Tumor-Nodes-Metastasis; BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin; HR, hazard ratio; CI, confidence interval; NA, not available.

The association between ACTB expression and lenvatinib efficacy in patients with HCC

According to the sample size calculation, a total of 173 patients were allocated (43 in the lenvatinib group and 130 in the control group), with a recorded power value of 0.8015 (Table S7). We subsequently conducted a retrospective analysis to assess the predictive value of *ACTB* in identifying HCC patients who would benefit from lenvatinib treatment. A total of 256 patients who underwent surgery for HCC at a single academic institution were included in the study. These patients were categorized into two groups: those who received treatment with lenvatinib and those who did not (Table S1). The variables to be matched were also screened using STATA software. Following PSM analysis, the control group comprised 164 participants who were matched with 61 participants in the lenvatinib group (*Figure 1A*). The results also indicated that there were no significant differences in case composition and variables between matched and original cases stratified by baseline parameters (Figure S1). Although there was no statistically significant difference between patients with and without cirrhosis, we further stratified cases without cirrhosis and found that those with low levels of inflammatory fibrosis had a longer time in therapeutic range (TTR) compared to those with high levels of inflammatory fibrosis and cirrhotic patients in both the lenvatinib and control groups (Figure S2A,S2B, Table S8). The expression of *ACTB* varied across different stages of inflammation and fibrosis, with a statistically significant correlation observed between *ACTB* expression and GS score in cancer tissue (r=0.228, P<0.001) (Figure S2C). However, no obvious correlation was found between *ACTB* expression and GS score in normal tissue (P=0.243) (Figure S2D). Patients with well-differentiated tissue in the lenvatinib and control groups exhibited a longer TTR compared to those with moderate and poor differentiation, as shown in Figure S2E,S2F. Subsequently, we performed IHC staining to assess the expression of *ACTB* in primary HCC specimens. The Cox proportional hazard model with an interaction term in the cohort was employed to evaluate the statistically significant interaction effect of continuous levels of *ACTB* and therapy on patient outcome. The results indicate a significant interaction (P=0.014) (*Figure 2A*) To determine the optimal cutoff threshold for *ACTB* levels, we employed X-Tile to traverse all *ACTB* expression values in ascending order and identify the cutoff point that best separates patient into low- or high-*ACTB* expression groups. We then evaluated the significance of lenvatinib

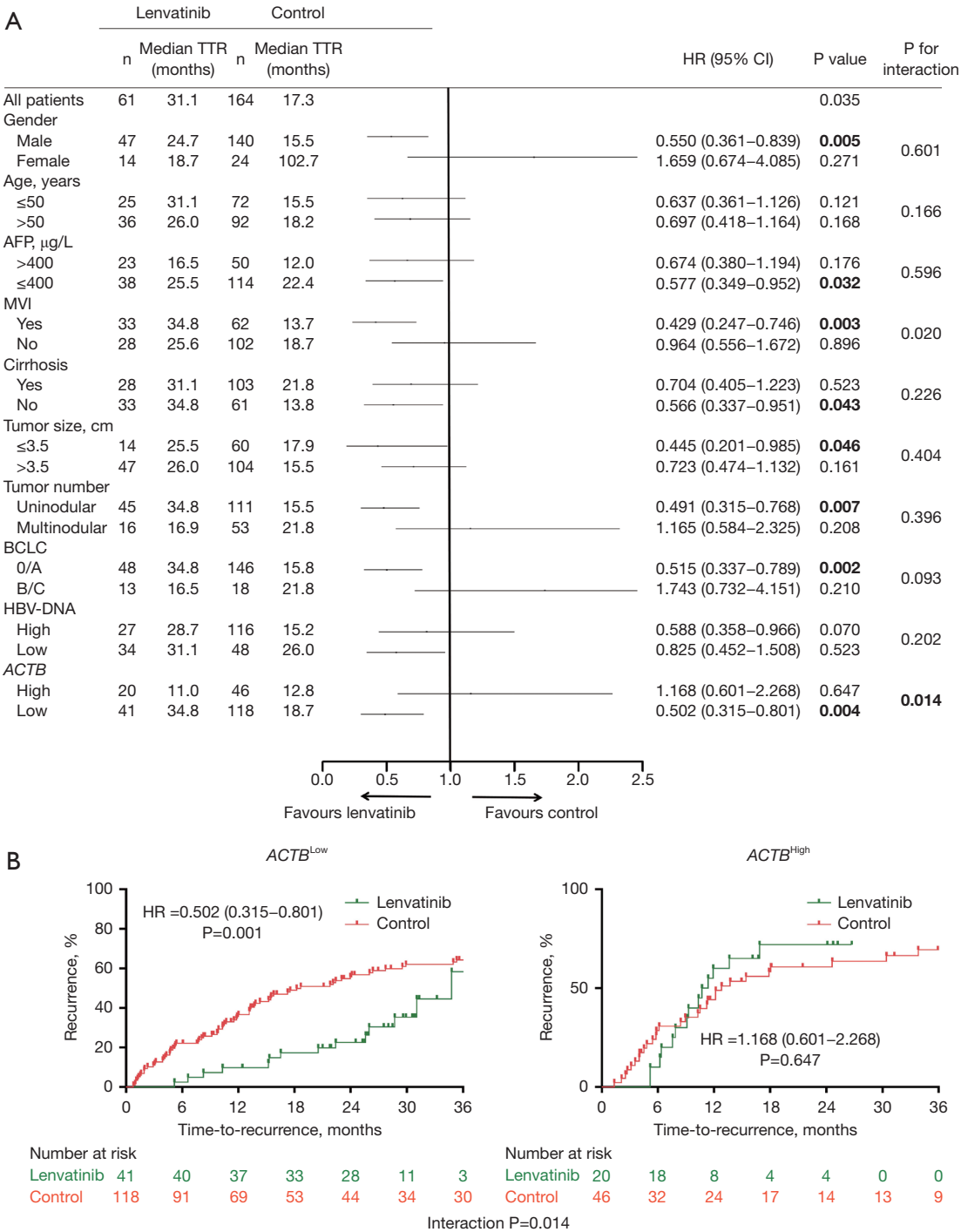


Figure 2 The association between baseline characteristics and lenvatinib benefit in HBV-related HCC patients. (A) Subgroup analysis of TTR in HCC patients was conducted using Cox regression, taking into account baseline characteristics and *ACTB* levels. The P values for the interactions between treatment and *ACTB* levels or clinical variables were also presented; (B) The TTR of $ACTB^{Low}$ or $ACTB^{High}$ was compared between the lenvatinib and control groups in HCC patients, aiming to evaluate the efficacy of lenvatinib treatment. The P value for the interaction between treatment and *ACTB* levels is presented below. TTR, time to recurrence; MVI, macroscopic vascular invasion; BCLC, Barcelona Clinic Liver Cancer Staging; HBV, hepatitis B virus; *ACTB*, beta-actin; HR, hazard ratio; CI, confidence interval; HCC, hepatocellular carcinoma.

benefit against control within each group. The groups exhibiting statistically significant variations in TTR were those with low expression of *ACTB* protein. A threshold point (88.4) was identified as having the greatest significance and used to further classify patients into two groups: $ACTB^{High}$ versus $ACTB^{Low}$, comprising 29.3% and 70.7% of the total population, respectively (*Figure 2A*). *ACTB* levels in the cohort were compared with clinical characteristics of the patients, revealing a significant correlation between *ACTB* levels and cirrhosis as well as HBV-DNA (Table S9). HCC patients exhibiting low levels of *ACTB* demonstrated a superior TTR response to lenvatinib therapy (HR =0.502, P=0.001) (*Figure 2B*). However, our findings indicate that patients with low *ACTB* expression exhibited significantly improved TTR when treated with lenvatinib compared to those with high *ACTB* expression, while no significant improvement in TTR was observed in the control group (*Figure 1B*). These findings suggest that lenvatinib adjuvant therapy did not demonstrate a significant improvement in TTR for all patients, but may provide benefits for those with $ACTB^{Low}$, BCLC 0/A and Child-Pugh A (*Table 1*).

Additionally, we constructed a nomogram that combined the Child-Pugh stage, the BCLC stage, and the *ACTB* score in the lenvatinib group (*Figure 3A*). Calibration plots showed that the nomogram compared favorably with an ideal model in the lenvatinib group at 0.5, 1 and 2 years (*Figure 3B-3D*). The C-index of the nomogram for predicting survival was 0.76 [95% confidence interval (CI): 0.71–0.84].

The predictive value of ACTB in stratifying lenvatinib benefit among patients with different prognostic factors

A precise postoperative adjuvant treatment tailored to individual HCC patients heavily relies on the accurate classification of patients using an optimal combination of biomarkers and clinical factors (36). We evaluated the efficacy of lenvatinib in $ACTB^{High}$ and $ACTB^{Low}$ patients stratified by prognostic variables. The median dosage of lenvatinib was 12 mg/day in both the $ACTB^{High}$ and $ACTB^{Low}$ expression groups, with a median duration of administration of 13.6 months in the former group and 13.1 months in the latter. For patients with low *ACTB* levels, lenvatinib therapy improved their TTR, while for those with high *ACTB* levels, there was no significant improvement in most categories, as illustrated in Figure S3. Notably, a significant number of benefits from lenvatinib were observed in the $ACTB^{Low}$ cohort among male patients (Figure S3A), with MVI (Figure S3D), non-cirrhosis (Figure S3E), tumor size ≤3.5 or >3.5 cm (Figure S3G,S3H), uninodular (Figure S3I) and BCLC stage 0/A (Figure S3K). The results suggest that the integration of $ACTB^{Low}$ with any of these variables may facilitate the identification of individuals who are most likely to benefit from lenvatinib treatment.

An increase in the expression of ACTB was observed in HCC specimens

To determine the proportion of patients who may derive therapeutic benefits from lenvatinib based on their *ACTB* levels, we subsequently conducted an analysis of *ACTB* expression in both HCC and adjacent para-tumoral normal liver tissues. The results presented in *Figure 4A,4B* demonstrate a significant upregulation of *ACTB* mRNA transcripts in HCC patient samples compared to adjacent para-tumoral normal tissues. The downregulation of *ACTB* mRNA did not show a significant difference in TTR within the control group, whereas its downregulation in the lenvatinib group was associated with a prolonged TTR (*Figure 4C,4D*). Furthermore, IHC assessment of HCC sections and adjacent normal liver tissues revealed a significant elevation in the levels of *ACTB* protein within HCC specimens. Furthermore, IHC staining of corresponding HCC and adjacent normal liver tissues demonstrated that only 18.2% of patients displayed downregulation of *ACTB* in their HCC specimens (*Figure 4E*).

Low levels of ACTB are associated with increased sensitivity to lenvatinib in HCC PDX models

Subsequently, the correlation between *ACTB* levels and lenvatinib response *in vivo* was validated through PDX models (*Figure 5*). Primary HCCs collected from six distinct patients who were not part of the cohort are detailed in Table S3. The IHC staining score threshold of 90.5 was utilized to distinguish HCCs with high- and low-*ACTB* scores. After PDXs with primary HCC was treated with lenvatinib or vehicle for 21 days, it was intuitively demonstrated that the expression of *ACTB* had an influence on the sensitivity of lenvatinib in HCC PDX model (*Figure 6*). The results indicated that lenvatinib did not demonstrate any therapeutic efficacy on the PDXs derived

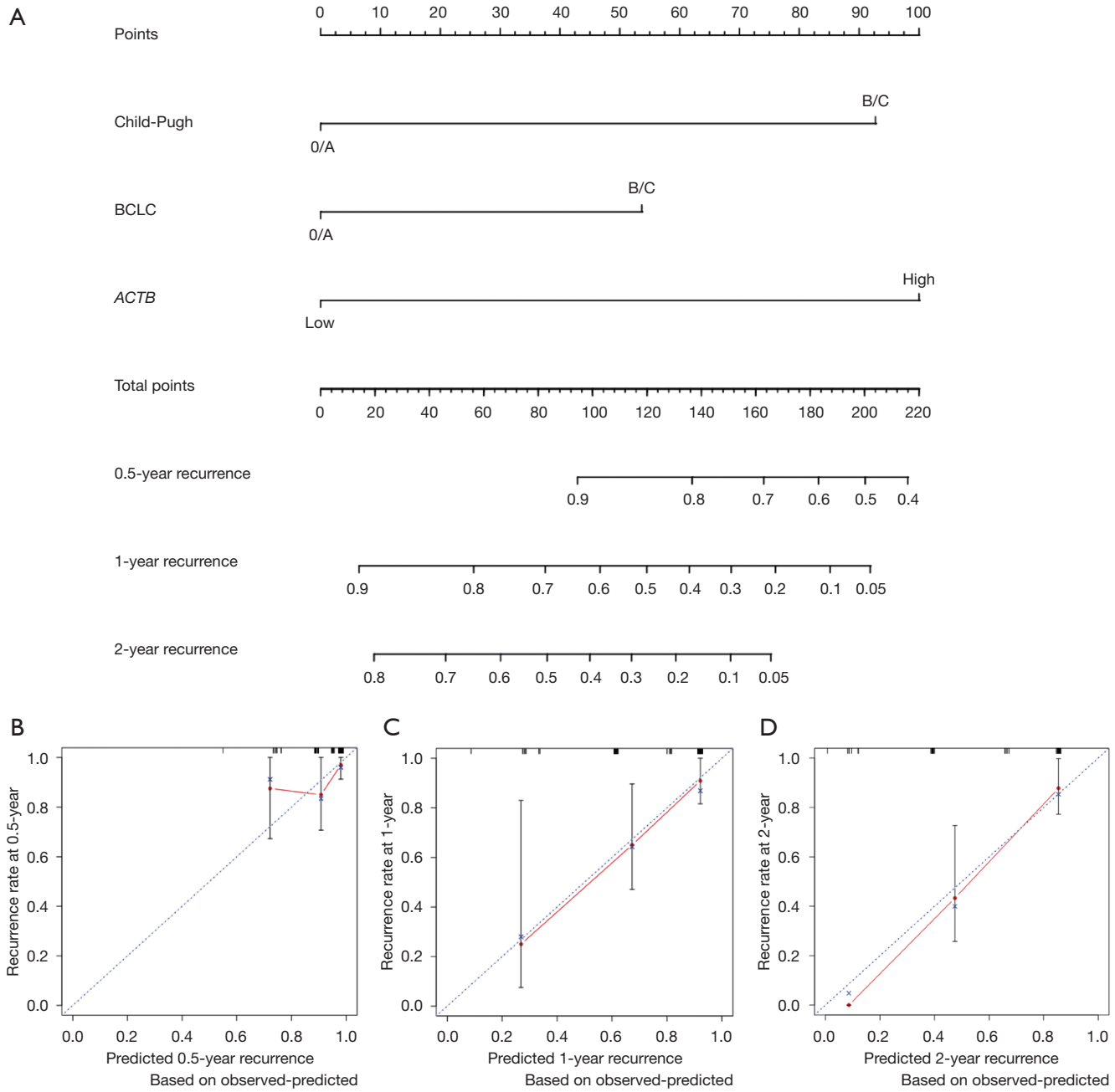


Figure 3 Establishing and validating a nomogram that combines the Child-Pugh stage, the BCLC stage, and the *ACTB* score to predict TTR in lenvatinib group. (A) The position of each variable is found on the corresponding axis, a line is drawn to the Points axis for the number of points, the points from the four variables are added together, and finally a line is drawn from the Total points axis to determine the 0.5-, 1-, and 2-year recurrence probabilities at the bottom; (B-D) Calibration of the nomogram in terms of agreement between predicted and observed 0.5-, 1-, and 2-year recurrence. Model performance is shown relative to the 45° line, representing the performance of an ideal nomogram for which the predicted outcome perfectly corresponds with the actual outcome at 1st and 2nd year. BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin; TTR, time to recurrence.

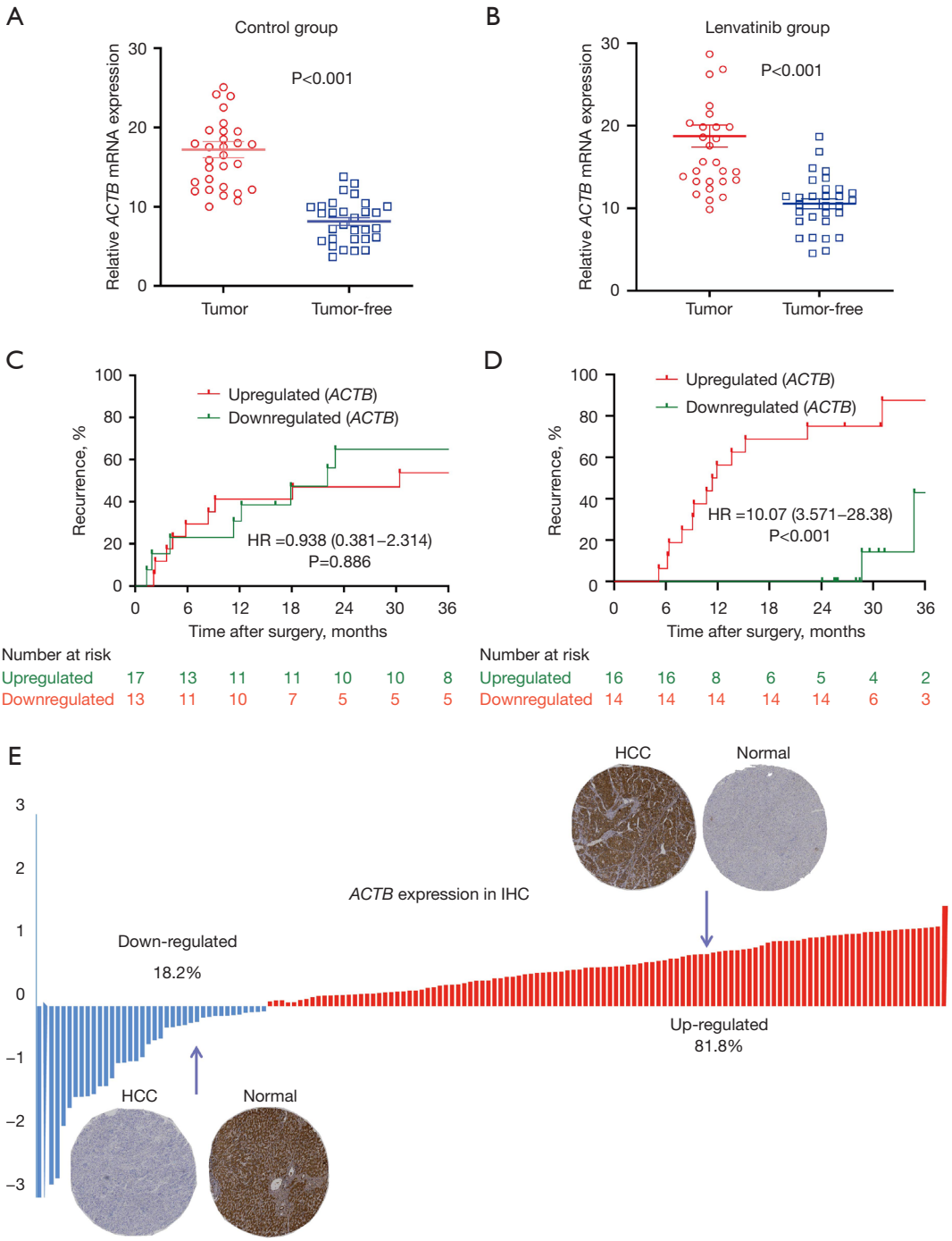
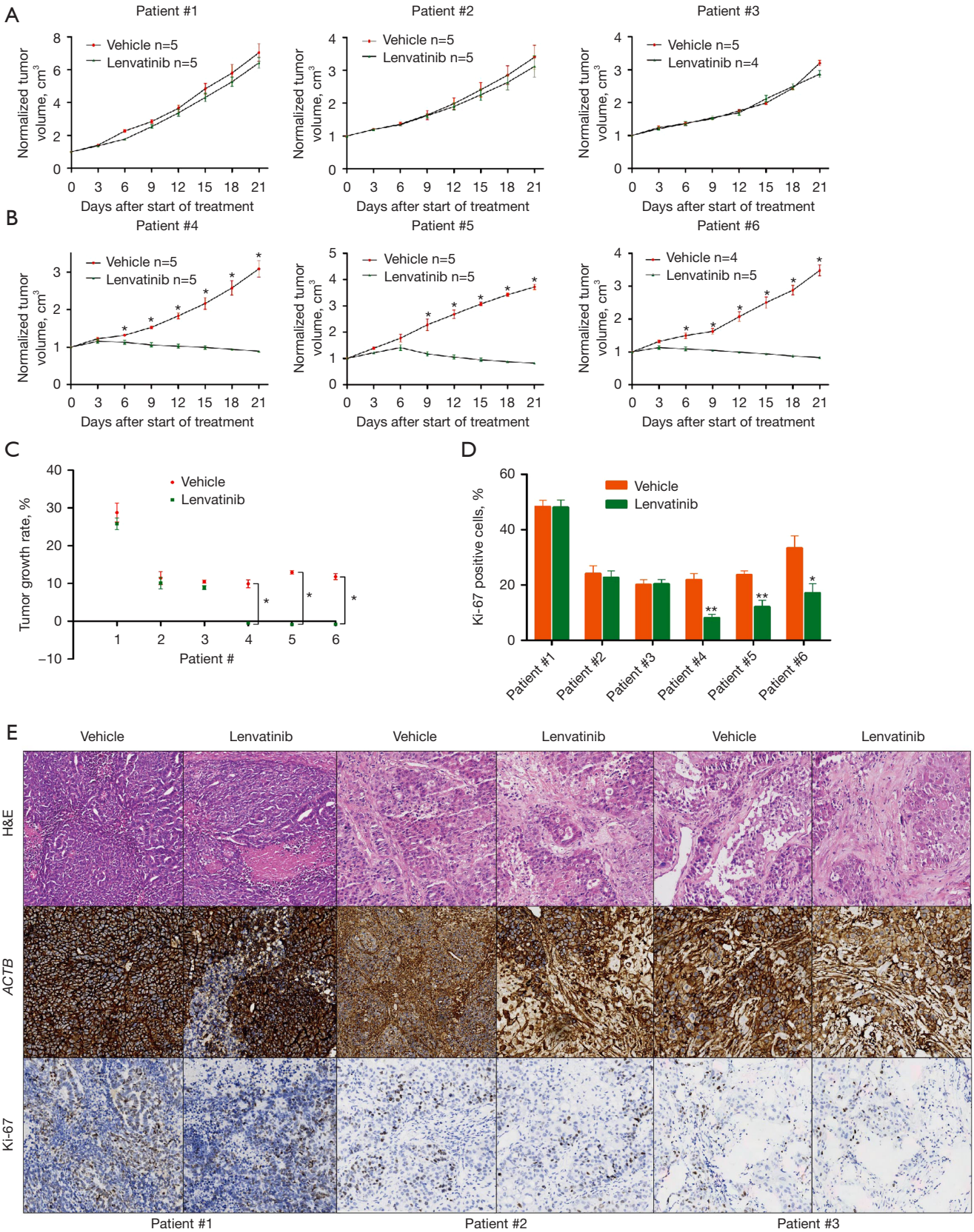


Figure 4 Elevated *ACTB* expression was observed in HCC specimens. (A,B) The expression of *ACTB* mRNA was assessed via RT-PCR in HCC and para-tumoral normal tissues from 30 patients in both control and lenvatinib groups. Compared to para-tumoral normal tissues, up-regulation of *ACTB* was observed in 81.8% of HCC cases; (C,D) the patients whose *ACTB* mRNA was downregulated in the lenvatinib group were labeled as the "Downregulated" group, while those with upregulated mRNA were labeled as the "Upregulated" group. TTR Kaplan-Meier analysis was then performed; (E) the expression of *ACTB* was analyzed in 225 patients with HCC and para-tumoral normal tissues using IHC staining, with representative images shown (×40). *ACTB*, beta-actin; HR, hazard ratio; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; RT-PCR, real-time polymerase chain reaction; TTR, time to recurrence.



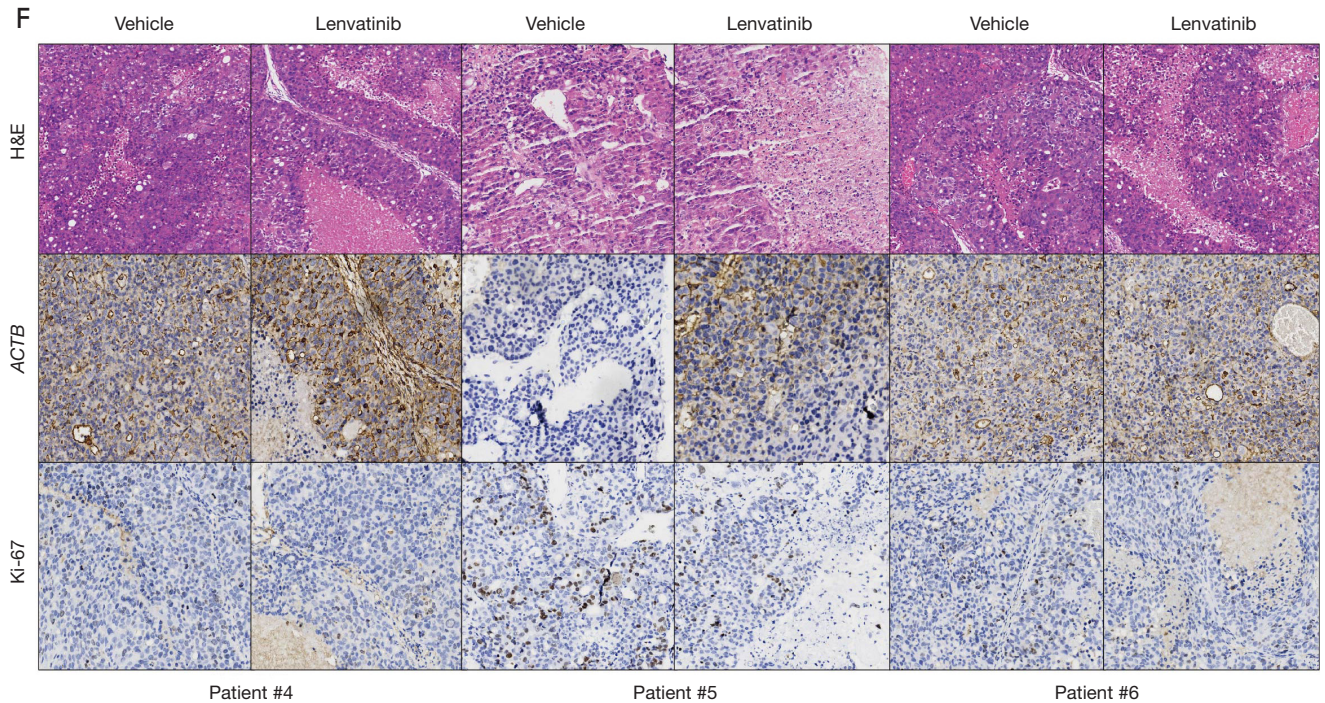


Figure 5 Low levels of *ACTB* are associated with increased sensitivity to lenvatinib in HCC PDX models. PDXs derived from the primary HCCs with high (A) or low (B) *ACTB* levels were treated with lenvatinib or vehicle for 21 days. The growth of the xenograft was monitored. The data were expressed as the mean ± SEM. (C) The tumor growth rates of PDXs with high or low levels of *ACTB* expression (Patients #1–3 and Patients #4–6, respectively) were presented alongside representative views of primary HCCs' *ACTB* expression as indicated by IHC staining. Mean ± SEM values were used to express the data, and a scale bar of 100 μm was included. (D) The proportion of the Ki-67 positive cells. *, P<0.05; **, P<0.005. (E) PDXs derived from patients with high *ACTB* levels indicated for treatment with either vehicle or lenvatinib were subjected to HE, *ACTB*, and Ki-67 staining. The views of the representatives were demonstrated (×100). (F) PDXs derived from patients with low *ACTB* levels indicated for treatment with either vehicle or lenvatinib were subjected to HE, *ACTB*, and Ki-67 staining. The views of the representatives were demonstrated (×100). HE, hematoxylin and eosin staining; *ACTB*, beta-actin; HCC, hepatocellular carcinoma; PDX, patient-derived xenograft; SEM, standard error of mean; IHC, immunohistochemistry.

from primary (HCCs of Patients #1–3, who exhibited elevated levels of *ACTB* (*Figures 5A,6A,6C,6E*). However, treatment with lenvatinib resulted in nearly complete inhibition of PDX development in tumors (Patients #4–6) exhibiting reduced *ACTB* levels, as compared to the vehicle control (*Figures 5B,6B,6D,6F*). Tumor growth rate analyses were performed on these PDXs, yielding consistent results (*Figure 5C*). Furthermore, a reduction in Ki-67 expression, which is a hallmark of proliferating cells, was only observed in PDXs obtained from tumors with low levels of *ACTB* when treated with lenvatinib (as shown in *Figure 5D-5F*). This implies that HCC patients with decreased *ACTB* expression may exhibit heightened responsiveness to lenvatinib therapy. These results additionally suggest that the levels of *ACTB* in patient tumors could serve as a valuable indicator of lenvatinib efficacy.

Discussion

This study aimed to predict HBV-related HCC recurrence post-partial hepatectomy and lenvatinib therapy. The focus was on identifying a predictive biomarker for personalized HCC treatment. In 2018, lenvatinib was approved as an alternative to Sorafenib and the first-line option for targeted therapy of advanced liver cancer. A multicenter phase III clinical trial published this year confirmed that lenvatinib combined with TACE can significantly improve the median OS of patients with advanced liver

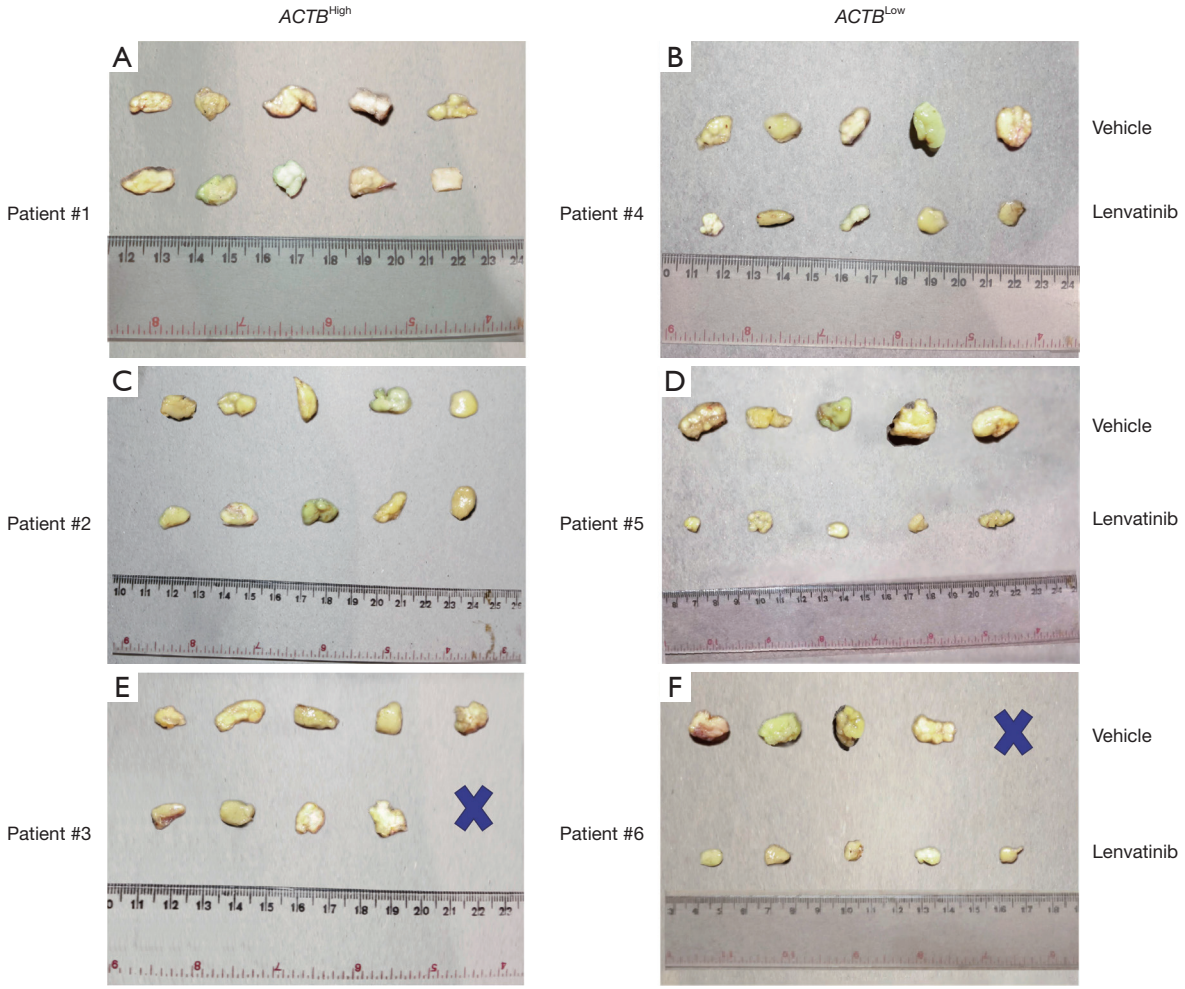


Figure 6 The expression of *ACTB* has an impact on the sensitivity of lenvatinib in HCC PDX models. (A,C,E) PDXs derived from primary HCCs exhibiting high levels of *ACTB* were subjected to treatment with lenvatinib or vehicle for a duration of 21 days; (B,D,F) PDXs derived from primary HCCs exhibiting low levels of *ACTB* were subjected to treatment with lenvatinib or vehicle for a duration of 21 days. *ACTB*, beta-actin; HCC, hepatocellular carcinoma; PDX, patient-derived xenograft.

cancer, lenvatinib combined with immune checkpoint inhibitors (ICIs) can bring survival benefits to patients with liver cancer in second-line and second-line treatment (37,38). Based on the fact that there is no clear biomarker during the use of lenvatinib, propensity score matching analyzed patients with or without lenvatinib, using *ACTB* expression levels in HCC samples. The study employed a nomogram, C-index, and calibration curve for predictive modeling. Results showed that early-stage HCC patients with lower *ACTB* levels responded better to lenvatinib therapy. Reduced *ACTB* expression correlated with favorable lenvatinib outcomes. *ACTB*, along with Child-Pugh and BCLC stage, independently predicted TTR in the lenvatinib cohort. The nomogram's predictions aligned well with actual observations. Lower *ACTB* levels in early-stage HCC patients indicate a more beneficial response to lenvatinib, suggesting its potential as a predictive biomarker for treatment stratification. Further validation in independent cohorts is essential for precision treatment in post-hepatectomy HCC patients.

Lenvatinib has been recently discovered to reduce tumor PD-L1 levels and inhibit Treg differentiation, thereby enhancing the efficacy of anti-PD-1 through FGFR4 inhibition (12,39). A stabilized inverse probability of

treatment weighting (SIPTW)-weighted multivariate Cox regression analysis showed that continuous use of lenvatinib can prolong the OS and PFS in patients with unresectable HCC (u-HCC). The duration of lenvatinib was a protective factor of OS. In addition, the Subpopulation Treatment Effect Pattern Plot (STEPP) analysis showed that with the increase of anti-PD-1 treatment cycle, the 12-month survival rate increased slowly, and patients who had been treated with anti-PD-1 for more than five cycles may benefit most from the combined treatment (40). In other research studies, lenvatinib has demonstrated its ability to inhibit FGFR1–3 signaling and reduce cancer stem-like cells in HCC (13). Nonetheless, there is currently no clinically validated biomarker available for predicting response to lenvatinib or other targeted therapies, and none of the targets of lenvatinib have been demonstrated to be useful in patient stratification or anticipating prognosis. As demonstrated by this study, the correlation between *ACTB*, a cytoskeletal structural protein, and lenvatinib response as well as patient recurrence is significant. Our research has demonstrated that low *ACTB* expression is a predictive factor for improved adjuvant lenvatinib outcomes in specific subgroups of cases, including male patients with MVI, non-cirrhosis, tumor size ≤3.5 or >3.5 cm, unimodular and BCLC stage 0/A. This indicates that this marker is only applicable to certain cases rather than all. Besides, HCC patients with low levels of *ACTB* may benefit from lenvatinib therapy. However, in patients with multinodular, regardless of their *ACTB* levels, lenvatinib did not improve outcomes. This suggests that either *ACTB* is not a predictive factor for this patient group or that lenvatinib monotherapy should not be used as first-line adjuvant therapy for advanced HCC patients after surgery.

The protein *ACTB*, identified as a highly conserved structural component of the cytoskeleton, plays a crucial role in cell growth and migration (41). According to a recent research report, *ACTB* exhibits differential expression and plays a crucial role in various human diseases, particularly cancer (10,42-45). The levels of *ACTB* protein in renal cell carcinoma cell lines and tissues are significantly elevated compared to those observed in normal controls (46). In a recent study, Braoudaki *et al.* have discovered that the *ACTB* protein plays a pivotal role in leukemia prognosis and may serve as a biomarker for distinguishing leukemia aggressiveness or inhibitor proteins in patients with high-risk acute lymphoblastic leukemia (HR-ALL). *ACTB* molecules exhibit the ability to discriminate between high- and low-risk leukemias, irrespective of their tissue origins (47). The high expression of *ACTB* alone did not correlate with a shorter TTR. However, elevated *ACTB* expression following adjuvant lenvatinib treatment post-surgery was indicative of poor therapeutic response. Furthermore, our findings suggest that *ACTB* may serve as a useful biomarker for predicting the efficacy of lenvatinib adjuvant therapy, rather than directly predicting TTR in patients after surgery. The mRNA transcript levels of *ACTB* were found to be significantly higher in HCC samples compared to those detected in para-tumoral normal tissues. While the expression of *ACTB* mRNA did not serve as a reliable predictor for TTR among postoperative patients in the control group, downregulation of *ACTB* mRNA was associated with longer TTR in the lenvatinib group. In addition, immunohistochemical staining of paired HCC and adjacent normal liver tissues demonstrated that only 18.2% of patients exhibited decreased expression of *ACTB* in their HCC specimens. The modulation of tumor cell adhesion and motility, which are critical for tumor growth and metastasis, is well known to be influenced by the cytoskeletal structure and actin microfilament system (48). The localization, polymerization, cytoskeletal organization and *ACTB* overexpression are all implicated in the promotion of colon cancer cell motility and metastasis. Differential distribution of *ACTB* in the perinuclear region and at the leading edge of cells may impact tumor cell migration plasticity and polarity, thereby influencing tumor metastasis (49). Furthermore, the upregulation and aggregation of *ACTB* in pseudopodia may facilitate tumor cell invasion (50). The expression of *ACTB* was dysregulated in various stages of HCC invasiveness and TNM classification, with ACTB mRNA 3'-UTR promoting HCC proliferation and invasion by AGO2-involved miR-1 and miR-29a degradation, thus enhancing the expression of miR-1 target gene *MET* and miR-29a target gene *MCL1* (51-53). Although the correlation between *ACTB* and tumor progression has been demonstrated in numerous studies, the impact of adjuvant therapy on *ACTB* expression levels remains unclear. This study highlights the evident advantages of lenvatinib in patients with $ACTB^{Low}$. Nevertheless, PDX models derived from $ACTB^{Low}$ patients exhibited superior responsiveness to lenvatinib adjuvant therapy compared to those derived from $ACTB^{High}$ patients. Patients with low expression of *ACTB* in the TNM I/II or BCLC A groups of HCC patients exhibit a superior

response to lenvatinib. However, in the PDX model, cases with TNM III or BCLC stage B also demonstrate favorable outcomes when *ACTB* expression is diminished. It is possible that the tumor tissue exhibiting low *ACTB* expression may be more responsive to lenvatinib, thereby resulting in a superior therapeutic outcome in the model. However, patients with advanced HCC may have a more intricate mechanism of drug resistance, resulting in limited efficacy of postoperative adjuvant therapy for lenvatinib monotherapy regardless of high or low *ACTB* expression. The drug resistance of Ranvartinib is caused by many reasons, involving many pathways, such as VEGF/VEGF receptor (VEGFR) pathway, RET signaling pathway, FGFR signaling pathway and so on. A combination of drugs is often used to overcome lenvatinib resistance in clinical practice, the combination of targeted and immune drugs, such as Ranvartinib combined with Pabolizumab, can accelerate the necrosis of tumor tissue and significantly reduce the level of tumor biomarkers such as AFP. The proportion of complete response (CR) and partial response (PR) in patients with PD-1 monoclonal antibody and Ranvartinib conversion therapy is also relatively high. (11,54). Meanwhile, biomarkers can help clinicians during treatment selection for individualized therapy, the classification of patients based on their low-*ACTB* levels could be a viable approach to enhance the response rates of lenvatinib in precision therapy for HCC.

Recent research has identified MVI, high levels of AFP, elevated HBV-DNA, multiple tumors, cirrhosis and satellite nodules as independent factors for predicting the risk of postoperative recurrence in HCC (6). A recent survey demonstrated that MVI is associated with aggressive biological features of HCC, lenvatinib can reduce postoperative recurrence and improve long-term survival in patients with HCC and MVI after curative hepatectomy, especially in cases with microvascular invasion where there is a higher risk of tumor spread (55). In our research, it was innovatively found that patients with HBV-related HCC used lenvatinib after radical surgery. Among people with microvascular invasion, the $ACTB^{low}$ group often had a better long-term prognosis. Therefore, we suspect that MVI and *ACTB* may jointly predict the long-term survival rate of patients who use lenvatinib after operation. The natural history of human HBV infection varies greatly among different genotypes. This variability includes: the main mode of transmission, the time of hepatitis B early antibody (HBeAg) seroconversion, the speed and severity of liver disease progression (including the possibility of progression to HCC and serological clearance of HBsAg), and the diversity of HBV can lead to differences in the natural history, disease progression and treatment response of patients with chronic infection, especially in the treatment of IFN-α. A study on patients with chronic hepatitis B treated with tenofovir showed that HBsAg was almost eliminated. This is caused by HBV's unique replication cycle and cell host factors. The mechanism of HBV-induced liver cancer usually includes direct induction of carcinogenic signal pathway, induction of chronic inflammation and subsequent cirrhosis. Therefore, the diversity of viruses must be considered when developing new treatment schemes (56). We further stratified cases without cirrhosis and found that patients with low levels of inflammatory fibrosis had longer TTR than patients with high levels and cirrhosis both in lenvatinib and control group (Figure S2A,S2B). The analyses also revealed no discernible differences in case composition between matched and original cases, stratified by baseline parameters. The implementation of postoperative adjuvant therapy represents a novel approach to reducing recurrence. According to a multicenter investigation, lenvatinib can be safely and effectively administered in patients with HCC regardless of their age (57). A retrospective exploratory analysis of the REFLECT trial revealed that patients who achieved an ORR demonstrated a significantly prolonged OS. It has been reported that a distinct trend in favor of lenvatinib over sorafenib (HR =0.82; 95% CI: 0.60–1.15) was observed among HBV-positive patients, indicating that lenvatinib may be the optimal treatment option for this patient population in approximately 59% of cases compared to only 1% of patients who received sorafenib therapy (58). The cost-utility analysis demonstrated that lenvatinib exhibited comparable clinical efficacy to sorafenib at a lower cost, suggesting that it may represent a cost-effective option for the treatment of u-HCC (59,60). The correlation between *ACTB* and objective radiological response was not established in this study. However, we observed a significant prolongation in recurrence time among adjuvant lenvatinib-treated $ACTB^{Low}$ patients. Additionally, our findings suggest that lenvatinib effectively suppresses the subsequent growth of $ACTB^{Low}$ PDXs rather than completely eradicating them. To minimize variations in tumor tissue characteristics, we endeavor to procure patient samples with consistent tissue structure types. Specifically, the HCC tissues under investigation exhibit a thick trabecular pattern and moderate

differentiation. The aforementioned findings underscore the potential therapeutic efficacy of *ACTB*. Besides, the recurrence benefit observed in $ACTB^{Low}$ HCC patients may be attributed to lenvatinib's ability to inhibit tumor growth and prevent HCC progression. Through Cox regression analysis, it was determined that Child-Pugh stage, BCLC and *ACTB* levels serve as independent risk factors, which have also been incorporated in this nomogram. Our proposed nomogram can efficiently predict the recurrence of HCC patients treated with lenvatinib post-surgery quite accurately.

There are certain limitations to the current study. Firstly, the decision-making process for Chinese patients regarding adjuvant treatment with lenvatinib in HCC setting is intricate. In addition to the doctor's prescription, patient compliance is also influenced by various factors such as the cost of lenvatinib therapy. To be candid, this was an observational study, thus the participants were recruited retrospectively and there was limited information available regarding the specific reasons for patients' receipt or non-receipt of lenvatinib treatment. Secondly, as lenvatinib has only been recently administered to HCC patients, we have solely obtained information on recurrence and not yet acquired data on OS of patients. Therefore, our study can only focus on the analysis of recurrence. Thirdly, the cohort consisted of patients with prior HBV infection while those with concurrent HCV infection were excluded from the study. Given the significant role of HCV in hepatocarcinogenesis, particularly in Western populations, it remains uncertain whether this biomarker is applicable to individuals of Western descent. Fourthly, the lenvatinib trial excluded patients who did not receive concurrent adjuvant therapy, and the control group was unable to select concurrent patients for comparison with the lenvatinib group. Finally, it should be noted that this study solely relies on a primary cohort and lacks a validation cohort, which may potentially introduce some limitations to the findings.

Conclusions

In summary, our study has demonstrated the predictive role of *ACTB* in identifying patients with high recurrence risk HCC who are likely to benefit from lenvatinib treatment. We propose that reduced levels of *ACTB* could serve as a novel and viable biomarker for personalized therapy with lenvatinib. To our knowledge, this is the first study to establish a correlation between a lenvatinib biomarker and its response to adjuvant therapy in early-stage HBV-related HCC. This has significant implications for personalized treatment with lenvatinib.

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-942/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-942/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-942/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-942/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional ethics board of Eastern Hepatobiliary Surgery Hospital (No. EHBHKY2023-K036-P001) and informed consent was obtained from all individual participants.

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Supplementary

Table S1 Baseline characteristics of total patients with HCC

Characteristics	Subgroup	Lenvatinib (n=76)	Control (n=180)	P value[†]
Gender	Male	59	153	0.153
	Female	17	27	
Age (years)	≤50	26	76	0.232
	>50	50	104	
MVI	Yes	48	81	0.008
	No	28	99	
Tumor size (cm)	≤3.5	22	46	0.575
	>3.5	54	134	
Tumor number	Uninodular	54	120	0.491
	Multinodular	22	60	
HBV-DNA	<1,000	27	52	0.320
	≥1,000	49	126	
Cirrhosis	Yes	34	116	0.003
	No	42	64	
BCLC	0/A	49	121	0.671
	B/C	27	59	
ACTB	High	33	48	0.016
	Low	42	122	

[†], P values were calculated using Chi-squared test. HCC, hepatocellular carcinoma; MVI, macroscopic vascular invasion; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin.

Table S2 Baseline characteristics of patients matched by propensity score

Characteristics	Subgroup	Lenvatinib (n=61)	Control (n=164)	P value[†]
Gender	Male	47	140	0.139
	Female	14	24	
Age (years)	≤50	25	72	0.694
	>50	36	92	
MVI	Yes	33	62	0.028
	No	28	102	
Tumor size (cm)	≤3.5	14	60	0.053
	>3.5	47	104	
Tumor number	Uninodular	45	111	0.379
	Multinodular	16	53	
HBV-DNA	<1,000	34	48	<0.001
	≥1,000	27	116	
Cirrhosis	Yes	33	103	0.235
	No	28	61	
BCLC	0/A	48	146	0.046
	B/C	13	18	
ACTB	High	20	46	0.488
	Low	41	118	

[†], P values were calculated using Chi-squared test. MVI, macroscopic vascular invasion; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin.

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Table S3 Clinical characteristics of patients whose primary HCCs were utilized as PDX models

Variables	Patient #1	Patient #2	Patient #3	Patient #4	Patient #5	Patient #6
Gender	Female	Male	Male	Female	Male	Female
Age (years)	51	71	53	58	38	65
HBsAg	Positive	Positive	Positive	Positive	Positive	Positive
AFP (μg/L)	≤400	≤400	≤400	>400	>400	≤400
Satellite	No	Yes	No	No	Yes	Yes
MVI	No	No	No	Yes	No	Yes
Tumor size (cm)	>3.5	>3.5	>3.5	≤3.5	>3.5	>3.5
Single or multiple	Single	Single	Multiple	Multiple	Multiple	Multiple
BCLC	A	A	A	A	B	A
TNM	I	I	II	II	III	II
Child-Pugh	A	A	A	A	A	A
ACTB expression[†]	High	High	High	Low	Low	Low
ACTB expression (tumor *vs.* normal)[‡]	Up-regulated	Up-regulated	Up-regulated	Down-regulated	Down-regulated	Down-regulated

[†], *ACTB* expression in the primary HCCs was analyzed by IHC staining, the cutoff point of IHC staining density score to separate *ACTB* high and low HCCs was 88.4; [‡], the expression of *ACTB* in HCC and para-tumoral normal tissues was examined by IHC staining. HCC, hepatocellular carcinoma; PDX, patient-derived xenograft; HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; MVI, macroscopic vascular invasion; BCLC, Barcelona Clinic Liver Cancer Staging; TNM, Tumor-Nodes-Metastasis; *ACTB*, beta-actin; IHC, immunohistochemistry.

Table S4 Univariate and multivariate analysis of TTR of matched patients

Variables	Subgroup	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value[†]
Gender	Female vs. male	0.859 (0.543–1.359)	0.517	–	–
Age (years)	≤50 vs. >50	0.937 (0.665–1.319)	0.708	–	–
Tumor size (cm)	>3.5 vs. ≤3.5	1.185 (0.819–1.713)	0.367	–	–
Tumor number	Multinodular vs. uninodular	1.220 (0.847–1.755)	0.285	–	–
MVI	Yes vs. no	1.133 (0.799–1.605)	0.485	–	–
AFP (μg/L)	>400 vs. ≤400	1.819 (1.281–2.584)	0.001	1.850 (1.295–2.643)	0.001
Child-Pugh	A vs. B	0.925 (0.432–1.980)	0.840	–	–
TNM	III/IV vs. I/II	1.520 (0.995–2.321)	0.053	–	–
BCLC	B/C vs. 0/A	1.274 (0.805–2.015)	0.301	–	–
Treatment	Lenvatinib vs. control	0.638 (0.419–0.972)	0.037	0.597 (0.391–0.913)	0.017
ACTB	High vs. low	1.590 (1.107–2.283)	0.012	1.463 (1.016–2.107)	0.041

[†], P values were calculated using Chi-squared test. TTR, time to recurrence; MVI, macroscopic vascular invasion; AFP, α-fetoprotein; TNM, Tumor-Nodes-Metastasis; BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin; HR, hazard ratio; CI, confidence interval.

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Table S5 The HBV infection status and utilization of antiviral drugs among patients with HBV-related HCC

Characteristics	Subgroup	Lenvatinib group (n=61)	Control group (n=164)	P value†
HBsAg	Positive	61	164	NA
	Negative	0	0	
HBcAb	Positive	60	90	<0.001
	Negative	1	74	
HBeAg	Positive	25	76	0.473
	Negative	36	88	
HBV-DNA (IU/mL)	<1,000	34	48	<0.001
	≥1,000	27	116	
Anti-HBV treatment	Entecavir	34	79	0.531
	Tenofovir disoproxil	15	52	
	Tenofovir alafenamide	12	33	

†, P values were calculated using Chi-squared test. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBsAg, Hepatitis B surface antigen; HBcAb, hepatitis B core antibody; HBeAg, hepatitis B early antibody; NA, not available.

Table S6 Baseline characteristics of patients using lenvatinib after partial hepatectomy

Characteristics	Subgroup	Low ABCT (n=41)	High ABCT (n=20)	P value†
Gender	Male	10	4	>0.99
	Female	31	16	
Age (years)	≤50	18	7	0.586
	>50	23	13	
MVI	Yes	24	9	0.414
	No	17	11	
Child-Pugh	A	39	19	>0.99
	B	2	1	
Tumor size (cm)	≤3.5	9	5	>0.99
	>3.5	32	15	
Tumor number	Uninodular	30	15	>0.99
	Multinodular	11	5	
BCLC	0/A	33	15	0.741
	B/C	8	5	

†, P values were calculated using Chi-squared test. MVI, macroscopic vascular invasion; BCLC, Barcelona Clinic Liver Cancer Staging; *ACTB*, beta-actin.

Table S7 Numeric results for the log rank test in terms of sample size

Variables	Values
Power	0.8015
N1 (lenvatinib group)	43
N2 (control group)	130
N (total)	173
HR	0.556
Ctrl Med TTR time (M1) (months)	17.3
Trt Med TTR time (M2) (months)	31.1
Ctrl loss	0
Trt loss	0
Ctrl to Trt	0
Trt to Ctrl	0
Alpha	0.05
Beta	0.1989

HR, hazard ratio; Ctrl, control; Med, media; TTR, time to recurrence; Trt, treatment.

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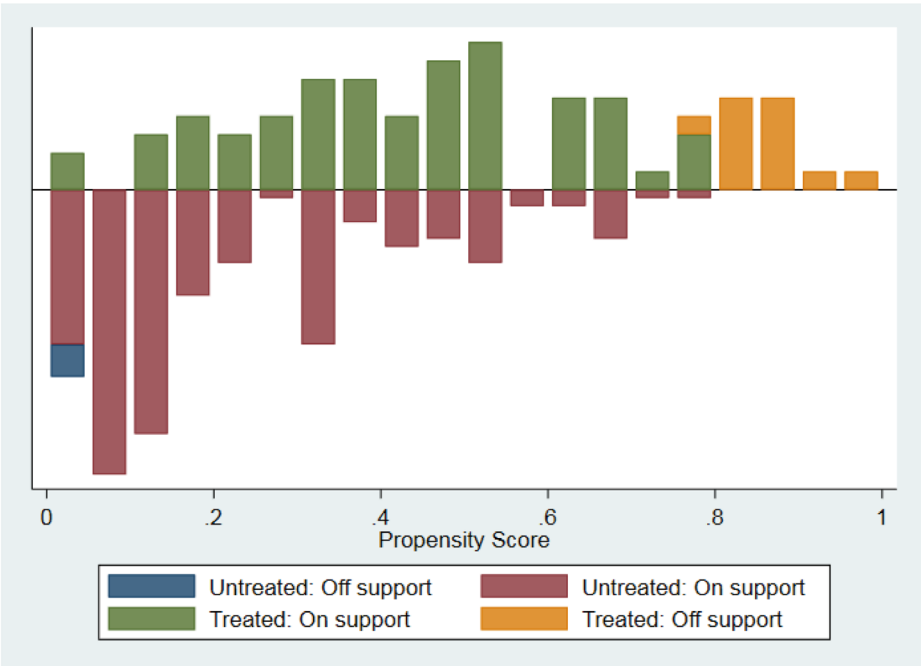
A

Variable	Sample	Lenvatinib Group	Control Group	Difference	S.E.	T-stat	p value
TTR	Total	21.021	21.293	-0.272	2.378	-0.11	0.912
	Matched	21.005	19.468	1.537	2.767	0.56	0.576

B



C

Variable	Mean			t-test	
	Lenvatinib	Control	% bias	t	[a]p>|t|
Gender	0.230	0.179	13.0	0.69	0.493
Age(year)	0.590	0.609	-3.9	-0.21	0.831
MVI	0.541	0.561	-4.2	-0.23	0.822
Tumor size(cm)	0.770	0.762	1.9	0.11	0.916
Tumor Number	0.262	0.240	4.7	0.28	0.783
HBV-DNA (IU/mL)	0.443	0.425	3.8	0.20	0.845
Cirrhosis	0.459	0.549	-18.4	-0.99	0.323
BCLC	0.213	0.213	0.0	0.00	1.000
ACTB	0.213	0.254	-9.1	-0.53	0.596

Figure S1 The experimental outcomes of patients pre- and post-matching. (A) The effects of patient matching on experimental outcomes; (B) the propensity score of patients; (C) the hypothesis test for the balance of variables pre- and post-matching in baseline characteristics of patients with HCC. [a], P values were calculated using *t*-test. TTR, time to recurrence; S.E., standard error; MVI, macroscopic vascular invasion; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer Staging; ACTB, beta-actin.

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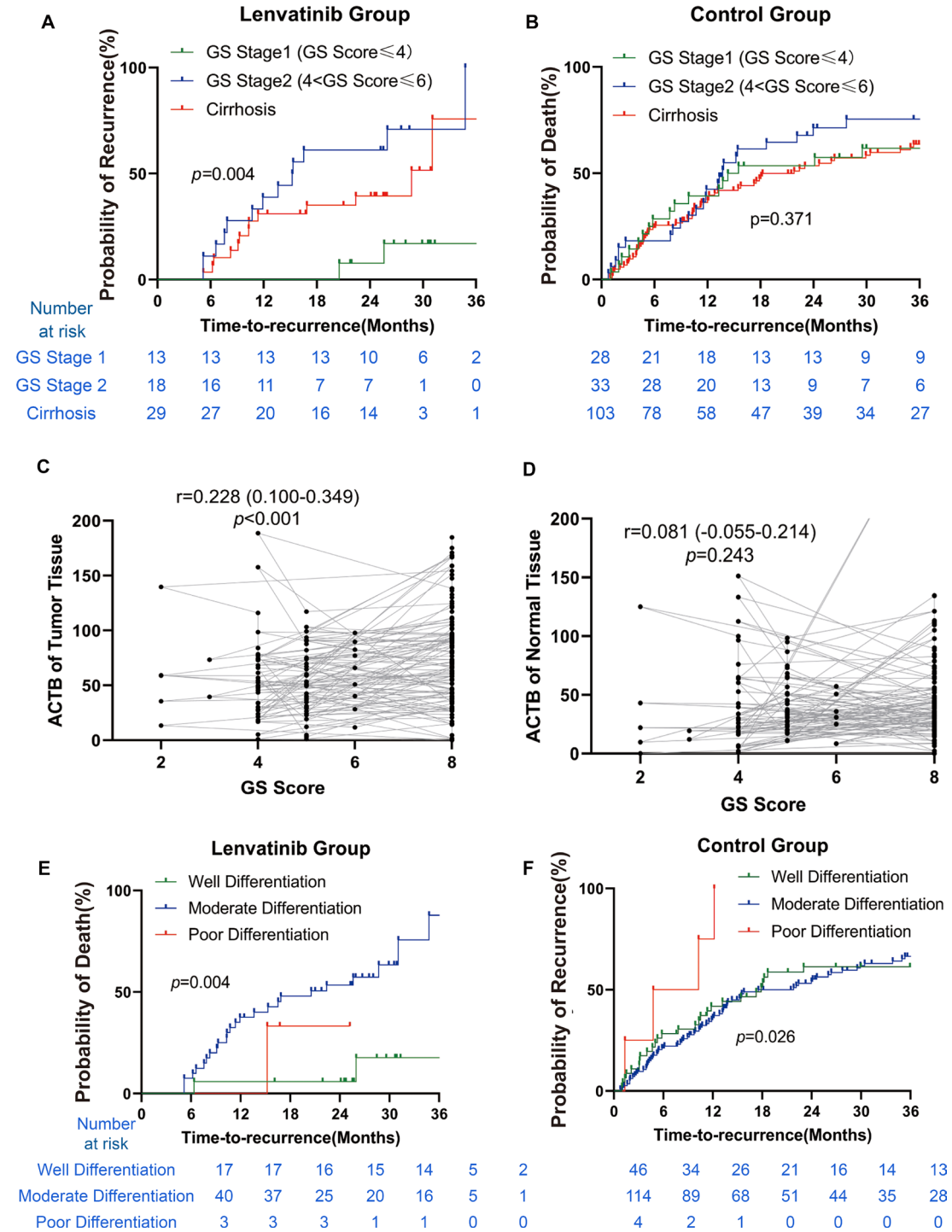

Figure S2 Analysis of hepatic inflammation, fibrosis and histological differentiation in patients. (A,B) The TTR were compared between the different stages of hepatic inflammation, fibrosis, or cirrhosis in lenvatinib and control groups; (C,D) correlation between *ACTB* expression and GS score in tumor or tumor tissues; (E,F) the TTR were compared between the different stages of histological differentiation in lenvatinib and control group. GS, Glasgow University Score for Liver Pathology staging system; *ACTB*, beta-actin.

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Table S8 Hepatic inflammation, fibrosis, and histological differentiation of patients.

Characteristics	Subgroup	Lenvatinib group (n=61)	Control group (n=164)	P value[†]
Inflammation and fibrosis staging (GS system)	G1S1	2	5	0.264
	G1S2	2	3	
	G2S2	13	22	
	G2S3	9	14	
	G3S2	4	6	
	G3S3	2	2	
	Cirrhosis	29	112	
Histological differentiation	Well	11	44	0.385
	Moderate	48	116	
	Poor	2	4	

[†], P values were calculated using Chi-squared test. GS, Glasgow University Score for Liver Pathology staging system.

Table S9 Correlations between *ACTB* and patient characteristics in patients.

Characteristics	Subgroup	*ACTB* low (n=159)	*ACTB* high (n=66)	P value[†]
Gender	Male	133	54	0.739
	Female	26	12	
Age (years)	≤50	70	27	0.667
	>50	89	39	
MVI	Yes	67	28	0.968
	No	92	38	
Tumor size (cm)	≤3.5	51	23	0.687
	>3.5	108	43	
Tumor number	Uninodular	111	45	0.809
	Multinodular	48	21	
HBV-DNA	<1,000	66	16	0.014
	≥1,000	93	50	
Cirrhosis	Yes	80	51	<0.001
	No	79	15	
BCLC	0/A	141	53	0.097
	B/C	18	13	

[†], P values were calculated using Chi-squared test. *ACTB*, beta-actin; MVI, macroscopic vascular invasion; HBV, hepatitis B virus; BCLC, Barcelona Clinic Liver Cancer Staging.

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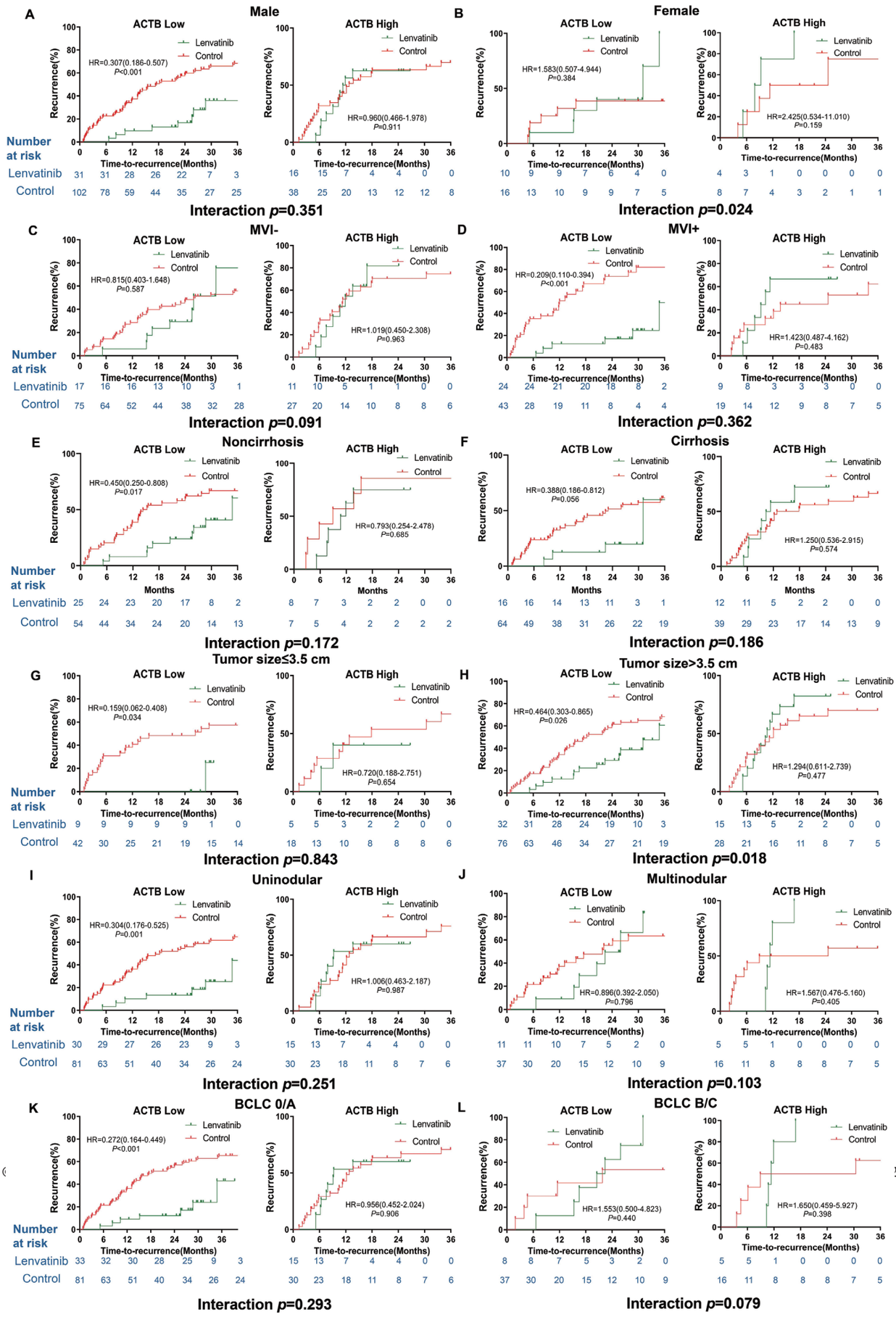

Figure S3 The predictive value of *ACTB* in stratifying patients by prognostic factors for lenvatinib benefit. (A,B) The TTR of male and female patients, stratified by *ACTB* levels, was compared between the lenvatinib and control groups using Kaplan-Meier analysis; (C,D) the TTR of patients with and without MVI, stratified by *ACTB* levels, was compared using Kaplan-Meier analysis between the lenvatinib and control groups; (E,F) the TTR of patients' non-cirrhosis and cirrhosis stratified by *ACTB* levels, was compared using Kaplan-Meier analysis between the lenvatinib and control groups; (G,H) the TTR of patients' tumor size is small and large stratified by *ACTB* levels, was compared using Kaplan-Meier analysis between the lenvatinib and control groups; (I,J) the TTR of patients uninodular and multinodular tumors stratified by *ACTB* levels, was compared using Kaplan-Meier analysis between the lenvatinib and control groups; (K,L) the TTR of patients BCLC 0/A and BCLC B/C stratified by *ACTB* levels, was compared using Kaplan-Meier analysis between the lenvatinib and control groups. The P value of interaction between treatment and *ACTB* levels was shown below. *ACTB*, beta-actin; HR, hazard ratio; MVI, macroscopic vascular invasion; BCLC, Barcelona Clinic Liver Cancer Staging; TTR, time to recurrence.

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