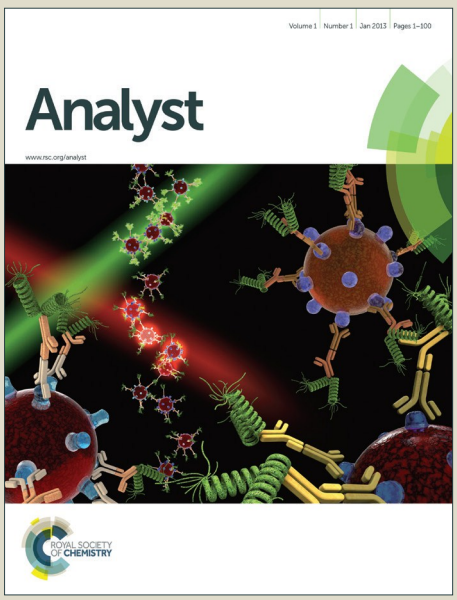
Analyst

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Serum Protein Biomarkers Relevant to Hepatocellular Carcinoma and Their Detection

Eric Waidely[1], A. O. Al-Yuobi[2], A. S. Bashammakh[2], M. S. El-Shahawi[2], Roger M. Leblanc[1]*

[1]*Department of Chemistry, University of Miami, 1301 Memorial Drive, Cox Science Center, Coral Gables, FL 33146, USA.*

[2]*Department of Chemistry, King Abdulaziz University, P.O. Box 80200, Jeddah 21589, Kingdom of Saudi Arabia*

Corresponding Author: **Roger M. Leblanc**

Email: **rml@miami.edu**

Phone: (305) 284-2194

Key Terms: Immunoassay, Biosensing, Radioimmunoassay, ELISA, α-fetoprotein (AFP), glypican-3 (GPC3), golgi protein 73 (GP73), α-L-fucosidase (AFU), des-γ-carboxyprothrombin (DCP), γ-glutamyl transferase (GGT) and squamous cell carcinoma antigen (SCCA)

Abstract

Hepatocellular carcinoma (HCC) is one of the most recurrent and lethal cancers worldwide. The low survival rate of this particular strain of carcinoma is largely due to the late stages at which diagnosis is determined. Tumorigenesis of hepatocellular carcinoma is most frequently made through ultrasonography, magnetic resonance imaging and computerized tomography scans, however, these methods are poor for detection of early tumor development. This review presents alternative hepatocellular carcinoma detection techniques through the use of protein and enzyme/isozyme biomarkers. The detection methods used to determine serum levels of α-fetoprotein (AFP), glypican-3 (GPC3), golgi protein 73 (GP73), α-L-fucosidase (AFU), des-γ-carboxyprothrombin (DCP), γ-glutamyl transferase (GGT) and squamous cell carcinoma antigen (SCCA) are presented and each marker's respective validity in the diagnosis of hepatocellular carcinoma is evaluated.

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer related death worldwide, killing a majority of people affected within a year of diagnosis[1]. Approximately 90-95% of worldwide liver cancers are HCC.[2] The main risk factors involved in HCC onset are hepatitis and cirrhosis[3]. In countries where hepatitis and cirrhosis are frequent, HCC is not only one of the most prevalent found cancers but is one of the most recurrent causes of death[4].

Neoplasm formations in the liver are generally detected through ultrasonography, magnetic resonance imaging (MRI) or an abdominal computerized tomography (CT) scan[5]. While these methods are accurate in detection of HCC, they often fail to discover tumor formations until the later stages. It is therefore of interest to establish ways to detect tumor formation in the earlier stages to screen at risk populations.

The use of biosensing has shown promising results in early cancer detection and in some cases can even help determine the prognosis of disease[6-8]. Biosensors are developed by combining a biological component with a physiochemical detector. The biological component is an analyte found within the body which is referred to as a biomarker. Biomarkers are any measurable substance found within an organism whose presence can be indicative of a disease or infection. The use of biomarkers has greatly advanced early cancer detection, which generates better survival rates. The physiochemical detectors chosen vary by the analyte being detected. Some of the more common forms of biosensing for cancers involve the use of spectroscopy[9,10], ELISA[11,12] or using potentiometric techniques[13].

HCC biomarkers can be divided into four categories: embryonic and glycoprotein antigens; enzymes and isozymes; genes; and cytokines[2]. Recent data suggests that genes and cytokines could potentially be the most sensitive for detection, however, further research needs to be performed to defend these claims[14]. As such, this review will focus on the more thoroughly investigated protein and enzyme biomarkers. Serum alpha-fetoprotein (AFP) is the most universally used antigen. AFP is an embryonic protein that is typically only produced by the fetal liver. Research has shown that serum AFP levels can be drastically elevated in individuals expressing HCC, leading to potential cancer detection[14]. However, AFP is not a perfect diagnostic test as 40% of individuals with early HCC development express normal or acceptable

AFP levels[15,16]. Alpha-L-Fucosidase (AFU), a lysosomal enzyme present in all mammalian cells, may offer a new scaffold for HCC detection. It has been found that alpha-L-fucosidase can diagnose 85% of HCC patients up to 6 months before it is detectable by ultrasonography[17].

Developing a standard biosensor for HCC has proven to be a difficult task. While several biomarkers appear to be relevant in HCC diagnosis, a consistent detection method has yet to be determined, which has limited the clinical use of HCC biomarkers. The most widely explored ways to detect early tumor growth for HCC have been through protein biomarkers, predominantly AFP. This review will discuss the current protein and enzyme biomarkers found to be relevant to HCC, with a focus on the methods used to quantify each biomarkers serum levels and what these levels mean in regards to neoplasm formations.

Embryonic and Glycoprotein Antigens

Alpha-fetoprotein (AFP): Alpha-fetoprotein is currently the most widely used tumor biomarker for HCC. AFP was first discovered in 1956 by Bergstrand and Czar through electrophoresis of human fetus serum proteins[18]. Early AFP synthesis occurs in the yolk sacs and then later on is produced by the fetal liver. The synthesis of AFP reaches levels as high as 3 g/L at around 12-16 weeks of gestation, however, post-birth serum levels drop rapidly to 10 μg/L≥ within the first 18 months[19]. In healthy adults, serum AFP levels typically fall into the range of 5-10 μg/L[20].

Consisting of 591 amino acids, AFP is a 70 kDa oncofetal glycoprotein[21] that contains a single asparagine-linked carbohydrate chain[22]. Being a glycoprotein, AFP exists as a heterogeneous entity with several different glycoforms found based upon affinity towards different lectins or electrophoretic separation[23,24]. Lens culinaris agglutinin (LCA) is able to distinguish AFP into three different isoforms: AFP-L1 (LCA nonreactive), AFP-L2

(intermediate reactive) and AFP-L3 (LCA-affinitive)[25]. The AFP-L3 glycoform appears to be produced by cancer cells, potentially allowing for discrimination between chronic liver diseases and HCC at lower serum levels (400≥ ng/mL)[26].

Elevated AFP levels were first associated with tumor development in mice by Abelev *et al.* in 1963[27]. Taratinov later found AFP in the serum of patients with hepatoma, leading to its current use as a biomarker for HCC[28]. The first quantitative serum assay for AFP was developed in 1971[20] and has since been extensively studied[29-31]. The best analysis methods for AFP are all based on modified immunoassays and can be found in *Table 1*.

Immunoassay	**Antibody-Label**	**Detection Limit (µg/L)**	**Inter-assay CV**	**Intra-Assay CV**	**References**
Radioimmunoassay	Radioactive Isotope (^{125}I)	1-16	3.4-8.8 %	2.7-5.2%	20 32
Nephelometry	Latex particle	5.0	8.4-10%	5.1-10%	33
ELISA	Enzyme	3.0	3.5-16%	6.9-15%	34-37
Microparticle Enzyme Immunoassay	Microparticle + Enzyme	0.5	6.8%	4.9%	38
Electrochemiluminescence	Ruthenium(II)-tris(bipyridyl) complex	0.4	3.5-4.6%	2.3-4.0%	39
Immunoradiometric assay	Radioactive Isotope (^{125}I)	0.2	3.0-10%	3.0-6.0%	40

Table 1. Overview of immunoassays for AFP serum concentration measurements.

As *Table* 1 suggests, radioimmunoassays for AFP offer the lowest detection limit. The radioimmunoassay involves labeling known quantities of AFP with $Na^{125}I$. The radioactive iodine isotope binds to tyrosine residues located on AFP and the labeled antigen is then incubated with known quantities of AFP specific antibodies and loaded into a column. Upon addition of serum samples, the unlabeled AFP found in sera will displace the radioactive labeled AFP, causing it to elute from the column. The quantity of labeled AFP eluted can be quantified with a gamma counter and allow for indirect measurements of the concentration of AFP found in the serum samples[20]. While radioimmunoassays for AFP offer great sensitivity, they suffer from long preparation times, large amounts of required dilutions and separations, and radiation damage. A more commonly implemented detection technique for serum AFP is ELISA. Although *Table* 1 portrays ELISA as one of the weaker methods in regards to detection limit, it has benefits through quick detection time, longer shelf life, and simpler detection techniques. The exact ELISA format varies but most employ isolation of target antigen onto a plastic bead or plate followed by incubation with the enzyme-labeled antibody. The enzyme labels chosen are most commonly horseradish peroxidase[36] and alkaline phosphatase[34]. Upon binding of the enzyme-labeled antibody, excess antibody is washed from the plastic surface and an enzyme specific chromogen substrate is added. The enzyme interacts with the chromogen substrate, producing a fluorescent or colored product which can be used to quantify the amount of antigen present. Another factor to take into account when determining which assay to use is the variability that can arise from experiment to experiment. As shown in *Table 1*, the inter-assay and intra-assay CV for all the immunoassays listed shows little variance between each assay type.

Although *Table* 1 would suggest that using an immunoradiometric scaffold is the best for serum AFP measurements, there are many variables to consider beyond the detection limit for the assay: including time, cost, cut-off point and ease of measurement. The cut-off point is used as an indicator that serum levels are above the norm and is the set point at which the distinction between healthy and diseased occurs. It is therefore not entirely relevant to obtain the lowest detection limit, but rather to obtain the highest sensitivity and specificity for the disease being detected. In diagnosing cases of HCC, the serum concentration of 20 ng/mL of AFP is the most universally used cut-off point; however this cut-off point has been found to fluctuate between ethnic groups[41,42]. *Table 2* comprises of the most common AFP serum concentrations used in HCC diagnosis along with the respective sensitivity and specificity as found from a statistical study by Trevisani *et al*[43]. The sensitivity of the assay measures the true positive rate while the specificity measures the false positive rate caused by other disorders expressing increased serum levels. As *Table* 2 depicts, higher AFP cut-off values become more specific for HCC but the measurement loses its sensitivity. The sensitivity of detection decreases at higher cut-off values due to the low serum levels of AFP expressed. Many individuals with HCC express only slight elevation of AFP while 80% of the smaller cases (tumors <3cm) show no elevation whatsoever, causing higher cut-off values to be less effective[44]. The specificity increases due to false positives from alternative viral etiology and other liver diseases that cause elevation in AFP becoming less relevant at such high serum levels[45].

AFP cut-off (ng/mL)	Sensitivity (%)	Specificity (%)
16	62.4	89.4
20	60.0	90.6
100	31.2	98.8

200	22.4	99.4
400	17.1	99.4

Table 2. Sensitivity and specificity of HCC diagnosis at the five most common used cut-off values of serum AFP levels.

The heterogeneous nature of AFP has shown promising results in increasing the sensitivity and specificity of HCC diagnosis. As noted, total AFP levels can be divided into three different types, based upon each isoforms affinity towards LCA. The LCA-bound glycoform, AFP-L3, is able to detect HCC at the cut-off value of 15% with a sensitivity of 96.9% and a specificity of 92%[46]. However, the most promising results come not from using AFP-L3 alone, but rather combining AFP-L3 percentages with the total serum levels of AFP. Leerapun *et al.*[47] found that using a cut-off value between 10-200 ng/mL of total AFP and a cut-off of 35% AFP-L3, the specificity of HCC detection reaches 100%.

Glypican-3 (GPC3): Glypican-3 is a membrane-bound heparan sulfate proteoglycan belonging to a family of six similar cell-surface proteins[48]. Each member of this family possesses a similar size and structure, ranging between 60-70 kDa[49]. Glypicans are bound to the cell membrane via a glycosyl-phosphatidylinositol anchor[50]. While the exact function of glypicans is unknown, they are believed to interact with various growth factors[49]. During fetal development, GPC3 can be found within the liver, lungs and kidneys. In adult tissues, small traces can be found within the kidney while none is detected in any other adult tissue. However, GPC3 has been found to be expressed by some neoplasms, suggesting this oncofetal protein has potential use as a biomarker.

Hsu et al.[51] found that the mRNA of GPC3 was detectable in 74.8% of HCC patients while only 3.2% expression was observed in noncancerous livers. Soon after, it was found that GPC3 mRNA was expressed in 75% of HCC cases while negligible expression was detected in

normal, cirrhotic and focal nodular hyperplasia livers[52]. These findings led future groups to analyze GPC3 levels and assess its relevance as a novel HCC biomarker. Up to now, the most commonly used detection technique for GPC3 serum levels is ELISA with cut-off values ranging from 3.9 pg/mL to 300 ng/mL[53].

Capurro et al.[54] presented the first assay analyzing GPC3 levels in the serum of healthy and HCC patients using a sandwich ELISA with horseradish peroxidase labeled antibodies and hydrogen peroxide with o-phenylenediamine as the substrate. The sandwich ELISA used involved placing monoclonal antibodies specific for GPC3 in 96-well plates followed by 24 hour incubation with serum samples. After thorough washing, a polyclonal antibody labeled with horseradish peroxidase is then added and the enzymatic activity is used to produce a calibration curve that can determine GPC3 serum levels. It was found that GPC3 was expressed in 72% of HCC patients while AFP was only expressed in 59% at the cut-off of 20 ng/mL, and an even lower 32% of patients when using the more sensitive cut-off of 100 ng/mL. As is the case for AFP detection, various cut-off points have been used in correlating GPC3 serum levels with HCC diagnosis. However, in the case of GPC3, there has yet to be a standard cut-off point determined that maximizes sensitivity and specificity. Currently, the cut-off values used vary from as low as 3.9 pg/mL all the way to 300 ng/mL, with the sensitivity of GPC3 ranging from 36-65% and specificities ranging from 65-100%[50,54-57]. While GPC3 shows promise in being more sensitive and specific than serum AFP, a meta-analysis performed by Liu et al.[53] concludes that the current data presented is flawed, presenting issues in sample size, patient selection, serum control and the heterogenous nature in experimental techniques. Future research will need to be conducted to determine the merit of GPC3 as a replacement or supplement to AFP in HCC diagnostics.

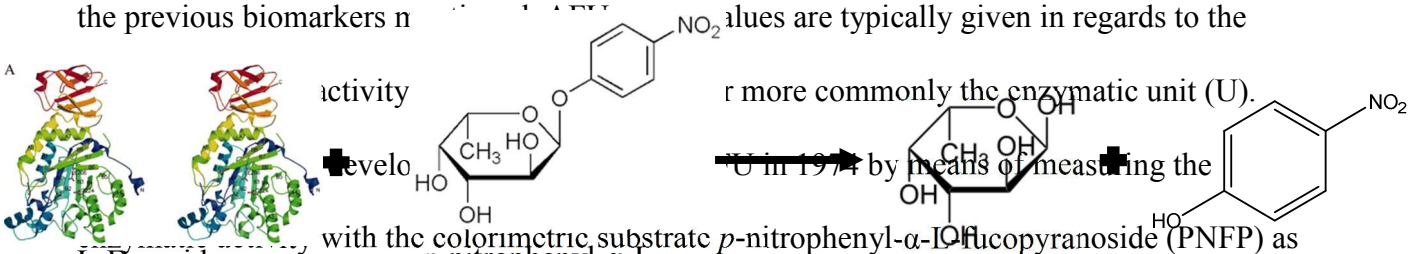
Golgi Protein 73 (GP73): Golgi protein 73 (GP73, also referred to as Golph2), is a 400 amino acid, 73-kDa transmembrane glycoprotein typically found within the *cis*-Golgi complex[58]. GP73 possesses several glycosylation sites, however, the role of these sites and the protein itself are unclear[59]. GP73 is primarily expressed in epithelial cells but has been found to be upregulated in hepatocytes in patients suffering from both viral and non-viral liver diseases[60]. Studies of human serum samples confirmed that GP73 is expressed in cases of HCC, leading to its current use as a potential predicative biomarker[61].

The primary detection techniques for GP73 include western blot, immunoblotting and ELISA. Marrero et al.[62] presented one of the first human serum assays of GP73 using an immunoblot assay. Zhou et al.[63] performed a meta-analysis on GP73 assays compared with AFP. GP73 sensitivities range from 69-95% while the specificities range from 35-97%. These values compromise results from all three assay types used and suggest a large amount of heterogeneity between each assay type. While higher sensitivity and specificity has been reported for GP73 which might suggest it is a possible replacement to AFP, further research needs to be done to confirm the consistency of this marker.

Enzymes and isozymes

Alpha-L-Fucosidase (AFU): Alpha-L-fucosidase is a lysosomal enzyme that catalyzes the hydrolytic cleavage of fucose-containing molecules[64]. Mammalian α-L-fucosidases are glycoproteins, possessing many different isoforms of ~54 kDa with optimial activity between pH 4 and 6.5[65]. AFU is present in low concentrations in all animal tissues but have been found to be overexpressed in cancerous tissue, especially HCC[66].

Due to the heterogeneous nature of α-L-fucosidases, an exact molecular weight is not available and as such other means of quantifying serum levels must be employed. In contrary to the previous biomarkers n alues are typically given in regards to the activity r more commonly the enzymatic unit (U). evelo U in 1974 by means of measuring the with the colorimetric substrate *p*-nitrophenyl-α-L-fucopyranoside (PNFP) as depicted in **Figure 1**[64]. In brief, the enzymatic activity is measured at a pH of 5.5 in an acetate



Scheme 1: Schematic of the assay developed through the hydrolysis of *p*-nitrophenyl-α-L-fucopyranoside by AFU to produce α-L-fucose and the colored product *p*-nitrophenol.

buffer and the absorbance of the cleaved *p*-nitrophenol is observed at 400 nm. The connection between AFU serum levels and HCC onset was not made until ten years later when Deugnier et al.[68] used a previously described AFU assay[69] to establish a correlation between high AFU levels and carcinoma.

To date, the most commonly used detection technique is the above mentioned assay using the colorimetric substrate PNFP. Many research groups have used this approach to determine the onset of HCC with sensitivities ranging from 60-90% and specificities ranging from 55-98%[4,70-74]. An alternative method was proposed by Wang and Cao[75] by chlorinating the 2 position of the standard substrate *p*-nitrophenyl-α-L-fucopyranoside to produce 2-chloro-4-nitrophenyl-α-L-fucoside. The addition of a chlorine group to the substrate leads to an increased cleavage rate and produces a faster assay with sensitivity for HCC detection reported at 81.8%

and specificity of 85.4%. While the standard approach for AFU detection appears to show promise in HCC detection, it is still affected by incubation times, indirect measurements via catalytic rates and interference caused by the overlap in yellow color of the substrate collected and that of the inherently yellow color of serum samples[13].

Des-γ-carboxyprothrombin (DCP): Des-γ-carboxyprothrombin is an abnormal, inactive form of prothrombin that is also referred to as PIVKA-II (protein induced by vitamin K absence or antagonist-II)[76]. DCP is characterized by the lack of carboxylation of the 10 glutamic acid residues typically found on the N-terminus and necessary for binding Ca^{2+} ions during the coagulation process[77]. Prothrombin is produced within the liver and healthy individuals usually do not express DCP.

Blanchard et al.[78] developed the first assay for DCP detection using a radioimmunoassay technique with ^{125}I labeling. The assay developed was a competitive assay, involving the addition of ^{125}I labeled DCP to polyclonal antibody specific for abnormal prothrombin. As serum is added with the native unlabeled DCP, the ^{125}I-DCP is displaced from the antibody and eluted for detection Liebman et al.[79] used this radioimmunoassay to measure the plasma DCP levels in patients with primary HCC and found significant elevation in 91% of the patients measured, leading to further research of this potential new biomarker. These findings led Motohara et al.[80] to develop an ELISA to investigate the validity of DCP as a new biomarker for HCC. The ELISA was established using a monoclonal antibody specific for PIVKA-II as a capture antibody and plates containing this antibody were incubated with plasma samples. After proper incubation, the plates are washed and treated with a second antibody labeled with horseradish peroxidase. After incubating the second antibody followed by thorough washing, 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] is added to measure the activity of the attached

enzyme. The ELISA technique proved to be quite sensitive with a detection limit found to be 0.13 U/mL[80]. Fujiyama et al.[81] then set out to use the above mentioned ELISA technique to study the correlation between DCP serum levels and HCC. It was found that 63% of patients with HCC show DCP levels >0.1 U/mL while 48% of patients expressed values >0.3 U/mL.

The future of DCP as a biomarker for HCC detection relies on its effectiveness when compared to the standard marker AFP[82]. Mita et al.[83] compared the detection capability of DCP with AFP using an ELISA approach for both measurements. Of the 91 patients with HCC measured, 62% had DCP levels above the cutoff 40 mU/mL while only 47% had AFP levels above the standard cutoff of 20 ng/mL. While these results are promising for the future of using DCP as an HCC biomarker, there are complications that could limit its use as a universal marker for all cases of HCC. While the exact cause of the production of abnormal prothrombin is unclear, it has been found to be elevated not only in cases of HCC but also in patients treated with coumarin anticoagulants, which could limit its effectiveness in detecting HCC universally[79].

γ-Glutamyl transferase (GGT): γ-Glutamyl transferase (also referred to as γ-glutamyl transpeptidase) is a membrane bound enzyme found in trace amounts in most animal tissues with the highest level of activity being found in the kidney[84,85]. GGT exists as a glycoprotein with various isoenzymes possible depending on the extent of carbohydrate binding[86]. The exact physiological function is not definitive but it has been found to aid in amino acid transport[87], cleavage of glutathione[88], and transfer of the γ-glutamyl group to acceptor amino acids[89]. Normal levels of GGT typically range between 3.2-24.8 mU/mL but this value has been observed to elevate significantly with various liver diseases leading many groups to evaluate its clinical relevance as a biomarker for HCC[90].

Goldbarg et al.[91] developed the first assay for GGT using a colorimetric set up. Applying a process developed by Bratton and Marshall[92], the colorimetric assay utilizes the chromogenic substrate N-(DL-γ-glutamyl)aniline to measure GGT. GGT transfers the γ-glutamyl moiety to an acceptor amino acid, releasing free aniline. The solution is then treated with sodium nitrite and N-(1-naphthyl)ethylenediamine dihydrochloride leading to diazotization of the released aniline substrate, producing a blue azo dye which can then be quantified using a photoelectric colorimeter to determine the enzymatic activity.

While the colorimetric assay was able to measure serum GGT levels in various human tissues, the values produced combine all isoforms of GGT which could hinder the sensitivity and specificity for HCC diagnosis. In fact, it is the GGT-II isoform that has been found as the diagnostic standard for cases of hepatoma. To overcome the issues that arise from GGT heterogeneity, many groups have set out to separate the various isoforms through electrophoretic methods with agarose gel[93], starch gel[94], cellulose acetate[95], polyacrylamide gel[96-98] and paper[99] prior to measuring enzymatic activity. Xu et al.[100] utilized vertical slab stage electrophoresis on polyacrylamide gel to separate GGT into 9-11 activity bands. They then adopted a modified version on the aforementioned colorimetric assay using γ-glutamyl *p*-nitroanlide as the enzyme substrate, glycyl glycine as an amino acid acceptor for the γ-glutmayl moiety, and N-(1-naphthyl)ethylenediamine dihydrochloride to produce a red azo dye through diazotization with free *p*-nitroanilide[90]. GGT-II appears as the second separation band and was found to have a sensitivity of 90% and a specificity of 97.1% for hepatoma cases, while it is expressed in only 3.1% of other liver diseases[100]. Cui et al.[101] set out to replicate these findings and while they observed a lower sensitivity and specificity, 74% and 82.2% respectively, their findings still show promise for GGT-II as a supplemental biomarker in HCC detection.

Squamous Cell Carcinoma Antigen (SCCA): Squamous cell carcinoma antigen is a tumor associated protein belonging to a family of high molecular weight serine protease inhibitors[102]. SCCA has been isolated into two isoforms: SCCA1 and SCCA2[103]. Both isoforms are expressed in a layer of the squamous epithelium and overexpression has been associated with tumorigenesis[104]. The two isolated isoforms have been found to protect neoplastic cells from apoptosis while SCCA1 has been found to promote tumor growth *in vivo*[105-107]. There is a strong association with the presence of SCCA in many different forms of cancer, including HCC, leading various groups to investigate its viability as a potential biomarker.

Kato and Torigoe[108] presented the first isolation and immunoassay for SCCA using a radioimmunoassay approach for associating SCCA with the onset of cervical squamous cell carcinoma. Pontisso et al.[109] were the first to establish serum SCCA as a biomarker for HCC using an ELISA with horseradish peroxidase and found SCCA was expressed in 85% of HCC cases. Further groups investigated serum SCCA levels using a similar method with sensitivities ranging from 18-84% and specifities ranging from 27-73%[110-114]. While serum SCCA may prove to be a valid supplementary biomarker for HCC diagnosis, more promising results have been found by quantifying serum SCCA-IgM immunocomplexes found in circulation. Beneduce et al.[115] found that by combining SCCA-IgM with AFP that the sensitivity of HCC detection was 70% while the specificity reached 100%. Using an ELISA technique, SCCA-IgM produces sensitivities ranging from 52-89% and specificities from 49-100%[111,115-118].

Combined Tests:

While promising results have been shown in testing for one biomarker at a time, many groups have found that the sensitivity of detection can be raised by detecting multiple biomarkers

at a time. **Figure 2** portrays the sensitivity and specificity of HCC detection by using the serum marker AFP combined with GPC3[119], DCP[101,120], GGT[101,121], and AFU[71,121]. In each case, combining an additional biomarker with AFP for HCC diagnosis leads to an increase in the sensitivity of detection, however, the specificity of detection takes a hit. W specificity is not ideal, it is possible that the specificity could be increased by varying the cut-off values used. As shown ble 2, th cificity of AFP tion incr as the cut-off value is raised. Each of om is at the c f value c L, so it is possible the specificity ect be by usin y h cut-off value.

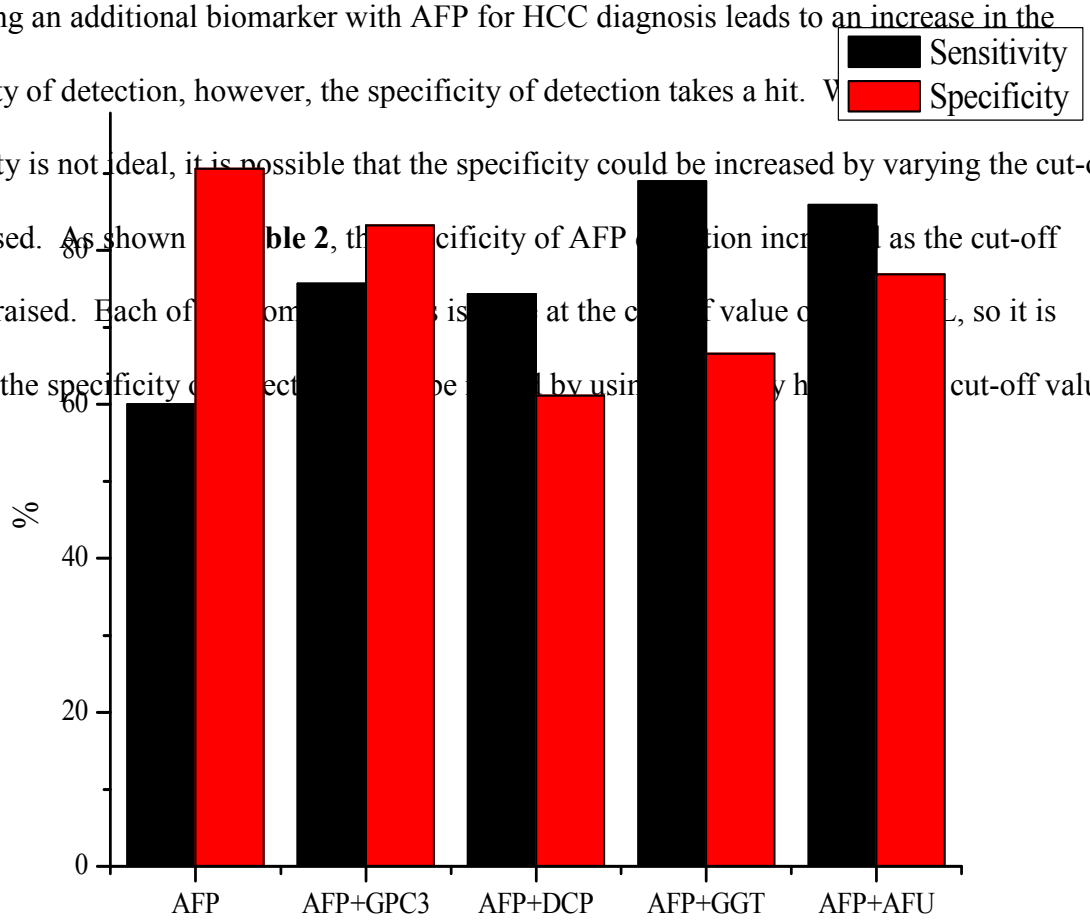


Figure 2: Sensitivity and specificity of combined detection with AFP and GPC3, DCP, GGT, and AFU.

Conclusion:

In summation, the detection techniques and the relevance of protein and enzyme biomarkers for hepatocellular carcinoma has been evaluated. While radioimmunoassays typically produce the lowest detection limits for protein biomarkers for HCC, the complications and difficulty that arise from these measurements has limited its use in serum biomarker measurements. Instead, the most commonly used technique for biomarker evaluation is an ELISA approach with horseradish peroxidase. AFP remains the gold standard for HCC diagnosis but cancer detection proves to be too complicated to be limited to a single marker. Variables such as geographical location, ethnicity, and preexisting viral etiology cause variations for each biomarker. The future of HCC diagnosis will likely lie in combination measurements by evaluating several of the above mentioned biomarkers simultaneously, rather than simply using one.

References

(1) Dai, L.; Lei, N.; Liu, M.; Zhang, J.-Y. *Experimental Hematology & Oncology* **2013**, *2*, 15.
(2) Zhao, Y.; Ju, Q.; Li, G. *Mol. Clin. Oncol.* **2013**, *1*, 593.
(3) Shiraha, H.; Yamamoto, K.; Namba, M. *Int. J. Oncol.* **2013**, *42*, 1133.
(4) Tangkijvanich, P.; Tosukhowong, P.; Bunyongyod, P.; Lertmaharit, S.; Hanvjvatvong, O.; Kullavanijaya, P.; Poovorawan, Y. *Southeast Asian J. Trop. Med. Public Health* **1999**, *30*, 110.
(5) Tejeda-Maldonado, J.; García-Juárez, I.; Aguirre-Valadez, J.; González-Aguirre, A.; Vilatobá-Chapa, M.; Armengol-Alonso, A.; Escobar-Penagos, F.; Torre, A.; Sánchez-Ávila, J.; Luis Carrillo-Pérez, D. *World J. Hepatol.* **2015**, *7*, 362.
(6) Ijichi, M.; Takayama, T.; Matsumura, M.; Shiratori, Y.; Omata, M.; Makuuchi, M. *Hepatology* **2002**, *35*, 853.
(7) Okuda, H.; Nakanishi, T.; Takatsu, K.; Saito, A.; Hayashi, N.; Yamamoto, M.; Takasaki, K. E. N.; Nakano, M. *J. Gastroenterol. Hepatol.* **2002**, *17*, 772.
(8) Tang, W.; Kokudo, N.; Sugawara, Y.; Guo, Q.; Imamura, H.; Sano, K.; Karako, H.; Qu, X.; Nakata, M.; Makuuchi, M. *Oncol. Rep.* **2005**, *13*, 25.
(9) Chen, Z.; Ren, X.; Meng, X.; Zhang, Y.; Chen, D.; Tang, F. *Anal. Chem.* **2012**, *84*, 4077.

(10) Tsay, G. C.; Dawson, G. *Anal. Biochem.* **1976**, *78*, 423.
(11) Tjalsma, H. *Expert Rev. Proteomic* **2010**, *7*, 879.
(12) Nilsson, B. *Curr. Opin Immunol.* **1989**, *2*, 898.
(13) Othman, A. M.; El-Houseini, M. E.; El-Sofy, M. S.; Aboul-Enein, H. Y. *Anal Bioanal Chem* **2011**, *400*, 787.
(14) Zhou, L.; Liu, J.; Luo, F. *World J. Gastroenterol.* **2006**, *12*, 1175.
(15) Choi, J. Y.; Jung, S. W.; Kim, H. Y.; Kim, M.; Kim, Y.; Kim, D. G.; Oh, E.-J. *World J. Gastroenterol.* **2013**, *19*, 339.
(16) Attia, M. S.; Othman, A. M.; Aboaly, M. M.; Abdel-Mottaleb, M. S. A. *Anal. Chem.* **2010**, *82*, 6230.
(17) El-Houseini, M. E.; Sherbiny, M. E.; El-Din Awad, M. M.; Amer, M. A.; Saad El Din, A. H.; Hussein, T. D. *J. Egypt Natl. Cancer Inst.* **2001**, *13*, 277.
(18) Bergstrand, C. G.; Czar, B. *Scand. J. Clin. Lab. Inv.* **1956**, *8*, 174.
(19) Debruyne, E. N.; Delanghe, J. R. *Clin. Chim. Acta* **2008**, *395*, 19.
(20) Ruoslahti, E.; Seppälä, M. *Int. J. Cancer* **1971**, *8*, 374.
(21) Mizejewski, G. J. *Exp. Biol. Med.* **1997**, *215*, 333.
(22) Yoshima, H.; Mizuochi, T.; Ishii, M.; Kobata, A. *Cancer Res.* **1980**, *40*, 4276.
(23) BrĘBorowicz, J.; Mackiewicz, A.; BrĘBorowicz, D. In *Scand. J. Immunol.*; Blackwell Publishing Ltd: 1981; Vol. 14, p 15.
(24) Alpert, E.; Drysdale, J. W.; Isselbacher, K. J.; Schur, P. H. *J. Biol. Chem.* **1972**, *247*, 3792.
(25) Johnson, P. J.; Poon, T. C. W.; Hjelm, N. M.; Ho, C. S.; Blake, C.; Ho, S. K. W. *Br J Cancer* **2000**, *83*, 1330.
(26) Du, M.-Q.; Hutchinson, W. L.; Johnson, P. J.; Williams, R. *Cancer* **1991**, *67*, 476.
(27) Abelev, G. I.; Perova, S. D.; Khramkova, N. I.; Postnikova, Z. A.; Irlin, I. S. *Transplantation* **1963**, *1*, 174.
(28) Tatarinov, Y. *Vopr. Med. Khim.* **1964**, *10*, 90.
(29) Alpert, M. E.; Uriel, J.; de Nechaud, B. *New Engl. J. Med.* **1968**, *278*, 984.
(30) Alpert, E.; Hershberg, R.; Schur, P. H.; Isselbacher, K. J. *Gastroenterology* **1971**, *61*, 137.
(31) Gupta, S.; Bent, S.; Kohlwes, J. *Ann. Intern. Med.* **2003**, *139*, 46.
(32) Chayvialle, J. A.; Ganguli, P. C. *Lancet* **1973**, *1*, 1355.
(33) Bernard, D. R.; Delanghe, J. R.; De Buyzere, M. L.; Leroux-Roels, G. G. *Eur. J. Clin. Chem. Clin.* **1996**, *34*, 847.
(34) Chan, D. W.; Kelsten, M.; Rock, R.; Bruzek, D. *Clin. Chem.* **1986**, *32*, 1318.
(35) Belanger, L.; Hamel, D.; Dufour, D.; Pouliot, M. *Clin. Chem.* **1976**, *22*, 198.
(36) Mancal, P.; Srámek, M.; Malbohan, I.; Simek, L. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1988**, *32*, 209.
(37) Maiolini, R.; Masseyeff, R. *J. Immunol. Methods* **1975**, *8*, 223.
(38) Fiore, M.; Mitchell, J.; Doan, T.; Nelson, R.; Winter, G.; Grandone, C.; Zeng, K.; Haraden, R.; Smith, J.; Harris, K.; Leszczynski, J.; Berry, D.; Safford, S.; Barnes, G.; Scholnick, A.; Ludington, K. *Clin. Chem.* **1988**, *34*, 1726.
(39) Blackburn, G. F.; Shah, H. P.; Kenten, J. H.; Leland, J.; Kamin, R. A.; Link, J.; Peterman, J.; Powell, M. J.; Shah, A.; Talley, D. B.; Tyagi, S. K.; Wilkins, E.; Wu, T. G.; Massey, R. J. *Clin. Chem.* **1991**, *37*, 1534.
(40) Suzuki, M. *Radioisotopes* **1988**, *37*, 225.

(41) Taketa, K.; Okada, S.; Win, N.; Hlaing, N. K. T.; Win, K. M. *Acta Med. Okayama* **2002**, *56*, 317.

(42) Soresi, M.; Magliarisi, C.; Campagna, P.; Leto, G.; Bonfissuto, G.; Riili, A.; Carroccio, A.; Sesti, R.; Tripi, S.; Montalto, G. *Anticancer Res.* **2003**, *23*, 1747.

(43) Trevisani, F.; D'Intino, P. E.; Morselli-Labate, A. M.; Mazzella, G.; Accogli, E.; Caraceni, P.; Domenicali, M.; De Notariis, S.; Roda, E.; Bernardi, M. *J. Hepatol.* **2001**, *34*, 570.

(44) Saffroy, R.; Pham, P.; Reffas, M.; Takka, M.; Lemoine, A.; Debuire, B. *Clin. Chem. Lab Med.* **2007**, *45*, 1169.

(45) Nguyen, M. H.; Garcia, R. T.; Simpson, P. W.; Wright, T. L.; Keeffe, E. B. *Hepatology* **2002**, *36*, 410.

(46) Singhal, A.; Jayaraman, M.; Dhanasekaran, D. N.; Kohli, V. *Crit. Rev. Oncol. Hematol.* **2012**, *82*, 116.

(47) Leerapun, A.; Suravarapu, S. V.; Bida, J. P.; Clark, R. J.; Sanders, E. L.; Mettler, T. A.; Stadheim, L. M.; Aderca, I.; Moser, C. D.; Nagorney, D. M.; LaRusso, N. F.; de Groen, P. C.; Menon, K. V. N.; Lazaridis, K. N.; Gores, G. J.; Charlton, M. R.; Roberts, R. O.; Therneau, T. M.; Katzmann, J. A.; Roberts, L. R. *Clin. Gastroenterol. Hepatol.* **2007**, *5*, 394.

(48) Wang, S. K.; Zynger, D. L.; Hes, O.; Yang, X. J. *Adv. Anat. Pathol.* **2014**, *21*, 450.

(49) Filmus, J.; Selleck, S. B. *J. Clin. Invest.* **2001**, *108*, 497.

(50) Nakatsura, T.; Yoshitake, Y.; Senju, S.; Monji, M.; Komori, H.; Motomura, Y.; Hosaka, S.; Beppu, T.; Ishiko, T.; Kamohara, H.; Ashihara, H.; Katagiri, T.; Furukawa, Y.; Fujiyama, S.; Ogawa, M.; Nakamura, Y.; Nishimura, Y. *Biochem. Biophys. Res. Commun.* **2003**, *306*, 16.

(51) Hsu, H.-C.; Cheng, W.; Lai, P.-L. *Cancer Res.* **1997**, *57*, 5179.

(52) Zhu, Z.; Friess, H.; Wang, L.; Abou-Shady, M.; Zimmermann, A.; Lander, A.; Korc, M.; Kleeff, J.; Buchler, M. *Gut* **2001**, *48*, 558.

(53) Liu, X.-F.; Hu, Z.-D.; Liu, X.-C.; Cao, Y.; Ding, C.-M.; Hu, C.-J. *Clin. Biochem.* **2014**, *47*, 196.

(54) Capurro, M.; Wanless, I. R.; Sherman, M.; Deboer, G.; Shi, W.; Miyoshi, E.; Filmus, J. *Gastroenterology* **2003**, *125*, 89.

(55) Liu, H.; Li, P.; Zahai, Y.; Qu, C. F.; Zhang, L. J.; Tan, Y. F.; Li, N.; Ding, G. D. *World J. Gastroenterol.* **2010**, *16*, 4410.

(56) Tangkijvanich, P.; Chanmee, T.; Komtong, S.; Mahachai, V.; Wisedopas, N.; Pothacharoen, P.; Kongtawelert, P. *J. Gastroenterol. Hepatol.* **2010**, *25*, 129.

(57) Yasuda, E.; Kumada, T.; Toyoda, H.; Kaneoka, Y.; Maeda, A.; Okuda, S.; Yoshimi, N.; Kozawa, O. *Hepatol. Res.* **2010**, *40*, 477.

(58) Fimmel, C. J.; Wright, L. *Hepatology* **2009**, *49*, 1421.

(59) Kladney, R. D.; Bulla, G. A.; Guo, L.; Mason, A. L.; Tollefson, A. E.; Simon, D. J.; Koutoubi, Z.; Fimmel, C. J. *Gene* **2000**, *249*, 53.

(60) Kladney, R. D.; Cui, X.; Bulla, G. A.; Brunt, E. M.; Fimmel, C. J. *Hepatology* **2002**, *35*, 1431.

(61) Block, T. M.; Comunale, M. A.; Lowman, M.; Steel, L. F.; Romano, P. R.; Fimmel, C.; Tennant, B. C.; London, W. T.; Evans, A. A.; Blumberg, B. S.; Dwek, R. A.; Mattu, T. S.; Mehta, A. S. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 779.

(62) Marrero, J. A.; Romano, P. R.; Nikolaeva, O.; Steel, L.; Mehta, A.; Fimmel, C. J.; Comunale, M. A.; D'Amelio, A.; Lok, A. S.; Block, T. M. *J. Hepatol.* **2005**, *43*, 1007.

(63) Zhou, Y.; Yin, X.; Ying, J.; Zhang, B. *BMC Cancer* **2012**, *12*, 17.
(64) Sulzenbacher, G.; Bignon, C.; Nishimura, T.; Tarling, C. A.; Withers, S. G.; Henrissat, B.; Bourne, Y. *J. Biol. Chem.* **2004**, *279*, 13119.
(65) Cordero, O. J.; Merino, A.; de la Cadena, M. P.; Bugía, B.; Nogueira, M.; Viñuela, J. E.; Martínez-Zorzano, V. S.; de Carlos, A.; Rodríguez-Berrocal, F. J. *Eur. J. Biochem.* **2001**, *268*, 3321.
(66) Levvy, G. A.; McAllan, A. *Biochem. J.* **1961**, *80*, 435.
(67) Avila, J.; Convit, J. *Biochim. Biophys. Acta* **1974**, *358*, 308.
(68) Deugnier, Y.; David, V.; Brissot, P.; Mabo, P.; Delamaire, D.; Messner, M.; Bourel, M.; Legall, J.-Y. *Hepatology* **1984**, *4*, 889.
(69) Troost, J.; van der Heijden, M. C. M.; Staal, G. E. J. *Clin. Chim. Acta* **1976**, *73*, 329.
(70) Bukofzer, S.; Stass, P. M.; Kew, M. C.; de Beer, M.; Groeneveld, H. T. *Br. J. Cancer* **1989**, *59*, 417.
(71) El-Houseini, M. E.; Mohammed, M. S.; Elshemey, W. M.; Hussein, T. D.; Desouky, O. S.; Elsayed, A. A. *Cancer Control* **2005**, *12*, 248.
(72) El-Tayeh, S. F.; Hussein, T. D.; El-Houseini, M. E.; Amer, M. A.; El-Sherbini, M.; Elshemey, W. M. *Disease markers* **2012**, *32*, 255.
(73) Giardina, M. G.; Matarazzo, M.; Morante, R.; Lucariello, A.; Varriale, A.; Guardasole, V.; De Marco, G. *Cancer* **1998**, *83*, 2468.
(74) Montaser, M. F.; Sakr, M. A.; Khalifa, M. O. *Arab Journal of Gastroenterology* **2012**, *13*, 9.
(75) Wang, J.-J.; Cao, E.-H. *Clin. Chim. Acta* **2004**, *347*, 103.
(76) Stefaniuk, P.; Cianciara, J.; Wiercinska-Drapalo, A. *World J. Gastroenterol.* **2010**, *16*, 418.
(77) Liebman, H. A.; Furie, B. C.; Furie, B. *Hepatology* **1982**, *2*, 488.
(78) Blanchard, R. A.; Furie, B. C.; Jorgensen, M.; Kruger, S. F.; Furie, B. *N. Engl. J. Med.* **1981**, *305*, 242.
(79) Liebman, H. A.; Furie, B. C.; Tong, M. J.; Blanchard, R. A.; Lo, K.-J.; Lee, S.-D.; Coleman, M. S.; Furie, B. *N. Engl. J. Med.* **1984**, *310*, 1427.
(80) Motohara, K.; Endo, F.; Matsuda, I. *Lancet*, *326*, 242.
(81) Fujiyama, S.; Morishita, T.; Hashiguchi, O.; Sato, T. *Cancer* **1988**, *61*, 1621.
(82) Abu El Makarem, M. *Hepat. Mon.* **2012**, *12*, e6122.
(83) Mita, Y.; Aoyagi, Y.; Yanagi, M.; Suda, T.; Suzuki, Y.; Asakura, H. *Cancer* **1998**, *82*, 1643.
(84) Hanigan, M. H.; Pilot, H. C. *Carcinogenesis* **1985**, *6*, 165.
(85) Horiuchi, S.; Inoue, M.; Morino, Y. *Eur. J. Biochem.* **1978**, *87*, 429.
(86) Matsuda, Y.; Tsuji, A.; Katunuma, N. *J. Biochem.* **1983**, *93*, 1427.
(87) Meister, A. *Science* **1973**, *180*, 33.
(88) Curthoys, N. P.; Hughey, R. P. *Enzyme* **1979**, *24*, 383.
(89) Hanes, C. S.; Hird, F. J. R.; Isherwood, F. A. *Biochem. J.* **1952**, *51*, 25.
(90) Szasz, G. *Clin. Chem.* **1969**, *15*, 124.
(91) Goldbarg, J. A.; Friedman, O. M.; Pineda, E. P.; Smith, E. E.; Chatterji, R.; Stein, E. H.; Rutenburg, A. M. *Arch. Biochem. Biophys.* **1960**, *91*, 61.
(92) Bratton, A. C.; Marshall, E. K. *J. Biol. Chem.* **1939**, *128*, 537.
(93) Hetland, Ö.; Andersson, T. R.; Gerner, T. *Clin. Chim. Acta* **1975**, *62*, 425.

(94) Orłowski, M.; Szczeklik, A. *Clin. Chim. Acta* **1967**, *15*, 387.
(95) Meng, X. Y.; Xu, K. C.; Yang, Z. H.; Huang, Y. J.; Rong, Z. H.; Liu, Y. H.; Ge, Z. J.; Cheng, M. Y.; Zhang, J. L.; Wan, H. M. *Jiangsu Med. J.* **1982**, *8*, 8.
(96) Sawaby, N.; Nakagen, M.; Yoneda, M.; Makino, H.; Kameda, S.; Kobayashi, K.; Sugioka, G.; Hattori, N.; Ishii, M. *Acta Hepatil. Jap.* **1977**, *18*, 101.
(97) Suzuki, H. *Gastroenterol. Jap.* **1981**, *16*, 422.
(98) Kojima, J.; Kantani, M.; Nakamura, N.; Kashiwagi, T.; Tohijoh, F.; Akiyama, M. *Clin. Chim. Acta* **1980**, *106*, 165.
(99) Kokot, F.; Kuska, J. *Clin. Chim. Acta* **1965**, *11*, 118.
(100) Xu, K.-C.; Meng, X.-Y.; Shi, Y.-C.; Ge, Z.-J.; Ye, L.; Yu, Z.-J.; Yang, D.-M. *Int. J. Cancer* **1985**, *36*, 667.
(101) Cui, R.; He, J.; Zhang, F.; Wang, B.; Ding, H.; Shen, H.; Li, Y.; Chen, X. *Br. J. Cancer* **2003**, *88*, 1878.
(102) Suminami, Y.; Kishi, F.; Sekiguchi, K.; Kato, H. *Biochem. Biophys. Res. Commun.* **1991**, *181*, 51.
(103) Giannelli, G.; Antonaci, S. *Digest. Liver Dis.* **2006**, *38*, 854.
(104) Kato, H.; Suehiro, Y.; Morioka, H.; Torigoe, T.; Myoga, A.; Sekiguchi, K.; Ikeda, I. *Jpn. J. Cancer Res.* **1987**, *78*, 1246.
(105) Schneider, S. S.; Schick, K. E.; Fish, E.; Miller, J. C.; Pena, D. S.; Treter, S. M.; Hui, G. A.; Silverman, A. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 3147.
(106) Suminami, Y.; Nagashima, S.; Vujanovic, N. L.; Hirabayashi, K.; Kato, H.; Whiteside, T. L. *Br. J. Cancer* **2000**, *82*, 981.
(107) Suminami, Y.; Shigeki, N.; Murakami, A.; Nawata, S.; Gondo, T.; Hirakawa, H.; Numa, F.; Silverman, G. A.; Kato, H. *Cancer Res.* **2001**, *61*, 176.
(108) Kato, H.; Torigoe, T. *Cancer* **1977**, *40*, 1621.
(109) Pontisso, P.; Calabrese, F.; Benvegnù, L.; Lise, M.; Belluco, C.; Ruvoletto, M. G.; De Falco, S.; Marino, M.; Valente, M.; Nitti, D.; Gatta, A.; Fassina, G. *Br. J. Cancer* **2004**, *90*, 833.
(110) Giannelli, G.; Marinosci, F.; Trerotoli, P.; Volpe, A.; Quaranta, M.; Dentico, P.; Antonaci, S. *Int. J. Cancer* **2005**, *117*, 506.
(111) Giannelli, G.; Fransvea, E.; Trerotoli, P.; Beaugrand, M.; Marinosci, F.; Lupo, L.; Nkontchou, G.; Dentico, P.; Antonaci, S. *Clin. Chim. Acta* **2007**, *383*, 147.
(112) Hussein, M.; Ibrahim, A.; Abdella, H.; Montasser, I.; Hassan, M. *Indian J. Cancer* **2008**, *45*, 167.
(113) Beale, G.; Chattopadhyay, D.; Gray, J.; Stewart, S.; Hudson, M.; Day, C.; Trerotoli, P.; Giannelli, G.; Manas, D.; Reeves, H. *BMC Cancer* **2008**, *8*, 200.
(114) Soyemi, O.; Otegbayo, J.; Ola, S.; Akere, A.; Soyemi, T. *BMC Research Notes* **2012**, *5*, 403.
(115) Beneduce, L.; Castaldi, F.; Marino, M.; Quarta, S.; Ruvoletto, M.; Benvegnù, L.; Calabrese, F.; Gatta, A.; Pontisso, P.; Fassina, G. *Cancer* **2005**, *103*, 2558.
(116) Pontisso, P.; Quarta, S.; Caberlotto, C.; Beneduce, L.; Marino, M.; Bernardinello, E.; Tono, N.; Fassina, G.; Cavalletto, L.; Gatta, A.; Chemello, L. *International Journal of Cancer* **2006**, *119*, 735.
(117) Pozzan, C.; Cardin, R.; Piciocchi, M.; Cazzagon, N.; Maddalo, G.; Vanin, V.; Giacomin, A.; Pontisso, P.; Cillo, U.; Farinati, F. *J. Gastroenterol. Hepatol.* **2014**, *29*, 1637.

(118) Mossad, N.; Mahmoud, E.; Osman, E.; Mahmoud, S.; Shousha, H. *Tumor Biol.* **2014**, *35*, 11559.

(119) Jia, X.; Liu, J.; Gao, Y.; Huang, Y.; Zhi, D. *Arch. Med. Res.* **2014**, *45*, 580.

(120) Sterling, R. K.; Jeffers, L.; Gordon, F.; Venook, A. P.; Reddy, K. R.; Satomura, S.; Kanke, F.; Schwartz, M. E.; Sherman, M. *Clin. Gastroenterol. Hepatol.* **2009**, *7*, 104.

(121) Zhu, J.; Jiang, F.; Ni, H.-B.; Xiao, M.-B.; Chen, B.-Y.; Ni, W.-K.; Lu, C.-H.; Ni, R.-Z. *Exp. Ther. Med,* **2013**, *5*, 89.