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Differential phosphopeptide expression in a benign breast tissue, and triple-negative primary and metastatic breast cancer tissues from the same African-American woman by LC-LTQ/FT-ICR mass spectrometry

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ABSTRACT

African-American women have a higher risk for developing triple-negative breast cancer (TNBC). Lacking the expression of receptors for estrogen and progesterone, and without human epidermal growth factor 2 receptor gene amplification, TNBC is a very aggressive type of breast cancer with a high likelihood of metastasis and recurrence. Specific therapeutic targets for this aggressive disease remain to be identified. Phosphorylation, a post-translational modification that adds one or more phosphate groups to a protein, plays a key role in the activation and deactivation of a protein's cellular function. Here, we report the first systematic phosphoproteomic analysis of a benign breast tissue, a primary breast cancer tissue, and a metastatic breast cancer tissue from the same African-American woman. Differential phosphoprotein levels were measured with reversed-phase nano-liquid chromatography coupled to a hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance mass spectrometer (LC-LTQ/FT-ICR MS). Five proteins were found to be highly phosphorylated in the metastatic site whereas six proteins were highly phosphorylated in the cancer site of the TNBC patient. Identified phosphoproteins are known to be involved in breast cancer signal transduction pathways and these results may identify new diagnostic and therapeutic targets for TNBC.

1. Introduction

Estrogen and progesterone are hormones involved in the growth and proliferation of breast cells [1]. Healthy breast cells express the estrogen (ER+) and progesterone receptors (PR+). Upon binding of their respective hormones, these receptors localize to the nucleus where they modulate DNA transcription. Breast cancer cells that test negative for estrogen receptor (ER−), progesterone receptor (PR−), and human epidermal growth factor receptor 2 (HER-2−) are called triple-negative breast cancer (TNBC). Because of this lack of expression, TNBC is not responsive to hormonal therapy or therapies that target the HER-2 receptor. TNBC comprises 10–20% of breast cancers [2,3] and has a higher rate of metastasis and recurrence than other types of breast cancer [4].

Although the overall incidence of breast cancer is lower in African-American women than in Caucasian-American women [5], breast cancer mortality is higher in African-Americans, regardless of age and body-mass index [6]. This increased mortality is partially attributed to the prevalence of TNBC in the African-American population, which is 3-fold higher than in Caucasian-American women [6]. To date, prognostic markers for TNBC have been limited to analysis of a few receptors, as well as cytokeratins [7]. There are several targeted breast cancer therapies in development [8]. However, none of these therapies are intended for specific treatment of TNBC, highlighting a need for the identification of biomarkers for this disease.

Phosphorylation is the most common type of post-translational protein modification. Phosphorylation is the addition of a phosphate group to a serine, threonine, or tyrosine residue on a target

Abbreviations: CHAPS, cholamidopropyl dimethylammonio propanesulfonate hydrate; CLTC, isoform 1 of clathrin heavy chain 1; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; HDGF, hepatoma-derived growth factor; HMGA1, HMG-Y of high mobility group protein HMG-I/HMG-Y; hnRNP A1, isoform A1–B of heterogeneous nuclear ribonucleoprotein A1; HSP, heat-shock protein; LC-LTQ/FT-ICR MS, reversed-phase nano-liquid chromatography coupled to a hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance mass spectrometry; MMP2, matrix metalloproteinase-2; SRRM2, isoform 1 of serine/arginine repetitive matrix protein 2; TNBC, triple-negative breast cancer; TRIM28, isoform 2 of transcription intermediary factor 1-beta.

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protein by a protein kinase [9] and acts as an "on/off" switch regulating protein activity. By means of signal transduction, this activity in turn affects numerous cellular functions including metabolism, growth, differentiation, and membrane transport. Understanding the changes in phosphorylation in different stages of breast cancer may help elucidate the signaling pathways and proteins responsible for disease progression. Here, the differential expression of phosphoproteins in benign breast tissue, primary breast cancer tissue, and metastatic breast cancer tissue from the same African-American woman with TNBC is reported. From these findings, it may be possible to identify targets for TNBC therapy by understanding the distinctive nature this cancer elicits at a molecular level.

2. Materials and methods

All solvents, methanol, acetonitrile, and water were purchased from J.T. Baker (Philipsburg, NJ) at HPLC grade. Trypsin, formic acid, ammonium bicarbonate, potassium dihydrogen phosphate, calcium chloride, acetic acid, Tris, and cholamidopropyl dimethylammonio propanesulfonate hydrate (CHAPS) were purchased from Sigma–Aldrich (St. Louis, MO). The protease and phosphatase inhibitor cocktail was purchased from Thermo-Pierce (Rockford, IL), Phos-TiO_2 (5 μm, Titansphere) was purchased from GL Sciences (Torrance, CA), and urea was purchased from Fisher Scientific (Pittsburgh, PA).

2.1. Tissue sample preparation

Provided by the Southern Division of the Cooperative Human Tissue Network, three breast tissue samples of normal, tumor, and metastatic to lymph node pathologies were sectioned from a 51-year old African-American woman's left breast. The patient's pathology report indicated that the specimens were negative for ER, PR, and HER-2 receptors. Tissues were prepared on ice [10] with a cold tissue lysis buffer composed of 50 mM Tris–HCl pH 7.4, 150 mM sodium chloride, 8 M urea, 4% CHAPS, and 2× protease/phosphatase inhibitor (EDTA free). Protein concentration was determined with a Bicinchoninic Acid Protein Assay Kit (Pierce, Rockford, IL). From each of the three samples, a volume equivalent to 2 mg of protein was pipetted into microtubes and vacuum dried for subsequent analysis.

2.2. Protein sample preparation, gel electrophoresis, and digestion

Soluble proteins underwent reduction of disulfide bonds with 10 mM dithiothreitol for 35 min at 56 °C and alkylation of free sulfhydryl groups with 55 mM iodoacetamide for 35 min at room temperature, in the dark, with continuous shaking. Extracted proteins were denatured at 90 °C for 10 min and separated by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis with a Bis–Tris 4–12% gradient gel (Invitrogen, Carlsbad, CA). The gel was stained with Coomassie blue (Invitrogen) and cut into ten bands according to molecular weight. In-gel trypsin digestion followed a standard protocol, but with larger volume in 15 mL tubes. Briefly, gel bands were excised into approximately 1×1 mm cubes, washed with 40% acetonitrile/50 mM NH_4HCO_3, and dried under vacuum. Trypsin was applied to each gel piece at high enzyme:substrate ratio (~1:30) to improve cleavage efficiency, and digestion was carried out for 18 h at 37 °C. The digests were then extracted with 70/25/5% acetonitrile/H_2O/formic acid, speed vacuumed to dryness, and stored at −80 °C for subsequent analysis.

2.3. Phosphopeptide enrichment

A Phos-TiO_2 (5 μm, Titansphere) spin column was preconditioned with acetonitrile via centrifugation. Tryptic peptides were dissolved in 50 μL of 0.1% trifluoroacetic acid (TFA), 25% lactic acid, and 60% acetonitrile, and non-specifically bound peptides removed by washing three times with 0.5% TFA and 80% acetonitrile. Phosphopeptides were eluted with 5% aqueous ammonium hydroxide and 5% aqueous pyrrolidine solutions [11].

2.4. Liquid chromatography and mass spectrometry

Prior to mass spectrometry, tryptic digests were separated on-line by reversed-phase nano-liquid chromatography (10 μL sample) with a C_{18} capillary column (New Objective, Woburn, MA). An Eksigent NanoLC (Dublin, CA) delivered a 90 min gradient (2–50% B) at 400 nL/min (solution A: 0.5% formic acid in 2% aqueous methanol; solution B: 0.5% formic acid in 98% aqueous methanol) [12].

Mass spectrometry was performed with a modified hybrid linear quadrupole ion trap/FT-ICR mass spectrometer (LTQ-FT; Thermo Fisher Corp., Bremen, Germany) equipped with an actively shielded 14.5 Tesla superconducting magnet (Magnex, Oxford, UK) [13]. External calibration was based on the quadrupolar trapping potential approximation [14]. The sample analysis was operated in top-ten data-dependent mode: high resolution MS for precursor ion mass measurement and low resolution LTQ MS/MS for product ion mass spectra. For each precursor ion measurement, 3×10^6 ions were accumulated in the LTQ prior to transfer to the ICR cell. The top ten most abundant ions were fragmented by collision-induced dissociation in the LTQ for low-resolution MS/MS (2 microscans, 10,000 target ions, 2.0 Da isolation width, 35.0 normalized collision energy, 0.250 activation Q, 30 ms activation period, and dynamic exclusion list size of 60 for 1 min). Automatic gain control (3×10^6 ions) resulted in less than 0.5 ppm rms mass error with external calibration. Data was collected with Xcalibur software (Thermo).

2.5. Data analysis and informatics

Raw data was extracted with a custom peak picking algorithm at a 10% signal-to-noise threshold (personal communication with Dr. Jarrod Marto). The resulting files were searched with MASCOT (Matrix Science, Cambridge, UK) against the IPI human database v3.30 (http://www.ebi.ac.uk/Databases/) and IPI human random database with a precursor ion tolerance of 2.0 ppm. Oxidation of methionine, iodoacetamide derivatized cysteine, and phosphorylated serine, threonine, and tyrosine were specified as variable modifications.

Scaffold (version 2.05, Proteome Software Inc., Portland, OR) served to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [15]. Phosphoprotein identifications were accepted if they could be established at greater than 99.0% probability and contained at least one identified phosphopeptide and one non-phosphopeptide (not necessarily the same segment). Protein probabilities were assigned by the Protein Prophet algorithm [16]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony.

2.6. Signaling pathway analysis

The NCI-Nature signaling pathway database batch query tool generated the described signaling pathways [17]. Pathways were

ranked by the probability that they included the phosphoproteins from the phosphoprotein data set: the lower the *p*-value, the higher the probability that the data set is biased toward a particular pathway. The parameters used to generate the *p*-values were the size of the query set, the number of biomolecules in a particular pathway, and the total number of biomolecules in the database. The *p*-value calculation for the batch query tool is based on a hypergeometric cumulative distribution function and is raw (i.e., not corrected for multiple-hypothesis testing). All NCI-Nature database entries were included in determining the *p*-value, and only pathways with $p < 0.05$ were included.

3. Results and discussion

14.5 Tesla LC-LTQ/FT-ICR MS provides 4-fold better ion mass accuracy and 2-fold higher resolving power than a similar 7 Tesla instrument operated at the same LC sampling rate [18]. Although a similar phosphopeptide enrichment method and 7 Tesla LC-LTQ/FT-ICR MS has previously been used to determine phosphorylation sites [19], to our knowledge there has been no differential phosphorylation expression comparison of tissues from a TNBC patient. Here, from the three tissue sections, we identified a total of 174 proteins, 52 of which were phosphoproteins (see Supplementary Tables S1 and S2). To increase stringency, only those phosphoproteins whose digested peptides were available in the flow-through after phosphopeptide enrichment were considered for further analysis. Table 1 shows the number of phosphopeptides per identified phosphoprotein in each of the three tissue sections and was constructed according to the total MS spectral count (or LC fractions) to determine the differential phosphopeptide expression level and group the phosphoproteins accordingly. Phosphorylation at a given serine or threonine position is typically less than 100%. If partial phosphorylation occurs at multiple sites, rather than only a single site, then phosphorylated peptides will appear in many more LC fractions.

Many identified phosphoproteins in Table 1 affect, or are affected, by breast cancer progression. For instance, hepatoma-derived growth factor (HDGF) is essential for breast tumorigenesis and is down-regulated by the estrogen receptor [20]. Isoform 1 of serine/arginine repetitive matrix protein 2 (SRRM2) gene is upregulated by testosterone in T47D breast cancer cells [21]. According to UniProtKB/Swiss-Prot, SRRM2 is also known to be phosphorylated upon DNA damage, but the phosphorylation site is not among those listed in UniProt or among those in phosphosite.org. Isoform HMG-Y of high mobility group protein HMG-I/HMG-Y (HMGA1), a regulator of gene transcription overexpressed in breast cancer [22], was recently found in human cancer cell lines to positively regulate the insulin-like growth factor-I receptor gene [23] that plays an important role in regulation of normal growth,

Table 1
Number of phosphopeptides identified for each phosphoprotein from each of the three tissue sections: Normal, invasive ductal carcinoma (Cancer), and metastatic ductal carcinoma to lymph node sample (Met.). The table also includes the molecular mass (M) and the accession number from the IPI human database from Scaffold. A number in parenthesis indicates that more than 1 accession number is related to the identified peptides. The number in brackets under the phosphopeptide number is the number, provided from Scaffold, of LC fractions in which that phosphopeptide was identified. Higher number of LC fractions means higher phosphopeptide expression level, whether at one, or several, phosphorylation sites.

Identified phosphoproteins	Accession number	M (kDa)	Number of phosphopeptides in		
			Normal	Cancer	Met.
LMNA Progerin	IPI00644087	69	1 [5]	1 [1]	0
HSP90AB1 85 kDa protein	IPI00334775 (+1)	85	1 [4]	0	0[a]
PTRF isoform 1 of polymerase I and transcript release factor	IPI00176903	43	2 [3]	0	0
AHNAK nucleoprotein isoform 1	IPI00021812	629	2 [4]	0	0
SEPT2 septin-2	IPI00014177 (+1)	42	1 [3]	0	0[a]
SRRM2 isoform 1 of serine/arginine repetitive matrix protein 2	IPI00782992	300	0[a]	0[a]	1 [1]
HDGF hepatoma-derived growth factor	IPI00514330	26	0[a]	2 [3]	2 [6]
SFRS11 Splicing factor arginine/serine-rich 11	IPI00464952 (+2)	54	0[a]	0[a]	1 [1]
SRRM1 serine/arginine repetitive matrix 1	IPI00328293 (+1)	103	0[a]	0[a]	[2]
HMGA1 isoform HMG-Y of high mobility group protein HMG-I/HMG-Y	IPI00177716	11	0[a]	0[a]	1 [2]
TRIM28 isoform 2 of transcription intermediary factor 1-beta	IPI00438230	80	0[a]	1 [1]	0[a]
HSP90AA1 heat shock protein HSP 90-alpha 2	IPI00382470(+4)	98	0	3 [3]	0[a]
HNRPC isoform C1 of heterogeneous nuclear ribonucleoproteins C1/C2	IPI00216592 (+3)	32	1 [3]	3 [8]	2 [3]
HNRPA1 isoform A1–B of heterogeneous nuclear ribonucleoprotein A1	IPI00215965(+2)	39	0	1 [2]	0
CLTC isoform 1 of clathrin heavy chain 1	IPI00024067	192	0	1 [1]	0[a]
MYH9 myosin-9	IPI00019502	227	1 [1]	1 [18]	0
CANX calnexin precursor	IPI00020984	68	1 [1]	1	0 [1]

[a] Protein expressed at a level below reliable detection from flow-through of phosphopeptides enrichment from that tissue site.

Table 2
Signal pathways for all 52 identified phosphoproteins determined by the NCI-Nature signaling pathway database batch query tool. Only those proteins with p-values < 0.05 are included. The pathway name, phosphoprotein gene symbol, p-value, and the citations for breast cancer related signal pathways are listed. Phosphoprotein gene symbols in bold are those identified in Table 1, whereas those not in bold are phosphoproteins not found in the flow-through following phosphopeptide enrichment.

Pathway Name	Phosphoprotein gene symbol	p-value	Citations: is pathway related to breast cancer?
Integrins in angiogenesis	**HSP90AA1**, IRS1, TLN1	3.02E-04	[34]
Paxillin-dependent events mediated by a4b1	ITGB1, TLN1	1.28E-03	[35]
Presenilin action in Notch and Wnt signaling	APC, CSNK2A1	7.23E-03	[36]
Arf6 trafficking events	**CLTC**, ITGB1	8.47E-03	[37]
Signaling events mediated by PTP1B	IRS1, YBX1	8.79E-03	[38]
Signaling events mediated by focal adhesion kinase	ITGB1, TLN1	1.27E-02	[39]
IL4-mediated signaling events	**HMGA1**, IRS1	1.38E-02	[40]
Regulation of Telomerase	**HNRNPC**, **HSP90AA1**	1.55E-02	[41]
CDC42 signaling events	APC, **SEPT2**	1.59E-02	[42]
Validated targets of C-MYC transcriptional activation	**HMGA1**, **HSP90AA1**	2.36E-02	[43]
ErbB receptor signaling network	**HSP90AA1**	4.45E-02	[44]
Atypical NF-kappaB pathway	CSNK2A1	4.72E-02	[45]

development, and carcinogenesis [24]. HMGA1 inhibits p53-dependent apoptosis, crucial for DNA-repair, by modulating both transcription of p53 target genes and cytoplasmic relocalization of homeodomain-interacting protein kinase 2 enhancing carcinogenesis [25].

HMGA1 protein is involved in signal pathways known to affect breast cancer (Table 2). Table 1 lists those phosphoproteins that exhibit the highest number of phosphopeptides from the metastasis site relative to the tumor and normal tissue sites. Phosphorylation of HDGF protein occurs within the tumor site but is even more pronounced in the metastatic site for all of the above mentioned proteins in Table 1 (SRRM2 and HMGA1, including HDGF). To summarize, these results suggest that phosphorylation of such proteins directly affects tumor progression in the TNBC patient, including proteins involved in tumorigenesis [26], DNA damage, gene transcription, apoptosis, and carcinogenesis.

Furthermore, many identified phosphoproteins in Table 1 contribute to the tumor phenotype. For instance, the decrease in sumoylation by doxorubicin of isoform 2 of transcription intermediary factor 1-beta (TRIM28) protein has been found essential to induce p21 expression, and thus cell growth inhibition in MCF-7 breast cancer cells [27]. In patients with invasive breast cancers, the co-expression of heat-shock protein (HSP) 90 alpha with an overexpressed kinase, or along with the loss of a phosphatase and tensin homologue deletion on chromosome 10, is a putative molecular prognostic marker to predict early relapse [28]. Moreover, HSP90-alpha, which is expressed extracellularly from breast cancer cells, interacts with matrix metalloproteinase-2 (MMP2). This interaction promotes MMP2 activation, which is crucial for tumor invasiveness [29]. HSP90-alpha is also involved in several signal pathways related to breast cancer (Table 2). Although isoform A1–B of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) protein has not been reported in breast cancer literature, its gene, *HNRPA1*, was found among the genes that discriminate ER+ from ER− breast tumors [30]. Isoform 1 of clathrin heavy chain 1 (CLTC) gene is one of a set of new genes shown to be overexpressed because of the chromosomal region 17q23 amplification in breast cancer and thus proposed to contribute to the tumor phenotype [31]. It is also involved in a breast cancer-related signal pathway (Table 2). Myosin-9 has been identified as a candidate breast cancer gene in a systematic analysis of the breast cancer genome [32]. Interestingly, these phosphoproteins exhibited no phosphopeptides in the metastasis site and had a higher number of phosphopeptides in the tumor site than in the normal tissue site (Table 1). Increased number of phosphopeptides in the tumor site implies that phosphorylation of such proteins, which contributes to a tumor phenotype, is necessary for cancer onset in TNBC. Targeted dephosphorylation of such proteins, such as TRIM28, HSP90-alpha, hnRNP A1, CLTC, and myosin-9, in breast cancer cells may help arrest cancer progression in TNBC [33].

Comparison of the phosphopeptide expression levels in three tissue sites from the same TNBC African-American woman reveals phosphorylation and dephosphorylation activities associated with TNBC tumor and cancer progression (Table 1). Several of the presently identified phosphoproteins were known to affect breast cancer, and the additional proteins listed in Table 1 provide new targets for TNBC research. The second group of five phosphoproteins in Table 1 is highly phosphorylated in metastasis, correlating their function to increased aggressiveness of the breast cancer cells. The third group of six phosphoproteins in Table 1 is also important because they are highly phosphorylated in the tumor site, and targeted dephosphorylation of such proteins in metastatic and cancer cells may arrest cancer progression in TNBC.

Conflict of interest

The authors declare that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.057.

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