Study of Protein Differential Abundance Between Invasive and Metastatic Breast Tumor Cells Using Laser Capture Microdissection, Isotopic Labeling and NanoLC-MS/MS

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Introduction

The need for homogeneous samples of diseased and control tissue is critical for the study of proteomics. This need has led many research efforts to use cultured cell lines. However, it was found recently that patterns of protein expression could dramatically change during cell culture growth 1-3, which would eventually affect the relevance of such data as it pertains to the *in vivo* milieu. Laser capture microdissection (LCM) is a technique that can selectively extract cells of interest from their native tissue environment 4, and thus provide homogeneous and representative samples from heterogeneous tissue sections. We have previously reported preliminary results studying the stages of breast cancer by comparing normal with DCIS and metastatic LCM breast cells 5. In this work, we have applied the improved sample preparation protocol to the study of invasive and metastatic breast tumor cells using two patient cases. This work has led to the identification of approximately 1,600 proteins. 29 and 35 proteins were found with over 3-fold differential abundance between invasive and metastatic cells from the two patients, among which 6 proteins were common to both. Of the latter, three cell-cell junction proteins were found differentially degraded and two S100 proteins, related to the inhibition of cell migration, were down-regulated in metastatic cells.

Method

30,000-50,000 breast tumor cells at invasive and metastatic stages were collected using laser capture microdissection (LCM) from two different patient tissues. The cell samples were prepared following the procedures described in Figure 1. Briefly, the cells were first lysed, and the total protein concentration measured. The same amount of total protein from different stages was separated using SDS-PAGE into 7 fractions representing different molecular weight (MW) ranges. The tryptic digests of these 7 fractions from specific stages were labeled with ^{16}O or ^{18}O. Following these steps, the mixtures of the corresponding digest samples from given MW sections were analyzed using nanoLC-MSMS on a linear ion trap - Fourier transform ICR mass spectrometer (LTQ-FTMS). Protein quantitative ratios were measured based on the isotopic ratios calculated for the identified peptides.

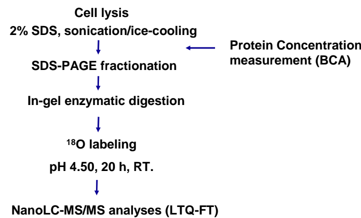

Figure 1 Sample Preparation Procedure for LCM Samples

Results

1. Comparison of Different Staining Methods on Peptide Identification

1) **In-solution digestion method:** Hematoxylin stained tissue led to only half peptide id compared to non-stained tissue; H&E only 1/3 of the peptide id from non-stained tissue.
2) **Acid/acetone wash to remove eosin after LCM**
3) **Results:** Similar number of peptides were identified from differentially stained tissues using SDS-PAGE as a fractionation method.
4) **Conclusions**: H&E staining, no problem!

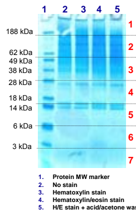

Figure 2 1D gel of Differentially Stained Mouse Liver Tissue Cell Lysates

Table 1 Number of Unique Peptides Identified from Three Gel Bands in the SDS-PAGE Gel of Differentially Stained Tissue Sections

Gel band	No stain (control)	Hematoxylin	H&E	H&E + acid/acetone wash
2	2412 (2321 [a])	2269	2393 (2350 [a])	2513 (2488 [a])
3	1851	1779	1849	1967
4	NA	721	790	NA

a number of unique peptides identified from the second analysis of the same sample.

2. Study of Invasive and Metastatic Stage of Breast Cancer

1) 30,000-50,000 LCM breast tumor cells at invasive and metastatic stages were studied using the methodology shown in Figure 1
2) The results are summarized in Table 2, with 1,008 and 1,559 unique proteins from each patient case.
3) The distribution of these identified proteins is shown in Figure 4. Twenty-four percent of the proteins belong to nuclear and 21% were from mitochondrion. Nine percent were membrane proteins, which demonstrated the effective cell lysis and protein solubilization in sample preparation.
4) Some previously reported cancer marker proteins were found with multiple peptides among the identified proteins. List of them is shown in Table 3.

Table 2 Results of Identified Peptides and Proteins from the Study of LCM Invasive and Metastatic Cells

Patient Cases	Case 131	Case 361
Total protein loading	9.1 μg	11.4 μg
Unique protein ID	1,008	1,559
Protein id with 1 pep	NA	172 (11%)
Unique peptide ID	3,310	5,778
Protein with >3 fold abundance ratio	29 (2.9%)	35 (2.2%)
Common proteins	6	

Note: Protein ID resulted from ProteinProphet probability ≥ 0.99
Peptide ID, Xcorr ≥ 1.8, 2.5, 3.75 for 1+, 2+ and 3+ ions; ΔCn ≥ 0.08, Rsp ≤ 5

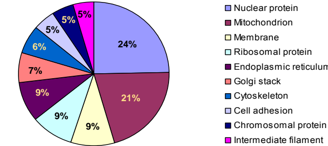

Figure 3 Distribution of Identified proteins in the cell

Table 3 Selected Identified Known Cancer Protein Markers [a]

	Unique peptide	Protein
1	20	**Receptor tyrosine-protein kinase erbB-2**
2	2	**Receptor tyrosine-protein kinase erbB-1**
3	28	**Creatine kinase, B chain**
4	7	**Epithelial-cadherin (E-cadherin)**
5	9	**Beta-catenin**
6	12	**CD166 antigen (ALCAM)**
7	10	**Membrane associated progesterone receptor component 2**
8	7	**Progesterone receptor-associated p48 protein**
9	4	**Membrane associated progesterone receptor component 1**
10	29	**Platelet-derived endothelial cell growth factor**
11	10	**Apoptosis regulator BAX**
12	6	**Plasminogen activator inhibitor-3**

a Esteva, F. J.; Hortobagyi, G. N., 109-118. Epub 2004 Mar 2011.

3. Proteins with differential abundance

Table 4 S100 Proteins Identified from Patient Case 361

Proteins	Unique Peptides	Ratio (Met/Inv)	Function
S100A4	2	0.3	tumor-promoting activity by inhibiting P53
S100A6	1	0.7	modulating calcium signaling that regulate the cell cycle
S100A7 (psorisan)	2	0.09	stimulates activity of Jab1, chemoattractant for CD4+ cells (T cells)
S100A8 * (calgranulin A)	3	0.19	migration inhibitory factor-related protein 8
S100A9 * (calgranulin B)	3	0.12	migration inhibitory factor-related protein 9
S100C	1	1.21	inhibition of tumor-cell motility
S100A13	1	0.38	release of fibroblast growth factor-1
S100P	2	0.90	
S100F	1	#	

* Common to patient cases 131 and 361.
# The peptide identified for this protein was the C-terminal peptide, which was not ^{18}O labeled, and therefore the relative quantitation ratio could not be determined.

Calgranulin A
ALNSIIDVYHK

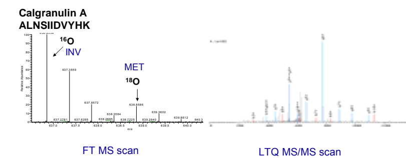

Figure 4 MS and MS/MS Spectra of a Peptide from Calgranulin A

Table 5 Differential Degradation of Desmosomal Proteins

Proteins	Unique Peptide ID (2 cases)	Ratio (Metastatic/Invasive)	
		Patient 131	Patient 361
Desmoglein 1 (113 kDa)*	12	4.5	7.1
Junction Plakoglobin (81 kDa)*	4	15.5	4.1
Desmoplakin (DP) (331 kDa)*	19	7.6	8.8
Plakophilin 1 (83 kDa)	5	47.0	
Desmocollin 1A/1B (100 kDa)	2	25.4	

* Common to patient case 131 and 361.

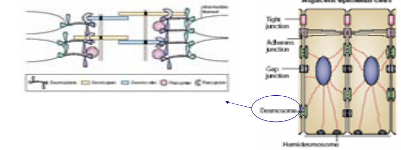

Figure 5 Structure of Desmosome in the Cell-Cell Junction

(Ref: Green, K. J.; Gaudry, C. A. Nat Rev Mol Cell Biol 2000, 1, 208-216.)

Desmoglein 1
ASAISVTVLNVIEGPVFRPGSK

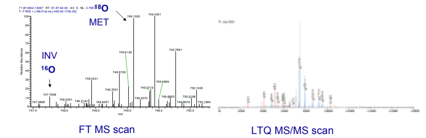

Figure 6 MS and MS/MS Spectra of a Peptide from Desmoglein 1

Conclusions

30,000-50,000 invasive and metastatic cells were collected using LCM from two patient tissues. Approximately 1,600 proteins were identified from the study. Using $^{16}O/^{18}O$ labeling and nanoLC-MS/MS, a group of desmosome proteins were found degraded in the tumor cells and the degradation was more active in the metastatic cells. On the other hand, another group of S100 proteins were observed down-regulated in metastatic cells compared to invasive cells. The S100 proteins are generally calcium-binding proteins, which are involved in the regulation of cell migration. Further experiments related to the validation of the potential marker proteins will be carried out using antibody-based methods such as Western blotting or synthetic isotopically labeled peptides.

References

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