



Typing of colon and lung adenocarcinoma by high throughput imaging mass spectrometry☆

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ABSTRACT

In advanced tumor stages, diagnosis is frequently made from metastatic tumor tissue. In some cases, the identification of the tumor of origin may be difficult by histology alone. In this setting, immunohistochemical and molecular biological methods are often required. In a subset of tumors definite diagnosis cannot be achieved. Thus, additional new diagnostic methods are required for precise tumor subtyping. Mass spectrometric methods are of special interest for the discrimination of different tumor types. We investigated whether it is possible to discern adenocarcinomas of colon and lung using high-throughput imaging mass spectrometry on formalin-fixed paraffin-embedded tissue microarrays. 101 primary adenocarcinoma of the colon and 91 primary adenocarcinoma of the lung were used to train a Linear Discriminant Analysis model. Results were validated on an independent set of 116 colonic and 75 lung adenocarcinomas. In the validation cohort 109 of 116 patients with colonic and 67 of 75 patients with lung adenocarcinomas were correctly classified.

The ability to define proteomic profiles capable to discern different tumor types promises a valuable tool in cancer diagnostics and might complement current approaches. This article is part of a Special Issue entitled: MALDI Imaging, edited by Dr. Corinna Henkel and Prof. Peter Hoffmann.

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1. Introduction

In advanced tumor stages diagnosis is made from metastatic tumor tissue. Distinguishing the most common metastatic adenocarcinomas (colon, kidney, breast, pancreas, lung, stomach and ovary) from each other is daily routine in clinical pathology [1]. The main metastatic localizations in the human body are liver, lung and bone. Despite histological, immunohistological (IHC) and molecular pathological techniques, the primary site of 3–5% of all epithelial tumors cannot be determined (cancer of unknown primary, CUP) [2]. Among these, 80% represent adenocarcinomas [1]. Interestingly, lung cancer is the main causative occult primary for bone metastases and, due to novel treatment options, has to be diagnosed with a high degree of certainty [3]. Adenocarcinoma is the most common histologic type in lung cancer [4]. Since therapy relies

on the determination of the primary tumor site (tissue of origin), exact classification of the tumors is mandatory. Besides IHC, molecular methods have been included as a diagnostic tool [5]. Mass spectrometry and especially imaging mass spectrometry (IMS) techniques are promising candidates to define the tissue of origin since hundreds of peptides or proteins can be analyzed at the same time. Furthermore, it may be an advantage that tissue integrity is preserved [6]. IMS allows correlation of specific tissue structures with a peptide- or protein pattern. In the present large-scale study, we analyzed tissue microarrays (TMAs) to investigate whether the discrimination of colon and lung primary tumors is possible by IMS. Furthermore, we tested our algorithm on a whole tissue slide of a case of metastasized colon cancer to the lung and on a whole slide of a primary lung adenocarcinoma. We demonstrate that the differentiation between adenocarcinomas from colon and lung based on a proteomic pattern acquired by IMS on formalin-fixed paraffin embedded (FFPE) TMAs is possible with high accuracy.

2. Materials and methods

2.1. Tissue samples and tissue microarray

We investigated a cohort of 383 FFPE tissue specimens from individual patients with primary adenocarcinoma of the colon ($n = 217$) and primary adenocarcinoma of the lung ($n = 166$). FFPE TMAs have been created from both tissue types. Lung TMA samples were provided by the Institute of Pathology, University Hospital of Heidelberg, colon TMAs were provided by the Institute of Pathology, Hospital Aschaffenburg, Germany and the Institute of Pathology, University Hospital “Carl Gustav Carus” of Dresden, Germany. Diagnosis was confirmed in each tissue core by histopathological and IHC investigations. Prior to TMA construction, a hematoxylin and eosin (H&E)-stained section of each block was analyzed in order to select representative tumor-containing regions. A TMA machine was used to extract a tandem 1.0 mm cylindrical core sample from each tissue donor block. The cohort was randomly divided in two sample groups: one sample group to build a classification model and a second sample group to validate the model. Additionally, two whole tissue sections, one with primary adenocarcinoma of the lung and one with a colon cancer metastasis in the lung were analyzed to test if the differentiation of both entities is possible on metastases. Furthermore, two individual FFPE tissue sections (one with adenocarcinoma of the colon and one with adenocarcinoma of the lung) were collected for identification analysis. Tissues were processed according to the Declaration of Helsinki. The local ethics committee of the University Hospital of Heidelberg, University Hospital of Dresden, as well as the Hospital of Aschaffenburg approved the study and informed consent was given by all participants.

2.2. Sample preparation

5 μm thick serial sections of each FFPE TMA were cut and mounted onto conductive indium tin oxide (ITO)-coated glass slides (Delta Technologies Ltd., Loveland, U.S.A.). Sample slides were processed for dewaxing with xylene (Fischer Scientific GmbH, Schwerte, Germany), rehydrating through graded ethanol (Fischer Scientific GmbH), and for heat induced antigen retrieval in Tris-HCl (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) buffer (10 mM, pH 9.0) at 95 °C for 20 min, as previously described [7]. Sections were subsequently on-tissue digested with trypsin (Promega GmbH, Mannheim, Germany) in a humidity chamber at 37 °C for 1.5 h. Trypsin solution was prepared in 200 μL 40 mM ammonium bicarbonate (Sigma-Aldrich Chemie GmbH) and 10% v/v HPLC grade acetonitrile (ACN, Fischer Scientific GmbH) to a final concentration of 0.1 $\mu\text{g}/\mu\text{L}$ and deposited onto the sections using an automatic reagent sprayer (ImagePrep, Bruker Daltonik GmbH, Bremen, Germany) in 25 cycles with a fix nebulization time of 1.2 s. A solution of 7 mg/mL of alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma-Aldrich Chemie GmbH) in 50/50 ACN/0.5%

trifluoroacetic (TFA, Fischer Scientific GmbH) was then applied onto the digested sections using the same ImagePrep device with an optimized Bruker Daltonik default method for sensor controlled nebulization of the matrix.

2.3. IMS analysis

IMS was performed using an Autoflex Speed TOF/TOF mass spectrometer (Bruker Daltonik GmbH) operated in reflectron mode with positive polarity. Each mass spectrum was automatically generated using the auto-execute software in FlexControl (Bruker Daltonik GmbH) in the range of m/z 700–4500 at spatial resolution of 150 μm . A peptide calibration standard mix including angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, and somatostatin 28 (Bruker Daltonik GmbH) was used for external calibration. Datasets were processed for baseline subtraction using the algorithm TopHat, chemical noise smoothing (5 m/z width), and peak alignment using a default statistical peptide internal mass spectrum calibration method of flex Analysis 3.3 software (Bruker Daltonik GmbH). Following the IMS measurement, matrix was removed from each section by two washes in 100% methanol (Fischer Scientific GmbH) for 5 min and H&E staining was performed for pathological examination to confirm the presence of tumor and to correlate IMS data with morphology.

2.4. Statistical analysis and classification

Histological cancer regions were marked by two pathologists (M.K., J.K.). The diagnosis was established by histology and IHC where necessary. The annotated sections were superimposed with MSI data, and mass spectra of regions of interest were collected for classification analysis. We used Linear Discriminant Analysis (LDA) (SCiLS Lab, SCiLS GmbH, Bremen, Germany) to generate the classification model. The training set included 101 colon and 91 lung primary carcinoma tissues. The LDA model was then applied to an independent set including 116 colon and 75 lung carcinoma tissue cores for validation. The cohorts are summarized in Table 1.

2.5. Protein identification by LC-MS/MS

One serial section (5 μm thick) from a colon primary adenocarcinoma was prepared for identification analysis. Peptide extraction was performed by pipetting up and down (ten times) on the regions of interest with 5 μL of 10% ACN first and then with 5 μL of 50% ACN extraction buffer. The extracts were collected in Maxymum Recovery tubes (Axygen, Aalst, Belgium) that allow low protein binding and lyophilized using a speedvac (Savant™ SPD 121P Thermo Fischer Scientific, Waltham, USA). The enriched extract was desalted using a Zip Tip C18 cartridge (Millipore, Overijse, Belgium) according to the manufacturer's instructions and resuspended in 10 μL of 0.1% formic acid solution. The sample was separated by liquid chromatography (LC; ACQUITY UPLC M-Class, Waters) using an ACQUITY UPLC M-Class HSS T3 Column, 1.8 μm , 75 $\mu\text{m} \times 250 \text{ mm}$ (Waters) with a flow rate of 600 nL/min. Solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN) were used with a linear gradient as follows: 0 min, 99% A; 1 min, 99% A; 5 min, 93% A; 140 min, 65% A, 150 min. The LC eluent was then electrosprayed from the analytical column at 2.1 kV voltage through the liquid junction of the nanospray source. The chromatography

Table 1
Samples used for LDA based classification model.

	Colon	Lung	Total number of patients
Patients in training set	101	91	192
Patients in validation set	116	75	191
Total number of patients	217	166	383

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