Diagnostic Value of Serum Glypican 3 in Hepatocellular Carcinoma: A Case Control Study of Patients Seen at the University College Hospital, Ibadan, Nigeria

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Abstract
Hepatocellular carcinoma (HCC) is a major health problem which has been increasing in Nigeria with a doubling in the incidence rate in the past 10 years. Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP). The reported sensitivity and specificity of AFP are not sufficient for early diagnosis, and so additional and more sensitive markers are needed. One of such tumor markers is serum Glypican 3 (GPC3). So, this study aimed to determine the frequency of GPC3 in apparently healthy controls and patients with HCC, comparing the diagnostic efficacy of GPC3 to AFP in HCC and to assess the diagnostic accuracy of combined AFP and GPC3 in the diagnosis of HCC. Fifty patients with clinical, radiological and/or histological features of HCC, and fifty apparently healthy controls who were HBsAg and anti-HCV negative and had normal abdominal ultrasonography scan were recruited for the study. Serum estimation of AFP and GPC-3 were done for all the subjects. The sensitivity and specificity of GPC3 was 54 % and 44% respectively based on a receiver operating characteristic(ROC) curve –derived optimum cut-off level >20 ng/ml. The sensitivity and specificity of AFP were 64% and 100% at an ROC derived optimum cut-off level of 20 ng/ml. The area under the receiver operating characteristic curve (AUROC) for AFP was 0.69 and was significantly larger than that of GPC3 which was 0.42(p <0.001). The diagnostic value of GPC3 in our patients with advanced hepatocellular carcinoma is poor in comparison with AFP.

Keywords: Glypican 3, Diagnostic value, Hepatocellular carcinoma, Nigeria
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1. Introduction
Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the second leading cause of cancer death worldwide. According to the GLOBOCAN report, there were estimated 782,000 liver cancer cases and 746,000 liver cancer related deaths in 2012\(^1\).

HCC is a major health problem, with more than 500,000 cases diagnosed annually\(^2\). The burden of HCC has been increasing in Sub-Saharan Africa with a steady increase in the incidence rate in the past 10 years\(^3\). Current diagnosis of HCC relies on clinical information, liver imaging, measurement of serum alpha-fetoprotein (AFP), \(^5\) and/or histology. The measurement of AFP may serve as an important tool in screening for HCC; however some reports have indicated that it has limited utility in differentiating HCC from benign hepatic disorders.\(^6\) Its high false-positive and false-negative rates, elevated levels in patients with acute exacerbation of viral hepatitis and in tumors other than HCC e.g. testicular tumors \(^6\) are noted as drawbacks.

Glypican-3(GPC3) is a member of the glypican family of heparan-sulfate proteoglycans (HSPGs), which is bound to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor. It is normally expressed in fetal liver and placenta and also has negligible expression in normal adult liver. GPC3 is highly expressed in HCC, and it has been discovered as a potential serological and histochemical marker, specific for the differentiation between the early stage of HCC formation and its precancerous state\(^1\). In 1997, Hsu\(^8\) et al first reported that mRNA and protein levels of GPC3 were upregulated to a greater extent in most HCCs than in normal liver, cholangiocarcinoma and metastatic carcinomas of the liver.

Thereafter, there have been series of studies comparing the sensitivity and specificity of AFP and GPC3 in an attempt to aid early diagnosis. Many of the studies have shown GPC3 to be more sensitive than AFP\(^9\), \(^10\), \(^11\). However series of studies have disputed these findings; Yasuda\(^9\) et al, evaluated the usefulness of GPC3 for the diagnosis of HCC in comparison with the three standard tumor markers (AFP, AFP-L3 and DCP). Authors observed that serum GPC3 concentration showed no increase in patients with HCC; rather, it was higher in patients without HCC. In addition, serum GPC3 did not correlate with the stage of HCC, suggesting that the level did not reflect the progression of HCC.\(^12\) In spite of the extensive studies on this tumour marker especially in the last decade, to date there is no report known to the authors of GPC3 expression in HCCs among Africans.

The aim of this study was to determine the frequency of GPC3 in apparently healthy controls and patients with HCC, compare the diagnostic efficacy of GPC3 to AFP in HCC and to assess the diagnostic accuracy of combined AFP and GPC3 in the diagnosis of HCC.
2. Patients and Methods

This was a cross-sectional case–control study, carried out in the Gastroenterology and Liver Unit of the Department of Medicine, University College Hospital (UCH), Ibadan, South-West Nigeria.

Patients were consenting adults with clinical, sonological and/or histological features of HCC, presenting to the Gastroenterology Unit at the Medical Outpatient Department (MOP) of UCH or admitted to the medical wards of the institution. Controls were apparently healthy patients’ relatives and volunteers such as members of staff of the hospital. Patients and controls for the study were recruited from March 2015 to November 2015. Fifty of these subjects were cases with clinical, radiological and/or histological features of HCC (30% of the cases were diagnosed via histology), while fifty of the subjects were apparently healthy individuals who were not reactive to HBsAg and anti-HCV. Ethical approval was sought and obtained from the joint UI/UCH Ethical Review Committee (Registration number NHREC/05/01/2008A) Informed consent was obtained from all the patients and controls before enrolling them for the study.

A full history and detailed clinical examination was done on all the patients. Laboratory tests carried out included hepatitis B surface antigen and hepatitis C antibody tests. Liver function tests including prothrombin time were done by conventional methods. All the patients and controls had abdominal ultrasound. Patients with evidence of malignancies outside the liver or metastasis to the liver, as determined by clinical, radiological and/or histological features, as well as those who had had chemotherapy were excluded from the study. All the subjects recruited for this study were native Nigerians.

Fifteen millilitres of venous blood was collected from all the subjects and sera stored at −20 °C until they were analyzed for the tumour markers. The analysis for both tumour markers (GPC3 and AFP) was carried out at the Virology research laboratory of the University of Ibadan, Ibadan.

2.1 AFP Assay

AFP was tested using commercially available immuno-enzymometric assay kit manufactured by INTECO Diagnostics, UK Ltd., London. The test was carried out as per manufacturer’s instructions. The upper limit of normal for the kits as given by the manufacturer was 10 IU/ml.

2.2 GPC3 Assay

The GPC3 values were obtained using GPC3 enzyme Immunoassay test kit (Konobiotech, China). The tests were carried out as per the manufacturer’s instructions, which involve using anti-GPC3 monoclonal antibody coated on the inner surface of the micro cup. GPC3 in the sample was captured and allowed to react with enzyme-labelled horse radish peroxidase. When substrate solution is added to the reaction product, the enzyme reaction develops colour. GPC3 concentration was determined from the absorbance of the coloured solution.

2.3 Data Analysis

The data was analyzed using the Statistical Package for Social Sciences (SPSS) version 21. Simple descriptive statistics were computed in the form of frequencies and percentages. In case of continuous variables were expressed as means. The significant level was set at p<0.05. Independent sample Pearson t-test was done to study the difference in the means of serum levels of AFP and Glypican 3 between cases and controls. This was graphically represented using a box plot. The sensitivity, specificity and positive predictive value were calculated for the various biomarkers using a 2x2 contingency table.

3. Results

A total of 100 adult Nigerians were studied, comprising fifty subjects with clinical, radiological and/or histological features of HCC (30% of the cases were diagnosed by histology), and fifty apparently healthy controls who were not reactive to HBsAg and anti-HCV. The mean age of the cases was 49.12±15.08 yrs, while that of the controls was 43.94±12.42 yrs. There was no significant age difference between the cases and the controls. (p=0.064) There was a male predominance among the cases, 41 males (82%) vs 9 females (18%) while controls were made up of 25 males (50%) and 25 females (50%). Thirty five (70%) of the cases were positive for HBsAg, while 2(4%) were positive for anti-HCV. This is shown in Table 1.
Table 1: Demographic and clinical characteristics of HCC cases and controls

| Variables                  | Cases              | Controls             | *p-value |
|----------------------------|--------------------|----------------------|----------|
| Mean Age (years)           | 49.12±15.08        | 43.94±12.42          | p = 0.064|
| Gender M:F                 | 41:9               | 25:25                | p = 0.001|
| Positive for HBsAg         | 35(70 %)           | 0(0.00 %)            |          |
| Positive for Anti-HCV      | 2 (4 %)            | 0(0.00 %)            |          |

3.1 Expression of AFP and GPC3 in Patients and controls

Serum AFP levels were measured in the same set of serum samples. The level of serum AFP in patients with HCC (162.62±185.49)ng/ml was significantly higher than those of healthy controls (2.94±2.06) ng/ml (p <0.001) (Table 2). The box plot also showed that the cases had a much higher mean serum AFP level (162.6) ng/ml compared to the controls (2.9) ng/ml (Fig. 1) while the receiver operating characteristic curve (Fig. 3) for serum AFP had an inflection at the point of optimal sensitivity of 68% and specificity of 99%, at the cutoff value of 15ng/ml. Serum GPC3 levels were also measured in the same set of serum samples. The level of serum GPC3 in patients with HCC (27.51±23.69) was lower than in healthy controls (48.03±56.57) (p=0.20). (Table 2) The box plot did not show any significant difference in the mean serum GPC3 level among the cases and controls (Fig. 4) while the receiver operating characteristic curve (Fig. 3) for serum GPC3 reflected its poor accuracy as a screening test for HCC, compared to AFP.

Table 3: GPC3 versus AFP in differentiation of patients with HCC from those without liver disease

| GPC3(IU/ml) | Sensitivity (%) | Specificity (%) |
|-------------|-----------------|-----------------|
| > 20        | 100             | 26.7            |
| AFP(IU/ml)  |                 |                 |
| > 10        | 74.2            | 85.0            |
| > 20        | 64.0            | 100             |
| > 100       | 42.0            | 100             |
| > 200       | 38.0            | 100             |
| > 300       | 29.0            | 98.3            |

Figure 1: Box plot of the serum AFP levels of the HCC cases and controls.
Figure 2: Box plot of the serum GPC3 levels of the HCC cases and controls.

Figure 3: ROC Curves for GPC3 and AFP in the diagnosis of HCC. The area under the ROC curve (AUROC) for GPC3 in the diagnosis of HCC is 0.423 (95% CI 0.310-0.536) compared to the AUROC for AFP in the diagnosis of HCC 0.866 (95% CI 0.780-0.926)

3.2 Sensitivity and specificity of AFP and GPC3
The sensitivity and specificity of AFP at different cut-off points and GPC3 at 20ng/ml are presented in table 3.

4. Discussion
The burden of HCC in sub-Saharan Africa continues to progressively worsen and mortality is usually among male patients in their prime.

Currently, the measurement of serum AFP level has been the only marker routinely used for detecting and monitoring HCC. Although, AFP has high sensitivity in detecting HCC, it equally exhibits high false-positivity. In our study, the sensitivity and specificity of AFP in differentiating HCC from healthy controls at a cut-off value
of 20 ng/mL were 64% and 100%, respectively.

In this study, serum GPC3 levels were increased in 54% of patients with HCC and in 56% of subjects without liver disease. Thus, the sensitivity and the specificity of this serum maker in differentiating HCC from normal controls were 53% and 44%, respectively. This finding is in agreement with previous reports from a recent study which evaluated the usefulness of GPC3 for the diagnosis of HCC in comparison with the three standard tumor markers (AFP, AFP-L3 and DCP). Authors observed that, serum GPC3 concentration showed no increase in patients with HCC; rather, it was higher in patients without HCC. In addition, serum GPC3 did not correlate with the stage of HCC, suggesting that the level did not reflect the progression of HCC.

They however attributed this to the use of the commercially available kit (BioMosaicos) for the measurement of serum GPC3 in the study. The Kit uses the anti-GPC3 monoclonal antibody “clone 1G12” that recognizes the last 70 amino acids of the C-terminal of the core protein (amino acids 491–560). This C-terminal region of GPC3 binds to the cell membrane and might not be released into the serum. Several other previous studies have also obtained results indicating poor diagnostic value of GPC3 for HCC as in the present study.

These previous studies and our results showed the poor sensitivity of GPC3 for diagnosing HCC, which might suggest low clinical diagnostic value of serum GPC3 for HCC. Although, it is possible that the concentration of GPC3 is lower in the serum than in tumor tissues in patients with HCC.

Furthermore, we also observed an elevation in serum GPC3 in controls and this has been observed in several studies. Some authors assumed that GPC3 could be produced by nonmalignant or premalignant liver cells. In addition, several recent studies have confirmed the expression of GPC3 in regenerative nodules, hepatitis C samples, and even in normal liver tissues. Although, none of our controls appeared to have any of these conditions.

In this present study, the expression of serum GPC3 was higher in the controls than in the cases (mean serum GPC3 27.5±23.69 and controls 48.03±56.56) which showed a poor diagnostic accuracy despite the fact that most of the cases had advanced HCC. Some of the reasons for this include the fact that no widely accepted cut off for GPC3 in the serum has been set. In most studies discussed earlier on, the serum GPC3 level ranged from 3.9pg/ml to 300ng/ml. It might therefore be safe to assume that in our study, the levels in the controls were in the normal ranges, while the levels seen in HCC cases were reduced probably due to an advanced disease. However, this needs further evaluation. Three of the four studies mentioned earlier which observed poor GPC3 expression were conducted in patients with mostly advanced disease. Lee et al found that GPC3 had a good diagnostic accuracy in small tumours (<3cm), while it had a poor sensitivity and specificity in larger tumours.

The GPC3 assay kit is another possible source of inter study result variations. The kit used in our study was a commercial kit from KonobioTech. The laboratory had used several other kits from this same manufacturer without any problem. The cold chain was not broken as the kit was kept within the temperature 2-8 °C from the manufacturers till its use in the research laboratory. The expiry date was October 2015 and the study was concluded by November 2015, and to ascertain the validity of the kit, three standard sera were used; a strong positive, a weak positive and a negative standard. The manufacturer’s protocol was followed. However, different kits have been available for the study of serum Glypican 3 and a recent study showed some of the difficulties that could arise from the assay variations.

The nature of plasma GPC3 may impact ELISA utility. Plasma GPC3 can be produced when an extracellular lipase (Notum) cleaves the GPI anchor of membrane bound GPC3 or when secreted, possibly by endogenous Notum or GPI-phospholipase D by the cell. Various species of GPC3 may also be found, as Hippo et al was able to detect a 50-kDa fragment produced by a cleavage in the COOH-terminal in addition to the commonly detected glycanated GPC3. Furthermore, plasma GPC3 competitively binds with several growth factors, which could interfere with antibody binding. Given these possibilities, it is clear that a uniform understanding of plasma GPC3 has not been reached.

In this study, Alpha fetoprotein values were frequently elevated in patients with HCC. AFP values >200 ng/ml was seen in 38% (19 cases) compared to controls. This is significantly lower than the 51.7% and 45.2% obtained by Soyemi et al and Oghoh. However, it is slightly higher than the 32% obtained by Ola et al. It however validates previous studies that have found that not all patients with hepatocellular carcinoma elaborate this tumor marker. These findings may be due to the differences in the sample sizes. The works of Soyemi and Oghoh had fewer number of cases than in this present study. The mean AFP value was 162.62±185.49 ng/ml, which compares to the AFP values in Nigerian patients with liver disease by Ola et al.

Furthermore, Ette et al noted the high sensitivity of des-gamma-carboxyprothrombin (DCP) in African patients relative to AFP in detecting HCC (especially tumours greater than 3cm) without combining both tumour markers.

HBV infection was the main risk factor associated with HCC in this study, with 70% of the patients being positive for HBsAg. This is comparable with the work of Olubuyide et al in the same study location about two decades earlier, in which a prevalence of 70% was reported among patients with HCC. Other authors have found the prevalence of HBV among patients with HCC to be 61%, 61.5% and 67% respectively.
5. Conclusion
In this study, serum level of GPC3 was higher in healthy controls than in patients with HCC and its diagnostic value was found to be poor in comparison with AFP in patients with advanced hepatocellular carcinoma. Also, the accuracy of combined GPC3 and AFP for the diagnosis of HCC was poor.

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