Improved detection of the MUC1 cancer antigen CA 15-3 by ALYGNSA fluorimmunoassay

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ABSTRACT

Breast cancer is the second leading cause of cancer-related deaths in women worldwide; a prime cancer biomarker to aid in the diagnosis, directed treatment, clinical management, and reoccurrence of this cancer is a MUC1 peptide fragment: cancer antigen 15-3 (CA 15-3). Herein, an immuno-fluorescence assay for CA 15-3 was developed; this ALYGNSA system consists of a protein biolinker (Protein G') adsorbed onto Poly (methyl methacrylate) (PMMA). The unique interaction of Protein G' with PMMA, a thermoplastic polymer has been demonstrated to improve human IgG capture antibody alignment/ orientation and result in greater assay sensitivity. Indeed a previous report (HEALTH 1 325 - 329, 2009) on the shed extracellular domain of HER-2/neu revealed a 10-fold increase in sensitivity of the ALYGSNA assay over a control ELISA assay. Results from this ALYGNSA assay study revealed that a 16-fold increase in detection (≤0.94 U/mL) of CA 15-3 was found in comparison to a commercial control ELISA kit (≤15 U/mL). In conclusion, this enhanced sensitivity of the ALYGNSA assay for CA 15-3, may provide insights into the role/function of this biomarker in normal, as well as, breast cancer and other epithelial cancers.

Keywords: CA 15-3; Cancer Antigen 15-3; Epithelial Tumor Antigen; MUC1; Breast Cancer Marker; ELISA; Fluorescent Immunoassay; ALYGNSA

1. INTRODUCTION

Breast cancer (BC) is the second leading cause of cancer-related deaths in women worldwide after lung cancer and is the most frequently diagnosed form of cancer among American women [1]. The most frequently used and best known biomarker is Her2 [2]; this oncoprotein is cell-membrane bound but its extracellular domain is shed into circulation making it a potent biomarker used to monitor the response to treatment, and to detect recurrences in patients with diagnosed breast carcinoma [3,4]. However, this biomarker is only expressed in 20% of all breast tumors [5]; therefore additional BC markers are rigorously being explored [6]. Mucins are promising candidates [7] in particular serum CA 15-3, a MUC1 Tumor Marker; this cancer antigen is overexpressed in >90% of breast carcinomas and metastases [8,9].

The mucin protein product encoded by the MUC1 gene contains approximately 50% carbohydrate by weight with a relative molecular mass of 400 kDa [10]. This cell surface mucin transmembrane glycoprotein, is expressed at the apical surface of most epithelia (e.g. mammary gland, female reproductive tract, stomach, etc.) in normal tissue [11]. It is comprised of three structural domains: a large and heavily O-glycosylated extracellular segment (exo-domain), a hydrophobic type 1 transmembrane region, and a short cytoplasmic tail domain involved in several signaling processes [12]. However, in cancerous tissue, this MUC-1 biomarker expression can be detected on the entire cell membrane due to transformation and loss of polarity [13,14]. After transport to the cell membrane, it undergoes proteolytic cleavage in which the soluble form of the large ectodomain is released into circulation [15]. The tumor marker antigen CA15-3, which corresponds to an immuno-dominant epitope in the extracellular portion of the membrane bound mucin MUC1, is shed into the bloodstream. An increase in the serum CA 15-3 shed ectodomain is associated with progression of carcinoma in patients diagnosed with breast cancer [16]. Levels of CA 15-3 measured greater than 35 U/mL are indicative of the potential progression and recurrence of breast cancer [17-19].

In this present study, an assay was developed for the detection of the breast cancer biomarker CA 15-3 utilizing the ALYGNSA system consisting of a protein biolinker (Protein G') adsorbed onto Poly (methyl methacrylate) (PMMA). The unique interaction of Protein G' with PMMA, a thermoplastic polymer, has been demonstrated to improve human IgG capture antibody align-

ment/orientation [20] and deliver greater sensitivity in the detection of several cancer biomarkers [21-24], including an additional breast cancer biomarker HER-2/neu [21] and an additional mucin MUC16 (CA 125) [22]. Herein, this same ALYGNSA assay system will be employed to measure CA 15-3 and compare the findings to a commercial control ELISA kit.

2. MATERIALS AND METHODS

2.1. CA 15-3 ELISA Assay

This CA 15-3 ELISA assay utilizes the "sandwich" principle, where a capture antibody is directly adsorbed onto a substrate. The detector antibody is labeled with an enzyme, which upon addition of the substrate, produces a colored product quantifiable by absorbance analysis. The ELISA kit for detection of CA 15-3 purchased from BioQuant contained: microwells pre-coated with murine monoclonal anti-CA15-3 antibody, sample diluent, enzyme conjugate diluent, enzyme conjugate concentrate, tetramethylbenzidine (TMB) solution and stop solution. A range of CA 15-3 reference standards (15 - 240 U/mL) provided were used directly (undiluted). This assay was performed, as closely as possible, to manufacturer's instructions. First, 200 μL of CA15-3 standards was dispensed into the appropriate microwells, gently mixed for 10 seconds, and incubated at 37˚C for 1 hour. The wells were rinsed 5 times with dH$_2$O, and 200 μL of enzyme conjugate reagent was dispensed into each well followed by mixing (10 seconds) and incubation (37˚C for 1 hour). The wells were re-washed and 100 μL of TMB reagent was dispensed into each well and gently mixed for 10 seconds. The wells were incubated at room temperature in the dark for 20 minutes, and then finally, 100 μL of stop solution was added to each well to terminate the reaction. The plate was gently mixed for 30 seconds then read at 450 nm with a Bio-Rad microtiter plate reader.

2.2. CA 15-3 ALYGNSA Assay

A "sandwich" fluoroimmunoassay which exploits unique polymer–protein noncovalent interactions between a recombinant Protein G' biolinker and a poly (methyl methacrylate) (PMMA) surface has been termed the ALYGNSA antibody-orientation system. Protein and antibody reagents used in ALYGNSA assay were: recombinant Protein G' (Pierce), Cancer Antigen 15-3 (CA 15-3) monoclonal antibodies (capture and detector) (Fitzgerald, clone# M002204, M002208) and CA 15-3 antigen also from Fitzgerald. The Pierce 660nm Protein Assay (Product #s: 22660, 22662) microplate format was utilized to verify protein concentrations of the CA 15-3 antibodies. The Thermo Scientific Micro BCA Protein

Assay Kit (Product #: 23235) was used to verify recombinant Protein G' and CA 15-3 protein concentrations. Poly (methyl methacrylate) (PMMA) (Sigma) was used to coat polypropylene plates (Corning). The ALYGNSA assay protocol followed previously reported methods [25]. Briefly, the PMMA plates were coated with recombinant Protein G' (1 μg/mL at 50 μL/well) and incubated overnight at 4˚C. After washing once with TBST, the plates were coated with the capture CA 15-3 antibody at 5 μg/mL at 50 μL/well and incubated overnight at 4˚C. After washing, the plates were blocked with 5% non-fat dry milk (NFDM) for 1 hour at room temperature and washed. The CA 15-3 antigen was diluted to 60 U/mL in PBS and 100 μL was applied to the top rows of each plate. A 1:1 serial dilution in PBS was performed and carried out to 0.94 U/mL. The plates were then incubated for 2 hours at room temperature, and then washed. The fluorescently labeled CA 15-3 detector antibody prepared by the DyLight 488 Microscale Antibody Labeling Kit as recently described [25] was diluted to 5 μg/mL, and applied at 50 μL/well. Following incubation for 1 hour at room temperature and a repeat washing, the plates were read for fluorescence at 485/523 nm on a BioTek Microplate Reader.

3. RESULTS and DISCUSSION

3.1. ELISA and ALYGNSA Immunoassays

Enzyme-Linked Immunosorbant Assay (ELISA) is a common immunochemical colorimetric method used to detect cancer biomarkers in biological fluids, such as serum [16,26]. The CA 15-3 ELISA assay utilizes the "sandwich" principle, where a capture antibody is directly adsorbed onto a substrate. The detector antibody is labeled with an enzyme, which upon addition of the substrate, produces a colored product quantifiable by absorbance analysis. A comparison of 15 commercial immunoassays for detection of CA15-3 (MUC1) in serum has been reported with manufacturer cut-off ranging from 23 – 39 (U/mL) [27]. A BioQuant ELISA kit was employed in the present study. After multiple runs of this commercial CA 15-3 ELISA kit assay, the L̲imit O̲f D̲etection (LOD) for CA 15-3 protein, in our hands, was determined to be ≤15 U/mL (**Figure 1**; **Table 1**); it is interesting to note that this is below the cutoff of 17 U/mL (reported range 5 - 47 U/mL) for the non-cancerous state [28].

For more efficacy of the use of proteins and antibodies, an alternative form of this assay, a fluorescent ALYGNSA assay was employed [20]. The ALYGNSA assay format is essentially the same as the colorimetric ELISA; to further enhance sensitivity of the assay, a properly
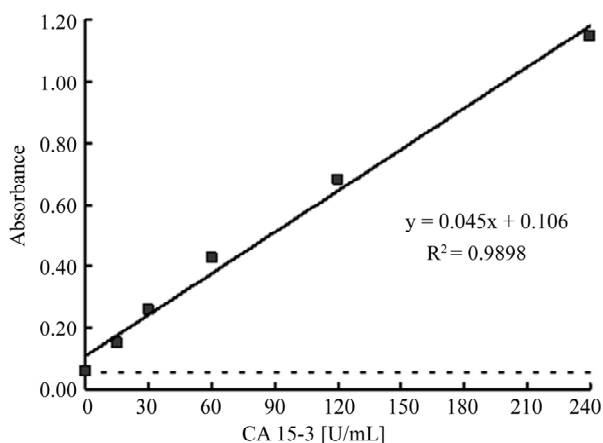
Figure 1. BioQuant CA 15-3 ELISA: Results for assay (-■-■-) and baseline (------) points. The standard deviation values are presented in **Table 1**. Each data point represents the average of five replicates. The LOD was determined by the blank plus two times the standard deviation of the blank value and was 15 U/mL.

Table 1. BioQuant CA 15-3 ELISA.

[CA 15-3] U/mL	Absorbance	±SD	%CV
240	1.020	0.21	20
120	0.628	0.08	13
60	0.426	0.00	0
30	0.225	0.05	22
15*	**0.138**	**0.02**	**12**
0	0.054	0.01	9

*Level of Detection (LOD) was ≤ 15 U/mL and was defined as the blank + 2 standard deviations (SD) [24].The intra-assay variability (%CV) or [(standard deviation / mean) × 100] was calculated based on the difference in multiple measurements.

aligned capture antibody is the primary key. To successfully achieve this "alignment", a recombinant *Streptococcal* protein G' is utilized. Protein G' binds to the Fc region of the capture antibody, in this case murine IgG, allowing optimal interaction of the antigen-binding arms with antigens, and in turn, increases the detection ability of the antibody. The ALYGNSA assay for CA 15-3 detection employs a sandwich assay protocol similar to the commercial ELISA described above. Multiple determinations with the CA 15-3 ALYGNSA assay system revealed an assay LOD of ≤0.94 U/mL for detection of CA 15-3 (**Figure 2**; **Table 2**). These results indicated a 16-fold greater sensitivity for the CA 15-3 ALYGNSA assays as compared to the BioQuant ELISA.

3.2. Structure of Extracellular Domain of MUC1: Potential for Vaccine Development

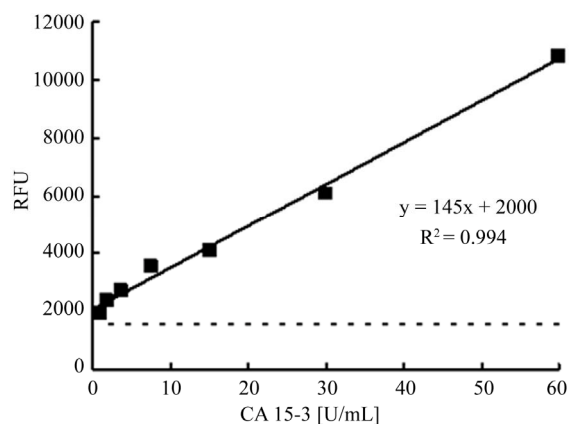The large extracellular tandem repeat domain is highly

Figure 2. CA 15-3 ALYGNSA Assay: ALYGNSA assay results (-■-■-) and baseline (------) points. The standard deviation values are presented in **Table 2**. Each data point represents the average of six replicates. The LOD, as defined previously in **Figure 1** was 0.94 U/mL.

Table 2. CA 15-3 ALYGNSA assay.

[CA 15-3] U/mL	Fluorescence	±SD	%CV
60	11480	947	8
30	6570	619	11
15	5010	47	1
7.5	3720	693	19
3.8	2900	690	24
0.94*	**1750**	**415**	**24**
0	1550	49	3

*Level of Detection (LOD) was ≤ 0.94 U/mL as defined earlier in **Table 1**. The %CV was calculated as described in **Table 1**; value and SD of blank were consistent with previously published reports [21-24].

O-glycosylated and alterations in glycosylation have been shown in epithelial cancer cells [12,29]. Repeats of the core protein give rise to a relatively rigid, linear structure protruding 200 - 500 nm above the cell surface [15,30]. The structure of the extracellular subunit includes a region of nearly identical tandem repeats of 20 amino acids, with variable number (20 - 125) of repeats (VNTR). This highly conserved tandem repeat region of CA 15-3 has been proposed to be evaluated as a vaccine for breast cancer by a number of clinical trials [7,16,31]. Vaccine development requires a dynamic detection range. Improved sensitivity as seen with our assay is vital for the success of these new treatment options.

3.3. MUC1/CA 15-3 Function: Improved Research Capability through Increased Sensitivity

The MUC1 gene encodes the CA 15-3 protein that is generally expressed in different levels on the apical sur-

face of many normal and malignant epithelial cells (expression over entire cell surface), including in the mammary gland, female reproductive tract, lung, kidney, stomach, gall bladder and pancreas, as well as, some non-epithelial cell types [7-9,31,32]. The precise function of CA 15-3 is unclear, although in general, the physiological function of mucins is in lubrication and hydration of cell surfaces, protection of proteins and cells from proteolysis and in the protection of tissues from microbial attack. However, other evidence suggests CA 15-3 appears to play a role in cell-adhesion, where it modulates cell-cell and cell-extracellular matrix (ECM) interactions. In addition to its function as a protective barrier with adhesion-modulating properties, the CA 15-3 cytoplasmic tail has the potential role in cell signal transduction and has been reported to contribute to metastases [32,33]. Increased assay sensitivity offers improved expression analysis for researchers working to identify the function of mucin protein MUC1. Further understanding of this protein may lead to the development of personalized treatment and preventative medicines.

4. CONCLUSIONS

In summary, our work utilized a fluorescent ELISA incorporating the newly developed ALYGNSA antibody-orientation system has revealed a 16-fold increase in sensitivity (≤0.94 U/mL) of CA 15-3 when compared to a commercial ELISA kit (≤15 U/mL). The ALYGNSA assay could aid in evaluation and detection of CA 15-3 under normal conditions, be useful for surveillance of patients diagnosed with breast cancer and to monitor the course of therapy in advanced disease. Furthermore, an increase in sensitivity of the CA 15-3 assay for detection of the shed extracellular domain containing the VNTR region, may provide additional insights into the role/function and clinical assessment of CA 15-3 in breast cancer and other epithelial expressing this protein. Finally, this system has the potential to be incorporated into a cost-effective biosensor device [34]. *This noninvasive serum-based test platform would assist in earlystage cancer diagnosis allowing the clinician to respond in a proactive rather than a reactive manner.*

5. ACKNOWLEDGEMENTS

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