Development and Validation of a Two-Site Immunoradiometric assay for Glypican-3 in plasma: Implication in Diagnosis of Hepatocellular Carcinoma

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This work aimed to set-up and evaluate a lab-made immunoradiometric assay for the detection of plasma glypican-3 (GPC3) in comparison with a commercial ELISA kit and to use them to evaluate the diagnostic potential of GPC3 in HCC patients. Anti-GPC3 monoclonal antibodies were radio-iodinated and used with a second antibody in the IRMA assay. The study included 450 subjects in 3 groups, 150 HCC patients, 150 hepatitis-C-virus (HCV) patients and 150 normal healthy subjects. Plasma GPC3 was assayed by our lab-made IRMA and by a commercial ELISA kit, along with alpha-fetoprotein (AFP). We were able to set-up an IRMA assay to measure plasma GPC3 and applied it to diagnose HCC patients. When compared to a ELISA kit, our IRMA assay showed much better performance characteristics on ROC curves with much higher area under the curves, sensitivities and specificities when diagnosing HCC patients from normal controls or HCV patients. Using our IRMA assay, showed that GPC3 is a good diagnostic indicator for HCC with 1.4 ng/ml cutoff for controls and at a cutoff value of 1.55 ng/ml it was able to discriminate HCC and HCV patients. GPC3 measurement was much better than AFP for the diagnosis of HCC.

Key words: HCC, radio-iodination, chloramines-T, Glypican-3, immunoradiometric assay

INTRODUCTION

Hepatocellular carcinoma (HCC) is a fatal cancer whose incidence is increasing sharply worldwide, especially in Egypt. HCC is sorted fifth as the most widespread neoplasm worldwide, and third as the most prevalent cause of cancer-related death (Jemal, 2011). Despite the great progress that was made in HCC treatment, its prognosis remains poor because HCC is usually diagnosed at a later stage.

Tests for HCC screening depends on serum markers and imaging examinations. Ultrasonography (US) is the most vastly used imaging test, however, it greatly depends on the operator's experience. In addition, HCC usually develops on a cirrhotic grounding, which may preclude the identification of small tumors by US. Among serological biomarkers, alpha-fetoprotein (AFP) is the most extended marker for HCC screening, but it is not sufficiently sensitive or specific (Ferrin, 2015). Thus, there is necessity to develop new indicators for early HCC diagnosis.

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Glypican-3 (GPC3) belongs to the heparan sulfate proteoglycan family. It is a cell surface protein which could be cleaved off to release a soluble 40-KDa fragment that can be detected in blood. GPC3 is broadly expressed in human embryos and plays a capital role in morphogenesis and growth. It is detectable in fetal liver starting from embryonic weeks 18 till 30, but cannot be identified in any of the normal adult hepatic tissue (Filmus, 2008; Wu, 2016). In 1997, it was reported for the first time that levels of GPC3 mRNA and protein were upregulated in most HCCs (Hsu, 1997). Since then, the diagnostic potential of GPC3 has been widely studied in HCC, and many of these studies confirmed that GPC3 could be a useful biomarker for HCC when measured serologically or immunohistochemically.

The current work aims to set up and evaluate a lab-made immunoradiometric assay for the detection of GPC3 in plasma in comparison with a commercial ELISA kit and to use them to evaluate the diagnostic potential of GPC3 in HCC patients.

SUBJECTS AND METHODS

Samples

The study included 450 subjects divided into three groups, group 1 included 150 patients recently diagnosed with HCC; group 2 included 150 patients with chronic HCV; and group 3 included 150 normal healthy individuals. Groups 2 and 3 subjects were selected of matched age and sex as group 1. Group 1 patients were collected from those admitted to the Cancer Management and Research Department, while group 2 patients were selected from those admitted to the Internal Medicine Department, Medical Research Institute, Alexandria University.

The study was approved by the local ethics committee and all subjects signed an informed consent prior to sample collection. From each subject involved in the study, a single blood sample was drawn on tubes containing EDTA as anticoagulant. Blood was centrifuged immediately at 4°C and plasma was isolated and stored in -80°C until used.

The median age of the HCC group was 42 y, with a range of 24-70 y, while that of the HCV group was 60 y, with a range of 44–73 y. The control group median was 50 y with a range of 35–62 y. However, no significant difference was found regarding age between the 3 groups (p=0.103). The male/female ratio of the HCC group was 4.8:1 (16 males and 26 females) and subjects of the two other groups were selected to match the male/female ratio of the HCC group (p=0.861).

Radiolabeling of monoclonal anti-GPC3 antibody

1G12 Monoclonal antibody (MAb) to human GPC3 (BioMosaics Inc., Burlington, VT, USA), 0.5 mg/ml PBS, was radiolabeled by Chloramine-T method (Marchalonis, 1969). All reagents were prepared in PBS (pH 7.2) containing sodium azide 0.02%. In a rectivial following the reagents were added in quick succession, MAb (10 μg in 20 μl), 125I as NaI (PerkinElmer Health Sciences, Groningen, Netherlands) 0.5 mCi in 0.5 μl and Chloramine-T trihydrate (Sigma-Aldrich, USA) 20μg in 20 μl, with continuous agitation for 30 seconds. The reaction was terminated by the addition of L-Cysteine (Sigma-Aldrich, USA) 20 μg in 20 μl and potassium iodide (Oxford Laboratories, UK) 20 μg in 20 μl.

Purification of labeled antibody

Labeled MAb was separated from non-labeled MAb by gel filtration chromatography using Sephadex G-25 (Sigma-Aldrich, USA). The column was packed with Sephadex, and the void volume and elution volume measured by blue dextran 2000 (Sigma-Aldrich, USA). The reaction mixture was applied to the column and allowed to pass through; the eluted product was collected in 115fractions,0.5 ml each. In each fraction, radioactivity and protein concentration were measured. Radioactivity showed two peaks and protein showed one peak that matched the first peak of radioactivity, as shown in figure 1. The fractions that contained both radioactivity and protein content were pooled together. The specific activity of the labeled MAb was 6.3×10^5 Bq/μg MAb (17 μCi/μg MAb). Immunoreactivity of the labeled MAb was verified.

Setting up of GPC3 Immunoradiometric Assay (IRMA)

Standards and controls were prepared by adding a known amount of Recombinant human GPC3 protein (ab220562) (Abcam plc, Hong Kong), which has a molecular weight of 62 kDa and amino acid sequence from 25 to 559. Recombinant GPC3 was reconstituted in PBS (pH 7.2) to make a 1 mg/mL stock solution. Standards and controls were prepared by dilution of stock solution using plasma of normal subjects that did not show any detectable GPC3, to final concentrations of 0, 0.156, 0.312, 0.625, 1.25, 2.50, 5, 10 and 20 ng/ml as standards and 0.5, 3.5 and 15 ng/ml as controls.

For the double antibody IRMA assay, a second antibody, Polyclonal bovine anti-mouse IgG antibody (XG-6148Bt) (ProSci Incorporated, California, USA) 0.5 mg/0.75 ml, was used.

All standards, controls and samples were assayed in duplicates. Clear Falcon™ Round-Bottom Polypropylene Tubes (Fisher Scientific Co. LLC, Pittsburgh, USA) were prepared. 35 μl of each standard, control or sample were pipetted into their respective tubes, then 30 μl of 125I-labeled MAb were pipetted into each tube, vortexed and incubated over-night at 4°C. 50 μl of polyclonal antibody and 100 μl of polyethylene glycol 4000 (Alpha Chemika, Maharashtra, India) (30% w/v in PBS) were added to each
tube, vortexed and incubated for 2 hours at 37°C then over-night at 4°C. All tubes were centrifuged at 3500 rpm for 30 min then decanted, washed with 1ml PBS, centrifuged at 3500 rpm for 30 min then decanted thoroughly and finally counted for 1 min/tube in a gamma counter (WIZARD® Automatic Gamma Counter, PerkinElmer, Waltham, MA, USA). Standards were used to construct a standard curve drawn as %B/T radioactivity on Y-axis vs GPC3 concentration on X-axis, figure 2A. Concentrations of controls and samples were determined by interpolation from the standard curve.

**Measurement of GPC3 by ELISA**

GPC3 was also measured by an Enzyme-linked Immunosorbent Assay (ELISA) Kit (Uscn - LifeScience Inc., Wuhan, China). According to the manufacturer's instructions, 100µL of standards, blank or samples were added into their respective wells, covered and incubated for 2 hours at 37°C. Wells were aspirated and washed then 100µL of Detection Reagent A working solution was added to each well and incubated for 1 hour at 37°C. Wells were aspirated and washed then 100µL of Detection Reagent B working solution was added to each well, incubated for 30 minutes at 37°C. Wells were aspirated and washed then 90µL of Substrate Solution was added to each well and incubated for 15 minutes at 37°C. 50µL of Stop Solution was added to each well then, the optical density (OD) of the wells was immediately read at 450nm. A standard curve was drawn and control and sample concentrations were determined by interpolation from the standard curve, figure 2B.
Measurement of AFP

AFP was assessed in all samples using Immunoradiometric Assay (IRMA) kit (BioSource Europe S.A.-Nivelles-Belgium) which is a two-step assay based on coated tube separation whose lower detection limit was 0.5 IU/ml. According to the manufacturer’s instructions; 50 µl of each calibrator, control or sample were dispensed into respective tubes and incubated for 1 hr at room temperature. Tubes were decanted and washed and 100 µl of anti-AFP-125I tracer were added into each tube and incubated for 1 hr at room temperature. Tubes were decanted, washed and counted on gamma counter. A standard curve was constructed and unknown samples were depicted by interpolation from the curve.

Quality testing parameters of GPC3 assays

Analytical sensitivity: The assay detection limit is the lowest measurable concentration distinguishable from zero. It was determined by measuring the assay standard zero 20 times in the same assay and the detection limit would be the value corresponding to the counts of 2 times of SD above the mean (M±2SD) of the zero standard.

Precision and accuracy: Intra-assay precision was studied by assaying specimens from two normal individuals (1.5 and 12 ng/ml) 25 times, in the same assay, while Inter-assay precision was calculated by assaying specimens from two normal individuals (1.5 and 12 ng/ml) in 20 consecutive assays. The coefficient of variation (CV) was calculated as SD/mean×100.

Linearity and analytical recovery: Three samples with concentrations over 10ng/ml were diluted 1:2, 1:4, and 1:8. The percent recovery was determined after measurement of the diluted samples. Sample spiking recovery was determined by adding two different amounts of GPC3 into three patient plasma samples with known GPC3 values. The percentage of sample spike recovery was calculated following the assay of the spiked samples in comparison with the expected value.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software package, version 22.0 (SPSS Inc., Chicago, IL, USA).

Data were expressed as ranges and mean ± standard deviation (M±SD). Kruskal-Wallis was used to examine the difference among three groups. Wilcoxon rank-sum test was used for multiple pair-wise comparisons. Receiver–operating characteristics (ROC) curves were performed to define the optimal cut-off values, and to assess sensitivity, specificity, and respective areas under the curve (AUCs) with 95% confidence interval (CI). Differences between two independent groups were compared with the Mann-Whitney U test. The Spearman’ rank correlation coefficient was used to evaluate the correlations. For all tests, a p-value of ≤ 0.05 was considered as statistically significant.

RESULTS

Performance characteristics of GPC3 assays

For the preparation of the IRMA kit, MAb was radiolabeled by 125I, to a very high specific activity of 6.3×105 Bq/µg MAb (17 µCi/µg MAb). In the working solution of the MAb used for the assay, the total count/tube was 75,000±1,200 CPM. A typical IRMA standard curve is shown in Figure2A. The affinity-purified antibodies used in the assay, either as capture antibody or as 125I-labeled antibody, ensured the strong immunoreaction of antigen-antibody binding and low background of 114±62 CPM (M±SD) which comprised 0.15% from the total, while the maximum binding reached up to 40% of the total count.

The analytical sensitivity of the IRMA kit, expressed as the minimum detectable limit, was 0.006 ng/ml when 35 µl sample is used, while sensitivity of the ELISA kit was 0.057 ng/ml. For IRMA assay, the inter-assay CVs were 6.3% and 2.5% and the intra-assay CVs were 6.9% and 2.7% for concentrations 1.5 and 12 ng/ml; respectively. For the ELISA kit, the inter-assay CVs were 10.0% and 6.3% and the intra-assay CVs were 11.5% and 7.1% for concentrations 1.5 and 12 ng/ml; respectively.

The percent recovery was determined after measurement of the diluted samples. Within the assay measurement range of 0.1-20 ng/ml, satisfactory assay linear recoveries of 94-107% were observed for the IRMA assay and 81-105% for the ELISA kit. Sample spiking recovery was determined as the percentage of sample spike recovery in comparison with the expected value. Recoveries from 99.3 to 108.5% were observed for IRMA and 85-104% for ELISA.

Thus, the performance characteristics of the lab-made IRMA assay were satisfactory for the measurement of GPC3 concentration in plasma of HCC and HCV patients and in control subjects.

Subjects involved in the study

The study involved 450 subjects divided into three groups, group 1 included 150 patients recently diagnosed with HCC; group 2 included 150 patients with chronic HCV; and group 3 included 150 normal healthy subjects.

Clinicopathological parameters of patients of the HCC group are presented in table 1. Only 6.7% of the cases were of stage I, with 36.0%, 31.3% and 26.0% were of stages II, III and IV respectively. 62.0% of HCC patients...
had ascites and 28.7% had lymph node metastasis. Also 29 patients (19.3%) had distant metastasis. Patients of the HCC group had significantly higher Child-Pugh score than HCV patients (p<0.001), as more than half HCV patients were of score A where half of HCC patients had score C, as presented in table 2.

Table 1: clinicopathological parameters of HCC patients group.

<table>
<thead>
<tr>
<th>Clinical Stage</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>6.7</td>
</tr>
<tr>
<td>II</td>
<td>54</td>
<td>36.0</td>
</tr>
<tr>
<td>III</td>
<td>47</td>
<td>31.3</td>
</tr>
<tr>
<td>IV</td>
<td>39</td>
<td>26.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ascites</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>-ve</td>
<td>57</td>
<td>38.0</td>
</tr>
<tr>
<td>+ve</td>
<td>93</td>
<td>62.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymph Node involvement</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>-ve</td>
<td>107</td>
<td>71.3</td>
</tr>
<tr>
<td>+ve</td>
<td>43</td>
<td>28.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>121</td>
<td>80.7</td>
</tr>
<tr>
<td>Yes</td>
<td>29</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Table 2: comparison of Child-Pugh score distribution in HCV and HCC patients groups.

<table>
<thead>
<tr>
<th>Child-Pugh score</th>
<th>HCV</th>
<th>HCC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>83</td>
<td>55.3</td>
<td>23</td>
</tr>
<tr>
<td>B</td>
<td>49</td>
<td>32.7</td>
<td>51</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>12.0</td>
<td>76</td>
</tr>
</tbody>
</table>

A: mild, B: moderate and C: severe

Table 3: GPC3 levels as measured by IRMA and ELISA and AFP levels indifferent study groups.

<table>
<thead>
<tr>
<th>GPC3 ELISA (ng/ml)</th>
<th>GPC3 IRMA (ng/ml)</th>
<th>AFP (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range/M±SD</td>
<td>P1/P2</td>
<td>Range/M±SD</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 – 16.50</td>
<td></td>
<td>0.13 – 2.20</td>
</tr>
<tr>
<td>5.84 ± 6.05</td>
<td></td>
<td>0.95 ± 0.60</td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.27 – 19.50</td>
<td>P1=0.038</td>
<td>0.14 – 2.70</td>
</tr>
<tr>
<td>8.32 ± 5.38</td>
<td></td>
<td>1.41 ± 0.75</td>
</tr>
<tr>
<td>HCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.30 – 19.0</td>
<td>P1=0.002</td>
<td>0.67 – 5.70</td>
</tr>
<tr>
<td>10.93 ± 3.99</td>
<td>P2=0.031</td>
<td>2.79 ± 1.18</td>
</tr>
<tr>
<td>p</td>
<td>0.002</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

IRMA/ELISA correlation Control: r_s=0.411, p=0.024

IRMA/AFP correlation HCC/HCV: r_s=0.146, p=0.442

IRMA/AFP correlation r_s=0.203, p=0.186

p: significance for Kruskal Wallis test for comparing different studied groups
p_1: significance for Mann Whitney U test for comparison with the control group
p_2: significance for Mann Whitney U test for comparison of HCV and HCC groups

Diagnostic significance of GPC3 and AFP

As indicated by ROC curves, GPC3 measurement by IRMA had a much higher AUC, sensitivity and specificity than GPC3 measured by ELISA, figure 3. As a marker for liver diseases, GPC3 measured by IRMA was superior (p<0.001), with an AUC of 0.881. At a cutoff value of 1.4 ng/ml for normal control subjects, the sensitivity of the test was 90% and the specificity was 67%. As a marker of HCC, assaying GPC3 by IRMA was also superior (p<0.001) with an AUC of 0.802. At a cut off value 1.55 ng/ml, the sensitivity and specificity of the test were 71% and 78%, respectively.

GPC3 measurement

GPC3 levels as assayed by IRMA in control subjects ranged 0.13-2.20 ng/ml with a mean of 0.95 ng/ml, while in HCV patients its range was 0.14-2.79 ng/ml with a mean of 1.41 ng/ml and in HCC patients GPC3 range was 0.67-5.70 ng/ml and the mean were 2.79 ng/ml. A significant difference was found between all three groups (p<0.001). GPC3 levels as assayed by ELISA in control subjects ranged 0.0-16.5 ng/ml with mean of 5.84 ng/ml, while in HCV patients its range was 1.27-19.5 ng/ml with a mean of 8.32 ng/ml and in HCC patients GPC3 range was 3.30-19.0 ng/ml and the mean were 10.93 ng/ml. A significant difference was found between all three groups (p=0.002), table 3.

GPC3 levels assayed by IRMA were so much lower than the levels assayed by ELISA but the levels by the two methods were significantly correlated with each other only in the control group (r_s=0.411 and p=0.024) but such correlation was missing in the HCC and HCV groups (r_s=0.146, p=0.442), table 3.

AFP in the control group ranged 0-8.16 IU/ml, with a mean of 2.71 IU/ml. The HCV group was significantly higher than controls (p<0.001) with a values ranging 2.58-81.7 IU/ml and a mean of 25.22IU/ml. HCC patients group were significantly higher than controls (p<0.001) and HCV groups (p<0.001), with a range of 1.60-1027.0 IU/ml and a mean of 523.16 IU/ml. GPC3 levels measured by IRMA did not show any significant correlation with AFP levels (r_s=0.203 and p=0.168), table 3.
Development and Validation of a Two-Site Immunoradiometric assay for Glypican-3 in plasma: Implication in Diagnosis of Hepatocellular Carcinoma

Figure 3: ROC curves of GPC3 measured by IRMA and by ELISA and of AFP as diagnostic tests for HCC patients.

GPC3 measured by ELISA showed lower AUCs than IRMA, but still with significant asymptomatic difference. For differentiating liver-diseased from control subjects, the AUC was 0.701 (p=0.012) and at a cutoff value of 2.4 ng/ml the test had a sensitivity of 73% and specificity of 62%. To differentiate HCC patients, the AUC was 0.698 (p=0.012) with an optimum cutoff value of 7.0 ng/ml at which the sensitivity and specificity were 65% and 64%.

As for AFP, it was a significant diagnostic marker for liver disease (p=0.011) with an AUC of 0.73, and at a cutoff value of 7.6 IU/ml its sensitivity and specificity were 70% and 71%. But as a marker for HCC, with an AUC of 0.51 it was not significant (p=0.146), where it was unable to differentiate HCC from HCV patients.

Correlation between GPC3 and clinicopathological parameters

GPC3 measured by IRMA was not significantly correlated with stage (p=0.185), lymph node metastasis (0.948), Ascites (p=0.085) or distant metastasis (p=0.833). However, it showed a significant relation with Child-Pugh classification (r_s=0.437, p=0.001) as GPC3 levels were significantly different in different Child-Pugh classes (p<0.001) where it showed a tendency to increase with increasing class, table 3.

Table 3: correlation between GPC3 levels measured by IRMA and Child-Pugh classes.

<table>
<thead>
<tr>
<th>GPC3 (ng/ml)</th>
<th>Child-Pugh class</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Min. – Max.</td>
<td>0.14 – 3.90</td>
<td>0.21 – 4.70</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.53 ± 0.88</td>
<td>2.15 ± 1.09</td>
</tr>
<tr>
<td>r_s=0.437, p&lt;0.001</td>
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<td></td>
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</tbody>
</table>

DISCUSSION

Imunoassays use the highly specific antigen-antibodies reaction to target specific molecules of interest and analyze their concentration in a sample. Yalow and Berson were the first to develop a radioimmunoassay (RIA) in 1959 (Yalow, 1959), then Immunoradiometric Assay (IRMA) has been introduced by Miles and Hales in 1968 (Miles, 1968). IRMA was preferred to RIA, being a direct assay rather than a back-titration of the analyte, however, there is always the risk of losing antibody affinity during the process of radio-iodination. IRMA introduced incredible sensitivity, specificity and precision, but had the major disadvantage of using radioactivity.

Engvall and Perlman described enzyme-linked immunosorbent assay (ELISA) for the first time in 1971, whereby quantification of antigens is done using an anti-immunoglobulin antibody linked to an enzyme (Engvall, 1971). This method evaded using radioactivity, the obvious disadvantage of IRMA, with acceptable sensitivity and specificity and so its use expended on the expense of RIA and IRMA. However, ELISA, being dependent on an enzymatic reaction and the ultimate signal being a color, was susceptible to variations due to time, temperature, pH, inhibitors, etc. These factors could greatly affect the reproducibility of ELISA.

We have developed and validated a simple, double antibody IRMA for direct and precise measurement of GPC3 in plasma. The detection limit of the assay (0.006 ng/ml) allows direct measurement of low, physiological GPC3 concentrations. The performance characteristics of the IRMA assay proved it to be a valuable method for the quantitative measurement of GPC3. The high affinity MAb and the high specific activity of the iodinated product insured maximum sensitivity and specificity of the assay. Upon comparing IRMA and ELISA three differences have been demonstrated. First, the repeatability of IRMA was superior to that of ELISA, where the CVs of our IRMA were lower than those of commercial ELISA. Second, the correlation between measurements of the two techniques was limited to low GPC3 levels and was not found in higher concentrations. Finally, the sensitivity, specificity and diagnostic performance indicated by ROC curve AUCs of the IRMA measurements were superior to those of ELISA.
Some reports have previously attempted to compare radiometric assays to ELISA, the majority of them found that ELISA could be satisfactory, but the performance criteria of radiometric assays have been found to be much higher than ELISA (Beuvery, 1984; Grange, 2014). Others, however, indicated that ELISA and radiometric assays are comparable and their results were proportional, so they reported ELISA, as a technique not using radioactivity to be preferable (Kristensen, 1992; Booth 1982).

Hepatocellular carcinomas are marked by rapid growth rate and due to the lack of accurate ways of diagnosis in the early stages, the prognosis and the survival rate of patients remain poor. Techniques for HCC surveillance and diagnosis that are currently used in clinical practice have certain limitations that may be inherent to the tumor development. Though the sensitivity of serum AFP is limited, it is still regarded as the most useful marker for HCC. However, it cannot discriminate between benign and malignant liver diseases (Films, 2004, Shirakawa, 2009). GPC3 is abundantly expressed in fetal liver, inactive in normal mature liver, and reactivated in HCC (Zhou, 2017). The GPC3 gene was identified as one of the most overexpressed genes in HCC, with immuno-histochemical staining of GPC3 showing 83.4% sensitivity in HCC. The transition from premalignant lesions to HCC is associated with a sharp increase of GPC3 expression in a majority of cases. GPC3 may promote HCC growth by stimulating the autocrine/paracrine canonical Wnt signaling (Wang, 2010).

Recently, several studies evaluated serum GPC3 as a marker for HCC, however, its diagnostic value in HCC patients remains controversial. Some reports found that GPC3 is a more sensitive and specific serum marker than AFP (Capurro, 2003; Hippo, 2004; Tangkijvanich, 2010; Qiao, 2011; Chen, 2013). Conversely, others showed that GPC3 is not a good serum marker for HCC (Yasuda 2010; Ozkan; 2011; Wang, 2014; Jia, 2016). Even among those who reported GPC3 to be a good serological marker, a huge discrepancy in results existed, which is most likely to result from either different method used to detect GPC3, different antibodies used which recognize different epitopes, varying cutoff values or HCC of different etiologies. This was confirmed by the results of the current study, where assaying GPC3 by IRMA resulted in a much lower range and much higher sensitivity and specificity than GPC3 assay by ELISA.

GPC3 levels measured by IRMA was significantly higher in HCC patients than in healthy controls, that was in accordance with most published reports and several meta-analyses (Capurro, 2003; Hippo, 2004; Tangkijvanich, 2010; Qiao, 2011; Zhou, 2017; Liu, 2015). The IRMA results showed that the optimum cutoff in normal controls was 1.4 ng/ml, at which level, its sensitivity and specificity to detect liver-diseased subjects were 90% and 67%; respectively. But since most liver diseases, most notably HCV, are likely to illicit GPC3 increase, the more important aspect was if the test would discriminate HCC from HCV patients. ROC curve proved the capability of the assay to discriminate HCV from HCC patients, and at a cutoff value of 1.55 ng/ml the sensitivity and specificity of the test were 71% and 78%; respectively.

GPC3 was a much better indicator than AFP and not just because of the much higher AUC, sensitivity and specificity, but also because AFP failed to differentiate between HCC and HCV patients. No significant correlation was found between preoperative serum GPC3 levels and AFP levels which was in accordance with all previous studies (Capurro, 2003; Hippo, 2004; Tangkijvanich, 2010; Qiao, 2011). Interestingly, the addition of GPC3 and AFP did not result in any significant increase in sensitivity or specificity than GPC3 alone. GPC3 levels did not show any significant correlation with most of the clinicopathological features of HCC including tumor grade, lymph node involvement, ascites and distant metastasis. Only Child-Pugh score was found significantly correlated with GPC3 levels in HCC patients, which was in agreement with the report of Haruyama et al. Since child-pugh score is mainly a prognostic indicator, GPC3 should be expected to hold a promise as a prognostic marker.

In conclusion, we were able to set up an IRMA assay to measure GPC3 in plasma and successfully applied it in the measurement of GPC3 levels to diagnose HCC patients. When compared to a commercial ELISA kit, our IRMA assay showed much better performance characteristics than ELISA on ROC curves with much higher area under the curves, sensitivities and specificities when diagnosing HCC patients from normal controls or HCV patients. Using our IRMA assay, showed that GPC3 is a good diagnostic indicator for HCC with 1.4 ng/ml cutoff value for controls and 1.55 ng/ml to discriminate HCC and HCV patients. GPC3 measurement was much better than AFP for the diagnosis of HCC.

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