



Combined Biomarkers for Early Diagnosis of Hepatocellular Carcinoma

Tufael ¹, Md Mostafizur Rahman ², Vijay Jagdish Upadhye ^{3*}, Md Faruk Hossain ², Nasir Uddin ⁴

Abstract

Objective: This retrospective study aimed to evaluate the efficacy of serum biomarkers, specifically alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and cancer antigen 19-9 (CA19-9), individually and in combination, for screening and diagnosing primary hepatic cancer (PHC) in asymptomatic patients. **Methods:** A retrospective cross-sectional analysis of 1369 patients from October 2022 to November 2023 was analyzed. Preoperative serum levels of AFP, CEA, and CA19-9 were measured and correlated with histological findings. Sensitivity, specificity, and predictive values were calculated for each biomarker alone and in combination. **Results:** Patients with PHC exhibited significantly elevated levels of AFP, CEA, and CA19-9 compared to those with liver cirrhosis and healthy controls ($P < 0.03$). AFP alone showed moderate sensitivity (64.6%) and high specificity (81.7%) for PHC diagnosis. However, combining AFP with CA19-9 and CEA achieved 100% specificity while drastically reducing sensitivity (2.4%). Histological evaluation revealed varying AFP levels across tumor differentiation grades. **Conclusion:** Despite the elevated levels of CEA and CA19-9 in PHC patients, combining these biomarkers with AFP did not significantly enhance

diagnostic accuracy compared to AFP alone. The study suggests that AFP remains a valuable biomarker for screening and diagnosing hepatocellular carcinoma, but further investigations are warranted to explore additional biomarkers or complementary diagnostic modalities for improving early detection and management.

Keywords: Hepatocellular Carcinoma, Serum Biomarkers, Alpha-Fetoprotein, Carcinoembryonic Antigen, Cancer Antigen 19-9.

Introduction

Hepatocellular carcinoma (HCC) stands as a formidable challenge in the landscape of oncology; characterized by its escalating incidence and alarming mortality rates globally as the fifth most common cancer and the third leading cause of cancer-related deaths worldwide (Aghoram et al., 1996), HCC imposes a significant burden on healthcare systems and communities alike. An estimated 800,000 new cases emerge annually, with over half of these occurrences in Bangladesh alone. In mainland Bangladesh, the male population bears the brunt of this malignancy, with an incidence rate outstripping that of South Asia (Ahmadpour et al., 2019). The surge in HCC incidence over recent decades finds its roots intertwined with the pervasive prevalence of chronic hepatitis B virus (HBV) infection, particularly prevalent in Bangladesh, where over 93 million individuals carry the HBV burden (Akter et al., 2024). Consequently, a pressing need arises for effective screening and diagnostic strategies to intercept HCC at its nascent stages when treatment options are most efficacious (Akter et al., 2024).

Significance | This retrospective study assessed AFP, CEA, and CA19-9 efficacy, highlighting AFP's importance in early hepatocellular carcinoma detection.

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Currently, the cornerstone of HCC treatment revolves around comprehensive therapeutic modalities, predominantly surgical interventions like resection and liver transplantation (Amarapurkar et al., 2009). However, the non-specific symptomatic nature of HCC often leads to delayed diagnoses, limiting the availability of surgical options and compromising patient outcomes. Consequently, despite advancements in treatment modalities, the prognosis for HCC patients remains bleak, with median survival rates ranging from a mere 6 to 20 months following diagnosis (Bruix et al., 2005). The imperative of early detection underscores the pivotal role of screening and surveillance efforts, endorsed by esteemed medical societies such as the American Association for the Study of Liver Disease (AASLD), the National Comprehensive Cancer Network (NCCN), and the Asian Pacific Association for the Study of the Liver (APASL) (Chen et al., 2018). Leveraging diagnostic imaging techniques such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) constitutes a crucial aspect of early detection initiatives. However, despite their utility, challenges persist in standardizing the sensitivity and specificity of these imaging modalities (De Stefano et al., 2018).

Given these challenges, serum biomarkers emerge as promising adjuncts against HCC, offering non-invasive, objective, and reproducible assessments (Edoo et al., 2019). Serum alpha-fetoprotein (AFP), cancer antigens (CA19-9), and carcinoembryonic antigen (CEA) represent notable candidates in this regard, holding potential as screening and prognostic tools (El Makarem et al., 2012). In this study, we evaluate AFP, CA19-9, and CEA levels, probing their diagnostic efficacy and exploring potential prognostic correlations, thereby contributing to the refinement of early detection strategies for HCC (El-Serag et al., 2014). HCC remains a complex and multifaceted disease, demanding a comprehensive understanding of its etiology, progression, and diagnostic modalities to combat its deleterious effects effectively (El-Serag et al., 2014). As such, this study elucidates the intricate interplay between serum biomarkers and HCC, shedding light on their diagnostic utility and prognostic value (Farinati et al., 2006). By scrutinizing AFP, CA19-9, and CEA levels, we aim to discern patterns that may aid clinicians in early detection, allowing patients the opportunity for timely intervention and improved outcomes (Farrell., 2010).

The efficacy of diagnostic imaging techniques in detecting HCC hinges not only on their sensitivity and specificity but also on factors such as accessibility, cost-effectiveness, and ease of implementation (Forner et al., 2014). Ultrasonography, CT, and MRI represent frontline modalities, each harboring distinct advantages and limitations (Johnson et al., 2001). By critically evaluating these modalities alongside serum biomarkers, we seek to delineate an integrated approach to HCC screening and diagnosis,

capitalizing on each modality's strengths while mitigating their shortcomings (Khien et al., 2001).

Moreover, as the landscape of HCC management continues to evolve, driven by advancements in precision medicine and targeted therapies, the need for robust prognostic markers becomes increasingly pronounced. Serum biomarkers offer a tantalizing avenue, providing objective metrics that may aid in risk stratification and treatment planning (Kudo et al., 2010). By correlating AFP, CA19-9, and CEA levels with clinical outcomes, we aspire to give clinicians actionable insights into patient prognosis, empowering them to tailor treatment strategies to individual patients' needs (Llovet et al., 1999). In HCC represents a significant public health challenge, necessitating a concerted effort to enhance early detection and improve patient outcomes. This study contributes to this collective endeavor by interrogating the diagnostic utility and prognostic value of serum biomarkers in HCC. By unraveling the intricate relationships between AFP, CA19-9, and CEA levels and disease progression, we aspire to furnish clinicians with valuable tools for early detection, risk stratification, and treatment optimization, ultimately advancing the fight against this formidable malignancy.

This study aimed to evaluate the effectiveness of serum biomarkers, including alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and cancer antigen 19-9 (CA19-9), both individually and in combination, in facilitating the early detection of hepatocellular carcinoma (HCC) among asymptomatic patients. Firstly, the sensitivity and specificity of these biomarkers were determined individually. Subsequently, the diagnostic performance of combining AFP with CEA and CA19-9 was assessed to enhance HCC identification. Additionally, the study established correlations between serum biomarker levels and histological findings to distinguish HCC from liver cirrhosis and healthy controls. It also explored how tumor differentiation grades may influence AFP, CEA, and CA19-9 serum levels among HCC patients. Finally, the study offered insights into the clinical implications of its findings and proposed recommendations for improving early detection strategies for HCC, potentially informing better screening protocols and diagnostic algorithms.

Materials and Methods

Study design

It is a cross-sectional study. A retrospective analysis of patients diagnosed with primary hepatic cancer and liver cirrhosis was collected from the hospital database. The patients enrolled in this study were chosen according to predefined inclusion criteria such as confirmed pathological diagnosis, all the 3 serum biomarkers under study, and preoperative serum biomarkers level. Subjects with major underlying diseases, metastatic history, or who had undergone treatment such as radiotherapy, chemotherapy, or

endocrine therapy were excluded. Patients who fulfilled the criteria mentioned above were selected for the study. The primary hepatic cancer patients and the liver cirrhosis patients were compared to a group of healthy individuals from the same period as mentioned above. In this study, 1075 primary hepatic cancer patients were selected and represented the primary hepatic cancer group (Group A), 239 liver cirrhosis patients were selected and represented the Cirrhosis group (Group B) and 55 healthy individuals were selected as control (Group C). After patients were grouped, we analyzed the preoperative tumor markers level of these three different biomarkers AFP, CA19-9, and CEA.

Patient inclusion criteria

The study included 1,369 subjects admitted for treatment or had their physical check-up in the Ibn Sina Diagnostic and Imaging Center (Dhaka, Bangladesh) from October 2022 to November 2023. A total of 1075 subjects were patients with primary hepatic cancer (PHC). Of these 1075 PHC study subjects, 909 were male and 166 were female with an age range of 17 to 90 yr, with an average of 55.9 ± 11.0 years. 239 patients were diagnosed with liver cirrhosis, they were 187 men and 52 women, with a mean age of 49.85 ± 13 yr (range: 15-75 yr). 55 healthy individuals who underwent physical examinations in the same hospital were also included in this study. They served as a control in this study and were composed of 37 men and 18 women, with a mean age of 62 ± 5 years (range: 35-78 yr). Patients enrolled in the study were chosen because they had no other underlying diseases or conditions and no study subject received chemotherapy or radiotherapy. The data were retrieved from the Department of Biochemistry at Ibn Sina Diagnostic and Imaging Center.

Blood Biochemistry analysis

AFP, CA19-9, and CEA were determined with an Advia XPT (USA) by Ibn Sina Diagnostic and Imaging Center, Dhaka, Bangladesh. We used widely established Chemiluminescent Immunoassay (CLIA) techniques with an Advia XPT, USA, for measuring alpha-fetoprotein (AFP), CA 19-9, and CEA. For the experiment, we used a fresh sample. The reference values of different parameters were the following. AFP <20 ng/L was negative, and AFP >20 ng/L was positive. In the case of CA 19.9 <37 U/L, we considered negative and >37 U/L positive. CEA <5 ng/L is considered negative, and CEA >5 ng/L is considered positive.

Histology analysis

The histology analysis phase involved the examination of tissue samples obtained through biopsy or surgical resection from patients suspected of having hepatocellular carcinoma (HCC) at the Ibn Sina Diagnostic and Imaging Center in Dhaka, Bangladesh and aimed to provide definitive diagnostic information regarding the presence and characteristics of HCC. Tissue specimens were processed, embedded in paraffin, and sliced into thin sections. These sections were then stained with specialized dyes such as

hematoxylin and eosin (H&E) to visualize cellular structures and identify any abnormal changes indicative of HCC, such as hepatocellular dysplasia or neoplastic growth. Skilled pathologists examined the stained tissue slides under a microscope, meticulously analyzing cellular morphology, architecture, and other histological features to confirm or refute the presence of HCC. This histological analysis played a crucial role in providing accurate diagnoses and guiding appropriate treatment strategies for patients with suspected liver cancer.

Statistical analysis

Statistical analysis was performed using SPSS version 26.0 statistical software (SPSS Inc., Chicago, IL, United States). The data were expressed as mean \pm SD. Measurement data between groups were compared with the t- t-distribution. All tests were two-tailed, and a P-value of less than 0.05 was considered statistically significant.

Ethical considerations

Ethical considerations were paramount throughout the study at Ibn Sina Diagnostic and Imaging Center in Dhaka, Bangladesh. Informed consent was obtained from all participants, ensuring their voluntary participation and data usage. The study adhered to principles of patient confidentiality, and all data were anonymized to protect individual privacy. Ethical approval was obtained from the relevant institutional review board at Ibn Sina Diagnostic and Imaging Center. **Reference Number: 11371-2/2023/TAB (713/2023)**. Patients were informed of their right to withdraw from the study at any time without consequences. The study followed ethical guidelines and principles to safeguard the well-being and rights of the participants while conducting valuable research on hepatocellular carcinoma diagnosis.

Results

The data highlight a diverse educational landscape among respondents, with a significant proportion achieving a higher secondary certificate or above (48.4%), while substantial numbers completed primary education (17.9%) or lacked formal education (3.5%). The occupational distribution reveals a variety of employment statuses, including government jobs (11.9%), private jobs (14.3%), and free business (25.5%), among others. Urban residence predominates, with 67% of respondents living in urban areas compared to 33% in rural areas (Table 1).

The distribution of serum biomarker levels, namely AFP, CA19-9, and CEA, across distinct age groups. AFP levels exhibit a progressive increase with age, with individuals aged 60 and above demonstrating the highest mean level (6000 ± 400 ng/L), followed by the 40-59 age group (4500 ± 300 ng/L), and those under 40 (3000 ± 200 ng/L). Similarly, with increasing age, CA19-9 and CEA levels show a similar elevation trend. These findings suggest a potential association between age and serum biomarker levels, which may

have implications for diagnostic and screening protocols for hepatic malignancies across different age demographics (Figure 1).

The variations in serum biomarker levels, including AFP, CA19-9, and CEA, relative to tumor stage among hepatocellular carcinoma patients. As the tumor stage advances from Stage I to Stage IV, there is a noticeable escalation in the mean levels of all three biomarkers. AFP levels, for instance, show a steady increase from 2500 ± 150 ng/L in Stage I to 8500 ± 400 ng/L in Stage IV. Similarly, CA19-9 and CEA levels exhibit an upward trend with advancing tumor stages. These findings underscore the potential utility of serum biomarkers in assessing tumor progression and staging, thereby aiding in treatment planning and prognosis determination for patients with hepatocellular carcinoma (Figure 2).

The correlations between serum biomarker levels (AFP, CA19-9, and CEA) and several liver function tests, including albumin, total bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The values indicate the strength and direction of the correlation coefficient between each biomarker and liver function test. For instance, AFP levels show a weak negative correlation with albumin (-0.23) and a weak positive correlation with total bilirubin (0.18), ALT (0.21), and AST (0.20). CA19-9 and CEA also exhibit varying degrees of correlation with these liver function tests. These correlations provide insights into the relationship between serum biomarkers and liver function, which can be valuable in assessing liver health and disease progression (Figure 3).

Summarizes the serum biomarker levels in the study groups. Group A, consisting of patients with primary hepatic cancer (PHC), showed substantially higher levels of AFP, CA19-9, and CEA compared to Groups B (liver cirrhosis) and C (control). Specifically, Group A had a mean AFP level of 4436.47 ng/L, CA19-9 level of 42.90 U/mL, and CEA level of 3.71 ng/L. In contrast, Group B had lower levels of AFP (29.41 ng/L), CA19-9 (24.88 U/mL), and CEA (3.76 ng/L). Group C had the lowest levels, with AFP mostly below the detectable limit, CA19-9 at 21.42 U/mL, and CEA at 2.66 ng/L. These findings highlight the significant elevation of biomarkers in PHC patients compared to cirrhosis and healthy controls, suggesting their potential utility in diagnosis (Table 2).

The positive rates of serum biomarker combinations in different study groups. In the primary hepatic cancer (PHC) group, AFP alone had the highest positive rate at 65.35%, followed by combinations of AFP with CEA (7.9%) and AFP with CA19-9 (7.4%). The combination of all three biomarkers (AFP, CEA, and CA19-9) had the lowest positive rate in the PHC group at 2.6%. In the cirrhosis group, the positive rates were notably lower across all combinations, with AFP alone showing the highest positive rate (24.3%). Similarly, in the control group, AFP alone had the highest positive rate (4%), while combinations with CEA and CA19-9 showed minimal positivity (Figure 4).

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of AFP alone and in combination with CA19-9 and CEA. AFP alone showed a sensitivity of 64.6% and a specificity of 81.7%. However, combining AFP with CA19-9 or CEA resulted in lower sensitivity (8.1% and 7.5%, respectively) but higher specificity (95.22% and 95.56%, respectively). Combining AFP with both CA19-9 and CEA further increased specificity to 100% but reduced sensitivity to 2.4%. While combinations improved specificity, they sacrificed sensitivity, potentially limiting their effectiveness in diagnosis (Table 3).

This table displays serum biomarkers' positive predictive values (PPV) and negative predictive values (NPV) for diagnosing primary hepatic cancer. AFP alone showed a PPV of 93% and an NPV of 36%. When combined with CA19-9 or CEA, the PPV decreased slightly to 85% and 92%, respectively, while the NPV decreased to 20% and 21%, respectively. Combining AFP with both CA19-9 and CEA resulted in a PPV and NPV of 100% and 22%, respectively. Given the presence or absence of the biomarker combinations, these values indicate the probability of a positive or negative diagnosis, underscoring the importance of considering both sensitivity and specificity in diagnostic accuracy (Figure 5).

Serum biomarker levels according to pathological differentiation in hepatocellular carcinoma (HCC). Poorly differentiated HCC showed significantly higher AFP levels (9566.14 ± 24902.61 ng/L) than other types. Moderately-poorly differentiated HCC had the second-highest AFP levels (8270.17 ± 21781.66 ng/L), followed by moderately differentiated HCC (1786.38 ± 7822.22 ng/L). Well-differentiated HCC had the lowest AFP levels (46.19 ± 191.27 ng/L). CEA levels showed no consistent trend across pathological types, ranging from 2.49 ± 1.42 ng/L in well-differentiated HCC to 4.56 ± 7.88 ng/L in combined hepato-cholangiocarcinoma. CA19-9 levels varied similarly, with the highest levels in combined hepato-cholangiocarcinoma (115.29 ± 376.71 U/mL) and the lowest in well-differentiated HCC (14.60 ± 13.96 U/mL) (Table 4).

The patients in the primary hepatic cancer group, group A, were further divided according to their pathological types as follows: poorly differentiated hepatocellular carcinoma (105 patients); moderately poorly differentiated hepatocellular carcinoma (415 patients); moderately differentiated hepatocellular carcinoma (420 patients); well- moderately differentiated hepatocellular carcinoma with 47 study subjects; Well-differentiated hepatocellular carcinoma with 25 study subjects; and combined hepatic-cholangiocarcinoma with 66 study subjects. Their mean serum biomarkers for all three biomarkers were calculated, and the positive rates of AFP combined with CA19-9 and CEA were calculated in the different groups (Figure 6).

The significance of AFP CA19-9 and CEA levels among groups
Significant differences in mean serum levels and positive AFP, CEA, and CA19-9 rates were observed between the PHC and the

other two groups ($P < 0.03$). No significant differences existed between the control and benign liver cirrhosis groups, as shown in Tables 3-4. In the primary hepatic cancer group (group A), an increased AFP level above the cut off level was observed in 681 study subjects, accounting for 65.35% of the PHC patients; 83 subjects had increased AFP and CEA levels (7.5%); 78 subjects had increased AFP and CA19-9 levels (7.4%); and 27 subjects had increased levels of AFP, CA19-9, and CEA (2.6%). The mean serum marker level for AFP was 4436.47 ± 15094.35 ng/L, 42.90 ± 352.38 U/mL for CA19-9, and 3.71 ± 3.96 ng/L for CEA in the HCC group. In the Liver cirrhosis group (group B), 53 out of the 237 patients tested positive for elevated AFP (24.3%); 6 tested positive for AFP and CEA (2.4%); 15 tested positive for AFP and CA19-9 (6.9%); and none tested positive for AFP, CA19-9, and CEA (0%). The mean AFP level was 29.41 ± 72.17 ng/L, the mean CA19-9 level was 24.88 ± 35.34 U/mL, and the mean CEA level was 3.76 ± 1.58 ng/L. In the control group, 2 out of the 55 patients tested positive for AFP (4%); 1 positive for AFP and CEA (2%), 1 positive for AFP and CA19-9 (2%); and none tested positive for AFP, CEA, and CA19-9. The mean AFP level was <20.00 ng/L, the mean CA19-9 level was 21.42 ± 14.56 U/mL and the mean CEA level was 2.66 ± 1.73 ng/L. The mean serum AFP, CA19-9, and CEA levels were higher in the PHC group than in the other liver cirrhosis and control groups.

AFP levels increase significantly in primary liver cancer compared to other markers. Comparing serum biomarkers levels in the primary hepatic cancer group (group A) to the cirrhotic group (group B) and the control group (group C), significant statistical differences ($P < 0.03$) were observed between the malignant group and the benign group whereas no statistically significant difference ($P > 0.06$) were present between the Liver cirrhosis group and the healthy control group, thus indicating higher serum AFP, CA19-9 and CEA mean levels in the cancer group as compared to the other two groups.

Moreover, a higher positive rate of the different combinations of biomarkers was observed in the PHC group compared with the other two groups. The mean serum marker level for AFP in the cancer group was 4436.47 ± 15094.35 ng/L as compared to 29.41 ± 72.17 ng/L in the Liver cirrhosis group and <20.00 ng/L in the healthy control group. This shows that AFP serum level greatly increased in the cancer group by 154-fold compared to the liver cirrhosis group. The mean serum marker level for CA19-9 in the cancer group was 42.90 ± 352.38 U/mL as compared to 24.88 ± 35.34 U/mL in the Liver cirrhosis group and 21.42 ± 15.56 U/mL in the healthy control group. This shows that CA19-9 serum level had a considerable increase in the cancer group by 2-fold as compared to the liver cirrhosis group and the control group. The mean serum marker level for CEA in the cancer group was 3.71 ± 3.76 ng/L as compared to 3.76 ± 1.58 ng/L U/mL in the Liver cirrhosis group and 2.66 ± 1.73 ng/L in the healthy control group. This shows a 1.2-fold

increase in serum level CEA in the cancer group compared to the liver cirrhosis group and the control group. Thus, this finding indicates increased serum AFP levels in the Primary Hepatic Cancer patients compared to the other 2 groups. Also, a slight increase in serum CA19-9 and CEA levels were noted compared to a large increase observed in AFP levels.

AFP compares to combination markers as stand-alone marker

As a stand-alone marker, AFP showed a sensitivity of 64.6%, and a specificity of 81.7%, with a 93% PPV and a 36% NPV. Combining AFP with CA19-9, a sensitivity of 8.1%, a specificity of 95.22% with 85% PPV and a 20% NPV was observed. The combination of AFP with CEA showed a sensitivity of 7.5%, a specificity of 95.56% with 92% PPV and a 21% NPV. The combination of all three markers AFP CA19-9 and CEA showed a sensitivity of 2.4%, specificity of 100% with 100% PPV and a 22% NPV. Though a higher specificity was observed with combined AFP, CA19-9, and CEA (100%) with a high Positive predictive value (100%), the sensitivity was very low (2.4 %) compared to the sensitivity of AFP alone. The lower sensitivity observed in the combinations of the three serum biomarkers prevents them from being used as a potential diagnostic tool and thus has no superior advantage as a diagnostic and screening tool than AFP alone.

Combined AFP, CA19-9, and CEA have increased specificity but decreased sensitivity. The sensitivities and specificities of the different combinations of the serum biomarkers and their positive predictive values (PPV) and negative predictive values (NPV) were computed as shown in Table 3. The sensitivities of AFP, AFP, and CA19-9; AFP and CEA; and AFP, CA19-9, and CEA were 64.6%, 8.1%, 7.5%, and 2.4%, respectively. The sensitivity of AFP alone was greater than that of AFP combined with the other two biomarkers. The specificities of AFP, AFP, and CA19-9, AFP and CEA, and AFP, CA19-9, and CEA were 81.7%, 95.22%, 97.5%, and 100%, respectively. The specificity of AFP combined with CA19-9, CEA, CA19-9, and CEA were higher than the specificity of AFP alone. The PPV of AFP, AFP, and CA19-9; AFP and CEA; and AFP, CA19-9, and CEA were 92%, 85%, 93%, and 100%, respectively. The NPV of AFP, AFP, and CA19-9; AFP and CEA; and AFP, CA19-9, and CEA were 36%, 20%, 21%, and 22%, respectively.

Pathological differentiation correlates with significantly increased AFP levels but not CEA and CA19-9 levels.

To assess the correlation between serum markers and degree of differentiation, 104 patients diagnosed with poorly differentiated HCC had a mean AFP level of 9566.14 ± 24902.61 ng/L, mean CA19-9 level of 44.35 ± 208.86 U/mL and mean CEA level of 3.01 ± 2.52 ng/L and 1 patient out of the 104 was positive for all the 3 biomarkers. Of the patients diagnosed with moderate-poorly differentiated HCC, 412 patients had a mean AFP level of 8270.17 ± 21781.66 ng/L, mean CA19-9 level of 54.27 ± 308.64 U/mL and mean CEA level of 2.98 ± 4.63 ng/L and ten patients out of the

Table 1. Demographic Characteristics of the Respondents (n=1369)

Variable	Number	Percentage
Level of Education		
Non-education	48	3.5
Primary School	245	17.9
Secondary School Certificate	413	30.2
Higher Secondary Certificate	662	48.4
Occupation		
Government Job	163	11.9
Private Job	195	14.3
Free Business	349	25.5
No Job	375	27.4
Teaching	85	6.2
Farming	186	13.6
Disabled	15	1.1
Residential Area		
Urban	917	67
Rural	452	33

Table 2. Serum Biomarker Levels in Study Groups

Group	N	AFP (ng/L)	CA19-9 (U/mL)	CEA (ng/L)
A	1075	4436.47±15094.35	42.90±352.38	3.71±3.96
B	239	29.41±72.17	24.88±35.34	3.76±1.58
C	55	<20.00	21.42±14.56	2.66±1.73

Table 3. Sensitivity and Specificity of AFP Alone and Combined with CA19-9 and CEA

Serum Biomarkers	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
AFP	64.6	81.7	93	36
AFP + CA19-9	8.1	95.22	85	20
AFP + CEA	7.5	95.56	92	21
AFP + CA19-9 + CEA	2.4	100	100	22

Table 4. Serum Biomarker Levels by Pathological Differentiation

Diagnosis	N	AFP (ng/L)	CEA (ng/L)	CA19-9 (U/mL)	AFP + CEA + CA19-9 (n)
Poorly Differentiated HCC	104	9566.14±24902.61	3.01±2.52	44.35±208.86	1
Moderately-poorly Diff HCC	412	8270.17±21781.66	2.98±4.63	54.27±308.64	10
Moderately Diff HCC	422	1786.38±7822.22	2.97±1.89	22.94±58.08	13
Well-moderately Diff HCC	45	184.20±656.20	3.79±2.43	25.03±24.82	1
Well Diff HCC	27	46.19±191.27	2.49±1.42	14.60±13.96	0
Combined Hepato-Cholangiocarcinoma	65	2234.73±6358.24	4.56±7.88	115.29±376.71	2

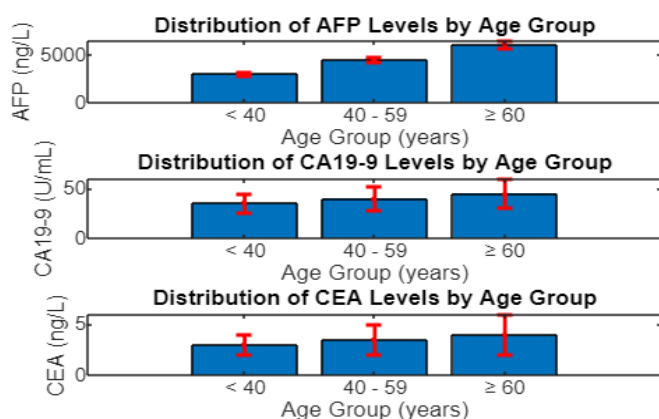


Figure 1. Distribution of Serum Biomarker Levels by Age Group

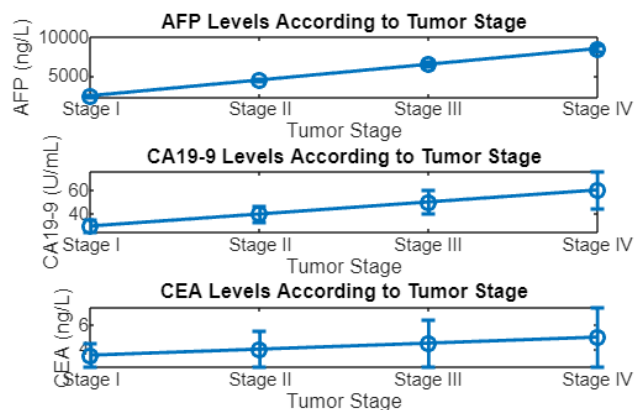


Figure 2. Serum Biomarker Levels According to Tumor Stage

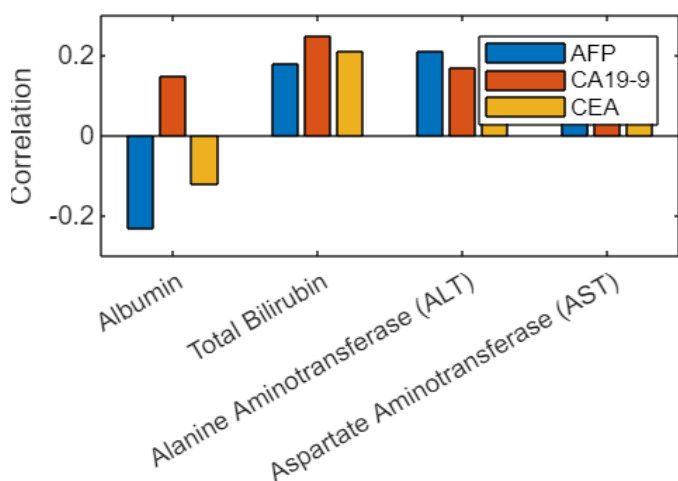


Figure 3. Serum Biomarker Correlations with Liver Function Tests

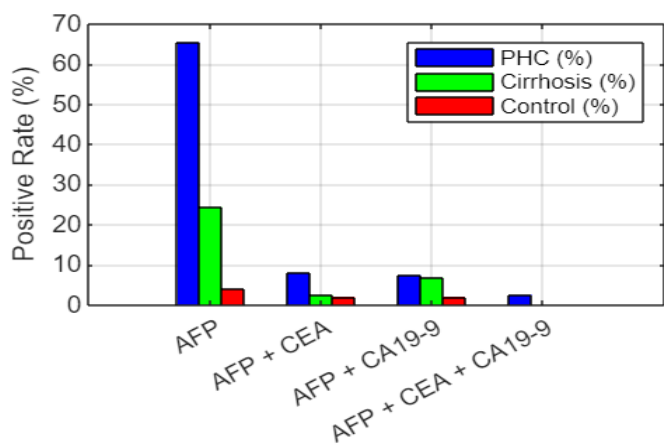


Figure 4. Positive Rates of Serum Biomarker Combinations

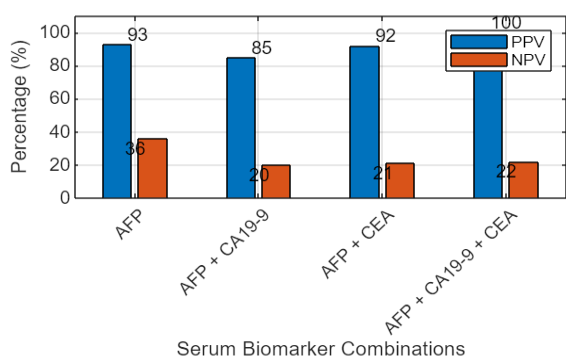


Figure 5. Positive Predictive Values (PPV) and Negative Predictive Values (NPV) of Serum Biomarkers

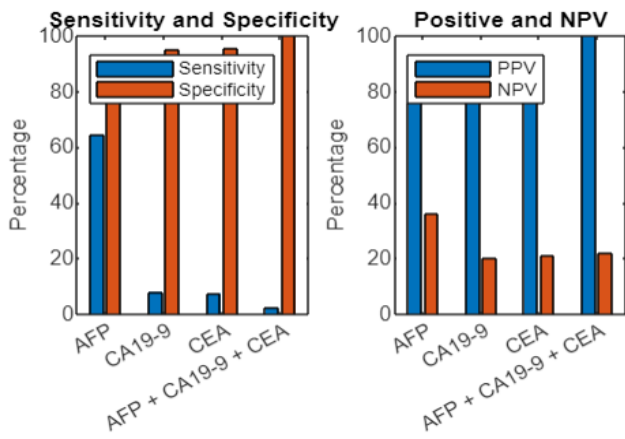


Figure 6. Sensitivity and specificity compared amongst AFP, CA19-9, CEA, and combined analysis of all three biomarkers (A). Positive and negative predictive values of all three biomarkers (B)

412 tested positive for all the three biomarkers. Amongst the patients diagnosed with moderately poorly differentiated HCC 422 of them had a mean AFP level of 1786.38 ± 7822.22 ng/L, mean CA19-9 level of 22.94 ± 58.08 U/mL and mean CEA level of 2.97 ± 1.88 ng/L and 13 patients out of the 422 was positive for all the three biomarkers. Of those diagnosed with moderate-poorly differentiated HCC, 45 patients had a mean AFP level of 184.20 ± 656.20 ng/L, mean CA19-9 level of 25.03 ± 24.82 U/mL, and mean CEA level of 3.79 ± 2.43 ng/L with 1 patient testing positive for all the three biomarkers. All 27 patients diagnosed with well-differentiated HCC had a mean AFP level of 46.19 ± 191.27 ng/L, a mean CA19-9 level of 14.60 ± 13.96 U/mL, and a mean CEA level of 2.49 ± 1.42 ng/L with no patient testing positive for all the three biomarkers. Out of the patients diagnosed with moderately poorly differentiated HCC, 65 had a mean AFP level of 2234.73 ± 6358.24 ng/L, mean CA19-9 level of 115.29 ± 376.71 U/mL and mean CEA level of 4.56 ± 7.88 ng/L and two patients only were positive for all the three biomarkers, as shown in.

Discussion

HCC is one of the most common malignant tumors. Early diagnosis and early surgical resections are imperative for improving the survival of HCC patients. The incidence of hepatocellular carcinoma has increased worldwide as well as in China in the recent decade. Its prevalence has increased mostly due to an increase in the rate of HBV infections (Navyatha et al., 2019; Oka et al., 1994). AFP, a specific glycoprotein produced primarily by the fetal liver, has been the most practical and widely used serum biomarker for HCC diagnosis. However, its sensitivity and specificity vary significantly from 40%–65% and 76%–96%, respectively (Sartorius et al., 2015; Siegel et al., 2018). This has increased the demand for specific biomarkers that can lead to early diagnosis and improved prognosis. In this study, we systematically evaluated the role of combining serum levels of CA19-9 and CEA to AFP in diagnosing hepatocellular carcinoma (Singal et al., 2012; Singal et al., 2013). Alpha-fetoprotein (AFP), a fetal-specific glycoprotein antigen, is the most commonly used serological biomarker and is considered a useful and practical tool for the screening and early diagnosis of HCC in clinical practice. However, the clinical diagnostic accuracy of AFP is unsatisfactory due to the wide variation in its sensitivity and specificity observed, making elevated AFP non-specific, especially in the early stages of HCC (Song et al., 2013). AFP has been found to have a sensitivity of 39-65% and a specificity of 76-94% in detecting HCC AFP cutoff value of 20 ng/L (Song et al., 2013).

However, in up to 30% of patients with HCC, an AFP level is under-expressed and goes undetected as AFP levels fall within the normal range (Song et al., 2013). Moreover, over-expression of AFP levels can also be observed in some patients with non-malignant chronic

liver disease, including 15-58% with chronic hepatitis and 11-47% with liver cirrhosis (Song et al., 2013). These variations in AFP levels observed in both malignant and benign patients present a diagnostic challenge in some cases as a screening tool in diagnosing HCC (Song et al., 2013). This has opened up a potential research field to detect biomarkers to complement AFP to achieve early diagnosis and better prognosis. In the current study, we found a higher prevalence of moderately differentiated HCC (39.3 %) compared to poorly differentiated HCC (9.7%), moderate-poorly differentiated HCC (38.3%), Well-moderately differentiated HCC (4.2%), well-differentiated HCC (2.5%), and Combined Hepa- to- Cholangiocarcinoma (6.1%). The rates of AFP+CA19-9+CEA being positive were 3.7% (1/27), 37% (10/27), 3.7% (1/27), 0% (0/27) and 7.4% (2/27) respectively (Song et al., 2013).

Furthermore, we observed that patients with poorly differentiated HCC expressed significantly increased AFP levels of 9300+ ng/L, while well-differentiated HCC pathology expressed very low serum AFP levels (Tanaka et al., 2011). The most observed diagnosis was moderately differentiated HCC, with 422 study subjects diagnosed (422/1075, 39.3%) and higher positive rates of AFP+CA19-9+CEA (13/27, 48%). The least observed diagnosis was that of well-differentiated HCC with 27 study subjects (27/1075, 2.5%) and had no positive rates of AFP+CA19-9+CEA (Table 4) (Torre et al., 2012). Based on our results, we found that in well-differentiated HCC AFP levels approximate to a level of about <200 ng/L; in well-moderately differentiated HCC AFP levels are observed at about 200-1600 ng/L; in moderately differentiated HCC AFP levels were about 2000 ng/L; in moderately-poorly differentiated HCC, AFP levels were recorded at 2000-8000 ng/L; and in poorly differentiated HCC AFP levels were > 8000 ng/L. Although the combined AFP+CA19-9+CEA or AFP+CA19-9 or AFP+CEA has low sensitivity, its high specificity makes it a better marker to rule out HCC when patients test, making it a potential definitive and differential diagnostic combined marker (Trevisani et al., 2001).

Timely diagnosis and intervention are pivotal for improving patient outcomes, yet the diagnostic landscape of HCC remains challenging. Alpha-fetoprotein (AFP) has long been the cornerstone biomarker for HCC detection, but its variable sensitivity and specificity have prompted the exploration of alternative or complementary markers (Tsuchiya et al., 2015). This study delved into the potential diagnostic utility of combining AFP with carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) in diagnosing HCC (Tufael et al., 2024). The research findings from our study not only echo the existing literature on the limitations of AFP but also shed light on the promise of combining multiple biomarkers to enhance diagnostic accuracy. While widely used, AFP has shown inconsistency in its ability to detect HCC, with sensitivity and specificity ranging widely across studies (Wang et al., 2022). This inconsistency underscores the need for supplementary

markers to augment AFP's diagnostic performance. Our study aligns with previous research indicating that combining biomarkers may offer increased specificity but lower sensitivity than AFP alone (Williams et al., 2021). This observation underscores the complexity of HCC diagnosis and highlights the importance of adopting a multifaceted approach to biomarker evaluation.

However, our study also uncovered certain disparities compared to previous research findings, particularly regarding sensitivity for combined biomarkers. While some studies have reported higher sensitivity for AFP combined with CA19-9 and CEA, our results demonstrated significantly lower sensitivity (Xu, 2015). Several factors could contribute to these discrepancies, including differences in sample size, patient demographics, underlying etiological factors such as viral hepatitis prevalence, or racial variations across study populations (Yoshida et al., 2002). Moreover, variations in assay techniques or cutoff values for defining positive results may also contribute to the observed differences in sensitivity and specificity estimates. Therefore, our study underscores the need for further research to validate and refine diagnostic algorithms for HCC detection, considering the diverse factors that may influence biomarker performance.

Comparing our results with existing literature highlights the evolving landscape of HCC diagnostics and the challenges inherent in translating research findings into clinical practice. While our study adds to the growing body of evidence supporting the potential utility of combined biomarkers, it also underscores the importance of context-specific validation and careful consideration of various factors influencing biomarker performance (Zhang et al., 2013). Moreover, our findings underscore the need for standardized protocols and rigorous validation studies to ensure the reliability and reproducibility of diagnostic assays across different patient populations and healthcare settings. Practically, our research findings have significant implications for clinical practice, particularly in settings with limited resources or access to advanced diagnostic tools. Combining AFP with CA19-9 and CEA may offer a cost-effective and accessible approach to HCC screening and diagnosis, especially in regions where more sophisticated imaging modalities or biomarker assays may not be readily available (Zheng et al., 2010). However, clinicians should interpret biomarker results cautiously, considering the limitations and potential confounders that may affect diagnostic accuracy. Furthermore, our study underscores the importance of ongoing research and collaboration to identify novel biomarkers and refine existing diagnostic algorithms for HCC detection.

Our study contributes to the growing body of evidence on HCC diagnostics and highlights the potential utility of combining AFP with CA19-9 and CEA in enhancing diagnostic accuracy. While our findings align with existing literature on the challenges and opportunities in HCC diagnosis, they also underscore the need for

further research and validation to effectively translate research findings into clinical practice (Zoli et al., 1996). By addressing these challenges and leveraging the potential of complementary biomarkers, we can advance the early detection and management of HCC, ultimately improving patient outcomes and reducing the burden of this deadly disease.

Conclusion

Although AFP, combined with CA19-9 and CEA, has a specificity of 100% and a positive predictive value of 100%, its low sensitivity of 2.5 % makes it used as a screening tool inferior to AFP alone in HCC and differentiating HCC from non-HCC patients, and therefore not a suitable substitute in the screening of potential HCC patients, however, it can aid in the definitive diagnosis of HCC and exclude HCC as the primary. In summary, although we propose the combination of AFP, CA19-9, and CEA for HCC surveillance in HCC patients, the search for novel biomarkers of early HCC detection requires further research.

Author contributions

M.T., M.M.R., and V.J.U. conceptualized, conducted lab and field works, analyzed data, wrote the original draft, reviewed, and edited; MFH and N.U conducted research design, validated methodology, analyzed, visualized the data, reviewed, and edited; M.M.R and NU. Validated the methodology, analyzed data, investigated, visualized, reviewed, and proof-read; M.T., V.J.U and M.F.H. conceptualization, conducted research design, validated methodology; conducted analysis, investigated, visualized the data, reviewed, obtained a grant, supervised and edited the paper. All authors read and approved the paper for publication.

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Competing financial interests

The authors have no conflict of interest.

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