Protein Modifications as Potential Biomarkers in Breast Cancer

Hongjun Jin and Richard C. Zangar
Cell Biology and Biochemistry Group, Fundamental and Computational Sciences Directorate, Pacific Northwest National Laboratory, PO Box 999, 902 Battelle Blvd, Richland, WA 99352.

Abstract: A variety of post-translational protein modifications (PTMs) are known to be altered as a result of cancer development. Thus, these PTMs are potentially useful biomarkers for breast cancer. Mass spectrometry, antibody microarrays and immunohistochemistry techniques have shown promise for identifying changes in PTMs. In this review, we summarize the current literature on PTMs identified in the plasma and tumor tissue of breast-cancer patients or in breast cell lines. We also discuss some of the analytical techniques currently being used to evaluate PTMs.

Keywords: PTMs, post-translational modifications, breast cancer, biomarkers
**Introduction**

Viable cells maintain membrane integrity, cytoskeleton morphology and proliferation status based on changes in protein structure and function. The complexity of regulation of so many different biomolecules goes beyond the “central dogma” of biochemistry, which implies that one gene encodes for one protein. This complexity of regulation not only results from variable mRNA splicing and DNA transcription, such that one gene can produce many mRNA and protein sequences, but also because one protein sequence can have multiple functions as a result of covalent modifications after synthesis. These post-translational modifications (PTMs) include phosphorylation, methylation, glycosylation, acetylation, oxidation and ubiquitinylation. During cancer progression, many PTMs contribute to abnormal cellular proliferation, adhesion characteristics and morphology.\(^1\) In breast cancer, recent studies suggest that PTM profiles can be used as “biochemical footprints” for tracking and verifying the function and activity of key cellular signaling pathways.\(^5\)–\(^7\) This conclusion suggests that, for early detection, PTMs may be useful biomarkers.

Breast cancer is the second most common type of cancer (after lung cancer), and the fifth most common cause of cancer death. According to the American Cancer Society, in 2008, an estimated 182,460 new cases of invasive breast cancer were diagnosed among US women. Approximately 40,480 of these women are expected to die from this disease (http://www.cancer.org/downloads/STT/2008CAFFfinalsecured.pdf). Like many other cancers, breast cancer is the result of multiple environmental and hereditary factors. Although risk factors such as lesions to DNA, failure of immune surveillance, abnormal growth factor signaling, and inherited or somatic genetic defects (e.g. in p53, BRCA1, BRCA2 genes) are associated with breast cancer development, the cause of any individual breast cancer case is typically unknown. As many studies have suggested, changes in gene expression levels for breast cancer may not fully reflect the true state of cancer progression or development.\(^5\)–\(^8\)\(^,\)\(^9\) This conclusion suggests that many of the differences between normal and cancer tissue may be caused by PTMs.\(^1\)\(^,\)\(^3\)\(^,\)\(^5\)\(^,\)\(^6\)\(^,\)\(^8\)\(^–\)\(^10\)

This review mainly focuses on the most recent publications on PTMs (especially oxidation and glycosylation) discovered in blood or tissue from breast cancer patients or from breast cancer cell lines. For more general reviews of PTMs, see prior reviews.\(^1\)\(^–\)\(^4\)\(^,\)\(^7\)\(^,\)\(^11\)

**Enzymatic PTMs**

Covalent modification of one or more amino acids of a given protein can dramatically alter the biological function of that protein. The likelihood that a particular reactive protein residue will undergo a modification reaction is influenced by the spatial orientation of that amino acid residue(s) in the protein, and is influenced by the adjacent amino acids, which can alter reactivity of the susceptible amino acid by influencing its electrophilic nature. Specific enzymes commonly catalyze these reactions. For example phosphorylation (phosphokinase), methylation (methylase), acetylation (acetyltransferase), and glycosylation (glycosyltransferases) are PTMs that are mediated by the indicated enzyme.\(^1\)\(^–\)\(^4\)\(^,\)\(^7\)\(^,\)\(^11\) Many PTMs also result from spontaneous reaction of susceptible residues with certain reactive chemical agents. For example, glycation (commonly called advanced glycation end product, or AGE) is the result of an activated sugar molecule, such as fructose or glucose, bonding to a protein without direct enzymatic involvement.\(^12\) For other PTMs, although enzymes may play an important role in producing the reactive molecule that results in the protein modification, the covalent modification occurs spontaneously without enzymatic activity. For the purposes of this review, we define these PTMs as non-enzymatic if an enzyme is not required for the actual protein modification. For example, peroxynitrite directly reacts with proteins to form nitrotyrosine.\(^13\)–\(^15\) Although peroxynitrite formation appears to require enzymatic production of reactive precursors, its binding to proteins is non-enzymatic, and therefore we consider nitrotyrosine to be a non-enzymatic PTM.

**Phosphorylation**

Phosphorylation is well recognized as a key regulator of enzyme activity. As the extensive research in protein phosphorylation has been carefully reviewed by others,\(^16\)–\(^18\) we only briefly cover this topic here. Abnormal phosphorylation of defined signal transduction pathways can alter the growth properties of breast tumors. With the use of sequence-specific antibodies against phosphorylation sites, analysis of protein
phosphorylation profiles allows one to determine the activation status of signaling pathways, which can provide valuable prognostic insights.\textsuperscript{19–21} Atsriku et al undertook a systematic mapping of PTMs in the human estrogen receptor alpha (ER-\(\alpha\)) in the MCF7 breast cancer cell line. They applied HPLC-ESI and MALDI-MS techniques to identify the phosphorylation sites on the estrogen receptors in these cells.\textsuperscript{22} Several novel phosphorylated serine residues were identified. The use of both HPLC-ESI and MALDI gave higher sequence coverage than either approach alone. Nine phosphorylated serine residues were identified, three of which were previously unreported.\textsuperscript{22}

### Acetylation

Histone acetyltransferases and histone deacetylases modify histones by adding or removing an acetyl group from the e-amino group of lysines within a conserved lysine motif. Histone acetylation results in changes in chromatin structure in response to specific endocrine signaling in several cancers, including breast cancer. Recent studies found that acetylation of the ER is mediated by histone acetylases.\textsuperscript{23–25} The acetylation of ER-\(\alpha\) alters its function in estrogen-dependent signaling.\textsuperscript{25,24} The regulation of ER by deacetylation provides a direct link between intracellular metabolism and hormone signaling.\textsuperscript{25,26} Wang et al\textsuperscript{27} showed that the acetylation of ER-\(\alpha\) alters its function \textit{in vitro} and \textit{in vivo}. These researchers also found that p300 selectively and directly acetylated the ER-\(\alpha\) at lysine residues within the ER-\(\alpha\) hinge/ligand-binding domain. Substitution of these residues with charged or polar residues dramatically enhanced ER-\(\alpha\) hormone sensitivity without affecting induction by MAPK signaling.\textsuperscript{27} These results suggest that ER-\(\alpha\) acetylation normally suppresses ligand sensitivity.

### Glycosylation

Cancer cells commonly have unusually high levels of certain types of tumor-associated glycans.\textsuperscript{78} Specific antibodies are available for these unusual carbohydrate residues, and there is considerable evidence that these glycans are increased in breast cancer.\textsuperscript{12,29,30} Differences in protein glycosylation commonly result from differences in the activities and subcellular (primarily Golgi and endoplasmic reticulum) localization of glycosyltransferases that determine the amounts of specific glycans.\textsuperscript{31–33} Several glycosylation modifications, such as TF and Tn antigens, certain Lewis antigens and Globe H (summarized in Table 1), are commonly associated with a variety of different cancers, including breast cancer. Glycoprotein analysis by mass spectrometry (MS) of biological samples, such as blood serum, is hampered by glycan complexity and the low concentrations of the potentially informative glycopeptides and proteins. Most MS-based studies have limited their analysis to glycosylation residues after cleavage of the glycans from the proteins. As such, these studies can identify global changes in glycosylation, but do not provide information on which proteins are modified.

Changes in glycosylation for cancer cells include both reductions and increases in naturally occurring glycans, as well as increases in glycans primarily restricted to embryonic tissues.\textsuperscript{34,35} One of the most common changes is an increase in the side branching of N-linked glycans.\textsuperscript{36} This increased branching is often attributed to increased activity of N-acetylgalactosaminyltransferase V (GlcNAc-T V, also known as MGAT5; the enzyme that leads to \(\beta1,6\)GlcNAc branching).\textsuperscript{37–40} The increased branching creates additional sites for terminal sialic acid residues, which, in combination with up-regulation of sialyltransferases, leads to an increase in global sialylation.\textsuperscript{41}

In addition to changes in glycan core structures, altered terminal structures are commonly associated with malignant breast cancer.\textsuperscript{42–47} Glycosyltransferases (e.g. sialyltransferases and fucosyltransferases) involved in adding terminating residues to glycans tend to be over-expressed in breast cancer tissue.\textsuperscript{20,30,48–66} The increase in activity of these glycosyltransferases, in turn, leads to an increase of certain terminal glycans. Glycan residues commonly found on transformed cells include sialyl Lewis x, sialyl Tn, Globo H, Lewis y and polysialic acid. Many of these glycans are observed in malignant breast tissues (summarized in Table 1).

### Non-Enzymatic PTMs

#### Oxidation

It has been hypothesized that cancer development is a process that is similar to “wounds that never heal”.\textsuperscript{67–70} Various studies have suggested that inflammation, which increases oxidative stress, is associated with cancer development or metastasis.\textsuperscript{67,71–74}
Both mouse models and human-pathology studies suggest that there is a strong immune response in the early stages of breast cancer that disappears in more advanced disease.\textsuperscript{72,75} Consistent with this observation, tumor levels of nitrotyrosine (nTyr), which are believed to be indicative of NO and superoxide levels, have been reported to be increased in the early breast cancer, but not in more advanced disease.\textsuperscript{72,75} The NO and superoxide may be produced by activated macrophages. Therefore, localized oxidative stress associated with the immune response to breast cancer might result in modifications of proteins secreted by the breast cancer cells that could be used to detect early disease. Reactive oxygen species (ROS) also regulate the synthesis and secretion of many receptor ligands (e.g. growth factors and chemokines).\textsuperscript{76–78} These factors regulate important processes in epithelial cancers, including the ligand-dependent activation of the proliferation (MAPK/Erk) and anti-apoptosis (PI3K/Akt) pathways.\textsuperscript{79–81} Therefore, proteins modified by ROS may be useful biomarkers that can provide insight into molecular processes occurring in tumors. The oxidative stress associated with the immune response results in protein modifications that may be useful in detecting early breast cancer.

An increase in 4-hydroxynonenal (4-HNE) adducts has also been reported in early breast cancer.\textsuperscript{82,83} 4-HNE is a non-enzymatic byproduct of lipid peroxides.\textsuperscript{71} Lipid peroxidation and HNE adducts may result from oxidative stress associated with the immune response.\textsuperscript{71,72} There is, however, also evidence that the intracellular redox environment is altered in breast cancer.\textsuperscript{84–87} Potentially leading to a variety of PTMs. Notably, levels of reduced glutathione (GSH) have been reported to be altered in breast cancer tissue.\textsuperscript{88–91} The literature on oxidative modifications (i.e. on 4-HNE, nTyr and GSH adducts) is summarized in Table 2. Each of these oxidative modifications represents a different cellular process; that is, 4-HNE adducts are a byproduct of lipid peroxidation, nTyr commonly results from an increase in NO (produced by either macrophages or breast epithelial cells) and GSH protein adducts can be indicative of intracellular oxidative stress, especially in the endoplasmic reticulum.\textsuperscript{71,72,88–92}

### Advanced Glycation End

Oxidative and carbonyl stress may contribute to the progression of cancer; on the other hand, these modifications may have some antiproliferative effects. Tesarova et al\textsuperscript{12} reported that serum levels of AGEs, carboxymethyllysine and advanced oxidation protein products (AOPP) in 86 patients with breast cancer and in 14 healthy age-matched control women could be subdivided based on the clinical stage, histological grading, and expression of hormone and Her2 receptors. Breast cancer patients had higher serum concentrations of AGEs even in the early stages of this disease; patients with advanced breast cancer (stages III and IV) had significantly higher AGE levels, not only compared to controls, but also compared to stages I and II breast cancer cases.\textsuperscript{12,70,93} Serum levels of AOPP were higher in patients having...
only weakly positive expression of Her2 compared to controls and in patients having the highest Her2 expression. These authors concluded that breast cancer patients had an early increase of AGEs (a marker of the carbonyl stress) followed by further increase of AGEs and elevation of AOPP (a marker of oxidative stress) in more advanced disease. As the clinical significance of these observations is currently uncertain, further studies are needed to validate these results in terms of the usefulness of AGE in the early detection of breast cancer.

### Methods for PTM Discovery and Analysis

**Mass-spectrometry-based proteomics**

Given the complexity and low abundance of the PTM samples, PTM analysis is still an analytical challenge. Various mass spectrometry (MS) technologies, including ion trap, time-of-flight (TOF), Orbitrap, and Fourier transform ion cyclotron resonance (FTICR), as well as hybrid configurations coupled with MALDI have been used for PTM detection in breast cancer studies (Table 1). Recent applications commonly include multi-stage separation, purification and enrichment of the PTM-containing peptides or proteins. The most frequently used proteomics approaches for PTM analysis may be MALDI-TOF, electrospray ionization tandem MS that uses LTQ–Orbitrap instrumentation, and surface-enhanced laser desorption/ionization (SELDI)-MS. For the MALDI and SELDI approaches, the profile of peak intensities in case and control samples are typically compared with the goal of defining a pattern that can segregate the sample types. Many analyses of PTMs in serum samples from breast cancer patients have been recently reported (Tables 1–3).

Most PTMs are present at low levels in cells and tissues, and are therefore difficult to detect by MS. For this reason, modification-specific analytical strategies that are designed to improve sensitivity and specificity have been employed to enrich and concentrate a specific class of PTM in complex biological samples. PTM peptide enrichment can employ either affinity or chemical methods. During the MS analysis, multi-stage MS techniques that further fragment suspected PTM peptides can improved confidence in peptide identification. Identification of PTMs commonly requires specialized bioinformatics tools, the validation of results by replicate analyses and follow-up biological experiments. Such PTM-specific methods can be combined with semi-quantitative techniques, including

### Table 2. Summary of recent oxidation PTMs in breast cancer studies.

| Oxidation PTMs                  | Targets                  | Methods            | References   |
|---------------------------------|--------------------------|--------------------|--------------|
| Total Oxidation                 | Blood, NAF proteins; Cytochrome P450 | MS, IHC            | 12,85,106–110,126,127 |
| Nitrotyrosine                   | VEGF-C                   | IHC                | 128,129      |
| Nitrotyrosine                   | NF-κB                    | Gel shift          | 130          |
| Nitrotyrosine                   | CXCR4, hyaluronan Serum proteins | IHC                | 72,75,114,128,129 |
| Thiobarbituric acid reactive substances (TBARS) | Lipid                  | HPLC              | 88,89,131    |
| Conjugated dienes (CD)          | Serum proteins           | HPLC              | 88,89        |
| Glutathione (GSH)               | Serum proteins           | Enzymatic measurements | 88,90,91,132,133 |
| 4-hydroxy-2-nonenal (4HNE)      | p53                      | MS, Immunoassay    | 82,83        |
| 3-Chloro Tyrosine*              | Chronic rhinosinusitis   | MS                 | 134          |
| 3-Bromo Tyrosine*               | Chronic rhinosinusitis   | MS                 | 134          |
| Advanced Glycation End (AGE)    | sRAGE glyoxalase I       | IHC                | 12,70,93     |

*Not from breast cancer studies.
stable-isotope labeling and peptide-intensity profiling. PTM-targeted methods have also been combined with subcellular fractionation to obtain biological insights about in the roles of specific organelles.113–117

ELISA Microarray
The microarray sandwich ELISA is an exceptionally sensitive analytical technique that can accurately measure individual protein concentrations down to the low or sub-pg/ml range.115,117–120 Adapted from the conventional sandwich ELISA, the ELISA microarray commonly uses complementary pairs of capture and detection antibodies (or, for glycan analysis, lectins) to measure trace antigens in complex biological fluids. 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 very little sample (20 µl, or less, of diluted sample per multiplexed analysis, after at least a 5-fold dilution), thereby allowing the screening of many PTMs using very small sample volumes. Even so, there are several challenges for ELISA microarray analysis. One challenge is the need for highly specific antibodies. There is limited commercial availability of good antibodies for many PTMs. Classical strategies of antibody generation by animal immunization may not result in high-quality antibodies for the targeted PTM. The second challenge is the potential for cross reactivity with nonspecific antigens.

Immunohistochemistry
Immunohistochemistry (IHC) has been widely used for evaluating PTMs in breast cancer.113,116,121 To identify PTMs as potential tumor markers, IHC offers a rapid method for comparing PTM levels in cancer tissue and adjacent normal tissue. Altered expression and PTM of several proteins using immunoblot analysis and IHC have been reported by several research groups (Tables 1–3). For example, modification of the beta subunit of prolyl-4-hydroxylase and of annexin A2 in tumor tissues was confirmed by immunoblot and immunohistochemistry.122 The determination of nitrotyrosine levels by IHC of breast cancer carcinoma tissue has been reported.75 A drawback of IHC in PTM analysis is the difficulty in quantifying the results.

Conclusion
Plasma-, tissue- or cell-based studies for PTM biomarkers in breast cancer have provided promising data. Several PTMs can only be readily detected in breast cancer tissue but not in normal breast. In particular, glycosylation and oxidative modifications appear to have potential as biomarkers. These results suggest that levels of certain PTMs may be indicative of breast cancer progression or development, although the data on which proteins are actually modified is still very limited. Once this deficit is addressed, we conclude that the post-translational modifications on specific proteins may be useful as biomarkers for breast cancer.
Acknowledgements

We thank Ms. Julie Wiley for the critical editing of this manuscript. This review was supported by the NIH grants U01 CA117378, R01 EB006177 and U54 ES016015.

Abbreviations

AGEs, advanced glycation end products; BRCA, breast-cancer susceptibility gene; CD, conjugated dienes; DCIS, ductal carcinoma in situ; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; 4-HNE, 4-hydroxynonenal; HPLC-ESI, high-performance liquid chromatography-electrospray ionization-mass spectrometry; IHC, immunohistochemistry; GlcNAc, N-acetylglucosamine; MGAT5, N-acetylglucosaminyltransferase V; GSH, glutathione; GSTP1, glutathione S-transferase P1; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; LCIS, lobular carcinoma in situ; MALDI, matrix-assisted laser desorption/ionization-mass spectrometry; NO, nitric oxide; nTyr, 3-nitrotyrosine; RASSF1A, RAS association family 1 gene; PI3K/Akt, phosphoinositide-3-kinase/protein kinase B; PTMs, post-translational modifications; ProMAT, Protein Microarray Analysis Tool; ROS, reactive oxygen species; sLeα, sialyl lewis a; sLeβ, sialyl lewis x; sTn, sialyl Tn; Leβ, Lewis y; SELDI, surface-enhanced laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; TBARS, thiobarbituric acid reactive substances; TF, Thomsen-Friedenreich.

Disclosures

The authors report no conflicts of interest.

References

1. Golka A, Guerini D. The O-linked N-acetylglucosamine modification in cellular signalling and the immune system.  ‘Protein modifications: beyond the usual suspects’ review series. EMBO Rep. 2008 August;9(8):748–53.
2. Hoffman MD, Sniatynski MJ, Kast J. Current approaches for global post-translational modification discovery and mass spectrometric analysis. Anal Chim Acta. 2008 October 3;627(1):50–61.
3. Janke C, Rogowski K, van DJ. Polyglutamylation: a fine-regulator of protein function? ‘Protein Modifications: beyond the usual suspects’ review series. EMBO Rep. 2008 July;9(7):636–41.
4. Krueger KE, Srivastava S. Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. Mol Cell Proteomics. 2006 October;5(10):1799–810.
5. Hanash SM, Pitteri SJ, Faca VM. Mining the plasma proteome for cancer biomarkers. Nature. 2008 April 3;452(7187):571–9.
6. Jensen ON. Interpreting the protein language using proteomics. Nat Rev Mol Cell Biol. 2006 June;7(6):391–403.
7. Spickett CM, Pitt AR, Morrice N, Kolch W. Proteomic analysis of phosphorylation, oxidation and nitrosylation in signal transduction. Biochim Biophys Acta. 2006 December;1764(12):1823–41.
8. Chin L, Gray JW. Translating insights from the cancer genome into clinical practice. Nature. 2008 April 3;452(7187):553–63.
9. Sawyers CL. The cancer biomarker problem. Nature. 2008 April 3;452(7187):548–52.
10. Sawyers CL. Cancer: mixing cocktails. Nature. 2007 October 25;449(7165):993–6.
11. Souza JM, Pelleffo G, Radi R. Protein tyrosine nitration—functional alteration or just a biomarker? Free Radic Biol Med. 2008 August 15;45(4):357–66.
12. Tesarova P, Kalousova M, Trnkova B, et al. Carbonyl and oxidative stress in patients with breast cancer—is there a relation to the stage of the disease? Neoplasma. 2007;54(3):219–24.
13. Beekman JS. Peroxynitrite versus hydroxyl radical: the role of nitric oxide in superoxide-dependent cerebral injury. Ann NY Acad Sci. 1994 November 17;738:69–75.
14. Beekman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. Methods Enzymol. 1994;233:229–40.
15. Beekman JS, Chen J, Crow JP, Ye YZ. Reactions of nitric oxide, superoxide and peroxynitrite with superoxide dismutase in neurodegeneration. Prog Brain Res. 1994;103:371–80.
16. Lange CA, Sartorius CA, Bdel-Hafiz H, Spillman MA, Horwitz KB, Jacobsen BM. Progesterone receptor action: translating studies in breast cancer models to clinical insights. Adv Exp Med Biol. 2008;630:94–111.
17. Ouchi T. BRCA1 phosphorylation: biological consequences. Cancer Biol Ther. 2006 May;5(5):470–5.
18. Glover JN, Williams RS, Lee MS. Interactions between BRCT repeats and phosphoproteins: tangled up in two. Trends Biochem Sci. 2004 November;29(11):579–85.
19. Vazquez-Martin A, Oliveras-Ferraros C, Colomer R, Brunet J, Menendez JA. Low-scale phosphoproteome analyses identify the mTOR effector p70 S6 kinase 1 as a specific biomarker of the dual-HER1/HER2 tyrosine kinase inhibitor lapatinib (Tykerb) in human breast carcinoma cells. Ann Oncol. 2008 June;19(6):1097–109.
20. Cai Y, Parra I, Zhang M, et al. Elevated expression of mitogen-activated protein kinase phosphatase 3 in breast tumors: a mechanism of tamoxifen resistance. Cancer Res. 2006 June 1;66(11):5950–9.
21. Ouyang X, Gulliford T, Zhang H, Smith G, Huang E, Epstein RJ. Association of ErbB2 Ser1113 phosphorylation with epidermal growth factor receptor co-expression and poor prognosis in human breast cancer. Mol Cell Biochem. 2001 February;218(1–2):47–54.
22. Atsriku C, Britton DJ, Held JM, et al. Systematic mapping of posttranslational modifications in human estrogen receptor alpha, with emphasis on novel phosphorylation sites. Mol Cell Proteomics. 2008 November 3.
23. Duong V, Breit C, Altucci L, et al. Specific activity of class II histone deacetylases in human breast cancer cells. Mol Cancer Res. 2008 December;6(12):1908–19.
24. Popov VM, Wang C, Shirley LA, et al. The functional significance of nuclear receptor acetylation. Steroids. 2007 February;72(2):221–30.
25. Fu M, Wang C, Zhang X, Pestell RG. Acetylation of nuclear receptors in cellular growth and apoptosis. Biochem Pharmacol. 2004 September 15;68(6):1199–208.
26. Margueron R, Duong V, Bonnet S, et al. Histone deacetylase alpha levels modulate the transcriptional activity of partial antiestrogens. J Mol Endocrinol. 2004 April;32(2):583–94.
27. Wang C, Fu M, Angeletti RH, et al. Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem. 2001 May 25;276(21):18375–83.
28. Hakomori S. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol. 2001;491:369–402.
29. Kumar SR, Sauter ER, Quinn TP, Deutscher SL. Thomsen-Friedenreich and Tn antigens in nipple fluid: carbohydrate biomarkers for breast cancer detection. Clin Cancer Res. 2005 October 1;11(19 Pt 1):6868–71.
Maccioni HJ. Glycosylation of glycolipids in the Golgi complex. J Neurochem. 2000 November;73(3):1191–90.

Czapinski JL, Bertozzi CR. Synthetic glycobiology: Exploits in the Golgi compartment. Curr Opin Chem Biol. 2006 December;10(6):645–51.

Dube DH, Bertozzi CR. Glycans in cancer and inflammation—potential for therapeutics and diagnostics. Nat Rev Drug Discov. 2005 June;4(6):477–88.

Sudhir-Jayaraman TE, Wang D, Steinman L, et al. Enhanced immune recognition of cryptic glycan markers in human tumors. Cancer Res. 2009 March 1;69(5):2018–25.

Bosques CJ, Raguram S, Sasisekharan R. The sweet side of biomarker discovery. Nat Biotechnol. 2006 September;24(9):1100–11.

Lau KS, Dennis JW. N-Glycans in cancer progression. Glycobiology. 2008 October;18(10):750–60.

Solemani L, Roder JC, Dennis JW, Lipina T. Beta N-acetylglucosaminyltransferase localizes throughout the Golgi and is responsible for the synthesis of the CMP-Neu5 Ac: GalNAc alpha2,6-sialyltransferase (ST6GalNac I) cDNA. Glycoconj J. 2001 November;18(11):1283–93.

Duke DH, Bertozzi CR. Glycans in tumor antigens. J Biol Chem. 2005 August;280(30):29216–27.

Dodson-Davis TE, Wang D, Steinman L, et al. Enhanced immune recognition of cryptic glycan markers in human tumors. Cancer Res. 2009 March 1;69(5):2018–25.

Bosques CJ, Raguram S, Sasisekharan R. The sweet side of biomarker discovery. Nat Biotechnol. 2006 September;24(9):1100–11.

Lau KS, Dennis JW. N-Glycans in cancer progression. Glycobiology. 2008 October;18(10):750–60.

Solemani L, Roder JC, Dennis JW, Lipina T. Beta N-acetylglucosaminyltransferase V (Mgat5) deficiency reduces the depression-like phenotype in mice. Gene Brain Behav. 2008 April;7(3):334–43.

Dennis JW, Granvoldy M, Warren CE. Glycoprotein glycosylation and cancer progression. Biochim Biophys Acta. 1999 December 6;1473(1):21–34.

Dennis JW, Laferte S, Waghorne C, Breitman ML, Kerbel RS. Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science. 1987 May 1;236(4801):582–5.

Kim YJ, Varki A. Perspectives on the significance of altered glycosylation in breast cancer. Breast Cancer Res. 2005;7(5):R780–7.

Clarke RL, Reith DE, Persaud V, D'Souza KM, Groves FL, Thaker S. Expression of Lewis y/b antigens is associated with decreased survival in lymph node negative breast carcinomas. Breast Cancer Res. 2005;7(5):334–9.

Gilewski T, Ragupathi G, Bhuta S, et al. Immunization of metastatic breast cancer patients with clustered sTn-KLH conjugate plus the immunomodulatory QS-21. Breast Cancer Res Treat. 2006 February 10;99(1):3586–94.

Dennis JW, Laferte S, Waghorne C, Breitman ML, Kerbel RS. Beta 1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science. 1987 May 1;236(4801):582–5.

Kim YJ, Varki A. Perspectives on the significance of altered glycosylation in breast cancer. Breast Cancer Res. 2005;7(5):R780–7.

Clarke RL, Reith DE, Persaud V, D'Souza KM, Groves FL, Thaker S. Expression of Lewis y/b antigens is associated with decreased survival in lymph node negative breast carcinomas. Breast Cancer Res. 2005;7(5):334–9.

Gilewski T, Ragupathi G, Bhuta S, et al. Immunization of metastatic breast cancer patients with clustered sTn-KLH conjugate plus the immunomodulatory QS-21. Breast Cancer Res Treat. 2006 February 10;99(1):3586–94.
74. Kariratla P, Mantyniemi A, Kang SW, Kinnula VL, Soini Y. Peroxiredoxins in breast carcinoma. *Clin Cancer Res.* 2003 August 15;9(9):3418–24.

75. Kariratla P, Soini Y, Auvinen P, Tammi R, Tammi M, Kosma VM. Hyaluronic in breast cancer: correlations with nitric oxide synthases and tyrosine nitrosylation. *J Histochem Cytochem.* 2007 December;55(12): 1191–8.

76. Murillo MM, Carmona-Cuenca I, Del CG, et al. Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor-kappaB-dependent mechanism. *Biochem J.* 2007 July 15; 405(2):251–9.

77. Clempus RF, Griendling KK. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res.* 2006 July 15;71(2):216–25.

78. Kim J, Lin J, Adam RM, Lamb C, Shively SB, Freeman MR. An oxidative stress response mechanism mediates chelerythrine-induced heparin-binding EGF-like growth factor ectodomain shedding. *J Cell Biochem.* 2005 January 1;94(1):39–49.

79. Chiarugi P, Fiaschi T. Redox signalling in anchorage-dependent cell growth. *Cell Signal.* 2007 April;19(4):672–82.

80. Fruehauf JP, Meyskens FL Jr. Reactive oxygen species: a breath of life or death? *Clin Cancer Res.* 2007 February 1;13(3):785–94.

81. Giannoni E, Burcchi F, Raueg I, Ramponi G, Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol Cell Biol.* 2005 August; 25(15):6391–403.

82. Steiner C, Peters WH, Gallagher EP, Magee P, Rowland I, Pool-Zobel BL. Post-translational modification of proteins during intermittent hypoxia. *Pflugers Arch.* 2007 March;283(3):738–48.

83. Albright CD, Klem E, Shah AA, Gallagher P. Breast cancer cell-targeted oxidative stress: enhancement of cancer cell uptake of conjugated linoleic acid, activation of p53, and inhibition of proliferation. *Exp Mol Pathol.* 2005 October;79(2):118–25.

84. Kuo PL, Chen CY, Tzeng TF, Lin CC, Hsu YL. Involvement of reactive oxygen species/c-Jun NH(2)-terminal kinase pathway in kotomolide A induces apoptosis in human breast cancer cells. *Toxicol Appl Pharmacol.* 2008 June 1;229(2):215–26.

85. Kabat GC, Rohan TE. Does excess iron play a role in breast carcinogenesis? An unresolved hypothesis. *Cancer Causes Control.* 2007 December;18(10):1047–53.

86. Khan GN, Merajver SD. Modulation of angiogenesis for cancer prevention: strategies based on antioxidants and copper deficiency. *Curr Pharm Des.* 2007;13(35):3584–90.

87. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. *Clin Cancer Res.* 2008;14(1):27–36.

88. Rajneesh CP, Manimaran A, Sasikala KR, Adaikappan P. Lipid peroxidation and nuclear factor-kappaB-dependent mechanism. *Invest Neoplasma.* 2007 December;23(8):720–5.

89. Lopez-Sanchez LM, Muntane J, de la MM, Rodriguez-Ariz A. Unraveling the S-nitrosoproteome: tools and strategies. *Proteomics.* 2009 February;9(4):308–18.

90. Arnold JN, Saldova R, Hamid UM, Rudd PM. Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics.* 2008 August;8(16):3284–93.

91. Kumar GK, Prabhakar NR. Post-translational modification of proteins during intermittent hypoxia. *Respir Physiol Neurobiol.* 2008 December 10; 164(1–2):272–6.

92. Temporini C, Calleri E, Massolini G, Caccialanza G. Integrated analytical strategies for the study of phosphorylation and glycosylation in proteins. *Mass Spectrom Rev.* 2008 May;27(3):207–36.

93. Wissner J, Premslr T, Sickmann A. Application of electron transfer dissociation (ETD) for the analysis of posttranslational modifications. *Proteomics.* 2008 November;8(21):4466–83.

94. Bigelow DJ. Nitrotyrosine-modified SERCA2: a cellular sensor of reactive nitrogen species. *Pflugers Arch.* 2009 January;457(3):701–10.

95. Wu S, Lourette NM, Tolic N, et al. An Integrated Top-Down and Bottom-Up Strategy for Broadly Characterizing Protein Isomers and Modifications. *J Proteome Res.* 2009 February 10.

96. Bigelow DJ, Qian WJ. Quantitative proteome mapping of nitrotyrosines. *Methods Enzymol.* 2008;440:191–205.

97. Livesay EA, Tang K, Taylor BK, et al. Fully automated four-column capillary LC-MS system for maximizing throughput in proteomic analyses. *Anal Chem.* 2008 January;80(1):294–302.

98. Marginean I, Kelly RT, Prior DC, LaMarche BL, Tang K, Smith RD. Analytical characterization of the electrosprey ion source in the nanoflow regime. *Anal Chem.* 2008 September;80(17):6573–9.

99. Smallwood HS, Lourette NM, Boschek CB, et al. Identification of a denitrase activity against calmodulin in activated macrophages using high-field liquid chromatography—FTICR mass spectrometry. *Biochemistry.* 2007 September 18;46(37):10498–505.

100. Yang ZP, Harris LE, Palmer-Troy DE, Hancock WS. Multitecin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. *Clinical Chemistry.* 2006 October;52(10):1897–905.

101. Barciszewska AM, Murawa D, Gawronska I, Nowak S, Barciszewska MZ. Analysis of 5-methylcytosine in DNA of breast cancer tissue: correlation with clinical and prognostic characteristics. *Ann Oncol.* 2007 March;28(3):738–48.

102. Kirmiz C, Li B, An HJ, et al. A serum glycomics approach to breast cancer diagnosis. *BMC Cancer.* 2007 December;1191–8.

103. Cao Y, Karsten U, Hilgers J. Immunohistochemical characterization of serum glycomics approach for quantifying expression of nuclear proteins assessed by multiplexed electrosprey ionization mass spectrometry. *Proteomics.* 2008 September;8(16):3284–93.

104. Carskiewicz DM, Tsoi A, Hsu YL. Involvement of nitric oxide synthase I in thyroid cancer cell growth and estrogen receptor pathways in breast cancer. *Breast Cancer Res Treat.* 2007 October;105(2):R89.

105. Kato I, Chen G, Djuric Z. Non-steroidal anti-inflammatory drug (NSAID) use and levels of a lipid oxidation marker in plasma and nipple aspirate fluids. *Breast Cancer Res Treat.* 2006 May;97(2):145–8.

106. Barciszewska MZ. Analysis of 5-methylcytosine in DNA of breast cancer tissue: correlation with clinical and prognostic characteristics. Phase II clinical trial. *Ann Oncol.* 2007 March;18(3):446–72.

107. Marx C, Yau C, Banwait S, et al. Proteasome-regulated ERBB2 and estrogen receptor pathways in breast cancer patients. *J Cell Biochem.* 2006 October;98(3):765–70.

108. Marx C, Yau C, Banwait S, et al. Proteasome-regulated ERBB2 and estrogen receptor pathways in breast cancer. *Mol Pharm.* 2007 June;71(6):1525–34.

109. Kirmiz C, Li B, An HJ, et al. A serum glycomics approach to breast cancer diagnosis and progesterone receptor levels in breast cancer therapy. *Oncol Rep.* 2005 May;13(5):847–51.

110. Cao Y, Karsten U, Hilgers J. Immunohistochemical characterization of a panel of 56 antibodies with normal human small intestine, colon, and breast tissues. *Tumour Biol.* 1998;19 Suppl 1:188–98.

111. Kitts C, Li B, An HJ, et al. A serum glycomics approach to breast cancer biomarkers. *Mol Cell Proteomics.* 2007 January;6(1):43–55.

112. Kiyosawa Z, Mechref Y, Kang P, et al. Breast cancer diagnosis and prognosis through quantitative measurements of serum glycan profiles. *Clin Chem.* 2008 July;54(7):1166–75.

113. Rexhepaj E, Brennan DJ, Holloway P, et al. Novel image analysis strategies for the study of phosphorylation and glycosylation in proteins. *J Histochem Cytochem.* 2009 February 10.

114. Yasuoka H, Tsujimoto M, Yoshidome K, et al. Cytoplasmic CXCRR4 expression in breast cancer: induction by nitric oxide and correlation with lymph node metastasis and poor prognosis. *BMC Cancer.* 2008 November;8(1):340.
115. Chen S, LaRoche T, Hamelink D, et al. Multiplexed analysis of glycan variation on native proteins captured by antibody microarrays. Nat Methods. 2007 May;4(5):437–44.

116. Zhou B, Phan V, Liu X, Juhasz A, Chu PG, Yen Y. Production of a monoclonal antibody against the lRRM2 subunit of ribonucleotide reductase and immunohistochemistry study of human cancer tissues. *Hyridoma (Larchmt).* 2006 October;25(5):264–70.

117. Li C, Simeone DM, Brenner DE, et al. Pancreatic Cancer Serum Detection Using a Lectin/Glyco-antibody Array Method. *J Proteome Res.* 2009 February 6;8(2):483–92.

118. Zangar RC, Varnum SM, Covington CY, Smith RD. A rational approach for discovering and validating cancer markers in very small samples using mass spectrometry and ELISA microarray. *Dis Markers.* 2004;20(3):135–48.

119. Varnum SM, Covington CC, Woodbury RL, et al. Proteomic characterization of nipple aspirate fluid: identification of potential biomarkers of breast cancer. *Breast Cancer Res Treat.* 2003 July;80(1):87–97.

120. Woodbury RL, Varnum SM, Zangar RC. Elevated HGF levels in sera from breast cancer patients detected using a protein microarray ELISA. *J Proteome Res.* 2002 May;1(3):233–7.

121. Yaziji H, Taylor CR, Goldstein NS, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. *Appl Immunohistochem Mol Morphol.* 2008 December;16(6):513–20.

122. Tomonaga T, Matsushita K, Yamaguchi S, et al. Identification of altered protein expression and post-translational modifications in primary colorectal cancer by using agarose two-dimensional gel electrophoresis. *Clin Cancer Res.* 2004 March 15;10(6):2007–14.

123. Sikut R, Zhang K, Baeckstrom D, Hansson GC. Distinct sub-populations of carcinoma-associated MUC1 mucins as detected by the monoclonal antibody 9H8 and antibodies against the sialyl-Lewis a and sialyl-Lewis x epitopes in the circulation of breast-cancer patients. *Int J Cancer.* 1996 May 29;66(5):617–23.

124. Martersteck CM, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. Unique alpha 2, 8-poly-sialylated glycoproteins in breast cancer and leukemia cells. *Glycobiology.* 1996 April;6(4):289–301.

125. Zhang S, Cordon-Cardo C, Zhang HS, et al. Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. *Int J Cancer.* 1997 September 26;73(1):42–9.

126. Cribb AE, Knight MJ, Dryer D, et al. Role of polymorphic human cytochrome P450 enzymes in estrone oxidation. *Cancer Epidemiol Biomarkers Prev.* 2006 March;15(3):551–8.

127. Bransfield LA, Rennie A, Visvanathan K, et al. Formation of two novel estrogen guanine adducts and HPLC/MS detection of 4-hydroxyestradiol-N7-guanine in human urine. *Chem Res Toxicol.* 2008 August;21(8):1622–30.

128. Samozu M, Brennan ML, To V, et al. Association between nitrotyrosine levels and microvascular density in human breast cancer. *Breast Cancer Res Treat.* 2002 June;73(1):271–8.

129. Nakamura Y, Yusaoku H, Tsujimoto M, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. *Clin Cancer Res.* 2006 February 15;12(4):1201–7.

130. Yakovlev VA, Barani JI, Rabender CS, et al. Tyrosine nitration of Ikapbeta: a novel mechanism for NF-kappaB activation. *Biochemistry.* 2007 October 23;46(42):11671–83.

131. Agnoletto MH, Guecheva TN, Donde F, et al. Association of low repair efficiency with high hormone receptors expression and SOD activity in breast cancer patients. *Clin Biochem.* 2007 November;40(16–17):1252–8.

132. Yosh G, Hardas S, Sultana R, St Clair DK, Vore M, Butterfield DA. Glutathione elevation by gamma-glutamyl cysteine ethyl ester as a potential therapeutic strategy for preventing oxidative stress in brain mediated by in vivo administration of adriamycin: Implication for chemoresistance. *J Neurosci Res.* 2007 February 15;85(3):497–503.

133. Gouaze V, ndrieu-Abadie N, Cuilllier O, et al. Glutathione peroxidase-1 protects from CD95-induced apoptosis. *J Biol Chem.* 2002 November 8;277(45):42867–74.

134. Citardi MJ, Song W, Batra PS, Lanza DC, Hazen SL. Characterization of oxidative pathways in chronic rhinosinusitis and sinonasal polyposis. *Am J Rhinol.* 2006 May;20(3):153–9.

135. Wu RC, Smith CL, O’Malley BW. Transcriptional regulation by steroid receptor coactivator phosphorylation. *Endocrine Reviews.* 2005 May;26(3):393–9.

136. Zhang S, Cordon-Cardo C, Zhang HS, et al. Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. *Int J Cancer.* 2004 March 15;10(6):2007–14.

137. Pfister S, Rea S, Taipale M, et al. The histone acetyltransferase hMOF is frequently downregulated in primary breast carcinoma and medulloblastoma and constitutes a biomarker for clinical outcome in medulloblastoma. *Int J Cancer.* 2008 March 15;122(6):1207–13.

138. Feng W, Lu Z, Luo RZ, et al. Multiple histone deacetylates repress tumor suppressor gene ARHI in breast cancer. *Int J Cancer.* 2007 April 15;120(8):1664–8.

139. Pledgie-Tracey A, Sobolewski MD, Davidson NE. Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines. *Mol Cancer Ther.* 2007 March;6(3):1013–21.

140. Krusche CA, Wulfing P, Kersting C, et al. Histone deacetylase-1 and -3 protein expression and post-translational modifications in primary colorectal cancer by using agarose two-dimensional gel electrophoresis. *Clin Cancer Res.* 2004 March 15;10(6):2007–14.

141. Sikut R, Zhang K, Baeckstrom D, Hansson GC. Distinct sub-populations of carcinoma-associated MUC1 mucins as detected by the monoclonal antibody 9H8 and antibodies against the sialyl-Lewis a and sialyl-Lewis x epitopes in the circulation of breast-cancer patients. *Int J Cancer.* 1996 May 29;66(5):617–23.

142. Martersteck CM, Kedersha NL, Drapp DA, Tsui TG, Colley KJ. Unique alpha 2, 8-poly-sialylated glycoproteins in breast cancer and leukemia cells. *Glycobiology.* 1996 April;6(4):289–301.

143. Zhang S, Cordon-Cardo C, Zhang HS, et al. Selection of tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. *Int J Cancer.* 1997 September 26;73(1):42–9.

144. Cribb AE, Knight MJ, Dryer D, et al. Role of polymorphic human cytochrome P450 enzymes in estrone oxidation. *Cancer Epidemiol Biomarkers Prev.* 2006 March;15(3):551–8.

145. Bransfield LA, Rennie A, Visvanathan K, et al. Formation of two novel estrogen guanine adducts and HPLC/MS detection of 4-hydroxyestradiol-N7-guanine in human urine. *Chem Res Toxicol.* 2008 August;21(8):1622–30.

146. Samozu M, Brennan ML, To V, et al. Association between nitrotyrosine levels and microvascular density in human breast cancer. *Breast Cancer Res Treat.* 2002 June;73(1):271–8.

147. Nakamura Y, Yusaoku H, Tsujimoto M, et al. Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. *Clin Cancer Res.* 2006 February 15;12(4):1201–7.

148. Yakovlev VA, Barani JI, Rabender CS, et al. Tyrosine nitration of IkappaBalpha: a novel mechanism for NF-kappaB activation. *Biochemistry.* 2007 October 23;46(42):11671–83.

149. Agnoletto MH, Guecheva TN, Donde F, et al. Association of low repair efficiency with high hormone receptors expression and SOD activity in breast cancer patients. *Clin Biochem.* 2007 November;40(16–17):1252–8.

150. Yosh G, Hardas S, Sultana R, St Clair DK, Vore M, Butterfield DA. Glutathione elevation by gamma-glutamyl cysteine ethyl ester as a potential therapeutic strategy for preventing oxidative stress in brain mediated by in vivo administration of adriamycin: Implication for chemoresistance. *J Neurosci Res.* 2007 February 15;85(3):497–503.

151. Gouaze V, ndrieu-Abadie N, Cuilllier O, et al. Glutathione peroxidase-1 protects from CD95-induced apoptosis. *J Biol Chem.* 2002 November 8;277(45):42867–74.

Publish with Libertas Academica and every scientist working in your field can read your article

“I would like to say that this is the most author-friendly editing process I have experienced in over 150 publications. Thank you most sincerely.”

“The communication between your staff and me has been terrific. Whenever progress is made with the manuscript, I receive notice. Quite honestly, I’ve never had such complete communication with a journal.”

“LA is different, and hopefully represents a kind of scientific publication machinery that removes the hurdles from free flow of scientific thought.”

**Your paper will be:**
- Available to your entire community free of charge
- Fairly and quickly peer reviewed
- Yours! You retain copyright

http://www.la-press.com