Fine-needle aspiration in PreservCyt®: a novel and reproducible method for possible ancillary proteomic pattern expression of breast neoplasms by SELDI-TOF

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Proteomic profiles of tumor protein expression by the surface enhanced laser desorption-ionization time of flight (SELDI-TOF) methodology have been shown to have a potential usefulness for protein discovery as well as screening, diagnosis, prognosis and therapeutic considerations of cancer from several organ systems. Fine-needle aspiration (FNA) specimens from tumor samples is an accepted method to diagnose the cells of interest but often can be a limited assessment due to quantity of the sample. The current use of fresh or rapidly frozen specimens for proteomic profiling can be burdensome for clinicians to collect and submit specimens. The current study tests the hypothesis that placement of FNA and other cytological material in PreservCyt® may be an acceptable method of sample handling for protein profiling evaluation by this method though it may not be suitable for true protein discovery or characterization. Excised fresh breast tissue for evaluation and/or treatment of a variety of breast lesions were sampled by FNA technique and placed into PreservCyt®. These samples were then homogenized under denaturing conditions and evaluated by the SELDI-TOF methodology. Most samples collected showed a satisfactory quantity of protein for analysis by the SELDI-TOF methodology. Protein patterns from a variety of benign and malignant lesions revealed reproducible patterns on triplicate testing. Benign lesions had similar protein patterns across age groups in this limited series that may have potential diagnostic significance. In conclusion, FNA of breast tissue placed in PreservCyt® is a potentially acceptable method of sample handling for evaluation by the SELDI-TOF methodology for establishment of reproducible protein patterns. Preliminary results from a spectrum of breast lesions suggest these patterns may have potential for ancillary testing for diagnostic consideration of breast lesions. This collection methodology could simplify sample gathering for further testing of all types of cytological specimens by the SELDI-TOF methodology. Larger studies will be needed to assess this methodology as a diagnostic aid.

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Fine-needle aspiration (FNA) is a well-accepted method of obtaining cells for the diagnosis of tumors for routine cytological evaluation.¹ However, clinician performed aspirates, those aspirates without immediate interpretation, or without collection by special methodology may fall short of being diag-

nostic. This may be due to nondefinitive cytological features or due to insufficient sampling for applying currently accepted ancillary testing such as immunohistochemical staining or immunocytochemical staining with proper controls. Current ancillary testing also does not appear conclusive for invasive *vs in situ* lesions of the breast² thus resulting in a nationwide decline in utilization of aspiration as a diagnostic technique for breast cancer diagnosis and current clinician therapeutic decision making (ie, sentinel node biopsy). This has resulted in a more costly and invasive approach by image-guided core sampling for all lesions of the breast even when breast lesions are palpable. Thus, a search for a better ancillary testing method to accompany the cytological appraisal of the FNA that can become

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timely, cost efficient, as well as comparing diagnostically favorable or surpassing the sensitivity/specificity of core-biopsy or excision of breast lesions continues.

Proteomic profiling is a relatively new methodology for assessing cancer by the proteins expressed by the neoplastic cells and/or the host response.³ Surface enhanced laser desorption-ionization time of flight (SELDI-TOF) methodology from Ciphergen Corporation is currently one of the methods for proteomic profiling. Most reports have been done on serum samples, fresh or frozen body fluids and nonfixed tissue.^{4,5} It is known that various preservatives crosslink proteins making them difficult to evaluate by SELDI-TOF methodology and interferes with protein characterization and identification. This is especially true of formalin-fixed tissues or alcohol-fixed cytology specimens. Laser capture of the cells of interest from frozen tissue blocks or archival material has been attempted but is time consuming and not practical for routine specimens in the context of timely diagnosis. The formalin-fixed archival material as noted previously also greatly interferes with the protein evaluation due to cross-linking of proteins. However, Fetsch *et al*,⁶ have shown successful proteomic profile pattern analysis of rapid Romanowsky-stained cytocentrifuged FNA that had been fixed in methanol. Many of the collection methods currently being utilized for proteomic evaluation necessitate special handling that limits their practicality for everyday use, especially for clinicians not within the academic community or near a testing-site that utilizes proteomic evaluation of patient material. A major roadblock for proving the capabilities of proteomic profiling is the gathering of sufficient number and variety of tumor samples to evaluate in a statistically significant manner. If evaluation of tumor proteomic profiling is to be done on a large scale or later offered as a practical ancillary test for routine clinical specimens then a simple and convenient methodology of collection must be developed that will assure a reproducible diagnostic pattern from the material collected.

Studies have shown that fine-needle technique concentrates the cells of interest in what best can be described as inexpensive and quick method or 'the poor man's laser capture' of cells.¹ However, it is uncertain if diagnostic proteomic profiles or patterns must be based upon having actual tumor cells present to evaluate or whether just the proteins expressed by the tumor cells and/or the host milieu are adequate for diagnostic significance based upon the serological proteomic studies.⁷

Our study as a pilot project was designed to evaluate the feasibility of cytological specimens being placed in PreservCyt® as a practical collection methodology for utilizing proteomic profiling as an ancillary testing method of cytological specimens. PreservCyt® is primarily a methanol-based fixative that is readily available in many clinicians' offices.

Secondly we wished to see if we could obtain reproducible patterns that allowed for separation of benign and malignant specimens for diagnosis.

Materials and methods

Surgical breast tissue specimens for evaluation of radiological or palpable abnormalities and/or prior diagnosed breast cancers undergoing definitive therapy that were received as fresh specimens at University Health System, San Antonio, Texas from April 2002 to August 2002 underwent aspiration of visible gross masses or random aspiration sampling if no gross lesion was identified with #22 gauge needles (MOL, LJF). The aspirations were directly rinsed within PreservCyt® solution (Cytoc Corporation, Boxborough, MA, USA) and stored at room temperature for periods of 3 days up to 6 weeks. To harvest the tissue for a long-term storage, the samples were pelleted by centrifugation from the PreservCyt® suspensions and washed once in phosphate-buffered saline (PBS). Small pellets (about 5–50 mg wet tissue) were then homogenized on ice in 100 μ l urea/CHAPS buffer with protease and phosphatase inhibitors. Clear lysates were collected by centrifugation and stored in small aliquots at -80°C . Two 5 μ l aliquots were used to determine protein concentration using BCA assay (Pierce). Immobilized metal affinity arrays (IMAC3/Cu) were activated according to the manufacturer's protocol and diluted samples were applied in duplicate to the ProteinChip array by a 96-well bioprocessor (Ciphergen Biosystems, Inc., Fremont, CA, USA) in a Biomek2000 robotic workstation (Beckman Coulter). Activation and sample loading was accomplished within 4 h time frame. Proteins were detected with the ProteinChip Reader PBS II. Average time for processing one ProteinChip® in the ProteinChip Reader PBS II was about 10 min. Each sample was analyzed in three separate runs. Further proteomic data mining was performed with the Biomarker Wizard software. *M/Z* ranges from 10 000 to 32 000 were compared by the Biomarker Wizard software that had been separated into malignant and benign categories based upon the histological specimen diagnosis. Histological diagnoses with pertinent prognostic data stripped of patient identifiers were also compared with the SELDI-TOF patterns visually (LJF) to evaluate for reproducible differences in the protein profiles between the variety of benign and malignant diagnoses in this limited series.

Results

Our initial run of 26 fresh surgical cases produced 24 useable FNA protein samples (two were lost in transport or lacking adequate protein by BCA assay). The 24 cases that contained adequate protein by BCA assay included seven benign breast lesions and

11 malignant or premalignant breast lesions. The other six cases were three benign and three malignant samplings from sites other than the breast. The histological classified benign breast lesions consisted of ductal and lobular hyperplasia (one), minimal focus of 'atypical' hyperplasia (one), apocrine metaplasia (one), fibroadenoma (three), fat necrosis and fibrosis (one). Those lesions histologically classified as malignant consisted of ductal carcinoma (six), DCIS (two), lobular carcinoma (two), LCIS (one). (See Figure 1 for histological diagnosis, grading, staging and patient demographics with SELDI-TOF patterns.)

Protein quantities measured by BCA assay revealed quantities of protein from 0.05 to 2.2 mg/ml harvested from the PreservCyt® samples.

M/Z ranges from 10 000 to 32 000 when compared by the Biomarker Wizard software revealed differences in the *M/Z* ranging from 10 000 to 32 000 in the breast cancer *vs* the nonmalignant breast biopsies.

Visual comparison of protein profiles revealed reproducible similar patterns across age groups for the majority of the benign diagnoses though not definitively separating the subsets of benign lesion types (Figure 2). Protein profiles for the malignant lesions appeared visually quite dissimilar from the benign patterns with suggestion of variations between the subtypes of carcinomas (Figure 3), grade (Figure 4) as well as between *in situ* and invasive categories (Figure 5) when compared with the histological diagnoses though not with sufficient cases for statistical analysis or validity. One sample was found to lack measurable protein peaks on all three runs (sample #27) for unknown reasons that appeared unrelated to storage time in the PreservCyt® solution. A similar 'nonpattern' also occurred on one of the runs of case #9 while run 3 revealed distinct protein peaks. Both showed protein levels felt to be adequate by the BCA assay. This would suggest that the problem was unrelated to sampling limitation and might be secondary to application of specimen to the chip, chip manufacturing variation or other proteomic process problem. It is known by prior studies that chip to chip variation or chip surface activation may result in test to test variation. Since time storage interval in PreservCyt® solution and BCA assays did not differ from specimens #9 and 27 with those specimens with distinct pattern we felt collection methodology was not the source of the nonpattern error.

Discussion

Our preliminary results reveal reproducible protein profile patterns by SELDI-TOF methodology of cytological samples placed in PreservCyt® on three separate runs of the same sample. When compared to the histological diagnoses of the concurrent surgical specimens there were similar patterns

expressed by all benign lesions across age and patient differences. This suggests potential diagnostic usefulness in separating benign from malignant lesions of the breast by this methodology in the majority of specimens when BCA assay reveals adequate protein sampling. Sample and test size is too limited in this pilot study to show if the additional protein peaks within the protein profile patterns will have diagnostic or prognostic significance when compared to histological type or grade of breast cancer.

These limited results warrant further analysis of a larger cohort of patients for evaluation and validation of this methodology to evaluate proteomic's pattern assisting FNA or other cytology diagnosis. We are currently collecting parallel specimens in buffered normal saline to compare with the PreservCyt® fixed specimens as well as to utilize for novel protein identification. We are also currently preparing a direct smear from one aspirate of each case to compare with protein quantitation (currently 130 breast lesions collected with 30 controls for statistical analysis to be published at a later date). These initial samples have also been preserved to evaluate consistency between new chip batches as well as with newer generation profiling technology (ie: Q-STAR).

Assuming sample number of 20–40 specimens for an automated proteomics lab, the above procedures can be accomplished easily within a day even by a less-experienced investigator. The system (Biomex2000 in conjunction with ProteinChip Reader PBS II) offers much higher throughput capability. If following wider testing and validation by other laboratories of this methodology, a likely scenario for sample handling could include tissue collection in PreservCyt® at a remote site, and shipment by surface mail to the analytical laboratory within days to a few weeks from the time of the biopsy. Further controlled testing of the limits of storage time, temperature, chip batch variation will all be necessary and could be problematic. The proteomics laboratory could process the tissue upon receipt and store it as a frozen homogenate for months at –80°C. With the above preprocessing of PreservCyt® specimens, multiple batches of ProteinChips® (multiples of 12) can be handled in bioprocessor and hundreds of samples can be fully analyzed in a few days, providing diagnostic information by proteomic patterns that could possibly then be correlated with the cytological or histological diagnoses especially for 'atypical' lesions when a more definitive diagnosis is needed. Extensive further controlled testing of this methodology must be done prior to consideration for patient care.

Conclusions

Cytological specimens placed in PreservCyt® appear capable of allowing a reproducible protein

Case #	Pt. Sex	PT. Age	Tumor Location	Histological Diagnosis	IP or Prognostic Markers	Aspirate Sample	SELDI-TOF Patterns
1	M	59	Mediastinum	Metastatic poorly differentiated adenocarcinoma mucin positive, favored lung primary	Elevated CEA	OK	
2	F	50	Breast	Invasive ductal carcinoma, grade 2/3, 3 of 7 nodes positive, Stage IIA	ER/PR +, Her-2 -, p53 -, Ki67 07%	Lost	NONE
3	F	81	Lymph node, neck	Metast adenoca with papillary features	Thyroglobulin & TTF-1 Negative	OK	
4	F	41	Breast	DCIS with comedonecrosis, nuclear grade 2/3, TisN0Mx	4 sentinel nodes negative, no other markers	OK	
5a	M	39	Colon	Adenomatous polyps	None	OK	
5b	M	39	Rectum	AIS arising in villous adenoma, Tis,N0,MX	Post adjuvant radiation/chemo, CEA not elevated	OK	
6	M	58	Colon / appendix	AICI from perforated appendix,	No CEA done	OK	

Figure 1 Table of surgical pathology diagnosis and proteomic patterns by FNA.

				NEOM			
9	F	72	Breast	NEOM, Ductal hyperplasia, single focus of atypical hyperplasia, fibrocystic changes		Varies	
10	F	52	Breast	Ductal carcinoma, grade 3/3, TcNOMX	ER/PR -, Her-2 +, p53 -, Ki67 64.5%	OK	
11	F	57	Breast	Usual hyperplasia, fibroadenoma, apocrine metaplasia, prior biopsy site		OK	
12	F	52	Breast	Ductal & lobular hyperplasia, recent excision	Sentinel node negative	OK	
13	F	50	Breast	Ductal hyperplasia & fibroadenoma	None	OK	
14	F	37	Breast	Ductal carcinoma, grade 3/3	ER/PR -, HER2 -, p53 +, Ki67=75%	OK	

Figure 1 (Continued).

15	F	42	Breast	Lobular carcinoma, 1.2cm diameter, node negative	ER+,PR+, Her2 -, p53 -, Ki67=15%	OK	
16	F	70	Breast	Lobular carcinoma, 1 cm lesion, negative sentinel node	ER/PR +, Her-2 -, p53 -, Ki67 10%	OK mislabel	
17	F	68	Breast	NEOM, apocrine metaplasia, fibrosis	None	Not run	Run1 ? Run3 ?
18	F	31	Breast	Fibroadenoma	None	OK mislabel	
19	F	27	Colon	NEOM, healed perforation tract	Prior trauma	OK	
20	F	84	Breast	NEOM, fat necrosis, 5 sentinel nodes negative	Prior ductal cancer (recent)	OK	
21	F	37	Breast	NEOM, Apocrine metaplasia & fibrosis	None	OK	
22	F	58	Breast	Invasive ductal carcinoma, grade 3/3, N0 (sentinel nodes)	ER/PR -, Her2-, p53+, Ki67=62%	OK	

Figure 1 (Continued).

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pattern by SELDI-TOF methodology that may have diagnostic significance. Preservation of quantifiable protein for a period of up to 6 weeks at room temperature was documented. The proteomic patterns from this collection and evaluation method suggests this may be a helpful ancillary method with diagnostic potential to improve the specificity of cytopathology interpretation once sufficient

testing can be done for statistical analysis. The results warrant further analysis of a larger cohorts of breast cytological specimens as well as from a variety of organ sites and cytological sample types such as bronchoalveolar lavages, urines, etc utilizing this simple transport system to determine if this will be a continuing reproducible and future costeffective methodology for ancillary testing of

Figure 2 Benign breast peak patterns. All benign histological diagnoses had similar proteomic patterns by this methodology.

Figure 3 Protein patterns lobular vs ductal carcinoma. In this limited case study invasive carcinomas had variable patterns but distinctly different from benign cases.

Figure 4 Protein patterns low grade vs higher grade lobular carcinoma. Increasing tumor grade appeared to have increasing protein peaks within this limited series.

cytology samples beyond breast FNA. We would strongly caution against the use of the proteomic profile as an ancillary test of cytology specimens currently until wider testing/validation of this

methodology and confirmation that earlier problems with chip batch reproducibility problems are resolved.

Figure 5 Protein patterns *in situ* vs invasive. In this limited series invasive carcinomas appeared to have a more complicated protein peak profile as compared to *in situ* lesions.

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