Austin Biomarkers & Diagnosis

Open Access

Austin Publishing Group

Mini Review

Application of Mass Spectrometry for Identification of Biomarkers in Formaldehyde-Fixed Paraffin Embedded Specimens

Tatsuaki Tsuruyama[1,3]*, Yu Kakimoto[2] and Yuka Yajima[3]

[1]Department of Diagnostic Pathology, Kyoto University Graduate School of Medicine, Japan

[2]Department of Forensic Medicine, Tokai University, Japan

[3]Department of Anatomic, Kyoto University, Japan

***Corresponding author:** Tatsuaki Tsuruyama, Department of Diagnostic Pathology, Kyoto University Graduate School of Medicine, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan, Tel: +81-75-753-4473; Fax: +81-75-753-4493; Email: tsuruyam@kuhp.kyoto-u.ac.jp

Received: October 16, 2014; **Accepted:** November 07, 2014; **Published:** November 13, 2014

Abstract

Mass Spectrometry (MS) has recently been utilized for the identification of biomarkers in peripheral blood and in urine, whereas its application in tissue samples remains limited. In this review article, we introduce a novel application of MS using archived Formalin-Fixed Paraffin-Embedded (FFPE) tissue. This method addresses the significant technical challenges for protein ionization, including amino acid residue modification of proteins. While various immunochemical pretreatments for enhancing the ionization signal of peptides have been reported, significant developments have yet to be achieved. We performed a simplified chemical pretreatment method for preparing tissue sections involving heating in acetonitrile-containing buffer under airtight and pressurized conditions. Analysis revealed that the number and intensity of ionized peptide peaks obtained from pretreated tissue were significantly higher than from untreated (control) tissue. This highly sensitive treatment may enable MALDI-MS and LC/MS (liquid chromatography/MS) analyses of archived pathological FFPE samples in the hospital, leading to the identification of new biomarkers.

Keywords: Mass spectrometry; FFPE; MALDI-TOF MS; Imaging

Introduction

Human tissues are usually preserved as formalin-fixed paraffin-embedded (FFPE) samples in repositories. Formaldehyde cross links amino acid residues such as arginine in proteins by methylene-bridging, thereby preventing autolysis and decomposition. However, this is a critical issue for follow-up MS studies, since the bridging makes it difficult to extract proteins and ionize peptides in samples. Furthermore, during immunohistochemical analysis of pathological samples, formalin-fixed tissues are typically subjected to an antigen retrieval protocol, which includes protein digestion, microwave heating, or boiling. In MS analysis, enzymatic digestion using tyrosine has been used to enhance ionization by fragmentation. However, enzymatic digestion is usually insufficient to achieve strong MS signal intensities, and Signal/Noise (S/N) ratios remain low. There are a number of reports discussing new procedures for preparing FFPE sections for MALDI-MS involving immuno histochemical pretreatment [1]. Here, we will introduce our protocols for identification of peptides using FFPE. Since immunohistochemical antigen retrieval methods involve the use of detergents, the introduction of noise in the signal is inevitable. For this reason, we aimed to develop other protocols to enhance ionization of embedded peptides in samples.

Laser-capture micro dissection

Combining micro-dissection of tissue samples with LC/MS spectrometry is a standard protocol for the analysis of FFPE tissues. We also applied this methodology using Electro Spray Ionization (ESI) with LC/MS. The identification of disease lesions during microscopic observation of frozen samples was more difficult compared to FFPE due to the loss of histologic structure. This point is of practical importance in the use of frozen samples. Specifically, during the micro-dissection of heart and brain, the infarction or tumor lesion was frequently not detectable. Thus, FFPE is more useful for stable and reproducible dissection for proteomics than expected. Serial sections of diagnostic slides were mounted on Frame Slides (Micro Dissect GmbH, Her born, Germany) and stained with hematoxylin. Stained slides were air-dried and tissue sample was sampled microscopically using w an LMD 6500(Leica Microsystems, Wetzlar, Germany) instrument to capture defined areas. First, the subjective lesion in the FFPE tissue (10 mm^2 in total) was collected into a 500-µL well. Each micro dissected sample was suspended in 20 µL of 0.1 mol/L NH_4HCO_3 containing 30% (v/v) CH_3CN in the wells, and then centrifuged at 10,000 x g for 1 min. Tubes were heated at 95°C for 90 min. After cooling, the tissues were digested with trypsin at 37°C overnight, and then heated to 95°C for 5 min for enzyme deactivation. After drying, samples were resuspended in 0.1% trifluoroacetic acid with 2% CH_3CN and the final protein concentration was adjusted to 0.2 µg/µL. Un expectedly, 1.0×10^4 peptides and $1.0\times10^{2-3}$ proteins were identified in the various formaldehyde FFPE tissues including lung, synovium, pancreas, and brain; similar numbers were obtained with frozen samples. Most of the detected proteins were cytoskeletal proteins, including beta-tubulin 3, immunoglobulin, and vimentin. Using LC/MS, we identified Sorbin and SH3 domain-containing protein-2 (SORBS2) as a novel marker of acute myocardial infarction [2,3].

Tissue pretreatment for MS imaging

We recently developed a simple in situ pretreatment technique

Citation: Tsuruyama T, Kakimoto Y and Yajima Y. Application of Mass Spectrometry for Identification of Biomarkers in Formaldehyde-Fixed Paraffin Embedded Specimens. Austin Biomark Diagn. 2014;1(1): 3.

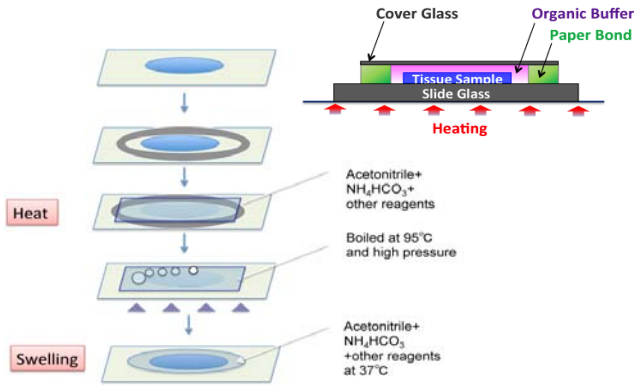

Figure 1: Schematic representation of the swelling and steam pretreatment in FFPE tissue sections.

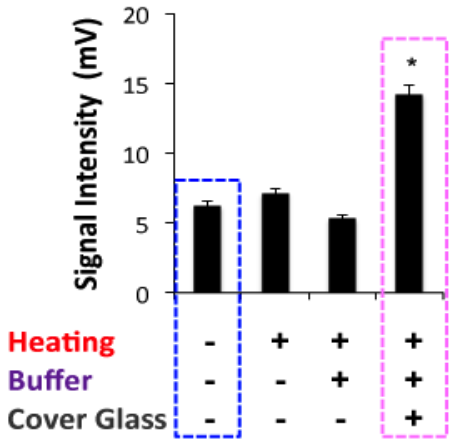

Figure 2: Effect of heating process on the average signal intensity across the 500–4000m/z range in analyses of tissue sections (mean ± SD, n = 6). H2A was identified as a marker of colonic adenocarcinoma.

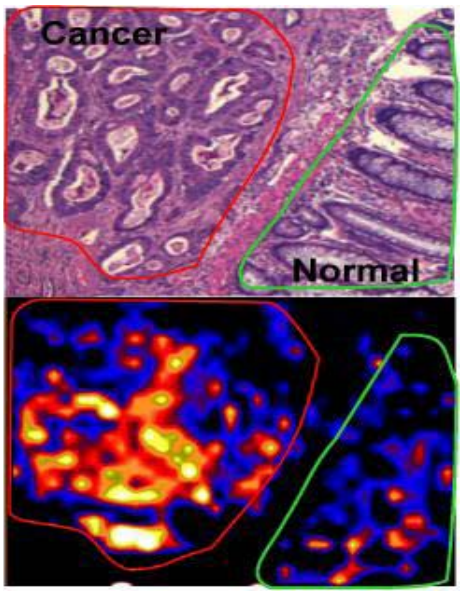

Figure 3: MS imaging of colon adenocarcinoma. Cancerous and normal areas are indicated by red and blue lines, respectively, in the Hematoxylin-eosinstained specimen (upper) and MS imaging (lower).

for preparing FFPE sections for MALDI/TOF-MS imaging. This novel imaging method involves pretreating tissues at high pressure and temperature for a short period. Pretreatment enhances the permeability of FFPE tissues for hydrophilization but does not cause tissue damage. The swelling procedure may enhance the crystallization of the mixed peptide and matrix component for MALDI/TOF-MS analysis. FFPE tissues subjected to this method showed a significant increase, 100-fold at maximum, in MS signal intensity for MS imaging. During the process, the buffer permeated the specimen until swelling reached equilibrium. The tissue section on the slide was encircled with a paper bond that was removable with the following procedure (Ta-100; Kokuyo, Osaka, Japan). Sample glass slide was incubated in situ for 1 h at 37 °C in buffer containing 0.1 M NH_4HCO_3 and 30% (v/v) CH_3CN. After removal of the buffer, the chamber was filled with a volume of buffer sufficient to cover the sample. The slide was then covered with an airtight layer of aluminum foil and heated at 94°C on an aluminum hot plate for in situ hybridization (DAKO, Glostrup, Denmark) (Figure 1). 200µl of 0.05µg/µl trypsin (Promega, WI, USA) solution containing 2.5mM NH_4HCO_3 and 10% (v/v) CH_3HN was added to the chamber for protein digestion, and the slide was incubated at 37°C overnight. The matrix solution for peptide ionization was a solution of 2,5-dihydroxybenzoic acid (DHB) in 50% methanol, which was suitable for analysis of peptides. The matrix was deposited in 15-nL droplets at a spatial interval of 250 µm. After drying at room temperature, mass spectra were acquired using a MALDI TOF/TOF mass spectrometer (AXIMA Performance; Shimadzu, Kyoto, Japan) equipped with a nitrogen laser. Spectra were acquired in positive ion mode over the range m/z500–3000. The signal intensity corresponding to m/z 850 was significantly increased by the pre-treatment (Figures 2-4) [4]. Using this method, we successfully identified a specific protein, histone H2A, which is highly expressed in cancerous tissue.

Conclusion

In this review article, we demonstrated that our novel method significantly enhances the signal intensity in MS analyses of FFPE tissues. This novel method for protein extraction from FFPE enabled proteomic analysis using LC/MS. Label-free LC-MS accompanied by precise laser micro dissection enabled *in situ* proteomic analysis that corresponded with pathological findings. Among the significant proteomic changes observed, SORBS2 was detected at high levels in the serum of patients with cardiac infarction [3]. This observation suggests that SORBS2is released to peripheral blood, indicating that this protein may represent a diagnostic biomarker.

Our treatment improved the applicability of FFPE specimens by swelling the deparaffinized sample and increasing tissue permeability. The high-pressure steaming procedure enhanced signal intensities. Long-term incubation in swollen vaporized buffer resulted in the development of enlarged spaces between collagen and other connective tissue fibers in the extracellular matrix, which is thought to enhance the permeability of tissue sections and matrix crystallization with peptides for MS imaging analysis. This physicochemical treatment of the tissue contributes to the increased availability of FFPE sample peptides. Using our methodology, histone H2A and SORBS2 were identified as potential markers of cancerous lesions and infarction, respectively. Previously, the over expression of histone H2A in colon

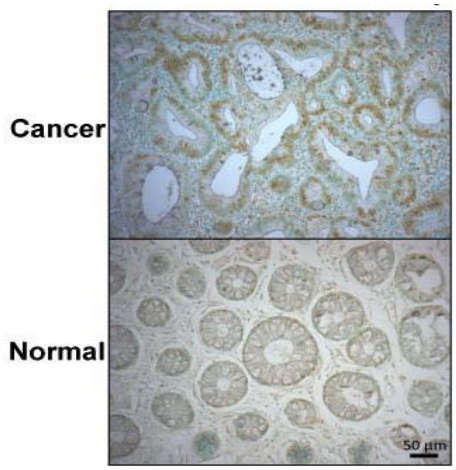

Figure 4: Immunohistochemical analysis of cancer (colonic adenocarcinoma) using anti-histone H2A. A significant increase in H2A signals was observed in the above image (x200, original magnification).

cancer cells has been reported in studies using LC/MS [5]. In addition to the development of the MS pretreatment, statistical analysis of our proteomic data has proven critical for the identification of useful biomarkers.

Results of our novel protocol indicate that MS imaging can be a powerful tool for *in situ* screening of disease biomarkers. Target peptides can then be further identified from proteins using MS/MS, thereby providing more specific information.

Considering the vast numbers of FFPE specimens that are stored in repositories, our new pretreatment method may significantly expand the number of samples available for MS-based proteomic analysis. In conclusion, FFPE tissues will be more available for MALDI-MS analysis in future.

References

1. Giusti L, Lucacchini A. Proteomic studies of formalin-fixed paraffin-embedded tissues. Expert Rev. Proteomics. 2013; 10: 165-177.
2. Kakimoto Y, Tsuruyama T, Miyao M, Abiru H, Sumiyoshi S, Kotani H, et al. The effectiveness and limitations of triphenyltetrazolium chloride to detect acute myocardial infarction at forensic autopsy. Am J Forensic Med Pathol. 2013; 34: 242-247.
3. Kakimoto Y, Ito S, Abiru H, Kotani H, Ozeki M, Tamaki K, et al. Sorbin and SH3 domain-containing protein 2 is released from infarcted heart in the very early phase: proteomic analysis of cardiac tissues from patients. J Am Heart Assoc. 2013; 2: e000565.
4. Kakimoto Y, Tsuruyama T, Yamamoto T, Furuta M, Kotani H, Ozeki M, et al. Novel in situ pretreatment method for significantly enhancing the signal in MALDI-TOF MS of formalin-fixed paraffin-embedded tissue sections. PLoS One. 2012; 7: e41607.
5. Naldi M, Andrisano V, Fiori J, Calonghi N, Pagnotta E, Parolin C, et al. Histone proteins determined in a human colon cancer by high-performance liquid chromatography and mass spectrometry. J Chromatogr A. 2006; 1129: 73-81.

Austin Biomark Diagn - Volume 1 Issue 1 - 2014
Submit your Manuscript | www.austinpublishinggroup.com

Citation: Tsuruyama T, Kakimoto Y and Yajima Y. Application of Mass Spectrometry for Identification of Biomarkers in Formaldehyde-Fixed Paraffin Embedded Specimens. Austin Biomark Diagn. 2014;1(1): 3.