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تقييم التعبير الجيني لـ miR-21 و miR-122 ومستويات بروتينات AFP , DCP , GPC3 كمؤشرات حيوية تشخيصية في سرطان الخلايا الكبدية

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المستخلص:

يُعد سرطان الخلايا الكبدية (HCC) أكثر أنواع سرطان الكبد شيوعًا، ويظهر عادةً لدى الأفراد المصابين بأمراض الكبد المزمنة الناتجة عن عدوى فيروس التهاب الكبد أو تليف الكبد. ويُمثل التشخيص المبكر لسرطان الخلايا الكبدية ومراقبة الاستجابة للعلاج تحديات سريرية كبيرة. تبحث هذه الدراسة في الأهمية السريرية للعلامات الحيوية AFP وDCP وGPC3 وmiR-21 وmiR-122 في التشخيص المبكر لسرطان الخلايا الكبدية ودورها المحتمل في مراقبة الاستجابة للعلاج. تم مشاركة ١٨٠ مشاركًا وتقسيمهم إلى ثلاث مجموعات، بواقع ٦٠ فردًا في كل مجموعة: مرضى سرطان الخلايا الكبدية الذين يتلقون العلاج، ومرضى سرطان الخلايا الكبدية الذين لا يتلقون العلاج، وأفراد أصحاء كمجموعة ضابطة. تم قياس مستويات AFP وDCP وGPC3 في مصل الدم باستخدام طريقة ELISA، بينما تم تحديد مستويات التعبير عن miRNA-21 وmiR-122 باستخدام تقنية qRT-PCR. أظهرت النتائج ارتفاعًا ملحوظًا في مستويات miRNA-21 وAFP وDCP وGPC3، وانخفاضًا ملحوظًا في مستوى miR-122 لدى مرضى سرطان الخلايا الكبدية مقارنةً بالمجموعة الضابطة، مع وجود فروق واضحة بين المجموعتين المعالجة وغير المعالجة.

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تشير هذه النتائج إلى أن miRNA−21 وmiR−122 وAFP وDCP وGPC3 قد تُمثل مؤشرات حيوية قيّمة لتشخيص سرطان الخلايا الكبدية ومراقبة الاستجابة للعلاج.

الكلمات المفتاحية: سرطان الخلايا الكبدية، المؤشرات الحيوية التشخيصية miR−122 و miRNA−21

Assessment of miR-21 and miR-122 gene expression and AFP, DCP, and GPC3 protein levels as diagnostic biomarkers in hepatocellular carcinoma

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Abstract

The most common type of liver cancer is hepatocellular carcinoma (HCC), which typically occurs in people with chronic liver disease caused by infection with hepatitis viruses or cirrhosis. Diagnostic issues of HCC and evaluation of therapeutic efficacy are large challenges. The aim of this study is to explore the clinical significance of AFP, DCP, GPC3, miR-21, and miR-122 in early detection of hepatocellular carcinoma (HCC) and their role in monitoring therapeutic responses. A total of 180 participants were selected and divided into three groups; treated HCC patients (60 persons), untreated HCC patients (60 persons) and healthy controls (60 persons). AFP, DCP, and GPC3 were quantified in the serum by ELISA method and the expression of miR-21 and miR-122 by q-RT-PCR. Results showed that miRNA-21, AFP, DCP, and GPC3 were significantly higher in HCC patients, and miR-122 was significantly lower in HCC patients compared to

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controls. The outcomes also demonstrate that the treated group differs noticeably from the untreated group. Results suggest that miRNA-21, miR-122, AFP, DCP, and GPC3 could be important biomarkers for the diagnosis of HCC and evaluation of response to therapy.

Keywords: Hepatocellular carcinoma, diagnostic biomarkers, miR-122, and miR-21.

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer that arises from hepatocytes and accounts for approximately 80% of liver cancer cases (Al-Gazally et al., 2023). Dysplastic lesions, which frequently develop in chronic inflammatory liver disease or hepatitis, are the defining features of the early phases of HCC. These lesions contribute to fibrosis and, subsequently, cirrhosis, which ultimately affects liver function and frequently results in death (Alaee et al., 2023). The primary causes of liver injury are viral hepatitis infection, excessive alcohol consumption, nonalcoholic steatohepatitis (NASH), autoimmune hepatitis, aflatoxins exposure, obesity, and diabetes (Ascha et al., 2010). Nevertheless, the early detection of HCC and the monitoring of therapy response are significant clinical challenges. The most frequently employed serologic biomarker for HCC is alpha-fetoprotein (AFP). AFP is a significant plasma protein that is synthesized by the fetal liver during the early stages of life and disappears from the serum shortly after birth (Bandiera et al., 2015; Capurro et al., 2003).

Nevertheless, the serum levels of AFP can fluctuate in specific circumstances, including fatty liver, steatohepatitis, alcohol abuse, liver injury or regeneration, chronic or active hepatitis, hepatoblastoma, hepatic necrosis, HCV-induced liver cirrhosis without HCC, and primary and metastatic liver cancer (Charrière et al., 2016; Faraziet al., 2012). Des-gamma carboxyprothrombin (DCP), also named protein induced by vitamin K absence/antagonist-II (PIVKA-II), is an aberrant prothrombin that is produced by malignant hepatocytes as a consequence of impaired vitamin K-dependent γ-carboxylation during post-translational modification (Gao et al.,

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2012). In 1984, DCP was initially identified as a serum marker in patients with liver cancer. Subsequent investigations have shown that DCP levels are elevated in over 90% of HCC patients, including numerous AFP-negative cases, indicating that it is a valuable biomarker for both diagnostic and prognostic purposes (Guo et al., 2017). Glypican-3 (GPC3), a cell surface-anchored proteoglycan, is glycosyl-phosphatidylinositol-linked to the plasma membrane and serves as a critical molecular regulator of a variety of cellular processes, such as proliferation, differentiation, migration, and adhesion (Huang et al., 2015). GPC3 is a promising biomarker for the diagnosis, progression, and treatment of HCC, as it is significantly expressed during fetal liver development but is absent in normal adult liver tissue (Jemal et al., 2011). MiRNAs are small non-coding RNAs that modulate protein expression by suppressing translation, inducing mRNA cleavage, and promoting mRNA degradation (miRBase, 2022). In HCC, miR-21 has become one of the most consistently upregulated oncogenic miRNAs among the miRNAs. Karakatsanis et al. (2013) suggest that the increased expression of miR-21 may be a promising biomarker that is associated with specific characteristics of HCC, including tumor size, venous invasion, and liver cirrhosis. By targeting numerous tumor suppressor genes and promoting processes such as invasion, metastasis, and epithelial-to-mesenchymal transition (EMT), miR-21 contributes to the aggressive phenotype of HCC (Laudadio et al., 2012). miR-122 functions primarily as a tumor suppressor in HCC, in contrast to the oncogenic role of miRNA-21. miR-122 is the most prevalent miRNA in the liver and is responsible for a variety of liver-specific functions, such as hepatitis virus replication, cellular growth and differentiation, and metabolism. Recent studies have shown that miR-122 is aberrantly regulated in the development of HCC (Libbrecht et al., 2006). In hepatocellular carcinoma (HCC), apoptosis and cell cycle arrest may be induced by the overexpression of miR-122, which inhibits cyclin G1 and Bcl2-like protein 2 (BCL2L2) (Manuc et al., 2020). miR-122 is up-regulated and leads to cell cycle arrest and suppression of HCC cell proliferation through the Wnt/B-catenin-transcription factor (TCF) signaling pathway (Naraki et al., 2002). Significant reduction in the expression levels of miR-

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122 has been reported in the primary HCC tumors compared to cirrhotic liver and in HCC main tumors compared to adjacent normal liver tissue (Panneerselvam et al., 2023). The aim of the present study is to evaluate the clinical relevance of combining traditional serum markers (AFP, DCP, and GPC3) with molecular markers (miR-21 and miR-122) for diagnosis and evaluation of therapeutic response in HCC patients.

Materials and Methods

Study Design

Most cases of HCC are associated with hepatitis B or C virus infection, alcohol-induced cirrhosis, or other chemical carcinogens. A case-control study was performed with a total of 180 patients who were split into three groups, each comprising 60 patients. Group 1 consisted of HCC patients who were currently undergoing treatment; patients received trans arterial chemical embolization. Patients also received systemic chemotherapy and radiotherapy; group 2 consisted of HCC patients who had been newly diagnosed, and group 3 consisted of individuals who appeared to be in good health and served as the control group. The age of the participants varied from 40 to 65 years. To mitigate potential confounding effects, each group was matched as closely as feasible in terms of age and sex. All patients in this investigation had to attend Al-Amal Hospital for Radiation and Nuclear Medicine. The sample size was determined using a statistical power analysis to ensure that it was sufficient to detect a statistically significant difference or association, with a significance level of 0.05. HCC was classified into the following Barcelona Clinic Liver Cancer (BCLC) stages shown in Table 1.

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Table1 - Barcelona Clinic Liver Cancer (BCLC)

Tumor features	stage
Single tumor or 2-3 tumors ≤3 cm	BCLC-A
2-3 tumors with a maximum diameter>3 cm or>3 tumors of any diameter	BCLC-B
Concomitant or isolated portal vein, hepatic vein, or vena cava tumor thrombus; bile duct tumor thrombi; preoperative tumor rupture; tumor metastasis to the lymph nodes; distant metastases	BCLC-C
Any	BCLC-D

COLLECTIONS OF SAMPLES

Venous blood samples of each participant (5ml) were collected and divided into two portions. The first portion was kept in a disposable gel tube and coagulated at room temperature for 10 min, then centrifuged at 4,000 rpm for 10 minutes to separate serum. The serum was then kept in a deep freezer until analysis by ELISA. The second portion was preserved in containers that contained TRIzol reagent and stored at -20°C until needed for RNA extraction and gene expression analysis.

MEASUREMENT OF SERUM AFP, DCP, AND GPC3 LEVELS

The serum levels of AFP, DCP, and GPC3 proteins were measured in all participants. Commercial ELISA kits from Sunlong (China) were used to conduct the measurements, according to the manufacturer's instructions, using the sandwich enzyme-linked immunosorbent assay technique (ELISA).

TOTAL RNA EXTRACTION

The manufacturer's instructions were followed to extract total RNA from blood samples using TRIzol reagent (Thermo Scientific, USA). Purity

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of the isolated RNA was determined by NanoDrop by assessing the absorbance ratio at 260/280 nm.

REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

The extracted RNA was reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (Applied Biosystems, USA) following the manufacturer's instructions. Using TaqMan miRNA assays, quantitative real-time polymerase chain reaction (qRT-PCR) was used to estimate the expression level of miRNA-21 and miR-122. Primers used in this study are shown in Table 2. The endogenous control for normalization was U6 small nuclear RNA (U6 snRNA). The relative expression levels of the miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2- Primer sequences of microRNAs (miR-21 and miR-122)

Sequence (5′→3′)	Primer Type	Target
TAGCTTATCAGACTGATGTTGA	**Forward**	**hsa-miR-21-5p**
TGGAGTGTGACAATGGTGTTTG	**Forward**	**hsa-miR-122-5p**

2.6. Inclusion Criteria

1-Patients aged 18 years or older.

2-Patients with a confirmed diagnosis of hepatocellular carcinoma (HCC) based on clinical, radiological, and/or histopathological criteria.

3-Patients who were diagnosed with or treated for HCC during the predefined study period.

4-Availability of the required clinical and laboratory data.

5-For prospective studies, patients who provide informed consent to participate in the study.

2.6. Exclusion Criteria

1-Patients without a confirmed diagnosis of HCC.

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2-Patients with another primary malignancy that may affect the study outcomes

3-Patients with HCC and insufficient clinical information or inadequate medical records.
4-Patients without the required biological samples or investigations.
5-Patients with mixed liver cancers, such as combined hepatocellular-cholangiocarcinoma (cHCC-CCA).

2.7.Statistical Analysis

GraphPad Prism version 8.0 was employed to conduct all statistical analyses. We conducted a One-Way ANOVA to evaluate the variation among various categories. Pearson's correlation coefficient quantified the correlations among parameters. Statistical significance was denoted by $p<0.05$, and the results were presented as the mean ± standard deviation (SD).

.Results

Quantification of serum AFP, DCP, and GPC3 levels

The levels of AFP, DCP, and GPC3 were significantly higher ($p < 0.0001$) in the newly diagnosed HCC group (ND) than in the control group, as demonstrated in Table 3. Further, the treated group exhibits a lower level of AFP, DCP, and GPC3 than the newly diagnosed HCC group, despite the fact that it remains significantly higher than the control group (Figure 1).

Table (3)- Assessment of AFP, DCP, and GPC3 levels in ND, treated, and control groups

Treated mean± SD	ND mean ±SD	Control mean± SD	Biomarker
5.863± 2.617	8.2531 ± 2.361	4.038± 5.2790	**AFP**
200.576±82.1736	282.879±93.4720	79.7267±33.330	**DCP**
15.385 ± 7.32647	25.501 ± 9.753	3.7721± 2.9210	**GPC3**

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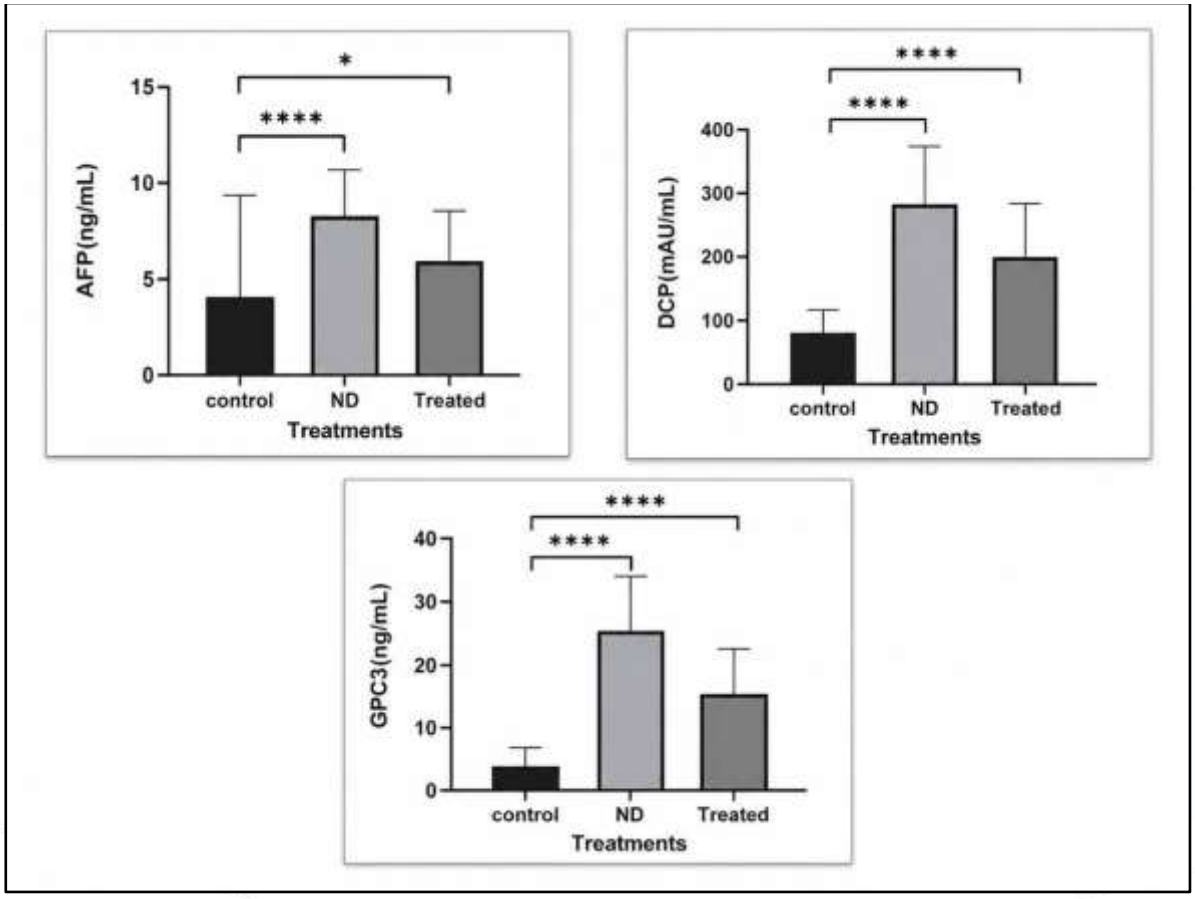

Figure1: Expression of AFP, DCP, and GPC3 in study groups.

CORRELATION ANALYSIS OF PROTEIN LEVELS AMONG STUDY GROUPS

The association between the protein biomarkers examined across the study groups was determined using Pearson's correlation analysis. In Table 4, the results indicate that there was no statistically significant correlation between the measured protein levels of the examined groups.

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Table 4: Correlation between AFP, DCP, and GPC3 in study groups.

Treated group	ND group	Control group	Correlation
0.010(p≥0.05)ns	-0.008(p≥0.05)ns	-0.056(p≥0.05)ns	**AFP vs DCP**
-0.140(p≥0.05)ns	0.019(p≥0.05)ns	-0.083(p≥0.05)ns	**AFP vs GPC3**
0.107(p≥0.05)ns	0.176(p≥0.05)ns	-0.119(p≥0.05)ns	**DCP vs GPC3**

ND: Newly diagnosed, NS: Non-significant

3.3. Serum Biomarker Measurements and Calculation of Scoring Models

were calculated for each biomarker and scoring model. Receiver operating characteristic (ROC) curve analysis was generated to determine the area under the curve (AUC) for each biomarker and model in detecting any-stage and early-stage HCC, and diagnostic performance was evaluated by calculating the sensitivity using different cut-off strategies. demonstrated good discriminatory ability between controls and treated subjects (AUC=0.7722), while its diagnostic performance was excellent in distinguishing HCC patients from controls (AUC=0.9142). The corresponding sensitivities were 85% and 90%, with specificities of 55% and 85%, respectively. Both ROC analyses were statistically significant (P<0.0001), indicating that AFP has stronger discriminatory and diagnostic performance for HCC than for the treated group, as shown in Figure 2.

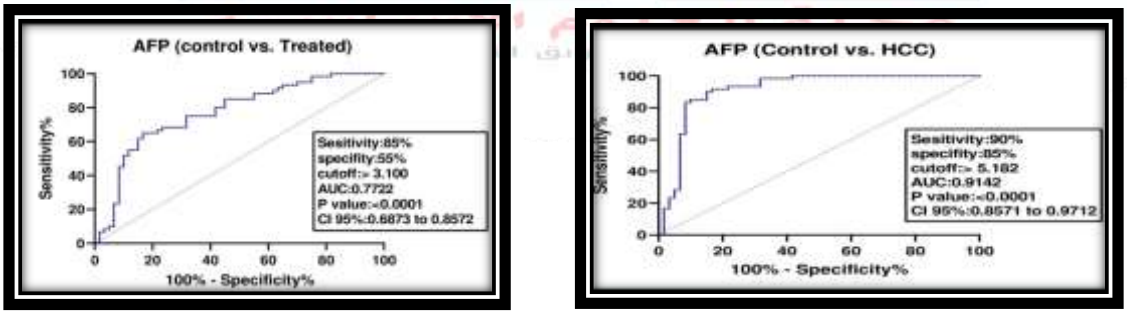

Figure 2: ROC Curves of AFP for Discrimination Between Control, Treated, and HCC Groups

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Results of ROC curve analysis showed that DCP had a high discriminatory ability between the study groups. For the comparison between the control and treated groups, the area under the curve (AUC) was 0.9344, with a sensitivity of 88.33% and specificity of 85% at a cutoff value of>104.8. The result was statistically significant (P<0.0001), with a 95% confidence interval of 0.8902–0.9787. In the comparison between the control group and HCC patients, DCP demonstrated a higher diagnostic performance, with an AUC of 0.9875, sensitivity of 95%, and specificity of 93% at a cutoff value of>131.4. This result was also highly statistically significant (P<0.0001), with a 95% confidence interval of 0.9744–1.000. Overall, these findings indicate that DCP demonstrated excellent discriminatory performance, with a higher diagnostic ability for distinguishing HCC patients from controls than for distinguishing the treated group from controls, as shown in (Figure 3).

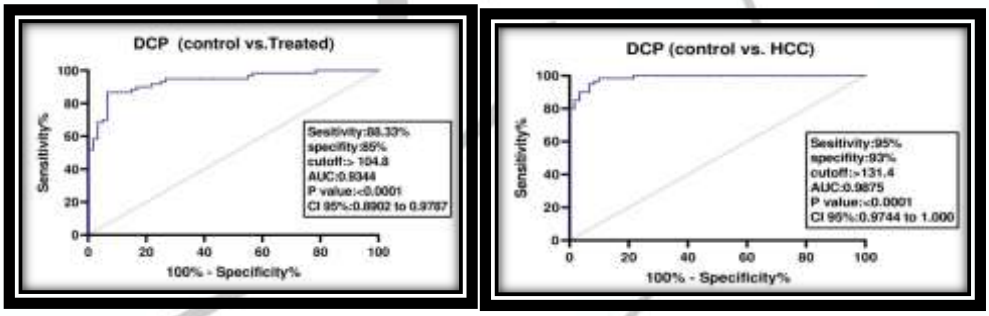

Figure 3: ROC Curves of DCP for Discrimination Between Control, Treated, and HCC Groups

ROC curve analysis showed that GPC3 had excellent diagnostic performance in distinguishing the studied groups. For control vs. treated, GPC3 demonstrated an AUC of 0.9653 (95% CI: 0.9352–0.9953, P < 0.0001), with 88.33% sensitivity and 90.00% specificity at a cutoff value of >7.519. For control vs. HCC, GPC3 showed an even higher diagnostic accuracy, with an AUC of 0.9942 (95% CI: 0.9848–1.000, P < 0.0001), 96.67% sensitivity, and 96.67% specificity at a cutoff value of >10.45. These findings indicate that GPC3 had excellent discriminatory ability, particularly in differentiating HCC patients from controls, as shown in (Figure 4).

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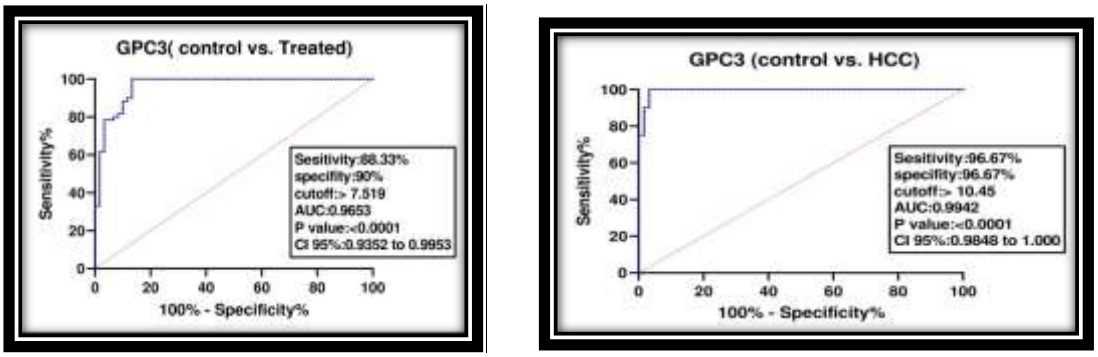

Figure 4: ROC Curves of GPC3 for Discrimination Between Control, Treated, and HCC Groups

3.4.GENE EXPRESSION ANALYSIS OF MIR-122 AND MIR-21

The newly diagnosed HCC group (ND) exhibited a significant decrease in the relative expression of miRNA-122 in comparison to the control group ($p < 0.0001$). Conversely, Table 5 illustrates that the treated group exhibited a substantial increase in miR-122 expression (1.59 ± 0.26, $p < 0.0001$).

Table 5- Relative expression of miR-122 among study groups.

Mean ± SD of Fold Change				
p Value	Treated	ND	Control	**Groups**
<0.0001	1.59 ±0.26c	0.02 ± 0.008b	1.0 ± 0.0a	

ND: Newly diagnosed, different letters (a, b, c) are significant at $p < 0.05$.

The newly diagnosed HCC group demonstrated a highly significant increase in miR-21 expression (44.42 ± 7.49-fold difference) in comparison to the control group. As illustrated in Table 6, the expression level in the treated group decreased to 9.53 ± 2.48 fold in comparison to the newly diagnosed HCC group, despite remaining significantly higher than the control group ($p<0.0001$).

Table 6- Relative expression of miR-21 among study groups.

Mean ± SD of Fold Change				
***p* Value**	**Treated**	**ND**	**Control**	**Groups**
<0.0001	**9.53 ± 2.48c**	**44.42 ± 7.49b**	**1.0 ± 0.0a**	

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ND: Newly diagnosed, different letters (a, b, c) are significant at $p < 0.05$.

.Discussion

In the present investigation, the potential clinical significance of biochemical (AFP, DCP, and GPC3) and molecular markers (miR-21 and miR-122) for early detection of HCC and for monitoring therapy responses was evaluated. Results showed that the molecular and protein biomarkers were considerably different in newly diagnosed HCC patients compared with treated patients and healthy subjects. This indicates that these biomarkers could be important in early detection and tracking of treatment response. Moreover, the newly diagnosed HCC group had significantly higher AFP levels than the control group, indicating that hepatocyte proliferation and tumour activity were increased. The reported reduction in the treated group may reflect a therapeutic response (post-conventional treatment) and reduced tumour burden. AFP is the commonest serological marker used for the diagnosis of HCC. Song et al. (2013) have demonstrated that increased blood AFP is related to the development and progression of HCC, as both a diagnostic and a prognostic indicator.

Another biomarker that is commonly studied for HCC diagnosis and the detection of cancer recurrence is DCP. However, some studies have indicated that simultaneous AFP and DCP evaluation would be more beneficial in diagnosing HCC than stand-alone AFP evaluation. Then, the DCP value was significantly raised in the newly diagnosed HCC group, which has confirmed its role as a tumor-related biomarker associated with abnormal prothrombin production by malignant hepatocytes. Further evidence of its efficacy in evaluating treatment response is the decrease in DCP levels in treated individuals. DCP is produced as a result of an impaired vitamin K–dependent carboxylation mechanism that in turn promotes the malignant proliferation of HCC cells (Sung et al., 2003). It is known that serum DCP levels in various liver diseases, either benign or malignant, are significantly different from normal levels and that it may be more sensitive than AFP (Tsai et al., 2009).

Compared to the healthy group, the newly diagnosed HCC group also shows a significant increase in GPC3. Sung et al. (2003) performed a study

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showing that GPC3 was overexpressed in the tissue samples collected from patients with HCC, and that GPC3 was secreted by the HCC-derived cell lines. In contrast, GPC3 is not expressed in hepatocytes of healthy people or hepatitis patients. GPC3 was reported to be more highly expressed in HCC tissues compared to benign tissues of the liver (Libbrecht et al., 2006). This expression was found to be correlated with the degree of dysplasia and the expression of HCC-related genes (Weitz et al., 1993).

Several miRNAs are particularly associated with HCC; previous studies have shown that miR-21 and miR-122 are the most common miRNAs in the HCC pathway (Wong et al., 2015). The results revealed that miR-21 was highly expressed in newly diagnosed HCC, and it is speculated to play an oncogenic role in regulating the growth, invasion, and apoptosis of HCC. Since the expression levels are reduced in the treated group, this could indicate that the treatment reduced tumor development to some extent.

MiR-21 is a tumor-promoting microRNA that is expressed in many human solid tumors, including stomach, colon, pancreatic, prostate, liver, lung, and breast tumors (Wang et al., 2020). MiR-21 is a well-established survival factor in the development of HCC and in liver damage. It was found that the serum and HCC tissues had an obviously high expression of miR-21. While expression of miR-21 in HCC tissues was not associated with overall survival, high expression of miR-21 is significantly associated with tumor development, and it may be a novel biomarker for HCC prognosis (Zhang et al., 2012).

However, miR-122 expression was down-regulated in newly diagnosed HCC compared to controls. miR-122 is a tumor suppressor miRNA involved in the regulation of intrahepatic metastasis of HCC (Zhou et al., 2018). Many studies have revealed the abnormal expression of miR-22 in many types of cancers such as HCC (Qiao et al, 2017). Ha et al. (2019) showed that decreased expression of miR-122 is commonly observed in HCCs and that miR-122 expression is related to cancer progression. Fang et al. (2022) discovered that miR-122 may be a potential biomarker and aid in the early detection of HCC.

Combining biochemical and molecular biomarkers led to better diagnostic results as compared to using single indicators. The combination of AFP, DCP, and GPC3 along with miR-21 and miR-122 can potentially provide a more specific and sensitive approach to the early detection and monitoring of HCC. Furthermore, these markers could prove useful in tracking the disease process and response to treatment, as there are differences between treated and untreated individuals. Overall, the findings from the current study highlight the importance of combining molecular markers with conventional serum markers for better diagnosis and management of HCC. There are many limitations to this research that should be taken into account. First, there might have been an impact of the relatively small sample size on the generalizability of the results. Secondly, this study did not involve a thorough analysis of the possible influence of different clinical and biological situations on the levels of expression of the blood-based miRNAs.

In the present study, AFP demonstrated good discriminatory ability between control and treated groups. In contrast, AFP showed excellent diagnostic performance in distinguishing HCC patients from controls. The lower specificity observed in the control versus treated comparison may be related to variations in AFP levels caused by underlying hepatic inflammation or liver disease, as AFP can be elevated in the absence of HCC. Moreover, the diagnostic performance of AFP can vary according to the selected cut-off value, tumor stage, and tumor size. These findings are consistent with previous studies reporting good discriminatory performance of AFP for HCC, although its sensitivity and specificity vary among different patient populations and cut-off values. Overall, the present findings suggest that AFP has a stronger diagnostic ability for distinguishing HCC patients from controls than for distinguishing treated patients from controls. (Lok et al., 2010.).

The present study demonstrated excellent diagnostic performance of DCP in distinguishing patients with hepatocellular carcinoma (HCC) from the control group, This indicates that DCP had a very high ability to discriminate between HCC patients and controls. These findings are consistent with previous evidence supporting DCP as a useful biomarker for HCC diagnosis.

The authors also reported that differences in assay methods, ethnicity, tumor size, and study design could contribute to variation in diagnostic performance among studies.

The higher AUC observed in the present study (0.9875) compared with the pooled AUC reported in previous studies may be related to differences in patient characteristics, sample size, disease stage, cutoff values, and assay methods. Another systematic review reported an AUC of 0.9002, with pooled sensitivity and specificity of 66% and 88%, respectively, further supporting the diagnostic value of DCP for HCC. Therefore, the high sensitivity, specificity, and AUC observed in the present study suggest that DCP has excellent discriminatory ability and may be a valuable biomarker for differentiating HCC patients from controls. (Liu et al., 2014.).

The high diagnostic performance of GPC3, particularly in the control vs. HCC comparison, can be explained by the abnormal overexpression of GPC3 during the development of hepatocellular carcinoma. GPC3 is highly expressed in HCC tissues and is considered a useful biomarker for distinguishing HCC from non-malignant liver tissue. (Jiang et al., 2022.).

.Conclusions

To sum up, AFP, DCP, GPC3, miR-21, and miR-122 revealed significant diagnostic potential for HCC. These can be used together to enhance the ability for early diagnosis and evaluation of treatment response in patients with HCC. Last but not least, further molecular and functional studies are warranted to gain insight into the mechanisms by which these biomarkers are associated with HCC pathogenesis and response to therapy.

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