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HCC-READ™ ERBB3 ELISA

For Sensitive and Specific Detection of Hepatocellular Carcinoma or Malignant Hepatoma

Abstract

According to Globocan 2012, liver cancer is ranked sixth in cancer incidence and second in cancer-related mortality worldwide. Hepatocellular carcinoma (HCC) forms 70% to 85 % of primary liver cancer. Currently, the most commonly used biomarker for detection of HCC is Alpha-Fetoprotein (AFP). However, a portion of HCC cases do not display elevated AFP level and a portion of non-HCC cases display an elevated AFP level, leading to low sensitivity of AFP. Hence, improvements to current screening methods or new biomarkers is needed for early detection of HCC. In this current study, we introduce ERBB3, a new potential biomarker for HCC, that is more superior to AFP in discriminating HCC from non-HCC cases. Also, Restalyst developed a new algorithm that incorporates both ERBB3 and AFP levels that has better sensitivity as compared to individual AFP results.

Literature Review

Epidemiology of Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) makes up 70% to 85% of primary liver cancer in most countries (Ahmed et al., 2008). Liver cancer is ranked the sixth most common type of cancer and the second leading cause of cancer-related death globally (Globocan, 2012). According to Globocan, 2012, the mortality rate is approximately 95% of the incidence rate in 2012, suggesting a poor prognosis.

HCC affects mainly Asians and Africans due to the higher rate of hepatitis B virus (HBV) infection and exposure to aflatoxin contaminated food (Venook, et al., 2010). Despite being less prevalent to HCC, Europe and the United States are noticing increasing HCC incidences that might be due to alcohol cirrhosis. (Venook, et al., 2010). This increase in HCC incidence has sparked greater interest in the early screening, diagnosis, surveillance, and treatment of HCC worldwide.

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Early Detection and Surveillance

Being asymptomatic at its early stage, HCC is often detected at an advanced stage, leading to poor prognosis (SEER, n.d.). As shown in SEER, n.d., the 5-year survival rate increases by three-fold or more when HCC is detected at its early stage as it is still confined to the primary site (localized).

Also, Schwartz & Carithers, 2016 cited these facts: In contrast to the declining death rates seen for all other common cancers (such as lung, breast, and prostate cancers), death rates from liver carcinoma in men increased by 2.8 percent per year and for women it increased by 3.4 percent per year globally.

As a result, high risk patient groups such as those with cirrhosis or chronic liver diseases are recommended to go for frequent surveillance to monitor their health conditions. The current surveillance programmes recommended by various professional organisation include ultrasonography (USG) with or without AFP or AFP-L3 at 6- month intervals (Fitzmorris and Singal, 2015). Despite being widely use for HCC screening, alpha-fetoprotein (AFP) does have its shortcomings as a serum biomarker (Asrih, et.al., 2013). A subset of HCC cases does not have elevated AFP levels, contributing to low sensitivity of AFP to detect HCC (Gomaa, et al., 2009). It has also been discovered that AFP is insufficient for HCC surveillance despite its combined use with USG (Fitzmorris and Singal, 2015).

Hence, we present here a unique biomarker from an academic medical centre of excellence for this discovery – ERBB3 (Receptor tyrosine-protein kinase erbB-3, also known as HER3 or human epidermal growth factor receptor 3.

Table 1. Surveillance Guidelines for HCC as recommended by various organizations. (Taken from Fitzmorris and Singal, 2015)

Organization	Target Population	Surveillance Method and Interval
AASLD	Cirrhotic patients, noncirrhotic HBV carriers with a family history of HCC, noncirrhotic Africans and African Americans with HBV, noncirrhotic Asian male HBV carriers past the age of 40 years, noncirrhotic Asian female HBV carriers past the age of 50 years	USG every 6 months
EASL	Cirrhotic patients, noncirrhotic HBV carriers with a family history of HCC, noncirrhotic HBV carriers with active hepatitis, noncirrhotic patients with chronic HCV and advanced liver fibrosis (F3)	USG every 6 months
APASL	Cirrhotic patients with HBV or HCV infection	USG plus AFP every 6 months
JSH	Cirrhotic patients, noncirrhotic patients with chronic HBV infection, noncirrhotic patients with chronic HCV infection	USG plus AFP/AFP-L3%/DCP every 3 to 6 months

AASLD, American Association for the Study of Liver Diseases; AFP, alpha-fetoprotein; AFP-L3%, Lens culinaris agglutinin A-reactive fraction of AFP; APASL, Asian Pacific Association for the Study of the Liver; DCP, serum des-carboxy prothrombin; EASL, European Association for the Study of the Liver; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; JSH, Japan Society of Hepatology; USG, ultrasonography.

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Restalyst Marker ERBB3

The human epidermal growth factor receptor 3 (ERBB3) protein, a member of ERBB family of receptor tyrosine kinase (RTK), plays a role in regulating cell proliferation, and differentiation (Casalini, et al., 2004). Deregulation of ERBB signalling is associated with several human cancers (Casalini, et al., 2004).

In a study conducted by Hsieh, et al., 2011, expression of ERBB3 proteins were found to be elevated in HCC-positive blood samples as compared to chronic hepatitis and cirrhosis samples, with an AUC of more than 0.95. Adding on, the same study also showed that the combination of ERBB3 and AFP further improved the discrimination of HCC from chronic liver condition (Hsieh, et al., 2011).

Our in-house evaluation supports the findings from Hsieh, et al, 2011. Clinical performance data derived from our novel product (HCC-READ™ ERBB3 ELISA) and the AFP levels of samples were combined and analysed using Restalyst's developed algorithm to determine the risk levels of HCC in patients.

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Materials and Methods

Clinical Samples

A total of 379 clinical samples (79 true-positive and 300 true-negative for hepatocellular carcinoma) were included in the clinical validation. Out of the 300 true-negative for hepatocellular carcinoma, 37 have chronic hepatitis.

Measurements of biomarkers – ERBB3 & AFP

The levels and the concentrations of ERBB3 in all clinical blood samples were determined HCC-READ™ ERBB3 ELISA. Capture antibodies specific to ERBB3 proteins are first coated onto microplates. Human blood samples and standard proteins are diluted and incubated in the microwells for 30 minutes, shaking at 350rpm at room temperature. Samples are recommended to perform in duplicates. After 30 minutes incubation, excess reagents are removed through washing with 1X Wash Buffer. Detection antibodies that are specific to ERBB3 proteins are diluted and introduced to the microwell for another 30 minutes, shaking at 350rpm at room temperature. During this incubation, antigen-antibody complexes are formed between the capture antibody,

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ERBB3 proteins and detection antibody. Excess detection antibodies are removed through washing steps. Secondary antibodies conjugated with horseradish peroxidase (HRP) are added to the microwell for 30 minutes, shaking at 350rpm at room temperature to detect these antigen-antibody complexes. Any excess secondary antibody is removed through washing steps. A blue colour reaction will occur when Tetramethylbenzidine (TMB) solution is added to the microwells for 15 minutes incubation in the dark. Addition of 1N sulphuric acid stops the reaction, turning the blue coloration to yellow. The microwells are then read with spectrophotometer or a microwell ELISA plate reader at 450 nm against a 620-630 nm reference filter which eliminated any possible causes of interferences. Results are measured in arbitrary REAAD™-units (RU).

The levels and the concentrations of AFP were determined using Roche Elecsys® AFP and results were measured in ng/ml. Other similar determination of AFP can be employed too.

Algorithm

The concentrations of ERBB3 & AFP, together with the age, gender, hepatitis status, were first evaluated and analysed using principal component analysis followed by discriminant function analysis. The concentrations of ERBB3 & AFP together with the age of the patient were further evaluated and analysed using binary logistic regression analysis.

Sensitivity and Specificity Determination

Receiver operating characteristic (ROC) curve was plotted to evaluate the usefulness of Restalyst's new algorithm in distinguishing HCC patients from non-HCC patients. The ROC curve is a graphical plot that plots true positive rate (Sensitivity) against false positive rate (1-Specificity) at various threshold. The area under ROC curve (AUC) predicts how well the algorithm is able to distinguish HCC patients from non-HCC patients. AUC values closer to 1, indicates that Restalyst's new algorithm is highly accurate in distinguishing HCC patients from non-HCC patients.

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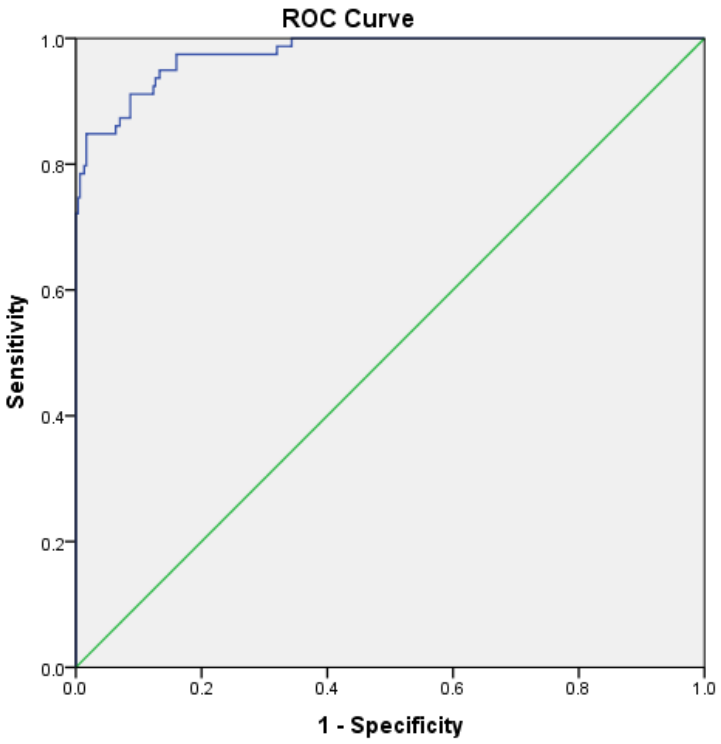
Performance Data

ROC

Results generated using Restalyst's new algorithm are used to plot Receiver operator characteristic (ROC) curve using SPSS software. Based on the ROC curve (Fig. 1), Restalyst's new algorithm is able to achieve a sensitivity and specificity of 91.1% and 91.3% respectively with an AUC of 0.976.

Fig. 1. The ROC curve below showing that Restalyst's new algorithm has an estimated sensitivity and specificity of 91.1% and 91.3% respectively with an AUC of 0.976.



Area Under the Curve

Test Result Variable(s): PP_AFP_ER_Age

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.976	0.008	0.000	0.961	0.991

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

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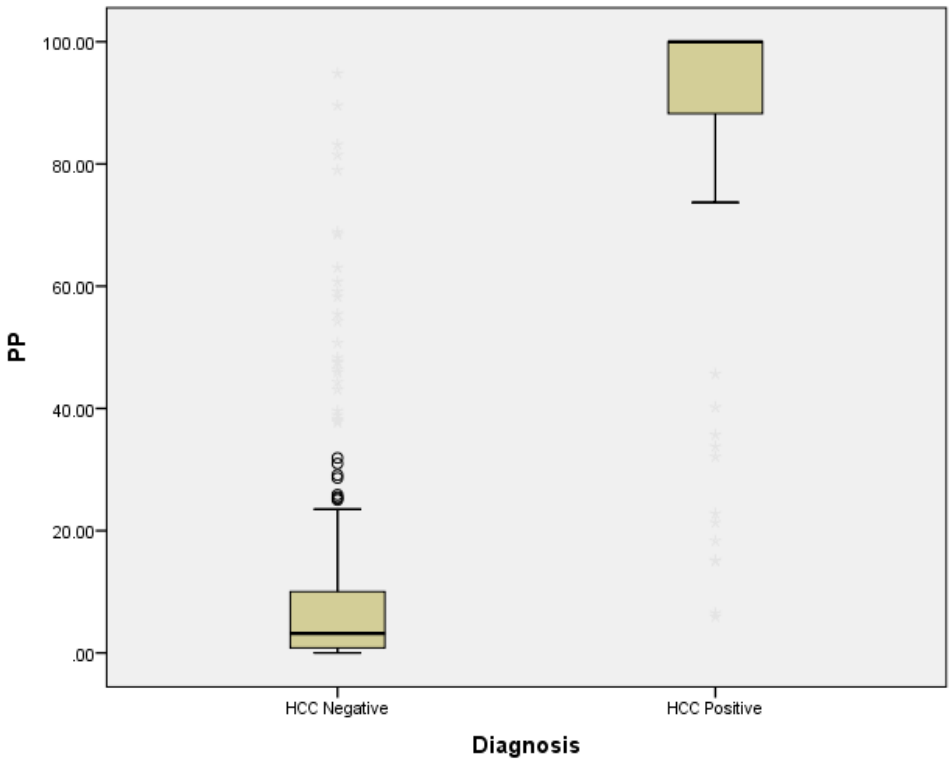
Evaluation between Hepatocellular Carcinoma and Non-Hepatocellular Carcinoma Subjects

The 379 clinical samples were further classified and evaluated into two different categories – hepatocellular carcinoma and non-hepatocellular carcinoma subjects (Fig. 2). Using Restalyst's new algorithm, it is able to clearly distinguish HCC cases from non-HCC cases.

Fig. 2. The box plot below shows the use of the new algorithm for 79 positive clinical samples gave a relatively higher average index as compared to the 300 negative clinical samples.



Discussion and Conclusion

The worldwide incidence of HCC is increasing due to its poor prognosis, and early detection of HCC is critically necessary to improve the overall survival rate. Although AFP is the most commonly used serological biomarkers for HCC detection, there is still room for improvement. Elevation of AFP is not only observed in HCC cases, but also in chronic liver disease and a portion of HCC cases do not display an elevated AFP level (Gomma, et al., 2009). All these contribute to the low sensitivity (39% - 65%) and specificity (76% - 94%) of AFP in HCC detection. Hence, there is a need for new discovery of serological biomarkers that can improve the current limitations.

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Hsieh, et al., 2011, found out that ERBB3, when use independently or in combination with AFP, was able to increase the early detection specifications of HCC. Restalyst's in-house evaluation and new algorithm further confirms the findings from Hsieh, et al, 2011. Additionally, we found that the combination of both biomarkers (AFP and ERBB3) showed a better sensitivity and specificity when discriminating HCC cases from non-HCC cases. Therefore, to conclude the current evaluation, the combined analysis of AFP and ERBB3 in the form of Restalyst's new algorithm has better performance as compared to current available screening methods such as AFP.

Bibliography

Asrih, M., Lenglet, S., Mach, F., & Montecucco, F. (2013, Jan 21). Alpha-fetoprotein: A controversial prognostic biomarker for small hepatocellular carcinoma. World Journal of Gastroenterology, 19(3), 328-330. Doi:10.3748/wjg.v19.i3.328

Ahmed, F., Perz, J., Jamison, P., Friedman, C., Bell, B. and Kwong, S. (2008). National Trends and Disparities in the Incidence of Hepatocellular Carcinoma, 1998 - 2003. Prev Chronic Dis, 5(3), p.A74.

Casalini, P., Iorio, M. V., Galmozzi, E., & Ménard, S. (2004). Role of HER receptors family in development and differentiation. Journal of cellular physiology, 200(3), 343-350.

Fitzmorris, P. and Singal, A. (2015). Surveillance and Diagnosis of Hepatocellular Carcinoma. Gastroenterol Hepatol, 11(1), pp.38 - 46.

Globocan. (2012). Retrieved October 25, 2017, from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

Gomaa, A. I., Khan, S. A., Leen, E. L., Waked, I., & Taylor-Robinson, S. D. (2009). Diagnosis of hepatocellular carcinoma. World Journal of Gastroenterology: WJG, 15(11), 1301.

Hsieh, S. Y., He, J. R., Yu, M. C., Lee, W. C., Chen, T. C., Lo, S. J., Bera, R., Sung, C. M., Chiu, C. T. (2011). Secreated ERBB3 isoforms are serum markers for early hepatoma in patients with chronic hepatitis and cirrhosis. Journal of proteome research, 10(10), 4715-4724.

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National Cancer Institute: Surveillance, Epidemiology and End Results (SEER) Program. (n.d.). Retrieved October 25, 2017, from https://seer.cancer.gov/statfacts/html/livibd.html

Schwartz, J. M., & Carithers, R. L. (2016). Epidemiology and etiologic associations of hepatocellular carcinoma. Retrieved from UpToDate.com: http://www.uptodate.com/contents/epidemiology-and-etiologic-associations-of-hepatocellular-carcinoma

Venook, A. P., Papandreou, C., Furuse, J., & de Guevara, L. L. (2010). The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. The oncologist, 15(Supplement 4), 5-13.