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.

**Translational Relevance**

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 PCAs-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 o-cyano-4-hydroxycinnamic acid (HCCA) was purchased from Bruker Daltonics. Trifluoroacetic acid (TFA) was purchased from Pierce Biotechnology. Rabbit monoclonal antibody to MEK2 (EP626Y) 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 dessicator 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.
Data processing and statistical analysis. 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 re-suspended 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 then 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).

6 Available as free software from http://www.MALDI-MSI.org.
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 semidry transfer method to Immobilon-P membranes (Millipore). The membranes were then incubated in Odyssey

**Fig. 1.** Direct tissue mass spectrometric analysis of human prostate tissue reveals cell-specific profiles. Frozen prostate tissue was subjected to MALDI-IMS as described. A, representative histologic image of H&E-stained prostate tissue showing areas of prostate adenocarcinoma (T), benign prostate glands (B), and benign stroma (S). B, resulting average spectra acquired from each region showing characteristic profiles for different cell types. Inset, expanded view of the mass range m/z 3,000 to 5,300 showing differences in the profiles for each cell type. C, MALDI-IMS of a single prostate tissue containing tumor and uninvolved regions. a, H&E image of a tissue specimen containing a defined area of PCa glands and benign glands. Insets, magnified (×10) views of each cell type. b, resulting two-dimensional ion density map showing high expression of a peak at m/z 4,355 (red) in the PCA area (inset is a scan of the tissue after matrix deposition). c, average spectra from a single spot obtained in the PCa (T) region and from the benign adjacent glandular (B) area displaying differential expression of the ion at m/z 4,355. D, MALDI-IMS two-dimensional ion density maps of a PCa-containing tissue and a benign prostate tissue. Circled, pathology-defined regions of PCa. Areas in red from the resulting MALDI-IMS indicate high expression of m/z 4,355. Top, spectra exported from representative regions of each tissue are shown in the m/z range of 4,000 to 4,600 and display the m/z 4,355 peak profile.
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 an 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 tissue sections 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 and clearly defined adjacent regions found adjacent to tumor in 14 of the 23 PCa 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. 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 the most significantly overexpressed in PCa-containing tissue regions (P = 2.76 × 10^{-16}). 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 intensity 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-observed profile.

MALDI-IMS using m/z 4,355 discriminates between cancer and uninvolved prostate tissue. 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 4,600 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
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 exchanger 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 Q9Y2I5) 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.
image derived from the parental m/z 4,355 as a comparison 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 PCa, 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 nonsmall 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.
The ERK5 pathway mediates normal cell-cell interactions during immune surveillance and is a critical regulator of cell invasion during tumor metastasis (reviewed in ref. 26). Indeed, the ERK5 pathway has been implicated in high-grade PCA. Specifically, an increase in MEK5 expression was associated with metastatic PCA, cell proliferation, matrix metalloproteinase-9 expression, and cell invasion (27). Strong MEK5 expression was also found to correlate with the presence of bony metastases and less favorable disease-specific survival. An additional report found significant correlation between ERK5 cytoplasmic overexpression, Gleason sum score, and less favorable disease-specific survival (28). The authors also found that ERK5 nuclear expression is significantly associated with the transition from hormone-sensitive to hormone-refractory disease. Clearly, our finding that MEKK2 is overexpressed in tumor compared with benign is consistent with established biological behavior of ERK5 signaling.

One study, examining the interactions of the MEK5 PB1 domain, found that both MEKK2 and ERK5 interact with the NH2-terminal extension of MEK5, suggesting that MEKK2 and ERK5 compete for binding to MEK5 rather than form a ternary complex (29). The PB1s are dimerization/oligomerization domains that are present in adaptor and scaffold proteins as well as kinases. PB1 domain–dependent MEKK2/3-MEK5 heterodimers provide a spatially organized signaling complex primed to activate ERK5 in response to activation of MEKK2 or MEKK3. No other MAPK cascade has been shown to form such a complex. Interestingly, the m/z 4,355 peak represents a peptide fragment that lies within the PB1 domain and may reflect molecular pathway changes indicative of PCA development.

Clearly, the utility of a tissue-assessed biomarker, such as MEKK2, is restricted to decisions made in pathology using biopsy or surgical material. The specific utility of a tumor-confined biomarker in PCA is limited, unless the expression varies with disease stage or prognosis. Our observation that the expression of the MEKK2 4,355 peptide is reduced in higher stage/grade disease (G = 4+3, pT3b) needs to be verified in a larger cohort with representation of more aggressive disease. In addition, such studies should include an assessment of the expression of MEKK2 with respect to observable disease outcome or surrogate clinical end points. We are particularly interested in determining if MEKK2 expression correlates with the presence of insignificant disease. In addition, differing thresholds for atypical lesions, assessment of Gleason score, and diagnosis of small cancers are known sources of analytic variability. In fact, second-opinion reviews suggest that reclassification of a malignant to benign lesion occurs in 1% to 2% of cases, and in up to 10% of cases, a change in Gleason score affecting clinical decision making may occur (30, 31). Thus, MEKK2 may provide for better staging/grading with potentially improved diagnostic uniformity.

Although our results show that MALDI-IMS can be a powerful biomarker discovery tool, in order for MALDI-IMS to be clinically useful it must contribute to the ability of pathologists and surgeons to make accurate diagnosis and prognosis. One attractive feature of the MALDI-IMS is the ability to display protein expression and spatial distribution as an intensity map, thus allowing for visual integration into the diagnostic workflow. However, the integration of MALDI-IMS into clinical diagnostics will ultimately depend on the competitive advantage over existing methods for assessing tissue biomarkers. Here, we specifically refer to immunohistochemistry of formalin-fixed tissues. Although immunohistochemistry is widely applied, and

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References


Clinical Cancer Research

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

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