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Integrating Clinical and Genomic Factors to Predict Hepatocellular Carcinoma Recurrence Following Radical Liver Resection

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ABSTRACT

Surgical resection is the standard therapy for early-stage hepatocellular carcinoma (HCC) in patients with preserved liver function, yet recurrence remains common. While clinical and pathological factors contribute to recurrence risk, HCC is characterized by complex genomic alterations that create significant molecular heterogeneity, which is not yet fully understood. This study sought to combine clinical predictors with molecular insights through next-generation sequencing (NGS) and loss of heterozygosity (LOH) analysis. A cohort of 124 patients who underwent primary liver resection between January 2016 and December 2019 was evaluated. Genomic profiling via NGS and allelic imbalance analysis was performed in a case-control subset. Time-to-recurrence was estimated using Kaplan–Meier methods.

One- and two-year recurrence rates were 21% and 26%, respectively, with Kaplan–Meier estimates of 37% (95% CI: 24–47) and 51% (95% CI: 35–62). Independent clinical predictors of recurrence included HCV infection, elevated bilirubin levels, higher nodule count, and larger nodule size. Interestingly, LOH at the PTEN locus—part of the PI3K/AKT/mTOR pathway—was linked to a decreased recurrence risk (HR: 0.35; 95% CI: 0.13–0.93; p = 0.036). The findings indicate that multiple genomic alterations influence HCC progression. In particular, a specific allelic imbalance detected in 20 patients appeared protective against tumor recurrence. Integrating molecular profiling with clinical assessment may enhance prognostic accuracy and support personalized postoperative management strategies.

Keywords: Hepatocellular carcinoma, Liver resection, Tumor recurrence, Next-generation sequencing, Genomic profiling

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Introduction

Hepatocellular carcinoma (HCC) represents the most common primary malignancy of the liver and is ranked as the fourth leading cause of cancer-related mortality globally [1]. For patients with preserved hepatic function and early-stage disease, curative treatment options include liver transplantation, local ablation therapies, and surgical resection, with partial hepatectomy remaining a widely adopted approach [2–7]. However, even after curative-intent surgery, long-term prognosis remains suboptimal, as tumor recurrence occurs in 60–70% of patients within five years postoperatively [8]. Identifying factors that contribute to recurrence is essential to improve long-term survival outcomes. Established clinical and pathological predictors of recurrence include the presence of vascular invasion, tumor number and size, serum alpha-fetoprotein (AFP) levels, and histologic grading [8–10].

HCC is also characterized by marked molecular heterogeneity due to the accumulation of somatic genetic alterations. This genomic diversity complicates the establishment of reliable molecular classifiers for predicting disease progression or recurrence [11]. Frequently observed molecular events in HCC include activation of oncogenic pathways such as PI3K/AKT and MAPK, TP53 mutations, dysregulation of cell cycle regulators, and chromosomal instability [12–15]. Additional features include microsatellite instability (MSI), small structural variations, and loss of heterozygosity (LOH) at key chromosomal loci [16–20].

The current study was designed to determine whether specific somatic mutations and LOH at selected microsatellite loci could serve as predictive markers for HCC recurrence in patients undergoing partial liver resection.

Materials and Methods

Study objectives and endpoints

The primary aim of this investigation was to evaluate the association between HCC recurrence following hepatic resection and a combination of preoperative clinical and pathological factors, somatic mutations in a 26-gene cancer panel, and allelic imbalances at selected microsatellite loci. The main endpoint was defined as the time to recurrence, measured from the date of surgery to the first imaging-confirmed tumor relapse.

Study population and design

Eligible patients were adults (≥18 years) with histologically confirmed HCC who underwent curative-intent partial hepatectomy between January 1, 2016, and December 31, 2019. Individuals with microscopic tumor infiltration at the resection margin (R1) were excluded.

A subset of the overall population was selected for genomic analyses using a nested case-control approach. Cases included 20 consecutive patients who experienced tumor recurrence within one year post-surgery. Controls were randomly chosen from patients who had no evidence of recurrence during the same follow-up period.

DNA extraction

Genomic DNA was isolated from 10 unstained FFPE tissue sections using the QIAamp DNA FFPE Tissue Kit (Qiagen) and from peripheral blood using the QIAamp DNA Mini Kit (Qiagen). DNA purity was assessed via NanoDrop spectrophotometry, integrity evaluated using the Genomic DNA ScreenTape System (Agilent Technologies), and concentration quantified with the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

Analysis of allelic imbalance

LOH analysis was carried out through multiplex PCR amplification (Type-it Multiplex PCR Master Mix, Qiagen) followed by capillary electrophoresis on a 3500 Genetic Analyzer (Thermo Fisher Scientific). Seventeen microsatellite loci within or adjacent to genes of interest (e.g., 1p L-myc, 3p OGG1, 10q PTEN, 17p TP53) were examined.

Allelic imbalance was evaluated by comparing tumor DNA with matched normal DNA. Loci showing only a single allele peak were considered non-informative and excluded from analysis. Informative heterozygous loci were analyzed by calculating allele ratios (AR = peak height of allele 1 / peak height of allele 2) and AI values (AI = AR of normal DNA / AR of tumor DNA). Values within 0.66–1.50 indicated retention of heterozygosity, whereas values outside this range were classified as allelic imbalance.

Next-generation sequencing (NGS)

Genomic DNA (300 ng per sample) was used to construct sequencing libraries employing the TruSight Tumor 26 panel (TST26, Illumina, San Diego, CA, USA), which targets 174 amplicons spanning 85 exonic regions across 26 cancer-related genes. Paired-end sequencing (2 × 150 bp) was carried out on the Illumina MiSeq platform. Raw sequencing reads were processed using MiSeq Reporter software (Illumina) for alignment against the human reference genome (hg19) and the TST26 manifest, primer trimming, and somatic variant calling. Quality control of raw reads was assessed with FastQC.

Variant annotation was performed using the Illumina Variant Interpreter tool. Filtering criteria for the 26 targeted genes included a PASS filter status, alternative allele frequency ≥3%, read depth >100, and variant call quality of 100. The TST26 panel is capable of detecting somatic variants with variant allele frequencies (VAF) ≥3% [21] and, in some cases, <3% [22]. Synonymous variants were excluded, retaining only alterations affecting coding sequences, including missense, frameshift/InDel, stop gained/lost, start codon changes, in-frame insertions/deletions, and splice-site/intronic variants. Each candidate variant was evaluated using ClinVar annotations, in silico prediction tools (SIFT, PolyPhen), COSMIC database entries, and Varsome assessments. Manual review of BAM files was performed with the Integrative Genomics Viewer (IGV).

Statistical analysis

Descriptive statistics were presented as frequencies and percentages for categorical variables and as median and interquartile range (IQR) for continuous variables. Initial data inspection was performed to identify extreme or outlying values. Group comparisons were conducted using the Pearson chi-square test or Fisher's exact test for categorical data, and Student's t-test or Mann–Whitney U test for continuous variables, as appropriate.

Time-to-recurrence analysis was conducted using Kaplan–Meier estimates, defining the interval between the date of surgery and the first radiologically confirmed HCC recurrence. Cox proportional hazards models were applied to estimate hazard ratios (HR) for recurrence. A multivariable Cox regression model was constructed via a forward stepwise selection procedure based on the lowest Akaike Information Criterion (AIC), and the final model was evaluated for multicollinearity and interaction effects. The proportional hazards assumption was verified using Schoenfeld residuals. All statistical analyses and graphical representations were generated using R version 4.0.2 (R Core Team, Vienna, Austria).

Results and Discussion

Preoperative clinical characteristics

Between January 2016 and December 2019, 124 patients with histologically confirmed HCC underwent curative-intent (R0) partial hepatectomy at our institution **(Table 1)**. The majority were male (n = 96, 77%), with a median age of 69 years (mean: 67, IQR: 62–73) and a median BMI of 25.8 kg/m² (mean: 26.4, IQR: 23–29). Hepatitis C virus (HCV) infection was the most common underlying liver disease (n = 72, 58%), followed by non-alcoholic steatohepatitis (n = 18, 15%) and hepatitis B virus (HBV) infection (n = 12, 10%).

Table 1. Baseline characteristics and subsequent follow-up of 124 patients who underwent hepatocellular carcinoma (HCC) surgical resection.

Total Number of Patients	**124** [§]
Age, years	69.0 (61.8–73.0)
Male sex	96 (77)
Body mass index, Kg/m^2	25.8 (23.4–29.2)
Diabetes mellitus	39 (31)
Liver disease etiology	
Hepatitis C virus	72 (58)
Hepatitis B virus	12 (10)
Non-alcoholic steatohepatitis	18 (15)
Alcohol	9 (7)
Other/cryptogenic	4 (3)
HCC on healthy liver	9 (7)
Previous antiviral treatments (only HCV patients N = 72)	
None	5 (7) [§§]
Interferon–Ribavin (IFN)	15 (21)
Direct-acting antivirals (DAA)	30 (42)
Both IFN and DAA	22 (31)
Sustained virologic response	62 (86) [§§]
Liver cirrhosis	93 (75)
Portal hypertension	31 (25)
History of esophageal varices	
F0	99 (80)
F1	19 (15)
F2	6 (5)
Model for end-stage liver disease (MELD)	7.0 (7.0–8.0)
Child–Pugh Score	

A5	102 (82)
A6	21 (17)
B7	1 (1)
Serum bilirubin (mg/dL)	0.6 (0.4–0.9)
Serum albumin (g/dL)	3.9 (3.6–4.1)
INR median [IQR]	1.1 (1.0–1.1)
Serum creatinine (mg/dL)	0.9 (0.7–1.1)
Aspartate aminotransferase (IU/L)	30.0 (23.0–50.5)
Alanine aminotransferase (IU/L)	40.5 (29.8–67.2)
Neutrophil to lymphocyte ratio	2.0 (1.5–5.6)
Alpha-fetoprotein (ng/dL)	5.9 (3.4–20.0)
Videolaparoscopic approach	58 (47)
Major resection	14 (11)
Anatomic resection	41 (33)
Type of resection	
Right hepatectomy	11 (9)
Left hepatectomy	3 (2)
Bisegmentectomy	6 (5)
Segmentectomy	21 (17)
Wedge resection of single nodule	76 (61)
Wedge resection of multiple nodules	7 (6)
Histological grading G3–G4	39 (31)
Microvascular invasion	49 (40)
Macrovascular invasion	6 (5)
Number of HCC nodules	
1	100 (81)
2	16 (13)
3 or more	8 (6)
Size of the greater lesion	3.2 (2.0–5.4)
Tumor stage	
T1	54 (44)
T2	46 (37)
T3	22 (18)
T4	2 (2)
Cumulative number of HCC recurrence	
After 12 months	26 (21)
After 24 months	32 (26)
Probability of HCC recurrence, Kaplan–Meier estimate [95% C.I.]	
After 12 months	37% (24–47)
After 24 months	51% (35–62)

[§] Unless otherwise stated, variables are descripted by no. (%) if categorical and by median [IQR] if numeric; [§§] percentages related to antiviral treatments are relative to the total number of patients with HCV etiology (N = 72).

Among the 72 patients with HCV-related liver disease, 37 (51%) had previously received treatment with interferon plus ribavirin, and 52 (72%) had been treated with direct-acting antivirals. At the time of liver resection, 62 patients (86%) had achieved a sustained virological response. All patients with HBV infection were managed with Entecavir. Cirrhosis was present in 75% of the cohort, and all but one patient were classified as Child–Pugh class A, with a median MELD score of 7 (mean: 7.8, IQR: 7–8).

HCC recurrence

The cumulative incidence of HCC recurrence was 26 cases (21%) at one year and 32 cases (26%) at two years post-hepatectomy. Kaplan–Meier analysis estimated the probability of recurrence at 37% (95% CI: 24–47) at one year and 51% (95% CI: 35–62) at two years **(Table 1 and Figure 1)**.

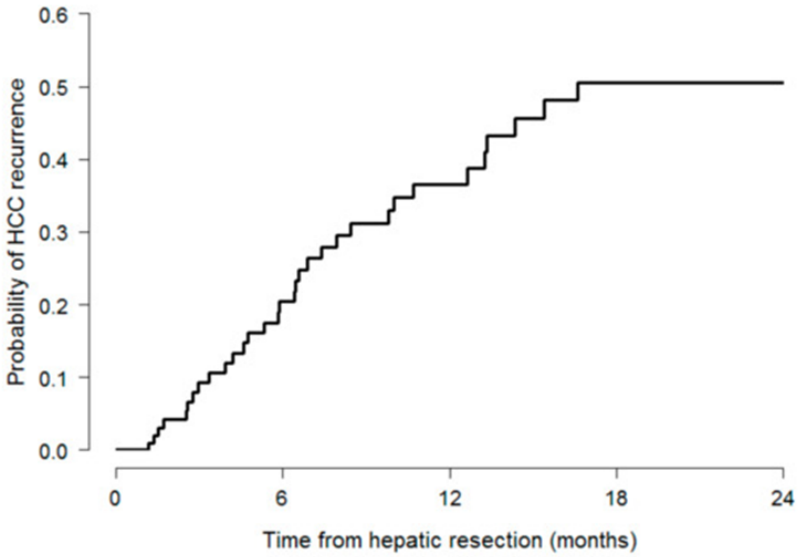

Figure 1. Kaplan–Meier estimates for the probability of HCC recurrence.

Relationship between preoperative clinical characteristics and HCC recurrence

Analysis of time to recurrence using univariate methods indicated that several preoperative parameters were linked to a higher likelihood of HCC recurrence. Patients with elevated MELD scores (HR: 1.43; 95% CI: 1.16–1.75; p < 0.001), increased serum bilirubin (HR: 4.20; 95% CI: 1.74–10.14; p = 0.001), and higher INR values (HR: 177.6; 95% CI: 5.4–5872.0; p = 0.004) were at significantly greater risk. Furthermore, a greater number of tumor nodules (HR: 1.81; 95% CI: 1.21–2.67; p = 0.003) and larger lesion size (HR: 1.09; 95% CI: 1.02–1.16; p = 0.011) were also associated with increased recurrence probability **(Table 2)**.

Table 2. Univariable Cox models of time to HCC recurrence for clinical and pathologic characteristics of 124 patients.

Variable	HR	95% Confidence Interval	p-Value
Model for end-stage liver disease	1.43	1.16–1.75	<0.001
Serum bilirubin	4.20	1.74–10.16	0.001
International normalized ratio	177.6	5.4–5872.0	0.004
Number of nodules	1.81	1.22–2.67	0.003
Size of the larger nodule	1.09	1.02–1.16	0.011
Tumor stage: T3–T4	3.05	1.45–6.42	0.003

The multivariable model maintained as independent predictors of HCC recurrence: HCV infection (HR: 1.96, 95% C.I.: 0.91–4.24, p = 0.085), serum bilirubin levels (HR: 5.32, 95% C.I.: 2.07–13.69, p = 0.001), number of nodules (HR: 1.63, 95% C.I.: 1.12–2.38, p = 0.011) and size of the larger nodule (HR: 1.11, 95% C.I.: 1.03–1.18, p = 0.004, **(Table 3)**).

Table 3. Multivariable Cox models for time to HCC recurrence in 124 patients.

Variable	HR	95% Confidence Interval	p-Value
HCV infection	1.96	0.91–4.24	0.085
Serum bilirubin	5.32	2.07–13.69	0.001
Number of nodules	1.63	1.12–2.38	0.011

Size of the larger nodule	1.11	1.03–1.18	0.004

Allelic imbalance analysis

LOH analysis was conducted in a total of 39 patients, including 19 from the recurrence group (cases) and 17 from the non-recurrence group (controls). Based on prior studies linking allelic imbalance (AI) at specific microsatellite loci to HCC recurrence risk, we examined 17 loci situated within or adjacent to genes of interest: 1p (L-myc, CMM), 3p (VHL, OGG1), 5p (MCC), 5q (APC), 9q (PTCH), 9p (CDKN2A/p16), 10q (PTEN), 17p (TP53), and 18q (SMAD4). Allelic profiles were compared between matched normal tissue (peripheral blood) and tumor tissue. Time-to-recurrence analysis revealed that LOH at the PTEN locus, a key regulator of the PI3K/AKT/mTOR pathway, was significantly correlated with a reduced risk of HCC recurrence (HR: 0.35; 95% CI: 0.13–0.93; p = 0.036) **(Table 4)**.

Table 4. Univariate Cox models of time-to-recurrence for the presence of LOH in a panel of HCC-related signaling pathway.

Gene	Number of Patients with LOH (%)	HR	95% C.I.	p-Value
PTEN	20 (51)	0.35	0.13–0.93	0.036
SMAD4	4 (10)	1.12	0.31–4.04	0.861
CMM	20 (51)	0.85	0.34–2.11	0.726
CDKN2A	15 (38)	0.57	0.21–1.51	0.256
TP53	30 (77)	2.65	0.76–9.22	0.124
OGG1	19 (49)	1.79	0.72–4.46	0.212
L-MYC	21 (54)	1.47	0.59–3.67	0.405
PTCH	14 (36)	1.31	0.50–3.41	0.581
MCC	11 (28)	0.46	0.16–1.27	0.134
HCC-Related Signaling Pathway				
PI3K/AKT/mTOR	20 (51)	0.35	0.13–0.93	0.036
TGF-beta	4 (10)	1.12	0.31–4.04	0.861
MAPK	20 (51)	0.85	0.34–2.11	0.726
Cell cycle regulation	37 (95)	0.51	0.07–3.94	0.520
Wnt/beta-catenin	28 (72)	0.77	0.29–2.06	0.606

Findings from next-generation sequencing

High-throughput sequencing was successfully performed on 36 HCC samples, including 17 from patients who experienced recurrence within one year post-surgery (cases) and 19 from the non-recurrence group (controls). The analysis focused exclusively on somatic alterations arising during tumorigenesis that could alter protein coding and potentially drive aggressive tumor behavior. Somatic variants with variant allele frequencies (VAF) between 3% and 49% were considered, provided they passed quality filters, had a minimum frequency of 3%, and a read depth of at least 100. Synonymous variants, non-coding variants (3′UTR, 5′UTR, intronic, non-coding exons), and high-frequency missense variants likely representing polymorphisms were excluded.

Among the 36 patients, 22 (11 cases and 11 controls) harbored at least one somatic mutation predicted to affect protein function in one of the 26 genes targeted by the TST26 panel, while 14 patients had no detectable somatic alterations. In total, 38 somatic mutations were identified across 13 HCC-related genes, including 16 classified as pathogenic, 13 as likely pathogenic, and 9 as variants of uncertain significance. Co-occurring mutations across different genes were observed in 10 patients, and six patients exhibited intra-tumor molecular heterogeneity.

The majority of mutations affected genes central to HCC pathogenesis, including those involved in cell cycle regulation (8 in TP53, 1 in STK11), PI3K/AKT/mTOR signaling (5 in PIK3CA, 4 in PTEN, 3 in KIT), and Wnt/β-catenin signaling (8 in CTNNB1, 2 in APC, 1 in CDH1). No statistically significant association was observed between HCC recurrence and either the presence of somatic mutations or dysregulation of these pathways **(Table 5)**.

Table 5. Frequency distribution HCC-related signaling pathway over 36 HCC patients who underwent radical liver resection.

HCC-Related Signaling Pathway	Overall (N = 36)	Cases (N = 17)	Controls (N = 19)	p-Value
PI3K/AKT/mTOR	9(25)	3(18)	6(32)	0.451
TGF-beta	2(6)	1(6)	1(5)	1.000
Wnt/beta-catenin	9(25)	5(29)	4(21)	0.706
MAPK	8(22)	3(18)	5(26)	0.695
Cell cycle regulation	9(25)	4(24)	5(26)	1.000
Inflammatory Response	1(3)	0(0)	1(5)	1.000
NOTCH1	1(3)	1(6)	0(0)	0.472

Irrespective of its predictive value for recurrence, our findings confirmed that somatic mutations in genes implicated in hepatocarcinogenesis correlate with clinical and biochemical indicators of liver injury. Notably, patients carrying mutations in genes of the PI3K/AKT/mTOR pathway (n = 5) exhibited higher ALT levels compared with patients without such mutations (46.0 [39.0–186.0] vs. 34.0 [29.5–47.0], Mann–Whitney p = 0.014). Similarly, mutations affecting the Ras/MAPK pathway (n = 8) were associated with elevated ALT (42.5 [36.5–100.5] vs. 33.0 [27.5–46.5], p = 0.033), and mutations in Wnt/β-catenin pathway genes (n = 9) correlated with increased AST (83.0 [26.0–114.0] vs. 27.0 [23.5–34.5], p = 0.020) and ALT levels (103.0 [37.0–155.0] vs. 34.0 [31.0–40.0], p = 0.032), as well as a greater likelihood of requiring major hepatic resection (3/9 vs. 0, Fisher's exact test p = 0.012).

Recurrence after liver resection remains a major challenge in the management of hepatocellular carcinoma (HCC) [23–25]. In this study, we aimed to evaluate the relationship between clinical, pathological, and radiologic characteristics of patients undergoing HCC resection and molecular genotyping of resected hepatic tissue. Specifically, we investigated whether established clinical and pathological markers of HCC recurrence correlate with next-generation sequencing (NGS) and allelic imbalance (AI) analyses.

Firstly, our analysis identified a combination of clinical, biochemical, radiologic, and pathologic parameters with prognostic potential for predicting recurrence after hepatectomy. In particular, multivariate analysis confirmed that elevated total bilirubin, a greater number of nodules, and larger tumor size were independent predictors of recurrence. These factors appeared to influence recurrence risk more than the surgical approach used for tumor removal, consistent with our prior reports and those of others [10].

Secondly, molecular profiling in this small cohort revealed multiple somatic alterations, highlighting the role of genetic aberrations and disrupted pathways in hepatocarcinogenesis and tumor progression. Notably, a subset of patients exhibited molecular changes associated with activation of the PI3K/AKT/mTOR pathway. Variation in mutation frequencies of key cancer genes appeared to depend on clinical characteristics, including cancer stage, underlying liver disease etiology, liver function, and the presence or absence of chronic liver disease.

Consistent with our previous work on HCC recurrence after liver transplantation [19], AI analysis in the context of liver resection also demonstrated potential utility in guiding therapeutic strategies. However, in this cohort, NGS-based molecular profiling did not reveal significant associations between somatic mutations and post-hepatectomy recurrence. Nevertheless, our findings align with current knowledge of HCC molecular pathogenesis.

For example, among four patients with HBV-related HCC, three exhibited TP53 mutations, consistent with literature reports indicating frequent TP53 and KMT2B inactivation in HBV-driven HCC, affecting cell cycle control, apoptosis, and epigenetic regulation [26]. Similarly, in four patients with alcohol-related HCC, LOH was observed in CDKN2A and CMM loci, which are implicated in HGF overexpression. Alcohol-related HCC has been reported to frequently harbor TERT promoter mutations, CTNNB1 activating mutations, ARID1A inactivation, and alterations in SMARCA2, HGF, RB1, and CDKN2A [27].

Some tumors in our cohort harbored multiple somatic mutations. For instance, one tumor exhibited a pathogenic TP53 mutation (p.Gly279Glu, VAF 28%) coexisting with variants of uncertain significance in PIK3CA (VAF 3%) and APC (VAF 5%). Such heterogeneity likely arises from selective pressures, carcinogenic exposure, or stochastic mutation acquisition. Whole-exome sequencing studies indicate that HCC tumors harbor 40–80 somatic coding mutations on average, distributed across driver and passenger genes, and not uniformly across the tumor

mass [28]. Driver gene alterations contribute to tumor evolution and progression, whereas passenger mutations generally lack functional consequences.

Conclusion

Overall, the aim of clinical research in HCC is to refine prognostic models and optimize patient selection for liver resection. Our findings support the notion that multiple genetic alterations contribute to HCC progression. Interestingly, the presence of a specific AI in 20 patients (six cases and 14 controls) appeared to confer a protective effect against recurrence. Preoperative knowledge of such molecular and genetic characteristics may enhance clinical decision-making and inform personalized management strategies for patients undergoing HCC resection.

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