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Approaching Solid Tumor Heterogeneity on a Cellular Basis by Tissue Proteomics Using Laser Capture Microdissection and Biological Mass Spectrometry

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Abstract

The purpose of this study was to examine solid tumor heterogeneity on a cellular basis using tissue proteomics that relies on a functional relationship between Laser Capture Microdissection (LCM) and biological mass spectrometry (MS). With the use of LCM, homogeneous regions of cells exhibiting uniform histology were isolated and captured from fresh frozen tissue specimens, which were obtained from a human lymph node containing breast carcinoma metastasis. Six specimens ~50 000 cell each (three from tumor proper and three from tumor stroma) were collected by LCM. Specimens were processed directly on LCM caps, using sonication in buffered methanol to lyse captured cells, solubilize, and digest extracted proteins. Prepared samples were analyzed by LC/MS/MS resulting in more than 500 unique protein identifications. Decoy database searching revealed a false-positive rate between 5 and 10%. Subcellular localization analysis for stromal cells revealed plasma membrane 14%, cytoplasm 39%, nucleus 11%, extracellular space 27%, and unknown 9%; and tumor cell results were 5%, 58%, 26%, 4%, and 7%, respectively. Western blot analysis confirmed specific linkage of validated proteins to underlying pathology and their potential role in solid tumor heterogeneity. With continued research and optimization of this method including analysis of additional clinical specimens, this approach may lead to an improved understanding of tumor heterogeneity, and serve as a platform for solid tumor biomarker discovery.

Keywords

laser capture microdissection (LCM); mass spectrometry (MS); solid tumor heterogeneity

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Supporting Information Available: Lists of 386 proteins identified in tumor proper and 164 proteins identified in tumor stroma. This material is available free of charge via the Internet at http://pubs.acs.org.

Introduction

Solid tumors are heterogeneous in composition. They are composed of cancer cells proper, along with stromal elements that collectively form a microenvironment, which is necessary to nurture the malignant process. Additionally, many of the stromal cells have been modified, thus, supporting the unique needs of the malignant state.[1] Since solid tumors can be composed of a variety of clones or subpopulations of cancer cells, these cells may differ among themselves in many properties, such as karyotype, growth rate, production and expression of cell surface markers, sensitivity to therapeutics (chemo, biologics, radiation), and so forth. Heterogeneity is not a unique property of tumors per se, since premalignant lesions and normal tissue are also composed of mixed subpopulations of cells.[2] Approaching solid tumors by reducing their complexity with up-front laser capture microdissection (LCM) should facilitate better characterization of tumorous tissue and may assist with biomarker discovery, by alleviating the dynamic range limitations of MS and the specificity of proteins to cell type of origin.

A number of scientific studies have employed LCM for cell capture and used mass spectrometry (MS) for protein identification. For instance, Li Zang et al.[3] developed a method for quantification of proteins from limited sample amounts from ductal carcinoma of the breast using LCM and LC-MS (LCQ, Thermo) and $^{16}O/^{18}O$ isotopic labeling. This study compared normal ductal epithelium versus metastatic ductal carcinoma. The number of cells collected by LCM was 10 000 and the number of identified proteins was 76. More recently, this same group[4] developed a method using LCM to isolate high-grade dysplastic and normal cells from ThinPrep slides prepared from cervical cytological specimens. This study collected 9 normal and 9 abnormal specimens, and each specimen contained ~12 000 cells. A 1D gel was used for separation prior to analysis by LC-MS (LTQ-FT MS, Thermo), and ~1000 proteins were reported identified.

Other interesting LCM-MS studies of note include the following. (i) Nano LC-FT-ICR analysis of ~3000 breast cancer cells via accurate mass and time (AMT) tag database that yielded 1003 protein identifications with two or more peptides, illustrating a substantial number of proteins can be detected from a limited number of cells using an AMT tag approach.[5] (ii) Formalin fixed paraffin embedded (FFPE) archival prostate cancer tissue from biopsy samples were analyzed by shotgun proteomics following an extraction procedure that disrupted cross-linked proteins, resulting in the identification of 428 prostate expressed proteins.[6] Work from other groups in applying a LCM sample approach to solution-based LC-MS/MS has also demonstrated applicability.[7–9]

The need for further proteomic method development is critical for attaining much needed goals such as the discovery of biomarkers for oncology. Therefore, the purpose of this study was to address solid tumor heterogeneity on a cellular basis and facilitate biomarker discovery for oncology through an optimized proteomic method based on a functional coupling of LCM with solution-based LC/MS-MS. Decomposition of solid tumors by LCM enables isolation of architectural features, thus, providing a more direct assessment of tumor heterogeneity, as well as an upstream localization of common cell types for subsequent MS analysis. Furthermore, this approach also helps to address the dynamic range (concentration), and specificity of proteins to cells of origin (biological localization). This present method that employs LCM coupled with biological MS resulted in an effective tissue proteomics approach able to address solid tumor heterogeneity, and may result in discovery of novel cancer biomarkers from clinically relevant samples.

Materials and Methods

Materials

Ammonium bicarbonate was purchased from Sigma (St. Louis, MO). Sequencing grade trypsin was obtained from Promega (Madison, WI). Trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Fluka (Milwaukee, WI). HPLC grade acetonitrile (CH_3CN) and methanol (CH_3OH) were obtained from EM Science (Darmstadt, Germany). Tris[2-carboxyethyl] phosphine (TCEP) was obtained from Pierce (Rockford, IL). Water was purified by a Barnstead Nanopure system (Dubuque, IA). CapSure Macro LCM Caps were purchased from MDS Analytical Technologies (Sunnyvale, CA). Mayer's Hematoxylin solution and Eosin Y solution (alcohol-based) were purchased from Sigma (St. Louis, MO). Scott's Tap Water Substitute Blueing Solution (magnesium sulfate buffered with sodium bicarbonate) was purchased from Fisher Scientific (Hampton, NH). One hundred percent ethanol (ethyl alcohol, absolute, 200 proof for molecular biology) was purchased from Sigma (St. Louis, MO). Solutions of 70% (v/v) and 95% (v/v) ethanol were established using Milli-Q-filter (Millipore, Billerica, MA) with purified H_2O. Xylene was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Laser-capture microdissection apparatus: PixCell IIe (Arcturus Molecular Devices now MDS Analytical Technologies, Sunnyvale, CA). Leica Cryostat CM 1850 UV was from Leica Microsystems (Wetzlar, Germany). Finally, uncoated, precleaned glass microscope slides, 25 × 75 mm were obtained from Fisher Scientific (Hampton, NH).

Antibodies

The primary antibodies used were (1) a rabbit polyclonal anti-Bax antibody (Cell Signaling, catalogue number 2772), diluted 1:1000; (2) a mouse monoclonal anti-keratin-19 antibody (Abcam, catalogue number ab7754), diluted 1:500; (3) a rabbit polyclonal anti-Histone H1.2 antibody (Abcam, catalogue number ab17677), diluted 1:500 with a rabbit polyclonal anti-Histone H1.1 antibody (Abcam, catalogue number ab17584), diluted 1:500; (4) a mouse monoclonal anti-E-cadherin antibody (BD Transduction Laboratories, catalogue number 610404), diluted 1:250; (5) a mouse monoclonal anti-Vimentin (Sigma, catalogue number V6389), diluted 1:100; (6) a mouse monoclonal anti-Annexin A5 (Santa Cruz Technologies, catalogue number SC-32321), diluted 1:100; (7) a rabbit polyclonal anti-beta Tubulin (Abcam, catalogue number ab6046), diluted 1:250; (8) a rabbit polyclonal anti-Histone H2A (BioVision, catalogue number 3621–100), diluted to 2 μg/mL; (9) a mouse monoclonal anti-alpha skeletal actin (Sigma, catalogue number A3853), diluted to 2 μg/mL; (10) a mouse monoclonal anti-alpha smooth muscle actin (Abcam, catalogue number ab7817), diluted 1:100; (11) a mouse monoclonal anti-Histones (Millipore, catalogue number MAB052), diluted 1:500. The peroxidase-labeled secondary antibodies used were goat anti-rabbit IgG (H + L) and goat anti-mouse IgG (H + L) from the Protein Detector Western Blot kit, TMB system, (KPL, Inc., Gaithersburg, MD).

LCM Sample Collection

A human lymph node containing breast carcinoma metastasis was obtained in accordance with the approval of the NCI/NIH Office of Human Subjects Review (OHSR) under the anonymous tissue acquisition protocol section. The tissue specimen was snap frozen and then embedded in cryostat mounting medium (TISSUE-Tek O.C.T., Sakura Finetek, Inc., Torrance, CA) and stored at −80 °C. Tissue sections (8 μm thick) were subsequently cut serially in a cryostat from the frozen tissue block. Each tissue section was immediately mounted on an uncoated, precleaned glass microscope slide (Fisher Scientific) and immediately stored again at −80 °C. Prior to LCM analysis, the slides stained by a standard hematoxylin and eosin (H&E) plus coverslip method were officially reviewed with a

pathologist in order to properly evaluate the histology, plan all LCM sessions, and guard against potential bias in the *z*-dimension of the tumor tissue plane.

At the time of LCM, the slides containing frozen sections were prepped with the following serial step procedure devised by the NCI LCM Core Facility. Frozen tissue slides were immediately fixed in 70% ethanol for 15 s, placed in deionized water for 30 s to remove OCT and rehydrate the tissue, placed in Mayer's Hematoxylin for 45 s to stain nuclei, placed in deionized water for 15 s to remove excess hematoxylin, placed in Scott's Tap Water for 15 s to change the hue of hematoxylin, placed in 70% ethanol for 15 s, and then placed in Eosin-Y for 3 s to stain cytoplasm. Then, dehydration steps were performed: 95% ethanol for 30 s twice, then 100% ethanol for 30 s twice. Ethanol was then removed through a 3 min bath in xylene.

Once air-dried, the slide/sections underwent LCM analysis with a PixCell IIe system (Arcturus Engineering now MDS Analytical Technologies). The standard LCM protocol[10] was followed, and approximately 50 000 cells of either breast tumor proper or stromal cells were laser captured with a 7.5 μm laser spot size, utilizing multiple caps.

Each cap was visualized via microscope to ensure the population of cells was greater than 98% homogeneous. The caps were then fitted with a 0.5 mL Eppendorf Microcentrifuge tube, labeled and stored at −80 °C.

Tissue Lysis and Digestion

The tissue lysis and digestion protocol was adapted from prior work.[11] Briefly, all caps were prepared in an identical manner. They were segregated by cell type (tumor vs stroma) but aggregated to achieve a total cell count of ~50 000 (50K cap set). Following cap removal from the Eppendorf tube, the polymer membrane was carefully peeled off the cap assembly and placed inside an Eppendorf tube. Cells were lysed by adding 50 μL of hypotonic buffer consisting of 12 mM NH_4HCO_3, 100% MeOH (v/v &equals 80/20), and 1 mM TCEP, final concentration, and then placed in a sonication bath for 20 min. The buffer was then adjusted to 50 mM NH_4HCO_3 (pH adjustment for trypsin to ~8.0) and tryptic digestion performed for 6 h at 37 °C with good table motion. A second trypsin digestion was then performed in a 60% MeOH/NH_4HCO_3 (v/v) buffer for 6 h at 37 °C. All digests were carefully transferred to a new tube and lyophilized to dryness. Each sample was rehydrated with 7 μL of 0.1% TFA and analyzed by LC-MS/MS.

Analysis of LCM Samples

Reversed-phase LC separations were carried out using a 75 μm i.d. × 10 cm-long fused silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) column with a flame-pulled tip (~5-7 μm orifice). The column was slurry-packed in-house with 5 μm, 300 Å pore size C-18 stationary phase (Vydac, Hercules, CA), using a slurry-packing pump (Model 1666, Alltech Associates, Deerfield, IL). After injecting 5 μL of sample, the column was washed for 30 min with 98% mobile phase A (0.1% FA in H_2O) and peptides were eluted using a linear step gradient from 2 to 40% mobile phase B (0.1% FA in CH_3CN) over 90 min and 60–98% mobile phase B over 10 min at a constant flow rate of 0.25 μL/min. The column was washed for 20 min with 98% mobile phase B and re-equilibrated with 2% mobile phase B for 30 min prior to subsequent sample loading. The reversed-phase column was coupled with a two-dimensional linear ion trap mass spectrometer (LTQ, ThermoElectron, San Jose, CA) for global shotgun proteomic analysis. The solvent system was delivered by an HP 1100 pump (Agilent Technolgies, Palo Alto, CA). A nanoelectrospray ionization source was employed, applying a potential of 1.7 kV, and capillary temperature of 160 °C. The mass spectrometer was operated in a data-dependent mode. The seven most abundant peptide molecular ions

detected by each MS survey scan were dynamically selected for MS/MS using collision-induced dissociation (CID) facilitated by a normalized collision energy of 36%. Dynamic exclusion was employed to avoid redundant acquisition of precursor ions previously selected for tandem MS.

Data Processing

The CID spectra were analyzed using a 40-node cluster computer following a Beowulf design model, running BioWorks/SEQUEST (ThermoElectron) via parallel virtual machine (PVM), against a nonredundant human proteome database (release date March 2008). The precursor ion tolerance was set to 1.5 Da, while fragment ion tolerance was 0.5 Da. Candidate peptides were required to possess tryptic termini at both ends and allowed a maximum of two missed cleavages. The following SEQUEST resholds were used for legitimately identified peptides: delta-correlation scores (dCn) ≥ 0.08 and charge state-dependent cross-correlation score (Xcorr) as follows: ≥ 2.1 for $[M + H]^{1+}$ peptides, ≥ 2.3 for $[M + 2H]^{2+}$ peptides, and ≥3.5 for $[M + 3H]^{3+}$ peptides. A final list of protein identifications was created adhering to the parsimony principle, reporting minimal number of protein identifications from a pool of uniquely identified peptides. Resultant raw data were subjected to false positive rate assessment via decoy (reverse) database analysis using in-house developed software. Finally, data were analyzed for biologic implications by Ingenuity Pathway Analysis (http://www.ingenuity.com), and the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov).

Western Immunoblot Analysis

A total of 20 μg of the tissue lysate was separated on 4–20% SDS–polyacrylamide gel (Invitrogen, catalogue number EC6025) and transferred onto a nitrocellulose membrane (Invitrogen, catalogue number IB3020-02) using the iBlot Gel Transfer system (Invitrogen). The membrane was first blocked with the 1× detector blocking solution for 1 h at room temperature. This was followed by incubation overnight at 4 °C with the primary antibody, then, for 2 h at room temperature with the peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by addition of the TMB (Tetramethylbenzidine) membrane peroxidase substrate forming an insoluble blue precipitate where the enzyme-labeled antibody is bound to the membrane through antigen–antibody complex (Protein Detector Western Blot Kit, TMB System, KPL, Inc., Gaithersburg, MD).

Results

In this study, a functional relationship between LCM and a shotgun proteomics method was used to study the heterogeneity present in a solid tumor, as well as discovery for potential biomarkers. Before initiating work with cancer related clinical specimens, preliminary studies using fresh frozen tissue from a human liver were conducted to assess a variety of experimental parameters and conditions, in order to begin to improve and optimize the sample prep and tissue extraction processes (data not shown). The optimized LCM sample preparation methodology was then applied to cancerous tissue, namely, fresh frozen tissue obtained from a human lymph node containing metastatic breast cancer. With the use of LCM, homogeneous regions of cells with uniform histology were isolated and captured from this human lymph node containing lesions from breast carcinoma metastasis.

Figure 1 shows the workflow used for experiments in this study. Fresh frozen tissue was cut on a cryostat (8 μm thickness) followed by tissue mounting and H&E staining with coverslip of selected slides. Histology review was performed, followed by a LCM planning session, both lead by a pathologist. Three samples, each containing approximately 50 000 cells, from tumor and stroma were analyzed. The polymer membranes were carefully removed from the

LCM cap and placed inside an Eppendorf tube. Hypotonic MeOH-based buffer along with sonication was employed for cell extraction, lysis and digestion. Samples were then prepared for MS analysis. Reversed-phase LC separation column was coupled with a LTQ for global shotgun proteomic analysis. CID spectra were analyzed on a 40-node cluster computer running BioWorks/SEQUEST. Figure 2 illustrates the LCM tissue extraction procedure. First, a region of interest is established around a histology section. Then a fill-pattern is applied within the region of interest in preparation for tissue extraction. Upon LCM cap removal, the selected groups of cells are captured in the LCM polymer membrane.

Experimental summaries of LCM-MS investigation of the human lymph node with metastatic breast carcinoma are shown in Table 1. Three samples, each containing approximately 50 000 cells from tumor and stroma (six samples total) yielded 531 unique proteins. High confidence measures were employed by including only proteins identified by ≥2 unique peptides. Additionally, a false positive rate assessment of peptide identification via decoy (reverse) database yielded a result of 7.9%. In summary, the examination of tumor proper resulted in the identification of 367 proteins (Supplementary Table 1 in Supporting Information), while study of tumor stroma yielded 164 protein identifications (Supplementary Table 2 in Supporting Information). Identified proteins were then subject to a set-based analysis. Figure 3 displays the results of this procedure. The values in this figure directly correspond to the column labeled "*Unique Proteins ID via ≥ 2 Peptides*" in Table 1. Of the 367 proteins germane to tumor, 250 were unique to tumor and 117 were also common with stroma. Regarding the 164 proteins relevant to stroma, 47 were unique to this tissue type.

Subcellular localization as well as protein functional analyses for both tumor and stroma were performed using Ingenuity Pathways Analysis. Results for subcellular analyses are displayed in Figures 4 and 5. Percentage composition for stromal cells was plasma membrane = 14%, cytoplasm = 39%, nucleus = 11%, extracellular space = 27% and unknown = 9%; and for tumor cells these results were 5%, 58%, 26%, 4%, and 7% respectively. Immediately noted are higher percentages of plasma membrane and extracellular space assignments for stroma. Regarding tumor samples, higher percentages of cytoplasm and nuclear assignments are noted as well. Figures 6 and 7 show the results for protein function analyses. Evident in this analysis are higher levels of enzyme assignments between stroma and tumor (16% vs 28%), and transcription regulator assignments (5% vs 8%).

Cross-validation studies using Western immunoblotting were performed on proteins identified in stroma, tumor, and both tissue histology types (common). Table 2 list the relevant tissue type, protein accession number, protein name, peptide count, and gene name for the 12 proteins for which westerns were performed. Figure 8 displays the Western blots of the six tumor related proteins, namely, Bax (20 kDa), Keratin 19 (40 kDa), Histone H1 (H1.1 33 kDa; H1.2 25 kDa), E-Cadherin (100 kDa), and Beta-Tubulin (50 kDa). Western blots of the two stroma related proteins, Histone H2A (16 kDa) and actin, alpha (40 kDa), are shown in Figure 9. Finally, Western blots of the four common (tumor and stroma) related proteins are shown in Figure 10, namely, Smooth Muscle Actin (40 kDa), Histone H4 (11 kDa), as well as Figure 7, which shows Vimentin (57 kDa), and Annexin A5 (35 kDa).

Discussion

Tumor heterogeneity is expressed in numerous ways. First clinically, patients categorically diagnosed with a specific tumor type will many times display a rather wide degree of variation in their response to therapies. Additionally, a therapy that was initially successful

many times will cease to be effective resulting in progression of disease for that same patient. Thus, tumors can display highly adaptive types of behaviors often evolving due to the pressure exerted from treatment (i.e., chemo, biologic, radiation), and in fact “rewire” their complex biologic circuits/networks, and render a successful resistance to treatment that was previously effective.

Second, tumors vary in their histology. A microscopic exam of solid tumor tissue reveals a wide array of cell types and features within these mixed patient populations. Cell specific attributes may include differences in morphology, degree of necrosis, vascularity, or quite often a zonal distribution of cellular nests or subpopulations.[12] These microscopic changes are a reflection of genomic alterations, which result in changes in DNA sequence, increased or decreased gene copy numbers, chromosomal aberrations, and DNA methylation modifications. Human malignancies develop and progress based on these molecular phenomena/events.[13] It is the specific protein manifestations reflected by aberrant genomic and epigenetic processes that particularly interest us in this study. Finally, the prevailing theories regarding the origin of solid tumor heterogeneity (stem cell theory versus clonal evolution hypothesis) may assist in devising approaches for improved understanding and possible exploitation for therapeutic benefit.

Solid tumor heterogeneity is a significant clinical problem in breast cancer. The clinical subtypes of breast cancer (i.e., hormone receptor (ER/PR) positive, HER-2 positive, or triple negative) are not single diseases. The diversity in molecular phenotypes as well as the range of clinical responses to identical medical therapy are common in breast cancer. Cancer risk, tumor initiation and progression, and the development of distinct molecular cancer subtypes are felt to be due to a variety of host factors, and thus the need for improved molecular diagnostics and tumor biology understanding.

Solid tumors are composed of an assortment of cell types, which have diverse morphologies, as well as distinct molecular phenotypes. The biological background of such heterogeneity is not well-understood. There are two accepted theories. Essentially, the stem cell theory suggests a program of aberrant differentiation, while the clonal evolution hypothesis supports competition among cellular neighbors, which results in the multitude of cell types present in a tumor. Both theories agree that tumors originate from a single cell that has acquired multiple mutations and gained an unlimited proliferative potential and or is resistant to apoptotic regulation.[2,14] Tumor heterogeneity and the diverse range of clinical manifestations are believed to be greatly influenced by the cell of origin, the particular genetic abnormalities, and the tumor–host microenvironment. Elegant work involving antibody-based approaches linked with genomic profiling and epigenetic techniques applied to breast tumor heterogeneity has begun to yield promising scientific and potential clinical insights.[15,16] Thus, a nonantibody based strategy employing LCM to decompose tumor architecture prior to MS analysis was the focus of this study.

Of the proteins strongly identified by MS in this study, 12 were cross verified with Western immunoblots based on their biological relevance in breast cancer and availability of antibodies. Keratin 19 (gene name KRT19) was found in tumor cells. It is the smallest known (40-kDa) acidic keratin, and is expressed often in epithelial cells in culture and in some carcinomas.[17] The biological significance of KRT19 in breast cancer has been conveyed on a genomic and mRNA transcript basis. Diversity in KRT19 gene expression has been found in lymph nodes of breast cancer patients.[18] The detection of KRT19 mRNA in peripheral blood (via circulating tumor cells assays) has been described to correlate with a poorer prognosis for breast cancer patients.[19]

Actin alpha was identified in stromal tissue in our study. The actin alpha gene (ACTA1) and KRT19 have been attributed to the stem cells of the mammary gland.[20] Cancer stem cells are cancer cells found in tumors, which possess stem cell characteristics (i.e., cell surface markers, self-renewal and differentiation). These cells are central figures in hypotheses concerning solid tumor heterogeneity, since they may give rise to a variety of cells within the solid tumor. These cells are premised to reside in tumors as distinct populations and thought to play significant roles in relapse and metastatic development. ACTA1 is seen overexpressed in breast cancer.[21] Certainly, improved understanding of cancer stem cells and key molecules associated with them is seminal to the development of new therapies and diagnostics, especially for patients with metastatic disease.

Four histone proteins were identified by MS and Western in this study. Their corresponding genes, HIST1H2AA, HIST1H1A, HIST1H1C, and HIST1H4B, are members of the extended major histocompatibility complex (xMHC), located on chromosome 6, and are essential for adaptive and innate immunity. It is well-established that cancer initiation and progression involves disorders of immunity, particularly immune surveillance and response. In addition to their vital role in transplant medicine (i.e., establishing a new immune system in a cancer patient), certain combinations (haplotypes) of xMHC loci are known to confer protection from, or susceptibility to, many diseases including most, if not all, autoimmune, inflammatory and infectious diseases.[22] The gene expression level of HIST1H1C has also been used in breast cancer to help separate the molecular phenotypes of invasive lobular carcinoma versus invasive ductal carcinoma.[23] Epigenetic phenomena such as loss of acetylation at Lys 16 and trimethylation at Lys 20 of histone H4 is a common hallmark of a number of human cancers, such as breast, colon, and lung.[24]

Alpha-actin, which was detected in stromal cells in our study, was recently found to be a new putative target of Akt signaling. As previously mentioned, molecular pathways are of major scientific and clinical interest in oncology. As more oncogenes and tumor suppresser genes are deciphered, the functions of their protein products are further defined, ultimately by fitting them into the deranged signaling pathways that are hijacked by the malignant transformation. A recent proteomic study employing co-immunoprecipitation showed applicability of alpha-actin as a potential target in breast cancer. This study focused on the Akt pathway, and studied the dynamic reorganization of the actin cytoskeleton, as germane to breast cancer cell migration. It is now thought alpha-actin may be a substrate of Akt kinase activity. Thus, alpha-actin may serve as a new functional target of Akt signaling.[25]

BAX is a pro-apoptotic bcl-2 family protein and in this study was indentified in tumor cells. By binding to the apoptosis repressor bcl-2, BAX helps to accelerate programmed cell death. Reduced expression of the BAX gene has been reported to be associated with poor response to chemotherapy and shorter survival times for women with metastatic breast cancer.[26] Although there has been debate in using BAX as a marker to predict a potential response to chemotherapy, it is still being considered as a marker to possibly select a patient for more intense treatment schedules.[27]

The epithelial cadherin precursor protein (E-cadherin) was identified in tumor cells. There are more than 20 recognized cadherins. The major epithelial cadherin, E-cadherin (gene name CDH1) participates in formation of the cytoplasmic cell adhesion complex. Compelling experimental evidence indicates that E-cadherin serves a potent invasion suppressor role. In addition, a tumor suppressor effect of E-cadherin has been suggested. In human cancers, partial or complete loss of E-cadherin expression correlates with malignancy. To investigate the molecular basis for the altered expression, Berx et al.[28] performed a PCR/SSCP mutation screen of the CDH1 gene in 49 breast cancer patients. No relevant DNA changes were encountered in any of 42 infiltrative ductal or medullary breast

carcinoma samples. In contrast, 4 of 7 infiltrative lobular breast carcinomas harbored protein truncation mutations (3 nonsense and 1 frameshift) in the extracellular part of the E-cadherin protein. Each of the 4 lobular carcinomas with E-cadherin mutations showed tumor-specific loss of heterozygosity of chromosomal region 16q22.1 containing the E-cadherin locus, thus, supporting the Knudson 2-hit hypothesis. Berx et al.[28] detected no E-cadherin expression in these 4 tumors by immunohistochemistry.[29]

Loss of E-cadherin (CDH1) function is thought to contribute to progression in breast cancer and other solid tumors by increasing proliferation, invasion, and/or metastasis. In some cases, the restoration of CDH1 function may be an important therapeutic option. There are reports of 69 somatic mutations of the CDH1 gene.[30] These comprised, in addition to a few missense mutations, mainly splice site mutations and truncation mutations caused by insertions, deletions, and nonsense mutations. There was a major difference in mutation type between diffuse gastric and infiltrative lobular breast cancers. In diffuse gastric tumors, the predominant defect was skipping of exons, which caused in-frame deletions. By contrast, most mutations found in infiltrating lobular breast cancers were out-of-frame mutations, which were predicted to yield secreted truncated E-cadherin fragments. In most cases, these mutations occurred in combination with loss of heterozygosity.[29,30]

Vimentin was identified in both tumor and stroma in this study. A small minority of ductal invasive breast carcinomas express vimentin in addition to keratins. The histogenesis of these cancers and the clinical significance of vimentin expression has remained unclear. From a formal point of view, myoepithelial histogenesis has been proposed and debated. Vimentin expression was interpreted by others as a sign of epithelial–mesenchymal transition, reflecting the final step of tumor dedifferentiation, which is generally associated with a high potential of tumor cell invasion. Vimentin-positive breast cancers are generally high grade with a high likelihood of metastasis.[31]

The annexin A5 protein was also detected in both tumor and stromal cells in our study. Annexin V radioisotopes (^{99m}Tc EC-annexin V) are undergoing interesting preliminary clinical studies (MD Anderson Cancer Center, Houston, TX). A recent human study had the translational aim to target early indications of apoptosis in breast cancer. The conclusion of this study, which involved 10 patients, showed apoptosis can be quantified using ^{99m}Tc EC-annexin V nuclear medicine imaging test, in 9 of 10 patients.[32]

Smooth muscle actin was detected in both tumor and stromal cells. Fibroblasts are abundant and essential stromal elements. Cancer associated fibroblasts are believed to promote tumor growth and progression. In breast and other epithelial tumors, reactive stroma is often adjacent to carcinoma cells and is believed to form in response to the malignant transformation of epithelial cells. Reactive stroma is characterized by activated fibroblast cells that express smooth muscle alpha actin (ACTA2), a modified extracellular matrix, and angiogenesis. Transcriptome analysis of invasive breast cancer has suggested that invasion occurs in part through the acquisition of a reactive phenotype in cancer associated fibroblasts.[33]

The tubulin beta-3 chain protein was detected in tumor cells in this study, and is the critical drug target for taxanes (paclitaxel and docetaxel), whose principle mechanism of action is the disruption of microtubule function. The antineoplastic effect of paclitaxel is mainly related to its ability to bind the beta subunit of tubulin, thus, preventing tubulin chain depolarization and inducing apoptosis. Antisense oligonucleotide (ODN) experiments confirmed that the inhibition of Class III beta-tubulin (TUBB3) could at least partially increase paclitaxel chemosensitivity. The hypothesis of a relationship between beta-tubulin tumor expression and paclitaxel clinical response has been recently verified in a series of 92

advanced breast cancer patients treated with a first line paclitaxel-based chemotherapy. In this study, 35% of patients with high Class III beta-tubulin expression showed progression of disease vs only 7% of patients with low expression. Therefore, this study suggests that Class III beta-tubulin tumor expression could be considered a predictive biomarker of paclitaxel-clinical resistance for breast cancer patients.[34]

It is evident that the majority of proteins cross-validated in this investigation do not belong to the low-abundance protein category. Hence, our future work will be focused on coupling present methodology with an additional mode of separation, aimed at enrichment of low-abundance proteins extracted from LCM-samples. Additionally, mass-spectrometry based tissue imaging can be used as complementary tool for spatial profiling of solid tumors in the context of tumor-specific marker identification.[35]

Clinical oncology is now being transformed from a subspecialty where patient treatment options are made in a categorical manner, to therapy assignments rationally derived from qualitative and quantitative molecular parameters. Additionally, emphasis in oncology is now focused on improved understanding of molecular pathways, and exploiting this for advanced therapeutics and novel diagnostic assays. In these regards, breast cancer treatment represents the model to which other cancer treatment regimens aspire. Central to this are rapidly evolving therapeutic strategies and guidelines based on hormone receptor and HER2 status, and in some cases gene profiling assays. To a large degree, breast cancer medication selection is a consequence, based on these defined molecular parameters obtained from the patient's tumor. Thus, therapy selection is more specific, with deliberate attempts to avoid toxicities from therapies not likely to be of benefit.[36,37]

As has been demonstrated in heart disease and HIV/AIDS, when robust biomarkers become available, remarkable clinical advancements occur quickly. Oncology is in desperate need of biomarkers for virtually all cancers, but especially solid tumors. The greatest potential for enabling biomarkers for cancer lies in improving the technology for protein biomarker discovery. Solid tumor heterogeneity is a perplexing scientific and clinical problem. Therefore, we sought to establish a new approach to solid tumor heterogeneity on a cellular basis, via analysis through an enhanced functional linkage between LCM and a solution-based MS method. Our sample prep technique employed organic solvent based lysis along with methanol facilitated solubilization and tryptic digestion.

In conclusion, the method described in this study, focused on solid tumor heterogeneity and potential cancer biomarker discovery, represents a powerful tool for proteomic profiling of human clinical specimens. The results of this investigation suggest that functional linkage between LCM and biological MS is of critical importance for proteomic profiling of the tumor and its stromal-based microenvironment. Cross-validation of selected protein species by independent immunoassay confirmed their presence in tumor proper and stroma, respectively, indicating potential applicability of the presented methodology as a supplementary tool for clinical molecular characterization of solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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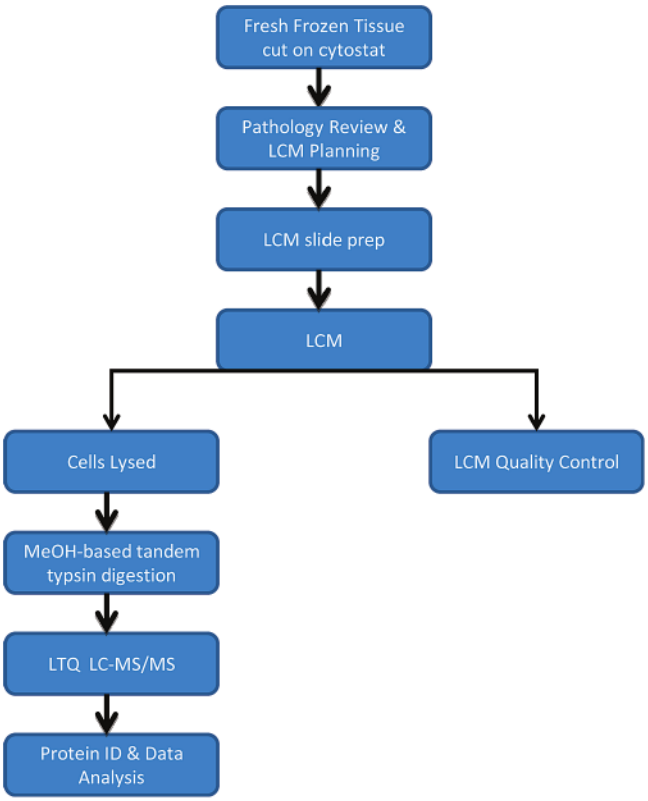

Figure 1.
LCM-MS workflow. Fresh frozen tissue was cut on a cryostat (8 μm thickness) followed by tissue mounting and H&E staining with coverslip of selected slides. A formal pathology review was performed by pathologist followed by LCM. The polymer membranes were carefully removed from the LCM cap and placed inside an Eppendorf tube. Hypotonic MeOH-based buffer along with sonication was employed for cell extraction, lysis and digestion. Samples were then prepared for MS analysis. Reverse-phase LC separation column was coupled with a LTQ for global shotgun proteomic analysis. CID spectra were analyzed on a 40-node cluster computer running BioWorks/SEQUEST.

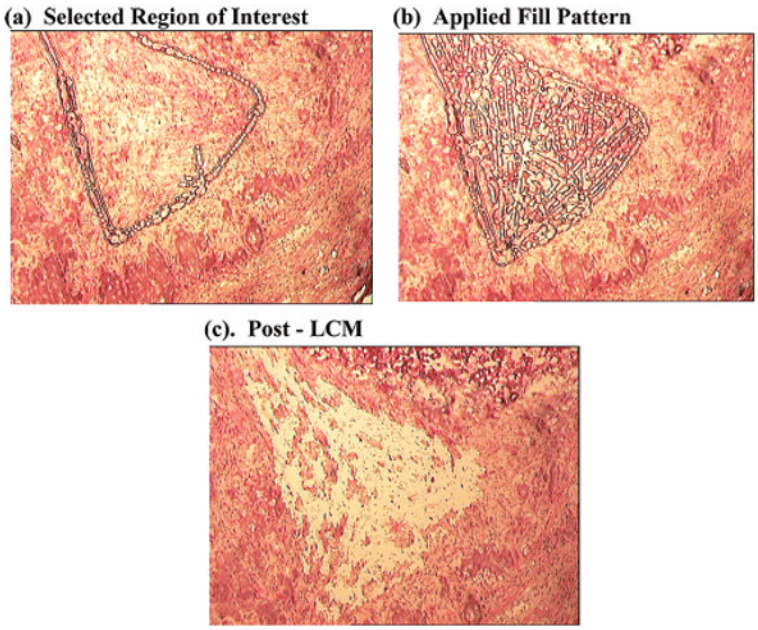

Figure 2.
The LCM workflow. LCM tissue extraction involves first establishing a histology area of interest (a), followed by a manually applied fill pattern to enable removal of a larger amount of cells (b). Upon removal of the LCM cap, the selected region of cells are extracted (c).

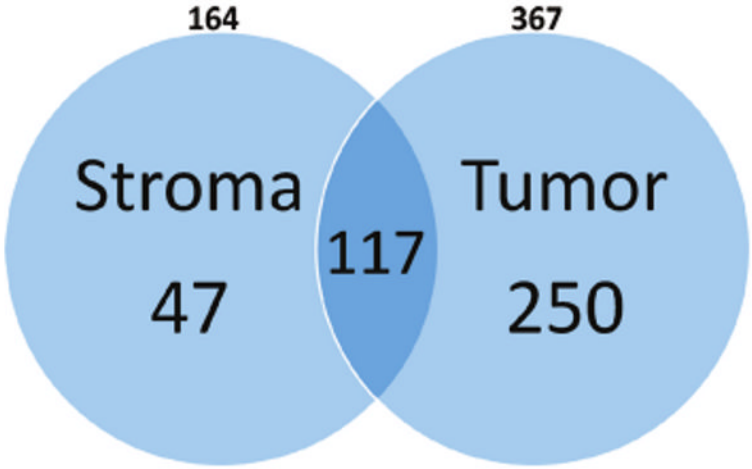

Figure 3.
Venn diagram depicting the relationship between proteins identified from tumor proper and tumor stroma. Of the 367 proteins strongly identified from tumor samples, 250 were unique to tumor, and 117 were common with stroma. Regarding the 164 proteins found in stroma, 47 were unique to this tissue.

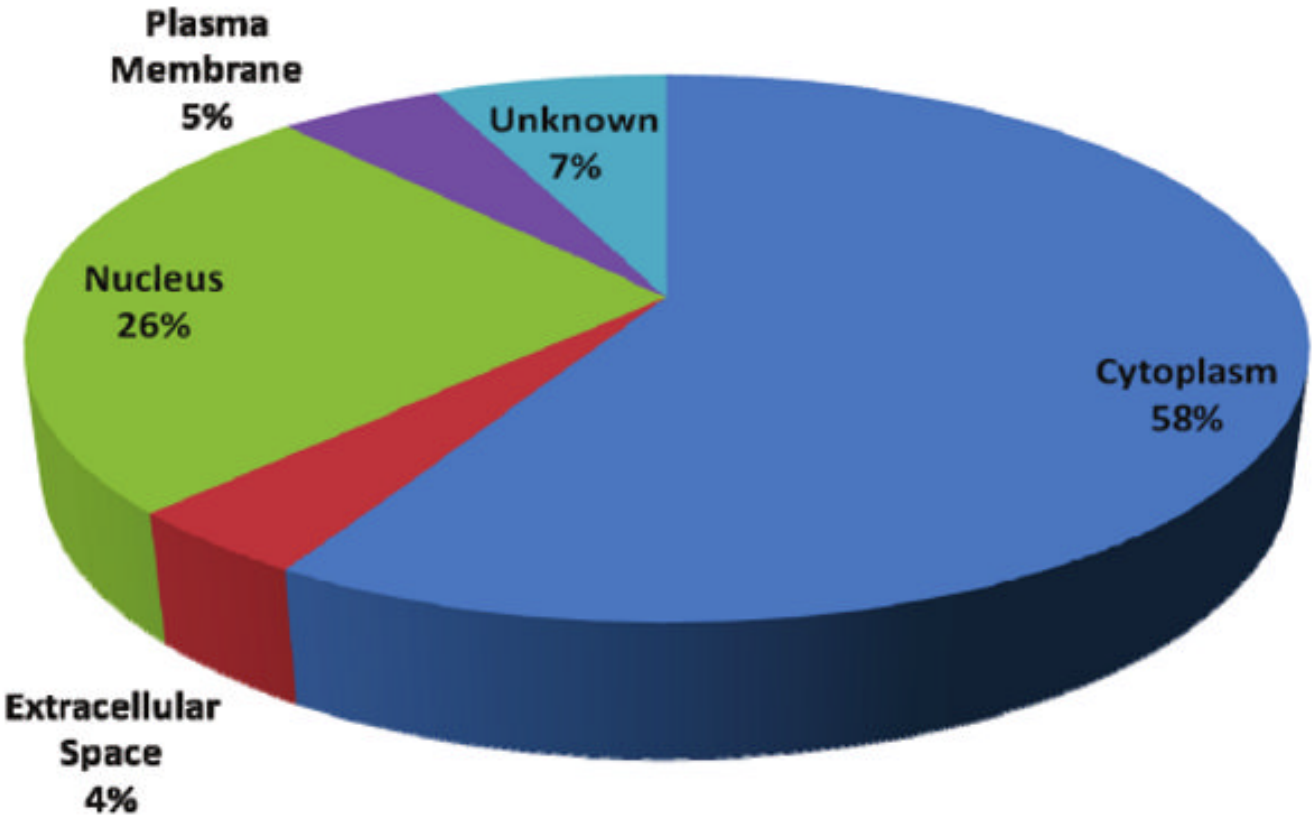

Figure 4.
Subcellular localization: tumor.

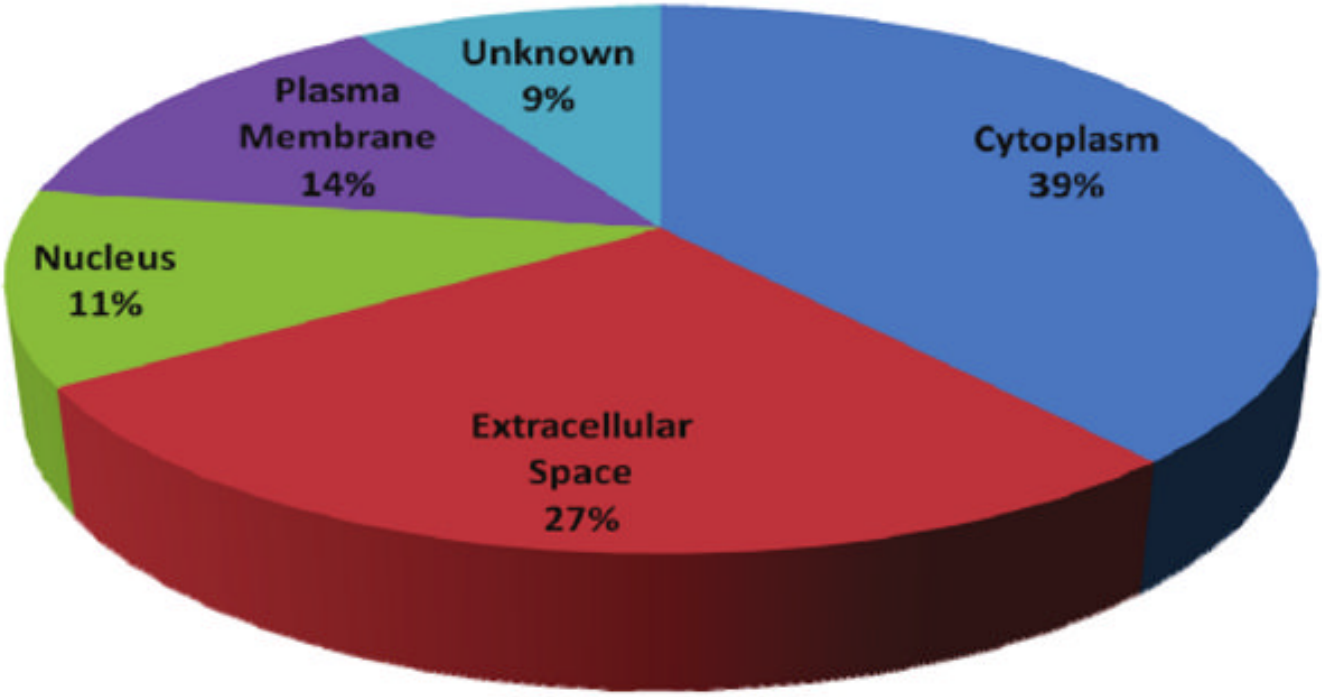

Figure 5.
Subcellular localization: stroma.

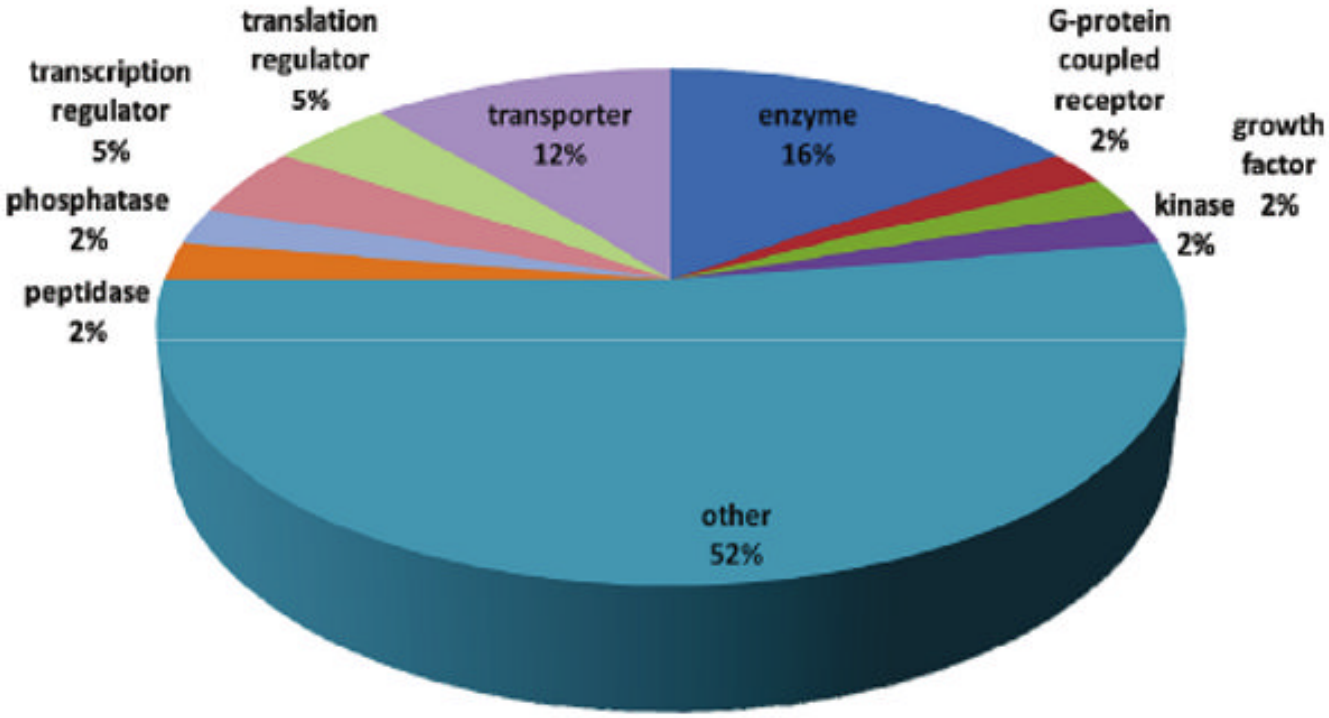

Figure 6.
Protein function: stroma.

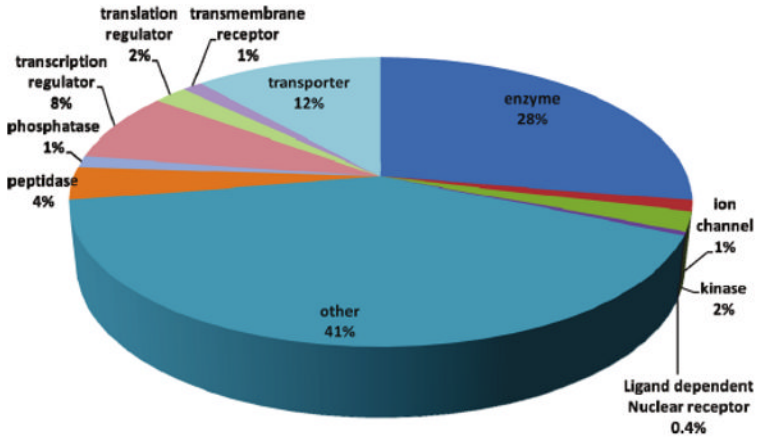

Figure 7.
Protein function: tumor.

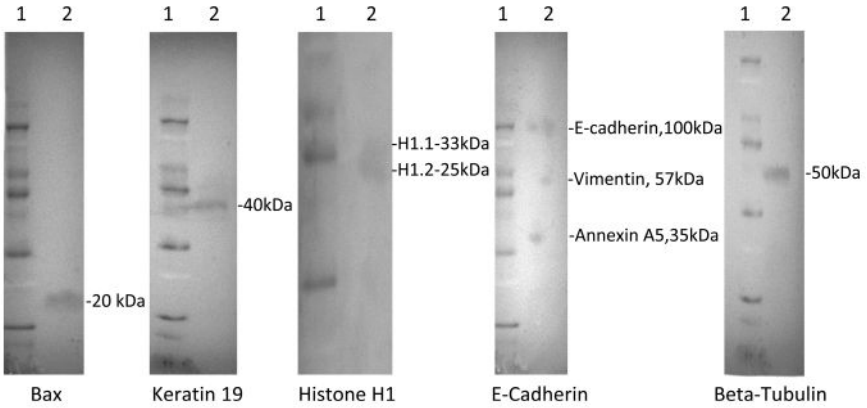

Figure 8.

Western blots of tumor related proteins. The six tumor related proteins are Bax, Keratin 19, Histone H1 (H1.1 and H1.2), E-Cadherin, Beta Tubulin. Note: this figure includes two proteins common to tumor and stroma, namely, Vimentin and Annexin A5. MW marker (1), blot (2).

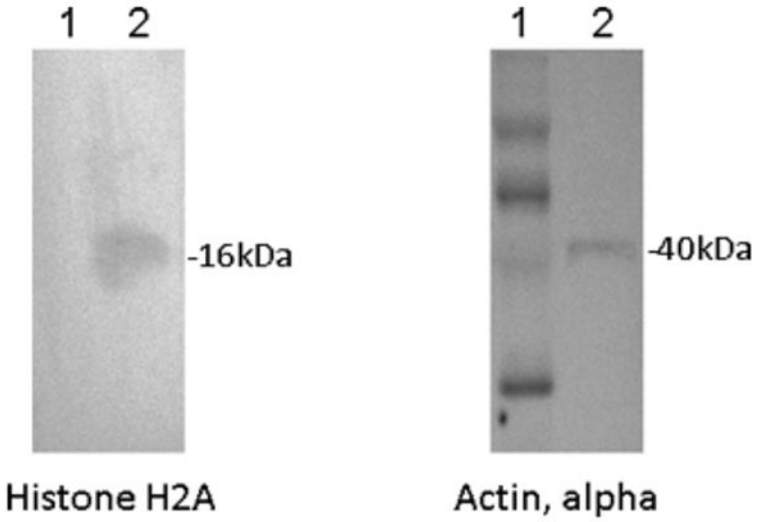

Figure 9.
Western blots of stroma related proteins. The two stroma related proteins are Histone H2A and Actin, alpha. MW marker (1), blot (2).

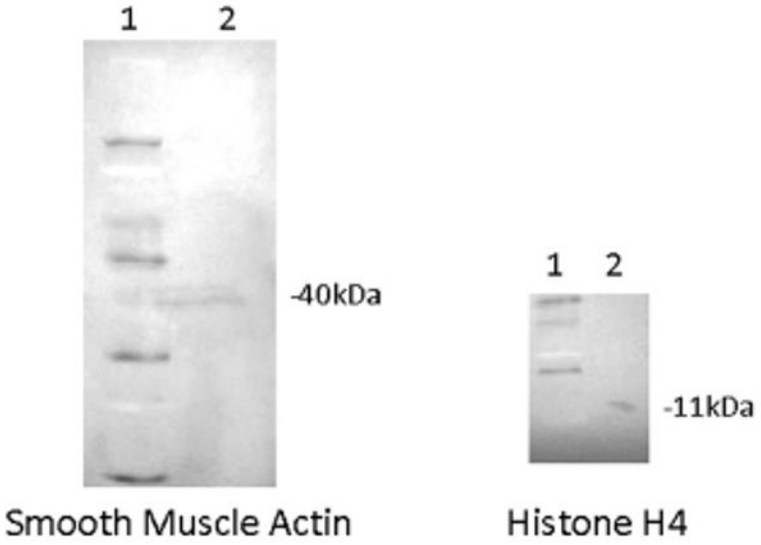

Figure 10.
Western blots of common (tumor and stroma) related proteins. This figure shows two of four common related proteins, namely, Smooth Muscle Actin and Histone H4. The other two common proteins (Vimentin and Annexin A5) are displayed in Figure 8. MW marker (1), blot (2).

Table 1

Summary of LCM-MS Analysis of Lymph Node with Breast Cancer Metastasis

tissue type	total peptides	unique peptides	total proteins	unique proteins ID via ≥2 peptides
Tumor	2656	1760	986	367
Stroma	1532	935	486	164
Total	4188	2695	1472	531

Table 2

Western Blots of Selected Proteins Identified in Tumor Proper and Stroma

tissue type	accession	protein	peptide count	gene name
Stroma	P68133	Actin, alpha skeletal muscle	2	ACTA1
Stroma	Q96QV6	Histone H2A type 1-A	4	HIST1H2AA
Tumor	Q13509	Tubulin beta-3 chain	2	TUBB3
Tumor	P08727	Keratin, type 1 cytoskeletal 19	20	KRT19
Tumor	Q02539	Histone H1.1	2	HIST1H1A
Tumor	P16403	Histone H1.2	2	HIST1H1C
Tumor	P12830	Epithelial cadherin precursor	2	CDH1
Tumor	Q07812	Apoptosis regulator BAX	2	BAX
Common	P08670	Vimentin	30	VIM
Common	P62736	Actin, aortic smooth muscle	16	ACTA2
Common	P62805	Histone H4	13	HIST1H4B
Common	P08758	Annexin A5	4	ANXA5