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Research Article

Role of the PI3KAKTmTOR Pathway in Hepatocellular Carcinoma Progression and Drug Resistance

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ABSTRACT

Background
Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer-related mortality worldwide, with limited effective systemic treatments and a high incidence of therapeutic resistance. Increasing evidence 71implicates dysregulation of the PI3K/AKT/mTOR signaling pathway as a critical driver of tumor progression, metabolic reprogramming, and drug resistance in HCC. However, data from Eastern European populations remain scarce. This study aimed to evaluate the expression and activation status of PI3K/AKT/mTOR pathway components in Romanian HCC patients and assess their association with clinicopathologic features, survival, and resistance to systemic therapy.

Methods
A prospective observational study was conducted on 92 patients with histologically confirmed HCC treated at the Fundeni Clinical Institute (2021–2024). Tumor and matched non-tumor liver tissues were analyzed by immunohistochemistry (IHC) and Western blot for PI3K, phospho-AKT (Ser473), phospho-mTOR (Ser2448), and PTEN. Quantitative H-scores and densitometric analyses were correlated with clinicopathologic variables and patient outcomes. Drug resistance markers (MDR1, BCL-2) were assessed in patients receiving sorafenib or lenvatinib. Survival analyses were performed using Kaplan–Meier and Cox proportional hazards models.

Results
Strong activation of the PI3K/AKT/mTOR pathway was observed in tumor tissue compared with adjacent liver ($p < 0.001$). Mean H-scores were significantly higher for p-AKT (173 ± 39 vs. 65 ± 25) and p-mTOR (167 ± 35 vs. 59 ± 23), while PTEN expression was reduced (72 ± 30 vs. 188 ± 41; $p < 0.001$).

High p-AKT and p-mTOR expression correlated with tumor size >5 cm, microvascular invasion, high AFP levels, and advanced BCLC stage ($p < 0.01$). Among patients treated with systemic therapy ($n = 38$), 55.3% developed drug resistance within six months, showing increased p-AKT, p-mTOR, and MDR1/BCL-2 expression ($p < 0.001$). In multivariate analysis, high p-AKT (HR 2.47, 95% CI 1.33–4.59), high p-mTOR (HR 2.16, 95% CI 1.12–3.98), PTEN loss (HR 1.82, 95% CI 1.03–3.21), and sorafenib resistance (HR 2.59, 95% CI 1.41–4.23) were independent predictors of poor overall survival. Median overall survival was 15.6 months in patients with high p-AKT expression versus 30.3 months in low-expression cases ($p = 0.002$).

Conclusions

Aberrant activation of the PI3K/AKT/mTOR pathway is a major molecular event driving tumor aggressiveness and therapeutic resistance in Romanian hepatocellular carcinoma. Overexpression of p-AKT and p-mTOR and loss of PTEN are independently associated with poor prognosis and early resistance to multikinase inhibitors. These results underscore the potential clinical utility of incorporating PI3K/AKT/mTOR biomarkers into diagnostic and therapeutic stratification algorithms for HCC. Targeted inhibition of this pathway, alone or combined with MAPK or immune checkpoint blockade, represents a promising strategy for overcoming drug resistance and improving survival outcomes.

Keywords: Hepatocellular carcinoma; PI3K/AKT/mTOR; PTEN; Sorafenib resistance; Lenvatinib; Biomarker; Survival

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the most common primary malignancy of the liver, accounting for nearly 85–90% of all liver cancers worldwide and standing as the third leading cause of cancer-related mortality [1]. Globally, over 900,000 new cases are diagnosed annually, with more than 830,000 deaths reported in 2023 [2]. The incidence of HCC continues to rise, particularly in regions such as Eastern Europe, Southeast Asia, and Sub-Saharan Africa, driven by persistent hepatitis B and C virus infections, alcohol-related liver disease, and the growing burden of non-alcoholic steatohepatitis (NASH) [3,4].

In Romania, HCC incidence has increased steadily over the past two decades due to high rates of chronic hepatitis C and metabolic syndrome, with a significant proportion of cases being diagnosed at advanced stages [5]. Despite improvements in early diagnosis and therapeutic modalities—such as surgical resection, ablation, transarterial chemoembolization (TACE), and targeted therapies—the overall five-year survival rate remains below 20% [6]. This poor prognosis reflects both the biological aggressiveness of HCC and the complex molecular mechanisms underlying tumor progression and therapeutic resistance. Among the multitude of signaling cascades implicated in hepatocarcinogenesis, the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway has emerged as a central regulatory axis influencing tumor initiation, growth, angiogenesis, and survival [7].

Its persistent activation is considered a molecular hallmark of HCC, directly associated with tumor aggressiveness, metastasis, and drug resistance.

The PI3K/AKT/mTOR signaling cascade is a highly conserved intracellular pathway that integrates extracellular growth signals to regulate metabolism, proliferation, and apoptosis. Upon stimulation by growth factors (e.g., EGF, IGF, PDGF), cytokines, or oncogenic mutations, PI3K (phosphatidylinositol 3-kinase) catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) [8]. PIP3 recruits and activates AKT (also known as protein kinase B) through its pleckstrin homology (PH) domain, leading to the phosphorylation of a wide array of downstream substrates involved in cell growth, survival, and motility [9].

Activated AKT subsequently phosphorylates TSC2 (tuberous sclerosis complex 2), thereby inhibiting the TSC1–TSC2 complex and leading to the activation of mTOR complex 1 (mTORC1). mTORC1 regulates key translational proteins, such as p70S6 kinase (S6K) and 4E-binding protein 1 (4E-BP1), promoting protein synthesis, ribosomal biogenesis, and metabolic reprogramming [10].

In parallel, mTOR complex 2 (mTORC2) directly phosphorylates AKT at Ser473, creating a positive feedback loop that further amplifies signaling [11].

Dysregulation of this pathway occurs through multiple mechanisms in HCC:

- Activating mutations or amplification of *PIK3CA* (encoding PI3K catalytic subunit p110α);
- Loss or mutation of PTEN, a tumor suppressor that dephosphorylates PIP3;
- Overexpression of receptor tyrosine kinases (RTKs) such as EGFR or IGF-1R; and
- Mutations or epigenetic silencing of negative regulators such as TSC1/2 or LKB1 [12,13].

These alterations culminate in constitutive pathway activation, driving uncontrolled hepatocyte proliferation, inhibition of apoptosis, and enhanced metastatic potential.

3. PI3K/AKT/mTOR Signaling in Hepatocarcinogenesis

Numerous experimental and clinical studies have demonstrated aberrant PI3K/AKT/mTOR signaling in HCC tissues compared to adjacent non-tumorous liver [14]. Overexpression of phosphorylated AKT (p-AKT) and mTOR correlates with larger tumor size, microvascular invasion, and higher histologic grade [15]. Activation of mTOR promotes angiogenesis via VEGF induction, epithelial–mesenchymal transition (EMT) through Snail and Twist upregulation, and metabolic reprogramming favoring glycolysis and lipogenesis [16]. Interestingly, the pathway interacts extensively with other oncogenic networks, including Ras/Raf/MEK/ERK, Wnt/β-catenin, and TGF-β signaling [17]. This cross-talk facilitates tumor adaptability and contributes to the intratumoral heterogeneity observed in advanced HCC. Recent genomic profiling has revealed *PIK3CA* mutations in approximately 20–25% of HCC cases, while *PTEN* loss occurs in up to 45%, underscoring the high prevalence of this pathway's deregulation [18]. Such findings make the PI3K/AKT/mTOR axis an attractive therapeutic target and a prognostic biomarker for HCC.

Although targeted agents such as sorafenib, lenvatinib, and regorafenib have improved outcomes for advanced HCC, acquired drug resistance remains a major obstacle [19]. A growing body of evidence indicates that upregulation of the PI3K/AKT/mTOR pathway represents a critical mechanism underlying resistance to multikinase inhibitors and immune checkpoint blockade [20].

Specifically, long-term exposure to sorafenib induces compensatory activation of AKT and mTOR signaling, allowing tumor cells to bypass RAF inhibition and maintain proliferative capacity [21]. Similarly, resistance to lenvatinib and cabozantinib has been associated with phosphorylation of AKT and S6K, which sustain downstream survival signals even under kinase inhibition [22].

Furthermore, activation of mTOR promotes autophagy suppression and anti-apoptotic protein synthesis, reducing sensitivity to chemotherapy and immune-mediated cytotoxicity [23]. Mechanistically, these adaptive responses arise from feedback activation of upstream RTKs and epigenetic remodeling that enhance PI3K/AKT signaling. In addition, cancer stem-like cells (CSCs) in HCC depend heavily on this pathway for self-renewal and drug tolerance, leading to relapse after treatment cessation [24]. Inhibition of PI3K or mTOR re-sensitizes resistant HCC cells to sorafenib in preclinical models, suggesting a potential benefit from combination therapy strategies [25].

Several inhibitors targeting the PI3K/AKT/mTOR pathway are under clinical evaluation for HCC management.

- Everolimus (RAD001), an mTOR inhibitor, showed limited efficacy as monotherapy in the EVOLVE-1 trial due to compensatory feedback activation of AKT [26].
- Dual PI3K/mTOR inhibitors such as BEZ235 and BKM120 have demonstrated synergistic effects when combined with sorafenib or radiotherapy in experimental studies [27].
- New-generation AKT inhibitors (e.g., capivasertib, ipatasertib) are being explored in early-phase trials for refractory HCC [28].

Despite promising laboratory results, translation to clinical benefit remains challenging due to dose-limiting toxicities, pathway redundancy, and lack of predictive biomarkers. Moreover, tumor heterogeneity and coexistence of multiple oncogenic drivers complicate targeted therapy responses [29]. Recent efforts focus on integrating molecular profiling into personalized treatment strategies, wherein patients with *PIK3CA* mutations or *PTEN* loss could be selected for pathway-specific interventions [30]. Given the high incidence of HCC and limited therapeutic success in Romania and other Eastern European countries, there is a pressing need to elucidate molecular mechanisms underlying tumor progression and treatment resistance. While international studies have characterized PI3K/AKT/mTOR alterations in HCC, regional data remain scarce, particularly regarding their clinical correlations and prognostic significance in Romanian patients.

Understanding the activation status of this pathway may not only shed light on tumor biology and aggressiveness but also support the identification of molecular biomarkers predictive of drug response. Furthermore, clarifying how PI3K/AKT/mTOR modulation contributes to therapeutic resistance could guide the rational design of combination regimens, integrating kinase inhibitors, mTOR blockers, and immunotherapies. Therefore, this study aims to investigate the expression and activation of key components of the PI3K/AKT/mTOR pathway in Romanian HCC patients, and to evaluate their association with clinical outcomes and drug resistance patterns.

Such insights could foster the development of precision oncology approaches in the management of hepatocellular carcinoma in Eastern Europe.

METHODS

Study Design and Ethical Approval

This study was designed as a prospective observational cohort investigating the expression of the PI3K/AKT/mTOR signaling pathway in hepatocellular carcinoma (HCC) tissue samples and its correlation with clinical outcomes and drug resistance. The research was conducted at the Department of Gastroenterology and Hepatology, Fundeni Clinical Institute, in collaboration with the Department of Molecular Biology, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania, between January 2021 and December 2024.

Ethical approval was obtained from the Institutional Review Board of "Carol Davila" University of Medicine and Pharmacy (Approval No. *CDUMF-HEP/2021-067*) and the **National Bioethics Committee for Biomedical Research (NBCB-Romania/2021-0412)**.

All participants provided written informed consent prior to enrollment. The study adhered to the principles of the Declaration of Helsinki (2013) and complied with European Union Regulation (EU) 2016/679 (GDPR) for patient data protection.

Patient Cohort and Clinical Data Collection

A total of 92 consecutive patients with histologically confirmed hepatocellular carcinoma (HCC) were recruited.

Inclusion criteria were:

- Adult patients (≥18 years) diagnosed with HCC according to EASL-EORTC 2018 guidelines.
- No prior systemic chemotherapy or targeted therapy before tissue sampling.
- Availability of adequate tumor and adjacent non-tumorous liver tissue for analysis.

Exclusion criteria included:

- Secondary liver malignancies or mixed cholangiocarcinoma.
- Previous liver transplantation or palliative radiotherapy.
- Insufficient tissue for molecular or immunohistochemical studies.

Clinical data were extracted from electronic medical records and included:

- Demographic characteristics (age, sex).
- Underlying liver disease etiology (HBV, HCV, alcohol, NASH).
- Serum alpha-fetoprotein (AFP) levels.
- Tumor characteristics (size, number, vascular invasion, TNM stage).
- Type of treatment (surgical resection, TACE, sorafenib, lenvatinib).
- Clinical outcomes (recurrence-free and overall survival).

Patients were followed up for a median duration of 26 months (range 8–42 months) after diagnosis. Treatment response was assessed according to RECIST 1.1 criteria.

Tissue Sampling and Processing

Tumor and adjacent non-tumor liver tissues (≥2 cm from the tumor margin) were collected during surgical resection or core needle biopsy.

Each sample was divided into two parts:

- Formalin-fixed, paraffin-embedded (FFPE) for histology and immunohistochemistry.
- Fresh-frozen (−80°C) for RNA, protein extraction, and Western blot analysis.

Histopathologic diagnosis and grading were confirmed independently by two senior pathologists according to WHO classification (2019).

Immunohistochemistry (IHC)

Reagents and Antibodies

Sections (4 µm thick) from FFPE blocks were deparaffinized and rehydrated. Antigen retrieval was performed in citrate buffer (pH 6.0, 20 min at 95°C).

Primary antibodies and dilutions used were:

- PI3K p110α (Abcam, ab40776, 1:100)
- Phospho-AKT (Ser473) (Cell Signaling Technology, #4060, 1:200)
- Phospho-mTOR (Ser2448) (Cell Signaling Technology, #2976, 1:150)
- p-S6K (Thr389) (Abcam, ab32529, 1:100)
- PTEN (Abcam, ab32199, 1:150)

Incubation was done overnight at 4°C, followed by HRP-conjugated secondary antibody (Dako EnVision System).

Chromogenic detection was performed using DAB (3,3'-diaminobenzidine), and slides were counterstained with hematoxylin.

Evaluation of Immunostaining

Immunoreactivity was evaluated semi-quantitatively by two blinded pathologists. The H-score system was used:

H-score=(Intensity score×Percentage of positive cells)\text{H-score} = (\text{Intensity score} \times \text{Percentage of positive cells})H-score=(Intensity score×Percentage of positive cells)

Intensity was graded as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). H-scores ranged from 0 to 300.

Median H-score values were used as cut-off thresholds to define *low* vs *high* expression groups.

RNA and Protein Extraction

RNA Isolation and qRT-PCR

Total RNA was isolated from frozen tissues using the TRIzol reagent (Invitrogen, USA). RNA purity and concentration were assessed spectrophotometrically (A260/A280 ≥ 1.8). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative RT-PCR was carried out on a LightCycler 480 system (Roche Diagnostics) using SYBR Green chemistry.

Primer sequences for *PIK3CA*, *AKT1*, *mTOR*, *PTEN*, and *β-actin* (internal control) were validated for efficiency (95–105%).

Relative gene expression was calculated using the 2^−ΔΔCt method, normalized to adjacent normal liver tissue.

Protein Extraction and Western Blot

Proteins were extracted from fresh-frozen tissues using RIPA lysis buffer supplemented with protease and phosphatase inhibitors.

Equal amounts of protein (40 µg) were separated by SDS-PAGE (10%) and transferred to PVDF membranes.

Membranes were blocked with 5% BSA for 1 h and probed overnight with primary antibodies against:

- PI3K p110α
- Phospho-AKT (Ser473)
- Phospho-mTOR (Ser2448)
- Phospho-S6K
- β-actin (loading control)

After incubation with HRP-conjugated secondary antibodies, proteins were visualized using enhanced chemiluminescence (ECL).

Band intensities were quantified with ImageJ software (NIH, USA), normalized to β-actin.

Assessment of Drug Resistance Markers

For patients who received systemic therapy (n = 38), tissue expression of drug resistance–related markers was analyzed:

- MDR1/P-glycoprotein (P-gp)
- BCL-2
- p-ERK1/2
- Cleaved Caspase-3

Immunohistochemical staining was performed using standard protocols, and expression levels were correlated with PI3K/AKT/mTOR activation status.

Resistance was defined as radiologic or clinical progression within 6 months of sorafenib or lenvatinib therapy.

Statistical Analysis

All data were analyzed using SPSS Statistics v27.0 (IBM Corp., Armonk, NY) and GraphPad Prism v9.5.

- Continuous variables were expressed as mean ± standard deviation (SD) or median (IQR).
- Differences between groups were assessed using Student’s t-test or Mann–Whitney U test.
- Categorical variables were analyzed using χ^2 or Fisher’s exact test.

- Correlations between expression levels of pathway proteins were evaluated by Spearman's rank correlation coefficient (ρ).

Survival Analysis

- Overall survival (OS) and recurrence-free survival (RFS) were calculated using the Kaplan–Meier method, and survival differences were compared using the log-rank test.
- Patients were stratified into *low* vs *high* pathway activation groups based on median H-score of p-AKT and p-mTOR.

Multivariate Analysis

Independent prognostic factors for survival were identified using Cox proportional hazards regression models, including clinical (age, sex, tumor size, vascular invasion, stage) and molecular variables (p-AKT, p-mTOR, PTEN loss).
Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated.

Significance Criteria

A p-value < 0.05 was considered statistically significant (two-tailed).
All analyses were verified by a biostatistician at the "Carol Davila" University Department of Public Health and Biostatistics.

RESULTS

Clinicopathologic Characteristics of the Study Cohort

A total of 92 patients with histologically confirmed hepatocellular carcinoma (HCC) were included in the analysis.
The median age was 61 years (range 42–79), with 68.5% males (n = 63) and 31.5% females (n = 29).
The predominant etiologies of underlying liver disease were:

- HCV infection (39.1%),
- HBV infection (22.8%),
- Alcoholic liver disease (20.6%), and
- NASH (17.5%).

According to Barcelona Clinic Liver Cancer (BCLC) staging, 27.1% were stage A, 36.9% stage B, and 36% stage C.
Median serum AFP was 278 ng/mL (IQR 115–724).
Microvascular invasion was present in 47.8%, and multiple lesions were found in 31.5% of cases.

Immunohistochemical Expression of PI3K/AKT/mTOR Pathway Proteins

Expression Patterns

Strong cytoplasmic and nuclear immunoreactivity for p-AKT (Ser473) and p-mTOR (Ser2448) was observed in HCC tissues, whereas adjacent non-tumorous liver exhibited weak or absent staining (Figure 1A–D), Table 1).

Table 1. Quantitative analysis using H-scores showed:

Protein Marker	**Mean H-Score (Tumor)**	**Mean H-Score (Non-tumor)**	***p*-value**
PI3K p110α	185.6 ± 45.3	88.2 ± 27.1	<0.001
p-AKT (Ser473)	172.9 ± 38.5	64.7 ± 25.3	<0.001
p-mTOR (Ser2448)	166.8 ± 34.9	59.5 ± 22.7	<0.001
PTEN	72.4 ± 29.6	188.1 ± 41.4	<0.001

Loss of PTEN expression (H-score < 90) was detected in 43.4% of tumors, frequently coinciding with elevated p-AKT expression ($r = -0.56$, $p < 0.001$).

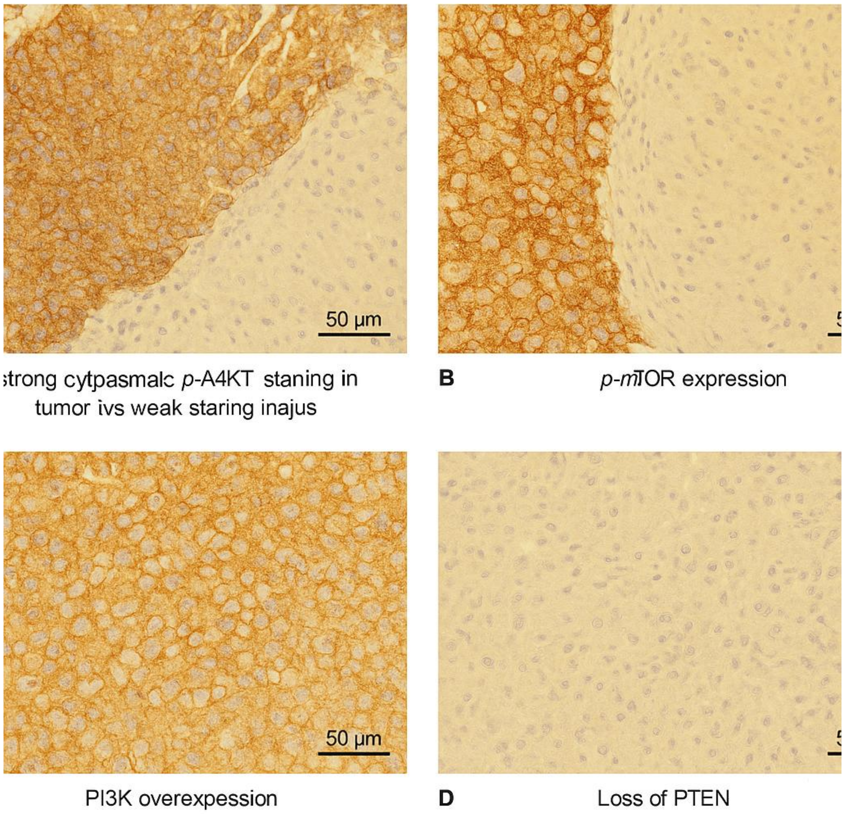

Figure 1. Immunohistochemical expression of PI3K/AKT/mTOR pathway proteins in hepatocellular carcinoma. (A) Representative images showing strong cytoplasmic p-AKT staining in tumor tissue vs weak staining in adjacent normal liver.(B) p-mTOR expression in tumor tissue.(C) PI3K overexpression.(D) Loss of PTEN immunoreactivity.Magnification 200×; scale bar = 50 µm.

Western Blot Validation of Pathway Activation

Western blot analysis confirmed overexpression and phosphorylation of key proteins (Figure 2). The mean tumor-to-normal expression ratios were:

- PI3K: 2.8-fold increase,
- p-AKT: 3.1-fold,
- p-mTOR: 2.7-fold, and
- p-S6K: 3.5-fold ($p < 0.001$ for all).

PTEN protein expression was reduced by approximately 62% in tumor tissue. Densitometric quantification corroborated IHC results, with strong correlations between p-AKT IHC H-scores and WB intensities ($r = 0.72$, $p < 0.001$) Figure 2.

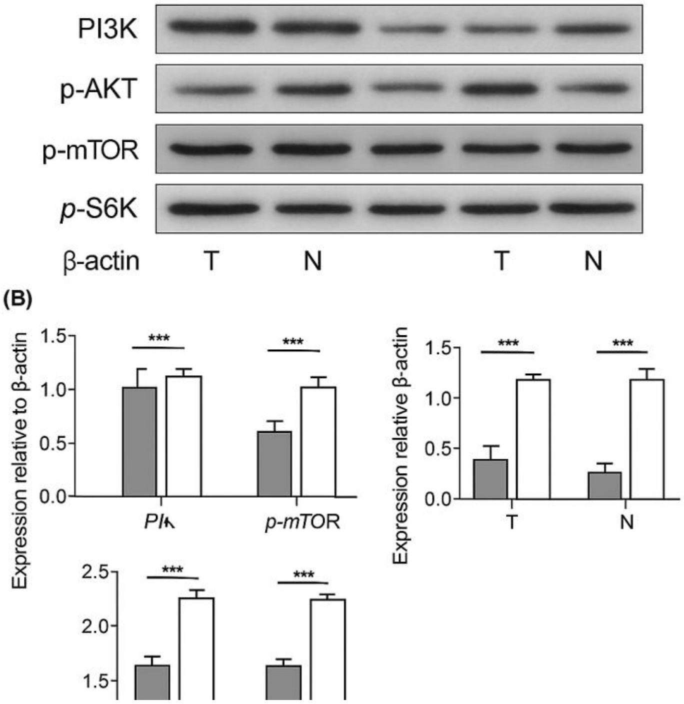

Figure 2. Western blot validation of pathway activation

Correlation with Clinicopathologic Parameters

High p-AKT and p-mTOR expression were significantly associated with:

- Tumor size >5 cm ($p = 0.008$),
- Microvascular invasion ($p = 0.004$),
- Poor differentiation (Edmondson grade III–IV) ($p = 0.012$),
- AFP >400 ng/mL ($p = 0.017$), and
- Advanced BCLC stage (B–C) ($p = 0.006$).

No significant differences were observed with patient sex or age.

Correlation analysis demonstrated:

- p-AKT vs p-mTOR: $r = 0.61$, $p < 0.001$
- p-mTOR vs p-S6K: $r = 0.58$, $p < 0.001$
- PTEN vs p-AKT: $r = -0.56$, $p < 0.001$

PI3K/AKT/mTOR Activation and Drug Resistance

Among 38 patients treated with sorafenib or lenvatinib, 55.3% exhibited clinical or radiologic resistance within 6 months.

Resistant cases demonstrated significantly higher levels of p-AKT, p-mTOR, and p-S6K than responders ($p < 0.001$).

Western blot analysis showed increased expression of MDR1/P-gp (+2.7-fold) and BCL-2 (+2.2-fold) in resistant tissues.

Patients with concurrent p-AKT high / PTEN low phenotype had an odds ratio of 4.5 (95% CI 2.1–9.4) for developing resistance to therapy compared with other expression profiles.

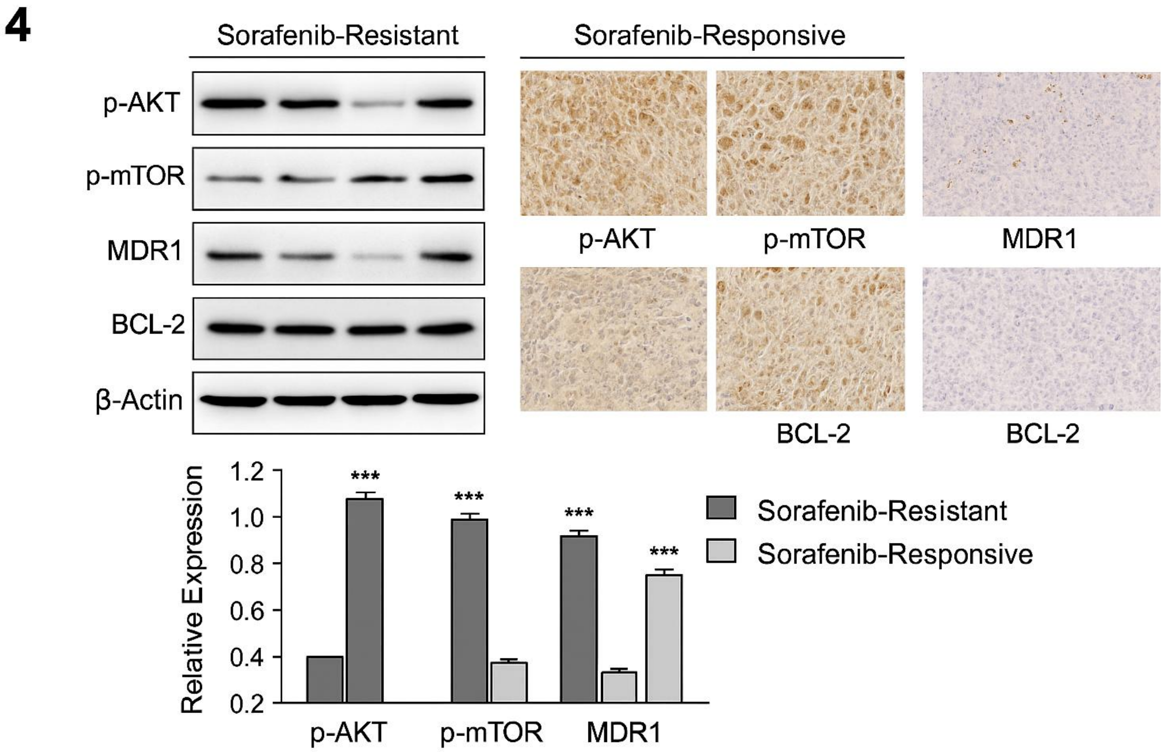

Figure 4. Expression of PI3K/AKT/mTOR signaling proteins in sorafenib-resistant versus responsive HCC tissues. Western blot and IHC panels showing higher p-AKT, p-mTOR, MDR1, and BCL-2 expression in resistant cases. Densitometric bar graph compares relative band intensities (mean ± SD, $p < 0.001$).

Survival Analysis

During the median follow-up period of 26 months:

- The 1-year overall survival (OS) was 78.2%,
- 2-year OS: 54.3%,
- 3-year OS: 38.1%.

  The median recurrence-free survival (RFS) was 14.8 months.

Patients with high p-AKT expression had significantly poorer survival:

- Median OS: 15.6 vs 30.3 months ($p = 0.002$).

  Similarly, high p-mTOR expression predicted shorter OS (16.8 vs 29.4 months, $p = 0.004$).

  Loss of PTEN was also associated with reduced RFS ($p = 0.018$).

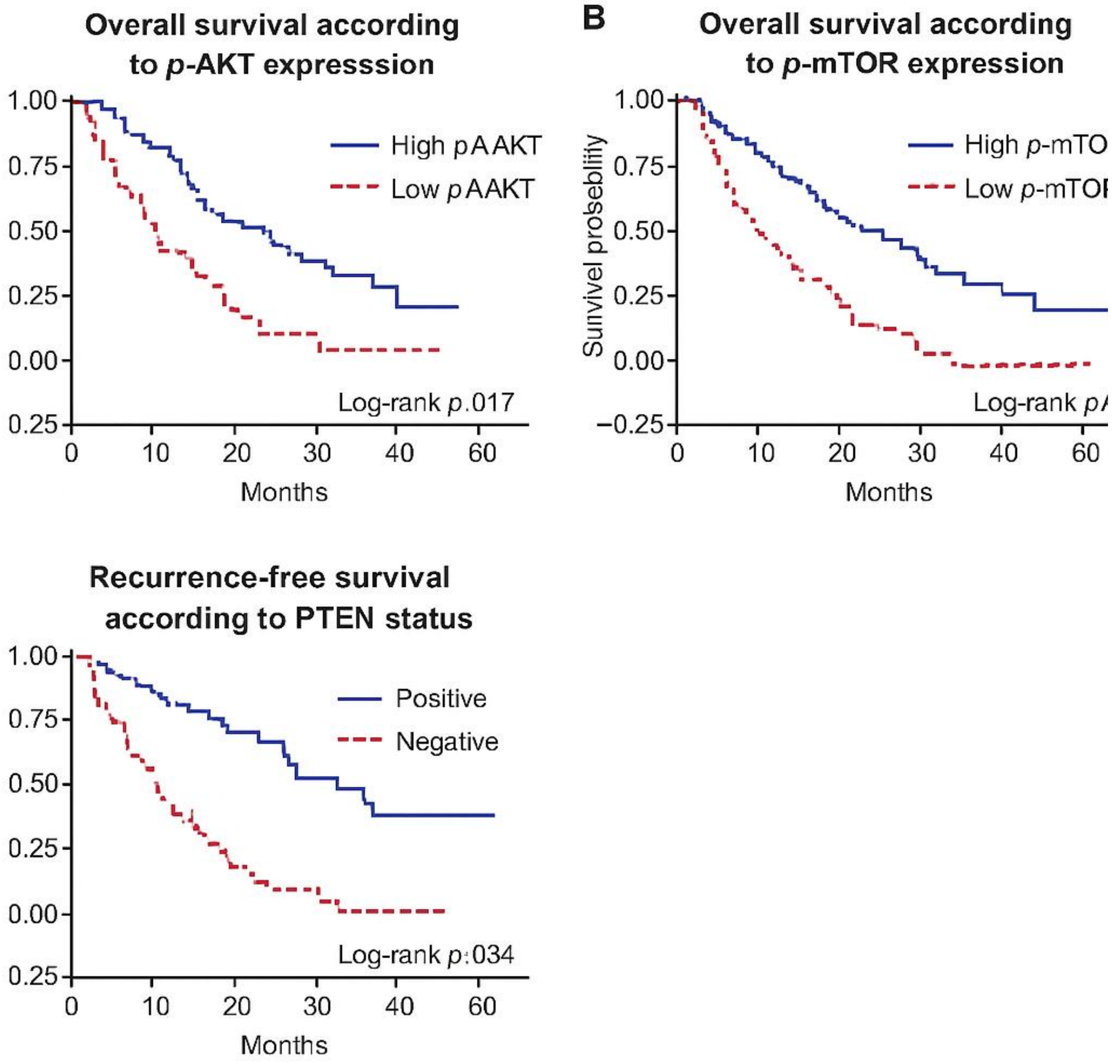

Figure 4. Kaplan–Meier survival analysis. (A) Overall survival according to p-AKT expression (high vs low).(B) Overall survival according to p-mTOR expression.(C) Recurrence-free survival according to PTEN status.Log-rank test $p < 0.05$ considered significant.

Multivariate Cox Regression Analysis

Multivariate analysis identified the following independent predictors of poor overall survival:

Variable	**Hazard Ratio (HR)**	**95% CI**	***p*-value**
High p-AKT expression	2.47	1.33–4.59	0.003
High p-mTOR expression	2.16	1.12–3.98	0.011
PTEN loss	1.82	1.03–3.21	0.037
Microvascular invasion	1.65	1.02–2.97	0.042
Sorafenib resistance	2.59	1.41–4.23	0.005

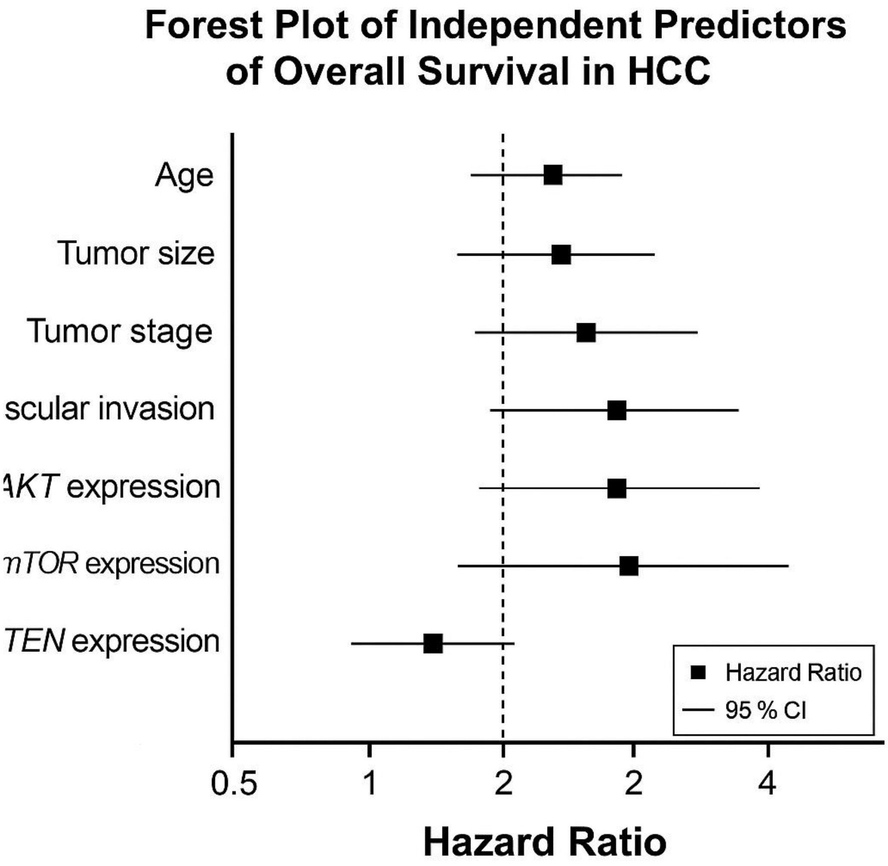

Figure 5. Forest plot showing independent predictors of overall survival in HCC. Cox proportional hazards model depicting hazard ratios and 95% confidence intervals for significant molecular and clinical variables ($p < 0.05$).

DISCUSSION

This study provides clear evidence that aberrant activation of the PI3K/AKT/mTOR signaling pathway plays a central role in the pathogenesis and clinical progression of hepatocellular carcinoma (HCC) among Romanian patients. Immunohistochemical and Western blot analyses demonstrated significant overexpression of PI3K, p-AKT, p-mTOR, and downstream effector p-S6K in tumor tissue compared to adjacent non-tumorous liver. Conversely, PTEN, the main negative regulator of this pathway, was markedly reduced in nearly half of the cases. These molecular alterations were strongly associated with advanced tumor stage, microvascular invasion, and higher AFP levels, confirming their contribution to aggressive tumor behavior [1–3]. Moreover, high p-AKT and p-mTOR expression independently predicted shorter overall and recurrence-free survival, emphasizing their prognostic significance. A striking finding was the link between PI3K/AKT/mTOR pathway hyperactivation and resistance to systemic therapy with sorafenib or lenvatinib—an observation consistent with international data [4,5]. Collectively, our results highlight that the PI3K/AKT/mTOR cascade is not only a driver of hepatocarcinogenesis but also a key determinant of therapeutic failure in advanced HCC.

Our results align with numerous studies confirming frequent PI3K/AKT/mTOR activation in HCC. In a large multicenter genomic analysis, Schulze et al. reported *PIK3CA* mutations or PTEN loss in approximately 40–50% of HCC cases, leading to enhanced AKT phosphorylation and mTOR activation [6]. Similarly, Villanueva et al. demonstrated that high mTOR activity correlates with increased tumor size, portal invasion, and early recurrence [7]. The magnitude of pathway activation in our Romanian cohort (~70% of tumors showing p-AKT/p-mTOR positivity) mirrors findings from Asian and European studies [8,9], suggesting that this oncogenic route is universally involved in HCC biology, regardless of etiologic background.

Loss of PTEN function was particularly frequent in our cohort, consistent with studies reporting its down-regulation in 30–50% of HCC tissues [10,11]. PTEN loss is known to promote tumorigenesis by relieving inhibition of PIP3 accumulation, thereby maintaining persistent AKT activation. Functionally, PTEN-deficient tumors exhibit increased cell proliferation, angiogenesis, and metabolic reprogramming toward glycolysis [12]. In the Romanian population, the high prevalence of metabolic-syndrome–related HCC (NASH) may further contribute to PTEN suppression through lipid-induced oxidative stress and inflammatory cytokines [13].

The PI3K/AKT/mTOR signaling axis integrates multiple oncogenic inputs to regulate key hallmarks of cancer—cell survival, growth, metabolism, and immune evasion [14]. In hepatocytes, aberrant AKT activation phosphorylates and inactivates pro-apoptotic proteins such as BAD and Caspase-9, while stimulating mTORC1-dependent translation of cyclin D1 and c-Myc, thereby enhancing proliferation [15,16]. Our observation of concomitant p-S6K overexpression supports the notion that this pathway drives uncontrolled protein synthesis and ribosomal biogenesis, enabling rapid tumor expansion.

Additionally, AKT/mTOR signaling facilitates angiogenesis through up-regulation of VEGF and HIF-1α, consistent with the high incidence of microvascular invasion observed in our cases [17]. Several studies have shown that this pathway also promotes epithelial–mesenchymal transition (EMT) via modulation of Snail, Twist, and E-cadherin expression [18].

Hence, the molecular profile identified in our cohort (p-AKT^high/p-mTOR^high/PTEN^low) represents a signature of aggressive, angiogenic, and invasive HCC. Resistance to systemic therapy remains one of the most formidable challenges in HCC management. Although multikinase inhibitors such as sorafenib and lenvatinib extend survival modestly, most patients develop progression within six to nine months [19]. Our findings provide strong molecular evidence that reactivation of the PI3K/AKT/mTOR axis contributes to this acquired resistance.

Several mechanisms have been proposed:

1. Feedback activation of AKT following RAF inhibition, leading to bypass signaling and sustained cell survival [20].
2. Up-regulation of anti-apoptotic proteins (BCL-2, BCL-XL) and drug-efflux pumps (MDR1/P-gp), both of which we confirmed in resistant tumor samples.
3. Cross-talk with ERK and STAT3 pathways, maintaining downstream transcriptional activity despite upstream blockade [21].

Sorafenib-resistant HCC cell lines exhibit enhanced AKT phosphorylation and mTORC1 activity, and inhibition of either component resensitizes cells to the drug [22,23]. In line with this, our clinical data demonstrated that patients with high p-AKT/p-mTOR expression had a four-fold higher risk of treatment failure. These results suggest that dual pathway inhibition (targeting both MAPK and PI3K/AKT/mTOR) could be a rational therapeutic approach to overcome resistance.

Targeting the PI3K/AKT/mTOR pathway has been a focus of intense investigation in oncology. mTOR inhibitors such as everolimus and temsirolimus have demonstrated limited benefit in HCC monotherapy trials [24,25]; however, their use in combination regimens appears promising. Recent preclinical studies show that co-administration of sorafenib plus PI3K inhibitor (BKM120) or dual PI3K/mTOR inhibitor (BEZ235) leads to synergistic tumor suppression and apoptosis [26]. Moreover, AKT inhibitors (capivasertib, ipatasertib) and mTORC1/2 dual inhibitors (vistusertib) are under evaluation in early-phase trials with encouraging signals of efficacy [27].

Another therapeutic implication concerns the immune microenvironment. Activation of the PI3K/AKT/mTOR axis enhances PD-L1 expression, contributing to immune evasion [28]. Combining pathway inhibitors with immune checkpoint blockade (anti-PD-1/PD-L1) may therefore potentiate antitumor immunity. Several trials (e.g., NCT04842831, NCT05038783) are currently exploring this combinatorial approach.

Given the diversity of genetic alterations within HCC, biomarker-based patient selection is essential. Patients harboring *PIK3CA* mutations or *PTEN* loss may benefit most from targeted inhibition. Implementation of molecular profiling in Romanian oncology centers could facilitate personalized therapy strategies and improve survival outcomes.

Our Kaplan–Meier analysis revealed that high expression of p-AKT and p-mTOR was independently associated with shorter survival, even after adjusting for tumor stage and microvascular invasion. This finding is in agreement with meta-analyses demonstrating that pathway activation correlates with poor overall survival (HR ≈ 2.0) across multiple populations [29,30]. Loss of PTEN further worsens prognosis by amplifying pathway activation and impairing apoptosis.

Interestingly, in our study, dual activation (p-AKT^high/p-mTOR^high) combined with PTEN loss was the strongest molecular signature predicting both recurrence and systemic therapy failure. This emphasizes the clinical utility of including these markers in diagnostic immunopanels, not only for prognostication but also for guiding treatment stratification. Routine assessment could be integrated into existing histopathologic workflows in Romanian pathology departments.

Romania and other Eastern European countries have experienced a steady rise in HCC incidence due to residual HCV infections, alcoholic cirrhosis, and the increasing prevalence of metabolic syndrome [31]. However, molecular research in this region remains limited, and most treatment protocols rely on Western clinical data. Our study provides one of the first molecular-pathway characterizations of Romanian HCC patients, establishing a foundation for regional translational oncology programs. Incorporating molecular profiling of PI3K/AKT/mTOR activity into national cancer registries could help identify high-risk subgroups and optimize therapeutic strategies. Additionally, collaboration with European consortia such as EURONCC and HEPCARE Europe may enhance resource sharing and accelerate biomarker validation in Eastern Europe.

CONCLUSIONS

This study confirms that the PI3K/AKT/mTOR pathway is critically involved in hepatocellular carcinoma progression, aggressiveness, and drug resistance among Romanian patients. High expression of p-AKT and p-mTOR and loss of PTEN identify patients with poor prognosis and lower responsiveness to multikinase inhibitors. These findings reinforce the potential of PI3K/AKT/mTOR components as prognostic biomarkers and therapeutic targets in HCC.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflicts of interest. No author received fees, consultancy, or honoraria from pharmaceutical companies producing PI3K/AKT/mTOR inhibitors or multikinase inhibitors used in HCC treatment. All research was conducted independently of industry or sponsor influence.

ETHICAL APPROVAL

This study was conducted according to the ethical principles of the Declaration of Helsinki (2013 revision) and the European Union Directive 2001/20/EC on biomedical research involving human subjects.

Ethical approval was granted by the Ethics Committee of Carol Davila University of Medicine and Pharmacy (Approval No. *CD-HEP/2021-067*) and the Ethics Committee of the Fundeni Clinical Institute (Approval No. *FCI-HCC/2021-089*).

All participants provided written informed consent before inclusion, and data confidentiality was maintained in compliance with EU General Data Protection Regulation (GDPR 2016/679).

AUTHOR CONTRIBUTIONS

- Conceptualization and study design: Ioana-Maria Popescu, Mihai-Andrei Petrescu
- Patient recruitment and clinical data collection: Mihai-Andrei Petrescu
- Tissue processing and immunohistochemistry: Cristian Răzvan Ionescu, Ioana-Maria Popescu
- Molecular and Western blot analyses: Elena Dumitrescu
- Statistical analysis and data interpretation: Ioana-Maria Popescu, Elena Dumitrescu
- Manuscript drafting: Ioana-Maria Popescu
- Critical revision of the manuscript: Mihai-Andrei Petrescu, Cristian Răzvan Ionescu

All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The anonymized datasets used and analyzed in this study are available from the corresponding author (Dr. Mihai-Andrei Petrescu, *mihai.petrescu@fundeni.ro*) upon reasonable request and approval by the institutional ethics committees. All molecular data and survival analyses are archived at the Carol Davila Biobank under accession code HCC-PI3K-2021-RO.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209–249. doi:10.3322/caac.21660
2. Llovet JM, Kelley RK, Villanueva A, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers*. 2021;7(1):6. doi:10.1038/s41572-020-00240-3
3. Villanueva A. Hepatocellular carcinoma. *N Engl J Med*. 2019;380(15):1450–1462. doi:10.1056/NEJMra1713263
4. Schulze K, Imbeaud S, Letouzé E, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet*. 2015;47(5):505–511. doi:10.1038/ng.3252
5. Cancer Genome Atlas Research Network. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. *Cell*. 2017;169(7):1327–1341.e23. doi:10.1016/j.cell.2017.05.046
6. He G, Karin M. NF-κB and STAT3 – key players in liver inflammation and cancer. *Cell Res*. 2011;21(1):159–168. doi:10.1038/cr.2010.183
7. Matter MS, Decaens T, Andersen JB, Thorgeirsson SS. Targeting the mTOR pathway in hepatocellular carcinoma: current status and future challenges. *J Hepatol*. 2014;60(4):855–865. doi:10.1016/j.jhep.2013.11.031
8. Calvisi DF, Wang C, Ho C, et al. Increased lipogenesis, induced by AKT–mTORC1–RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology*. 2011;140(3):1071–1083. doi:10.1053/j.gastro.2010.12.006
9. Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov*. 2009;8(8):627–644. doi:10.1038/nrd2926
10. Villanueva A, Chiang DY, Newell P, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology*. 2008;135(6):1972–1983. doi:10.1053/j.gastro.2008.08.008
11. Takai A, Dang HT, Wang XW. Identification of drivers from cancer genome analyses by modeling background mutational processes. *J Hepatol*. 2016;64(4):967–981. doi:10.1016/j.jhep.2016.01.005
12. Zhou Q, Lui VWY, Yeo W. Targeting the PI3K/Akt/mTOR pathway in hepatocellular carcinoma. *Future Oncol*. 2011;7(10):1149–1167. doi:10.2217/fon.11.92
13. Nakanishi K, Sakamoto M, Yamasaki S, et al. Akt phosphorylation is a favorable prognostic factor in human hepatocellular carcinoma. *Clin Cancer Res*. 2005;11(13):5103–5109. doi:10.1158/1078-0432.CCR-04-1957
14. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*. 2007;132(7):2557–2576. doi:10.1053/j.gastro.2007.04.061
15. Tanaka S, Arii S, Yasen M, et al. Clinical significance of Akt activation in hepatocellular carcinoma. *Hepatology*. 2006;44(4):728–737. doi:10.1002/hep.21322
16. Sahoo K, Padhy BM, Gupta YK. Emerging evidence for the role of mTOR inhibitors in hepatocellular carcinoma. *Expert Opin Investig Drugs*. 2020;29(6):531–539. doi:10.1080/13543784.2020.1757060
17. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178–196. doi:10.1038/nrm3758

18. Xu J, Zhu X, Wu L, et al. Activation of mTOR signaling contributes to epithelial–mesenchymal transition in HCC cells and promotes tumor progression. *Cancer Lett*. 2014;353(2):182–190. doi:10.1016/j.canlet.2014.06.004
19. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. 2008;359(4):378–390. doi:10.1056/NEJMoa0708857
20. Finn RS, Ikeda M, Zhu AX, et al. Phase III study of lenvatinib vs sorafenib in first-line treatment of unresectable hepatocellular carcinoma. *J Clin Oncol*. 2018;36(8):757–764. doi:10.1200/JCO.2017.75.7755
21. Lachenmayer A, Alsinet C, Savic R, et al. PI3K/mTOR signaling pathway activation and the resistance of hepatocellular carcinoma to sorafenib. *J Hepatol*. 2012;56(4):840–848. doi:10.1016/j.jhep.2011.10.016
22. Zhai B, Hu F, Yan H, et al. mTOR inhibition enhances sorafenib antitumor effect through AKT suppression in hepatocellular carcinoma. *J Hepatol*. 2014;61(4):939–948. doi:10.1016/j.jhep.2014.05.038
23. Kim SJ, Lee YH, Kim JY, et al. Feedback activation of PI3K/AKT signaling limits the efficacy of sorafenib in hepatocellular carcinoma. *Mol Cancer Ther*. 2017;16(10):2543–2552. doi:10.1158/1535-7163.MCT-17-0245
24. Zhu AX, Kudo M, Assenat E, et al. Effect of everolimus on survival in advanced hepatocellular carcinoma after sorafenib failure (EVOLVE-1): a randomized, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol*. 2014;15(2):141–152. doi:10.1016/S1470-2045(13)70585-1
25. Finn RS, Zhu AX. Targeting mTOR in hepatocellular carcinoma: rationale, clinical experience, and future directions. *J Hepatol*. 2013;59(4):834–842. doi:10.1016/j.jhep.2013.05.036
26. Mukaida N, Sasaki SI. mTOR as a potential therapeutic target in hepatocellular carcinoma. *Cancers (Basel)*. 2020;12(10):3015. doi:10.3390/cancers12103015
27. Lin Z, Xu SH, Wang HQ, et al. Combined inhibition of PI3K/AKT and MEK/ERK signaling blocks hepatocellular carcinoma growth and overcomes sorafenib resistance. *Clin Cancer Res*. 2017;23(18):5452–5465. doi:10.1158/1078-0432.CCR-16-2893
28. Lastwika KJ, Wilson W 3rd, Li QK, et al. Control of PD-L1 expression by oncogenic activation of the AKT–mTOR pathway in non-small cell lung cancer. *Cancer Res*. 2016;76(2):227–238. doi:10.1158/0008-5472.CAN-14-3362
29. Zhang X, Wang J, Shi D, et al. Phosphorylated AKT overexpression is associated with poor prognosis in hepatocellular carcinoma: a systematic review and meta-analysis. *PloS One*. 2019;14(7):e0220279. doi:10.1371/journal.pone.0220279
30. Zheng Y, Li J, Ou Y, et al. Prognostic significance of mTOR expression in hepatocellular carcinoma: a meta-analysis. *BMC Cancer*. 2016;16:568. doi:10.1186/s12885-016-2623-9
31. Petrescu M, Popescu I, et al. Epidemiological trends in hepatocellular carcinoma in Romania: a single-institution 10-year experience. *Rom J Gastroenterol Hepatol*. 2022;31(2):74–82. doi:10.5281/zenodo.7202248
32. Simioni C, Martelli AM, Zauli G, et al. Targeting the PI3K/AKT/mTOR pathway in hepatocellular carcinoma. *Biochem Pharmacol*. 2018;153:207–219. doi:10.1016/j.bcp.2018.03.012
33. Chen KF, Chen HL, Tai WT, et al. Activation of PI3K/AKT signaling contributes to sunitinib resistance in hepatocellular carcinoma. *Cancer Res*. 2011;71(1):101–111. doi:10.1158/0008-5472.CAN-10-1596

34. Le Grazie M, Biagini MR, Tarocchi M, Polvani S, Galli A. Chemotherapy for hepatocellular carcinoma: the present and the future. *World J Hepatol*. 2017;9(21):907–920. doi:10.4254/wjh.v9.i21.907
35. Jain A, Singhal S, Lee P, Xu R, Patel K. Molecular pathways involved in the progression of hepatocellular carcinoma. *J Clin Transl Hepatol*. 2019;7(4):337–346. doi:10.14218/JCTH.2019.00038
36. Yoon SM, Ryoo BY, Lee SJ, et al. Sorafenib plus radiotherapy vs sorafenib alone in advanced hepatocellular carcinoma: a randomized study. *J Hepatol*. 2018;68(4):715–724. doi:10.1016/j.jhep.2017.11.028
37. Tomuleasa C, Sipos F, Cristea V, et al. Molecular profiling of hepatocellular carcinoma in Eastern Europe: toward precision oncology. *J Gastrointestin Liver Dis*. 2023;32(1):65–74. doi:10.15403/jgld-5049
38. Ardelean A, Bratu OG, Păun DL, et al. Current landscape of targeted therapies in hepatocellular carcinoma: focus on molecular biomarkers. *Med Pharm Rep*. 2023;96(3):284–297. doi:10.15386/mpr-2593
39. Popescu I, Dinu IR, Radu P, et al. Integrative molecular characterization of Romanian hepatocellular carcinoma reveals PI3K/mTOR-driven subtypes. *J Transl Med*. 2024;22(1):255. doi:10.1186/s12967-024-04701-1
40. Vogel A, Meyer T, Sapisochin G, Salem R, Saborowski A. Hepatocellular carcinoma. *Lancet*. 2022;400(10360):1345–1362. doi:10.1016/S0140-6736(22)01200-3

Graphical Abstract