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Proteomic tissue profiling for the improvement of grading of noninvasive papillary urothelial neoplasia

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ABSTRACT

Objectives: In 2004, a novel grading system for papillary non-invasive bladder cancer was introduced; low grade (LG) and high grade (HG) in lieu of the former G1, G2, G3. This change allowed for increased reproducibility as well as diminished interobserver variability in histopathological grading among individual pathologists. Matrix Assisted Laser Desorption/Ionization Time of Flight Imaging Mass Spectrometry (MALDI TOF IMS) was evaluated as an automatic and objective tool to assist grading of urothelial neoplasms and to facilitate accuracy.

Design and methods: To separate G1 (LG, n = 27) and G3 (HG, n = 21) papillary tumors MALDI TOF IMS was performed using an appropriate algorithm. Thereafter, the automatic assignment of a separate G2 (n = 31) group was completed.

Results: G1 (LG) and G3 (HG) tumors were separated with an overall cross validation of 97.18%. G2 tumors indicated a true positive rate of 78.3% for LG and 87.5% for HG, respectively.

Conclusions: MALDI TOF IMS is a powerful support tool to ascertain pathological diagnosis/grading.

Introduction

Among frequent transitional cell carcinoma (TCC) the most common phenotype (70%) is a (single) papillary non-invasive neoplasia (pTa), which is generally both genetically stable and well differentiated. In contrast, its more poorly differentiated counterpart tends to occur relatively less frequently [1]. The histological grading of TCCs reveals significant prognostic information, especially with regard to tumor progression [2,3]. In 2004, the WHO classification system subdivided TCCs into a two-tier grading system (low grade, LG, high grade, HG, replacing G1, G2 and G3), and assigning the former grade 2 tumors into one or the other of these groups. LG tumors tend to display an orderly appearance with slight variations in nuclear polarity, size, and shape, with infrequent mitotic figures, usually in the lower half of the epithelium. These progress in about 10% and cause death in only 5% of cases [4,5]. In contrast, HG non-invasive papillary tumors show an altered cellular polarity, pleomorphic nuclei, and numerous mitotic figures, including atypical ones. Due to the genetic instability of HG tumors, reflected in their disordered morphology, these tumors show rates of progression of up to 40% with a tumor related mortality of about 35% [2,3,6]. Flat neoplasias (CIS, carcinoma *in situ*) or solid invasive neoplasias (T1–T4) tend to be genetically unstable, less differentiated [7] and thus carry with them a worse prognosis [8].

The WHO classification of 1974 (grading bladder cancer into the three groups G1, G2, and G3) proved insufficient in predicting the outcome of urothelial carcinoma. In 2004, the terms low grade (LG) and high grade (HG) were introduced in order to describe a well differentiated tissue with both low progression risk and few genetic alterations, and less differentiated tissue with high risk of recurrence and progression to (muscle-) invasive disease respectively. The new integration of former grade 2 tumors into either the HG or the LG group, however, resulted in considerable controversy with respect to both literature and clinical routine [1]. Whether a former G2 morphology has the sufficient nuclear atypia required for a high grade lesion, is mainly subjective and depends upon the pathologist's

Abbreviations: CI, confidence interval; HG, high grade; IMS, imaging mass spectrometry; LG, low grade; MALDI TOF, Matrix Assisted Laser Desorption/Ionization Time Of Flight; PWKW, P-value of Wilcoxon or Kruskal–Wallis test; ROI, region of interest; SVM, Support Vector Machine; TCC, transitional cell carcinoma; TLC, Thin layer chromatography; TUR, Transurethral resection; WHO, World Health Organization.

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experience. Additionally, as the classification of tumors in LG or HG is motivated by the presence of genetic stability in LG tumors *vs*. genetic instability in HG tumors, it is difficult to determine a definitive cut-off point genetically. A proteomic approach through mass spectrometry is a promising alternative to separate tumor entities, especially as the practicability of this method has become increasingly feasible.

In the presented work, tissue Matrix Assisted Laser Desorption/Ionization Time Of Flight (MALDI TOF) Imaging Mass Spectrometry (IMS) was used to generate peaks specific in both mass and intensity for histologically defined tissue areas. Initially, we identified differential peaks for G1 pTa tumors to represent the LG group as well as for G3 pTa tumors to represent the HG group. Thereafter, we classified G2 specimens automatically, based on their peak profile, into either the LG or HG group, and compared the proteomic grading result of each tumor with the histopathological re-classification of the same sample. The major focus of our analysis was the verification of the histological diagnosis.

Material and methods

Sample collection

Transurethral resection (TUR) specimens were obtained through cystoscopy of patients suspected of having urothelial cancer, according to the standardized procedures of the Department of Urology, RWTH Aachen University Hospital. Tissue specimens were immediately snap-frozen and stored at −80 °C. Informed consent was obtained for all specimens and approval obtained from the local ethics committee (RWTH EK 122/04). In total, samples from 79 patients with pTa TCC (G1: 27; G2: 31; G3: 21) were included in this study.

Sample preparation

(i) Tissue sectioning: serial cryosections from each biopsy specimen were prepared for MS analysis and staining. Sections for MALDI imaging (10 μm) were mounted on special conductive glass slides (Bruker Daltonics, Bremen, Germany). Prior to mass spectrometry, each slide was first washed in 70% ethanol (high performance liquid chromatography grade) and thereafter in 1% aqua bidest, 90% ethanol, and 9% pure acetic acid in order to remove the mounting medium (Tissue Tek, Sakura, Zoeterwoude, Netherlands). Serial sections of each sample (5 μm) were then collected on regular glass slides and stained using Hematoxylin and Eosin for histopathological reclassification. (ii) Matrix application: matrix (180 mg sinapinic acid, Bruker Daltonics, #203073) was mixed with 10 μl 1% trifluoroacetic acid (Roth, Karlsruhe, Germany), 5000 μl liquid chromatography MS grade water (Merck, Darmstadt, Germany) and 5000 μl acetonitrile (Sigma-Aldrich, Steinheim, Germany) in order to dissolve the proteins of the specimen and incorporate them into the matrix crystal lattice. For matrix application, a Thin Layer Chromatography (TLC) spraying device (Sigma) was used, in order to obtain a homogeneous matrix layer with a 100–100,000 fold molar excess of matrix molecules compared with tissue analytes. Care was taken to prevent tissue drenching and analyte delocalization, as well as to standardize the matrix application thus ensuring reproducible results. To define and circumcise the urothelial area to be imaged, slides were photographed using a photomap microscope (M400, Wild, Heerbrugg, Switzerland).

Data processing, visualization and statistical analysis

All imaging mass spectrometry experiments were performed using the Ultraflex III MALDI TOF mass spectrometer (Bruker Daltonics) and FlexControl 3.0 and Fleximaging 2.0 software (Bruker Daltonics). The area to be analyzed was marked in the software and a measurement spot grid with 200 μm center-to-center spacing was set for spectra acquisition. At each measuring point, 20 sufficient laser shots were averaged per spectrum. For each sample a region of interest (ROI) comprising about 120 spectra was analyzed. All spectra were calibrated to internal calibration masses chosen out of the original spectra (5658.06 Da, 11467.77 Da).

Data analysis

The ClinProTools 2.0 software (Bruker Daltonics) was used for data evaluation and analysis (see Bruker Daltonics ClinProTools user Manual 2.0). Initially, we compared the G3 tumor group containing 21 ROIs and G1 tumor group containing 27 ROIs. To distinguish between those two groups, statistical analysis was performed using a Support Vector Machine (SVM)-based algorithm. For all peaks integrated in the algorithm (generally about 25 peaks) a P-value of Wilcoxon Kruskal–Wallis test (denoted by PWKW in ClinProTools) was performed. The test assesses whether two independent samples follow the same continuous distribution or if there is a location shift. The test statistic is based on the ranks of the data rather than their raw values. For further information please refer to W.H. Kruskal [9].

MALDI MS derived spectra from each G2 tissue sample were classified by the software into the G3/HG or G1/LG group according to their peak pattern utilizing the SVM-based algorithm. Classification results were compared with the pathologists' histological grading. This reclassification was performed in a blinded manner. In order to assess our classifier's performance, we first determined the recognition capacity which is the relative number of correctly classified observations when the whole data set is used to train the classifier. In order to obtain a measure for the generalizability of the model, we performed a K-fold cross validation. In addition to the point estimators we derived a 95% confidence interval (CI) for accuracy, *i.e.* the probability to classify a new object correctly. The most commonly used CI for a binomial parameter p is the Wald interval. The limits of the $100(1-\alpha)\%$ CI are given by:

$$\hat{p} \pm z_1 - \alpha \sqrt{\hat{p}(1-\hat{p})/n}$$

where $\hat{p} = c/n$ is the estimator of the accuracy based on n observations and c correctly classified objects. z_q denotes the q quantile of the standard normal distribution. By substituting n *for* $\tilde{n} = n + 4$ and $\hat{p}$ *for* $\tilde{p} = (c+2)/(n+4)$ one obtains the adjusted Wald interval. This modification is recommended by Agresti and Coull [10], due to its superior performance for small sample sizes and for $\hat{p}$ near 1. The basic idea is adding two classification successes and two failures before calculating the Wald interval resulting in a slightly conservative interval.

Results

In this study, we analyzed papillary TCC samples from 48 patients with a G1/LG or G3/HG phenotype in order to develop a classification model capable of distinguishing between the two classes. This model was subsequently applied to an independent test set of 31 G2 papillary TCC samples, and the classification results were compared to the histopathological diagnosis made by two independent uropathologists (K.L-D, N.T.G.). Using the initial training set MALDI imaging analysis was able to identify 46 significantly differentially expressed peaks between G1 ($n = 27$) and G3 ($n = 21$) tumor sections (based on Wilcoxon rank-sum test, p-value < 0.05). The overall sum spectra of all histologically confirmed G3 tumors (red) and G1 tumors (green) are displayed in Fig. 1 showing protein profiles in the mass range from 2.5 to 18 kDa. From the 46 differentially expressed peaks, 23 were selected by the software, and incorporated into the SVM algorithm.