Biochimica et Biophysica Acta 1844 (2014) 1051–1058

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Identification and validation of novel candidate protein biomarkers for the detection of human gastric cancer ☆

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ARTICLE INFO

Article history:
Received 9 May 2013
Received in revised form 13 January 2014
Accepted 30 January 2014
Available online 7 February 2014

Keywords:
Biomarkers
Intestinal-type gastric adenocarcinoma
Serum proteomics
Post-translational modifications
Isoforms

ABSTRACT

The timely detection of gastric cancer will contribute significantly towards effective treatment and is aided by the availability and reliability of appropriate biomarkers. A combination of several biomarkers can improve the sensitivity and specificity of cancer detection and this work reports results from a panel of 4 proteins. By combining a validated preclinical mouse model with a proteomic approach we have recently discovered novel biomarkers for the detection of gastric cancer. Here, we investigate the specificity of four of those biomarkers (afamin, clusterin, VDBP and haptoglobin) for the detection of gastric cancer using two independent methods of validation: ELISA, and a non antibody based method: Multiple Reaction Monitoring with High Resolution Mass Spectrometry (MRM-HR). All four biomarkers reliably differentiated GC from benign patient serum, and also in a small cohort of 11 early stage cases. We also present a novel isoform specific biomarker alpha-1-antitrypsin (A1AT) that was identified using a mouse model for gastric cancer. This isoform is distinct in charge and mobility in a pH gradient and was validated using human samples by isoelectric focussing and Western-blot (IEF-WB). This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

1. Introduction

Gastric cancer (GC) is the fourth most common cancer in the world and the second leading cause of death due to cancer, reflecting the overall 5 year survival rate (5YSR) of 24% [1]. This poor outcome can be attributed to an extended asymptomatic period associated with this cancer, and difficulty in the detection of early stage gastric adenocarcinoma when treatment could improve long term survival of patients.

Endoscopic investigations are invasive, and given the lack of early symptoms for GC, not generally conducted until an advanced stage where the tumour has significant invasion [2]. While routine screening by gastric photofluorography in Japan has led to an improvement in the 5YSR by over 50% [3], the costs involved cannot be justified in Western countries where the incidence is low, or in those countries with lower GDP. In addition, the sensitivity of barium X-ray reaches only 39% for early stage cancer [4], leading to this being combined with esophagogastroduodenoscopy (EGD), the combination of which is in turn limited by the number of highly experienced endoscopists and the variability of the luminous intensity and quality of endoscopic images depending on differences in endoscope diameter [5]. Unfortunately, EGD is known to be an unpleasant experience for patients invoking anxiety, pharyngeal discomfort, nausea, the gag-reflex and choking, and has been associated with adverse incidents such as cardiovascular responses [6–8].

The incidence of GC in Asia and South America is similar to that in Japan [9], and a cost effective and non-invasive test to identify individuals at risk of GC for further referral for endoscopic biopsy could contribute significantly to improving survival and reducing the health cost burden associated with this disease. Current non-invasive tests for GC include serum pepsinogen in combination with *Helicobacter pylori* infection testing, gastrin 17 [9], carcinoembryonic antigen (CEA), carbohydrate antigen

Abbreviations: VDBP, vitamin D binding protein; MRM, multiple reaction monitoring; PTM, post translational modification; A1AT, alpha-1-antitrypsin; ToF, time of flight; MS, mass spectrometry; DIGE, difference gel electrophoresis

☆ This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

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1570-9639/$ – see front matter
http://dx.doi.org/10.1016/j.bbapap.2014.01.018

(CA) 19–9, CA72–4 [10], and alpha-fetoprotein (AFP) [11], however, all of the above tests provide relatively low sensitivity and specificity [12,13].

The advantages of multiple biomarkers over single biomarkers include: that protein biomarkers may be indicative of more than one disease and therefore not unique to a specific cancer e.g. Il6 is over expressed in oral, prostate, multiple myeloma and renal cell cancers [14]; single biomarkers often have inadequate predictive value e.g. about 70% for PSA [15]; a single biomarker for a given cancer may for a biochemical reason be poorly expressed in a particular patient but it is unlikely that an entire panel of protein biomarkers would fail to be expressed. It is acknowledged that at the early validation stage the use of multiple biomarkers is more expensive than a single biomarker. However, once clinical relevance is established, ELISA analysis can be integrated into routine clinical pathology platforms and multiplexed.

Few studies have looked at a suite of protein biomarkers that are differentially regulated and can show congruence of late-stage and early-stage GC biomarkers [16]. This is possibly because serum biomarkers that are able to distinguish early-stage GC are difficult to identify and validate due to the lack of serum samples from corresponding patients, the inherent difficulties of human genetic diversity and the relatively low abundance of potential serum biomarkers in comparison with the complex serum proteome [17]. Given the incidence of GC in densely populated low-income countries, a diagnostic test based on a suite of differentially regulated serum biomarkers providing good sensitivity and specificity, and that can detect early-stage GC would have international application.

Recently we reported the differential regulation of serum biomarkers in tumour-bearing versus tumour-free cohorts in a highly reproducible, preclinical validated mouse model for early stage GC [18]. We showed that the three candidate biomarker proteins afamin, clusterin and haptoglobin were individually superior to a current clinical marker CA72-4 in discriminating GC patients from healthy controls. Prior to this, and to the best of our knowledge, changes in serum levels of afamin and clusterin had not been implicated in the diagnosis of GC, thus potentially yielding novel gastric cancer biomarkers. Here we report differential regulation of 4 biomarkers, with the addition of vitamin D binding protein (VDBP) to those previously reported, in serum of an extended cohort of GC patients, including a small number ($n = 11$) of early gastric cancer patients. The biomarkers were validated using ELISA and MRM mass spectrometry.

Isoform specific biomarkers have been described in a number of cancers including epithelial ovarian cancer [19], and their relevance in the detection of cancer has been recently reviewed [20]. The difference in physico-chemical properties of the different isoforms has been mostly attributed to posttranslational modifications such as glycosylation. Although the identification of the modification is not absolutely necessary, it might provide important insight into the regulation of the protein of interest. The reliable quantification of protein isoforms is technically challenging and therefore limits their applicability in a clinical or diagnostic setting. However, we were able to confirm the specificity of an isoform specific biomarker using human serum by IEF-WB. Future advances in technologies are of critical importance to allow the use of this novel set of biomarkers.

The need for serum biomarkers is two-fold: firstly to reduce patient impact of screening techniques and the costs involved with such invasive measures; and secondly to enable highly specific detection of early stage GC such that treatments are highly effective thus improving %YSR's.

The well-being of patients is of utmost concern during detection and treatment of gastric and other forms of cancer at the late and early stages, and the high incidence of late stage versus early stage GC detection further highlights the need for a non-invasive, and thus low patient impact and sensitive screening technique.

2. Materials and methods

2.1. Patient samples

Samples were collected with approval from the Ethics Committees of the Peter MacCallum Cancer Centre (Melbourne, Australia), National University Hospital (Singapore) and the University of Adelaide (Adelaide, Australia). Serum samples were obtained from 37 (female $n = 10$, 69 ± 10 years; male $n = 17$, 66 ± 11 years) preoperative GC patients with intestinal type gastric adenocarcinoma (according to Lauren classification). Eleven of these were classified as early-stage gastric cancer (AJCC 6th Edition Stage I disease, with minimal depth of invasion into mucosa and no metastatic lymph nodes) (female $n = 6$, 67 ± 13 yrs; male $n = 5$, 60 ± 13 yrs). The benign gastrointestinal disease serum samples (female $n = 16$, 54 ± 3 yrs; male $n = 4$, 55 ± 5 yrs) were in the majority female, an acknowledged gap in the cohort. Demographic and clinical pathological details of patients from which serum for the ELISA analysis was obtained are shown in Tables 1A and 1B. The clinical and demographic information on the 10 serum samples used in determination of isoforms is shown in Table 2.

2.2. Enzyme-linked immunosorbent assays (ELISA)

ELISAs were performed in accordance with the manufacturer's recommendations. ELISA kits were obtained for: afamin (E92284Hu), clusterin (E91180Hu), haptoglobin (E90817Hu) and VDBP (E91810Hu) from USCN Life Science Inc., China. Serum protein concentrations were interpolated from kit-specific standard curves generated in GraphPad Prism (GraphPad Software). Unpaired Student's *t*-test (significance threshold of $p < 0.05$) and receiver operating characteristics (ROC) were performed in GraphPad Prism.

2.3. Multiple reaction monitoring high resolution mass spectrometry (MRM-HR)

Ten GC and 10 control serum samples were prepared for MRM analysis using QProteome spin columns, followed by an in-solution tryptic digestion for downstream MRM analysis following protocols provided by the Australian Proteome Analysis Facility (APAF) (for QProteome depletion) followed by digestion with trypsin (20 mg) at 37 °C for 6 h (enzyme/substrate ratio approximately 1:25) [21,22].

Samples were run on Triple TOF 5600 (AB Sciex) mass spectrometer with an Eksigent Ultra nano-LC system (Eksigent) with a SGE ProteCol C18, 300 Å, 3 μm, 150 μm × 10 cm column. Prior to analysis, samples were reconstituted in 100 μL of 2% acetonitrile, 0.1% formic acid and vortexed. Samples were then sonicated in a water-bath for 10 min and centrifuged at 12,000 *g* for 5 min prior to nano-LC for MRM-HR analysis. Samples were diluted 1:1 with 2% acetonitrile, 0.1% formic acid then injected (10 μL, full loop injection) onto a peptide trap (Michrom peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% acetonitrile at 5 μL/min for 10 min. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient from mobile phases A:B (98:2) to mobile phases A:B (60:40) at 600 nL/min over a 150 min period where mobile phase A was 0.1% formic acid and mobile phase B was 90% acetonitrile and 0.1% formic acid. The reverse phase nano-LC eluent was subject to positive ion nanoflow electrospray analysis. A TOF-MS scan was acquired (m/z 350–1500, 0.25 s) followed by product ion scans for 23 pre-selected precursor ions from 9 targeted proteins. The product ion scans were 100 ms in the mass range m/z 100–1500 with the total cycle time of 2.6 s. MRM-HR data were acquired twice for each sample with a blank run between samples.