

RESEARCH ARTICLE

Estimation of Leucine Aminopeptidase and 5-Nucleotidase Increases Alpha-Fetoprotein Sensitivity in Human Hepatocellular Carcinoma Cases

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Abstract

Purpose: To find parameters that can increase alpha-fetoprotein (AFP) sensitivity and so help in accurate diagnosis and rapid management of hepatocellular carcinoma (HCC), as AFP has limited utility of distinguishing HCC from benign hepatic disorders for its high false-positive and false negative rates. **Materials and Methods:** Serum levels of AFP, 5'-nucleotidase enzyme activity (5-NU) and leucine aminopeptidase enzyme (LAP) activity were measured in 40 individuals. **Results:** LAP and 5'NU were elevated in HCC at $p < 0.001$. Pearson correlation coefficients showed that changes in AFP exhibited positive correlation with both 5'-NU and LAP at ($p < 0.001$). The complementary use of LAP only with AFP resulted in an increase in sensitivity of AFP from 75% to 90% in detecting HCC. The complementary use of both LAP and 5-NU with AFP resulted in an increased sensitivity of AFP in detecting HCC from 75% to 95%. **Conclusions:** LAP and 5-FU can be determined in HCC patients in combination with AFP to improve its sensitivity and decrease false negative results.

Keywords: Hepatocellular carcinoma - alpha fetoprotein - leucine aminopeptidase - nucleotidase - sensitivity

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers worldwide. The disease is predominant in Asia and Africa, but its incidence is steadily increasing throughout the rest of the world. Most HCCs develop in patients with a history of chronic hepatitis or cirrhosis in which there is continuous inflammation and regeneration of hepatocytes. Unlike other solid malignancies, the coexistence of inflammation and cirrhosis makes the early diagnosis and prognostic assessment of HCC much more difficult (Gao et al., 2012).

HCC is a complex disease with a variety of underlying pathogenic anomalies caused by multiple risk factors. The lack of ideal biomarkers for HCC diagnosis, prognosis, and therapy have posed a major challenge to HCC management. These complications highlight the need to identify valuable biomarkers for the diagnosis and treatment of HCC. Surgical resection, liver transplantation and local ablation are considered curative therapeutic practices for HCC. Other modalities, such as targeted therapy and trans-arterial chemoembolization (TACE) are palliative treatments. Despite these curative or palliative treatments, prognosis is still poor due to underlying liver diseases and the unique biology of HCC. As a result,

biomarkers that better predict patients who are at higher risk of recurrence and poorer prognosis would help guide their treatment (Zhu et al., 2011). In addition to imaging techniques including ultrasonography, magnetic resonance imaging, and computerized tomography, the assessment of circulating biomarkers is used in the diagnosis of HCC without pathologic confirmation (Trinchet et al., 2011). Because of the ease of accessibility, circulating biomarkers such as alpha fetoprotein (AFP), VEGF, HGF, and TGF-beta are used as independent factors for HCC prognosis (Llovet et al., 2012; Wang et al., 2014) and are still preferred for prognostic prediction.

Since its first detection in the serum of HCC patients in 1964, AFP has been commonly used for diagnosis and surveillance of HCC (IuS, 1964), (Carr et al., 2014) and has been suggested as an independent indicator for prognosis thus far for patients at risk for HCC (Tangkijvanich et al., 2000; Zhou et al., 2006; Mittal et al., 2011). However, AFP level is also increased in benign liver disease (Taketa 1990). HCC patients with a high serum AFP level tend to have a shorter life expectancy. Alpha-fetoprotein was discovered in 1956 by Bergstrand and Czar using paper electrophoresis of human fetus serum proteins (Bergstrand and Czar 1956). However, it was only in 1963 that AFP gained interest after discovery of AFP in adults during

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carcinogenesis (Abelev et al., 1963). Up to date, the exact biological function of AFP remains unclear. Due to its similarity to albumin, AFP is suggested to function as a transport molecule for several different ligands such as bilirubin, fatty acids, retinoids, steroids, heavy metals, dyes, flavonoids, phytoestrogens, dioxin and various drugs (Schieving et al., 2014). AFP is also suggested to have an immunosuppressive activity and a role in regulating cell proliferation. Despite the uncertainty concerning its biological role, AFP is of diagnostic importance (Tian et al., 2011). Alpha-fetoprotein has been the most widely used serum marker for HCC diagnosis. However, its sensitivity for detecting HCC ranges between 25%-60%, and its specificity is also low because it can also be detected in patients with cirrhosis (11%-47%) and chronic hepatitis (15%-58%) (El-Serag et al., 2008; El-Attar et al., 2010; Xu et al., 2012). Increasing cut-off values can improve its specificity up to 76%, but on the other hand a corresponding decrease in sensitivity (maximum 60%) is observed (Lok et al., 2010; Bertino et al., 2012). Serum concentration of 20 ng/mL is the most commonly used cut-off value to distinguish HCC patients from healthy adults in clinical researches. However, some investigations have shown that the cut-off value reflects fluctuations among different ethnic groups (Soresi et al., 2003). Though the measurement of AFP serves as an important tool in screening HCC patients, some reports have indicated that it has a limited ability in distinguishing HCC from benign hepatic disorders due to its high false-positive and false negative rates. In addition, it has been shown that patients with acute exacerbation of viral hepatitis without HCC may also have markedly increased AFP levels (Richardson et al., 2012). All these drawbacks decrease the actual value of AFP as a diagnostic marker for HCC.

The 5'-nucleotidases represent a group of enzymes that catalyze the dephosphorylation of ribo- and deoxyribonucleoside monophosphate to the corresponding nucleosides and thus form the catabolic arm of substrate cycles that maintain balanced nucleotide pools (Augusto et al., 2013). To date, at least 5 human nucleotidases have been isolated and were shown to differ in their sub-cellular localization, substrate specificity and tissue distribution. CN1 (Cytosolic nucleotidase 1A, B), CN2 (Cytosolic nucleotidase II) and dNT1 (deoxynucleotidase I) are localized in the cytosol, whereas dNT2 (deoxynucleotidase II) is present in the mitochondria and as an ecto-enzyme on the outer plasma membrane (Mazzon et al., 2003; Hunsucker et al., 2005). 5'-nucleotidases have been suggested as more specific and sensitive markers for detecting hepatic metastasis than other liver enzymes (Fausther et al., 2012). Also breast cancer patients showed increased levels of serum 5'-nucleotidase (Chatterjee et al., 1976).

L-Leucine aminopeptidases (LAPs) are exopeptidases that catalyze the hydrolysis of leucine residues from the amino-termini of protein or peptide substrates (Matsui et al., 2006). LAPs show a high activity in liver as well as in duodenum, small intestine, pancreas, testis and stromal cells of the uterus. They represent a simple and reliable tool as a marker of hepatobiliary malignancy when compared to other conventional biochemical liver

function tests (Sathe and Taskar, 1982; Tian et al., 2014). Placental LAP (P-LAP) is the biomarker for the evaluation of ovarian epithelial malignancy and a target of molecular therapy (Mizutani et al., 2007).

Materials and Methods

Patient samples were obtained from Oncology institute in Minia. The study included 40 individuals. The first group included 20 patients suffering from hepatocellular carcinoma while the second group included 20 individuals of healthy volunteers. Using sterile vacutainer tubes, 10 ml venous blood were withdrawn from each patient and the withdrawn blood was allowed to clot. Serum was separated by centrifugation at 3000 rpm for 10 minutes. Serum was then divided into aliquots and frozen at -80°C for serological investigations. The following parameters were measured in serum: Alpha fetoprotein (AFP) level, 5'-Nucleotidase enzyme activity (5-NU), and Leucine aminopeptidase enzyme (LAP) activity.

All cases were subjected to the following investigations: complete history of patients through patient files, full clinical examination including general and abdominal examination with stress on the size of the liver and spleen. Abdominal ultra sonographic examination was performed to assess the status of the liver and spleen and detect the presence of ascites. Ultrasonographically-guided percutaneous needle was used to collect biopsies from the liver. Laboratory investigations included liver function tests with the aim of investigating the status of the liver in all patients and to exclude liver disease in control subjects. Liver function tests included levels of ALT, AST, ALP and total bilirubin.

Determination of serum alpha-fetoprotein (AFP) using ELISA technique

It was determined according to the method of (Wu et al., 1988; Bates 1991; Lee et al., 1991; Sato et al., 1993) using CALBIOTECH Alpha-Fetoprotein (AFP) ELISA kit (USA) following the instructions of the manufacturer.

Determination of serum 5'-nucleotidase (5'-NT) activity

5'-Nucleotidase (5'-NT) activity was determined depending on ATP hydrolysis using a modification of the method described by (Yegutkin 1997; Oses et al., 2004). The released inorganic phosphate (PPi) is determined quantitatively as the amount of chromophore formed with molybdate reagent, 1-amino-2-naphthol-4-sulfonic acid in bisulfite and 2-mercaptoethanol. PPi reacts with molybdate reagent to produce phosphomolybdate and PPi-molybdate complexes. 2-mercaptoethanol is responsible for color formation which has the peak absorption at 580 nm (Kuang et al., 2007). Serum total protein was determined according to the method of Gornall (Gornall et al., 1949) using commercially available kit (spectrum) using biuret reagent and standard bovine albumin.

Determination of serum leucine aminopeptidase (LAP) activity

leucine aminopeptidase (LAP) activity was determined by estimating the amount of B-naphthylamide liberated

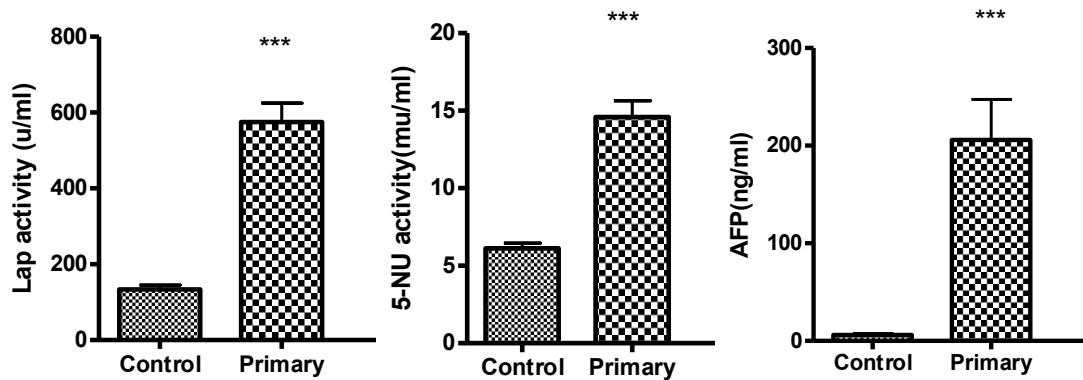


Figure 1. Serum Values of LAP Activity (u/ml), 5-NU Activity (mu/ml) and AFP (ng/ml) in Control Group to HCC Group

by the leucine aminopeptidase hydrolysis of L-leucyl-B-naphthylamide hydrochloride substrate followed by coupling with N-(1-naphthyl)ethylenediamine.2HCL then the produced color was measured colorimetrically (Martinek et al., 1964).

Statistical analysis

T test and Pearson correlation coefficients were done using SPSS and Graph pad prism software while sensitivity and specificity were calculated by equations:

$$\text{Sensitivity} = a/a+c$$

$$= a (\text{true positive})/a+c (\text{true positive} + \text{false negative})$$

$$\text{Specificity} = d/b+d$$

$$= d (\text{true negative})/b+d (\text{true negative} + \text{false positive})$$

Results

Liver function tests in control and HCC patients

All liver function tests of ALT, AST, ALP and total bilirubin were significantly ($p<0.001$) up-regulated in the HCC group compared to control (Table 1).

Table 1. Liver Function Tests of ALT, AST, ALP and Total Bilirubin Levels in HCC Versus Nonmalignant Group (Mean±S.E, N=20)

Liver function tests	Control	Primary HCC
ALT (U/L)	19.90±1.438	54.90±6.231***
AST (U/L)	22.50±1.309	96.55±20.51***
ALP (U/L)	53.80±4.370	99.35±11.00***
Total bilirubin (mg/dl)	0.5930±0.03102	1.423±0.1160***

Table 2. Pearson Correlation Coefficients Values and Significance of AFP Versus 5-NU and LAP

Correlation of AFP with other parameters	5-NU	LAP
	Pearson r	0.718
P value	$P<0.0001$	$P<0.0001$
significance	***	***

Table 3. AFP, LAP and 5-NU Sensitivity and Specificity in HCC Versus Nonmalignant Group

Cut off value	Sensitivity	Specificity
AFP < 20 ng/ml	75%	90%
LAP < 200 u/ml	85%	85%
5-NU < 8mu/ml	75%	75%
AFP and LAP	90%	80%
AFP and LAP and 5-NU	95%	65%

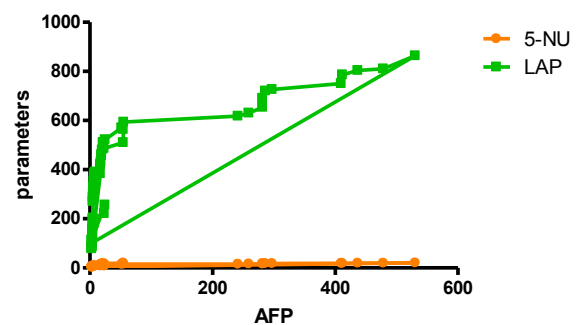


Figure 2. Pearson Correlation Coefficients of AFP Versus 5-NU and LAP

Regarding AFP level, a highly significant increase in serum alpha-fetoprotein (AFP) level was observed in HCC group at $P<0.001$ compared to normal healthy persons. With a cut-off value of AFP exceeding 20 ng/ml, the sensitivity and specificity for HCC group was 75% and 90% respectively. In addition to AFP, a significant increase in serum 5-NU activity was observed in HCC group ($P<0.001$) compared to normal healthy persons. With a cut-off value for 5-NU activity exceeding 8 mu/ml, both the sensitivity and specificity for HCC group were 75%. Also a highly significant increase in serum LAP activity was observed in HCC group ($P<0.001$) compared to healthy control persons. With a cut-off value for LAP activity exceeding 200 u/ml, the sensitivity and specificity for HCC group were equally 85% as shown in Figure (1).

Pearson correlation coefficients of AFP versus other parameters

Pearson correlation coefficients showed that changes in AFP exhibited positive correlation with both 5'-NU and LAP as shown in Figure 2, Table 2 and this correlation supports the use of these parameters to improve AFP sensitivity.

Serum values of all three parameters including AFP, LAP, and 5-NU in HCC patients were significantly higher compared to non-malignant group while sensitivity of LAP was higher than that of AFP in detecting HCC patients (85% versus 75% for AFP). Evaluating both LAP and AFP resulted in an increase in AFP sensitivity from 75% to 90% in detecting HCC, however, the specificity decreases to 65%. The complementary use of both LAP and 5-NU together with AFP resulted in an increase in sensitivity of AFP from 75% to 95% in detecting HCC (Table 3).

Discussion

Worldwide, hepatocellular carcinoma (HCC) is the 5th most common cancer and the 3rd most important cause of cancer mortality, with a 5-year survival rate of 7%. HCC is a disease with a very poor prognosis due to its resistance to conventional chemotherapy, as it is mostly diagnosed at an advanced stage when most potentially curative therapies are of limited efficacy (Sangro et al., 2011). Early detection of liver malignancy makes it possible to provide the patient with the most optimal therapy. Serum tumor markers have been used as an effective method for detecting malignant tumors for a long time, and they could be as valuable as ultrasonography and computer tomography in the diagnosis of HCC (Malaguarnera et al., 2010). Current conventional methods for diagnosis and screening include physical examination, ultrasound imaging and serum alpha-fetoprotein (AFP) concentration measurement in high-risk patients. Despite the fact that serum AFP is still the golden standard amongst diagnostic markers for HCC, its diagnostic value is more and more questionable, due to the poor sensitivity and specificity of the assay used (Gan et al., 2014). In addition, AFP has a limited utility in distinguishing HCC from benign hepatic disorders for its high false-positive and false negative rates as in case of patients with acute exacerbation of viral hepatitis but no HCC. Those patients may have markedly increased AFP levels (Bae et al., 2005). In 2003, Yao reported three cases with chronic hepatitis B infection who presented with initial serum AFP levels more than 1,000 ng/ml (Yao, 2003). There was no evidence of HCC based on multiple abdominal imaging studies. Initiation of antiviral drug led to rapid decrease in AFP levels in all three cases, which suggests that elevation of AFP was due to hepatic inflammation and viral replication. Similarly, Bae and co-workers reported 2 cases with HBV and HCV infection showing AFP levels that exceeded 4700 ng/ml (Bae et al., 2005) which decreased in response to antiviral therapy. Both findings reinforce the idea that AFP elevation was regarded to hepatic inflammation and viral replication declaring that measurement of AFP alone is not sufficient for the diagnosis of HCC. This study aimed to find parameters that can alleviate AFP sensitivity problem and hence provide an accurate diagnosis and rapid management of HCC. Our results showed higher sensitivity level of LAP (85%) in HCC patients compared to AFP (75%) indicating LAP ability to enhance liver cancer and metastasis. Recently, Tripathi and his work-team showed that over-expression of LAPs protein could enhance liver malignancy (Tripathi et al., 2013). Also he showed that knockdown of LAPs in HepG2 induced cell cycle arrest at G1/S checkpoint while overexpression of LAPs promoted tumorigenesis in Huh7 cells via accelerating G1/S phase transition. It was reported that upregulation of LAPs induced low expression of E-cadherin which is a cardinal regulator of epithelial-to-mesenchymal transition (EMT) and so advanced the mobility and aggressiveness of HCC cells (Zhai et al., 2014). LAPs are expressed in HCC tissues and cells and provide clear evidence for the important role of LAPs in HCC development and metastasis. Targeting

this protein may be of great value in the treatment of HCC (Tian et al., 2014).

Our results showed that 5'NU sensitivity was similar to AFP values (75%) in HCC patients. 5'-nucleotidases enzymes have been suggested to be more specific and sensitive than other liver enzymes in detecting hepatic metastases and hepatobiliary diseases (Hunsucker et al., 2005). Regarding correlation results of all parameters to AFP, changes in AFP exhibited positive correlation ($P < 0.0001$) with 5'-NU, LAP and this correlation results can support the use of these two parameters to increase AFP sensitivity.

The combined determination of LAP and AFP increased the sensitivity of the diagnosis of HCC patients to 90% while the simultaneous determination of both markers LAP and 5'NU with AFP all together improved the sensitivity of the diagnosis of HCC patients to 95% but decreased the specificity to 65%. The increased sensitivity value indicates the importance of co-determination of the three markers to reach more efficient diagnosis of HCC as sensitivity issue is greatly looked for in risky cases.

In conclusion, from our results we can conclude that the determination of the parameters LAP and 5-FU in combination with AFP can be used as a diagnostic tool in HCC patients to upgrade AFP's sensitivity to 95% and decrease its false negative results.

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