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ProteinChip® Array Analysis of Microdissected Colorectal Carcinoma and Associated Tumor Stroma Shows Specific Protein Bands in the 3.4 to 3.6 kDa Range

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Abstract. *Multiple pathways of carcinogenesis have been associated with colorectal carcinomas, including the adenoma-carcinoma sequence. The non polyposis coli gene has also been implicated in the pathogenesis of these tumors. Identification of the epithelial- mesenchymal interaction may help in understanding the pathways of invasion and may lead to the development of new, non-invasive tools for the diagnosis and prognosis of colon carcinomas. A ProteinChip® Array technology (SELDI=Surface Enhanced Laser Desorption Ionization) has been developed enabling analysis and profiling of complex protein mixtures from a few cells. This study describes the protein analysis of approximately 500-1000 freshly obtained cells from normal and malignant colonic epithelium and its associated stroma by SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry). Pure cell populations of normal and malignant epithelium as well as stroma (without tumor cells) were selected by microdissection from 9 patients. A pattern of 3 peptides of 3.48, 3.55 and 3.6 kDa, which were increased in the colon tumor epithelium and stroma compared to associated normal colon and stroma in all 9 patients, was observed. Coupling microdissection with SELDI represents a powerful tool to identify cell and tumor specific proteins and to understand molecular events underlying the invasive event in colorectal carcinomas. The presence of certain proteins in invasive carcinomas may lead to the development of non invasive biomarkers for the identification or detection of recurrence of colorectal malignancies.*

Colorectal carcinoma is one of the leading causes of cancer death in North America and Western Europe (1). In most cases tumor development is associated with adenomatous polyps. There is significant epithelial-stromal interaction which may inhibit invasion of malignant clones to a certain point. Multiple steps have been proposed in the development from non invasive to invasive clones, both on the basis of molecular changes and on the basis of protein interactions (2). Early diagnosis of colorectal carcinomas is of great importance as endoscopic removal of precursor lesions may eliminate the malignant potential of clinically harmless lesions. Serum markers, similar to markers for the diagnosis of prostatic carcinomas, are not available for the diagnosis of colorectal carcinomas. Therefore identification of novel biomarkers may represent a powerful tool for improving tumor diagnosis and surveillance.

Increasing emphasis is being laid upon the analysis of cellular proteomics, in addition to transcriptional studies with cDNA arrays, which can accelerate identification of new diagnostic markers (3). The classic 2-D polyacrylamide gel electrophoresis (2D PAGE) (4, 5) has been replaced by the application of automated systems such as the SELDI-TOF (6) by using a ProteinChip® (Ciphergen Biosystems, Fremont, CA, USA) array of addressable protein binding sites on a solid substrate. Captured individual proteins from complex mixtures are subsequently resolved by mass

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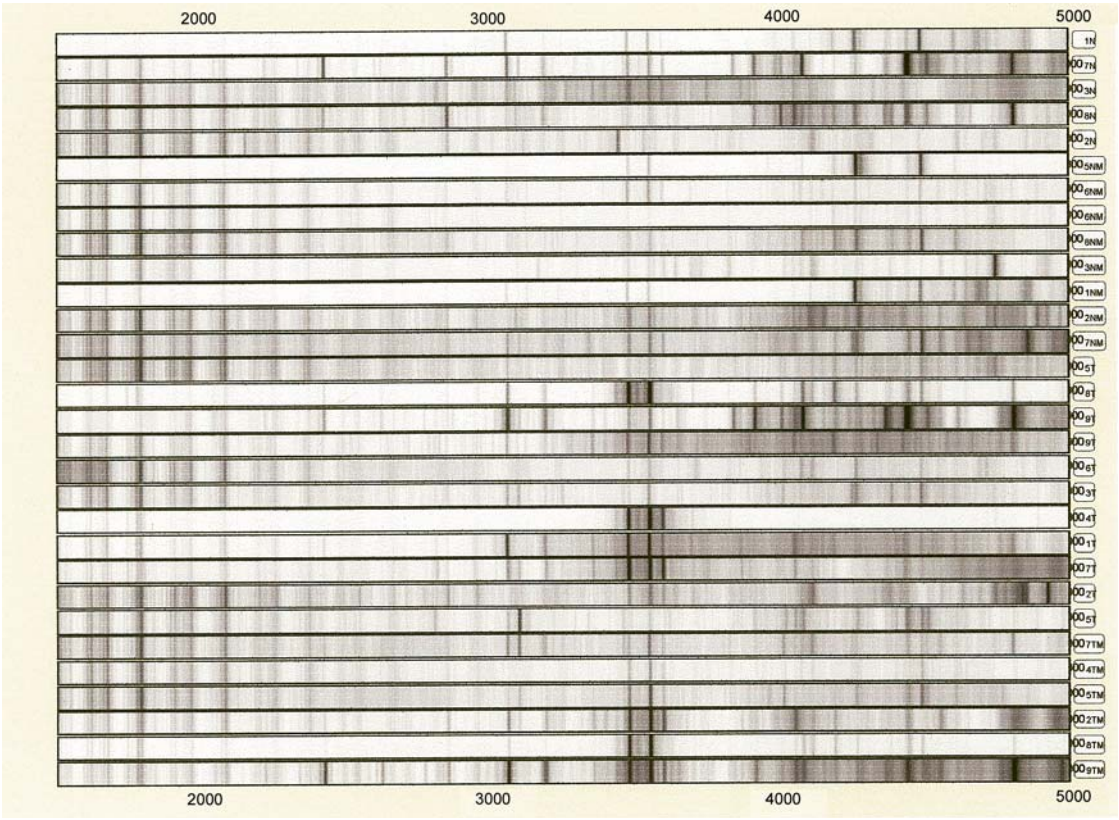

Figure 1. *The results in a gel view for the 1500-5000 Da/mass ranges.*

spectrometry. This fast novel technology combines several advantages over 2-D gels. Most importantly, it requires far less starting material, has a higher reproducibility and allows analysis of the presence of proteins in the femtomole range (7-9).

This report describes our initial studies using microdissection in conjunction with SELDI and a newly elaborated analysis software (Ciphergen Proteinchip software 3.02) to detect potential new diagnostic markers for the existence and invasive properties of colorectal carcinoma.

Materials and Methods

Tissue procurement. Colectomy specimens from patients with invasive colorectal carcinoma were obtained from 9 patients. All identifiers were removed from samples and they were analyzed anonymously. None of the patients had been treated with radio-, chemo-therapy prior to colectomy. A staff pathologist from the Department of Pathology, University of Pennsylvania, USA, was present at the time of tissue removal in the operation room. Tumor and normal tissue were immediately resected and submerged in RNA later® and frozen at -70˚C within 5 minutes. Further preparation of the organ by a surgical pathologist and histological evaluation guaranteed correct grading, TNM (tumor, node, metastasis) classification and application of all diagnostic standards.

Table I. *Summary of patient age and tumor stage.*

	Age	Tumor stage
Patient Control	73	pTis
Patient 1	58	pT2b pN0
Patient 2	59	pT3a pN0
Patient 3	64	pT2a pN0
Patient 4	62	pT2b pN1

Microdissection. Epithelial and underlying mesenchymal cells from both malignant and benign counterparts were microdissected. These included colonic epithelial cells and malignant epithelial tumor cells. The areas for microdissection chosen from underlying stroma in invasive carcinomas did not contain appreciable tumor cells. Microdissection was performed by preparing slides on the cryostat which were stained slightly. Based on careful review of histological sections, the cell samples contained more than 95% of desired cells.

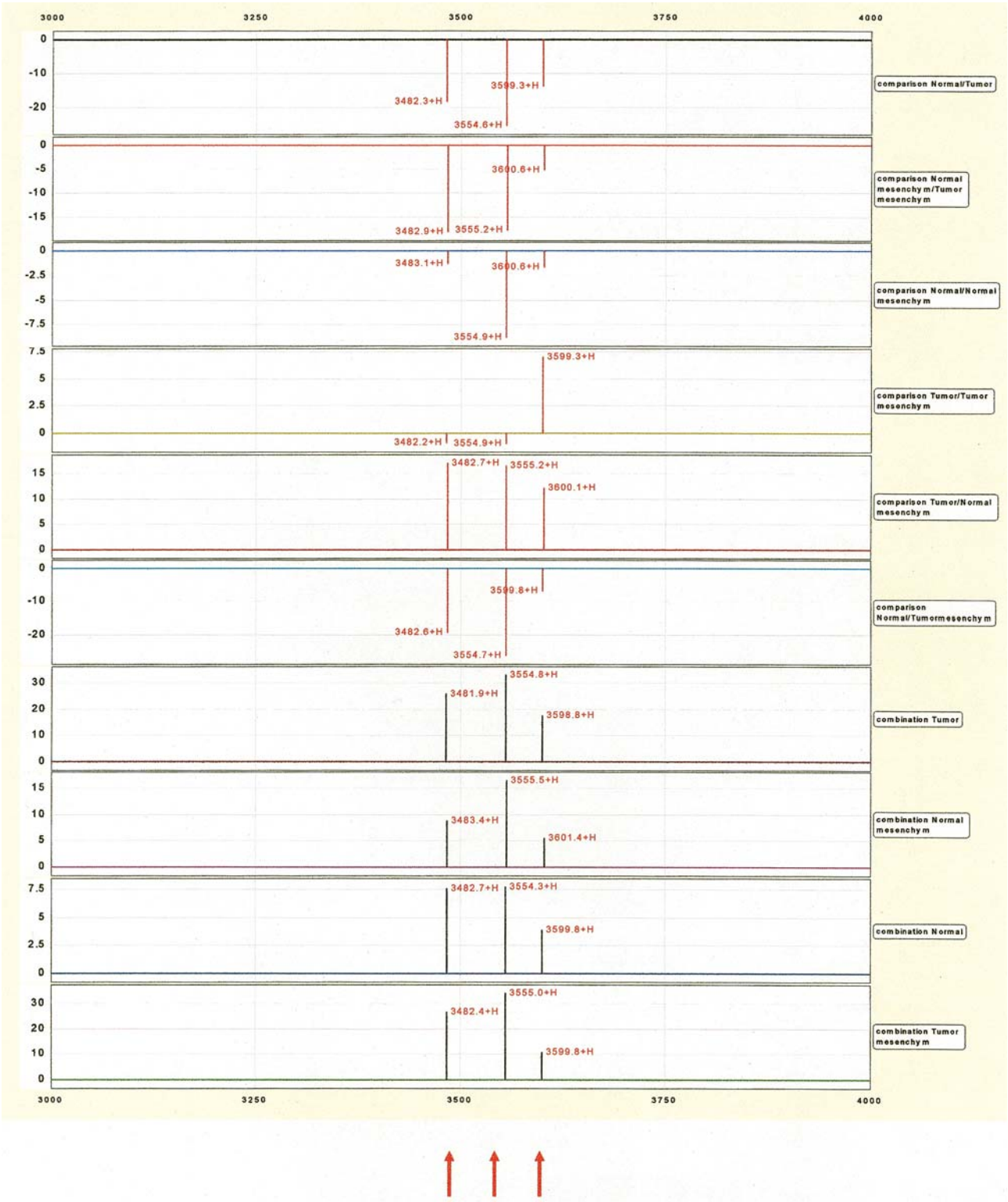

Figure 2. *The samples were grouped relative to the different sub-populations and subsequently combination maps were generated by the software combining all spectra of one subpopulation into one combination map. The resulting combination maps were then compared group-wise by substraction using Ciphergen software to produce comparison maps (T: tumor; NM: normal mesenchym adjacent to tumor).*

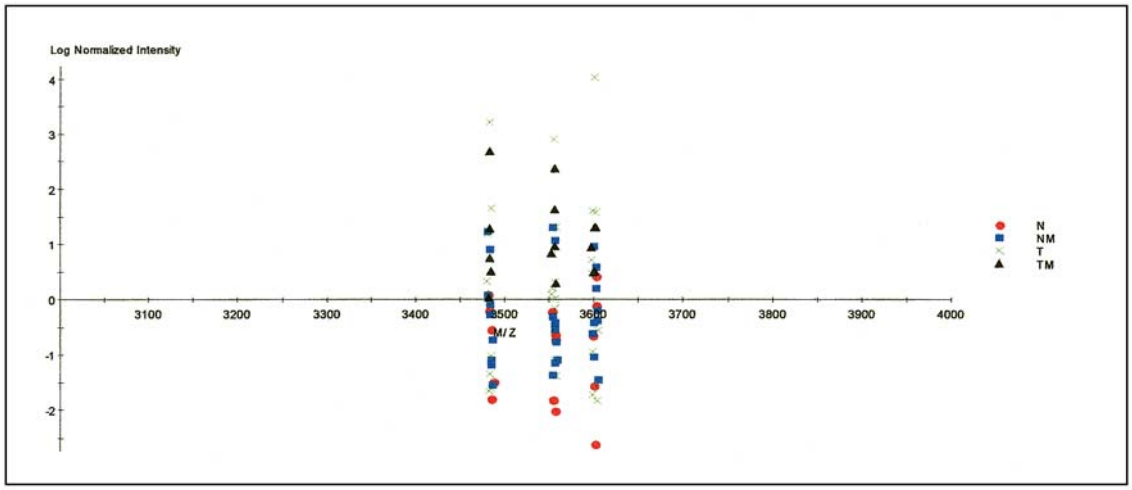

Figure 3. *Using the Biomarker wizard analysis, proteins of 3481, 3553 and 3601 Da have a higher intensity in tumor adjacent mesenchym (TM) and epithelial tumor elements (T) compared to normal epithelium (N) and normal stroma (NM).*

SELDI analyses of cell lysates. A total of 1000 microdissected cells were lysed in 15 μl of 50mM Hepes- buffer with 1% Triton X-100 (pH 7.4). The lysates were homogenized by vigorous vortexing, resuspended and subsequently centrifuged for 5 minutes at 3500 RPM with a bench top centrifuge. The process of sample preparation was performed according to the policy manual of the manufacturer and has been described in detail (10). The spots of a WCX2 ProteinChip® array were preincubated twice with 10 mM HCl and 10 mM ammonium acetate in PBS for 5 minutes each by shaking the whole ProteinChip in 20 ml of each of the solutions. Subsequently, a suitable plastic lid filled with a damp paper cloth was attached, generating a humidity chamber. The ProteinChips were incubated for 30 minutes at room temperature. The lysates were removed and the spots were washed three times with PBS + 0.05% Triton and then three times with high purity water using a 8-channel pipettor. Subsequently, the spots were air dried. Two applications of 0.8 ml saturated sinapinic acid (SPA, a laser desorption enhancing agent) dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid (TFA) in a 5-minute time-lag were performed. Air-drying finalized the sample preparation. Mass analysis of the ProteinChip® arrays was then performed in a Protein Biology System II ProteinChip® reader according to an automated data collection protocol. The instrument was operated with a source and detector voltage of 20 and 1.8 kV, respectively. Laser intensity was set to 270 a.u., (detector sensitivity to 5 a.u.), with an optimization range of the time lag focusing feature between 1000 Da and 20000 Da with a focus at 6000 Da. In total 221 laser shots were fired, 195 of them collected between the relative geometric positions 21 and 81 of the spots.

Data interpretation was performed using the ProteinChip® software version 3.02. In detail, the spectra obtained under similar experimental conditions from all sub-populations on WCX2 chips were imported in one experiment file and normalized to total ion current between 1500 Da and 20000 Da. A calculated normalization factor of 1.99 was applied to all spectra and the peaks were automatically labelled (automatic peak detection) with 50% detection sensitivity. The samples were grouped relative to the different sub-populations and subsequently combination maps were generated by software combining all spectra of one sub-population into one combination map. The following parameters were used: peak closeness 0.5% of mass, peak height computed as average of intensity. The resulting combination maps were then compared groupwise to produce comparison maps using the following parameters: peak closeness: 0.5%, peak height: intensity, peaks only in spectrum A: in blue color in positive direction, peaks only in spectrum B: in green color showing negative direction and peaks in spectrum A and B: in red color showing in both directions depending on the difference in peak height in the spectra A and B, respectively.

The mean intensities of the combination maps were exported and plotted *vs.* the sample groups for each peak described. Corresponding "gel"- and "spectra views" are shown and were magnified from the original spectra obtained.

Results

Patients. Patients age and TNM status are shown in Table I. The patients ranged in age from 43 to 88 years (mean 68.8). There were 7 males and 2 females. The tumors demonstrated moderate differentiation in 7 cases and signet ring features in 1 case. There was 1 patient with a Tis (*in situ*) N0 M0 lesion, 2 patients with T3 N0 MX lesions, 5 patients with T3 N1 MX tumors and one patient with a T3 N2 MX tumor.

Proteomics. Figure 1 demonstrates representative protein masscharge spectra showing protein gel views from 1.5-5 kDa from the 9 patients. For data analysis software features of the ProteinChip® software version 3.02 were used. The spectra obtained under similar experimental conditions

from all subpopulations were imported in one file and normalized to "total ion current" between 1.500 and 20000 Da/charge. A calculated normalization factor of 1.99 was applied to all spectra.

The peaks were automatically labelled ("automatic peak detection" feature) with medium detection sensitivity. The samples were grouped relative to the different subpopulations and subsequently combination maps were generated by the software, combining all spectra of one subpopulation into one combination map. The resulting combination maps were then compared groupwise to produce comparison maps. The results of the combination and comparison maps are shown in Figure 2.

We were able to detect a number of differentially-regulated proteins in the mass/charge range between 1.5 and 5 kDa. The most prominent up-regulated peaks in the low molecular weight mass range were peaks with a mass/charge of 3481, 3553, 3601 Da/ charge (mass error ± 1000 ppm due to calibration variation). The average relative intensity of the 3.6 kDa peak in the combined tumor glands sample was 14.97, compared with 3.91 in the normal epithelial cells. The average relative intensity of the 3.48 kDa peak in the combined tumor glands sample was 25.87, compared with 7.53 in the normal epithelial cells. The average relative intensity of the 3.55 kDa peak in the combined tumor glands samples was 33.08, compared with 7.7 in the normal epithelial cells. In the combined tumor stroma samples the average relative intensity of the 3.55 kDa peak was 34.01 *vs.* 16.45 for the same peak in the normal stroma cells, the relative intensity of the 3.6 kDa peak was 11.2 *vs*. 4.9 in the normal stroma. The average relative peak intensity of the 3.48 peak was 26.78 in the tumor stroma *vs.* 8.79 in the normal stroma.

Using the Biomarker wizard analysis, proteins of 3481, 3553 and 3601 Da/charge have a higher intensitiy in tumor-associated stroma and epithelial tumor elements compared to normal epithelium and normal stroma (Figure 3).

Discussion

The search for differences of protein expression between invasive and non invasive clones in colorectal carcinomas and between normal stroma and stroma containing invasive tumor will not only help in understanding carcinogenesis but identify biomarkers that could support tumor diagnosis and screening (11). Traditional 2-D gel analysis necessitates the presence of (at least 50000 cells) sizable amounts of protein. The necessity for larger amounts of tissue will subsequently lead to significant tissue contamination with unwanted cellular material. With the development of the SELDI technology only small amounts of target tissue is necessary, which can be easily obtained using laser supported microdissection (12).

Using the commercially available ProteinChip® software version 3.02 for microdissected colorectal carcinoma tissue, we identified several differences in protein mass spectra in benign *vs*. malignant epithelium and benign *vs*. tumor stroma. Specifically, there was a peptide pattern with molecular mass/charge ratios of 3.4-3.6 kDa. This was clearly differentially-expressed both in the malignant epithelial and stromal component as compared to the benign counterparts. This will allow further characterization of the peak at 3.4-3.6 kDa/charge for protein identification. Although the SELDI technique applied in this experiment does not allow identification of the target proteins specifically, this technique rapidly identifies the presence of such proteins which can subsequently be identified with direct mass sequencing technology recently developed at Ciphergen Biosystems Inc. Additionally, the SELDI technique applied in this experiment has demonstrated tumor-specific protein alterations, which, if proven to be present in a large number of cases, may lead to the development of specific tests to diagnose tumors with invasive potential, to the development of plasma tumor markers either for primary diagnosis or as a recurrence marker and, potentially, to specific drugs in the treatment of invasive colorectal carcinomas (13).

In summary, this study supports the applicability of this powerful new technology in conjunction with microdissection for protein profiling in different compartments of malignant tumors and associated normal counterparts, *i.e.* epithelial areas and stromal areas. Microdissection in conjunction with SELDI technology may generate important information allowing an understanding of colorectal carcinogenesis and the expression of specific proteins in the event of invasion. The results of this technique are highly reproducible and can be performed within a short time-frame. Not only fresh tissue and deep-frozen archival tissue is suitable for analysis, but also fixed specimens from cytology preparations (14).

The technique applied allows detection of proteins from complex mixtures of less than 1000 cells on a high throughput basis. The software used allows sensitive detection of relative peak height among all the spectra. Future studies are necessary to quantify the abnormal proteins found in different tissue sources and to evaluate whether the identified tissue proteins are also present in the plasma of the affected patients.

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