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A Quantitative Proteomic Analysis of FFPE Melanoma

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To the Editor,

Currently, melanoma is diagnosed based on microscopic features, and some of these attributes, including tumor thickness, ulceration, mitotic index, and extent of lymph node involvement, have prognostic significance (1). Patients with melanoma detected at an early stage undergo surgery to remove the primary tumor, but some patients progress to advanced stage disease despite treatment (2). Thus, there is a major need for the identification of prognostic biomarkers of melanoma. Unfortunately, biomarker studies using frozen tissue from primary human tumors are problematic, due to the inherent instability and tissue heterogeneity of the samples. In contrast, formalin-fixed paraffin embedded (FFPE) tissue is very stable and can be coupled with laser microdissection for targeted sample isolation. However, harvesting enough cells and extracting the cross-linked proteins has been challenging.

We describe an approach that successfully extracts sufficient amounts of protein from FFPE tissue for mass spectrometric analysis and for free quantification of identified proteins. Similar approaches have proven successful for the analysis of other FFPE samples (3,4,5). Our approach is the first described for the comprehensive analysis of melanoma and melanocytic nevi using a coupled method with gel electrophoresis and spectral counting. For this proof-of-principle analysis, FFPE patient samples were collected from a single melanocytic nevus and single example of metastatic melanoma. Approximately 100,000 cells of melanocytic nevus and metastatic melanoma lesions were harvested with a Leica AS LMD laser microdissector. Proteins were uncross-linked and extracted with the Liquid Tissue MS Protein Prep Kit (Expression Pathology). Equal amounts of the proteins were split into 3 gel lanes and were analyzed by Coomassie/SDS-PAGE, which revealed the extraction of micrograms of protein (Figure 1). Each gel lane was sliced into 17 bands of 2 mm each and digested with trypsin. Tryptic peptides from the 102 gel bands were analyzed by LC-MS/MS with a Thermo LTQ-XL mass spectrometer coupled to an Eksigent nanoLC-2D (6). We identified a total of 888 proteins (0.45% false discovery rate using a decoy database from 56,013 spectra). Relaxing the stringency of the protein identification to a false discovery rate of 1.7% provided for the identification of 1,167 unique proteins from 88,180 spectra.

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In order to determine whether a protein was differentially expressed between nevus and metastatic melanoma samples, a label-free approach based on spectral counting was used (7,8,9,10). Spectral count is the number of tandem mass spectra assigned to a given protein in all bands from a single gel lane. To determine the relative amount of a protein in a given gel lane, a normalized spectral abundance factor (NSAF) was calculated (7). The NSAF was calculated as follows:

$$(NSAF)_k = \frac{(\frac{SpC}{L})_k}{\sum_{i=1}^N (\frac{SpC}{L})_i}$$

where k is a given protein, SpC is the spectral count, L is the length of the protein, and N is all proteins identified in the gel lane. Plotting the frequency distribution of $\ln(NSAF)$ values clearly showed that the data followed a normal distribution as indicated by the fitting of a Gaussian curve with an R^2 value of 0.99 (Figure 2A). In accordance to t-test, there were 390 proteins out of 888 total proteins that were found differentially expressed ($p < 0.05$) between metastatic melanoma and nevus lesions. The distribution of the p -values from the t-test was then divided into bins of size 0.025 and the number of proteins for each bin plotted in a bar graph (Figure 2B). The 32 most significant proteins, according to the lowest p -value from the t-test and adjusted with Bonferroni correction, were visually inspected by hierarchical clustering (Figure 2C). Two proteins of particular interest, silver and fatty acid synthase (SILV and FASN), were found in the top 10 most significant proteins and appeared as up-regulated in metastatic melanoma as compared to melanocytic nevus. SILV and FASN both have been shown to be up-regulated in cancers, which makes them appropriate validation tools for this proof-of-principle study (11,12,13).

For validation, melanocytic nevus and melanoma samples were stained with either SILV or FASN antibodies and the samples were scored based on the intensity of the staining and the percentage of extent (Figures 3 & 4). The intensity of staining was scored as nil (0), low (1), medium (2), or high (3). The percentage of melanocyte or melanoma cell staining was scored as 0 (no staining), 1 (<25% staining), 2 (25–50%) and 3 (>50% staining). The intensity was multiplied by the percentage of extent and the following product is then categorized as such: 0–1 is 0; 2–3 is 1+; 4–5 is 2+; 6–9 is 3+. In general, most of benign lesions (benign nevi or dysplastic nevi) have low or no expression (0 or 1+) of SILV or FASN. Many of the melanomas have higher expression (2+ or 3+) of SILV or FAS. Table 1 shows the number of cases with each score for SILV and FASN indicating higher staining in melanoma compared to benign. Both SILV and FASN were found to be significantly different by Chi square analysis between melanoma and benign with p -values of >0.0001 and 0.0015, respectively.

In conclusion, we present an unbiased, high throughput and quantitative approach for the identification of proteins that are differentially expressed in metastatic melanoma. Using quantitative label-free mass spectrometry of laser microdissected samples, we have identified 390 proteins differentially expressed in melanoma. Two of these proteins, silver and fatty acid synthase, were validated as being up-regulated in melanoma. Our proof-of-principle analysis lays the foundation for an extensive examination of archived human melanoma tissues for the discovery of biomarkers that will help clinicians with diagnosis, prognosis and treatment of this cancer.

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Figure 1. Technical triplicate analyses of metastatic melanoma and nevus samples demonstrate the reproducibility of the quantitative mass spectrometric approach for the analysis of FFPE tissue samples

Proteins were extracted from one paraffin embedded nevus and metastatic melanoma sample. The samples were equally split into 3 aliquots and resolved by SDS-PAGE/ Coomassie. Each of the 6 gel lanes was entirely sliced into 17 bands, subjected to trypsin digestion, and peptides were analyzed by LC-MS/MS with a Thermo LTQ-XL mass spectrometer equipped with an Eksigent nano2D-LC. Proteins were identified with Mascot (95% confidence threshold). Pearson correlation analyses were performed to test reproducibility.

Figure 2. Label-free quantification of proteins identified from FFPE nevus and metastatic melanoma tissues

A) NSAF values of proteins identified from both FFPE nevus and metastatic melanoma tissues have a Gaussian distribution. The natural log of the NSAF values of all proteins identified are sorted into bins of 0.3. A Gaussian fit of the data is shown ($R^2=0.99$). B) P -values of proteins identified from both FFPE nevus and metastatic melanoma samples are sorted in bins of 0.025. Proteins found by t-test to be differently present between nevus and melanoma are shown shaded ($p<0.05$). C) Clustered heat map of the 32 most significant proteins differentially present between nevus and melanoma samples as determined by t-test and adjusted with Bonferroni correction ($p<0.05$).

Figure 3. SILV is up-regulated in melanoma

Immunohistochemical staining of benign melanocytic nevus (A) and malignant melanomas (B – D). Many of the melanoma cells have strong cytoplasmic staining of SILV. All of the pictures are taken at the same magnification (100×).

Figure 4. Fatty acid synthase is up-regulated in melanoma

Immunohistochemical staining of benign melanocytic nevus (A) and malignant melanoma (B). There are rare nevus cells with cytoplasmic staining whereas many of the melanoma cells are positive. The sebaceous glands are positive for FAS. Both pictures are taken at the same magnification (200 ×).

Table 1
Scoring of SILV and FASN staining

A category was assigned based on the intensity of staining multiplied by the percentage of extent. Both SILV and FASN are up-regulated in melanoma with a Chi square p-value of <0.0001 and 0.0015, respectively

Ab	Sample	0	1+	2+	3+
SILV	Benign or Dysplastic nevi	14	4	0	1
SILV	Melanoma	2	0	3	8
FASN	Benign or Dysplastic nevi	10	2	1	1
FASN	Melanoma	1	0	3	5