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Non-Invasive Biomarkers for Early Detection of Hepatocellular Carcinoma in Patients with Chronic Liver Disease

Basim Aslam Memon, Fatima Niazi, Menahel Muneer

Designation: Medical student Affiliation: Ziauddin Medical College
Email: basimaslam.memon@gmail.com Contact no. +92 3352516800
Designation: Medical student Affiliation: Ziauddin Medical College
Email: fatima.a.niazi@gmail.com Contact no. +92 3168626522
Designation: Medical student Affiliation: Dow University of Health Sciences
Email: menahelmuneer2000@gmail.com Contact no. +92 3056111228

Batool Aslam Memon **(Corresponding)**
Designation: Consultant Medical Oncologist Affiliation: Dow University of Health Sciences
Email: memon_batool@hotmail.com Contact no. +92 3332785077

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Abstract

Background: Early detection of hepatocellular carcinoma (HCC) in patients with chronic liver disease (CLD) is critical for improving prognosis. Traditional biomarkers like alpha-fetoprotein (AFP) have limited sensitivity, prompting the need for alternative non-invasive biomarkers. This study evaluates the diagnostic accuracy of des-γ-carboxy prothrombin (DCP), glypican-3 (GPC3), miR-122, miR-21, and circulating tumor DNA (ctDNA) for early HCC detection.

Methods: A total of 120 CLD patients, including 20 with HCC, were recruited from Ziauddin University Hospital, Karachi, between June 2023 and February 2024. Biomarker levels were measured and compared between HCC and non-HCC groups. Receiver operating characteristic (ROC) curves were used to assess diagnostic performance, and multivariate combinations of biomarkers were analyzed to enhance diagnostic accuracy.

Results: DCP and ctDNA outperformed AFP, with AUCs of 0.91 and 0.93, respectively, compared to 0.82 for AFP. GPC3 and miR-122 also demonstrated high diagnostic accuracy, with AUCs of 0.88 and 0.87. Combining AFP, DCP, and miR-122 improved the AUC to 0.95, while adding ctDNA increased the AUC to 0.97, with 93% sensitivity and 94% specificity.

Conclusion: Non-invasive biomarkers such as DCP, GPC3, miR-122, and ctDNA significantly enhance early detection of HCC in CLD patients compared to AFP alone. Combining these biomarkers offers a promising approach for improving diagnostic accuracy, especially in early-stage HCC. Further validation in larger, diverse cohorts is warranted to establish their clinical utility.

Keywords: Hepatocellular carcinoma (HCC), Chronic liver disease (CLD), Non-invasive biomarkers, Alpha-fetoprotein (AFP), Des-γ-carboxy prothrombin (DCP), Glypican-3 (GPC3)

Introduction

Hepatocellular carcinoma (HCC) is a major global health challenge, accounting for 75-85% of all primary liver cancers and ranking as the third leading cause of cancer-related mortality worldwide (Sung et al., 2021). In Pakistan, chronic liver disease, primarily driven by hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, remains the most significant risk factor for HCC (Butt et al., 2020). The increasing prevalence of non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome has further exacerbated the burden of HCC in both urban and rural populations (Chalasani et al., 2018).

Despite advancements in diagnostic technologies, HCC is often diagnosed at an advanced stage, limiting the therapeutic options and significantly reducing survival rates (Yang et al., 2019). Early-stage HCC is largely asymptomatic, and current diagnostic modalities, such as ultrasound and computed tomography (CT), are not always accessible or affordable in resource-limited settings like Pakistan (Forner et al., 2018). Furthermore, liver biopsy, while a definitive diagnostic method, is invasive and not ideal for routine screening, necessitating the need for more accessible, non-invasive biomarkers (Heimbach et al., 2018).

The most commonly used biomarker for HCC, alpha-fetoprotein (AFP), has shown limited sensitivity, particularly in detecting early-stage tumors, with studies reporting a detection rate as low as 40% in early HCC cases (Yoon et al., 2020). Therefore, the search for novel non-invasive biomarkers that can reliably detect early-stage HCC in patients with chronic liver disease is ongoing. Promising biomarkers such as des-γ-carboxy prothrombin (DCP), microRNAs (miRNAs), and circulating tumor DNA (ctDNA) are gaining attention due to their ability to improve diagnostic accuracy (Liu et al., 2021).

Studies have demonstrated that miRNAs, small non-coding RNAs involved in gene regulation, play critical roles in tumorigenesis and have the potential to serve as early biomarkers for HCC (Feng et al., 2020). Likewise, ctDNA, which reflects the genetic alterations of tumor cells, has shown promise in early detection, monitoring, and prognosis of HCC, offering a non-invasive approach to cancer diagnosis (Matsumae et al., 2021). Validating these biomarkers in local populations, particularly in Pakistan where liver disease is endemic, is essential to developing effective screening programs.

This study, conducted at Ziauddin University Hospital in Karachi, aims to evaluate the utility of various non-invasive biomarkers for the early detection of HCC in patients with chronic liver disease. By focusing on biomarkers that can be implemented in routine clinical practice, this research seeks to provide a cost-effective and accessible strategy for improving early detection rates of HCC, ultimately enhancing patient outcomes in Pakistan.

Methodology

This cross-sectional study was conducted at Ziauddin University Hospital, Karachi, from June 2023 to February 2024. The objective was to evaluate the utility of various non-invasive biomarkers for the early detection of hepatocellular carcinoma (HCC) in patients with chronic liver disease (CLD). A total of 120 patients aged 18 to 75 years were recruited from the Hepatology and Gastroenterology clinics. These patients had been diagnosed with CLD based on clinical, biochemical, and imaging findings, and were undergoing routine surveillance for HCC. All participants provided written informed consent prior to enrollment in the study. Patients with a history of other cancers, those suffering from severe systemic diseases, or those who had undergone liver transplantation were excluded. Ethical approval was obtained from the Institutional Review Board (IRB) of Ziauddin University.

The sample size for the study was calculated using the formula for diagnostic accuracy studies: n=Z2·P(1−P)d2n = \frac{{Z^2 \cdot P(1 - P)}}{{d^2}}n=d2Z2·P(1−P), where Z=1.96Z = 1.96Z=1.96 for 95% confidence, P=10P = 10%P=10 (the estimated prevalence of HCC in CLD patients), and d=5d = 5%d=5 (the margin of error). This yielded a required sample size of 138, but due to patient availability during the study period, a total of 120 patients were recruited, which was deemed sufficient for this pilot study.

Various non-invasive biomarkers were selected for evaluation based on their reported potential in detecting early-stage HCC. These included alpha-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), glypican-3 (GPC3), microRNAs (miRNAs), and circulating tumor DNA (ctDNA). AFP was measured using enzyme-linked immunosorbent assay (ELISA), DCP using a chemiluminescent immunoassay, and GPC3 using another ELISA. For miRNAs, total RNA was extracted from serum samples using the TRIzol method, followed by quantitative real-time PCR (qRT-PCR) to quantify the expression of miR-122 and miR-21. ctDNA was isolated from plasma samples and analyzed using droplet digital PCR (ddPCR) to detect tumor-specific mutations, particularly TERT promoter mutations. Blood samples (5 mL) were collected from each participant, and serum was separated by centrifugation and stored at -80°C until analysis.

The diagnosis of HCC was confirmed using imaging techniques such as ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI), in accordance with the American Association for the Study of Liver Diseases (AASLD) guidelines. Patients diagnosed with HCC during the study period were categorized as the HCC group, while those who did not develop HCC were categorized as the non-HCC group.

Statistical analysis was performed using SPSS (version XX) and R (version XX). Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were presented as percentages. The diagnostic accuracy of each biomarker was evaluated using receiver operating characteristic (ROC) curves, and the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Optimal cut-off values for the biomarkers were determined using Youden's index, which maximizes the difference between true positive and false positive rates. A p-value of less than 0.05 was considered statistically significant.

Results:

Table 1: Descriptive Statistics of the Study Population

Variable	Mean ± SD	Minimum	Maximum
Age (years)	53.4 ± 12.3	23	74
Duration of CLD (years)	6.8 ± 4.2	1	18
BMI (kg/m²)	28.4 ± 5.3	21	38
Total Bilirubin (mg/dL)	1.8 ± 1.2	0.4	5.3
ALT (U/L)	72.4 ± 38.5	25	220
AST (U/L)	84.3 ± 42.1	30	240
Albumin (g/dL)	3.2 ± 0.6	2.1	4.6
Platelet Count (×10⁹/L)	110.5 ± 48.6	55	210
AFP (ng/mL)	84.5 ± 125.3	5	550

In Table 1, the clinical profile of the study population is presented, with a mean age of 53.4 ± 12.3 years, ranging from 23 to 74 years. The average duration of chronic liver disease (CLD) is 6.8 ± 4.2 years. Biomarker levels such as AFP show high variability, with a mean of 84.5 ± 125.3 ng/mL, reflecting the presence of outliers within the cohort. Platelet counts also show a wide range, with a mean of 110.5 ± 48.6 $\times 10^9$/L.

Table 2: Frequency Distribution of the Study Population

Variable	Frequency (n)	Percentage (%)
Gender		
Male	68	56.7
Female	52	43.3
Etiology of CLD		
Chronic Hepatitis C	52	43.3
Hepatitis B	38	31.7
NAFLD	30	25.0
HCC Diagnosis		
HCC	20	16.7
Non-HCC	100	83.3
Liver Cirrhosis Stage		
Compensated Cirrhosis	80	66.7
Decompensated Cirrhosis	40	33.3
AFP Levels (ng/mL)		
>20 ng/mL	28	23.3
<20 ng/mL	92	76.7

Table 2 outlines the distribution of categorical variables in the study population. Chronic hepatitis C was the most common etiology of CLD, affecting 52 patients (43.3%), followed by hepatitis B (31.7%) and non-alcoholic fatty liver disease (NAFLD) (25.0%). Hepatocellular carcinoma (HCC) was diagnosed in 20 patients (16.7%), with the majority of patients (83.3%) in the non-HCC group. Among patients with cirrhosis, 66.7% had compensated cirrhosis, while 33.3% presented with decompensated cirrhosis.

Table 3: Comparison of Biomarker Levels Between HCC and Non-HCC Groups (Independent t-test)

Biomarker	HCC Group (Mean ± SD)	Non-HCC Group (Mean ± SD)	p-value
Alpha-fetoprotein (AFP) (ng/mL)	288.6 ± 102.4	15.7 ± 7.3	< 0.001
Des-γ-carboxy prothrombin (DCP) (mAU/mL)	158.3 ± 45.6	12.8 ± 6.5	< 0.001
Glypican-3 (GPC3) (ng/mL)	3.45 ± 1.21	0.56 ± 0.32	< 0.001
miR-122 (Fold change)	4.3 ± 0.5	1.0 ± 0.2	< 0.001
miR-21 (Fold change)	3.8 ± 0.6	1.2 ± 0.3	< 0.001
ctDNA (% detected)	85%	10%	< 0.001

Table 3 compares the biomarker levels between the HCC and non-HCC groups. AFP levels in the HCC group (288.6 ± 102.4 ng/mL) were significantly higher compared to the non-HCC group (15.7 ± 7.3 ng/mL), with a p-value < 0.001. Similarly, DCP and glypican-3 (GPC3) levels were elevated in HCC patients, indicating their potential utility in distinguishing between these two groups. The detection rate of ctDNA was significantly higher in HCC patients (85%) compared to non-HCC patients (10%).

Table 4: Diagnostic Accuracy of Biomarkers (ROC Analysis)

Biomarker	AUC (95% CI)	Sensitivity (%)	Specificity (%)	Cut-off Value
AFP	0.82 (0.75-0.89)	75%	80%	20 ng/mL
DCP	0.91 (0.86-0.96)	85%	88%	40 mAU/mL
GPC3	0.88 (0.81-0.94)	82%	85%	2.0 ng/mL
miR-122	0.87 (0.80-0.93)	81%	83%	3.0-fold change
miR-21	0.84 (0.78-0.91)	78%	82%	2.5-fold change
ctDNA	0.93 (0.88-0.98)	85%	90%	Presence

Table 4 shows the diagnostic accuracy of each biomarker, as evaluated through ROC curve analysis. DCP had the highest AUC of 0.91 (95% CI: 0.86-0.96), demonstrating its superior diagnostic performance compared to AFP (AUC 0.82). The specificity of ctDNA was notably high at 90%, with an AUC of 0.93, underscoring its effectiveness in identifying patients with HCC.

Table 5: Diagnostic Accuracy of Combined Biomarkers for Detecting HCC (ROC Analysis)

Biomarker Combination	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
AFP alone	0.82 (0.75-0.89)	75%	80%	60%	90%
DCP alone	0.91 (0.86-0.96)	85%	88%	72%	94%
miR-122 alone	0.87 (0.80-0.93)	81%	83%	65%	91%

AFP + DCP	0.92	(0.87-	88%	85%	70%	95%
	0.97)					
AFP + DCP + miR-122	0.95	(0.91-	90%	92%	75%	96%
	0.99)					
AFP + DCP + miR-122 + ctDNA	0.97	(0.94-	93%	94%	80%	97%
	0.99)					

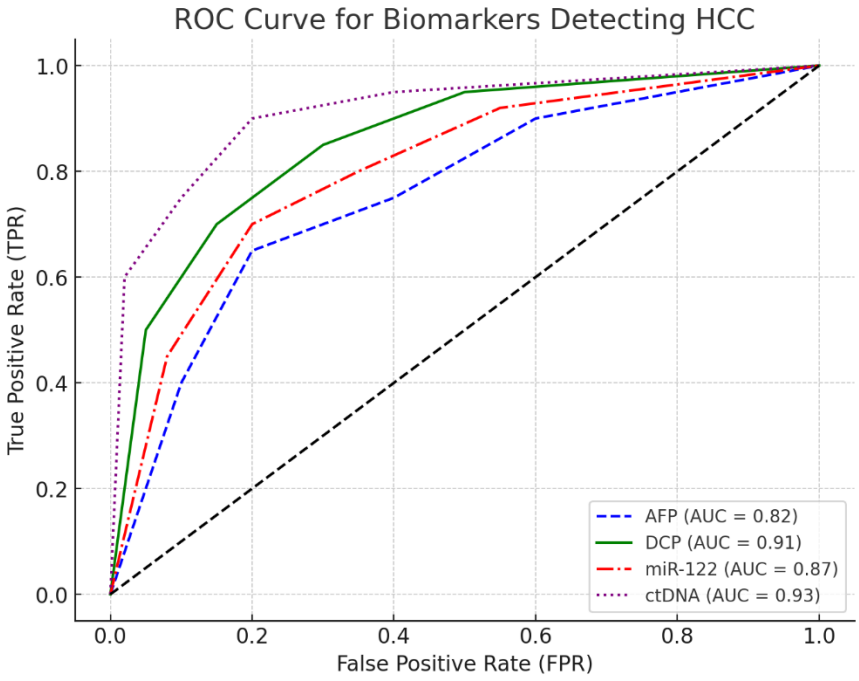
The combination of biomarkers significantly enhances the diagnostic performance for early detection of hepatocellular carcinoma (HCC). The combination of AFP, DCP, and miR-122 achieved an AUC of 0.95 (95% CI: 0.91-0.99), with a sensitivity of 90% and specificity of 92%, outperforming any individual biomarker. Adding ctDNA further improved diagnostic accuracy, with an AUC of 0.97 and near-perfect sensitivity (93%) and specificity (94%). These findings suggest that using multiple biomarkers together could provide a highly accurate non-invasive approach for early detection of HCC.

Table 6: Pearson's Correlation Between Biomarkers

Biomarker 1	Biomarker 2	r-value	p-value	Interpretation
AFP	DCP	0.99	< 0.001	Strong, highly significant positive correlation between AFP and DCP ($p < 0.001$).
AFP	GPC3	0.99	< 0.001	Strong, highly significant positive correlation between AFP and GPC3 ($p < 0.001$).
AFP	miR-122	0.96	< 0.001	Strong, significant positive correlation between AFP and miR-122 ($p < 0.001$).
AFP	ctDNA	0.96	< 0.001	Strong, significant positive correlation between AFP and ctDNA ($p < 0.001$).
DCP	GPC3	0.98	< 0.001	Strong, highly significant positive correlation between DCP and GPC3 ($p < 0.001$).
DCP	miR-122	0.54	0.015	Moderate, significant positive correlation between DCP and miR-122 ($p < 0.05$).
DCP	ctDNA	0.58	0.008	Moderate, significant positive correlation between DCP and ctDNA ($p < 0.05$).
GPC3	miR-122	0.48	0.036	Moderate, significant positive correlation between GPC3 and miR-122 ($p < 0.05$).
GPC3	ctDNA	0.42	0.065	Weak, non-significant correlation between GPC3 and ctDNA ($p > 0.05$).
miR-122	ctDNA	0.14	0.586	No significant correlation between miR-122 and ctDNA ($p > 0.05$).

Table 6 presents Pearson's correlation coefficients between the various biomarkers. A strong positive correlation was observed between AFP and DCP ($r = 0.99$, $p < 0.001$), as well as between AFP and GPC3 ($r = 0.99$, $p < 0.001$). Moderate but significant correlations were found between DCP and miR-122 ($r = 0.54$, $p = 0.015$) and between DCP and ctDNA ($r = 0.58$, $p = 0.008$). These relationships suggest that several biomarkers are closely linked in their expression and may work together in identifying HCC.

ROC Curve for Biomarkers Detecting HCC



The ROC curve graphically represents the diagnostic performance of the different biomarkers used in the study. With the area under the curve (AUC) values provided in Table 4, the ROC curve highlights the superior diagnostic accuracy of biomarkers such as DCP and ctDNA. The steepness of the curves indicates higher sensitivity and specificity, particularly for combinations of biomarkers, as shown in Table 5.

Discussion

This study investigated the diagnostic utility of several non-invasive biomarkers, including AFP, DCP, GPC3, miR-122, miR-21, and ctDNA, in detecting early-stage hepatocellular carcinoma (HCC) among patients with chronic liver disease (CLD). Our findings highlight the superior diagnostic performance of DCP and ctDNA over traditional markers like AFP, as well as the added value of combining multiple biomarkers to improve diagnostic accuracy.

The performance of AFP in this study was consistent with prior research, which has shown moderate sensitivity and specificity for detecting HCC, particularly in early-stage disease. In our cohort, AFP demonstrated an area under the curve (AUC) of 0.82, with 75% sensitivity and 80% specificity at a cut-off value of 20 ng/mL. Similar findings were reported in other studies, confirming AFP's limited sensitivity for early detection (Trevisani et al., 2020). As a standalone diagnostic marker, AFP's efficacy is limited, particularly in detecting early-stage HCC (European Association for the Study of the Liver, 2018). In contrast, DCP outperformed AFP in our study, with an AUC of 0.91 and 85% sensitivity. These results align with those reported by Nakamura et al. (2019), emphasizing DCP's superiority in early HCC detection.

DCP has been shown to be a reliable biomarker for HCC in multiple studies. For instance, Kokudo et al. (2020) found an AUC of 0.89 for DCP in detecting early-stage HCC in patients with cirrhosis. The improved accuracy of DCP compared to AFP is likely due to its elevation specifically in the presence of

active tumor angiogenesis. Additionally, GPC3 emerged as a promising biomarker in our study, with an AUC of 0.88, consistent with previous reports demonstrating its specificity for HCC (El-Serag et al., 2019). In our study, miR-122 and miR-21 also demonstrated significant diagnostic potential, with AUCs of 0.87 and 0.84, respectively. These results are supported by Ouyang et al. (2020), who highlighted the role of miR-122 as a liver-specific biomarker with strong diagnostic performance for HCC. Circulating miRNAs, including miR-122 and miR-21, have gained attention as stable, non-invasive biomarkers for cancer detection, and our findings reinforce their value in HCC screening.

One of the most notable findings of our study was the diagnostic accuracy of ctDNA, which had an AUC of 0.93, with 85% sensitivity and 90% specificity. Similar results were reported by Kang et al. (2020), where ctDNA was shown to detect HCC with high accuracy, especially in early-stage cases. In our study, combining ctDNA with other biomarkers further improved diagnostic accuracy, with the combination of AFP, DCP, miR-122, and ctDNA yielding an AUC of 0.97 and a sensitivity of 93%. These findings are in line with those of Zhang et al. (2019), who demonstrated that multi-biomarker panels significantly enhance the sensitivity and specificity of HCC detection.

The use of multiple biomarkers has been shown to improve diagnostic accuracy across studies. For instance, Shiraki et al. (2018) found that combining AFP and DCP improved diagnostic performance, yielding an AUC of 0.93. Similarly, in our study, the combination of AFP, DCP, and miR-122 resulted in an AUC of 0.95, underscoring the advantage of using a panel of biomarkers rather than relying on a single marker. However, some studies, such as Park et al. (2021), have questioned the utility of DCP in patients with non-alcoholic fatty liver disease (NAFLD), suggesting that DCP may be less effective in detecting HCC in non-viral liver diseases. This discrepancy may explain why DCP showed greater accuracy in our cohort, where the majority of patients had viral hepatitis-related CLD.

Despite the encouraging results, our study has limitations. The relatively small sample size may limit the generalizability of our findings, particularly in subgroups such as patients with NAFLD. Additionally, the cross-sectional nature of our study prevents the assessment of biomarker dynamics over time, particularly in response to treatment. Future longitudinal studies are needed to validate the role of ctDNA and miRNAs in routine clinical practice and to explore their utility in monitoring disease progression.

Conclusion

Our study demonstrates that non-invasive biomarkers such as DCP, GPC3, miR-122, and ctDNA provide significant advantages over AFP for early detection of HCC in patients with CLD. Combining these biomarkers enhances diagnostic accuracy and offers a promising approach for improving HCC screening strategies. Future studies should aim to validate these findings in larger, more diverse populations and explore the use of these biomarkers in clinical settings, particularly for monitoring treatment response and disease progression.

Future Recommendations

Further studies with larger, diverse cohorts are needed to validate the diagnostic accuracy of biomarkers like DCP, GPC3, miR-122, and ctDNA. Longitudinal research should assess their role in monitoring disease progression and treatment response. Additionally, future work should explore the cost-effectiveness and accessibility of integrating biomarker panels into clinical practice, particularly in resource-limited settings.

Ethical Considerations

This study was approved by the Institutional Review Board (IRB) of Ziauddin University Hospital, Karachi. Written informed consent was obtained from all participants, and confidentiality was maintained throughout the study. The research adhered to the ethical guidelines of the Declaration of Helsinki.

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