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14. ABSTRACT In this project, we focus on oxidation and glycosylation PTMs on proteins known to be secreted by the breast as candidate biomarkers for the early detection of breast cancer. ELISA microarray technology is employed to evaluate assays that have potential be used as breast cancer biomarkers. During the first year of this project we have validated a panel of ELISA capture antibodies for our PTM-ELISA microarray. And we tested 8 proposed PTM detection antibodies with clinical breast cancer plasma samples. Nitrotyrosine, bromotyrosine, chlorotyrosine, TF antigens, and AEG do not give significant difference in our test with clinical breast cancer samples. GSH, GloboH and 4-HNE were found to be altered with breast cancer plasmas samples. The sensitivity and the specificity of each assay and the combined assays were evaluated using the ROC analysis. In the best multivariate analysis, the AUC values ranged from 78% to 89% for 3 different PTMs (GSH, GloboH and 4-HNE). Overall, our data suggest that the PTM ELISA microarray platform is a promising tool for discovery and evaluation of biomarkers that have potential for the early detection of breast cancer.					
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Introduction

Breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death. In 2007, (the most recent year for which statistics are available), 202,964 women were diagnosed with breast cancer, and 40,598 women died from the disease (1). The number could be reduced by a more accurate screening method that can detect breast cancer in its earliest stages. Current screening methods, including clinical breast examination and conventional mammography, have high rates of false-positive and false negative results (2-5). Conventional enzyme-linked immunosorbent assay (ELISA) methods are widely used to screen many potential diseases based on changes in blood proteins. Changes in proteins identified by proteomic studies are largely different from those found by genomics studies (6, 7). This underscores the importance of performing biomarker screens at the protein level and suggests that many of the differences between normal and cancer samples are due to posttranslational modifications (PTMs) such as phosphorylation, glycosylation, oxidation, methylation, ubiquitinylation and acetylation (6-9). **In this project, we focus on oxidation and glycosylation PTMs on proteins known to be secreted by the breast as candidate biomarkers for the early detection of breast cancer.** ELISA microarray technology is employed to evaluate assays that have potential be used as breast cancer biomarkers (10-15). During the first year of this project we have validated a panel of ELISA capture antibodies for our PTM-ELISA microarray. And we tested 8 proposed PTM detection antibodies with clinical breast cancer plasma samples. We also developed one monoclonal antibody (patent pending, Appendix page 21) for the halotyrosine. We have developed our PTM ELISA microarray methods, published one chapter book (Appendix page, page 22), and submitted two manuscripts to high profile journals and presented (or going to, abstracts accepted) our results at international breast cancer conferences (Appendix page, page 11-12). And we are preparing new manuscript for the new discovered PTM biomarkers for breast cancer early detections.

Body

Develop ELISA microarray: an ideal tool for high-throughput, quantitative analysis of breast cancer biomarkers

The microarray sandwich ELISA is exceptionally sensitive, being able to accurately measure protein concentrations down to the sub-pg/ml range (14). Adapted from the conventional sandwich ELISA methods, the ELISA microchip also uses complementary pairs of capture and biotinylated detection antibodies to measure trace levels of antigens in blood. The microarray technique is also suited for targeted discovery research because of its ability to simultaneously conduct multiple assays. At the same time, this multiplex analysis requires much less sample (15-20 µl per chip, after a 5-fold dilution) than conventional ELISA, thereby allowing the screening of many PTMs using very small sample volumes. Using of the Protein Microarray Analysis Tool (ProMAT), which is a custom bioinformatics program developed by Dr. Zangar's group from our institute, allows for rapid analysis of the large quantity of ELISA microarray data (12, 13). The new ELISA microarray have developed for PTM analysis based on the current ELISA microarray platform, with the major difference being the detection antibody. That is, we use biotinylated antibodies that specifically recognize the PTMs. For this purpose,

we adapted the established capture antibodies from the panel and added more capture antibodies based on previous studies for those abundant antigens that only express in breast cancer plasma but not from healthy controls (15). We validated these capture antibodies by individual ELISA microarray and by multiplex combination. We tested them together for optimizing the specificity and sensitivity followed the standard protocol.

PTM detection antibodies

As we stated in our proposal, at the first year of this project, we have evaluated oxidation modifications using the new PTM microarray chip. For oxidative modifications, 4-hydroxynonenal (4HNE), nitrotyrosine, and glutathione (GSH) adducts are tested with commercial available antibodies and antigens to select the best specificity and sensitivities. And the small number of breast cancer plasmas samples was applied to establish the assays.

We also developed halotyrosine antibodies for the breast cancer early detection.

We also tested target unusual or abnormal glycosylation products that are reported increased in breast cancer, including Globo H (16-18), sialosyl-TF (19-22) and advanced glycation end products (AGE) (23, 24). However, we are lacking of specific antibodies for this task. We only got limited antibody resources for these PTMs. The only available antibody for GloboH is IgM species, the application for ELISA microarray is very limited. After careful optimizing the protocol, we tested a small number of breast cancer plasma samples, and we got good correlation and encourage us to test it on large amount of breast plasma samples. We tested sialosyl-TF and AGE products with small set of breast cancer samples, we found out that the sialosyl-TF and advanced glycation end products (AGE) are too low to detect or the sensitivity of these antibodies are not applicable in our ELISA microarray assays. So we have not applied these assays with large amount of breast cancer plasma samples.

Chip development

Previously, the lab has developed an ELISA microarray chip with a set of 20 assays for proteins known to be altered in blood from breast cancer patients (10, 13, 25). BSA, PBS, and IgG Fc fragment also are printed as negative controls. Green fluorescent protein (GFP) is spiked into each sample and used as an internal calibrant. Processing

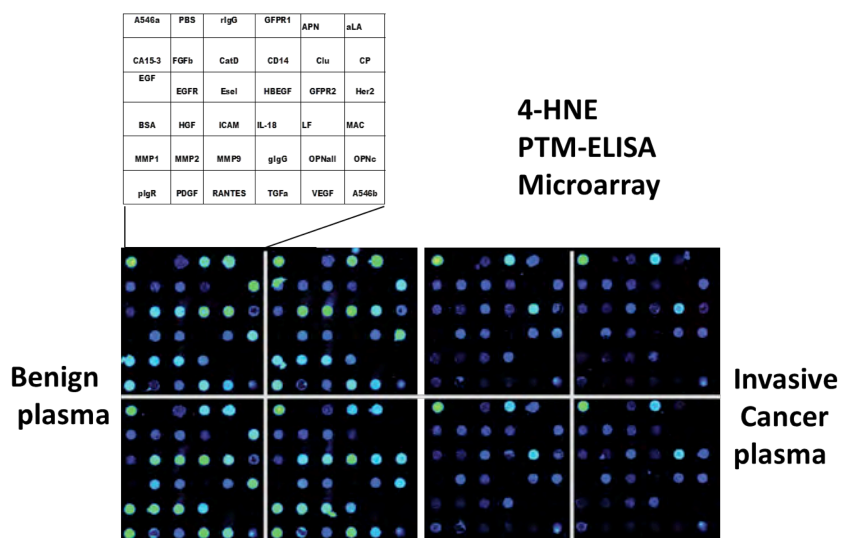


Figure 1 Printed capture antibody spots (200 microns each) were amplified from 4-HNE-PTM-ELISA microarray. One invasive breast cancer plasmas (right) and benign control plasma sample (left) were tested in same chip. Each sample was tested four replicate, like all assays, were printed once in each quadrant. In each quadrant 24 capture antibodies plus controls and calibrates were printed as the indicated arrays.

the breast cancer samples for 5 different PTMs and 27 different antigens have produced a total of 135 assays.

Oxidation biomarkers for early breast cancer

Our preliminary studies indicated there is a correlation between the breast cancer and the level of nitrotyrosine tested with human plasma samples. And we expanded this assay for several other similar studies for tyrosine modifications. Because tyrosines that are post-translationally modified by nitrating oxidants, brominating oxidants, and chloramination oxidants produced from macrophages, neutrophils or eosinophils to form nitrotyrosine, bromotyrosine and chlorotyrosine respectively, have relevance to breast cancers (26-32). For example, hypochlorous acid/hypochlorite adducts have been reported for breast cancer (33-36). Our studies suggest that circulating PTM levels can be used as a biomarker for endothelial cell dysfunction, which is of concern in several human diseases. We have submitted nitrotyrosine plasma samples studies to the journal of *Environmental Health Perspectives* (Appendix page 13), and bromotyrosine sputum samples studies to the Journal of *American Respiratory and Critical Care Medicine* (Appendix page 17) for the methods development.

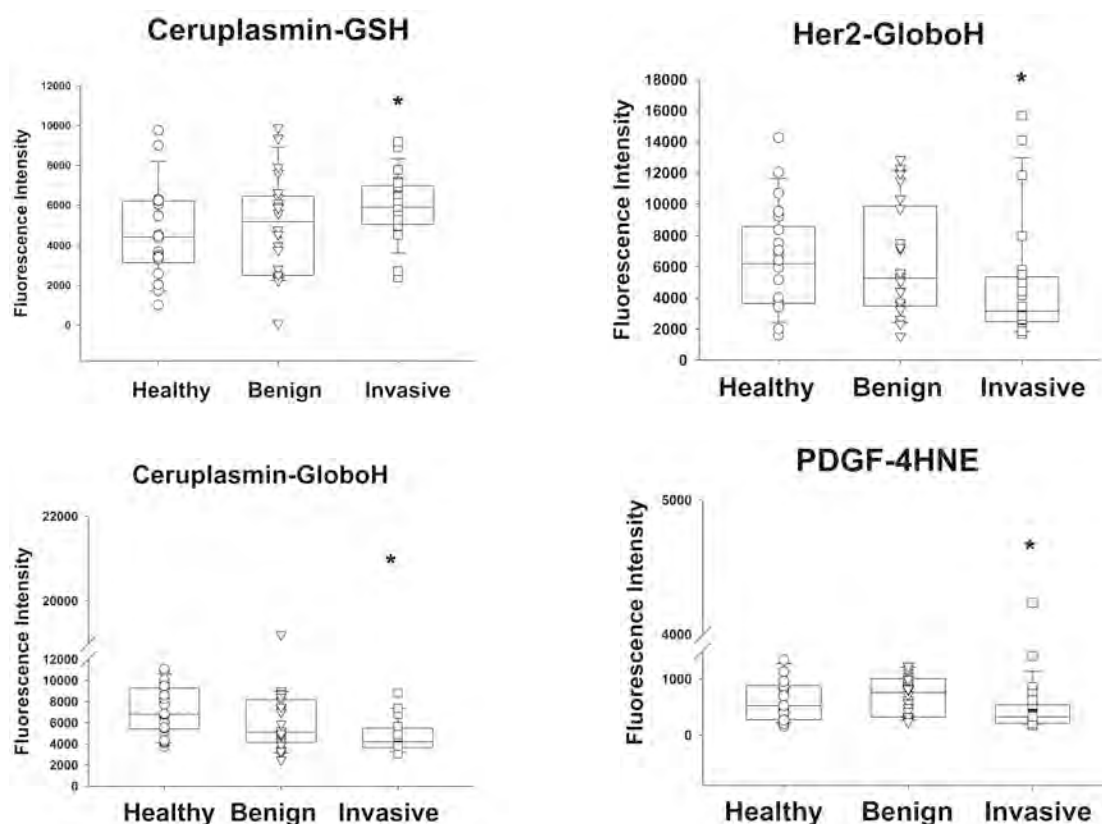


Figure 2. Box plots of PTM-ELISA microarray results. Selected PTMs (GSH, GloboH, and 4HNE) were representing significant increasing (Ceruplasmin-GSH) or decreasing (Her2-GloboH, Ceruplasmin-GloboH, PDGF-4HNE) post-translational modifications occurred in invasive breast cancer with circulating plasma proteins. “*” in the figures are indicating the significant difference between invasive cancer group to the healthy control group based statistical analysis (Box Cox ANOVAs p value <0.05).

4HNE is a non-enzymatic breakdown product of lipid peroxides (37). These modifications have been reported to be associated with oxidative enzymatic breakdown product of lipid peroxides (37). These modifications could be associated with oxidative stress associated with the immune response (30, 37). When we established this PTM-ELISA microarray assay, and tested with breast cancer plasma samples, we found 4HNE protein modification is alerted early breast cancer with several circulating proteins (i.e. PDGF, HGF) (Figure 1, 2,3, Appendix, page 11-12).

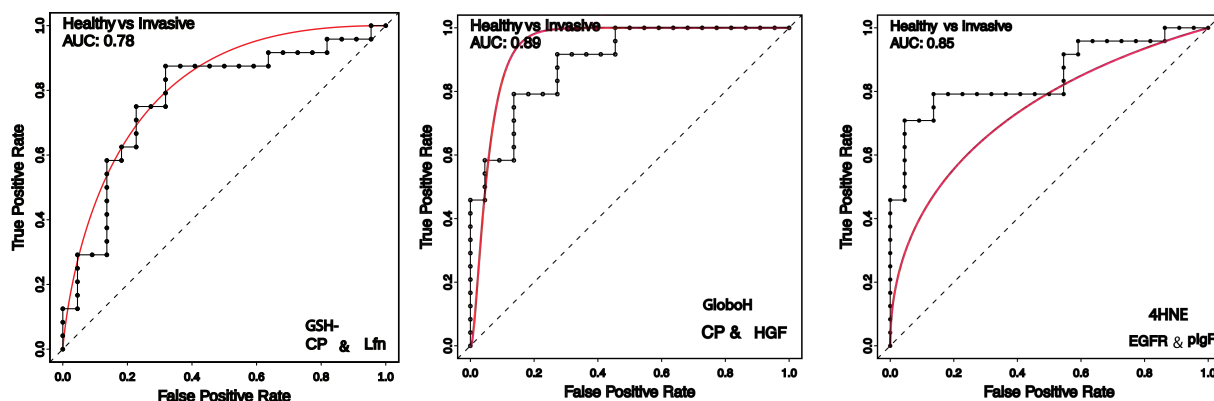


Figure 3 ROC (receiver operating characteristic) curve analysis for PTM-ELISA microarray results. Combination of two biomarkers within same PTM gives AUC (area under the curve) to 78% for GSH, 89% for GloboH and 85% for 4HNE to distinguish invasive breast cancer from the healthy controls.

Glutathione (GSH) is protein adduct which is an indicative of intracellular oxidative stress, especially in the endoplasmic reticulum. Growing evidence suggested that GSH adducts is useful indicator of breast cancer (38-40). We established this PTM-ELISA microarray chip, and tested it with clinical breast cancer plasma samples, we found GSH modification is altered with several circulating proteins (Figure 2, Figure 3, Appendix, page 11-12).

Glycosylation and early breast cancer

Cancer cells can express a high level of certain types of tumor-associated carbohydrate antigens (41). There is considerable evidence that these unusual glycosylation residues are associated with breast cancer (20, 24, 42, 43). The activities and Golgi localization of glycosyltransferases are the basis for the altered glycodynamics of cancer tissue, and determine the ranges and amounts of specific glycosylation produced. There are several cancer-associated structures, including the TF and Tn antigens (20, 44, 45), and certain Lewis antigens (46, 47), Globo H (18, 42, 48), that have been reported to be important in breast cancer. These structural changes can alter cellular function, including its adhesive properties and its potential to invade and metastasize (46). We developed GloboH-ELISA microarray assays, and tested it with clinical breast cancer samples. We found several circulating proteins containing this modification are altered in invasive cancer (Appendix, page 11-12). We are now in the preparation of the manuscript of this finding. For the other glycosylation modification PTM-ELISA-microarrays, we are lacking of specific antibodies currently, hopefully we can find applicable antibodies for those glycosylations in future.

Progress according to our Specific Aims and Statement of Work

Specific Aim 1) Develop antibody microarray chips that can be used to measure PTMs associated with glycosylation (GloboH, TF Antigens, AGEs) state or oxidative adducts (nitrotyrosine, 4-HNE, GSH). For this specific aim, we have successfully developed 5 PTM assays based on selected biomarker panels. These chips are targeting abundant proteins that have been identified in NAF and are also found in the circulation. The 5 PTM detection antibodies have been individually used with the full panel of 24 capture antibodies to detect specific PTMs on each of the antigens.

Specific Aim 2) Evaluate the utility of the panel of biomarkers developed in Aim 1 in a case-control study with plasma samples. We have tested our PTM-ELISA microarray chips on two sets of clinical plasma samples containing early breast cancer and benign controls. Totally 140 samples including 62 invasive breast cancer and 78 benign controls were tested. Each sample was tested in triplicate. So we have tested totally (24X5X140X3) 50400 sandwich ELISA for this project.

Statement of Work Task 1. Develop antibody microarray chips that can be used to measure PTMs associated with glycosylation (GloboH, TF Antigens, AGEs) state or Oxidation adducts (nTyr, 4-HNE, GSH). We have successfully developed microarray chips for GloboH, 4-HNE, GSH and nitrotyrosine. And we also developed halotyrosine antibody and halotyrosine microarray chips. Although we have not found any significant changes with nitrotyrosine or halotyrosine with tested breast cancer plasma samples, we may apply these chips with breast cancer tissue or other types samples in future.

Difficulties

Identifying protein biomarkers in serum or plasma remains a big challenge given the vast range of protein species and broad range concentrations. We intend to develop microarray chips to find whether oxidation and glycosylation modifications can be used for breast cancer detection biomarkers. The big difficulty for this type chip development is hard to find applicable antibodies for the PTM-ELISA. We developed a halotyrosine monoclonal antibody for the assay development, but we have not identified any plasma protein modification differ between breast cancer and benign controls using this antibody.

Key Research Accomplishments

1. Established new panel of 24 ELISA analysis for breast cancer studies;
2. Established PTM-ELISA microarray chips for nitrotyrosine, 4HNE, GSH, and GloboH
3. Developed one oxidation modification antibody, halotyrosine, and PTM-ELISA microarray chip using this antibody;
4. Tested the PTM-ELISA microarray chips on 140 clinical plasma samples

Reportable Outcomes

Published Peer Reviewed paper in “Cancer Biomarkers” IOP press.

Hongjun Jin and Richard C. Zangar Invited Review: Antibody microarray for high-throughput, multianalyte analysis *Cancer Biomarkers* 2010, 6:1-10

Manuscripts submitted:

Jin, H., B.J. Web-Robertson, E.S. Peterson, R. Tan, D.J. Bigelow, M.B. Scholand, J.R. Hoidl, J.G. Pounds, and R.C. Zangar Smoking and COPD have Opposite Effects on 3-Nitrotyrosine in Plasma Proteins ***Environmental Health Perspectives***, (Appendix, page 12)

Jin, H, T.S. Hallstrand, D.S. Daly, P. Nair, D.J. Bigelow, J. G. Pounds and R.C. Zangar Halotyrosine levels in induced sputum reflect eosinophil activity and indirect airway hyperresponsiveness ***American Journal of Respiratory and Critical Care Medicine*** (Appendix, page 16)

Posters

AACR 2011 Abstract #3182 (Accepted)PTM ELISA microarray for breast cancer biomarker discovery, AACR 102 Annual Meeting (April 2-6, 2011, at the Orange County Convention Center in Orlando, Florida) PTM ELISA Microarray for Breast Cancer Biomarker Discovery (Appendix, page 10)

Era of Hope 2011, Abstract (Accepted), Posttranslational Modifications of Specific Circulating Proteins are Promising Biomarkers for Breast Cancer Detection (Appendix, page 11)

Conclusion

To detect breast cancer in its earlier stages, oxidation and glycosylation modification PTMs are proposed as potential biomarkers with circulating human proteins. We developed 5 PTM-ELISA microarray chips (nitrotyrosine, halotyrosine, 4HNE, GSH and GloboH) compiled with an established panel of 24 capture antibodies compiled with previous breast cancer studies. Using these approaches, we identified several circulating human proteins-containing PTMs unique to the plasma of breast cancer patients. The potential impact of this work is that first, it provides a methodological platform for the study of post-translationally-modified proteins and secondly, through the development of assays to monitor these biomarkers in the plasma of breast cancer patients, it has the potential to offer opportunities for more timely therapeutic intervention by providing improved diagnostic capability with use of early disease biomarkers.

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Appendices

Accepted Conference Abstracts

AACR 2011

Abstract #3182 (Accepted)

PTM ELISA microarray for breast cancer biomarker discovery

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PTM ELISA Microarray for Breast Cancer Biomarker Discovery

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Post-translational modifications (PTMs) of proteins are known to be altered during breast cancer development. These PTMs are potentially useful biomarkers for breast cancer. In order to study the potential of PTMs in the early detection of breast cancer, ELISA microarray protocols for analyzing several PTMs in a panel of 24 proteins that are found in blood but known to be secreted by breast tissue and/or breast tumors. We evaluated these PTMs in a case-control study with 68 plasma samples. The control samples were from age-matched women with benign breast disease. The sensitivity and the specificity of each assay and the combined assays were evaluated using the areas under (AUC) the receiver operator curves. In the best multivariate analysis, the AUC values ranged from 77% to 87% for 4 different PTMs. Overall, our data suggest that the PTM ELISA microarray platform is a promising tool for discovery and evaluation of biomarkers that have potential for the early detection of breast cancer. (* This research is supported by NIH U01 CA117378 and The US Department of Defense BCRP Postdoctoral Fellowship (W81XWH-10-1-0031).

Era of Hope 2011

Abstract (Accepted)

Posttranslational Modifications of Specific Circulating Proteins are Promising Biomarkers for Breast Cancer Detection*

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Levels of post-translational protein modifications (PTMs) are known to be altered in breast cancer tissue. We therefore hypothesized that PTMs in proteins that are secreted by breast tissue could be useful circulating biomarkers for breast cancer. In order to study the potential of specific PTMs for the early detection of breast cancer, an ELISA microarray platform for the analysis of PTMs was developed. This platform included an ELISA microarray chip for 24 proteins that are detectable in blood but are secreted by breast tissue and/or breast tumors. For each of these 24 proteins, we measured the levels of four candidate PTMs in two independent sample sets with a total of 140 plasma samples. For both sample sets, all samples were collected at the time of biopsy, after referral from a positive screen (such as mammography). The samples were subsequently categorized as either benign controls or cancer cases based on the pathology report. The subjects in the two groups were age-matched. The sensitivity and the specificity of each assay and the combined assays were evaluated using the areas under the receiver operator curves (AUC). In an analysis of 4 markers, the best AUC value was 87%. Our study suggests that certain circulating proteins with specific oxidative modifications or glycosylation residues can be used to distinguish between women with early breast cancer and those with benign breast disease. Therefore, these biomarkers may have potential to distinguish between true positive and false positive results obtained with standard clinical screens, including mammography. (* This research is supported by NIH U01 CA117378 and The US Department of Defense BCRP Postdoctoral Fellowship (W81XWH-10-1-0031).

**Published Peer Reviewed paper in
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Antibody microarrays for high-throughput, multianalyte analysis

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Abstract. Enzyme-linked immunosorbent assay (ELISA) microarray technology promises to be a powerful tool for detecting and validating protein biomarkers, especially panels of biomarkers. ELISA microarrays are capable of high-throughput analysis of multiple proteins using small sample volumes. In this chapter we review the literature on the use of antibody microarrays for biomarker discovery and validation. We also described the methodologies we employ to obtain high-quality data through protocol optimization and data calibration.

Keywords: Antibody microarray, multianalyte, multiplex, high-throughput, biomarker, calibration

1. Introduction

Recent technological advances have improved the ability to globally analyze protein levels and gene expression patterns, thereby resulting in lists of candidate biomarkers [1–4]. For example, proteomic methods that employ mass spectrometry can now evaluate hundreds or thousands of proteins that may potentially function as disease markers [5–7]. Even so, this technology platform is generally limited by a combination of throughput, sensitivity and quantitative capabilities. To address the issue of sensitivity, sample fractionation, depletion of abundant proteins and/or protein enrichment are commonly employed [8,9]. Unfortunately, these protocols are commonly time-consuming and expensive, thus limiting the ability for high throughput analysis that is needed for clinical validation of biomarkers [10–12]. Enzyme-linked immunosorbent assay (ELISA) microarrays are one emerging technology that can simultaneously address the issues of sensitivity and throughput when undertaking targeted, multiplexed, preclinical validation studies. Another advantage

of this analytical method is that the methodology is directly comparable to traditional clinical analyses, and therefore the analysis can be easily converted into a standard clinical assay, should the validation step be successful.

Sandwich ELISAs use a matched set of capture and detection antibodies to measure each antigen (Fig. 1A). ELISA microarray analysis commonly uses sandwich ELISAs (Fig. 1B, Table 1). A variety of different platforms and substrata have been employed for multiplex ELISA analysis, including glass slides, beads, microplates, nitrocellulose membranes or 3-dimensional gel, but the basic sandwich ELISA analysis is conceptually the same regardless of the platform. For the multiplex platform, the various capture antibodies are physically separated either by printing in isolated spots or by attaching to different beads. To facilitate throughput and ease of analysis, purified antigens (which are used to generate the standard curves) are combined into a single mixture, as are all of the detection antibodies. Typically, without any detectable assay cross-reactivity of interference, multiple proteins can be simultaneously quantified within a single sample. It has been widely reported that the ELISA microarray is an efficient tool for measuring the levels of up to 50 proteins with excellent sensitive (~ 1 pg/ml), dynamic range (~ 3 logs), and low coefficients of variation between replicate measure-

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Manuscripts Submitted

Method development for nitrotyrosine-PTM-ELISA microarray

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Smoking and COPD have Opposite Effects on 3-Nitrotyrosine Levels of Plasma Proteins

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Abstract

BACKGROUND: Nitric oxide is a physiological regulator of endothelial function and hemodynamics. Oxidized products of nitric oxide can form nitrotyrosine, which is a marker of nitrative stress. Cigarette smoking decreases exhaled nitric oxide, and the underlying mechanism may be important in the cardiovascular toxicity of smoking, although it is not clear if this effect results from decreased nitric oxide production or increased oxidative degradation of nitric oxide to reactive nitrating species. These two processes would be expected to have opposite effects on nitrotyrosine levels, a marker of nitrative stress. **OBJECTIVE:** In this study, we determine the effects of cigarette smoking and chronic obstructive pulmonary disease (COPD) on circulating levels of nitrotyrosine, and thereby gain insight into the processes regulating nitrotyrosine formation. **METHODS:** A custom antibody microarray platform was developed to analyze the levels of 3-nitrotyrosine modifications on 24 proteins in plasma. In a cross-sectional study, plasma samples from 458 individuals were analyzed. **RESULTS:** Nitrotyrosine levels in circulating proteins were consistently reduced in smokers and former smokers but increased in COPD subjects. **CONCLUSIONS:** Smoking broadly suppresses the levels of 3-nitrotyrosine in plasma proteins, suggesting that cigarette smoke suppresses endothelial nitric oxide production. In contrast, the increase in nitrotyrosine levels in COPD patients most likely results from inflammatory processes. This study provides the first evidence that smoking has persistent effects on endothelial production of nitric oxide in humans, and provides insight into how smoking could induce endothelial dysfunction and a long-term increase in the risk of cardiovascular disease.

There was a persistent effect of smoking on nitrotyrosine, as evidenced by changes observed in former smokers. Although the former smokers in this study had quit smoking from 1 to 35 years prior to the blood sampling, we did not observe a significant correlation between the time since quitting and the levels of nitrotyrosine in the blood (see Supplementary Material, Figure 8). Previous studies have demonstrated that smoking has irreversible effects on genetic mutations and gene expression profiles in bronchial epithelial cells (Beane et al. 2007; Mao et al. 1997). These changes are detectable up to 20 years after smoking cessation, and are believed to have a role in the sustained effects of cigarette smoke exposure on lung cancer risk. Our study provides evidence that smoking has irreversible effects on endothelial cell function and, in particular, eNOS activity. Therefore, this study provides novel insight into how cigarette smoke exposure could induce persistent effects on the cardiovascular system and may help explain the sensitivity of the cardiovascular system to environmental smoke.

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