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Oxidatively Modified Proteins as Plasma Biomarkers in Breast Cancer

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Abstract

BACKGROUND—Post-translational protein modifications (PTMs) are increased in breast tumors.

OBJECTIVE—We explored whether PTMs on proteins secreted by the breast could be detected in plasma and potentially used for the early detection of breast cancer.

METHODS—We used a custom ELISA microarray platform to measure 4-hydroxynonenal (HNE), glutathione (GSH), nitrotyrosine and halotyrosine adducts in 27 secreted proteins, for a total of 108 candidate biomarkers. Two independent sets human plasma samples were measured, for a total of 160 samples. The results were analyzed for consistent cancer-associated changes across the two sample sets. Plasma samples for both cases and benign controls were collected at the time of tissue diagnosis after referral from a positive screen (such as mammography). The results from both studies were evaluated using ANOVA and t-tests or receiver operator curves (ROC).

RESULTS—Levels of GSH-modified ceruloplasmin and HNE-modified PDGF were significantly altered in plasma samples from cancer patients relative to benign controls. Healthy controls, which were only included in the first set of samples, were similar to the benign controls for both of these markers. A combination of three glutathionylated proteins had the best area under the ROC curve, with a value of 76%.

CONCLUSIONS—Specific PTMs in individual proteins may be useful for distinguishing between women with breast cancer and those with benign breast disease. These oxidative changes in plasma proteins may reflect redox changes in breast cancer. Additional studies on oxidative modifications in individual proteins are warranted.

Keywords

breast cancer; biomarkers; reactive oxygen species; protein adducts; hydroxynonenal; glutathione; plasma

Introduction

Breast cancer is the second most common cancer and the fifth most common cause of cancer death in the United States. In 2008, breast cancer resulted in 458,000 deaths worldwide

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according to World Health Organization [1]. Women in the United States have among the highest incidence of breast cancer in the world, with an approximate 1 in 8 lifetime risk of developing breast cancer and a 1 in 35 risk of death. In 2011, there were 230,480 newly diagnosed breast cancer cases in the United States, resulting in 57,650 deaths [2]. The mortality and morbidity associated with breast cancer likely could be reduced by complementary screening method such as circulating biomarkers.

Oxidative stress appears to be a risk factor for breast cancer and may contribute to the pathology of this disease [3]. Oxidative stress is increased in breast cancer [4–6] and the greatest increase is observed during early-stage disease [5]. Oxidative stress is an underlying factor in oxidative modifications of proteins, including proteins that are secreted into the blood. The primary goal of this study is to determine if oxidatively modified proteins have potential as biomarkers for the early detection of breast cancer regardless of the underlying mechanisms. Even so, oxidatively modified tumor proteins that are detected in blood could provide insight into molecular processes occurring in the tumor. Reactive oxygen species (ROS), which are responsible for oxidative protein modifications, regulate important processes in epithelial cancers such as activation of MAPK/Erk and PI3K/Akt pathways [7]. In cultured human mammary epithelial cells, we found that both of these signaling pathways and intracellular ROS regulate the secretion of a variety of autocrine factors, paracrine factors and matrix metalloproteases [8, 9]. Based on these studies, we hypothesized that alterations in cell signaling and oxidative stress associated with breast cancer would not only results in increased levels of certain proteins that are secreted into blood but that these proteins would have unusually high oxidation rates. As such, these oxidized proteins could serve as circulating breast cancer biomarkers.

In this study, we evaluate oxidative, post-translational protein modifications (PTMs) that are potentially characteristic of early breast cancer. Because we expect the modified proteins to be at low abundance, we use the sandwich enzyme-linked immunosorbent assays (ELISAs) because these assays have exceptional sensitivity and specificity. In the sandwich ELISA, one antibody is used to capture and concentrate the target protein and a second antibody selectively measures the oxidative modification. Although neither the individual proteins nor the modification may be unique to breast cancer, we hypothesized that an increase of both secretory and oxidative processes in breast cancer tissue would result in useful biomarkers. Our results suggest that oxidatively modified proteins are altered in plasma from breast cancer patients and that these proteins have potential to be used as biomarkers for this disease.

Materials and Methods

Human subjects

All subjects were recruited and samples were collected under Institutional Review Board (IRB)-approved protocols and informed consent at the Duke University. The Duke IRB protocols were subsequently reviewed and approved by the IRB at the Pacific Northwest National Laboratory prior to transfer and analysis of the samples. Two sets of plasma samples were analyzed for a total of 160 subjects. The first sample set contained plasma from 22 subjects with benign breast disease tumors, 24 subjects with invasive breast cancer and 22 subjects of healthy controls (total of 68 subjects). The second sample set contained plasma from 54 subjects with benign breast disease and 38 with invasive breast cancer (total of 92 subjects).

All plasma samples were collected in the same clinical setting, using the same protocol. All subjects had received a positive screen (e.g., mammography) and were therefore referred for an image-guided biopsy, at which time the blood samples were collected. Since it was

unknown until after the pathological analysis which subjects had breast cancer and which had benign disease, this study design minimizes the possibility of potentially confounding factors such as behavioral changes or emotional stress associated with knowledge of cancer by the subjects. Samples in the control groups were selected to correspond to the cancer cases based on the subject's age, body mass index (BMI), and race (Table 1).

Processing of plasma samples

Plasma samples were stored as individual aliquots at -80°C until use. Immediately before analysis, the samples were thawed on ice and centrifuged at $15,000\text{ g}$ for 20 min at 4°C to remove trace precipitates. The supernatant was diluted 5-fold in 0.1% BSA/PBS containing 1000 pg/mL of green fluorescent protein, which was used as the antigen in a calibrant sandwich ELISA to identify and normalize any chip-to-chip bias [10, 11]. The individual capture antibodies (listed in Supplementary Table 1) are typically saturated by low ng/mL concentrations of antigen, and we routinely dilute plasma samples 500-fold in order to get antigen concentrations in the usable range of the standard curve for the unmodified proteins. We previously demonstrated that capture antibodies for the plasma proteins are typically saturated at a 5-fold dilution of plasma [12]. As such, the 5-fold dilution of plasma used in this study is expected to saturate the capture antibodies, resulting in approximately equal masses of captured antigen on each spot. Thus, the PTM signal should be relative to a consistent mass of antigen and not be influenced by plasma variations in antigen concentrations.

ELISA microarray analysis

The basic ELISA microarray protocol has been previously described in detail [13]. In brief, 27 capture antibodies (listed in supplementary Table 1) were printed on each chip in quadruplicate spots such that one replicate spot per antibody was printed in each of 4 identical quadrants (Fig. 1). The commercial sources for these antibodies have been previously reported [12, 14]. A capture antibody for the green fluorescent protein, which was used as a sandwich ELISA calibration assay (see below), and orientation spots were also printed in quadruplicate on each chip (Fig. 1). The samples were blocked based on study group and then randomized for the ELISA analysis. The 4 PTM analyses were each conducted separately. Individual chips were first incubated with a diluted plasma sample from a single subject, with each sample analyzed on 3 identical chips for each of the 4 PTM analyses. Thus, this study represents data from 1800 ELISA chips and 48,000 ELISAs, without taking into account the 4 replicate spots for each assay on each chip. For each PTM analysis, all samples were simultaneously processed, thus eliminating concerns about reagent drift or other potential day-to-day variations. After washing, the chips were incubated with one of 4 biotinylated PTM detection antibodies (listed in supplementary Table 2) and a biotinylated detection antibody for green fluorescent protein. The biotin signal was amplified using the biotinyltyramide system [15]. Fluorescent labeling was produced using streptavidin conjugated with Alexa 546. After processing, the microarray slides were imaged with a fluorescent laser scanner (ScanArray Express HT, Perkin-Elmer, Downer Grove, IL, USA) and ScanArray Express software was used to quantify the fluorescent intensity of the spots. Data calibration across chips was undertaken using the data from the green fluorescent protein ELISA analysis using ProMAT Calibrator [10, 11] and then was processed using ProMAT [16]. We developed both of these programs as open-source programs in R that are freely available on the web (www.pnl.gov/statistics/ProMAT/).

Statistics

Results from within and across reagents and studies were statistically evaluated, individually and in combination, using ANOVA, t-tests and ROC analyses. First, promising reagent-

specific assays were identified by using Box-Cox transformed ELISA spot-fluorescence data and then comparing individual assays between the cancer and no-cancer groups by ANOVA. The ANOVA model included a two-level study factor to account for intensity offsets between studies while the intensities were Box-Cox transformed in order to produce normally distributed residuals of the same scale between studies. When P was less than 0.05 for the ANOVA analysis, statistical differences between individual groups were delineated by t-tests. In these cases, only the t-test p values are presented here. Assay results were then evaluated with ROC analyses of the individual assays and linear discriminant scores of assay combinations. ROC curves were computed using data from the two individual studies and the data combined across studies using the Moses algorithm [17]. These analyses were executed using R [18] with Box-Cox transform and linear discriminant algorithms from the MASS [19] library, and ROC algorithms from the ROCR library [20].

Results

GSH and HNE modifications in plasma proteins are altered in breast cancer

We analyzed 4 PTMs in 27 proteins for an analysis of 108 total candidate biomarkers. We used several criteria to identify likely candidate proteins for our analysis. First, we selected potential biomarkers based on our proteomics study that identify abundant proteins in nipple aspirate fluid, which is highly enriched in proteins secreted by the breast ductal tissue, the cells of which are the source of most breast cancers [21]. We also analyzed a number of secreted proteins reported by others to be produced by normal or cancerous breast tissue. Finally, we evaluated a selection of proteins that we identified as being secreted at higher levels in human mammary cells in response to modest changes in intracellular ROS or to epidermal-growth-factor signaling [8, 9, 22, 23].

No differences were observed for halotyrosine or nitrotyrosine protein modifications across the two groups. Although we anticipated that oxidative protein adducts would be increased in breast cancer patients, only glutathione adducts to ceruloplasmin were found at higher levels in the breast-cancer subjects compared to benign controls in both sets of samples (Fig. 2 and Table 2). In contrast, GSH adducts to hepatocyte growth factor were lower in the breast cancer subjects relative to the benign controls. We and others previously found that levels of circulating hepatocyte growth factor are increased in recurrent breast cancer patients relative to healthy controls [15, 24], suggesting that the decrease in oxidatively modified hepatocyte growth factor is not influenced by a decrease in total levels of this protein.

The levels of three HNE-adducted proteins (i.e., matrix metalloprotease 2, platelet-derived growth factor A [PDGF] and polyimmunoglobulin receptor) were significantly lower in breast cancer patients compared to the benign controls (Table 2). PDGF-HNE, which had the greatest difference between the two groups, is shown in Fig. 2.

Alterations in PTM combinations between breast cancer and healthy controls

For this comparison, we focused on differences between women with invasive cancer and those with no breast abnormality as determined by screening mammography. Results from the multiple PTM-modified proteins were combined using ROC analyses. For this analysis, we focused on either multiple proteins with the same PTM or on different PTMs found on a single protein. That is, we did not evaluate all possible combinations of PTMs across all proteins. The combination of three different proteins, ceruloplasmin, hepatocyte growth factor and clusterin, all with GSH adducts, produced an area under the ROC curve (AUC) of 76% (Fig. 3) when data from the two studies was combined. This result was the best observed for any three proteins with the same oxidative modification. In addition, the

combination of lactoferrin with either GSH or HNE modifications had an AUC value of 0.63 (Fig. 3). This result was the best observed for single proteins with more than one oxidative modification.

Discussion

Cancer has been compared to “a wound that never heals”, implying that chronic oxidative stress from inflammation is important in cancer development. In addition, other cancer-related processes can increase ROS in breast tumors, potentially altering gene expression and cellular function [25, 26]. The levels of individual PTMs are broadly increased in breast cancer tissue [4]. To determine if a specific protein modification could be a useful biomarker, we examined single oxidative modifications on individual proteins that are secreted by the breast. Because there is insufficient knowledge to predict which PTMs are likely to be most useful, we examined 108 combinations of oxidative protein modifications (4 modifications on each of 27 different proteins).

One oxidative modification we evaluated was HNE, which is a nonenzymatic byproduct of lipid peroxidation that spontaneously adducts to proteins. Protein-bound HNE in the tumor tissue is increased in more than one stage of breast cancer, but HNE levels are highest in early breast cancer relative to the adjacent tissue [5, 27] suggesting that HNE modifications may be particularly useful for detecting early-stage disease. Surprisingly, three proteins had changes in the levels of HNE adducts and in all cases HNE adducts were suppressed in the blood of breast cancer patients relative to benign controls. The meaning of this change remains uncertain and could relate to either decreased formation of these adducts or an increase in their plasma clearance in breast cancer patients.

In vitro, lipid peroxidation and the associated HNE adducts result from relatively severe oxidative stress that occurs when the cellular antioxidant defense system is overwhelmed. More subtle changes in the intracellular redox environment likely precede severe oxidative stress. Cysteine is the most readily oxidized amino acid and susceptible cysteine residues are oxidized under physiological redox conditions and are converted to GSH-cysteine protein adducts. We therefore examined GSH adducts as an indicator of subtle changes in redox status. Modest redox changes may contribute to breast cancer development and progression by altering intracellular signaling and cell-to-cell communication. For example, we found that modest increases in intracellular ROS production altered the expression and secretion of a variety of bioactive proteins in several epithelial cell lines (9). This study found that in human mammary cells, expression of low-levels of a ROS-generating protein increased secretion of TGF α , a ligand for the epidermal growth factor receptor, but suppressed secretion of matrix metalloproteinase 1. The general effects of ROS on protein secretion were conserved across epithelial cells from different tissues and mammalian species, but the individual secreted proteins varied with cell type.

We examined protein halotyrosine modifications, which are primarily the result of granulocyte peroxidase activities [28]. In a previous analysis of proteins in sputum, we found elevated levels of halogenated proteins in asthma patients with either eosinophilic or neutrophilic disease relative to healthy controls (unpublished data). However, there is a lack of evidence for a role for these granulocytes in breast cancer development. As such, we included the halotyrosine analysis as a type of negative control assay, anticipating that this protein modification would not be associated breast cancer patients. As we did not observe any changes in protein halogenation, our results provide additional evidence that these granulocytes are not associated with breast cancer.

Similar to halotyrosine, we found that protein nitrotyrosine levels were not significantly different between cancer patients and either benign or healthy controls. This relationship was not anticipated because nitrotyrosine modifications are commonly associated with many different human diseases, including breast cancer [29]. In breast tumors, increases in protein nitrotyrosine are found in the vicinity of infiltrating inflammatory cells [30]. We previously analyzed nitrotyrosine levels in individual proteins found in plasma from 458 subjects. This study found that nitrotyrosine levels were associated with chronic obstructive pulmonary disease and tobacco smoke exposure, suggesting that the nitration of plasma proteins is influenced both by inflammation and endothelial nitric oxide synthase activity, which appears to be suppressed by smoking [12]. These results suggest that multiple processes influence nitration levels of plasma proteins and that these processes may mask any cancer-related nitration changes in the proteins we analyzed.

Overall, the general markers of oxidative stress and changes in redox status, HNE, and GSH proved to be more promising cancer biomarkers than nitrotyrosine or halotyrosine. These latter two processes are more likely to be indicators of specific inflammatory processes while the first two processes are non-specific markers of changes in oxidative stress and redox status. Our most promising results were observed when data from several oxidatively modified proteins were combined, a result that is consistent with our prior analysis of unmodified proteins as breast cancer biomarkers [31]. Further studies are needed to replicate and confirm the results reported here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A546a	PBS	rlgG	GFPR1	APN	aLA
CA15-3	FGFb	CatD	CD14	Clu	CP
EGF	EGFR	Escl	HBEGF	GFPR2	Her2
BSA	HGF	ICAM	IL-18	LF	MAC
MMP1	MMP2	MMP9	glgG	OPNall	OPNc
plgR	PDGF	RANTES	TGFa	VEGF	A546b

Figure 1. Image of a single ELISA microarray

Each microarray chip contains 27 capture antibodies for assay proteins and additional control spots for orientation (A546) and antibodies for an internal calibration assay (GFP). All reagents were individually printed in quadruplicate (once in each quadrant). A single scanned chip is shown in the lower image, with crossed white lines added to separate the 4 identical quadrants. The color is artificial and corresponds to fluorescence signal intensity, which increases from black (background) to blue (weak fluorescent signal) to green (strong signal). The layout of a single quadrant is shown in the upper figure.

Figure 2. Representative graphs of normalized fluorescence data for oxidative modifications in individual proteins found in plasma

Data are shown for two independent sets of plasma samples (Study 1 and 2). Each datapoint represents the average raw fluorescence signal for triplicate analyses (one chip per analysis) of a single sample. Since the fluorescent values are in arbitrary units, the data was normalized based on the average value of all the assays for each of the two studies. Data values for each assay on each individual chip were calculated as the median value of 4 identical assay spots. Thus, each datapoint shown here represents data derived from 12 analyses. The central horizontal bar and cross bars represent the mean and the standard error, respectively. CP-GSH, glutathionylated ceruloplasmin; PDGF-4HNE, 4-hydroxynonenal-adducted platelet-derived growth factor.

Figure 3. The best ROC curves for plasma biomarkers on a single protein with different modifications or for a single modification on different proteins

Graphs represent comparisons between oxidatively modified protein levels, as measured in plasma, with comparisons between subjects with invasive breast cancer and those with benign breast disease. **A.** The combination of three different PTMs biomarkers: 4-hydroxynonenal (HNE) and glutathione (GSH) adducts on lactoferrin (Lfn) produced an area under the fitted ROC curve (AUC) of 63%. **B.** The combination of ceruloplasmin (Cp), lactoferrin (Lfn) and clusterin (Clu) modified with glutathione adducts (GSH) produced an AUC of 76%. The black and the blue lines represent the empirical data for the first and second sample sets, respectively, and the red line is a fitted curve derived from both sample sets.

Table 1

Subjects characteristics.

Sample Set 1				
	Healthy Controls	Benign Controls	Invasive Cancer	Total
N	22	22	24	68
Age (years)	46±16 ^a	52±14	50±8	50±13
Race (B/H/W ^b)	6/1/15	6/0/16	3/0/21	15/1/52
BMI (kg/m ²)	23±1	26±3	26±4	26±4
Sample Set 2				
	Healthy Controls	Benign Controls	Invasive Cancer	Total
N	0	54	38	92
Age (years)	NA	53±13	53±11	53±12
Race (B/H/W)	NA	0/0/54	0/0/38	0/0/92
BMI (kg/m ²)	NA	26±5	30±8	28±7

^b Mean±SE.^a Abbreviations: B, Black; H, Hispanic; W, White; BMI, Body Mass Index. NA, Not applicable.

Summary of t-test results for GSH and HNE oxidative modification on 26 individual proteins for data from two independent sets of plasma samples.

Table 2

Assay	First Study		Second study		Both	
	Benign	Cancer	Benign	Cancer	t-test	p value
	Signal	Signal	Signal	Signal		
GSH-APN	3827	4190	1582	2250	0.70	0.70
GSH-CA15-3	5831	5565	478	168	0.49	0.49
GSH-CatD	3497	2831	901	1370	0.56	0.56
GSH-CD14	4236	3283	1000	569	0.26	0.26
GSH-Clu	1701	2722	723	1265	0.11	0.11
GSH-CP	4960	5988	392	442	0.004	0.004
GSH-E-Selectin	834	850	880	1234	0.69	0.69
GSH-EGF	4965	3604	376	400	0.31	0.31
GSH-EGFR	457	390	690	981	0.82	0.82
GSH-bFGF	6747	7062	1021	834	0.56	0.56
GSH-HBEGF	1660	1598	1293	1504	0.89	0.89
GSH-Her2	1757	1585	438	369	0.70	0.70
GSH-HGF	3133	1654	783	661	0.02	0.02
GSH-ICAM	1900	1567	1375	863	0.73	0.73
GSH-IL-18	98	85	1111	1113	0.11	0.11
GSH-Lfn	11040	9062	352	311	0.27	0.27
GSH-MAC-2BP	299	210	303	392	0.65	0.65
GSH-MMP1	348	291	320	542	0.96	0.96
GSH-MMP2	257	188	266	390	0.37	0.37
GSH-MMP9	328	261	659	1214	0.46	0.46
GSH-OPN	105	122	697	660	0.57	0.57
GSH-PDGF	2065	1680	1459	1166	0.78	0.78
GSH-pIgR	344	323	607	1017	0.83	0.83
GSH-RANTES	377	433	2504	1738	0.90	0.90
GSH-TGFa	323	235	715	830	0.42	0.42

	First Study		Second study		Both	
	Benign	Cancer	Benign	Cancer	t-test	p value
	Signal	Signal	Signal	Signal		
Assay						
GSH-VEGF	143	104	243	296		0.18
HNE-APN	1952	928	1615	1499		0.93
HNE-CA15-3	568	553	2745	2371		0.39
HNE-CatD	656	641	634	599		0.89
HNE-CD14	1042	900	452	440		0.88
HNE-Clu	77	94	339	348		0.34
HNE-CP	632	616	267	275		0.37
HNE-E-selectin	1893	627	1267	1385		0.78
HNE-EGF	796	691	26673	25932		0.23
HNE-EGFR	1233	259	695	726		0.09
HNE-bFGF	231	1510	681	742		0.09
HNE-HB-EGF	2506	1133	864	899		0.46
HNE-Her2	855	799	1151	1127		0.96
HNE-HGF	2969	2348	932	699		0.18
HNE-ICAM	1069	879	1717	1421		0.40
HNE-IL-18	96	126	1353	1080		0.05
HNE-Lfn	2814	2380	2289	1845		0.18
GSH-MAC-2BP	3283	2024	806	764		0.32
HNE-MMP1	1212	216	711	713		0.05
HNE-MMP2	1232	198	724	644		0.039
HNE-MMP9	1034	244	618	690		0.23
HNE-OPN	86	105	352	411		0.18
HNE-PDGF	777	558	2063	1723		0.0013
HNE-pIgR	1693	326	799	794		0.009
HNE-RANTES	466	306	760	657		0.84
HNE-TGFa	1114	174	856	726		0.08
HNE-VEGF	752	145	642	579		0.17

No significant changes were observed for nitrotyrosine and halotyrosine P-values >0.05 are highlighted.