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GC-REAAD™ ITIH3 ELISA

For Sensitive and Accurate Detection of Gastric Carcinoma

Abstract

Gastric Carcinoma has one of the highest incidence rates and mortalities worldwide, especially in Asia. With diagnosis often made late in the stages of gastric carcinoma and thus the higher rates of unsuccessful treatments, early detection becomes a key measure for managing and improving the outcome of gastric carcinoma patients. An improved version of GC-REAAD™ developed by Restalyst, is introduced for sensitive and accurate detection of a patented protein— Inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), in human blood samples in a non-invasive way, unlike endoscopy which is invasive, within an assay time of approximately 2 hours. GC-REAAD™ is a sandwich ELISA IVD kit and it is validated using 869 clinical samples—consisting of 128 true-positive and 741 true-negative for gastric carcinoma. Based on the clinical validation performed, a receiver operating characteristics (ROC) curve (Hajian-Tilaki, 2013) of GC-REAAD™ gave a sensitivity of 90.6% and specificity of 90.3% with an AUC of 0.979.

Literature Review

Gastric Carcinoma

Globally, gastric carcinoma or also known as stomach cancer, are the major causes for cancer-related mortality. For example, in 2012, there were 952,000 new cases of stomach cancer, which is approximately 6.8% of the total new cancer cases (International Agency for Research for Cancer (IARC), 2012). At the local scale, as quoted from Singapore's Health Promotion Board article on stomach cancer, 'Cancer of the stomach is the fifth most common cancer in men and the seventh most common cancer in women in Singapore' (Health Promotion Board, Singapore, 2012). Further, incidence rate for stomach cancer are found to highest in Eastern Asia.

Screening & Diagnosis

Although there are effective treatments available for gastric carcinoma, diagnosis often occurs at the late stages of gastric carcinoma. At the late stages of gastric carcinoma, with symptoms and further complications, treatment often becomes more difficult and thus unsuccessful. Hence, early

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screening and diagnosis for gastric carcinoma becomes critical for proper management of gastric carcinoma (Jemal, et al., 2011).

At present, the conventional methods for screening and diagnosis include endoscopy—where a thin and flexible tube is inserted through the mouth and into the stomach, Barium meal—where X-ray is performed after the patient consumes a thick liquid (i.e. barium) which coats the lining of the oesophagus and stomach, and Computerised Tomography (CT) scan (Health Promotion Board, Singapore, 2012). Although these methods are widely used, they are procedures which are invasive and often costly.

ITIH3 (Inter-alpha-trypsin inhibitor heavy chain H3)

Inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) is a protein belonging to the family of inter-alpha-trypsin inhibitor that comprises a family of protease inhibitors that are found in the extracellular matrix of various organs including blood. An ability of these heavy chains is to link covalently to hyaluronic acid (HA)—a major component of the extracellular matrix (Huang, Yoneda, & Kimata, 2004).

ITIH3 was selected from a panel of protein markers that was screened for possible association with gastric carcinoma plasma detection; based on results from iTRAQ (Applied Biosytems, 2004), ITIH3 was found to be expressed relatively higher in mice with high tumor load as compared to control. ITIH3 also exhibits similar iTRAQ trends with human gastric carcinoma plasma data (Chong, et al., 2010). Additionally, this finding was substantiated with a screening test (i.e. immunoblotting with ITIH3 antibody) which was performed using 167 clinical plasma samples—consisting of 83 healthy and 84 gastric carcinoma subjects. This immunoblotting test achieved a ROC curve with an estimated maximal sensitivity and specificity of 96% and 66% respectively for ITIH3 in gastric carcinoma detection. It was also observed that the plasma from patients with early-stage gastric carcinoma had significantly ($p < 0.001$) higher levels of ITIH3 as compared to that from healthy subjects (Chong, et al., 2010).

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Materials and Methods

Clinical Samples

A total of 869 clinical samples (128 true-positive and 741 true negative for gastric carcinoma) were included in the clinical validation.

Measurement of biomarker – ITIH3

All clinical blood samples were tested for ITIH3 using GC-REAAD™ ITIH3 Sandwich ELISA. Capture antibodies specific to ITIH3 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 ITIH3 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, ITIH3 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).

Sensitivity and Specificity Determination

Receiver operating characteristics (ROC) curve was plotted to evaluated the usefulness of GC-REAAD™ ITIH3 ELISA in distinguishing gastric carcinoma from non-gastric carcinoma 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 GC-REAAD™ kit is able to distinguish GC patients from non-GC patients. AUC values closer to 1, indicates that GC-REAAD™ is highly accurate in distinguishing GC patients from non-GC patients.

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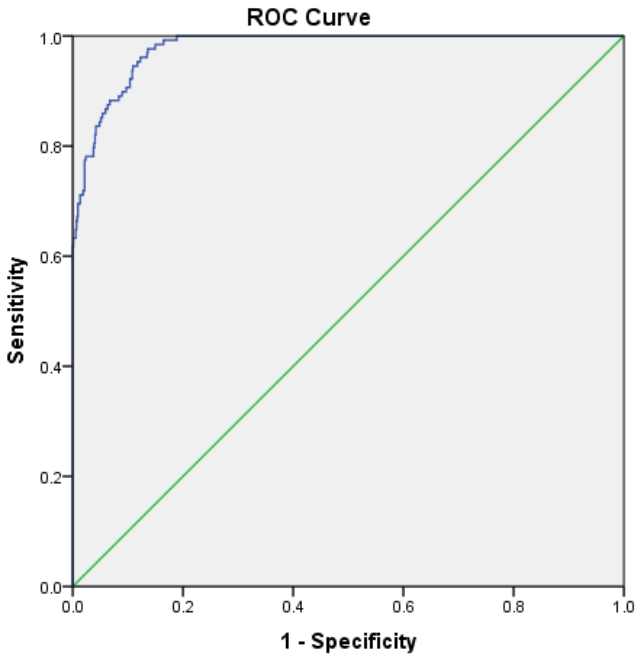
Performance Data

ROC

The kit was subjected to a clinical validation with 869 clinical samples which consisted of 128 true-positive and 741 true-negative for gastric carcinoma. Results generated are used to plot Receiver operator characteristic (ROC) curve using SPSS software. Based on the ROC Curve as shown below (Fig. 1), the kit has achieved a sensitivity of 90.6% and specificity of 90.3% with an AUC of 0.979.

Fig. 1. The ROC curve below showing that GC-READ™ has an estimated sensitivity and specificity of 90.6% and 90.3% respectively with an AUC of 0.979



Diagonal segments are produced by ties.

Area Under the Curve

Test Result Variable(s): Concentration

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.979	.004	.000	.970	.987

The test result variable(s): Sw_3A1_Atlas_100_Ratio has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

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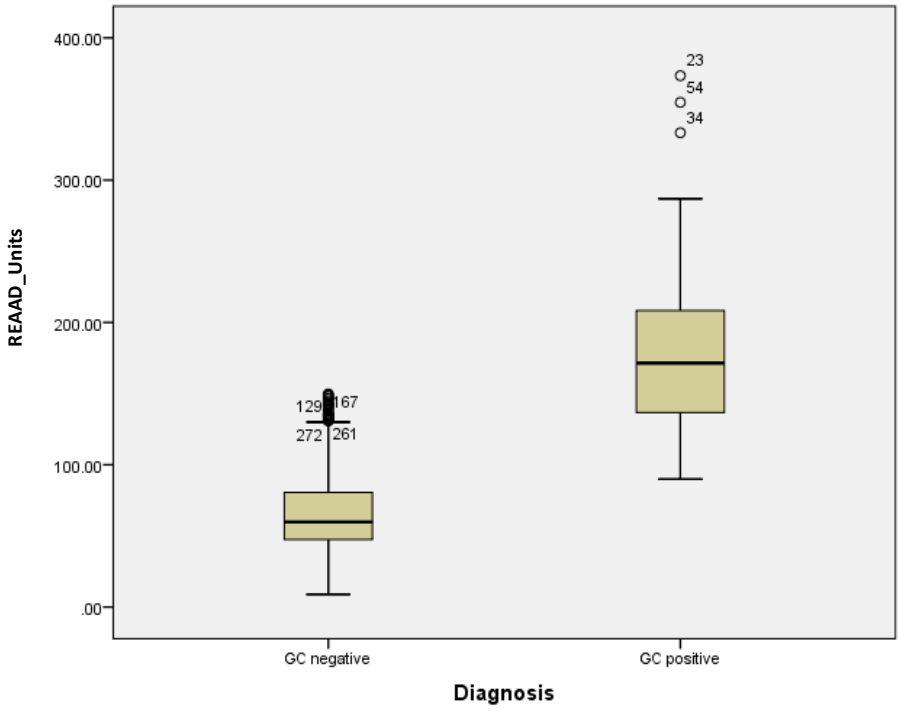
Evaluation between Gastric Carcinoma and Non-Gastric Carcinoma Subjects

The 869 clinical samples were further classified and evaluated into two different categories: gastric carcinoma and non-gastric carcinoma subjects.

Fig. 2. The plot below shows the spread of the ITIH3 concentration on GC-REAAD™. The 128 positive clinical samples gave a relatively higher average ITIH3 concentration as compared to the 741 negative clinical samples.



Discussion and Conclusion

In a review, conventional markers such as CEA, CA19-9, and CA72-4 were mentioned to be ineffective for the detection of gastric carcinoma, giving a sensitivity ranging from 16-63% (Ebert & Rocken, 2006). Presently, the gold standard method for diagnosis of gastric carcinoma is endoscopy—which is an invasive method with adverse side effects. Restalyst's GC-REAAD™ only requires small volumes of human blood samples and its testing procedures is less invasive as compared to endoscopy. GC-REAAD™ can work in complementary to existing methods for diagnosis, thereby providing further insights to aid in the diagnosis of gastric carcinoma.

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GC-READ™ has been validated using the 869 clinical samples that comprised of samples from both 128-gastric carcinoma-positive and 741 gastric carcinoma-negative patients. As shown in the results, GC-READ™ achieved a ROC which gave an estimated sensitivity and specificity of 90.6% and 90.3% respectively with an AUC of 0.979. Additionally, GC-READ™ is able to discern between gastric and non-gastric carcinoma subjects. In-house data from validation studies were submitted to health authorities for this licensing procedure.

Bibliography

Applied Biosytems. (2004). Protocol 16_iTRAQ labeling. Retrieved from Protocol 16_iTRAQ labeling: http://www.biotech.cornell.edu/sites/default/files/uploads/Documents/Proteomics_protocols/Protocol%2016_iTRAQ%20labeling.pdf

Chong, P., Lee, H., Zhou, J., SC, L., Loh, M., Wang, T., . . . Lim, Y. (2010). ITIH3 is a Potential Biomarker for Early Detection of Gastric Cancer. J Proteome Res., 9(7): 3671-3679.

Ebert, M., & Rocken, C. (2006). Molecular screening of gastric cancer by proteome analysis. Eur J Gastroenterol Hepatol., 18(8):847-853.

Hajian-Tilaki, K. (2013). Caspian J Intern Med. 2013 Spring; 4(2): 627–635. Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation.

Health Promotion Board, Singapore. (2012, April). Stomach Cancer. Retrieved from Health Promotion Board: hpb.gov.sg/HOPPortal/dandc-article/588

Huang, L., Yoneda, M., & Kimata, K. (2004). A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. J. Biol. Chem, 279 (37):38079-38082.

International Agency for Research for Cancer (IARC). (2012). Stomach Cancer-Estimated Incidence, Mortality and Prevalence Worldwide in 2012.

Jemal, A., Bray, F., Center, M., Ferlay, J., Ward, E., & Forman, D. (2011). Global Cancer Statistics. CA Cancer J Clin, 69-90.