Discrimination of bladder cancer cells from normal urothelial cells with high specificity and sensitivity: Combined application of AFM and modulated Raman spectroscopy

Elisabetta Canetta^{1,2,§}, Andrew Riches³, Eva Borger⁴, Simon Herrington³, Kishan Dholakia², Ashok K. Adya^{1,*}

¹ School of Science, Engineering and Technology, Abertay University, Bell Street, Dundee DD1 1HG, UK

² School of Physics and Astronomy, University of St Andrews, North Haugh, Fife KY16 9SS, UK

³ School of Medicine, University of St Andrews, St Andrews, Fife KY16 9TF, UK

⁴ School of Biology, University of St Andrews, St Andrews, Fife KY16 9TF, UK

* Corresponding author. Tel.: +44 1382 308653; Fax: +44 4382 8663; E-mail address:

ashok.adya@abertay.ac.uk

§ Current address: St Mary's University College, School of Sport, Health and Applied Science, Waldegrave Road, Strawberry Hill, Twickenham TW1 4SX

Abstract

Atomic force microscopy (AFM) and modulated Raman spectroscopy (MRS) were used to discriminate between living normal human urothelial cells (SV-HUC-1) and bladder tumour cells (MGH-U1) with high specificity and sensitivity. MGH-U1 cells were 1.5-fold smaller, 1.7-fold thicker and 1.4-fold rougher than normal SV-HUC-1 cells. The adhesion energy was 2.6-fold higher in the MGH-U1 cells compared to normal SV-HUC-1 cells, which possibly indicates bladder tumour cells to be more deformable than normal cells. The elastic modulus of MGH-U1 cells was 12-fold lower than SV-HUC-1 cells, suggesting a higher elasticity of the bladder cancer cell membranes. The biochemical fingerprints of cancer cells displayed a higher DNA and lipid content probably due to an increase in the nuclear to cytoplasm ratio. Normal cells were characterised by higher protein contents. AFM studies revealed decrease in the lateral dimensions and increase in thickness of cancer cells compared to normal cells which authenticate the observations from MRS. Nanostructural, nanomechanical and biochemical profiles of bladder cells provide qualitative and quantitative markers to differentiate between normal and cancerous cells at the single cellular level. AFM and MRS allow discrimination between adhesion energy, elasticity, and Raman spectra of SV-HUC-1 and MGH-U1 cells with high specificity (83%, 98%, and 95%) and sensitivity (97%, 93%, and 98%). Such single cell-level studies could have a pivotal impact in the development of AFM-Raman combined methodologies for cancer profiling and screening with translational significance.

Keywords: Atomic Force Microscopy, Modulated Raman Spectroscopy, Bladder cancer, Cytoskeleton organisation, Cell mechanics

1. Introduction

Bladder cancer is the fourth most common genito-urinary malignancy in males and the eleventh most common cancer in females. Although since the early 1990s the bladder cancer death rate has decreased by more than a third, ~100 people still die from bladder cancer every week making this tumour the eighth most common cause of cancer death.

Cancer transformation and progression can be strongly associated with changes in the structural, mechanical [1-4], and biochemical [5-8] properties of cells. The biomechanical features of cancer cells, such as their adhesion and elasticity, are known to have a strong impact on cell function, adherence, transformation, motility, and metastasis [3,9]. Such qualitative and quantitative markers are pivotal for the development of new clinical tools for early diagnosis of cancer [1-3] and cancer transformation, progression and metastasis [10].

Atomic Force Microscopy (AFM) has been extensively used in the past decade to determine the nano-biomechanical differences between cancer and normal cells [11-12]. Cancer and malignant cells of different types have consistently been found to be softer than normal and benign cells [13-15]. The changes in the nano-biomechanical phenotypes of cells during oncogenesis and cancer transformation and progression have been linked with the reorganisation of the cell cytoskeleton [16-17]. It is recognised that cell deformability and shape stability are governed by the integrity of the cytoskeleton [17-21]. The actin filaments in the superficial zone of the cell and the microtubule network in its deep zone [16] play a major role in cell biomechanics. Comparison of the mechanical phenotypes of either normal or cancer cells is of great relevance for translational research.

Raman spectroscopy (RS) is a powerful laser-based scattering technique that allows measuring the biochemical features of the specimen as well as identifying the structure of its molecules. The Raman spectrum of a specimen can be considered to be its intrinsic biochemical fingerprint [22-25]. In the last decade, RS has been used for the identification of

bladder and prostate normal and cancer cells [5,26-27] as well as normal, inflammatory, and cancer bladder tissues [6,7]. The disadvantage of RS is the dramatic limitation caused by strong fluorescence signals. A simple method for fluorescence suppression based on the continuous modulation of the excitation wavelength called modulated Raman spectroscopy (MRS) has been developed [28]. MRS has been successfully applied to the identification of human urothelial (SV-HUC-1) and bladder cancer cells (MGH) in urine samples [29]. In this paper, we present the combined application of AFM and MRS to discriminate between normal (SV-HUC-1) and malignant (MGH-U1) human bladder cell lines.

2. Materials and Methods

2.1 Cell lines

A human urothelial cell line (SV-HUC-1) was kindly supplied by Dr. Catherine Reznikoff, University of Wisconsin, Madison, U.S.A. Cells were cultured in the following medium: F-12 nutrient mixture of L Glutamine (Ham) GIBCO 21765 with added Human Insulin 5 µg/ml, Hydrocortisone 1 µg/ml, Transferrin 5 µg/ml, Glucose 2.7 mg/ml, non-essential amino acids 0.1 mM, Penicillin 100 µg/ml, Streptomycin 100 units/ml and foetal calf serum 1%. The SV-HUC-1 cells had been immortalised by transformation with simian virus 40 (SV40) [30]. A cell line derived from a recurrent human bladder tumour (MGH-U1) was kindly supplied by Professor John Masters, Prostate Cancer Research Centre, UCL Medical School, London. This MGH cell line is maintained in long-term culture [31]. It has been shown that the MGH cell line is the same as the T24 cell line, also derived from a recurrent bladder tumour [32]. MGH-U1 cells were cultured in the following medium: GIBCO D - MEM:F12 (1:1) with added Foetal calf serum 7% (Globepharm), Penicillin 100 µg/ml, L-Glutamine 2 mM and Streptomycin 100 units/ml.

2.2 Visualisation of F-actin filaments

MGH-U1 and SV-HUC-1 cells were plated in 8 well Lab-Tek slide chambers at low density. Cytochalasin D (Sigma C8273) was added at a concentration of 2 μ M and cells incubated for 3 hours along with untreated wells. The slides were then washed in Dulbeccos phosphate buffered saline (DPBS) and fixed in 4% paraformaldehyde for 10 minutes. Following washing in DPBS, cells were permeabilised in 0.1% Tween in DPBS for 5 minutes, washed again and blocked with 1% bovine serum albumin (BSA) for 40 minutes. After another wash, cells were stained with Alexa Fluor 594 phalloidin (Life Technologies A12381) using a 1:250 dilution of the stock in 1% BSA for 1 hour. Finally slides were washed in DPBS, mounted in Vectorshield mounting medium with DAPI and viewed using a Leica DM5500B fluorescence microscope to visualise the actin filaments.

2.3 Sample preparation for AFM

Cells from the two cell lines were plated into Lab-Tek slide chambers at appropriate cell densities (10^4 cells per ml) so that cells were well spread for the AFM studies. AFM studies were carried out using living cells in their culture medium. Cells from the SV-HUC-1 and MGH-U1 cell lines were prepared daily in triplicate and kept in the incubator at 37°C and 5% CO₂ until use.

2.4 Sample preparation for MRS

Cells from each cell line were fixed by adding PreservCyt (Cytect UK) at a ratio of 2 parts cell suspension in culture medium to 1 part PreservCyt. The cells were then pelleted by centrifugation at 600 g for 10 minutes and resuspended in PreservCyt for storage at 4°C. Samples for MRS analysis were washed in phosphate-buffered saline (PBS) and re-suspended in 100 μ l of PBS to concentrate the cells. The sample chamber used in the MRS

experiments was made by placing 80 μm deep vinyl spacer between a quartz slide and a quartz coverslip (UQG, UK). 20 μl of cell suspension was pipetted inside the chamber and the cells allowed to sediment onto the inverted quartz coverslip for ~ 30 minutes before starting the experiments.

2.5 Atomic force microscopy

The ultrastructure of living SV-HUC-1 and MGH-U1 cells were determined by using a commercial AFM instrument (Nanowizard I, JPK Instruments, Germany) in contact mode, and their nanomechanical properties were determined from the AFM force mapping (AFM-FM) data gathered soon after with the same instrument. All the AFM experiments were performed at room temperature in appropriate culture medium (see Section 2.1) for each cell type. AFM experiments were performed for a maximum of 2 hours on each of the two cell lines to ensure that the cells were alive for the duration of the experiments. After 2 hours the samples were discarded and a new sample (see Section 2.3) was used. The experiments were performed over different days to ensure the validity of the results. For AFM imaging and AFM-FM, Si_3N_4 cantilevers coated with Au reflective coating (MSCT, Bruker AFM probes) were used. These had a nominal spring constant of 0.01 N/m and a resonance frequency of 7 kHz. The cantilevers were 310 μm in length and 20 μm in width, and had a silicon pyramidal tip with a radius of curvature of ca. 10 nm and half-open angle of ca. 35° , microfabricated at their end. The cantilevers were calibrated using the thermal noise method [33] before each experiment to determine the exact spring constant, k , of the cantilever. The measured spring constant of the cantilever used ranged from 0.011 to 0.018 N/m. Knowing the exact value of k is essential for quantitative AFM-FM experiments.

The images of cells of interest were first captured by AFM raster scanning. The height and deflection images (512×512 pixels) were acquired at a scan speed of 0.5 Hz and a set point

of 2 nN to avoid any damage to the cell surface during scanning. The images were processed using the JPK data processing software 4.2.53 (JPK Instruments, Germany). AFM-FM experiments were carried out on the same cell soon after its image was acquired in order to relate the cell surface (e.g. morphology and roughness) to its nanomechanical (e.g. adhesion and elasticity) properties. AFM-FM experiments were conducted using the Force Mapping mode of the AFM instrument. In brief, 40 different $50 \times 50 \mu\text{m}^2$ force-volume maps over 10×10 point grids (100 force-displacement curves per map with a $5 \mu\text{m}$ step size) were recorded over the whole cell body. In each of the 100 force spectroscopy measurements, the tip was brought into contact with the specimen surface and then withdrawn from the surface at a load/unload speed of $0.5 \mu\text{m/s}$. A maximum loading force of 4 nN was applied which gave an indentation ranging from 270 nm to 950 nm depending upon the differences in the mechanical properties of different cells [21, 34].

2.5.1 Roughness and section analyses

The roughness and section analyses were carried out using the JPK data processing software 4.2.53. The surface roughness of SV-HUC-1 and MGH-U1 cells was analyzed by measuring the root-mean-square roughness, R_{rms} on raw AFM height images, i.e. images which did not undergo any filtering. For both SV-HUC-1 and MGH-U1, 40 cells were chosen randomly and the R_{rms} measured on 6 different areas ($4 \times 4 \mu\text{m}^2$) of the cell surface. The data were analyzed using the sampling theory as described elsewhere [35]. Section analysis on the AFM height images of SV-HUC-1 and MGH-U1 cells was also carried out to measure the dimensions (length and width) and thicknesses (peak-to-valley height) of the cells (Fig. 1). In AFM height images, the widths and lengths of samples are overestimated due to the effects of tip deconvolution. The widths and lengths were therefore estimated using the Stemmer & Engel method [36]. The histogram distributions of the roughness, length, width and thickness values

were obtained using R 3.0.1 [37] with a bin width of 25 nm for the roughness, 5 μm for length and width, and 1 μm for the thickness. To determine the underlying distribution of the roughness, the density probability functions of all the distributions were calculated through the Kernel density estimation non-parametric method and the density plot overlapped to the histogram of each distribution. This statistical analysis was performed using R 3.0.1 [37]. The statistical significance (set at $P \leq 0.05$) of nanostructural differences was determined with the Student's t-test using R 3.0.1 [37].

2.5.2 Nanomechanical properties

The AFM-FM data were analyzed using the JPK data processing software 4.2.53. The individual force-displacement curves were converted into force-distance (F - d) curves and the latter shifted to remove the offset. The adhesion energy (energy spent in debonding the AFM probe from the cell surface combined with the energy required to deform the cell near to its surface), W_{adh} , was calculated from the F - d curves (Fig. 2). The Young's (elastic) modulus, E , of the cell surface was determined by analyzing the indentation portion of the F - d curves (Fig. 2b) using an approach derived from the Sneddon's variation of the Hertz model [38]. Because in this model the contribution of the adhesive force to the contact area between the tip and the cell surface is neglected, E will be referred to as the effective modulus, E_{eff} , of the tip-surface system [39]. The histogram of the distributions of W_{adh} and E_{eff} were obtained using R 3.0.1 [37] with a bin width of 2×10^{-16} J for the adhesion energy and 1 kPa for the effective elastic modulus. To determine the underlying distribution of the roughness the density probability functions of the two distributions were calculated through the Kernel density estimation non-parametric method and the density plot overlapped to the histogram of each distribution. This statistical analysis was performed using R 3.0.1 [37]. The statistical significance (set at $P \leq 0.05$) of nanostructural differences was determined with the Student's t-test. The correlations between the nanomorphological and nanomechanical properties of

SV-HUC-1 and MGH-U1 cells were investigated by linear regression. The statistical analysis was performed using R 3.0.1 [37]. The ability of AFM force mapping experiments to identify SV-HUC-1 cells and MGH-U1 cells on the basis of their adhesive and elastic properties was also determined by calculating sensitivity (measure of the AFM-FM's ability to identify the positives, i.e. MGH-U1 cells in the cancer-normal bladder cells binary system) and specificity (measure of the AFM-FM's ability to identify negatives, i.e. SV-HUC-1 cells in the cancer-normal bladder cells binary system).

The first step in the calculation of sensitivity and specificity was the construction of the confusion matrix (table that permits to visualise the performance of the AFM-FM experiments in identifying correctly SV-HUC-1 and MGH-U1 cells – Table 1) by using the R package “mda” (mixture and flexible discriminant analysis) [37].

Table 1. Confusion matrix for the MGH-U1+SV-HUC-1 binary system

	Predicted classification	
True classification	MGH-U1	SV-HUC-1
MGH-U1	True positive	False positive
SV-HUC-1	False negative	True negative

The second step consisted in the calculation of sensitivity and specificity using R 3.0.1 [37]:

$$Sensitivity = \frac{number_of_true_positives}{number_of_true_positives + number_of_false_negatives} \quad (1)$$

$$Specificity = \frac{number_of_true_negatives}{number_of_true_negatives + number_of_false_positives} \quad (2)$$

2.6 Modulated Raman spectroscopy (MRS)

Raman spectra were acquired using a home-built MRS system. The basic principles of MRS have been described elsewhere [29]. Briefly, the MRS setup was equipped with a 785 nm tunable diode laser (Sacher Lasertechnik, TEC-520–0780-100). An external arbitrary waveform/function generator (Keithley 3390, 50 MHz) was connected to the tuneable laser to modulate the wavelength with a ramp wave of 50% symmetry, a modulation frequency of 40 mHz, and a modulation amplitude of 60 GHz which corresponds to 0.2 nm at 785 nm wavelength. 40 stacked spectra from 40 fixed SV-HUC-1 and 40 fixed MGH-U1 were acquired in the spectral region 500 to 2100 cm^{-1} with a laser exposure time of 5 s while the laser was modulated. The 40 stacked spectra were subsequently analysed using a MATLAB script (Mathworks, Natick, USA) based on the Principle Component Analysis (PCA) method for fluorescent background suppression [28] to obtain the MRS spectra of both types of cells. PCA was then performed to compare the modulated spectra of SV-HUC-1 and MGH-U1 cells.

3. Results and discussion

The nanostructural, nanomechanical and biochemical properties of normal urothelial cells (SV-HUC-1) and bladder cancer cells (MGH-U1) were investigated in order to establish a correlation between them.

3.1 Cancer and normal cell membrane nanostructures

The nanostructural properties of 40 living SV-HUC-1 and 40 living MGH-U1 cells were investigated by contact mode AFM imaging in their culture media (Fig. 3). The AFM height and deflection images of two different phenotypes of normal SV-HUC-1 cells [i.e. the typical flat appearing normal epithelial cells exhibiting low nuclear to cytoplasmic ratio (Figs 3a-c) and “umbrella”-like (Figs 3d-f) cells, so called not only because their morphology resembles an open umbrella but also because the surface area of these cells

further increases after liquid intake like an umbrella which has been completely open] and MGH-U1 (Figs 3g-l) cells show the finest and ultra nanoscopic details of cell shapes and surface structures. These structures can be directly related to the cellular cytoskeleton organisation. SV-HUC-1 cells (Figs 3a-f) are well spread with the SV-HUC-1 umbrella cells showing the typical triangular shape (Figs 3d-e). The typical epithelial normal SV-HUC-1 cells display well-organised F-actin filaments that run in parallel (see Fig. 3c). Although the umbrella-like normal cells also show F-actin filaments that run in parallel in most of the cellular body (Fig. 3d) but they slightly weave around closer to the edge of the cell (Fig. 3f). These cells also clearly show focal adhesion (FA) points (indicated by arrows in Figs 3b and e). FAs are known to regulate the cellular adhesion to the extra cellular matrix [40] and their presence in SV-HUC-1 cells possibly enables the spreading of urothelial cells [41] as clearly revealed by the AFM images (Figs 3a-e). Conversely, MGH-U1 cells (Figs 3g-l) exhibit a round or elliptical shape and show fewer F-actin filaments compared to normal cells. F-actin filaments in them are not well aligned and form either a net-like pattern (Fig 3i) or ridges (Fig 3l). MGH-U1 cells do not present many FAs but they show a very well defined lamellopodium (indicated by the arrow in Fig 3h) which probably provides greater mobility to MGH-U1 cells. Some of the cancer cells also display pseudopodia (indicated by arrows in Fig 3k) which can be another indication of the higher level of locomotion of MGH-U1 cells. The fact that the filaments observed in the AFM images were made of F-actin was confirmed by fluorescent microscopy. The MGH-U1 and SV-HUC-1 cells showed abundant F-actin filaments labelled with fluorescent phalloidin (Fig. 4a and c). The F-actin filaments were severely disrupted following pretreatment with cytochalasin D and form aggregates and foci (Fig. 4b and d).

The differences in the cytoskeleton organisation and in the morphology of normal and cancer cells were confirmed by AFM roughness (Fig. 5) and section analysis (Fig. 6). The

roughness of SV-HUC-1 and MGH-U1 cells was significantly different ($P = 0.04$). The histograms of the probability for the roughness from normal urothelial cells and the corresponding Kernel density plot (Fig. 5a) show that the roughness distribution is not normal. In particular, a broad and short “shoulder” is present on the left of the central peak which seems to correspond to a second population in the histogram. Hence, two populations with peaks at (153.68 ± 7.21) nm and (260.25 ± 8.19) nm seem to be present. This trend could be indicative of the different F-actin filament organisations (Figs. 3c and f) in the two different SV-HUC-1 phenotypes (i.e. the typical flat appearing normal epithelial cells and umbrella-like cells). In particular, the flat normal epithelial SV-HUC-1 cells seem to be ~ 41% less rough than the umbrella-like normal cells, possibly due to the more parallel and regular organisation of the F-actin filaments (Fig. 3c) compared to the woven F-actin filaments that are observed on the surface of the umbrella-like SV-HUC-1 cells (Fig. 3f). A similar trend was found for the histograms of probability for the roughness for bladder cancer cells and the corresponding Kernel density plot (Fig. 5b) that revealed the presence of a narrow and sharp “shoulder” on the right of the central peak and, therefore, showed the non-normality of the roughness distribution for MGH-U1 cells. This narrow shoulder seems to correspond to a second population in the histogram. These two populations are centred at (210.61 ± 15.25) nm and (271.65 ± 18.9) nm, respectively. The appearance of this two populations could be attributed to the heterogeneity in cytoskeleton disorganisation observed in cancer cells (Figs. 3i and l). Two main types of MGH-U1 cells were observed: cells with few woven F-actin filaments visible on their surfaces (Fig. 3i) and cells with a ridge-like organisation of the cytoskeleton (Fig. 3l). Interestingly, the F-actin seems to be less organised in the central region of the cancer cells (Figs. 3l) as has also been previously observed for breast cancer cells [13] and malignant thyroid cells [14]. The less number of F-actin filaments and the ridge-like cytoskeleton organisation could be attributed to the lower

amount of F-actin and higher amount of G-actin (globular form of actin) which is known to be associated with bladder carcinogenesis [42]. Cancer cells displaying a ridge-like organisation of the cytoskeleton were found to have a surface roughness $\sim 22\%$ higher than cancer cells showing only few woven F-actin filaments. Additionally, the lengths, widths, and thicknesses of SV-HUC-1 and MGH-U1 cells are found to be significantly different ($P = 0.001$). The histograms of length and width values from SV-HUC-1 cells (Figs. 6a and c) reveal a broad normal distribution for the length and for the width as shown by the absence of any “shoulder” in the corresponding density plots. These normal distributions are centred at $(71.32 \pm 5.03) \mu\text{m}$ for the length and $(17.45 \pm 0.58) \mu\text{m}$ for the width. The broadness of the distribution could be due the two different SV-HUC-1 phenotypes. Conversely, the histograms from MGH-U1 cells (Figs. 6b and d) show a narrow distribution for the length and for the width. These distributions are not normal as shown by the presence of little “shoulders” on the right of the central peaks. However, these shoulders do not correspond to a second population in the histograms for the length and width. The distributions are centred at $(35.1 \pm 0.9) \mu\text{m}$ for the length and $(24.55 \pm 2.98) \mu\text{m}$ for the width. This non-normality could be attributed to the heterogeneity of the MGH-U1 samples observed in Figs 3g-l. The histogram of thickness from SV-HUC-1 cells (Fig. 6e) displays a non-normal distribution as shown by the appearance of a “shoulder” on the right of the central peak. However, this shoulder does not correspond to a second population in the histogram. The distribution of the thickness of SV-HUC-1 is centred at $(5.26 \pm 0.29) \mu\text{m}$. Conversely, the histogram from MGH-U1 cells (Fig. 6f) reveals a broad normal thickness distribution centred at $(6.68 \pm 1.57) \mu\text{m}$. This seems to indicate heterogeneity in the normal and bladder cancer cell population in terms of cell thickness. Overall, the roughness and section analyses show that MGH-U1 cells are rougher, thicker and smaller in dimensions (area of normal cells: $\sim 1244.5 \mu\text{m}^2$, cancer cells: $\sim 861.7 \mu\text{m}^2$) than SV-HUC-1 cells, possibly due to the

reorganisation of the cytoskeleton in cancer cells. These findings are in agreement with the results obtained by Li et al. (2008) [13] and McEwen et al. (2013) [43] on human breast and lung cancer cells.

3.2 Nanomechanical properties of bladder cancer and normal urothelial cells

It is known that cellular nanostructures and nanomechanics are inter-related. In particular, F-actin reorganisation is strongly linked to cell migration [44] and enhanced cell motility [45] in cancer cells.

To investigate the link between morphology and nanomechanics of SV-HUC-1 and MGH-U1 cells, AFM indentation and stretching experiments were performed on the cells soon after visualising them by AFM imaging. This allowed a direct comparison between the nanostructural and nanomechanical properties of individual cells. Fig. 7 shows typical examples of adhesion energy and elastic maps of SV-HUC-1 and MGH-U1 cells. The colour scale goes from black to white and it covers a range $0 - 21 \times 10^{-16}$ J for the adhesion energy map and a range $0 - 37$ kPa for the elasticity map. It is interesting to note that the adhesion energy and elastic maps for the normal urothelial cells show a quite uniform distribution of adhesion energy ($\sim 6 \times 10^{-16}$ J) and elasticity (~ 14 kPa) all along the cell body in contrast to cancer cells which show a nonuniform distribution. This clearly indicates a homogenous level of deformability and elasticity of SV-HUC-1 cells. Conversely for cancer cells, while the adhesion energy ($\sim 12-17 \times 10^{-16}$ J) seems to be higher in the nuclear region, their elasticity (~ 5 kPa) seems to be lower in the nuclear region. This suggests yet again (see Section 3.1) an inhomogeneous degree of deformability and elasticity of cancer cells.

The adhesion energy, W_{adh} , and effective elastic modulus, E_{eff} , for SV-HUC-1 and MGH-U1 cells are found to be significantly different ($P = 0.001$). To visualise the skewness (i.e. departure from horizontal symmetry) and kurtosis (i.e. how tall and narrow the central peak is) or lack thereof in W_{adh} and E_{eff} for normal and cancer cells, box-plots were drawn (Figs.

8a and b). The line in the inter-quartile range (i.e. height of the box which accounts for the middle 50% of the sample distribution) represents the median of the sample. The median of the W_{adh} box-plot (Fig. 8a) is 8.17×10^{-16} J for SV-HUC-1 cells and 26.95×10^{-16} J for MGH-U1 cells. This suggests that cancer cells are more deformable and adhesive than the normal cells. W_{adh} for SV-HUC-1 and MGH-U1 cells are positively highly skewed as clearly indicated by the values for the skewness which are 0.3 and 1.5 for normal and cancer cells, respectively. The inter-quartile range is 11.81 for SV-HUC-1 cells and 6.92 for MGH-U1 cells which indicates a larger variability in W_{adh} for normal cells. Moreover, the kurtosis is 2.11 for normal cells and 4.87 for cancer cells which means that the central peak of the adhesion energy for normal cells is lower, broader and perhaps less distinct than for cancer cells. A dramatic difference between SV-HUC-1 and MGH-U1 cells can be observed in the box-plots for the Young's (elastic) modulus (Fig. 8b). Cancer cells are more elastic than their normal counterpart as also clearly shown by the median values: 27.57 kPa for SV-HUC-U1 cells and 2.46 kPa for MGH-U1 cells. E_{eff} for normal and cancer cells are positively skewed with a skewness of 0.28 and 1.54 for normal and cancer cells, respectively. Normal cells display a greater variability (inter-quartile range = 12.67) in their elasticity compared to cancer cells (inter-quartile range = 3.59). This is confirmed by the fact that the elasticity for MGH-U1 cells presents a taller and narrower central peak (kurtosis = 4.8) compared to SV-HUC-U1 cells (kurtosis = 2.48).

Although box-plots can clearly indicate the shapes of the W_{adh} and E_{eff} populations, they cannot provide information about the shapes of their distributions or reveal multimodality. W_{adh} histograms (Figs. 8c and e) display a non-normal adhesion energy distribution for normal and cancer cells as shown by the shoulders that are present in the corresponding Kernel density plots. These shoulders seem to correspond to a second population. Hence, two populations appear to be present with peaks at $(4.04 \pm 0.71) \times 10^{-16}$ J and $(9.91 \pm 0.87) \times 10^{-16}$

J for normal cells, and peaks at $(26.34 \pm 0.61) \times 10^{-16}$ J and $(44.59 \pm 0.94) \times 10^{-16}$ J for cancer cells. The E_{eff} histograms (Figs. 8d and f) show a broad normal elasticity distribution for SV-HUC-1 cells centred at (23.7 ± 1.2) kPa, and a non-normal distribution for cancer cells as shown by the presence of a narrow and sharp shoulder on the right of the central peak of the density plot (Fig. 8f). The shoulder seems to correspond to a second population. These two populations are centred at (1.68 ± 0.42) kPa and (3.48 ± 4.31) kPa for MGH-U1 cells. These results suggest that cancer cells are softer (lower E_{eff}) and more deformable (higher W_{adh}) than the normal cells. Moreover, the appearance of very broad normal distribution for the E_{eff} of SV-HUC-1 cells and two populations for the W_{adh} of SV-HUC-U1 and MGH-U1 cells and the E_{eff} distribution of MGH-U1 cells seem to suggest that the two different phenotypes for both SV-HUC-U1 and MGH-U1 cells have different deformability and elasticity. This may imply that elasticity and deformability are important nanomechanical markers that allow distinguishing clearly between normal and cancer cells. This is confirmed by high specificity and sensitivity values: 97% sensitivity and 83% specificity for the adhesion energy and 93% sensitivity and 98% specificity for the elasticity.

As previously observed (see Section 3.1) from the surface roughness (Figs. 5a and b) measurements, the presence of two populations for the W_{adh} and a very broad normal distribution for the E_{eff} distributions of normal cells could be due to the presence of two different SV-HUC-U1 phenotypes. Similarly, the appearance of two populations for the elasticity of cancer cells could be caused by heterogeneity in the cytoskeleton organisation. To verify these hypotheses, the correlation between surface roughness and elasticity was investigated (Fig 9a). Linear regression revealed a moderate negative correlation (correlation coefficient = -0.31) for normal cells which means that the rougher the cell surface, the more elastic the cell. Comparing this finding with the roughness analysis results (Fig. 5a) showed that umbrella-like cells, which displayed a rougher cell surface, are more elastic (lower E_{eff})

than the typical epithelial normal cells, which had a smoother surface. Normal umbrella-like urothelial cells are highly elastic cells whose cytoskeleton is rich in parallel running F-actin filaments as also shown by AFM images (Figs. 3d-f). Interestingly, the correlation between elasticity and roughness was not significant ($P = 0.053$) which indicated that the increase in elasticity of the cells was not purely due to the increase in cell roughness. Conversely, linear regression revealed a weak positive correlation (correlation coefficient = 0.26) for cancer cells: the rougher the cell surface, the less elastic (higher E_{eff}) or more stiffer it is. Roughness analysis (Fig. 5b) revealed that MGH-U1 cells with a ridge-like organisation of the cytoskeleton (e.g., cell 2 in Fig. 3h and 3j) had a rougher cells surface, whereas cancer cells exhibiting few woven F-actin filaments (e.g., cell 1 in Fig. 3h and 3i) had a smoother cell surface. Hence, the cancer cells with a ridge-like cytoskeleton organisation are stiffer (higher E_{eff}) than the MGH-U1 cells with few woven F-actin. Similar to SV-HUC-1, the correlation between elasticity and roughness for MGH-U1 was not significant ($P = 0.1$) which suggests that the increase in cancer cell stiffness (defined as ratio of stress to strain, i.e., deformation - measures the rigidity or lack of elasticity of the cell, and is inverse of softness) was not only caused by an increase in the cell roughness but also by some other factors.

To better understand the link between nanostructural and nanomechanical properties of normal and cancer bladder cells, the correlation between cell elasticity and thickness was established (Fig. 9c and d). Linear regression showed a similar trend: weak negative correlation (correlation coefficient = -0.2) for SV-HUC-U1 cells (the thicker the cell, the softer, i.e. the more elastic) and relatively strong positive correlation (correlation coefficient = 0.41) for MGH-U1 cells (the thicker the cell, the stiffer, i.e. the less elastic or more rigid). Hence, normal umbrella-like urothelial cells (Fig. 3d-e) are thicker and have a rougher cell surface, possibly due to the larger number of woven F-actin filaments as is also seen in AFM images (e.g., Fig. 3f). This type of cell is also more elastic as shown by the negative

correlation between thickness and surface roughness versus effective elastic modulus, E_{eff} (Figs. 9a-b). The correlation between the increase in elasticity and cell surface roughness could indicate that the F-actin filaments observed in the AFM images (Fig. 3f) are stress fibres (i.e. contractile bundles). When a controlled strain was applied to the SV-HUC-U1 cell during the indentation part of the AFM force mapping experiments, the contractile F-actin bundles did not allow the cell surface to deform too much, as also shown by the value of the adhesion energy, $W_{adh} = (5.64 \pm 0.98) \times 10^{-16}$ J, and therefore the cell could recover its original shape. This seems to confirm the hypothesis that the organisation of the filamentous F-actin cytoskeleton is strictly related to morphological and functional cell changes [46, 47]. The correlation between elasticity and thickness is not significant ($P = 0.1$) for SV-HUC-1 cells possibly indicating that the increase in elasticity of normal cells is not purely related to an increase in cell thickness but to an increase in both cell roughness and cell thickness. Conversely, the correlation between elasticity and cell thickness is significant ($P = 0.001$) for MGH-U1 cells, suggesting that the increase in cell stiffness is predominantly caused by an increase in cell thickness. These results seem to imply that the degree of elasticity of normal urothelial cells depends upon the roughness of its surface and the cell thickness, while for bladder cancer cells it mainly depends upon the cell thickness.

3.3 Modulated Raman spectral analysis of normal and cancer bladder cells

Average Modulated Raman (MR) spectra of SV-HUC-1 and MGH-U1 cells (Fig. 10a) show very clearly the Raman peaks of the chemical constituents of the cells allowing specific assignments of individual peaks to be made, based on previous data [23]. Normal SV-HUC-1 spectra are characterised by strong protein Raman peaks, such as those at 621, 954, 1004, 1030, 1050, 1131, 1463, and 1625 cm^{-1} as previously shown by Jess et al. [48] for cervical neoplasia. Spectra of normal cells also show a substantial peak at 710 cm^{-1} , which corresponds to C-N stretching vibrations in lipid. Moreover, a significant peak at 854 cm^{-1}

observed in the normal cell spectra can be assigned to C-C stretching mode in proline [47]. In contrast, cancer cell spectra show a significant decrease of proteins, such as the peak at 954 cm^{-1} corresponding to the C=O vibrations of the backbone of amide I (α -helix). However, bladder cancer spectra show a significant increase in DNA and RNA content as can be clearly seen by the presence of intense peaks at 645 , 1208 , and 1610 cm^{-1} which correspond to nucleotide chains [47,48]. The increase in DNA content in MGH-U1 cells seems to be confirmed by the higher relative intensity of the peaks pertaining to ring breathing modes in DNA bases, such as those at 727 , 785 , 1095 , and 1208 cm^{-1} . Cancer cell spectra also show an intense peak at 1736 cm^{-1} which could correspond to C-O vibrations, indicating an increase of the carbon-oxygen bonds.

To summarise, comparison of MR spectra of normal SV-HUC-1 and cancer MGH-U1 cells clearly indicate significantly higher DNA and lipid concentrations and lower concentrations of proteins in cancer cells. The higher DNA concentrations and lower protein contents observed in cancer MGH-U1 cells are consistent with changes associated with tumour development as previously observed by Jess et al. [48] for cervical neoplasia, Stone et al. [7], de Jong et al. [6], and Draga et al. [49] for malignant bladder tissues, and Harvey et al. [5,8] for fixed urological cells.

The increase in the intensity of DNA peaks and in particular of the phosphate group, PO_2^- backbone stretching of DNA (1095 cm^{-1}) observed in MGH-U1 cells is in agreement with the results obtained by Dovbeshko et al. [50] who reported an increase in this mode for cancer cells compared with normal cells due to spatial changes in the position and organisation of the phosphate group in the DNA of tumour cells. The higher DNA concentration in MGH-U1 cells is expected because of an increase in the nuclear to cytoplasm ratio caused by the decrease of lipids in the cytoplasm and the increase of DNA content in the nucleus that allows malignant cells to replicate very quickly [7].

PCA analysis was performed on the MR spectra of SV-HUC-1 and MGH-U1 cells (Fig. 10b) and it showed two clusters that clearly displayed the grouping effect of SV-HUC-1 and MGH-U1 cells, the similarity (tight clusters) within normal and cancer cell data sets and the dissimilarity (well separated clusters) between SV-HUC-1 and MGH-U1 cells. MRS could distinguish between normal and cancer cells with high sensitivity (98%) and specificity (95%). These results are in good agreement with those obtained for the nanomechanical properties of the normal and cancer cells, indicating that nanomechanical and biochemical features are important quantitative biomarkers.

4. Conclusions

The nanostructural, nanomechanical and biochemical properties of normal urothelial (SV-HUC-1) cells and bladder cancer (MGH-U1) cells have been investigated by AFM imaging, AFM force mapping and modulated Raman spectroscopy (MRS).

The main difference between normal and cancer cells is found to be the organisation of the cytoskeleton. SV-HUC-1 cells presented actin filaments running parallel across the cell body. Two different SV-HUC-1 phenotypes (the typical flat appearing normal epithelial type and umbrella type cells) were observed that differed mainly in their surface roughness (umbrella-like normal cells are ~41% rougher than the flat appearing normal epithelial cells). MGH-U1 cells displayed two organisations of the cytoskeleton: a ridge-like organisation and another in which only few woven actin filaments were visible. The cancer cells with a ridge-like cytoskeleton organisation had a surface roughness ~ 22% higher than cancer cells showing only few woven actin filaments. Overall, MGH-U1 cells presented ~ 23% rougher cell surface than SV-HUC-1 cells. MGH-U1 cells were ~ 40% thicker and ~31% smaller in dimensions than SV-HUC-1 cells, possibly due to the reorganisation of the cytoskeleton observed in cancer cells.

The two SV-HUC-1 phenotypes were found to have similar levels of deformability but different elasticity. The typical epithelial cells were ~ 37% stiffer than umbrella-like cells. This finding seems to indicate cell elasticity to be one of the main nanomechanical markers. MGH-U1 cells were ~ 93% softer and ~ 65% more deformable than SV-HUC-1 cells. These differences in the elasticity of normal and cancer cells seem to depend upon the cell roughness and thickness. SV-HUC-1 cells with a rougher cell surface and thicker (i.e. umbrella-like cells) were more elastic than normal (typical epithelial type) cells with smoother surface and flatter. In contrast, MGH-U1 cells with a rougher cell surface and thicker (i.e. ridge-like organisation of the cytoskeleton) were found to be stiffer than cancer cells with cytoskeleton organised in few woven F-actin filaments and flatter.

MR spectra of SV-HUC-1 and MGH-U1 cells indicated clear differences in their biochemical signatures. MGH-U1 cells had higher DNA and lipid content which could be caused by an increase of the nuclear to cytoplasm ratio as also suggested by the decrease in dimensions and increase in their thickness. SV-HUC-1 cells were characterised by higher protein contents.

The adhesion energy, elasticity, and MR spectra of SV-HUC-1 and MGH-U1 cells were identified with an efficiency of 91%, 96%, and 97%, respectively.

In conclusion, cell surface morphology (roughness and thickness) and mechanical properties, such as elasticity may serve as nanomechanical markers not only to identify cancer cells but also to distinguish between different phenotypes existing in normal cells and cancer cells. Moreover, the high sensitivity, specificity and accuracy obtained from the multivariate analyses on AFM nanomechanics and MRS data demonstrate the great potential of the AFM/Raman combination as an accurate clinical tool for the screening and profiling of cancer cells.

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References

1. Suresh S, Biomechanics and biophysics of cancer cells. *Acta Biomater* 2007; 3: 413-438; Nanomedicine: Elastic clues in cancer detection. *Nat Nanotech* 2007; 2: 748-749.
2. Cross SE, Jin YS, Rao J, Gimzewski JK, Nanomechanical analysis of cells from cancer patients. *Nat Nanotech* 2007; 2: 780-783.
3. Cross SE, Jin YS, Tondre J, Wong R, Rao J, Gimzewski JK, AFM-based analysis of human metastatic cancer cells. *Nanotechnology* 2008; 19: 384003.
4. Fritsch A, Höckel M, Kiessling T, Nnetu KD, Wetzel F, Zink M, Käs JA, Are biochemical changes necessary for tumour progression?. *Nat Phys* 2010; 6: 730-732.
5. Harvey TJ, Hughes C, Ward AD, Correia Faria E, Henderson A, Clarke NW, Brown MD, Snook RD, Gardner P, Classification of fixed urological cells using Raman tweezers. *J Biophotonics* 2009; 2: 47-69.
6. de Jong BWD, Bakker Schut TC, Maquelin K, van der Kwast T, Bangma CH, Kok DJ, Puppels GJ, Discrimination between nontumor bladder tissue and tumor by Raman spectroscopy. *Anal Chem* 2006; 78: 7761-7769.
7. Stone N, Hart Prieto MC, Crow P, Uff J, Ritchie AW, The use of Raman spectroscopy to provide an estimation of the gross biochemistry associated with urological pathologies. *Anal Bioanal Chem* 2007; 387: 1657-1668.

8. Harvey TJ, Correia Faria E, Henderson A, Gazi E, Ward AD, Clarke NW, Brown MD, Snook RD, Gardner P, Spectral discrimination of live prostate and bladder cancer cell lines using Raman optical tweezers. *J Biomed Opt* 2008; 13: 064004.
9. Lekka M, Pogoda K, Gostek J, Klymenko O, Prauzner-Bechciki S, Wiltowska-Zuber J, Jaczewska J, Lekki J, Stachura Z, Cancer cell recognition – Mechanical phenotype. *Micron* 2012; 43: 1259-1266.
10. Kumar S, Weaver V, Mechanics, malignancy, and metastasis: the force journey of a tumour cell. *Cancer Metastasis Rev* 2009; 28: 113-127.
11. Lekka M, Laidler P, Gil D, Lekki J, Stachura Z, Hryniewicz AZ, Elasticity of normal and cancerous human bladder cells studied by scanning force microscopy. *Eur Biophys J* 1999; 28: 312-316.
12. Sokolov I, Atomic force microscopy in cancer cell research, in “Cancer Nanotechnology”, Eds. H. Singh Nalwa and T. Webster, American Scientific Publishers’ Inc.; 2007. p. 1-17.
13. Li QS, Lee GYH, Ong CN, Lim CT, AFM indentation study of breast cancer cells. *Biochem Biophys Res Commun* 2008; 374: 609-613.
14. Prabhune M, Belge G, Dotzauer A, Bullerdiek J, Radmacher M, Comparison of mechanical properties of normal and malignant thyroid cells. *Micron* 2012; 43: 1267-1272.
15. Plodinec M, Loparic M, Monnier CA, Obermann EC, Zanetti-Dallenbach R, Oertle P, Hyotyla JT, Aebi U, Bentires-Alj M, Lim RYH, Schoenberger C, The nanomechanical signature of breast cancer. *Nat Nanotechn* 2012; 7: 757-765.
16. Kasas S, Wang X, Hirling H, Marsault R, Huni B, Yersin A, Regazzi R, Grenningloh G, Riederer B, Forrò L, Dietler G, Catsicas S, Superficial and deep changes of cellular mechanical properties following cytoskeleton disassembly. *Cell*

- Motil Cytoskel 2005; 62: 124-132.
17. Kumar S, Maxwell IZ, Heisterkamp A, Polte TR, Lele TP, Salanga M, Mazur E, Ingber DE, Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organisation, and extracellular matrix mechanics. *Biophys J* 2006; 90: 3762-3773.
 18. Ingber DE, Tensegrity: The architectural basis of cellular mechanotransduction. *Annu Rev Physiol* 1997; 59: 575-599; *Mechanobiology and diseases of mechanotransduction. Ann Med* 2003; 35: 564-577; Tensegrity I. Cell structure and hierarchical systems biology. *J Cell Sci* 2003; 116: 1157-1173.
 19. Radmacher M, Measuring the elastic properties of living cells by the atomic force microscope, in *Atomic Force Microscopy in Cell Biology*, Vol. 68, Eds. B.P. Jena and J.K.H. Hörber, Academic Press (London, UK); 2002. p. 67-90.
 20. Van Vliet KJ, Bao G, Suresh S, The biomechanics toolbox: experimental approach for living cells and biomolecules. *Acta Mater* 2003; 51: 5881-5905.
 21. Kuznetsova TG, Starodubtseva MN, Yegorenkov NI, Chizhik SA, Zhdanov RI, Atomic force microscopy probing of cell elasticity. *Micron* 2007; 38: 824-833.
 22. Cauberg ECC, de Bruin DM, Faber DJ, van Leeuwen TG, de ka Rosette JJMCH, de Reijke TM, A new generation of optical diagnostics for bladder cancer: technology, diagnostic accuracy, and future applications. *Eur Urol* 2009; 56: 287-297.
 23. Notingher I, Raman spectroscopy cell-based biosensors. *Sensors* 2007; 7: 1343-1358.
 24. Jess PRT, Garcés-Chávez V, Riches AC, Herrington CS, Dholakia K, Simultaneous Raman micro-spectroscopy of optically trapped and stacked cells. *J Raman Spectrosc* 2007; 38: 1082-1088.
 25. Rao S, Bálint S, Cossins B, Guallar V, Petrov D, Raman study of

- mechanically induced oxygenation state transition of red blood cells using optical tweezers. *Biophys J* 2009; 96: 209-216.
26. Crow P, Uff J.S, Farmer J.A, Wright M.P, Stone N, Optical diagnostics in urology: current applications and future prospects. *Brit J Urol Int* 2003; 92: 400-407.
 27. Crow P, Uff JS, Farmer JA, Wright MP, Stone N, The use of Raman spectroscopy to identify and characterise transitional cell carcinoma *in vitro*. *Brit J Urol Internat* 2004; 93: 1232-1236.
 28. Mazilu M, De Luca AC, Riches AC, Herrington CS, Dholakia K, Optimal algorithm for fluorescence suppression of modulated Raman Spectroscopy. *Opt Express* 2010; 18: 11382-11394.
 29. Canetta E, Mazilu M, De Luca AC, Carruthers AE, Dholakia K, Neilson S, Sargeant H, Briscoe T, Herrington CS, Riches AC, Modulated Raman spectroscopy for enhanced identification of bladder tumor cells in urine samples. *J Biomed Opt* 2011; 16: 037002.
 30. Christian BJ, Loretz LJ, Oberley TD, Reznikoff CA, Characterisation of human uroepithelial cells immortalised in vitro by simian virus 40. *Cancer Res* 1987; 47: 6066-6073.
 31. Masters JRW, Hepburn PJ, Human bladder cancer in vitro drug sensitivities: Range and stability in long-term culture. *Brit J Cancer* 1986; 54: 131-135.
 32. O'Toole CM, Povey S, Hepburn P, Franks LM, Identity of some human bladder cancer cell lines. *Nature* 1983; 301: 429-430.
 33. Hutter JL, Bechhoefer J, Calibration of atomic-force microscope tips. *Rev Sci Instrum* 1993; 64: 1868-1873.

34. Laurent VM, Kasas S, Yersin A, Schäffer TE, Catsicas S, Dietler G, Verkhovsky AB, Meister JJ, Gradient of rigidity in the lamellopodia of migrating cells revealed by atomic force microscopy. *Biophys J* 2005; 89: 667-675.
35. Adya AK, Canetta E, Walker G, Atomic force microscopic study of the influence of physical stresses on *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *FEMS Yeast Res* 2006; 6: 120-128.
36. Stemmer A, Engel A, Imaging biological macromolecules by STM: quantitative interpretation of topographs. *Ultramicroscopy* 1990; 34: 129-140.
37. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; 2013. URL <http://www.R-project.org/>
38. Sneddon IA, The relation between load and penetration in the axisymmetric Boussinesq problem for a punch of arbitrary profile. *Int J Eng Sci* 1965; 3: 47-57.
39. Canetta E, Marchal J, Lei CH, Deplace F, König AM, Creton C, Ouzineb K, Keddie JL, A Comparison of Tackified, Miniemulsion Core-Shell Acrylic Latex Films with Corresponding Particle-Blend Films: Structure-Property Relationships. *Langmuir* 2009; 25: 11021-11031.
40. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P, Molecular biology of the cell, 5th ed. Taylor and Francis Group; 2008.
41. Imani R, Kabaso D, Kreft ME, Gongadze E, Penic S, Elersic K, Kos A, Veranic P, Zorec R, Iglic A, Morphological alterations of T24 cells on flat and nanotubular TiO₂ surfaces. *Croat Med J* 2012; 53: 577-585.
42. Rao JY, Hemstreet III GP, Hurst RE, Bonner RB, Min KW, Jones PL, Cellular F-actin levels as a marker for cellular transformation: correlation with bladder cancer risk. *Cancer Res* 1991; 51: 2762-2767.

43. McEwen GD, Wu Y, Tang M, Qi X, Xiao Z, Baker SM, Yu T, Gilbertson TA, deWald DB, Zhou A, Subcellular spectroscopic markers, topography and nanomechanics of human lung cancer and breast cancer cells examined by combined confocal Raman microspectroscopy and atomic force microscopy. *Analyst* 2013; 138: 787-797.
44. Olson MF, Sahai E, The actin cytoskeleton in cancer motility. *Clin Exp Metastas* 2009; 26: 273-287.
45. Rotsch C, Radmacher M, Drug-induced changes of cytoskeletal structure and mechanics of fibroblasts: an atomic force microscopy study. *Biophys J* 2000; 78: 520-535.
46. Kumar S, Maxwell IZ, Heisterkamp A, Polte TR, Lele TP, Salanga M, Mazur E, Ingber DE, Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. *Biophys J* 2006; 90: 3762-3773.
47. Stricker J, Falzone T, Gardel ML, Mechanics of the F-actin cytoskeleton. *J Biomech* 2010; 43: 9-14.
48. Jess PRT, Smith DDW, Mazilu M, Dholakia K, Riches AC, Herrington CS, Early detection of cervical neoplasia by Raman spectroscopy. *Int J Cancer* 2007; 121: 2723-2728.
49. Draga ROP, Grimbergen MCM, Vijverberg PLM, van Swol CFP, Jonges TGN, Jummer JA, Bosch JLHR, In vivo bladder cancer diagnosis by high-volume Raman spectroscopy. *Anal Chem* 2010; 15: 5993-5999.
50. Dovbeshko GI, Gridina NY, Kruglova EB, Pashchuk OP, FTIR spectroscopy studies of nucleic acid damage. *Talanta* 2000; 53: 233-246.

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Normal urothelial (SV-HUC-1) cells

Bladder cancer (MGH-U1) cells

Cellular Length

Cellular Width

Cellular Thickness

Figure 6

Normal urothelial (SV-HUC-1) cells

Bladder cancer (MGH-U1) cells

AFM height image

Adhesion energy map

Elastic map

Figure 7

Figure 8

Figure 9

Figure 10

FIGURE CAPTIONS

Figure 1. (a) Section analysis on the AFM image of a living human urothelial cell (SV-HUC-1) under physiological conditions. The two arrows indicate how the cell width was measured using the JPK image processing software. The value measured for the cell width is also given. (b) Measure of the peak-to-valley height, H , and of the width (measured as full width at half maximum, FWHM) of the cell from cross-section analysis.

Figure 2. (a) Sketch of the steps of a force spectroscopy experiment showing contact between the AFM probe and the sample surface and subsequent force loading of the sample surface occurring during the approach cycle of the AFM experiment. On the retract cycle, the sample surface is stretched until the point of detachment is reached. (b) Example of a typical experimental F - d curve. The meanings of the elastic modulus, E , the maximum adhesive force, F_{max} , and the adhesion energy, W_{adh} are also identified.

Figure 3. (a) AFM height image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; height scale $0 - 7.2\ \mu\text{m}$), (b) AFM deflection image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; deflection scale $0 - 2.3\ \text{nN}$) of a normal typical urothelial cell (SV-HUC-1). The arrows indicate the focal adhesion points, and (c) high resolution deflection image ($8\ \mu\text{m} \times 8\ \mu\text{m}$ area; deflection scale $0 - 1.3\ \text{nN}$) of the marked zone of the SV-HUC-1 cell surface shown in (b). (d) AFM height image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; height scale $0 - 8.5\ \mu\text{m}$), (e) AFM deflection image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; deflection scale $0 - 1.7\ \text{nN}$) of a normal “umbrella”-like SV-HUC-1 (For definition of “umbrella-like” cells, see

Section 3.1). The arrows indicate the focal adhesion points, and (f) high resolution deflection image ($8\ \mu\text{m} \times 8\ \mu\text{m}$ area; deflection scale $0 - 0.9\ \text{nN}$) of the marked zone of the SV-HUC-1 cell surface shown in (e). (g) AFM height image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; height scale $0 - 9.4\ \mu\text{m}$), (h) AFM deflection image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; deflection scale $0 - 1.7\ \text{nN}$) of bladder cancer cells (MGH-U1). The arrow indicates the lamellopodium, and (i) high resolution deflection image ($8\ \mu\text{m} \times 8\ \mu\text{m}$ area; deflection scale $0 - 1.3\ \text{nN}$) of the marked zone of the MGH-U1 cell surface shown in (h).. (j) AFM height image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; height scale is $0 - 12.4\ \mu\text{m}$); (k) AFM deflection image ($60\ \mu\text{m} \times 60\ \mu\text{m}$ area; deflection scale is $0 - 1.1\ \text{nN}$) of a bladder cancer cell (MGH-U1). The arrows indicate pseudopodia, and (l) high resolution deflection image (area of $8\ \mu\text{m} \times 8\ \mu\text{m}$ and deflection scale of $0 - 0.7\ \text{nN}$) of the of the marked zone of the MGH-U1 cell surface shown in (k).

Figure 4. (a) MGH-U1 cells stained with Alexa Fluor 594 phalloidin illustrating the network of F-actin filaments; (b) MGH-U1 cells pre-incubated with cytochalasin D before staining with Alexa Fluor 594 phalloidin illustrating the disruption of the network of F-actin filaments; (c) SV-HUC-1 cells stained with Alexa Fluor 594 phalloidin illustrating the network of F-actin filaments; (d) SV-HUC-1 cells pre-incubated with cytochalasin D before staining with Alexa Fluor 594 phalloidin illustrating the disruption of the network of F-actin filaments. The scale bar is $20\ \mu$.

Figure 5. (a) Histogram of the roughness distribution for SV-HUC-1 cells and Kernel density plot (solid line). The density plot shows that the distribution is not normal due to the presence of a broad and short peak on the left of the central

peak. The non-normality of the roughness distribution could be caused by the two different phenotypes observed in the SV-HUC-1 cell line; (b) Histogram of the roughness distribution for MGH-U1 cells and Kernel density plot (solid line). The density plot shows that the distribution is not normal due to the presence of a narrow and sharp peak on the right of the central peak. This trend could be related to different cytoskeleton organisations in the MGH-U1 cell line.

Figure 6. (a) Histogram of the length distribution for SV-HUC-1 cells and corresponding Kernel density plot (solid line). The broad distribution could be attributed to the presence of two different phenotypes in the SV-HUC-1 cell line; (b) Histogram of the length distribution for MGH-U1 cells and corresponding density plot (solid line). The distribution displays a non-normal distribution, as indicated by the presence of a little “shoulder” on the right of the central peak of the density plot, possibly due to the heterogeneity of the sample; (c) Histogram of the width distribution for SV-HUC-1 cells and density plot (solid line). The distribution is normal (no appearance of any shoulder in the density plot) and broad, probably because of the two different phenotypes in the SV-HUC-1 cell lines as observed in (a); (d) Histogram of the width distribution and corresponding density plot (solid line) for MGH-U1 cells. The distribution is non-normal (as indicated by the presence of shoulders on the right of the central peak of the density plot) which could be caused by the heterogeneity of the sample; (e) Histogram of the thickness distribution and density plot (solid line) for SV-HUC-1 cells; (f) Histogram of the thickness distribution and density plot (solid line) for MGH-U1 cells. The distribution is quite broad probably due to the two different

organisations of the cell cytoskeleton as previously observed for the roughness distribution (Fig. 5b).

Figure 7. (a) Height AFM image of a SV-HUC-1 cell ($50\ \mu\text{m} \times 50\ \mu\text{m}$ area; height scale 0 - $7.2\ \mu\text{m}$); (b) Height AFM image of a MGH-U1 cell ($50\ \mu\text{m} \times 50\ \mu\text{m}$ area; height scale 0 - $10.5\ \mu\text{m}$); (c)-(d) Adhesion energy maps of the SV-HUC-1 cell shown in (a) and the MGH-U1 cell depicted in (b), respectively. Colour scale goes from black to white and covers a range $0 - 21 \times 10^{-16}\ \text{J}$; (e)-(f) Elastic map of the SV-HUC-1 cell shown in (a) and the MGH-U1 cell displayed in (b), respectively. Colour scale goes from black to white and covers a range $0 - 37\ \text{kPa}$.

Figure 8. (a) - (b) Comparison of the adhesion energy and effective elastic modulus values of SV-HUC-1 and MGH-U1 cells. The values are displayed as box-plots in which the line inside the box represents the median value. MGH-U1 cells are more deformable and softer than SV-HUC-1 cells; (c) Histogram of the adhesion energy distribution for SV-HUC-1 cells and the corresponding Kernel density plot (solid line). The distribution is not normal as shown by the “shoulder” on the right of the central peak. This could possibly be due to the two different phenotypes observed in the SV-HUC-1 cell line.; (d) The effective elastic modulus for SV-HUC-1 shows a broad normal distribution because no “shoulders” are present in the corresponding density plot (solid line). This trend could be caused by the two different phenotypes observed in the SV-HUC-1 cell line; (e) The adhesion energy distribution for MGH-U1 cells is not normal as indicated by the appearance of a “shoulder” in the density plot (solid line). This could possibly be due to the two different cytoskeletal organisation observed in the MGH-U1 cell line.; (f) The effective elastic modulus distribution for MGH-

U1 cells is not normal as shown by the presence of a narrow and sharp “shoulder” in the density plot (solid line). This trend could probably correspond to the two different MGH-U1 phenotypes.

Figure 9. (a) - (b) Correlation between elasticity and cell roughness for SV-HUC-1 and MGH-U1 cells. The correlation coefficient of the elasticity and cell roughness is -0.31 for SV-HUC-1 cells and +0.26 for MGH-U1 cells which indicates that the variables are moderately negatively correlated (the rougher the cell surface, the more elastic the cell) for SV-HUC-1 cells while they are weakly positively correlated (the rougher the cell, the stiffer it is) for MGH-U1 cells; (c)-(d) The correlation coefficient of the elasticity and thickness is -0.2 for SV-HUC-1 and +0.41 for MGH-U1 cells, indicating that the parameters are weakly negatively correlated (the thicker the cell, the more elastic it is) for SV-HUC-1 cells, whereas they show a relatively strong positive correlation (the thicker the cell, the stiffer it is) for MGH-U1 cells

Figure 10. (a) Mean modulated Raman spectra of normal SV-HUC-1 and cancer MGH-U1 cells. The spectrum is fluorescence-free, making it possible to distinguish clearly between the individual Raman peaks of the chemical constituents of the cells. (b) Principle Component Analysis (PCA) of all individual MR spectra for normal SV-HUC-1 and cancer MGH-U1 cells.