Enhanced immuno-detection of shed extracellular domain of HER-2/neu

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ABSTRACT

HER-2/neu oncogene is over-expressed and amplified in patients associated with metastatic breast cancer. An increased level (>15 ng/mL) in the shed extracellular domain (sECD-HER 2/neu) is indicative of the potential presence and associated progression of this disease. A fluorescent ELISA incorporating the newly developed ALYGNSA antibody-orientation system revealed a 10-fold increase in sensitivity (≤0.63 ng/mL) of sECD-HER 2/neu when compared to a control standard ELISA kit (≤7.5 ng/mL). This enhanced mode of detection has the potential to not only address breast and other cancers per se but also permit an in depth evaluation of “shed extracellular domains”, in general, and the role of these “proteolytic derived factors” in physiological signalling at normal levels.

Keywords: HER-2/neu; Shed Extracellular Domain; C-erbB2; Breast Cancer Marker; ELISA; Fluorescent immunoassay; ALYGNSA

1. BACKGROUND

Breast cancer, which is the most diagnosed form of cancer in women, will account for 27% of cancer cases in 2009 according to the American Cancer Society. The human epidermal growth factor HER-2 oncogene (c-erbB-2) has been implicated in this disease [1-4]. The HER-2/neu oncoprotein is amplified and over-expressed in 25 to 30 percent of patients with aggressive breast cancer. Further, the full-length receptor (p185HER2) undergoes a proteolytic cleavage resulting in the release of the soluble shed ECD/HER2 (sECD-HER 2/neu) fragment. The resultant truncated intracellular form containing the kinase domain is associated with enhanced signaling activity and consequently contributes to metastatic breast cancer [5]. The sECD/HER 2/neu fragment from the surface of breast cancer cells once shed into the blood of individuals can be quantified, making a useful breast cancer biomarker.

Measured levels of the sECD-HER 2/neu protein greater than 15 ng/ml were indicative of the potential presence and the associated progression of primary tumors to metastatic breast cancer [4,6,7]. Most commercially available assays use this concentration as a focal point leaving smaller amounts undetected. Since the over expression of HER-2/neu oncogene is a useful tool as a prognostic and predictive marker for breast cancer, development of a more sensitive biomarker assay appeared ideal for monitoring the progression, the early recurrence of metastatic breast cancer, and the response to therapy [5,8,9]. A more sensitive assay would also allow for the establishment of an individual’s baseline level of sECD-HER-2/neu protein through which more accurate detection of unexpected increase might be achieved. Finally, an improved assay may be useful to monitor the normal signaling activity of the Her-2/neu pathway.

A current, rapid method to detect Her-2/neu protein in biological fluids, such as serum, is the Enzyme-Linked Immunosorbant Assay (ELISA) [10,11]. A typical commercial HER-2/neu ELISA assay uses 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. In this study, an assay was developed for the detection of the sECD-HER-2/neu protein utilizing the ALYGNSA system consisting of a protein biolinker (Protein G’) and poly (methyl methacrylate) or PMMA, a thermoplastic polymer. The unique interaction of Protein G’ with PMMA has been demonstrated to improve human IgG capture antibody alignment/orientation [12] and provide greater sensitivity in detection of cancer biomarkers CA-125 [13] and PSA [14].

In this report, this system has been shown to detect an order of magnitude lower level of (sECD-HER 2/neu) than the commercial ELISA kit counterpart.

2. MATERIALS AND METHODS

2.1. ELISA Assay Method

Microwells coated with mouse monoclonal anti-HER-2/−/
neu protein antibody, HER-2/neu reference standards, sample diluent, detector antibody, substrate diluent, conjugate diluent, conjugate concentrate, substrate tablet, substrate diluent and stop solution were used in the HER-2/neu colorimetric ELISA (Siemens). The ELISA assay was performed according to manufacturer’s instructions. First, the HER-2/neu antigen reference sample was diluted to 9.21 ng/mL with sample diluent. The standards and control were dispensed into the appropriate wells at 100 μL/well and incubated at 37°C for 3 hours. The plate was rinsed three times with 350 μL/well volume of plate wash buffer. Then, 100 μL of detector antibody was dispensed into each well (except for the substrate blank wells) and incubated at 37°C for 1 hour. After washing, 100 μL of working conjugate solution was applied to all wells except for the substrate blank wells. Following incubation at room temperature for 30 minutes, 100 μL of working substrate solution was applied to all wells, including the substrate blank wells. After incubation at room temperature for 45 minutes in the dark, 100 μL/well of stop solution was applied to all the wells and the plate was read for absorbance at 498 nm on a BioRad plate reader.

### 2.2. ALYGNSA Assay

Protein and antibody reagents used in the *sECD-HER-2/neu* ALYGNSA assay were recombinant Protein G’ (Sigma), C-erbB-2 monoclonal antibody (Labvision, 

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**Figure 1.** Siemens *sECD-HER 2/neu* ELISA: Results for assay (closed squares) and baseline (open squares) points. The standard deviation values are presented in Table 1. The LOD was determined by the blank value +2 times the standard deviation. The intra-assay variability (%CV) or [standard deviation / mean] x 100] was calculated based on the difference in duplicate measurements. The highly linear relationship between the standards was confirmed by the coefficient of determination (R²) value of 0.998. Each data point represents the average of eight replicates.

**Figure 2.** *sECD-HER 2/neu* ALYGNSA: Results for assay (closed squares) and baseline (open squares) points. The standard deviation values are presented in Table 2. As with the Her-2/neu ELISA, the ALYGNSA method showed a highly linear response as indicated by the R² value of 0.997. Each data point represents the average of six replicates.

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**Table 1.** Siemens ELISA kit detection of *sECD-HER 2/neu*.  

<table>
<thead>
<tr>
<th>[ng/mL]</th>
<th>Absorbance ±SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>2.50 ± 0.25</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>1.80 ± 0.17</td>
<td>9.4</td>
</tr>
<tr>
<td>15</td>
<td>1.20 ± 0.10</td>
<td>8.3</td>
</tr>
<tr>
<td>7.5*</td>
<td>0.740 ± 0.12</td>
<td>16</td>
</tr>
<tr>
<td>2.5</td>
<td>0.410 ± 0.24</td>
<td>59</td>
</tr>
<tr>
<td>0</td>
<td>0.220 ± 0.18</td>
<td>82</td>
</tr>
</tbody>
</table>

* Level of Detection (LOD) was ≤7.5 ng/mL and was defined as the blank + 2 standard deviations (SD) [9]. The intra-assay variability (%CV) or [standard deviation / mean] x 100] was calculated based on the difference in duplicate measurements.

**Table 2.** *SECD-HER 2/neu* detection by ALYGNSA.  

<table>
<thead>
<tr>
<th>[ng/mL]</th>
<th>Fluorescence ±SD</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>27900 ± 1100</td>
<td>3.9</td>
</tr>
<tr>
<td>10</td>
<td>17100 ± 790</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>11900 ± 2300</td>
<td>19</td>
</tr>
<tr>
<td>2.5</td>
<td>10000 ± 1300</td>
<td>13</td>
</tr>
<tr>
<td>1.25</td>
<td>9080 ± 105</td>
<td>1.2</td>
</tr>
<tr>
<td>0.63</td>
<td>8330 ± 58</td>
<td>0.7</td>
</tr>
<tr>
<td>0.31</td>
<td>7450 ± 190</td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>4870 ± 1400</td>
<td>29</td>
</tr>
</tbody>
</table>

* Level of Detection (LOD) was ≤0.63 ng/mL as defined above. The %CV was also calculated; value and SD of blank were consistent with previously published reports [12,13].
clone c-erbB-2/HER-2/neu Ab 20) and HER-2/neu antigen, reference sample (Siemens). Poly (methyl methacrylate) (PMMA) (Sigma) was used to coat polystyrene plates (Corning). The coating buffer was phosphate buffered saline (PBS); pH 7.4. A PMMA solution in acetone prepared to a concentration of 10 mg/mL was solution cast onto polystyrene plates and incubated overnight at room temperature in a desiccator. After drying, the plates were washed with one volume of 1% SDS in PBS, pH 7.4 and 3 volumes of PBS. The PMMA plates were coated with recombinant Protein G' (1 μg/mL at 50 μL/well) and incubated overnight at 4°C. After washing, the plates were blocked with 5% NFDM at 150 μL/well for 2 hours at room temperature, and then re-washed. The plates were coated with the primary HER-2 antibody at 2 μg/mL at 50 μL/well and incubated overnight at 4°C. After washing, the plates were re-blocked and washed. The reference sample (antigen) was diluted to 20 ng/mL and 100 μL applied to the top rows of each plate. A 1:1 serial dilution in PBS was performed and carried out to 0.31 ng/mL. The plates were incubated for 1 hour at room temperature, and then washed. The HER-2/neu secondary antibody was labeled using DyLight 488 from Pierce. First, 8 μL of the Borate Buffer (0.67 M) was added to 100 μL of 1 mg/mL HER-2/neu antibody in PBS. Then, 100 μL of the prepared protein was added to the vial of DyLight Reagent, briefly centrifuged and incubated for 60 minutes at room temperature in the dark. One hundred microliters of the purification resin was added into the spin column and centrifuged for 1 minute at ~1,000 x g. In a new tube 100 μL of the labeling reaction was added to the spin column, with the purification resin and centrifuged for 1 minute at ~1,000 x g to collect the purified protein. This fluorescent-labeled secondary HER-2 antibody was diluted to 2 μg/mL, and applied at 50 μL/well. Following incubation for 1 hour at room temperature and washing, the plates were read for fluorescence on a BioTek Microplate Reader.

3. RESULTS

3.1. ELISA Assay

An ELISA kit (Siemens) served as a model for a commercially available method to detect sECD-Her-2/neu protein in breast cancer. A standard curve of absorbance versus concentration of sECD-HER 2/neu (ng/mL) was generated after multiple runs of the Siemens ELISA kit (Figure 1). The limit of detection (LOD) for sECD-Her-2/neu protein, in our hands, was determined to be ≤7.5 ng/mL (Table 1).

3.2. ALYGNSA Assay

The ALYGNSA assay for sECD-HER 2/neu detection employs a sandwich assay protocol similar to the ELISA method, except for the protein biolinker, Protein G', and the fluorescently labeled detector antibody; Fluorescence detection analysis is a more sensitive method of analysis than colorimetric detection employed in the ELISA method. Multiple determinations revealed an assay LOD of ≤0.63 ng/mL for sECD-HER 2/neu (Figure 2, Table 2) and, hence, a 10-fold greater sensitivity than the Siemens ELISA kit.

4. DISCUSSION

4.1. sECD-HER 2/neu Detection: Importance in Breast Cancer Treatment

HER-2/neu is over-expressed and amplified in patients with metastatic breast cancer. An increase serum level (>15 ng/mL) of its shed extracellular domain (sECD-HER 2/neu) is indicative of the potential presence and associated progression of this disease. In this report a newly developed ALYGNSA system has been shown to detect an order of magnitude lower level of sECD-HER 2/neu than the commercial ELISA kit counterpart. Prevention of HER-2/neu overexpression has been extensively demonstrated using novel HER-2/neu-blocking agents [8,15,16]. Monoclonal therapeutic antibodies known to bind to extracellular domains of HER-2/neu have been well characterized [17 and references therein]; they include: pertuzumab/Omnitarg™ which acts upon Domain II and is believed to hinder receptor dimerization; cetuximab/Erbitux™ which blocks essential structural transitions of Domain III; and perhaps the most well known and of greatest interest here, trastuzumab/Herceptin™ which binds to Domain IV blocking the action of sheddase and inhibiting sECD-HER 2/neu formation. Trastuzumab was the first FDA approved HER-2/neu monoclonal antibody used in the treatment of HER2/neu overexpression in breast cancer patients. sECD-HER 2/neu (>15 ng/mL) is currently used to select patients for therapy with trastuzumab.

4.2. sECD-HER 2/neu Quantitation: Detection and Treatment of Additional Cancers

Numerous reports have focused on evaluation of serum sECD-HER 2/neu levels in patients not only in breast cancer but also in several other tumor types including but not limited to ovarian [18] prostate [19], and lung cancer [20]. Each condition has a unique set of laboratory, pathological, and clinical factors essential to the detection, treatment, and prognosis. Greater detection, evaluation and standardization of sECD-HER 2/neu levels in serum, as well as other cancer biomarkers, may provide a universal approach to epidermal growth factor receptor (EGFR) related cancers [21,22].
4.3. sECD-HER 2/neu Detection: Assessment of Normal Physiological Processes

EGFR and the other three homologous members of the EGFR family of receptor tyrosine kinase (also referred to as the ErbB or HER family) are comprised of four members: EGFR, HER2, HER3, and HER4. Under normal physiological conditions, the EGFR family governs vital processes including cell growth, cell differentiation, and cellular migration (processes which are altered in cancerous states). Disruption of signaling from the receptors can result in aggressive diseases including many cancers (epithelial tumors) which could possibly lead to poorer outcomes. A recent review [16] has focused on the structural/functional aspects of ligand-induced ErbB receptor structural transitions and dimerization, and mechanistic modes of activation and inhibition. Further, a key part of the EGFR story involves sECD-HER 2/neu [23] and its proteolytic processing via protein ectodomain shedding by the ADAM family of proteins [24]. Post-translational processing of extracellular membrane components is a major mode of regulation [25]. The ALYGNSA assay, with its enhanced sensitivity, may be a key part of the EGFR story involving mechanistic modes of activation and inhibition.

5. CONCLUSIONS

This work utilized a fluorescent ELISA incorporating the newly developed ALYGNSA antibody-orientation system which revealed a 10-fold increase in sensitivity (≤0.63 ng/mL) of sECD-HER 2/neu when compared to a control standard ELISA kit (≤7.5 ng/mL). The ALYGNSA assay could aid in evaluation and detection of sECD-HER 2/neu under normal conditions and may provide additional insight into its role in disease states. This experimental success with analytical (buffer-based) samples should encourage future work with clinical (serum-based) samples.

6. ACKNOWLEDGMENT

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REFERENCES


