Imaging Mass Spectrometry of a Specific Fragment of Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase Kinase 2 Discriminates Cancer from Uninvolved Prostate Tissue

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Abstract

Purpose: Histopathology is the standard approach for tissue diagnostics and centerpiece of pathology. Although the current system provides prognostic information, there is need for molecular markers that enhance diagnosis and better predict clinical prognosis. The ability to localize disease-specific molecular changes in biopsy tissue would help improve critical pathology decision making. Direct profiling of proteins from tissue using matrix-assisted laser desorption/ionization imaging mass spectrometry has the potential to supplement morphology with underlying molecular detail.

Experimental Design: A discovery set of 11 prostate cancer (PCa)–containing and 10 benign prostate tissue sections was evaluated for protein expression differences. A separate validation set of 54 tissue sections (23 PCa and 31 benign) was used to verify the results. Cryosectioning was done to yield tissue sections analyzed by a pathologist to determine tissue morphology and mirror sections for imaging mass spectrometry. Spectra were acquired and the intensity of signals was plotted as a function of the location within the tissue.

Results: An expression profile was found that discriminates between PCa and normal tissue. The overexpression of a single ion at m/z 4,355 was able to discriminate cancer from uninvolved tissue. Tandem mass spectrometry identified this marker as a fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 2 (MEKK2). The ability of MEKK2 to discriminate tumor from normal cells was orthogonally confirmed.

Conclusions: This study highlights the potential of this approach to uncover molecular detail that can be correlated with pathology decision making. In addition, the identification of MEKK2 shows the ability to discover proteins of relevance to PCa biology. (Clin Cancer Res 2009;15(17):5541–51)

Prostate cancer (PCa) is one of the most common malignancies in the United States (1). It is clinically heterogeneous, with a highly variable natural history (2). The discovery and widespread use of serum prostate-specific antigen (PSA) monitoring for early detection has greatly changed the way PCa is diagnosed and treated. However, PSA lacks specificity as a screening tool for PCa, and there is really no lower limit of PSA that entirely excludes cancer (3). Thus, clinical decision making in PCa places a significant burden on biopsy results. Ultrasound-guided needle biopsy is the standard for diagnosis; however, a negative result does not exclude the presence of cancer. Both sampling and analytic variables account for false-negative results. In practice, false-negative results engender a need for repeat biopsies, which can delay diagnosis and treatment or unnecessarily subject cancer-free men to repeat biopsies and their attendant anxiety and risk (4, 5). The heterogeneity of
Prostate cancer (PCa) is a leading cause of death and morbidity in U.S. men. Despite consistent improvement in the management of this disease, there still remain unacceptable sequelae. It is estimated that upwards of 20% of patients with low-risk, clinically organ-confined PCAs ultimately progress with biochemical recurrence. In addition, upwards of 10% of men presenting for radical prostatectomy will have insignificant PCAs on prostatectomy pathology evaluation. Matrix-assisted laser desorption/ionization imaging mass spectrometry allows for the direct imaging of molecular detail within tissue. Such detail linked with histology could improve decision making about PCAs. Our study provides proof of principle that this technique can deliver spatially defined functionally significant molecular detail of patient tissues. We show the ability to reproducibly discriminate tumor from uninvolved tissue. Furthermore, the identification of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 2 shows that the molecular detail being probed by matrix-assisted laser desorption/ionization imaging mass spectrometry is not limited to traditional high-abundance proteins.

Materials and Methods

**Patients and tissue samples.** Patients were consented before undergoing radical prostatectomy at Sentara Norfolk General Hospital. Study protocols were approved by the institutional review board at Eastern Virginia Medical School. The age range of the patients was 46 to 82 y, with a mean age of 58.8 y who underwent surgery between 2003 and 2008. A total of 75 patients undergoing radical prostatectomy (21 for the discovery set and 54 for the validation set) were recruited for this study. Two cored specimens were harvested from each prostate immediately after removal of the gland. Each core is divided longitudinally to create mirrored cores; one was fixed and paraffin embedded and the other is embedded in OCT compound (Sakura Finetek USA) and frozen at 80°C. The frozen blocks yielded 41 sections (10 for the discovery set and 31 for the validation set) of benign tissue harvested from prostate tissue distal from the tumor site and 34 sections (11 for the discovery set and 23 for the validation set) of PCa-containing tissue. Of the 23 tumor-containing tissue sections in the validation set, 14 of these sections also harbored benign tissue adjacent to tumor. These were included as “benign adjacent” samples in the validation set. Cryosectioning was done on a Microm HM 505E cryostat at -20°C. A serial cryosection at 7 μm was stained with H&E as a guide and analyzed by a pathologist to determine tissue morphology. Two additional serial sections at 10 μm were mounted on conductive indium-tin oxide–coated glass slides (Bruker Daltonics) and used for MALDI-IMS.

**Materials.** Acetonitrile, ethanol, high-performance liquid chromatography–grade water, and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) were purchased from Sigma Chemical Co. The α-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Bruker Daltonics. Trifluoroacetic acid (TFA) was purchased from Pierce Biotechnology. Rabbit monoclonal antibody to MEKK2 (EP62 6Y) was purchased from Abcam.

**Tissue section preparation.** Immediately after sectioning, the indium-tin oxide–coated slides were washed and fixed with 70% ethanol and 95% ethanol for 30 s each (19). A water wash was done to remove residual embedding medium followed by a repeat of the ethanol washes of 70% and 95%. Slides were air dried and stored in a desiccator for 1 h before matrix deposition. A matrix solution of sinapinic acid (10 mg/mL) containing 75% acetonitrile and 0.13% TFA was sprayed uniformly over the tissue using an automated spraying device (ImagePrep workstation, Bruker Daltonics), which controls matrix deposition.
Microscopy

**Table 1. Characteristics of patient cohort**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Discovery</th>
<th>Validation</th>
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<tbody>
<tr>
<td><strong>PCa tissues</strong></td>
<td></td>
<td></td>
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<tr>
<td>Patients</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>59.4 (46-65)</td>
<td>58.8 (46-62)</td>
</tr>
<tr>
<td>Mean preoperative PSA, ng/mL (range)</td>
<td>7.97</td>
<td>11.1 (4.0-13.3, 2.4-69.6)</td>
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<tr>
<td>Pathologic classification</td>
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</tr>
<tr>
<td>pT2a</td>
<td>0</td>
<td>3</td>
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<tr>
<td>pT2b</td>
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<td>1</td>
</tr>
<tr>
<td>pT2c</td>
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<td>8</td>
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<tr>
<td>pT3a</td>
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<td>8</td>
</tr>
<tr>
<td>pT3b</td>
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<tr>
<td>Gleason score (section tested)</td>
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<td>9</td>
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<tr>
<td>Total patients/total sections tested</td>
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<td>23/23</td>
</tr>
<tr>
<td><strong>Benign tissues</strong></td>
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<td></td>
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<tr>
<td>Patients</td>
<td>10</td>
<td>31</td>
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<tr>
<td>Mean age (range)</td>
<td>58.4 (51-65)</td>
<td>58.8 (46-76)</td>
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<td>Mean preoperative PSA, ng/mL (range)</td>
<td>8.30</td>
<td>10.8 (4.8-15.5, 3.6-51.8)</td>
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<td>Pathologic classification</td>
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<td>pT2a</td>
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<tr>
<td>Total patients/total sections tested</td>
<td>10/10</td>
<td>31/31</td>
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<td><strong>Peptides differentially expressed</strong></td>
<td>Highly expressed in PCa cells m/z P</td>
<td>P 3,373, 2.81 E-08</td>
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<tr>
<td></td>
<td>Highly expressed in benign cells m/z P</td>
<td>4,274, 7.94 E-021</td>
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<td><strong>Correct classification</strong></td>
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<tr>
<td>PCa areas</td>
<td>Discovery set 85%</td>
<td>Validation set 81%</td>
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**Automated analysis of the spectral data was done to identify all differentially expressed peaks between cell types. Spectra derived from pathology-defined regions of interest (ROI) in each tissue were exported using the FlexImaging software for profile analysis. Baseline subtraction, normalization (to total ion current), peak detection, and spectral alignment were done using ClinProt Tools (Bruker Daltonics). A mass window of 0.3% and a signal to noise ratio of 3 were selected for peak detection. A genetic algorithm using k-nearest neighbors was used to obtain a classification between normal and PCa-containing tissue. The result of the genetic algorithm is the peak combination, which is proved to separate best between the different classes. Significant differences between groups were determined by Student’s t test. A P value of <0.01 was considered to indicate statistical significance. The predictive power of the putative biomarker to detect PCa tissue areas was tested using the area under the receiver operator characteristic (ROC) curve using SAS 9.1 Statistical Software (SAS Institute Cary, NC). The optimal cutoff point was defined as that point on the ROC curve that maximizes both sensitivity and specificity.**

**Tissue and cell culture lysates.** Tissue lysates were prepared from bulk frozen prostate tissue (~0.5 mm³) by homogenizing the samples in a small Dounce tube on ice with a solution of 20 mmol/L HEPES and 1% Triton X-100 (1 mL). Lysates were then sonicated at room temperature for 15 min and spun down at 14,000 rpm for 2 min to remove cellular debris. The lysates were then subjected to fractionation using weak cationic exchanger magnetic beads (Bruker Daltonics) according to the supplier's specifications. The bound peptides and proteins were eluted in 20 μL. Five microliters of this eluate were lyophilized and resuspended in 5 μL of HCCA matrix in 50% acetonitrile with 0.1% TFA. Lysates from the PCa cell lines (Du145, LNCaP, and PC-3) were prepared from 10⁶ cells in a lysis buffer containing 0.3% SDS, 3% DTT, and 30 mmol/L Tris (pH 7.5).

**MALDI-MS/MS and protein identification.** One microliter of each tissue lysate mixed with matrix was then spotted on a steel MALDI target. The mass profiles were recorded by MALDI-MS using the same acquisition parameters as for tissue imaging. Data were collected on the Ultraflex III in reflectron mode to verify the presence of the peak of interest. MS/MS analysis of the peak was then done in LIFT mode. An optimized high-mass LIFT method was used and externally calibrated with fragments from a peptide standard with parent masses in the mass range of 700 to 4,500 Da. A parent mass was selected and LIFT analysis (MS/MS) was done in the Ultraflex TOF-TOF. Peaks were labeled using FlexAnalysis software supplemented by manual picking using two criteria: peaks displayed a signal to noise of 3 and present in two of three spectral groups. A composite peak list was compiled, and database search was done using Mascot 2.2.03 with the following settings: MS Tol. of 50 ppm, MS/MS Tol. of 1.3 Da, no enzyme designation, and serine acetylation, using the National Center for Biotechnology Information database for human sequences with 20,080,125 entries.

Trypsin digestion was done on the tissue slices (10 μm) by spotting 0.5 μL of a solution of 0.769 μg/μL trypsin in 50 mmol/L ammonium bicarbonate (pH 8.0). The tissue slices were then incubated at 37 °C for 2 h in a humidity chamber. Following trypsin treatment, the tissue sections were spray coated with HCCA (7 mg/mL in 50% acetonitrile, 0.2% TFA). Data were collected across the tissue in reflection mode and converted to BioMap images.

**Immunohistochemistry.** Immunostaining of frozen specimens was done by the avidin-biotin peroxidase complex method using a Vectastain Elite ABC kit (Vector Laboratories). Briefly, frozen tissues were incubated in 0.3% hydrogen peroxide to block endogenous peroxidase activity for 15 min. Sections were then exposed to normal goat serum to block nonspecific binding, and endogenous avidin and biotin were blocked with an avidin-biotin blocking kit (Vector Laboratories).
Tissue sections were then incubated with rabbit monoclonal antibody to MEKK2 (EP626Y; Abcam) diluted 1:50 in PBS for 1 h at room temperature. Sections were then treated with biotinylated goat anti-rabbit immunoglobulin G (IgG), followed by treatment with avidin-biotin-peroxidase complex, and stained with IMPACT 3,3′-diaminobenzidine peroxidase substrate (Vector Laboratories) according to the supplier’s protocol. Counterstaining was done with Gill's hematoxylin.

Western Blot Analysis. A total of 30 μg (for cell lysates) or 100 μg (for tissue lysates) of protein were separated on a 4% to 12% SDS-PAGE gel and transferred by semidyry transfer method to Immobilon-P membranes (Millipore). The membranes were then incubated in Odyssey
differentially expressed ions were detected, which could be specific regions with designated cell types present in prostate Several peptide ions were found to discriminate PCa from be-

350 and 400 peaks within the mass range (Supplementary Fig. S1). On average, we could resolve between 2,000 and 20,000 peaks from our initial discovery set of 21 tissues, the ion at m/z 4,355 was the most significantly overexpressed in PCa-containing tissue regions (P = 2.76 × 10⁻¹⁰). We therefore wanted to evaluate the utility of this peak alone for the detection of PCa regions within prostate tissue via MALDI-IMS. Figure 1C is a representative image of a tissue section with one specific region of PCa and clearly defined adjacent regions of normal prostate glands. A higher magnification view of each cell type can be seen in the insets. Clearly evident from the ion density map, the m/z 4,355 peak was highly expressed in the PCa region compared with the surrounding tissue. Little to no expression is visible in the normal stroma or benign glandular regions. When representative spectra were exported from the specific regions (tumor versus benign), we can clearly observe a prominent peak at m/z 4,355 overexpressed in the PCa-obtained profile.

MALDI-IMS using m/z 4,355 can identify PCA-specific regions of prostate. When we examined the list of differentially expressed peaks from our initial discovery set of 21 tissues, the ion at m/z 4,355 was most significantly overexpressed in PCa-containing tissue regions across the separate validation sample set. Intensity values with respect to defined tissue regions found adjacent to tumor in 14 of the 23 PCa sections were then washed four times in PBST, rinsed in PBS, and scanned with the Odyssey IR imaging system (LI-COR Biosciences).

Results

Identification of an expression profile that discriminates between PCa and adjacent normal tissue. Our investigation into the presence of a PCa-specific protein/peptide expression profile was conducted on tissue sections from 75 prostates harvested from individuals undergoing radical prostatectomy. The patient and tissue sample characteristics of the discovery and validation cohort are presented in Table 1. Tissue sections were uniformly coated with matrix solution using an automated spraying device, and adjacent serial sections were stained with H&E for histopathology. Parallel-stained slides of each section were read by a genitourinary-trained pathologist, and the ROIs were designated. These ROIs contained prostate adenocarcinoma cell populations, benign epithelial cells, stromal cells, and benign epithelial cells adjacent to tumor cells.

In the initial discovery experiment, we analyzed 21 tissue sections (11 PCa and 10 benign). The resulting spectra were used to generate two-dimensional molecular maps of the peptides and proteins present in each tissue section, and automated analysis of the spectral data was done to identify differentially expressed peaks. The resolving power of our technique was comparable with laser capture microdissection capture of cells followed by MALDI-TOF analysis of the extracted proteins (Supplementary Fig. S1). On average, we could resolve between 350 and 400 peaks within the mass range m/z 2,000 to 20,000. Several peptide ions were found to discriminate PCa from benign tissue (Table 1). Examination by a pathologist reveals specific regions with designated cell types present in prostate tissue sections (Fig. 1A). This process is defined as pathology-designated ROI. In the mass range m/z 3,000 to 5,000, several differentially expressed ions were detected, which could be used to discriminate between PCa and adjacent benign regions (Fig. 1B, inset; Table 1). Of particular note are two peptide ions at average m/z of 4,027 and 4,355, which showed significant overexpression in PCa cells when compared with benign adjacent cell spectra. Another peak at m/z 4,274 was expressed in benign adjacent epithelial cells and stroma with little or no expression seen in PCa cells. Spectra derived from ROIs designated as tumor or benign from the initial 21 tissues examined were used to generate a classification algorithm using three m/z values (m/z 4,027, 4,274, and 4,355), which was capable of correctly classifying 85% of PCa tissue areas. Selected component ions with significant discriminating power were evaluated in these initial tissues and images derived around the pathology-designated ROI. This allowed for a visual determination of region-specific changes in peptide ion expression. Representative examples of images derived from mapping all three discriminating m/z are shown in Supplementary Fig. S2.

The classification ability of the same genetic algorithm derived from the discovery set using the three discriminatory peaks (m/z 4,027, 4,274, and 4,355) was then evaluated on the larger validation set. This set consisted of 23 tumor sections and 31 benign sections for a total of 54 sections (54 cancer-confirmed patients). The performance of the three-peak genetic algorithm in the validation set was comparable with that seen in the discovery set. Specifically, the PCA areas in the validation set could be correctly classified in 81% of the tissues tested (Table 1).

MALDI-IMS using m/z 4,355 can identify PCA-specific regions of prostate tissues. To evaluate the differential expression of m/z 4,355 between PCa and benign regions, we conducted an analysis of the validation set of prostate tissues. We examined the images produced from the ion density of the m/z 4,355 peak following the analysis of 23 PCa and 31 benign prostate tissue sections (distal from tumor site). We also examined an additional 14 benign prostate tissue regions found adjacent to tumor in 14 of the 23 PCa sections used for the validation set. Shown in Fig. 1D are representative ion images of the expression of the m/z 4,355 peak in tissues containing PCa and distal benign sections. The corresponding spectra in the representative region of m/z 4,000 to 6,000 are shown in the top panels above each image. A set value for the peak intensity threshold used to display the m/z 4,355 peak in each image was determined from the discovery set and applied to the validation set. This threshold represented the maximum peak intensity observed from the normalized intensity values obtained in PCa regions. Any pixel displaying an intensity greater than or equal to this set threshold was then considered high expression and is represented in the images obtained in the validation set. An intensity scale can be seen at the bottom right of Fig. 1C. We provide in Supplementary Fig. S3 a representative set of eight paired tissues. High expression was visible in each section, where PCa cells were present or visible throughout the tissue when no benign cells were present. In contrast, little to no expression of the m/z 4,355 ion was detected in sections containing benign cells only.

To illustrate the tissue-specific expression of m/z 4,355, we examined the intensity values with respect to defined tissue regions across the separate validation sample set. Intensity values for m/z 4,355, normalized to total ion current, were calculated.
Fig. 2. The normalized intensity values for m/z 4,355 can discriminate tumor from benign tissue. A, normalized average intensity values for m/z 4,355 in different prostate tissue areas. A total of 23 PCa-containing tissues, 14 benign adjacent, and 31 distal benign tissues were analyzed via MALDI-IMS. The resulting normalized average intensity values for the m/z 4,355 peak were plotted for PCa, benign adjacent, and benign distal regions. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. *, P = 3.2E-5; **, P = 4.0E-10. B, the predictive power of the putative biomarker to detect PCa tissue areas was tested using the area under the ROC curve. Averaged normalized intensity values obtained from MALDI-IMS spectra for PCa tissue were plotted according to Gleason grade (C) or pathologic stage (D). The high-grade samples (n = 8; from tissues with grade 4+4 [n = 4] and 4+5 [n = 4]) with pathologic stage pT3b (n = 5) or pT4 (n = 3) are included in the plots but were not part of the validation set.
for each tissue region and plotted: PCa, benign adjacent, and benign distal (Fig. 2A). The average normalized intensity of the m/z 4,355 peak in benign tissue found in the same section with PCa cells or benign prostate tissue from a section containing no PCa cells was 20.8 and 19.6, respectively, whereas for PCa regions the average intensity found was 41.1, representing a 2.1-fold increase. A ROC curve calculated from the average normalized intensity of each ROI (distal benign versus PCa) is shown in Fig. 2B. The optimal cutoff point for using the 4,355 peak as a biomarker for PCa in tissue sections was a normalized average intensity value of 33. This cutoff point was associated with a sensitivity of 90.3% and a specificity of 86.4% (area under curve AUC 0.960). To maximize sensitivity, a cutoff value of 23.8 was chosen, which represents a sensitivity of 96.8% and a specificity of 81.8%.

We next conducted a more detailed analysis of the expression of the m/z 4,355 by disease stage/grade. We plotted the normalized intensity values for m/z 4,355 by Gleason grade and pathologic stage. The results of this analysis can be seen in Fig. 2C and D. Tissues with a Gleason combined score of 3+3 had an averaged normalized intensity value for m/z 4,355 of 44.8, with 89% of the tissues exhibiting a value above the ROC cutoff of 23.8. Tissues with a Gleason score of 3+4 had an averaged normalized intensity value of 41.0, with 90% of these tissues displaying a value above the ROC cutoff. Tissues with a Gleason score of 4+3 had an averaged normalized intensity of 32.0, with

Fig. 3. Sequence identification of the peptide at m/z 4,355. A, spectra showing the peak profile of m/z 4,355 collected by direct acquisition from tissue or from tissue lysates as indicated in each panel in linear or reflectron mode. B, deconvoluted MS/MS spectra of monoisotopic peak (m/z 4,350.4) collected using TOF/TOF LIFT. Peaks attributed to internal fragments are labeled and indicated with arrows.
75% of tissues displaying a value above the ROC cutoff. This reduction in expression of the m/z 4,355 peak observed with increasing Gleason grade was also observed between pathologic stage. Although this reduction was not significant between Gleason scores, a significant reduction was seen between pathologic stages pT2 and pT3b as well as pT2a versus pT3b. Tissues from prostates designated as pT2a, pT2b, or pT2c had a normalized average intensity of 42.7, with 92.3% of the tissue samples above the cutoff. A similar trend is seen in tissues from prostates designated pT3a, which had an average intensity of 45.6 for m/z 4,355, with 87.5% of the tissues with a value above 23.8. If, however, the tissue specimen was procured from a prostate with a pathologic designation of pT3b indicating seminal vesicle invasion, the average normalized intensity dropped to 30.1, with only 28.6% of tissues having a value above the cutoff.

To further define the trend of decreasing expression of 4,355 with increasing stage/grade of disease, we examined an additional eight sections from more aggressive disease (Gleason 8/9/10 and pT4). The analysis was conducted as described above for the previous tissues, and the evaluation of 4,355 was conducted with the same cutoff score. As can be seen in Fig. 2C and D, the trend toward decreased expression was observed in higher grade/stage disease. Specifically, of the eight high-grade tissues tested, only two (25%) had expression above the cutoff value of 23.8. Of the high-grade cases, three were designated as pT4 and only one (33%) of these had a normalized intensity value for m/z 4,355 above the cutoff.

**Sequence identification of m/z 4,355 as a fragment of MEKK2.** Having established the differential expression of a peptide ion at m/z 4,355, we next set out to sequence identify the peptide. Lysates were prepared from four tissue samples: two PCa and two benign prostate. Sections from these tissues were examined in the initial MALDI-IMS analysis and found to have high or low expression of the peak at m/z 4,355. Protein lysates were incubated with weak cationic magnetic beads, and eluted fractions were shown to be enriched for the m/z 4,355 peak as measured by MALDI-TOF. As seen in Fig. 3A, the peptide ion captured from the weak cationic exchange fractionation of the PCa tissue lysate matches the mass detected directly from the tissue within an error of 0.26 Da, whereas no peptide ion was detected at this m/z in the enriched lysate from benign tissue. The lysate was then concentrated via lyophilization and prepared for MS/MS analysis as described. Figure 3B is a representative MS/MS spectra showing the fragmentation pattern of the parent ion (m/z 4350.4), in which several large internal fragments are observable. The composite fragmentation series gave a Mascot top score of 42.3 (1.3 Da, 50 ppm), with scores ≥37 indicating significant homology, and matched to a fragment of MEKK2 (Swiss-Prot entry Q9Y215) with S-acetylation at the NH2 terminus of the peptide (Supplementary Fig. S2). Of 84 total observed peaks, 79 could be accurately assigned to theoretical fragments of MEKK2. This sequence represents amino acid residues 26 to 61 in the 619- amino acid full-length protein and lies within the PhoX-and-Bem1 (PB1) domain of the molecule.

To further establish that the m/z 4,355 ion derived from tissue is a fragment of MEKK2, we did an in-tissue digest to analyze for the presence of predicted MEKK2 tryptic fragments. A tissue section previously found to have high expression of the m/z 4,355 peak in a PCa region was used for this analysis. Serial sections of the same tissue region were harvested and analyzed. One of the mirror sections was trypsin treated, whereas the adjacent mirror section was untreated and used as a control. Ion density maps were also generated using the indicated theoretical tryptic peptides. As seen in Fig. 4, specific theoretical masses of the predicted MEKK2 fragments after trypsinization could be detected in the trypsin-treated PCa tissue. The in-tissue trypsin-generated fragments matching the theoretical digest masses were not present in the mirror untreated section of the same PCa tissue (Fig. 4C). Furthermore, trypsin digestion of benign tissue sections did not generate observable fragment ions corresponding to theoretical MEKK2 cleavage products (Fig. 4B). We also show a representative ion density map.
With images derived from the tryptic peptides (Fig. 4A). This analysis shows that both the parental peptide and predicted peptide fragments display concordant tissue expression. **Expression of MEKK2 in PCA cell lines and tissues.** Western blot analysis was done on PCA and benign tissue extracts and three PCA cell lines (Du145, LNCaP, and PC-3). LNCaP cells originate from a lymph node metastatic lesion of human PCAs, and Du145 and PC-3 are human prostate adenocarcinoma cell lines metastatic to the brain and bone, respectively. The relative expression of MEKK2 in these systems is shown in Fig. 4D. All three prostate cell lines showed strong expression of full-length MEKK2 (70 kDa). This analysis revealed higher MEKK2 expression in the PCA tissues when compared with the expression seen in the benign tissues. Densitometry analysis indicated a 4.4-fold increased expression of MEKK2 in PCA tissue compared with benign tissue. **MEKK2 is overexpressed in PCA-specific regions of the prostate.** In some cases, the overexpression of a fragment of a protein may coincide with overexpression of the whole protein. If this is the case, then selective overexpression of MEKK2 in tissue would be further confirmation of our results. We examined the expression of MEKK2 in PCA tissue using immunohistochemistry. PCA-containing and uninvolved frozen tissues were stained for MEKK2 expression. The antibody used is specific for the NH₂-terminal portion of the MEKK2 protein where the PB1 domain is located and is the same one used above for the Western blot analysis (20). As seen in Fig. 5A, MEKK2 staining correlates with the presence of PCA in the tissue section. The ROI designated in the H&E panel was prescribed by a genitourinary-specialized pathologist as containing tumor. Additional prostate tissues were also stained, and magnified views of the stained PCA glands and benign tissue can be seen in Fig. 5B. In Fig. 5C, we show an analysis of sections designated as all tumor or all benign. The prostate tissues examined showed high levels of MEKK2 within involved tissue with predominantly cytoplasmic expression pattern. In contrast, benign glands displayed little to no MEKK2 expression.

**Discussion**

The correlation of molecular information to histopathologic structures offers tremendous potential for improved characterization of clinical tissues. The use of MALDI-IMS and profiling to obtain such molecular information in the form of protein and peptide distributions greatly enhances the ability to identify potential candidates for new specific biomarkers. In their groundbreaking study, Yanagisawa et al. (12) showed that m/z patterns obtained directly from lung tissue could be used to classify histologic groups and predict nodal involvement and survival in non–small cell lung cancer. Similar results were obtained for ovarian cancer in which peptide profiling by direct MALDI analysis of 25 ovary carcinomas (stages III and IV) and 23 benign tissues identified several peptides observed to only be present in carcinoma compared with benign samples (21). The most prevalent peptide was identified as a fragment of immunoproteasome PA28. Specific to PCA, MALDI-IMS revealed a specific expression pattern that was temporally associated with prostate development in an animal model (22), and in a separate study, a pattern of m/z was associated with pathogenesis of PCA in a cohort of 22 prostate tissue samples (17). In our current study, we used MALDI-IMS in an attempt to discriminate tumor from uninvolved prostate tissues. Unique to our study design was the use of a large tissue set that allows for a discovery group (11 PCA/10 uninvolved) and a separate test/validation group (23 PCA/31 uninvolved) for examining the performance of the selected biomarker. The performance of a single biomarker was associated with the corresponding image in which PCA tissue could be detected. We also sequence identify the m/z generating peptide as a fragment of MEKK2 and confirmed the differential expression of MEKK2 between tumor and benign tissue using immunohistochemistry and Western blot. Biochemical and genetic studies have shown that MAP3Ks are crucial in relaying distinct cell surface signals through various downstream MAPK pathways. MEKK2 is 1 of only 2 of the 20 known MAP3Ks, the other being MEKK3, which regulates the MEK5/ERK5 pathway (23–25). Growth factors and oxidative/osmotic stress have been shown to stimulate the three-tier ERK5 kinase module consisting of MEKK2/3, MEK5, and ERK5. MEKK2 and MEKK3 encode PB1 domains that selectively heterodimerize with the MEK5 PB1 domain to form a functional MEKK2 (or MEKK3)-MEK5-ERK5 ternary complex.
sues. Although immunohistochemistry is widely applied, and existing methods for assessing tissue biomarkers. Here, we spe-
cistics will ultimately depend on the competitive advantage over lowing for visual integration into the diagnostic workflow.

tive feature of the MALDI-IMS is the ability to display protein sur-gery to make accurate diagnosis and prognosis. One attrac-
cally useful it must contribute to the ability of pathologists and biomarker discovery tool, in order for MALDI-IMS to be clini-
ing may occur (30, 31). Thus, MEKK2 may provide for better
benign lesion occurs in 1% to 2% of cases, and in up to 10%
opinion reviews suggest that reclassification of a malignant to
mors and potential correlation with cancer stage/grade. We are cur-
E implant structural isoforms that may be of relevance to regulation of cellular signaling pathways. Alternatives should be explored, as the demands for precision and accuracy in our diagnostics will only increase as the number of targeted agents increases.

Imaging mass spectrometry has the potential to address some of the shortcomings of immunohistochemistry. For instance, because IMS directly measures the analyte, it is possible to obtain direct quantitation and the platform is amenable to multiplexed analysis. In addition, IMS can readily determine posttranslational modifications of proteins potentially providing information not obtained by indirect measurements. The ability to quantitate small amounts of intact or modified proteins becomes especially crucial in situations where small core biopsies of tumors are done, and clinical decision making begins before definitive excision when larger portions of tumors become available. In counterbalance to these advantages, there are several practical obstacles for the clinical application of IMS. Most prominent perhaps is that although MALDI-IMS on paraffin-embedded, formalin-fixed samples has been done (16), such efforts have met with limited success. Thus, the development and use of alternative fixatives is a critical area of investigation (32). We are currently investigating alternative fixation methods, including the commercially available UMFix approach developed at the University of Miami (33).

Our studies presented here highlight the potential for MALDI-IMS to be integrated into clinical decision making. Clearly, the specific utility of the m/z 4,355 generated by a peptide of MEKK2 will need to be established through future studies involving direct as-
sement of biopsy tissue and pathology outcome. However, our immunohistochemical analysis shows that MEKK2 is overex-
ressed in tumor-involved tissues within the prostate. Although ERK5 has been shown to correlate with high-grade disease, this is the first demonstration of MEKK2 overexpression in prostate tu-
ors and potential correlation with cancer stage/grade. We are cur-
rently examining the role of MEKK2 activation in respect to ERK5 activity and tumor progression. Overall, our findings suggest that application of MALDI-IMS to identify disease-specific molecular changes in tissue will provide molecular detail to improve the critical pathology decision-making process in PCA diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Imaging Mass Spectrometry of a Specific Fragment of Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase Kinase Kinase 2 Discriminates Cancer from Uninvolved Prostate Tissue

Lisa H. Cazares, Dean Troyer, Savvas Mendrinos, et al.


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