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Diagnostic value of alpha-fetoprotein and lens culinaris agglutinin-reactive Alpha-fetoprotein in liver cirrhosis and hepatocellular carcinoma patients in Pakistan

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most commonly encountered liver cancer. Because of latent HCV infections, there is projected rise in new HCC cases in Pakistan; with the onset of HCC several decades after initial infection has occurred. As it is diagnosed at very late stage, predicted outcome of HCC is poor. This results in effective medical intervention difficult. The purpose of this study is to establish a serum marker and/or panels of serum markers, that can detect the at risk patients with chronic liver disease, not only before but also after early development of HCC.

Methods: This study was conducted for ten months in Lahore General Hospital, Lahore, Punjab, Pakistan after taking the approval from the ethical board. A total of eighty patients (40 patients of HCC and 40 patients of Liver cirrhosis) were included using non-probability, consecutive sampling. AFP and AFP-L3 levels of these patients were measured. The data was analyzed using SPSS Ver. 25. A p-value of ≤0.05 was considered as statistically significant.

Results: In group 1, the mean age of patients was 60.02 ± 11.5 years and in group 2, it was 60.80 ± 7.42 years. In group 1, the mean of AFP was 64.93 ± 171.76 (ng/mL) and AFP-L3 was 0.56 ± 0.51(ng/mL). On the other hand, in group 2, the mean of AFP was 329.88 ± 231.55 (ng/mL) and AFP-L3 was 177.59 ± 104.10 (ng/mL). Significant association was found between AFP and AFP-L3 in both groups. Receiver operating characteristic (ROC) curve for AFP and AFP-L3 in relation to both groups obtained. The sensitivity of AFP was 85% and specificity was 80%. The sensitivity and specificity of AFP-L3 was 90 and 100%, respectively.

Conclusion: AFP-L3 has a better diagnostic value for Hepatocellular carcinoma than AFP.

Keywords: Biomarkers, Fetoprotein, Hepatocellular Carcinoma, Liver Cirrhosis

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Introduction

Liver cancer is a demanding universal health issue with an increase in frequency of disease globally. By the year 2025,An estimated over a million cases are expected, with the highest prevalence of cases and deaths in Asia Pacific region and Africa.[1] Hepatocellular carcinoma (HCC) is considered fifth most prevailing malignancy in men and seventh in women around the world.[2,3] It is one of the main reasons of malignancy across the globe.[4] According to World Health Organization (WHO), HCC is 4th primary cause of cancer- related mortality. [5]

Hepatocellular carcinoma (HCC) comprises of 90% of primary hepatic malignancy alone and thus, contributes significantly to overall disease load. Liver cirrhosis (LC) is the primary health risk for HCC worldwide. Most common risk factors of HCC are infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), congenital diseases such as alpha-1 antitrypsin deficiency and diabetes.[6-8]

Over the years, the HCC diagnostic surveillance has greatly improved, but it is often delayed, and thus compromising the curative treatments such as

resection. As such, it is crucial to detect HCC cases early to improve the outcome and prognosis of Hepatocellular carcinoma patients.[9]Most common methods used worldwide for HCC surveillance are ultrasound (US) and alpha-fetoprotein (AFP) testing, repeated every 3-6 months in high-risk patients.[10] In HCC surveillance programs, it is essential to include all patients with cirrhosis and non-cirrhotic HBV infected patients, who are susceptible of developing Hepatocellular carcinoma, however, approximately only 50% of the HCC cases are diagnosed with these programs.[11] For the exact diagnosis of HCC, patients having lesion of >1cm on US or AFP levels >20ng/mL on surveillance, should undertake a multiphasic CT and MRI.[12]

Alpha-fetoprotein (AFP) is a non-specific tumor marker for Hepatocellular carcinoma. It is one of earliest tumor marker to be detected. AFP is a conjugated protein and an oncofetal antigen.[13] However, over the last few years, controversy has arisen regarding use of AFP as a screening test for HCC. In approximately 40% of patients with HCC, alpha-fetoprotein levels are normal, especially at the beginning of disease i.e., it has low sensitivity. Patients with viral hepatitis, cirrhosis and cholangiocarcinoma show elevated levels of AFP, thus giving false-positive results.[14] This highlights the need for more accurate biomarkers to improve early HCC detection during surveillance.

Latest studies have documented that Lens culinaris agglutinin-reactive alpha-fetoprotein, also called as AFP-L3, may prove to be a better screening biologic marker for HCC detection. Alpha-fetoprotein can be fractioned by the technique of affinity capillary electrophoresis into 3 forms: L1, L2 and L3, developed on the basis of reactiveness with the lectin, Lens culinaris agglutinin (LCA). LCA is a carbohydrate binding protein separated from lentil seeds. AFP-L1 is non-LCA binding glycoform and is predominantly linked with non-malignant conditions of liver such as liver cirrhosis and chronic hepatitis B virus infection. AFP-L2 has an average capacity for binding to LCA and is mainly formed by yolk sac tumors. AFP-L3 binds firmly to LCA via an additional α 1 – 6 fucose residue attached at the reducing terminus of N-acetyl glucosamine and is produced only by cancerous liver cells. So, AFP-L3 is the main type of glycoform in individuals with HCC.[15]

Raised AFP-L3 levels (>10%) are affiliated with 7-fold increase in risk of developing HCC within the next 21 months and may also show increase levels 3-21 months before HCC is detected by imaging techniques (ultrasound and/or MRI). The AFP-L3 levels also distinguishes non-malignant hepatic disease from Hepatocellular carcinoma, as it is only produced by the malignant hepatocytes.[16]Though, the diagnostic sensitivity of AFP-L3 is low in patients with decreased levels of AFP but the specificity in patients with AFP-negative liver cancer is 85.1%.[17]Pakistan is among the countries with the highest HCV seroprevalence. A recent systematic review of all studies published during the years 2000 to 2013 estimated an overall HCV prevalence of 11.6% in the adult population, including 10.1% among blood donors, and 4.7% among pregnant women, and HCV genotype 3a prevalence was found to be the dominant genotype with 63.45%.[18,19] The pathogenesis of disease manifests that 55-89% of acute HCV infections convert to chronic infections, amongst which 2-24% will progress to liver cirrhosis over 20 years. In patients with liver cirrhosis, 1-4% develops HCC per annum.[20,21]Thus, the rationale of this study is to help in establishing a screening test for our country that has high sensitivity and specificity and at the same time it has reproducible results without any variability.

Methods

This cross sectional (analytic) comparative study was performed at the Lahore General Hospital, Lahore, from June 2022 to March 2023. Sample size was calculated by using sensitivity and specificity sample size calculator. A total of 80 patients, including 40 patients of liver cirrhosis and 40 patients of HCC who visited Lahore General Hospital, Lahore were enrolled .The present study was approved through the Medical Ethics Committee of Lahore General Hospital/Postgraduate Medical Institute, Lahore and is in accordance with the Declaration of Helsinki.(#UHS/Education/125-15/3410 dated 1-10-2019).

Liver cirrhosis was diagnosed based on clinical signs and symptoms, lab abnormalities (increase in aspartate aminotransferase (AST)/Alanine aminotransferase (ALT) ratio to more than one, decrease in plasma albumin to globulin ratio to more than one, increase in prothrombin time (PT), decrease in platelet count) and ultrasound findings. HCC was diagnosed based on ultrasound and CT (computed tomography) findings. Confirmed cases of Liver cirrhosis and HCC were included. Patients with metastatic liver cancer, fibro lamellar HCC and patients with elevated AFP due to conditions other than liver disease such as, non-seminomatous germ cell tumors and yolk sac tumors were excluded.

A total of 80 blood samples from Hepatocellular carcinoma patients and liver cirrhosis patients without HCC were collected through disposable syringe in the serum separator tubes. The collected blood samples were centrifuged at 60rpm for 2-3min.The supernatant i.e., serum was separated out and placed into separately labeled eppendorf tubes. The serum containing tubes were then stored in -80^{o} C freezer. AFP and AFP-L3 levels were done by Enzyme-linked immunosorbant assay (ELISA) using Cal biotech AFP ELISA Kit (Catalogue No. AF237T) and estimation of AFP-L3 levels were done by using BT LAB Human alpha-fetoprotein Lens culinaris agglutinin 3 ELISA kit (catalogue no E1671Hu) following manufacturer's instructions.

Data was entered in Microsoft excel and analyzed by using Statistical Package for the Social Science (SPSS) software version 25.0 (SPSS Inc., Chicago, IL, USA). The non-parametric data were described as median and interquartile range (IQR) and categorical data as frequency (n) and percentage (%). The differences between two groups were analyzed using the Kruskal-Wallis H test. Chi square was used to find out the p-values of variables. Receiver operating characteristic curve (ROC) analysis was used to compare clinical utility between AFP-L3 and AFP. P<0.05 was considered to be statistically significant.

Results

The median(IQR) of AFP levels in liver cirrhosis patients (group 1) was 0.82(7.22-0.13) ng/mL. The lowest and highest values were 0.008 and 588 ng/mL respectively. The data rejected normality (Shapiro-wilk test, p<0.0001). The median(IQR) of AFP levels in hepatocellular carcinoma patients (group 2) was 459(533-35.4) ng/mL. The lowest and highest values were 0.09 and 592 ng/mL respectively. The data rejected normality (Shapiro-wilk test, p<0.0001). Kruskal-Wallis test was performed to see if there were differences in AFP levels between group 1 and group 2. Alpha-fetoprotein levels were more raised in hepatocellular carcinoma patients as compared to liver cirrhosis patients and a statistically significant difference between two groups was found (Table-1).

Table-1: Descriptive statistics of AFP in liver cirrhosis and hepatocellular carcinoma patients

AFP	Group 1	Group 2	Test of significance
	LC	HCC	
Median	0.82 ng/mL	459 ng/mL	KW= 29.1408 p<0.000001
IQR	7.22-0.130	533.5-35.4	
Shapiro-Wilk test	W= 0.4140 p<0.0001	W= 0.8091 p<0.0001	

LC, liver cirrhosis; HCC, hepatocellular carcinoma; AFP, alphafetoprotein;
IQR, interquartile range; KW, Kruskal Wallis test; p-Value, probability value

The current study results of AFP and AFPL3 were plotted in table against gold standard test results (imaging studies) to calculate diagnostic accuracy of AFP and AFPL3 in HCC detection (Table-2).

Table-2: Table for calculation of diagnostic accuracy of AFP and AFP-L3

AFP	Disease Present(HCC)	Disease Absent (HCC)	TOTAL
Test Positive	34	8	42
Test Negative	6	32	38
TOTAL	**40**	**40**	**80**
AFPL3	**Disease Present(HCC)**	**Disease Absent(HCC)**	**TOTAL**
Test Positive	36	0	36
Test Negative	4	40	44
TOTAL	**40**	**40**	**80**

ROC curve for AFP in relation to both groups was obtained. At the cutoff point of ≥8.9 ng/ml for AFP, the sensitivity and specificity of AFP for detection of HCC was 85.2% and 76.5% respectively, with PPV of 80.95% and NPV of 84.21% and diagnostic accuracy was found to be 82.5% (Table-3, Figure-1).

Table-3: Receiver operator characteristics (ROC) analysis of AFP with respect to both groups

Statistics	Value
AUC	0.85
95% CI	0.757 to 0.923
p-Value	<0.0001
z statistic	7.442
Standard Error	0.0474
Sensitivity	85%
Specificity	80%
PPV	80.95%
NPV	84.21%

Accuracy	82.5%

AUC, area under curve; CI, confidence interval; p-Value, probability value;
PPV, positive predictive value; NPV, negative predictive

The median(IQR) of AFP-L3 levels in liver cirrhosis patients (group 1) was 0.40(0.75-0.26) ng/mL. The lowest and highest values were 0.03 and 2.5 ng/mL respectively. The data rejected normality (Shapiro-wilk test, p<0.0001). The median(IQR) of AFP-L3 levels in hepatocellular carcinoma patients (group 2) were 147(296-14.3) ng/mL. The lowest and highest values were 0.03 and 489 ng/mL respectively. The data rejected normality (Shapiro-wilk test, p<0.0001). Kruskal-Wallis test was performed to see if there were differences in AFP-L3 levels between group 1 and group 2. AFP-L3 levels were found to be more increased in hepatocellular carcinoma patients than in liver cirrhosis patients and there is a statistically significant difference between two groups (Table-4).

Table-4: Descriptive statistics of AFP-L3 in liver cirrhosis and hepatocellular carcinoma patients

AFP-L3	Group 1	Group 2	Test of significance
	LC	HCC	
Median	0.405 ng/mL	147 ng/mL	KW= 51.5984 p<0.000001
IQR	0.747-0.262	296.2-14.3	
Shapiro-Wilk test	W=0.7703 p<0.0001	W=0.919 p<0.0001	

LC, liver cirrhosis; HCC, hepatocellular carcinoma; AFP, alphafetoprotein;
IQR, interquartile range; KW, Kruskal Wallis test; p-Value, probability value

ROC curve for AFP-L3 in relation to both groups was also obtained. Keeping the cutoff point of ≥2.5 ng/ml for AFP-L3, the Sn, Sp, PPV, NPV and diagnostic accuracy of AFP-L3 was found to be 90%, 100%, 100%, 90.99% and 95% respectively (Table-5, Figure-1).

Table-5: Receiver operator characteristics (ROC) analysis of AFP-L3 with respect to both groups

Statistics	Value
AUC	0.967
95% CI	0.900 to 0.994
p-Value	<0.0001
z statistic	18.463
Standard Error	0.0253
Sensitivity	90.00%
Specificity	100%
PPV	100%
NPV	90.99%
Accuracy	95%

AUC, area under curve; CI, confidence interval; p-Value, probability value;
PPV, positive predictive value; NPV, negative predictive value.

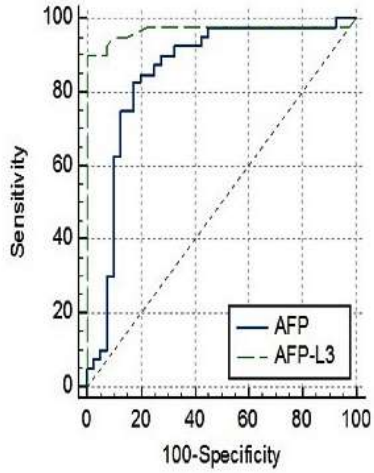

Figure-1: Receiver operating characteristics (ROC) curve analyses using AFP and AFP-L3 for discriminating Hepatocellular carcinoma patients from liver cirrhosis patients.

Discussion

In this study, in patients with LC, significantly lower mean AFP and AFP-L3 levels were found as compared to in HCC patients (p=<0.000001). A study by Cerban et al.[22] also reported similar results i.e: p<0.0001 for AFP and p<0.0001 for AFP-L3 among the LC and HCC patients. Lee et al.[23] also documented data similar to our data, in which p-value of AFP and AFP-L3 with respect to both stages was <0.001.

In this study, the sensitivity and specificity of AFP at cut off value of >8.9 ng/mL was 85% and 80% respectively, with diagnostic accuracy of 82.5%. The cut off value of AFP-L3 was >2.5 ng/ml. At this cut off value, the sensitivity of AFP-L3 was 90% and specificity was 100% with accuracy of 95%. Ibrahim et al.[24] also reported the similar results in their study with sensitivity of 80% and specificity 90% for AFP and 100% sensitivity and specificity of AFP-L3 with AUC of 0.8 for AFP and AUC of 1 for AFP-L3, but their cut-off value for AFP was >16.15 and >13.9 for

AFP-L3, which is higher than our results. The positive and negative predictive values and accuracy results were also very similar to our study.

In an earlier study by Lee et al.[23] AFP alone had a sensitivity of 78.8% and specificity of 60.8% with AUC of 0.77 and AFP-L3 alone had a sensitivity of 71.3% and specificity of 80.7% with AUC of 0.8. The slight difference may be due to the fact that they used much lower cut-off values than our study i.e; 4.2ng/mL for AFP and 0.3ng/mL for AFP-L3. Another possibility may be that they studied larger number of patients (n=462 liver cirrhosis patients without HCC and n=160 HCC patients), which included larger tumor size. They also reported lower positive predictive values for both AFP and AFP-L3 as compared to our study.

Song et al.[25] reported much lower sensitivities for both AFP and AFP-L3 to differentiate between liver cirrhosis patients with and without HCC i.e. 51.5% sensitivity of AFP and 28.3% sensitivity of AFP-L3 with AUC 0.76 and 0.62, respectively. They also reported lower negative predictive values for AFP and AFP-L3, 56.4% and 47.4%, as compared to our study.

Another study reported a sensitivity and specificity of 75% and 100%, respectively, for AFP but they use very high cut-off value of 128 ng/ml for AFP and AUC of AFP was also greater than our study i.e. 0.97. Their results of AFP-L3 were very similar to our study i.e. 97.5% sensitivity and 100% specificity with 98% accuracy at cut-off of 23 ng/mL and AUC of 1.[26]

Conclusion

In current study, levels of both alpha-fetoprotein and lens culinaris agglutinin reactive alpha-fetoprotein (AFP-L3) were found raised in Hepatocellular carcinoma patients but AFP-L3 showed greater sensitivity and specificity than AFP in detection of Hepatocellular carcinoma and it can be used as diagnostic biomarker for the detection of HCC in early stages.

Recommendations

Further studies are needed regarding the use of combination of these two markers for early detection of HCC and correlation of AFP-L3 levels with different stages of carcinoma.

Conflicts of Interest: None declared.

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B. Active Participation in Active Methodology
C. Interpretation/ Analysis and Discussion