Label-Free Molecular Fingerprint-Based Cytopathology

Screening and Diagnosis Research

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The gold standard for the bladder cancer diagnosis is cystoscopy, which is invasive, painful, and may be accompanied by health risks for the patients. The presented "label-free molecular fingerprint-based cytopathology" method uses spatially resolved Raman spectra as fingerprints for the biochemical composition of urine cells, classifying individual cells automatically in a bioinformatics approach.

Urine-based Test for Urothelial Carcinoma (UC)

Bladder cancer is the seventh most regularly-diagnosed cancer worldwide in the male population and UC is the major type. The recurrence rate of UC is very high and patients with a UC history repeatedly undergo follow-up examinations to monitor the recurrence for years after treatment.

Numerous non-invasive tests based on urine have been developed for monitoring UC recurrence and used as an adjunctive to cystoscopy, while these tests provide low sensitivity. For instance, voided urine cytology provides a sensitivity of ~84% for high-grade UC and very low sensitivity of ~16% for low-grade UC [1]. In addition to the presence of high inter-/intra-observer dilemma, the ability of urine cytology to detect recurrence of UC varied significantly between different institutions with a sensitivity of 38-65% [2]. Furthermore, the US Food and Drug Administration (FDA) approved non-invasive tests based on urine biomarkers including (NMP22) and UroVysion. However, these tests suffer from low sensitivity, providing no diagnostic certainty in excluding UC. Therefore, none of these tests has been recommended in the European Association of Urology guidelines [3]. The combination of cystoscopy and these non-invasive tests lead only to increase the costs without any significant improvement in the surveillance for recurrence. Therefore, there is an unmet medical need to develop urine-based tests with higher sensitivity, especially for early-stage, low-grade UC, and surveillance of patients with a UC history.

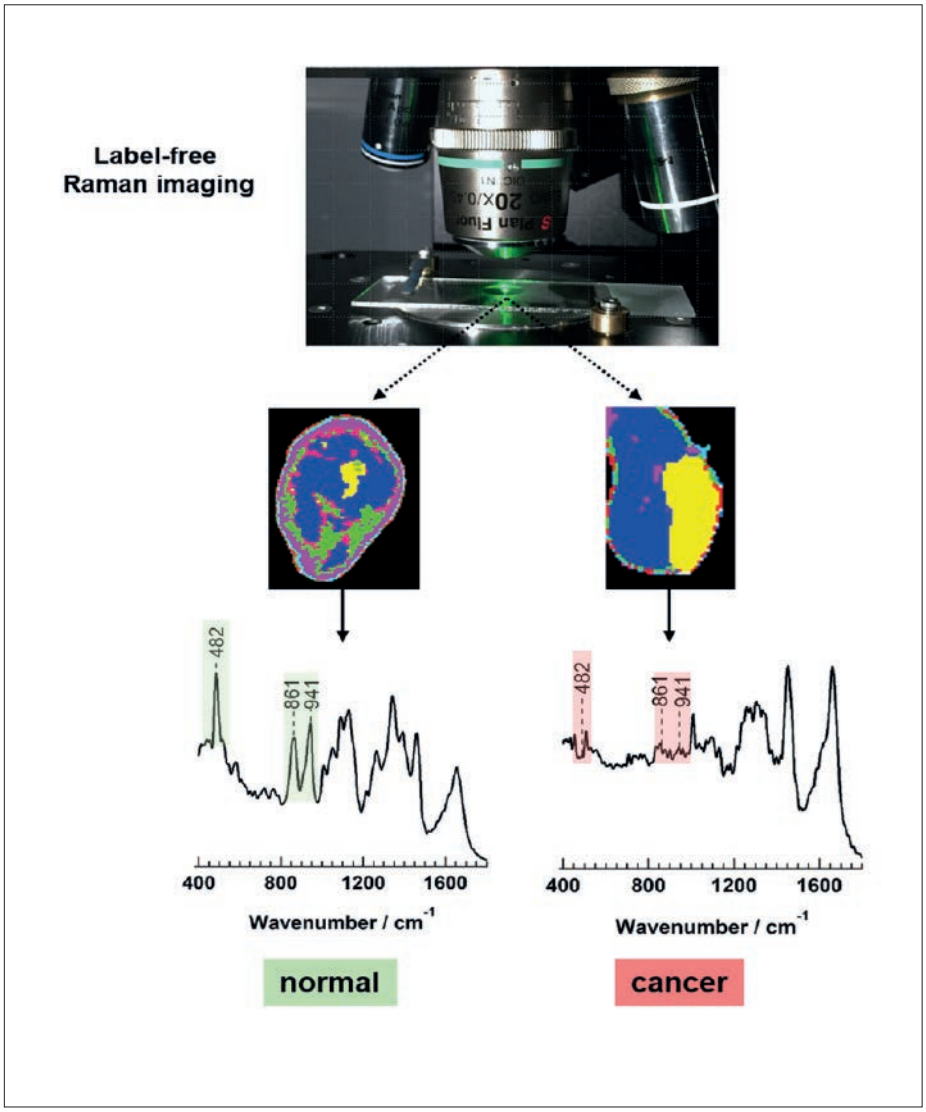

Fig. 1: Label-free Raman imaging of urothelial cells in an unstained microscopic slide of urine cytology from patients diagnosed with urocystitis and high-grade UC. The yellow colour in the cell images represents the nucleus. The Raman spectra provide molecular resolution through Raman marker bands that can be used to distinguish between normal and high-grade cancerous urothelial cells.

Raman-based Spectral Cytopathology

A label-free and inter-/intraobserver-independent non-invasive method with high accuracy for the detection of urothelial cancer cells in urine sediments using Raman spectral imaging and coherent anti-Stokes Raman scattering (CARS) has been developed [4]. Raman microscopic imaging characterizes cellular samples

with high spatial and lateral resolution. The spatially resolved spectra of the Raman microscopic images reflect the biochemical composition of the cell at the corresponding pixel positions (fig. 1). Raman microscopy has shown the ability to monitor the molecular changes in for example cells upon the progression of cancer, enabling the differentiation between cancerous and non-cancerous cells. CARS is a non-linear form of Raman microscopy that provides fast imaging up to video rate [5].

Essentially, the present novel method that differentiates between cancerous and non-cancerous urothelial cells, contains training and validation stages [4]. The objective of the training stage was to recognize the urothelial cells in urine sediments and to obtain their representative spectra that can be used to train a supervised classifier such as random forest (RF) or deep convolutional neural networks (DCNNs). In the training stage, the unstained microscopic slide of urine cytology was first measured by CARS imaging. This produces an image containing different types of urine cells including, normal or urocystitis, low-grade, and high-grade cancerous urothelial cells, squamous cells, leukocytes, erythrocytes, and bacteria. CARS images were used for the preselection of urothelial cells based on cell size and morphology in a similar way as that the pathologists using in hematoxylin-eosin (H&E)-stained images in urine cytology. For instance, the cancerous urothelial cells can be discriminated from urocystitis cells based on the shift in the ratio of the nucleus to the cytoplasm as well as the distortion of the nuclear morphology upon the progression of cancer [6]. Afterwards, Raman spectral imaging of these selected cells was acquired and an example of the Raman results of urocystitis and high-grade cancerous urothelial cell are shown in figure 2. Cell nuclei can be visualized in a label-free manner using the Raman marker band of DNA. Finally, cells were H&E-stained and a pathologist annotated the H&E-stained cells shown in figure 2, thus approving our preselection. It is apparent that the morphology of cellular nuclei produced by label-free Raman imaging is similar to that obtained with the H&E-stained images. The Raman results also revealed large spectral changes upon the progression of cancer and provided molecular resolution through Raman marker bands. For instance, the results indicate not only a decrease in the glycogen level but also an increase of fatty acids levels in cancerous urothelial cells in comparison to the urocystitis cells as expected. These metabolic changes enabled for automated identification of cancerous urothelial cells in urine with high accuracy [4].

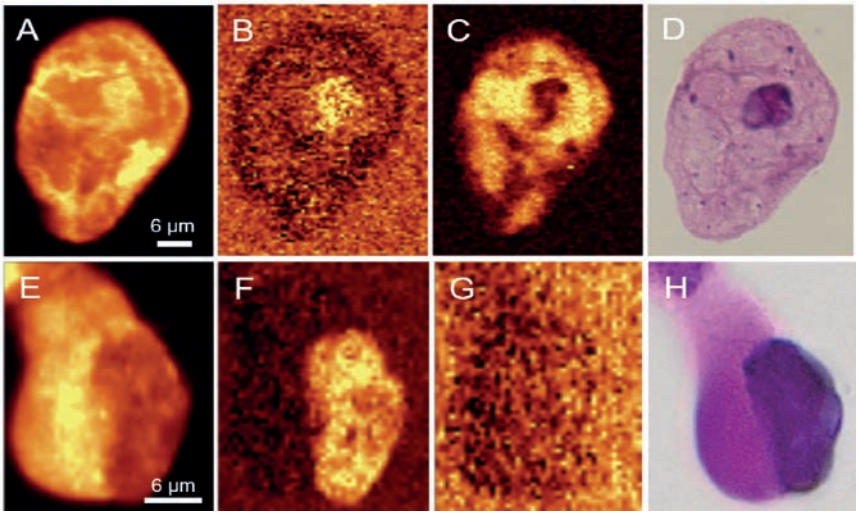

Fig. 2: Raman spectral imaging of urocystitis (A–C) and high-grade cancerous (E–G) urothelial cells from urine sediments. (A, E) Integrated Raman intensity images of cells in the 2800–3050 cm^{-1} regions. This wavenumber range displays the distribution of cellular components containing C–H moiety. (B, F) Integrated Raman intensity images of cells in the 785– 805 cm^{-1} regions. These mages display the nucleus of cells via the DNA marker band near 790 cm^{-1}. (C, G) Integrated Raman intensity images of cells in the 465-500 cm^{-1} regions. These mages display the distribution of glycogen in cells through a glycogen marker band near 482 cm^{-1}. These images clearly indicate that glycogen level is depleted in the cancerous urothelial cell in comparison with urocystitis cell. (D, H) H&E-stained images.

The Raman spectra or spectral images of cancerous and non-cancerous urothelial cells were obtained and used to train either RF [4,7] or DCNNs classifiers [8]. Urothelial cells in urine sediments, not included in the training stage, can then be identified in a label-free and automated manner in the validation stage by the trained classifiers. In addition, a patient is classified as "cancer" if most of the urothelial cells were considered as cancer cells. The validation of the results was performed by means of leave-one-patient-out cross-validation. The RF classifier was trained on individual pixel spectra of cytoplasm and nucleus of cancerous and non-cancerous urothelial cells, yielding an accuracy of 90% [4,7]. This accuracy was enhanced to 100% by using spatial bagging that is based on a statistical analysis of the majority vote over classification results obtained from individual pixel spectra [7]. We also have used another approach based on the extraction of morphological and textural features from Raman spectral images to differentiate between cancerous and non-cancerous cells. The RF classifier trained on these features produced an accuracy of 100%, while that trained on the features extracted from H&E-stained images yielded an accuracy of 90% [7]. These results indicate that Raman images carry more morphological and textural information than H&E-stained images, the current gold standard in cytopathology. Furthermore, we have performed DCNNs classifications and the results have shown that the accuracy of the classifications based on DCNNs exceeds other classifiers those described above. This is because the DCNNs classifications were based not only on the spectral information but also on morphological features of cells. The deep learning approach will significantly improve the application of Raman spectroscopy in diagnosis in the next few years.

Future Work and Perspective

The results have shown that Raman microscopy has the potential for the identification of urothelial cancer cells in urine sediments with high accuracy. Such kind of non-invasive tests based on urine with higher sensitivity, presumably also for early-stage and low-grade UC, will significantly improve the surveillance for years of the recurrence in patients with a previous history of UC. Consequently, this will reduce the need for the invasive discomfort test, cystoscopy, and decreasing the management cost of the patients. The current study represents small cohort measurements of urine sediments from pa-

tients diagnosed with urocystitis and high-grade UC. However, a large cohort study is necessary to provide not only reasonable statistics but also to overcome the heterogeneity in urine samples. In addition, the study should be extended to include urine sediments from patients in the early-stage and low-grade UC.

One disadvantage of Raman spectral imaging is that the Raman intensity is weak and this leads to a longer accumulation time to improve the signal-to-noise ratio of the Raman signals, making this method not suitable for measurements of a large cohort study. On the other hand, coherent Raman microscopic methods such as stimulated Raman scattering (SRS) or CARS provide fast imaging up to video rate and should be used in future. Similar to the Raman spectra, SRS spectra reflect the biochemical composition of the cells and the spectra provide information about the molecular changes in the cells upon the progression of cancer.

Acknowledgements

The original papers are published in Analytical Chemistry (doi: 10.1021/acs.analchem.7b01403), Journal of Chemometrics (doi: 10.1002/cem.2973), and Journal of Biophotonics (doi: 10.1002/jbio.201800022). This project was funded by the Protein Research Unit Ruhr within Europe (PURE), Ministry of Innovation, Science, and Research (MIWF) of North-Rhine Westphalia, Germany and European Regional Development Fund, European Union and North-Rhine Westphalia, Germany and German Social Accident Insurance (DGUV: UroFollow, FP-0241 BC).

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