RESEARCH ARTICLE

# Cytokeratin-18 in Diagnosis of HCC in Patients with Liver Cirrhosis

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## Abstract

**Background:** Hepatocellular carcinoma (HCC) is a common malignancy that occurs secondary to viral hepatitis B and C cirrhosis under the influence of environmental factors. In early stages, clinical diagnosis is often difficult and distinguishing HCC from cirrhosis and other hepatic masses by conventional tests is frequently not feasible. Physicians usually depend on measuring serum alpha-fetoprotein (AFP), but this marker has low sensitivity and specificity. The aim of this research was to determine any role of serum cytokeratin-18(Ck-18) as a marker for diagnosis of HCC in patients with liver cirrhosis. **Patients and methods:** We used ELISA to measure the serum levels of AFP and CK 18 in 60 Egyptian patients (30 cirrhotic and 30 with HCC) and 30 controls. **Results:** The Ck-18 level was significantly elevated in the HCC group (1247.8± 105.3U/L) when compared to the liver cirrhosis (834.1± 38.8 U/L) and control groups (265.2±83.1U/L). Ck-18 as a marker showed 95.6% sensitivity, 93.3% specificity and 98.8% accuracy. The mean serum AFP was 4901.4±2185.8ng/ml in the HCC group, 100.7±71.7 ng/ml in the cirrhotic group, and 4.0±1.2ng/ml in controls. AFP showed 55. 7% sensitivity, 97. 7% specificity and 84.4% accuracy. Combined use of both Ck-18 and AFP improved the sensitivity to 98%. **Conclusion:** Serum cytokeratin-18 level can be used as a diagnostic biomarker for HCC with a higher sensitivity than AFP.

**Keywords:** Cytokeratin-18- cirrhosis- HCC- Hepatocellular carcinoma- alpha fetoprotein

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## Introduction

Hepatocellular carcinoma (HCC) is a common malignancy in Egypt (El-Seragand Rudolph 2007). It occurs secondary to hepatitis C virus (HCV), hepatitis B virus (HBV) and environmental factors (Ziada et al., 2016, Zekri et al., 2012, El-Azm et al., 2013). It is important to know that, the earlier the diagnosis of HCC, the better its prognosis. Radiological investigations detect HCC usually after a period of the tumor onset (Maet al., 2008). Oncologists (without pathological confirmation) depend on alpha fetoprotein (AFP) beside the radiological findings for diagnosing HCC, but AFP has a lower sensitivity and may of normal values in HCC or may be elevated in another disease rather than HCC like liver cirrhosis, some benign hepatic focal lesions as well as non hepatic malignancies ( Xu et al., 2013). This makes AFP insufficient in HCC diagnosis with loss of the chance of effective HCC management. The use of several serum markers to detect the early diagnosis of HCC is highly recommended (Zhao et al., 2013). There are many trials to discover a more sensitive and specific marker for HCC diagnosis (Shi et al., 2014, El-Mashad et al., 2015, Ismaiel et al., 2015, Youns et al., 2013).

Cytokeratins are tissue or organ specific proteins that are found in the cytoplasm of epithelial tissues (Schweizer et al., 2006). Cytokeratine-18 (Ck-18) is a type 1 cytokeratin that represents about 5% of proteins in the liver and has potential role in hepatocyte apoptosis which mediates hepatocytes damage and morphological changes through a family of intracellular cysteine proteases, called caspases (mainly caspase-3). These caspases react on different substrates including cytokeratin-18 (the major intermediate filament protein in the liver), with a consequence of apoptotic changes and characteristic damage (Bantel et al., 2004; Danial and Korsmeyer, 2004). It has been confirmed that CK-18 secretion occurs in parallel with DNA synthesis, protein synthesis, and cell division and this suggests an important role of Ck-18 in carcinogenesis (Linder, 2007 and El-Zefzafy et al., 2015). Gonzalez- Quintela et al, (2006) noted high levels of Ck-18 in patients with hepatocellular carcinoma.

This study aimed to evaluate the role of serum Cytokeratin-18 (Ck-18) as a noninvasive marker for diagnosis of HCC in patients with liver cirrhosis.

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## Materials and Methods

*Patients and Methods*

This study was conducted on 60 patients and30 controls presenting to the Tropical Medicine and Clinical Oncology Departments, Tanta University Hospital, Faculty of Medicine, University of Tanta, Egypt. All patients and controls were aware about the study, and we obtained a written consent from each one, as well as the approval of the ethical committee. The studied patients and controls were divided into the following groups:

Group I (GI): Included 30 patients with liver cirrhosis, 16 of them were males (53.33%) and 14 females (46.67%). The mean age 50.13 ± 7.35. Twenty-two patients (73.33%) had HCV infection, while 8 patients (26.67%) showed HBV infection.

Group II (GII):Included 30 cirrhotic patients with HCC fulfilling HCC criteria on tri-phasic C.T. scan, 17 of them were males (56.67%) and 13 females (43.33%).Their mean age was 53.63± 5.85. Twenty patients (66.67%) with HCV infection, and10 patients (33.33%) with HBV infection.

Group III (GIII): Included 30 healthy individuals, 14 of them were males (46.66%) and 16 females (53.33%) with mean age 49.56 ± 7. 91. There was a non-significant difference regarding age and sex with the studied patients groups (GI and GII) (P> 0.05).

Exclusion criteria: A past history or evidence of other malignancies, autoimmune disorders, organ failure and other causes of cirrhosis (e.g. Alcoholic and non-alcoholic fatty liver diseases).

The studied groups were subjected to thorough history taking and clinical evaluation. Laboratory investigations included; complete blood count (CBC), liver function tests, viral hepatic markers (HBsAg, Anti HCV and PCR for HCV-RNA) and serum AFP was measured by Enzyme-linked immunosorbent assay (ELISA) (Macias-Rodriguez et al., 2000). All studied groups were subjected to abdominal ultrasound, while GI and GII were subjected to Tri-phasic CT scan abdomen. Liver biopsy and histopathological examination to confirm HCC diagnosis was done in GII.

Measurement of Serum Ck-18: Serum Ck-18 assay was detected using the available human cytokeratin-18 ELISA Kit supplied from DRG International Inc, USA. The results were expressed as U/L (Sumer et al., 2013).

*Sample collection and storage*

Peripheral blood (4ml) was collected from each subject for the isolation of serum and samples were allowed to clot for two hours at room temperature then sera were centrifuged for 20 minutes at approximately 1000 xg, and transferred into plain polypropylene tubes and stored at -20 °C until further processing.

Sample preparation: Samples were diluted 1000- fold with PBS (PH=7.0-7.2) as following:

Dilution (100- fold): 10 µL of sample was added to 990 µL of PBS (PH=7.0-7.2) and mixed together.

*Procedure*

1- All reagents and samples were brought to room temperature before use.

2- 100 µl of each standard ,blank and samples were added into appropriate wells, the plate was sealed with the adhesive seal provided and incubated for 2 hours at 37°C

3- The solutions were discarded and the plate was blotted on paper towels until all of the liquid is removed from the wells.

4- 100 µl of the working solution of detection antibody (Detection Reagent A) was added into each well; the plate was sealed and incubated at 37°C for 1 hours.

5- The solution was aspirated and washed as follow: 350 µL of 1Xwash buffer was added to each well being used and gently shaked 1~2 minutes, and then discarded. The remaining liquid was removed from all wells completely by snapping the plate onto absorbent paper. The wash step was repeated for more 3 times consecutively. After the last wash, any remaining wash buffer was removed by decanting. The plate was inverted and blotted against clean paper towels.

6- 100µL of Detection Reagent B working solution was added to each well. Incubated for 30 minutes at 37oC after covering it with the Plate sealer.

7- The aspiration/wash process was repeated for total 5 times.

8- 90µL of TMN Substrate Solution was added to each well. Covered with a new Plate sealer. Incubated for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protected from light. The liquid was turned blue by the addition of Substrate Solution.

9- 50µL of Stop Solution was added to each well. The liquid turned yellow by the addition of Stop solution. The liquid was mixed by tapping the side of the plate.

10- The absorbance in each well was read at 450 nm (using a reference wavelength of 540-570), within 30 minutes of adding the stop solution by an ELISA reader (Stat fax 2100) (Sumer et al., 2013).

*Calculation of results*

The standard curve was used to ascertain the concentration of cytokeratin- 18 in unknown specimens. The standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the cytokeratin-18 concentration in on the x-axis and a best fit curve was drawn through the points on the graph.

• The concentration of cytokeratin-18 for an unknown was determined by locating the absorbance for each unknown on the vertical axis of the graph then detected the intersecting point on the curve, and reading the concentration from the horizontal axis of the graph.

• The concentrations read from the standard curve were multiplied by the dilution factor (1000).

Statistical analysis: The results were expressed as mean ± standard deviation (SD).Comparison was performed using statistical package for social studies (SPSS). Comparison between two groups was performed using the t- test. Comparison of mean values between groups was performed using analysis of variance (ANOVA). When the value of ANOVA (F) was found significant, one way ANOVA on rank test (Tukey's test) was performed to compare each two groups and P value less than 0.05 was considered statistically significant. The diagnostic accuracies of AFP and serum CK 18 were determined by

receiver operator characteristic (ROC) curve analysis, reporting area under the curve (AUC) and its 95% confidence interval. The diagnostic cut off and the related sensitivity and specificity were determined.

## Results

Our study was conducted on 30 patients of liver cirrhosis (GI) and 30 patients of HCC and 30 healthy controls. The results of liver function tests showed a statistically significant difference between the two diseased groups (GI and GII) compared to group III of healthy controls (P<0.05). There was a significant decrease of hemoglobin (Hb) in the HCC group compared to the control group (P<0.05) without significant difference between GI andGII or between GI and GIII (P>0.05). The red blood cell (RBCs) and platelet counts were significantly lower in the diseased groups compared to controls (P<0.05) with a non-significant difference between the two diseased groups (P>0.05). There was a non-significant difference between all groups as regards the total leukocytic count (TLC) (P>0.05) (table1). Child-Pugh score of group I and II: in group, I there were 7 patients (23.3%) with Child score A, 9 patients (30%) were child B and 20 patients (46.67%) were child C while in group II; 8 patients (26.67 %) were child A, 22 patients (73.33%) were child B and 0 patients (0 %) were child C.

AFP showed a significant elevation in the HCC group (4901.367± 2185.800ng/ml) compared to the control group (4.033±1.191 ng/ml) and cirrhotic liver group (100.733±71.726 ng/ml) (P <0.05). The results of Ck-18 show there was a significant elevation in the mean serum CK -18 in the HCC group (1247.761 ± 105.31U/L) in relation to the control group (265.191 ± 83.1 U/L), and cirrhosis group (834.099 ± 38.84 U/L) (P< 0.001). Also, there was a significant elevation in the mean serum CK -18 in group I of liver cirrhosis in comparison to the group of healthy controls (P< 0.001) (Table 1).

The results of radiological examination of the studied groups showed portal vein dilatation was found in 23 patients (76.67%) of GI and 12 (40%) in GII with no portal vein thrombosis in both groups, splenomegaly was detected in 16 patients (53.33%) of GI and 11 patients (36.67%) of GII, ascites was found in 17 patients (56.67%)

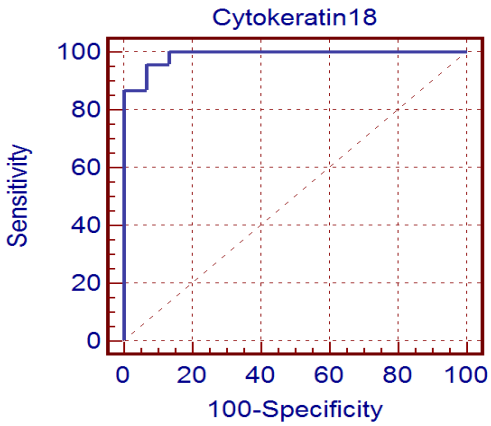


Figure 1. ROC Curve of Cytokeratin 18

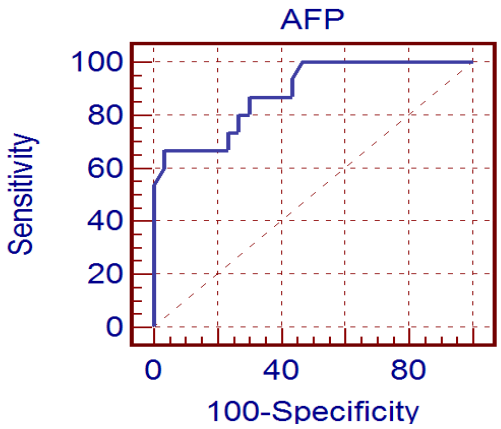


Figure 2. ROC Curve for AFP.

of group I, no abdominal lymph node enlargement was detected in studied groups. As regards number of focal lesions in patients of HCC group 19 patients (63.33 %) showed single hepatic focal lesion, 7 patients (23.33%) had 2 focal lesions and 4 patients (13.33%) had 3 focal lesions. Right lobe focal lesions were detected 22 patients (73.33%) and left lobe focal lesions were detected in 8 patients (33.33%) of group II (Table 2).

Table (3) shows comparison of the levels of Ck-18 among Child-Pugh classes in group I of liver cirrhosis with no statistically significant difference was detected (P = 0.112) however, a statistically significant differences were found when we compared Ck-18 levels between the HCC group and each Child-Pugh classes of group I (P< 0.001).

Table (4) shows a statistically significantly positive correlation between serum Ck-18 and AFP, ALT, AST, Child Pugh score, portal vein diameter, splenic size, size and number of the focal lesions. However a significant negative correlation was detected between Ck-18 levels and serum albumin and prothrombin activity (P<0.05).

Receiver Operator Characterizing (ROC) curve analysis: using ROC curve to differentiate between HCC and cirrhosis CK-18 showed the 95.6% sensitivity, 93.3% specificity and 98.8% accuracy (Figure1). When we used the curve for AFP to differentiate between HCC and cirrhosis we reported AFP to has 55.67% sensitivity, 97.67% specificity and 84.44% accuracy (Figure 2).

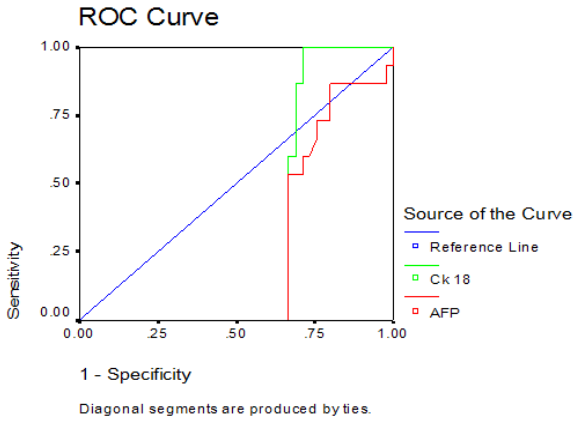


Figure 3. Roc Curve of AFP with CK -18

Table 1. Laboratory Data of All Studied Groups

| | GI<br>Liver cirrhosis | GII<br>HCC | GIII<br>Control | ANOVA | P |
|---|---|---|---|---|---|
| Total bilirubin (mg/dL) | 4.03±2.23 | 4.92±2.48 | 0.667±0.158 | F = 40.52<br>P <0.001* | P1>0.05<br>P2 <0.05*<br>P3 <0.05* |
| Direct bilirubin (mg/dL) | 2.34±1.3 | 2.71±1.37 | 0.35±0.08 | F = 40.6<br>P <0.001* | P1>0.05<br>P2 <0.05*<br>P3 <0.05* |
| ALT (IU /L) | 67.33±27.96 | 79.47±29.28 | 28.23±5.55 | F = 38.6<br>P <0.001* | P1>0.05<br>P2 <0.05*<br>P3 <0.05* |
| AST (IU /L) | 64.8±31.42 | 71.47±20.46 | 26.5±3.74 | F = 37.34<br>P <0.001* | P1>0.05<br>P2 <0.05*<br>P3 <0.05* |
| Albumin (g/dL) | 2.68±0.38 | 2.45±0.26 | 4.44±0.19 | F = 37.34<br>P <0.001* | P1 > 0.05<br>P2 < 0.05*<br>P3 <0.05* |
| Prothrombin % | 65.2±12.67 | 59.04±22.57 | 94.2±4.85 | F = 26.8<br>P <0.001* | P1 = 0.609<br>P2 <0.001*<br>P3 < 0.001* |
| INR | 1.475±0.28 | 1.719±0.6 | 1.047±0.07 | F = 13.45<br>P <0.001* | P1 =0.207<br>P2 = 0.005*<br>P3 <0.001* |
| Hemoglobin (Hb) (g/dl) | 11.076±1.7 | 9.89±2.13 | 12.813±1.57 | F = 6.891<br>P <0.001* | P1 = 0.288<br>P2 = 0.052<br>P3< 0.001* |
| Red blood cells (RBCs)x103/cc | 3.97±0.55 | 3.569±0.74 | 4.745±0.32 | F = 13.9<br>P <0.001* | P1 = 0.16<br>P2= 0.001*<br>P3 <0.001* |
| Plateletsx103/cc | 111.46±41.84 | 109.46±46.61 | 225.467±64.59 | F = 11.59<br>P <0.001* | P1 = 1<br>P2 = 0.002*<br>P3 = 0.001* |
| Total leukocytic count (TLC) (cell/C. mm) | 9073.3±4632.56 | 6290±3578.6 | 7713.33±1193.95 | F = 2.548<br>P =0.065 | P1 > 0.05<br>P2 > 0.05<br>P3 > 0.05 |
| AFP (ng/ml) | 100.733±71.726 | 4901.367±2185.8 | 4.033±1.191 | F =2.88<br>P= 0.04* | P1 < 0.05*<br>P2 < 0.05*<br>P3 < 0.05* |
| CK-18(U/L) | 834.099± 38.84 | 1247.761±105.3 | 265.191±83.1 | F =359<br>P<0.001* | P1 <0.001*<br>P2 <0.001*<br>P3 <0.001* |

ALT, Alanine aminotransferase; AST, aspartate aminotransferase; INR, nternational normalization ratio; AFP, alpha fetoprotein; CK-18, Cytokeratin-18; P1, GI versus GII; P2, GI versus GIII; P3, GII versus GIII; *, significant difference

Table 2. Radiological Data of Studied Groups

| | G I | GII | GIII |
|---|---|---|---|
| Portal vein dilatation | 23 (76.67%) | 12 (40%) | 0 (0%) |
| Portal vein invasion | 0 (0%) | 0 (0%) | 0 (0%) |
| Splenomegaly | 16 (53.33%) | 11 (36.67%) | 0 (0%) |
| Ascites | 17 (56.67%) | 0 (0%) | 0 (0%) |
| Abdominal LN enlargement | 0 (0%) | 0 (0%) | 0 (0%) |
| One focal lesion | - | 19 (63.33%) | - |
| Two focal lesions | - | 7 (23.33%) | - |
| Three focal lesions | - | 4 (13.33%) | - |
| Right lobe focal lesions | - | 22(73.33%) | - |
| Left lobe focal lesions | - | 8 (33.33%) | - |

LN, Lymph node

Moreover, we found that, the combined use of both serum

Table 3. Comparison of Ck-18 levels between Different Child-Pugh Classes in GI and between GII and Each Class in GI

| | GI (Liver cirrhosis) | | | | |
|---|---|---|---|---|---|
| | Child A | Child B | Child C | GII (HCC) | P |
| Ck-18(U/L) | 813.46±34.38 | 826.96±44.22 | 849±33.35 | 1247.761±105.3 | P1<0.001*<br>P2<0.001*<br>P3<0.001* |
| | | F= 2.376, P = 0.112 | | t 1= 10.52<br>t 2= 11.42<br>t3 = -13.56 | |

t1and P1, GII versus GI child A; t2 and P2, GII versus GI child B; t3 and P3, GII versus GI child; F and P comparison of Ck-18 between different Child classes in GI

Table 4. Correlation between Ck-18 Levels and Different Biochemical and Radiological Data

| Correlations | Cytokeratin-18 (U/L) | |
|---|---|---|
| | R | P-value |
| S.Albumin (gm/dl) | -0.497 | 0.001 * |
| ALT(IU /L) | 0.463 | 0.029 * |
| AST(IU /L) | 0.329 | 0.039 * |
| Prothrombin activity (%) | -0.464 | 0.001 * |
| AFP (ng/ml) | 0.386 | 0.035 * |
| Child-Pugh score | 0.362 | 0.049 * |
| Portal vein diameter (mm) | 0.411 | 0.008 * |
| Size of Spleen (cm) | 0.428 | 0.004 * |
| Focal lesion size by CT (cm) | 0.337 | 0.022 * |
| Focal lesion size number (by CT) | 0.905 | <0.001* |

CK 18 and AFP improve sensitivity to 98% (Figure 3).

## Discussion

Egypt showed a rise in HCC from 4.0% in 1993 to 7.3% in 2003, this increase is directly related to HCV, HBV and environmental factors (El-Zayadi et al., 2005 and El-Gaafary et al., 2005). Chronic HBV and HCV infection induce liver damage by many mechanisms, including cleavage of intracellular CK-18, which in turn accelerates liver cell apoptosis (Bantel et al., 2004; Danial and Korsmeyer, 2004). CK-18 has a pivotal role in cell division and synthesis of protein and DNA (El-Zefzafy et al., 2015). As a tumor marker, Ck-18 has been well studied in different cancers as esophageal squamous cell carcinoma, renal cell carcinoma, oral cavity carcinoma, lung cancer, human breast and colorectal cancer (Weng et al., 2012).

In that study, there was a significant elevation in serum AFP in HCC group compared with the control and cirrhosis groups but with low sensitivity. A low sensitivity of AFP was reported by different studies (Soresi et al., 2003; Oka et al., 1994 and Sanai et al., 2010). In patients with high risk of HCC, AFP is usually used for screening; however, serum AFP has a transient rise during exacerbation of hepatitis on top of chronic liver disease and slight increase in chronic hepatitis and cirrhosis causing diagnostic difficulties. Also, it may be normal in some HCC patients (Sherman, 2001).

Our results showed a significant increase in serum CK-18 in the cirrhosis group compared to controls. Abdel Haleem et al., (2013) reported significantly higher serum levels and hepatic expression of CK-18 in patients of chronic hepatitis compared to controls and a significant elevation in patients with liver cirrhosis compared to patients of chronic hepatitis and they hypothesized that this was due to the release of CK-18 during liver cell apoptosis and suggested that CK-18 may be useful for monitoring disease activity in chronic HCV and liver cirrhosis patients. This report is in accordance with previous reports of Diab et al., 2008 and Yilmaz et al., 2009 who suggested that CK-18 may have a role as a non-invasive biomarker for the diagnosis or staging of chronic liver diseases.

The current study showed a significant elevation in serum CK-18 among HCC patients compared to cirrhotics. These data are accepted by Gonzalez-Quintela et al., (2006). Moreover, Kronenberger et al., (2005) found that serum CK-18 level is raised in patients with chronic hepatitis and HCC than healthy controls, but higher in HCC patients than chronic hepatitis patients. Kakehashi et al., (2009) explained that CK-18 may drive neoplastic transformation of glutathione S-transferase in rat hepatocytes, causing HCC. Mu et al., (2014) also, found that cytokeratin-18 expression was significantly higher in six of the seven HCC cell lines examined than in the control cells by the use of immunofluorescence staining and microscopic examination. we compared the levels of Ck-18 among Child-Pugh classes in group I of liver cirrhosis and no statistically significant difference was detected (P = 0.112) however, a highly significant differences were found when we compared Ck-18 levels between the HCC group and each Child-Pugh classes of liver cirrhosis group (GI) (P< 0.001) this suggest that Ck-18 levels are significantly higher in HCC than all Child-Pugh classes of liver cirrhosis. Our results also, consistent with those of Waidmann et al., (2013) and Godin et al., 2015 who reported that, serum levels of CK-18 are increased in patients with HCC compared to those with cirrhosis and this suggests that, CK-18 measurement may improve non-invasive diagnosis of HCC Waidmann et al., (2013).

Analysis of the ROC curve showed CK-18 to have a better sensitivity and accuracy than AFP, while both have a nearly similar specificity. This is similar to the results of El-Zefzafy et al., (2015) who found the CK-18 levels to be significantly increased in HCC patients than in chronic

hepatitis or normal control. Also, they reported a better sensitivity of CK-18 (95%) than that of AFP (45%) with a similar specificity for both 96.7% for cK-18 and 96.6% for AFP. Additionally, we found a better sensitivity for combined use of Ck-18 and AFP (98%).

Recently, Lorente et al. 2016 evaluated the prognostic role of CK-18 in HCC patients before liver transplantation. They measured the level of CK-18 in patients with HCC before undergoing liver transplantation and they found that, survivors at one year after liver transplantation for HCC showed lower serum CK-18 levels before liver transplantation than non-surviving patients, denoting an association between serum CK-18 levels before liver transplantation and one-year survival after liver transplantation.

Serum cytokeratin-18 levels can be used as diagnostic biomarker of HCC with better sensitivity than AFP. The combined use of both CK-18 and AFP improve the sensitivity of HCC detection up to 98%.

*Recommendation*

Further large studies are needed to evaluate the prognostic significance of CK 18 in patients of HCC.

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