Microfluidic isolation and characterisation of bladder cancer cells from urine for early and non-invasive diagnosis of bladder cancer

Catarina Filipa Matoso Abreu, Instituto Superior Técnico, Portugal
catarina.abreu@tecnico.ulisboa.utl.pt

ABSTRACT

Bladder cancer has one of the highest recurrence rates among cancers and requires lifelong surveillance. Cystoscopy and urine cytology are the "gold standard" for bladder cancer detection but have well-known limitations. Thus, new methodologies to early identify and characterise various bladder cancers and their true biological potential are urgently needed. In this work, rare tumour-associated cells from body fluids of bladder cancer patients were isolated and characterised, using a microfluidic device. Importantly, cell capture was based on deformability and size, so that all cancer cells were trapped, regardless of their phenotype. Results show that in spiking experiments of HT1376 bladder cancer cells with peripheral blood mononuclear cells, the microfluidic platform reached an isolation efficiency of around 50% and an enrichment ratio of 22. Additionally, bladder wash and urine samples collected from bladder cancer patients subjected to transurethral resection were immunostained in situ with the following identifying markers: pan-cytokeratin (epithelial), vimentin (mesenchymal), survivin (malignancy), DAPI (nucleus) and CD45 (leukocyte). Notably, survivin, pan-cytokeratin and vimentin positive cells were found both in bladder wash and urine samples of a patient diagnosed with high-grade non-invasive papillary urothelial carcinoma (stage Ta). In contrast, for another patient diagnosed with low-grade carcinoma, survivin could not be detected in any sample, and vimentin positive cells were only present in the bladder wash. Overall, our findings revealed the phenotypic diversity of cancer cells in body fluids, likely relevant for patient treatment and follow up and pave the way for the realisation of a liquid biopsy for bladder cancer.

Keywords: Bladder cancer, Microfluidics, Liquid biopsy, Urine, Survivin

1. INTRODUCTION

Bladder cancer is the most common malignancy of the urinary tract, the 5th most common type of cancer in the Western World and the 9th Worldwide[1]. Cystoscopy adjunct with urine cytology are the gold standard for its detection. However, cystoscopy is an invasive and expensive diagnostic procedure with limited sensitivity for the detection of flat lesions, whereas urine cytology presents low sensitivity for low-grade papillary tumours. Even though several markers have been proposed over the years, including six FDA-approved marker tests, their use has not been implemented in clinical diagnosis due to their low sensitivities or high false positive rates[2–4]. This has contributed to the search of new biomarkers that may assist in early detection of recurrence. When combined, these biomarkers hold the potential of increasing the sensitivity and the specificity of urine cytology. Moreover, besides the identification of cancer cells, a more profound knowledge of the tumorigenic potential and cell phenotype is necessary in order to direct treatment.

Survivin is an apoptosis inhibitor protein expressed in the G2/M phase of the cell cycle. It is one of the most commonly overexpressed genes in cancer, being highly expressed in a variety of solid tumours and hematologic malignancies and absent or underexpressed in normal adult tissue, except for highly proliferative areas within normal tissues including vascular endothelial, hematopoietic and neural stem cells. Survivin expression can be detected in all bladder cancer cells but not in normal urothelium and is associated with disease recurrence, stage, progression and mortality[5–7].

Epithelial-to-mesenchymal transition (EMT) is a reversible process in which epithelial cells undergo changes in morphology, differentiation and motility and is highly associated to cancer progression and metastasis[8,9]. During this process, expression of epithelial markers such as E-cadherin and cytokeratin decreases while mesenchymal markers like vimentin and fibronectin[9] are upregulated. Markers of EMT are associated with muscle invasive bladder cancer, resistance to therapeutic agents and poor outcome[10]. By targeting these biomarkers, it is possible to assess the phenotype of a given cell and infer on the aggressiveness of the tumour.

Microfluidic devices have been intensively reported over the last years for their applicability in the isolation of rare cells, namely circulating tumour cells from blood. The same principles can be applied for rare cell isolation from other body fluids. Moreover, by targeting rare cells that have been in direct contact with the primary tumour site, their detection should be facilitated.

One of the major obstacles in voided urine cytology is that malignant cell origin cannot be identified. Cells present in urine can shed not only from bladder but also from any other organ of the

urinary tract, namely from the renal pelvis, ureter or urethra. To overcome this, bladder washes were used since they contain cells solely present in the bladder urothelium. By isolating these tumour cells in a microfluidic platform, direct comparison with the hospital cystoscopic and cytopathological evaluation is valid.

The aim of this project was to isolate and characterise distinct cancer cell populations from body fluids using a microfluidic device, with the long-term goal of developing a non-invasive diagnostic platform. Both bladder cell lines and clinical samples were used in this work to determine the potential of the microfluidic device. Cell phenotype was investigated by *in situ* immunocytochemistry using a panel of 5 distinct biomarkers.

2. MATERIALS AND METHODS

2.1. Master Fabrication

Master moulds were fabricated on 200 mm silicon wafers vapour primed with hexamethyldisilazane (HDMS) to improve photoresist (PR) adhesion. Wafers were spin coated with 2.2µm of AZP4110 positive PR (Microchemicals GmbH, Germany), deposited using a SUS MicroTec optical track. The pattern was transferred by Direct Write Laser lithography (DWL 2.0 Heidelberg, Germany). Sample was post baked and developed with AZ400K. The pattern was then etched by Silicon Deep Reactive Ion Etching (STPS Pegasus, United Kingdom) until the desired depth of 20 µm was reached. Once the etching was finished, the PR and Teflon residues were stripped using oxygen plasma in the STPS Pegasus machine. Finally, the wafer was diced into the individual masters using a DAD 3350 Dicing Saw (Disco, Japan) and cleaned with Isopropyl alcohol, rinsed with water and dried at 150ºC on a hot plate. All master wafers were treated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane (97%, Sigma-Aldrich) by vapour deposition to hydrophobize the surface.

2.2. Microfluidic Device Fabrication

Microfluidic devices were fabricated by polydimethylsiloxane (PDMS) soft-lithography. PDMS was prepared as a two-part system with mix ratio of 10:1 (w/w) base/curing agent (Sylgard 184 Silicon Elastomer kit, Dow Corning) and centrifuged at 3000xg for 5 minutes. PDMS was poured over the master and degassed using a desiccator. PDMS curing process occurred at 65ºC for 2 hours. Replica was cut with the aid of a scalpel. Inlet and outlet ports were made using a 1.5mm biopsy punch (Miltex). For the bonding, 76x100x1.0 mm glass slides (Knittel Glass) and PDMS replica surfaces were activated by weak oxygen plasma for 15 seconds using plasma cleaner (PDC-002-CE, Harrick Plasma). Tubing was performed with 0.51x1.52 mm Ethyl-Vynil-Acetate microtube (Cole Parmer Instruments). To increase channel wettability, 250 µl of ethanol 70% (v/v) was pumped at 100 mbar using a pressure pump (Mitos P-pump range, dolomite). Channels were rinsed with 250 µl of Phosphate Buffered Saline (PBS) (Phosphate Buffered Saline tablet, 10 mM, pH 7.4, Sigma Aldrich) at 200 mbar. The surface was passivated with 250 µl of 1% (w/v) Pluronic F-127, Sigma Aldrich) in PBS at 300 mbar. Devices were incubated overnight at 4ºC with parafilm sealed tubing and under tab water.

2.3. Cell Culture and Sample Preparation

2.3.1. Model system for bladder cancer

HT1376 bladder carcinoma cells (kindly provided by Dr. Alexandre Ferreira, IPO, Porto) were cultured in T-25 cell culture flasks (working volume of 5 ml) in complete Gibco RPMI 1640 Medium (1X) + GlutaMAX™ (ThermoFisher Scientific) supplemented with 10% (v/v) Heat-inactivated Fetal Bovine Serum (FBS) (Gibco) and 1% (v/v) Penicillin Streptomycin (Pen Strep, Gibco) and incubated in a humidified 37ºC/5% CO_2 chamber (Binder) until 80% confluent (around 3.00×10^6 cells). Cells were then harvested and either used for the experiments or maintained in culture. For sub-culturing, cells were washed with 5 ml of PBS pH 7.4 (1X) (Gibco) and incubated with 10% (v/v) of 0.25% Trypsin-EDTA (1X) (Gibco) in a humidified 37ºC/5% CO_2 incubator for 5 minutes for cell detachment. Trypsin was inactivated by adding 5 ml of complete growth medium. Finally, cells were counted in a Neubauer chamber using the Trypan blue dye (Sigma)-exclusion assay, and used in subsequent experiments.

2.3.2. Peripheral Blood Mononuclear Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were obtained from blood collected on the day of the experiment from healthy volunteers, by density gradient centrifugation. Briefly, one unit of blood sample (3 ml) was carefully layered over one unit of pre-warmed Histopaque-1077 (Sigma Aldrich) without mixing the two solutions. Sample was centrifuged (Centrifuge 5810 R, Eppendorf) at 650xg for 10 minutes without brake to avoid instantaneous mixing of the two layers. The PBMC layer was collected with a 15 ml plastic Pasteur pipette, transferred to a new 15 ml falcon tube, washed with 5 ml of PBS-2%BSA and centrifuged again at 300xg for 10 min. After discarding the supernatant, cells were resuspended in 1 ml of PBS-2%BSA. For PBMC counting, Tuerk solution (Fluka Analytical) was used. Cells were diluted at a ratio of 1:10 of

Tuerk and a 10 µl aliquot counted in neubauer chamber.

2.4. Microfluidic Device Performance

2.4.1. Capture Efficiency

For positive control experiments, HT1376 cells were harvested as described above and stained with the cell-permeable dye Calcein AM (Sigma Aldrich) in order to visually count viable cells under the fluorescence microscope. Approximately $1x10^6$ cells in 800 µl of PBS were mixed with 1µl of 4µM calcein and incubated in a humidified chamber 37ºC/ 5% CO_2 for 20 minutes followed by 3 washing steps at 1000xg for 5 min with PBS. The supernatant was discarded and cells resuspended in a final volume of 1 ml of PBS, to achieve a cellular concentration of $1x10^6$ cells/ mL. Next, serial dilutions were prepared so that a cell suspension of approximately 1000 HT1376 cells in 500 µL of PBS was obtained. This solution was then run in the microfluidic device at either 200 or 300 mbar using a pressure pump while monitored under the inverted optical fluorescence microscope (Nikon Eclipse MA200). To avoid any sudden increases in pressure and consequentially flow rate, pressure was gradually increased at 50 mbar intervals. At the end of the process, the microfluidic device was washed with 500 µL of PBS and cells fixed during 20 min with 250 µL of 4% (w/v) formaldehyde solution (Formalin, Sigma). A final washing step was performed to remove the fixative from the channels. Capturing efficiency of the microfluidic platform was determined by counting fluorescent entrapped cells.

2.4.2. PBMC Retention

PBMCs were obtained following the sample preparation protocol and similarly to HT1376 cells, were stained with Calcein-AM as described above to enable accurate detection of isolation yields. For the negative control experiments, serial dilutions were prepared so that a cell suspension of approximately 10,000 PBMCs in 500 µL of PBS was obtained. The cells suspension was then run under the inverted optical fluorescence microscope by the same protocol as described in section 2.4.1. PBMC retention was determined by counting stained cells trapped on the microposts under the fluorescence microscope.

2.4.3. Spiking Experiments

In order to determine isolation efficiency, purity and enrichment ratio of the device, 1000 HT1376 bladder cancer cells pre-stained with calcein were spiked to unlabelled 10,000 PBMCs in a total volume of 500 µl of PBS. Cell suspension was then processed through the device at 200 mbar, and cells washed with 500µl of PBS, fixed with 250 µl of formalin for 20 min and washed again with 500 µL of PBS. Finally, the microfluidic platform was extensively examined and the number of labelled and unlabelled cells counted and registered.

2.5. Clinical Samples

Bladder washes and urine samples were collected at Centro Hospitalar do Porto-Hospital Geral de Santo António (CHP-HSA), from patients who previously underwent diagnostic or follow-up cystoscopy. Patients aged <18 years or patients who had received previous treatment with local or systemic chemotherapy or immunotherapy were excluded from this study.

Catheterized urine samples were collected immediately before surgery and transferred into 50 ml plastic falcons pre-filled with 5 ml of FBS. Bladder washes were collected in parallel, as a part of the TURBT protocol. After the cystoscope was inserted through the urethra into the bladder, saline buffer was irrigated within the bladder and 45 ml were collected into 50 ml plastic falcons pre-filled with 5 ml of FBS. Samples were processed on the same day of collection at the International Iberian Nanotechnology Laboratory (INL) in Braga. This study was approved by the Ethics Committee of the Hospital and sample collection performed after written informed consent was obtained from all subjects.

2.6. Clinical Sample Processing

2.6.1. Bladder Wash Pre-processing

Samples were pelleted by centrifugation (1000xg for 5 min) and resuspended in 1 ml of PBS-2%BSA. Red blood cell lysis was performed through the addition of 2 ml of lysis buffer (145mM Ammonium chloride (NH_4Cl), 10mM Potassium chloride (KCl) and 0.1mM EDTA) and after a 5 min incubation at room temperature, cells were centrifuged for 5 min at 300xg. Cells were then resuspended in 1 ml of TrypLE™ Select enzyme solution (ThermoFisher Scientific) and incubated for 5 min at RT to promote dissociation of possible cell aggregates present. Enzyme inactivation was performed by diluting cells in 1 ml of PBS-2%BSA.

Bladder washes were then pelleted via centrifugation (1000xg for 5 min), resuspended in a final volume of 10 ml of PBS-2%BSA and filtered through cell strainers (first 70 µm and then 40 µm) to remove any cell aggregates that might clog the device. In order to concentrate the sample, the filtered cell suspension was centrifuged at 1000xg for 5 min and cell pellet resuspended in 1 ml of PBS-2%BSA.

2.6.2. Urine Pre-processing

Non-voided urine samples were pre-processed as the bladder washes, including cell filtration using strainers with different pore sizes (70 and 40 µm). The final cell suspension was centrifuged at 1000xg

for 5 min, and the pellet resuspended in 1 ml of RPMI complete medium with 10% DMSO and immediately frozen at -80ºC until further use. At the time of analysis, cells were thawed, resuspended in 5 ml of PBS-2%BSA, centrifuged at 1000xg for 5 min, resuspended again in 3 ml of PBS-2%BSA and passed through the 40 µm cell strainer. Finally, the sample was concentrated by centrifugation at 1000xg for 5 min and pellet resuspended in 1 ml PBS-2%BSA to run in the device.

2.6.3. Cell isolation in the microfluidic device

All samples were visually monitored by microscopy during the microfluidic isolation to ensure clogging did not occur and also to analyse cell behaviour and distribution throughout the separation process. Pre-processed bladder washes or urine samples were flown through the device at 200 mbar, and washed with 500 µl of PBS. Prior to sample fixation, and if necessary, 250 µl of TrypLE™ reagent was pumped into the device to dissociate cell aggregates and left to incubate for 5 minutes. Samples were washed with 250 µl of PBS and then fixed with 250 µl of Formalin solution during 20 minutes before a final washing step. Tubing tips were sealed with parafilm and devices immersed in water for storage at 4ºC until the immunostaining analysis.

2.7. Immunocytochemistry

2.7.1. Survivin primary antibody conjugation

Anti-Survivin monoclonal antibody (Clone 9H18L32, ThermoFisher Scientific) was concentrated from 0.5 mg/ml to 1mg/ml using the AbSelect™ Antibody Concentration & Clean-up Kit (InnovaBiosciences) and labelled with DyLight® 755 (Lightning-Link® Rapid Conjugation System, InnovaBiosciences), a far red fluorescence emitting fluorophore.

2.7.2. Antibodies for immunocytochemistry

Anti-cytokeratin-pan-FITC (1:100, Clone C-11, Sigma), anti-CD326-Alexa Fluor 488 (EpCAM) (1:50, Clone 9C4, Biolegend) to identify epithelial cells; anti-CD45-cy5 (1:25, Clone HI30, Abcam) to identify white blood cells; anti-vimentin-eFluor 570 (1:50, Clone V9, eBioscience) for mesenchymal-like cells; anti-survivin-Dylight 755 (1:25, Clone 9H18L32, Invitrogen, InnovaBiosciences) as a tumour cell identifying marker. 4',6-Diamidino-2-Phenylindole (DAPI) (1:1000 in ethanol, Sigma) was used to mark the nucleus.

2.7.3. Immunocytochemistry in Microfluidic Devices

Firstly, permeabilization of fixed cells retained in the device was induced by pumping 250 µl of 0.5% Triton X-100 in PBS into the microfluidic platform with incubation for 15 minutes at RT. Channels were rinsed with 250 µl of PBS and non-specific antibody binding blocked with 250 µl of PBS-2%BSA for 30 min. Entrapped cells were stained with anti-cytokeratin-pan-FITC, anti-CD45-cy5, anti-vimentin-eFluor 570, anti-survivin-Dylight 755 and DAPI in total volume of 250 µl of PBS-2%BSA. Both ends of tubing were sealed with parafilm and devices stored at 4ºC immersed in water and protected from light until imaging analysis by fluorescence microscopy.

3. RESULTS AND DISCUSSION

3.1. Device Characterisation

In this project, a label-free microfluidic platform was designed, by the group, for size-based isolation and enrichment of cancer cells. The design consists in five rows of posts, with increasingly narrower gap widths (50, 20, 15, 10 and 5 µm) to separate cells according to their size and deformability.

3.2. Microfluidic Device Performance

3.2.1. Cancer cell isolation efficiency

To evaluate the capture efficiency of tumour cells in the microfluidic device, experiments using a low number of HT1376 cells (1000 cells per 500 µl of PBS) were conducted. PBS, commonly used in various cell culture applications, was used as a vehicle solution as it also closely mimics the saline buffer used to collect bladder washings during cystoscopy. Bladder cancer cells were pre-labelled with calcein and cell suspensions were run at constant pressure differentials (200 or 300 mbar). Cells trapped on the microposts were counted by observation using fluorescence microscopy, and capture efficiency was determined using Equation 1.

$$Capture\ efficiency\ (\%) = \frac{(HT1376\ cells)_{trapped}}{(HT1376\ cells)_{inlet}} \times 100 \qquad (1)$$

HT1376 bladder cancer cells were successfully captured at 200 and 300 mbar. However, a quantitative analysis over 3 independent experiments (**Figure 1.A**) indicates higher cell retention (37%) at 200 mbar, while only 16% of cells were captured at 300 mbar input pressure. This discrepancy can be explained by increased hydrodynamic forces acting on the cells trapped on the posts at higher pressure values, which causes cells to deform and squeeze between posts. If the inlet pressure were too high, it would ultimately result in unspecific separation regardless of cell size or deformability and cells would flow though the device, with minimal retention.

3.2.2. PBMC retention

Since bladder cancer is associated with haematuria (blood in the urine) and inflammation, other cell types, namely white and red blood cells, are

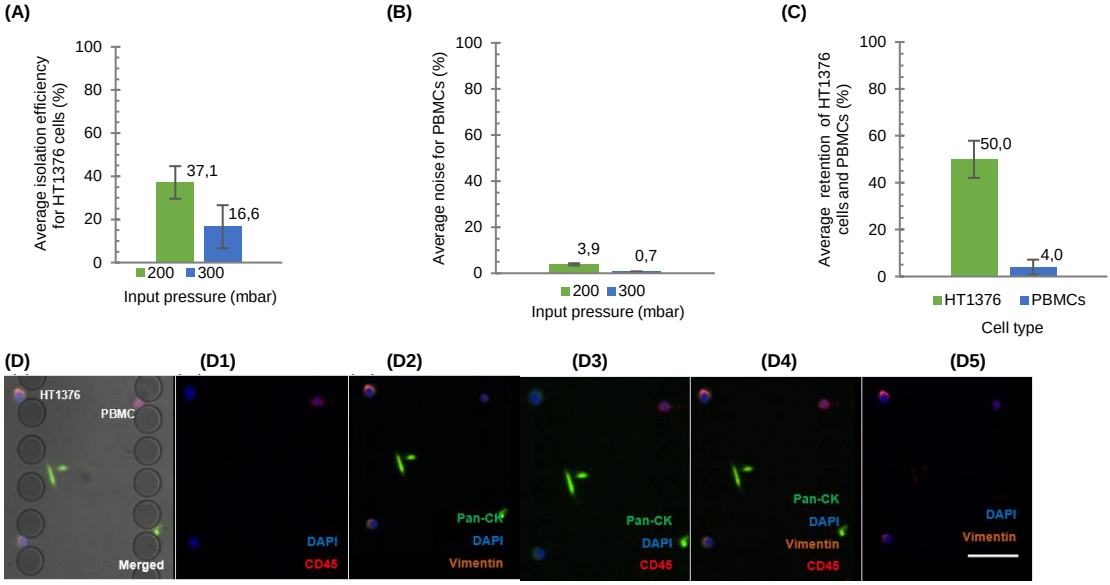

Figure 1. Device performance control tests to assess isolation efficiency as well as immunocytochemistry performed in situ.
(A) *Quantification (%) of H1376 bladder cancer cell capture at two different inlet pressures.* ***(B)*** *Average noise for peripheral blood mononuclear cells (PBMCs) (%) in function of the input pressure (mbar).* ***(C)*** *Average noise for peripheral blood mononuclear cells (PBMCs) (%) in function of the input pressure (mbar).* ***(D)*** *Immunocytochemistry performed inside the microfluidic device for retained HT1376 cancer cells and PBMCs. Scale bar: 50 µm*

expected to be present in bladder washes or urine of patients. Therefore, the retention of unwanted blood cells in the device was also ascertained. To address this, PBMCs were isolated from peripheral blood of healthy donors and 10,000 cells pre-labelled with calcein in 500 µl of PBS were run through the microfluidic device. PBMCs, which comprise lymphocytes and monocytes range between 7 and 15 µm in diameter and are highly deformable cells. Hence, at high pressure conditions, the majority is expected to escape the microposts.

Figure 1.B shows that the average PBMC retention, determined using Equation 2, was indeed residual in both conditions. Thus, taking into consideration that the better capture efficiency data obtained was at 200 mbar, this was selected as optimal input pressure and therefore used in all subsequent experiments.

$$Noise\ (\%) = \frac{(PBMCs)_{trapped}}{(PBMCs)_{inlet}} \times 100 \qquad (2)$$

3.2.3. Spiking Experiments

To evaluate device performance in terms of cancer cell capture purity, 1000 HT1376 cancer cells pre-labelled with calcein were spiked in 10,000 of unstained PBMCs, in a total volume of 500 µl of PBS, loaded and run in the microfluidic platform. Results show that, for spiking experiments, the average percentage of leukocytes retained in the system remains around 4%. Importantly however, the average percentage of cancer cell retention increased up to 50% (**Figure 1.C**) in comparison to unmixed HT1376 cells (**Figure 1.A**), possibly due to hindrance caused by leukocytes. More importantly, an average purity of 62% was achieved, as determined by Equation 3.

$$Purity\ (\%) = \frac{(HT1376\ cells)_{trapped}}{(HT1376\ cells)_{trapped} + (PBMCs)_{trapped}} \times 100 \qquad (3)$$

This result indicates that immunocytochemistry can be accurately performed *in situ* for the characterisation of isolated cancer cells. Also, the system allowed the concentration of cancer cells in comparison to the sample by a factor of 22, determined by Equation 4.

$$Enrichment\ ratio = \frac{(HT1376\ cells)_{trapped}/(PBMCs)_{trapped}}{(HT1376\ cells)_{inlet}/(PBMCs)_{inlet}} \qquad (4)$$

3.3. Phenotypic characterisation of HT1376 bladder cancer cells

In order to identify rare tumour associated cells in clinical samples, and more importantly, their different phenotypes (epithelial, mesenchymal or stem cell-like), specific tumour cell markers need to be used and their cellular expression investigated. Hence, we started by characterising cells of the bladder HT1376 line for the following identifying markers: EpCAM and cytokeratins as epithelial, N-cadherin and Vimentin, as mesenchymal and CD44 and CD133 as stem cell-like markers. In parallel, the hematopoietic marker CD45 was also included as a negative control.

Fluorescence activated cell sorting (FACS) was previously performed in the group in order to assess the level of expression of the different biomarkers (data not shown). The FACS analysis revealed that, in HT1376 cells, EpCAM expression was negligible. However high expression of pan-cytokeratin (pan-CK) was detected. Regarding the mesenchymal

markers, no significant expression of N-cadherin was observed, contrarily to vimentin which was highly expressed. Within the panel of stem cell-like markers, it was found that this cell line did not express CD133 but had substantial expression of CD44.

Given the limitations of conventional fluorescence microscopes, at this stage, only 4 biomarkers were selected for the immunocytochemical analysis in the microfluidic device. A nuclear marker, DAPI, was necessary to ensure that the fluorescence signal was derived from a nucleated cell and not from unspecific debris or PDMS autofluorescence. As epithelial and mesenchymal markers are desired for the phenotype characterisation, allowing an appropriate assessment of the tumour aggressiveness, pan-CK and Vimentin fluorescently labelled antibodies were included in the biomarker panel. Finally, CD45 was used to identify blood cells that might be present in the sample. Since lymphocytes also express vimentin, the incorporation of CD45 prevented false-positive signals from Vimentin$^+$ CD45$^+$.

Having confirmed immunofluorescence detection of the selected biomarkers in the HT1376 cells and human PBMCs in microwell plates, as it was obtained for FACS, we next investigated whether the same could be observed when cells are trapped inside the microfluidic device. The possibility of performing the immunostaining inside the platform allows the evaluation of the expression of the different biomarkers in each cell, which is essential to accurately define distinct cell types present in the sample and retained in the system as well as identify different cancer cell populations, regarding their epithelial/mesenchymal phenotype.

Figure 1.D shows a photomicrograph of a control spiking experiment, in which the retained cells were immunostained *in situ* with DAPI, pan-cytokeratin, vimentin and CD45 according to section 2.7.3. This analysis led us to some important findings: firstly, as it can be observed, the two distinct cell populations can be accurately characterised and distinguished by immunocytochemistry inside the device. The HT1376 cells are shown to express pan-CK, vimentin$^+$, DAPI$^+$ and CD45$^-$ whereas human PBMCs are pan-cytokeratin$^-$, vimentin$^+$, DAPI$^+$ and CD45$^+$. This finding is extremely important since a clear visualisation of the several biomarkers is required for phenotype characterisation and cell type discrimination. Secondly, a cell distribution pattern can be identified. PBMCs that remain trapped on the device are scant and when present remain on the last row of posts (5 µm gap). On the other hand, HT1376 cells are isolated on previous rows, mainly on the 3rd or 4th rows (15 and 10 µm gap, respectively).

Even though the selected biomarker panel allowed the identification of different cell types in control samples, and is commonly used to identify circulating tumour cells in blood of cancer patients, analysis of other body fluids such as bladder washes or urine poses additional challenges. The previous biomarker panel was enough for cell characterisation due to the fact that control samples used in this study consisted solely of human bladder cancer cells and PBMCs. In bladder washes or urine, a larger number of cell types are expected to be present, particularly normal urothelial cells, which display an epithelial phenotype, and shed from the bladder wall during the washing step of the cystoscopic evaluation[2]. Hence, it is imperative to include an additional biomarker to distinguish normal from malignant bladder cells.

Survivin, an apoptosis inhibitor, is highly expressed in a large variety of solid tumours and hematologic malignancies and is absent or under expressed in normal adult tissues. In bladder cancer, it has been reported in a large number of studies as a potential diagnostic biomarker, particularly in the detection of early stage and low-grade tumours, which is the main limitation of urine cytology[5,7,11]. Thus, it was selected and included in the biomarker panel to specifically detect bladder cells of malignant origin.

Prior to assessing clinical samples, the expression levels of the survivin were evaluated on control HT1376 cells. **Figure 2** represents a photomicrograph of the HT1376 cell line stained for the nucleus (DAPI) and a tumourigenic biomarker (Survivin).

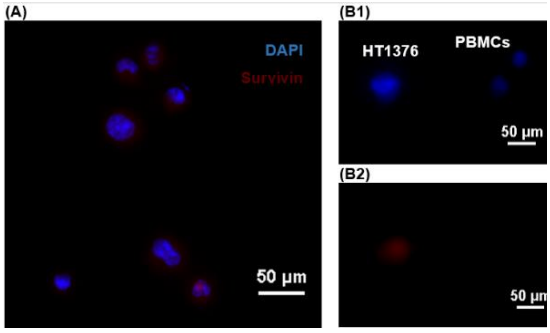

Figure 2. Survivin immunostaining of the HT1376 bladder cancer cell line and peripheral blood mononuclear cells in tissue culture wells.
(A) *HT1376 bladder cancer cells stained for DAPI (blue) and Survivin (far red).*
(B) *HT1376 bladder cancer cells spiked in PBMCs for the evaluation of the survivin fluorescence intensity obtained for the different cell types.*

As illustrated in **Figure 2.B**, positive survivin expression was observed in spiked HT1376 cells when compared to white blood cells. This result was of extreme importance for the project progression. Considering that the antibody-fluorophore conjugate was done in the laboratory and that the microscope was not originally optimised for far red observation, the presence of far red fluorescence indicates that this conjugation process was a success. More importantly, high survivin expression was observed for the HT1376 cancer cells whereas no expression was visible for PBMCs.

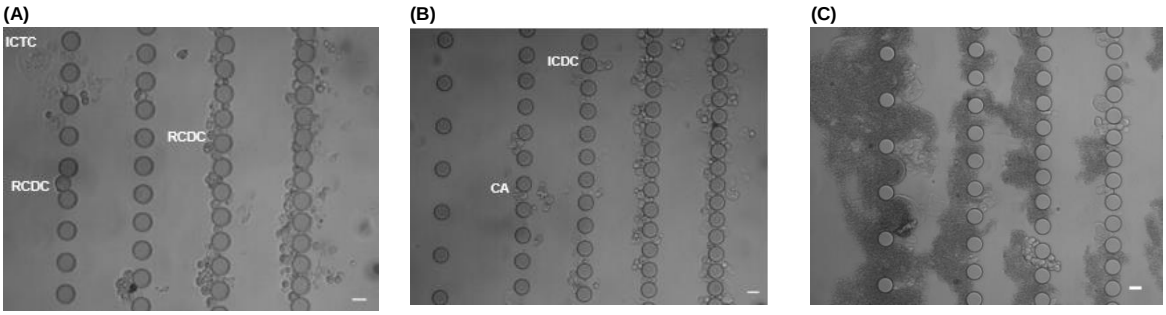

Figure 3. Bladder wash processing in the microfluidic devices for patient 6 (A-B) and patient 1 (C).
(A) *Photomicrograph of one region of the bladder wash microdevice where irregular shaped cells with translucent cytoplasm (ICTC) are isolated in the first rows. Round cells with defined membrane and dense cytoplasm and various sizes (RCDC) represent the majority of the isolated cells.* ***(B)*** *Different region of the same device, in which it is visible the isolation of cell aggregates (CA) and irregular shaped cells with dense cytoplasm (ICDC).* ***(C)*** *Bladder wash of patient 1 where debris and different cell morphologies are explicit. Scale bar 20 µm.*

Prompt by these great results, we then moved to clinical sample analysis using this panel of five biomarkers. Identification of cancer cells (Survivin+/CD45-) as well as their phenotypic characterisation (pan-cytokeratin and vimentin) was pursued.

3.4. Bladder cancer cell detection and phenotypic analysis

Bladder washes and urine samples from 6 patients were processed and immunostained in the microfluidic device. However, due to time and technical constrains, not all could be fully analysed at the time of submission of this thesis. The results obtained for both bladder washes and urine samples from patients 1 and 6 will be thoroughly described and discussed below.

3.4.1. Bladder Wash analysis

Patient 6 was the first patient in which the staining and phenotypic analysis was performed. **Figure 3** represents two different sections of the microfluidic device at the end of the experiment.

In the first row of posts in **Figure 3.A**, it is possible to visualise the presence of two different cell types: round with defined membrane and dense cytoplasm (RCDC), with sizes equal or superior to 20 µm, and translucent irregular cells (ICTC). Regarding the latter, it is clear that these cells are highly deformable and are able to squeeze through the first line of posts until the following row (15 µm gaps). **Figure 3.B** represents a different section in which no cells were entrapped on the first row and only clusters and cells with a dense cytoplasm were retained. As it is visible, the majority of cell clusters are isolated in the last two rows (10 and 5 µm). The same can be verified for patient 1, still it is impossible to make a better assessment due to the submerging extent of debris (**Figure 3.C**).

Immunocytochemistry *in situ* with the optimised panel of biomarkers, tested in control samples (DAPI, pan-CK, vimentin, survivin and CD45), was incorporated to perform phenotypical analysis. **Figure 4.** was adjusted in order to present the fluorescence signals of DAPI-blue, pan-CK-green, vimentin-orange, since it was seen that the CD45 expression was negligible. The observation of **Figure 4.** indicates the presence of an elevated cell number retained in the system, particularly in the 5 and 10 µm gap rows, with minimum debris present in the system. Pan-CK staining is observed that the majority of the cells. Further observation suggests that the cells present in the 20 and 15 µm gap rows, particularly the round and dense cytoplasm cells as well as the cell aggregates, present a clear epithelial phenotype due to the intensity of the obtained signal. As you move further from the 20 µm rows, the green fluorescence intensity diminishes and is only significant for some isolated cells.

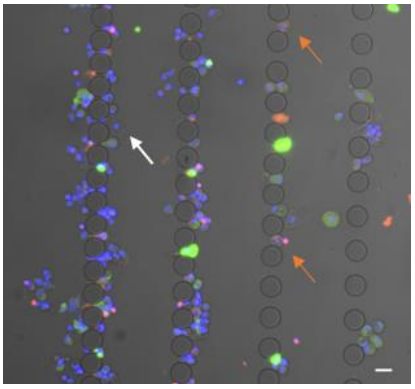

Figure 4. Bright Field and fluorescence channels for DAPI, pan-cytokeratin, and vimentin. of one section of the bladder wash device of patient 6. Scale 20 µm

When assessing the mesenchymal phenotype, it is possible to observe that the majority of the cells express vimentin, however the signal intensity is not as strong as the green signal. Even though the signal appears to be stronger for the 5 and 10 µm rows, in the 15 µm gap row, there are two cells that appear to have a significant vimentin expression (marked with orange arrows).

Finally, the survivin immunostaining indicative of a tumourigenic phenotype was assessed. However, signal obtained was faint, most possibly due to technical constrains, as only 30% of the light can be detected at this wavelength by the camera. It should be noted though that this was the only possible

fluorophore that could be used taking into consideration the other biomarkers. To improve the detection capacity, analysis at a higher magnification (60x) was performed from this moment on, for the evaluation of the survivin biomarker.

Interestingly, in the last row of posts, it is visible the existence of a cell, (marked by the white) arrow, that does not express any of the biomarkers used in this panel. Considering that the specimens where directly removed from the bladder during the cystoscopy, it would be expected that it would present an epithelial or hematological cell phenotype. This raises the question of which is the phenotypic characterisation of this cell and it would be interesting to evaluate other biomarkers or search for specific mutations on that particular cell.

Analysis at a higher magnification (60x), improved the signal detection, as the light was focus directly on the cell of interest. Strikingly, as illustrated in **Figure 5**, a survivin positive cell was detected in the bladder wash sample from patient 6, along with the pan-cytokeratin and vimentin, indicating that this cell might be undergoing an EMT process. A very interesting aspect of this cell is the binucleation that is visually clear with the DAPI staining which is indicative of mitotic dysfunction and commonly associated with malignancy[12].

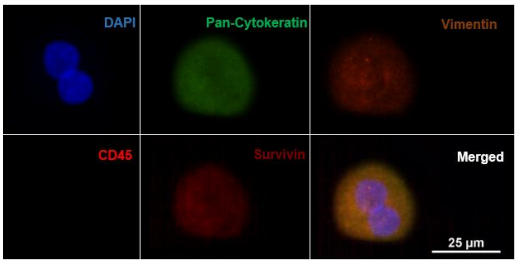

Figure 5. Survivin-positive cell isolated in the bladder wash microfluidic device of patient 6.

In **Figure 6**, different cell types with distinguished phenotypes can be seen trapped in the device.

Although these cells are very similar in size, with approximately 20 µm diameter, it is evident the presence of differential expression. For instance, the well-defined circular cell with a regular nucleus (a), has a significant expression of pan-cytokeratin coupled with intense expression of vimentin. On the other hand, the cell (b) mildly expresses pan-cytokeratin and presents a significant vimentin expression and also an irregular nucleus with small cytoplasm. In both cell types it is visible the expression of survivin, indicating that these are cancerous cells. Cell (c) has an increased nucleus to cytoplasm ratio and no expression of epithelial markers and an intense expression of vimentin. The slight expression of CD45 and lack of expression of survivin indicates that this cell is most likely a lymphocyte.

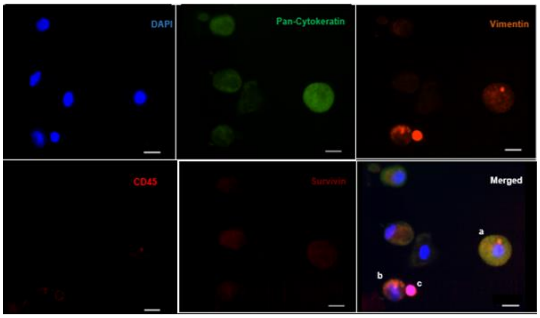

Figure 6. Cells present in the bladder wash of patient 6 expressing different phenotypes. Scale bar 10 µm

Contrarily to patient 6, analysis of microfluidic isolated cells from patient 1 was not so successful most likely due to the large amounts of debris present. Still, cells positive for pan-cytokeratin and vimentin expression were found (**Figure 7**). Nevertheless, vimentin expression in cells from patient 1 was lower than that detected in cells from patient 6, particularly in the case of multinucleated cells. No surviving positive cells were observed for patient 1, in contrast to patient 6. This difference may be related to the disease state of the patients or alternatively to sample processing conditions that were not ideal for this patient.

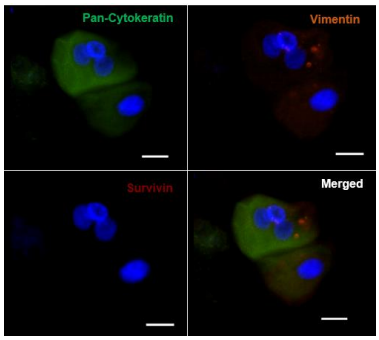

Figure 7. Potential cancer cell isolated from the bladder wash of patient 1. Scale bar: 10 µm

3.4.2. Urine analysis

Cell morphology in urine samples was remarkably similar to that of bladder wash samples, as observed in **Figure 8.** This was somehow expected since urine was collected though a catheter, thus cells present in the specimen should only be derived from the bladder. In voided urine though other cells from the ureter or urethra should also be present[2]. The main difference observed between the two samples was the presence of larger cell debris in non-voided urine, possibly due to a long presence of urine in the bladder.

Figures 8.A/D illustrate cell heterogeneity found after urine processing, which was equivalent to that of bladder wash samples (**Figure 3**).

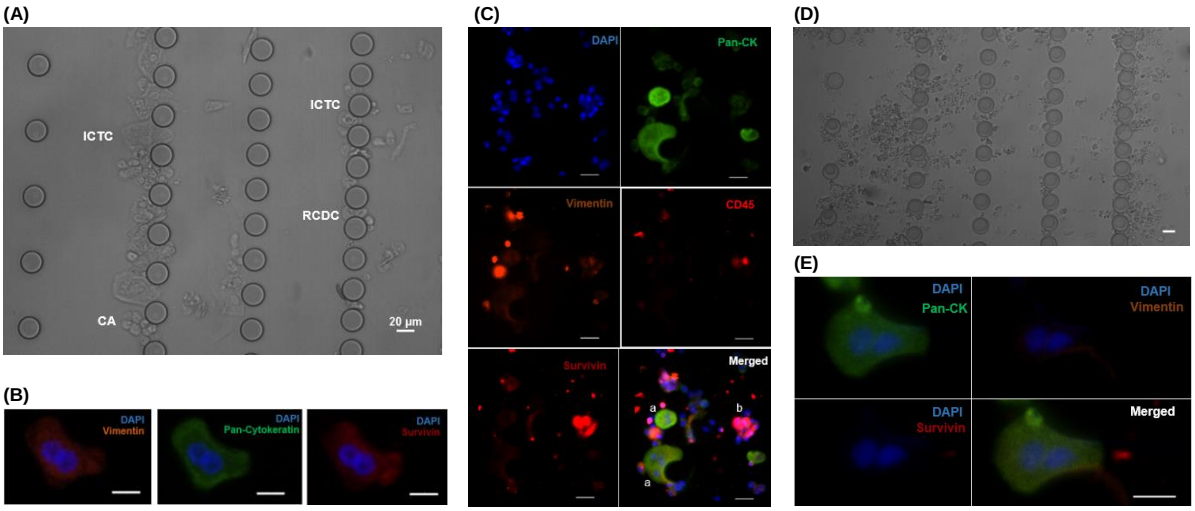

Figure 8. Urine processing and immunocytochemistry performed for retained cell in the system for patients 6 and 1.
(A) *Isolation pattern of different cell morphologies after 20 minutes of urine processing in the device for patient 6.* ***(B)*** *Photomicrograph of a survivin positive cell isolated in the urine of patient 6. Scale bar 10 µm.* ***(C)*** *Different cell phenotypes present in the urine of patient 6. Scale bar: 20 µm* ***(D)*** *Cells isolated from the urine of patient 1 on the device. Scale bar: 20 µm.* ***(E)*** *Multinucleated cell without vimentin or survivin expression isolated from the urine of patient 1 on the device. Scale bar 10 µm.*

After the evaluation of the different phenotypes in bladder washes, the same procedure was conducted for the urine samples. A detailed analysis at the single cell level revealed the presence of cell expressing pan-cytokeratin, vimentin and, remarkably, also survivin for patient 6 (**Figure 9.B)**. Of note, this cell is also binucleated, similarly to another cell found in the bladder wash sample collected from this patient (**Figure 5)**. This extraordinary finding shows that a correlation between data from bladder washes and urine can be established. More importantly, it demonstrates that cancer cells can be targeted in urine.

In **Figure 8.C**, different cell populations are found in the same row of posts. In this figure, survivin expression is obtained for some pan-cytokeratin$^+$, vimentin$^+$ cells (a), however, high survivin expression is also obtained for some vimentin$^+$, CD45$^+$cells (b). This is consistent with previous studies reporting survivin expression by hematological cells[11]. In some areas of the device single-cell isolation in height was not so successful, as evidenced in this figure, highlighting the need for critical evaluation of the data when interpreting results.

When analysing the urine sample from patient 1, vast amounts of cellular debris were found (**Figure 8.D)**, although at much lesser extent than its bladder wash counterpart (**Figure 3.C**). This was most likely due to the pre-processing protocol optimisation.

In contrast to patient 6 samples, no significant vimentin expression was detected in samples from patient 1. Also, multinucleated cells were particularly checked for survivin expression, as previously found for patient 6. As shown in **Figure 8.E** this binucleated cell exhibited high levels of pan-cytokeratin expression and no expression of vimentin or survivin.

This may be indicative of the presence of a superficial urothelial cell (umbrella cell) which possesses round and frequently multiple nuclei[2]. Still, further studies are required, ideally including additional markers and a larger patient cohort.

3.5. Pathology Results and Final Remarks

After sample processing and subsequent phenotypic analysis, the laboratory results were crosschecked with data kindly provided by the pathology unit of CHP-HAS. **Table 1** summarises the main findings obtained for bladder washes and urine using the microfluidic platform as well as the pathology diagnostic.

According to the medical evaluation, both patients have non-invasive papillary carcinoma (stage Ta), however there are important differences among them that must be considered: Patient 6 has a high-grade tumour, which is usually poorly differentiated and indicative of a poor prognosis. On the other hand, patient 1 has a low-grade tumour, which has less risk of recurrence and invasiveness.

Taking into account the pathology report, this project provides some interesting results. The low number of vimentin$^+$ cells in patient 1 and of those, the reduced expression levels observed are consistent low-grade tumours. In addition, these cells are highly differentiated, exhibiting an epithelial phenotype. Moreover, no detection of survivin may be related with the low-stage and low-grade of the tumour. Nonetheless, survivin expression in some cells cannot be ruled out, as only 30% of survivin signal is detected by the microscope used, due to technical constrains.

Table 1. Demographic characteristics and biomarkers in bladder wash and urinary cells from patients with transitional cell bladder tumours in TURBT

Patient ID	Gender/Age	Pathology	Biomarkers					Result
			DAPI	Pan-CK	Vimentin	Survivin	CD45	
6	F/57	Stage Ta High-grade	+	+	-	-	-	Normal urothelial cell
			+	+	-	+	-	Epithelial cancer cell
			+	+	+	-	-	Cell in EMT
			+	+	+	+	-	Cancer cell in EMT
			+	-	+	-	-	Mesenchymal cell
			+	-	+	+	-	Mesenchymal cancer cell
			+	-	+	-	+	Hematologic cell
			+	-	+	+	+	Hematologic cell
1	M/78	Stage Ta Low-grade	+	+	-	-	-	Normal urothelial cell
			+	+	+	-	-	Cell in EMT
			+	-	+	-	+	Hematologic cell

F – Female
M – Male

In contrast, for patient 6 distinct and varied cell phenotypes are observed. The patient samples were positive for survivin staining, indicating that those isolated cells are most likely malignant. This is further corroborated by the pathology report (patient 6: high-grade vs patient 1: low-grade) and by the disparate results observed for patient 1 (no surviving expression), which may be related to tumour aggressiveness. Furthermore, the expression of the different biomarkers analysed (pan-cytokeratin, vimentin and survivin) was higher in multinucleated cells. Also, pan-cytokeratin expression was lower in Vimentin$^+$ cells, as compared to pan-cytokeratin$^+$/Vimentin$^-$ cells. This downregulation of epithelial markers and concomitant expression of mesenchymal ones, indicates a possible epithelial-to-mesenchymal transition, a process associated with tumour progression. Likewise, it is interesting to note that, in this patient, vimentin$^+$/survivin$^+$ cells lacking pan-cytokeratin expression were detected. These are typically mesenchymal-like cells which likely underwent EMT. Together, these observations are relevant and if further validated have an impact for therapeutic reasoning and patient follow-up.

4. CONCLUSIONS

This platform is capable of isolating cells from bladder washes and urine with distinct phenotypes. More importantly, urine, which is the ultimate goal, for the development of non-invasive diagnosis, proved that it can be used for targeting rare cells. The selected panel of biomarkers may be able to distinguish low from high-grade carcinomas, however, further studies need to be performed and a larger patient cohort analysed.

In future work, normal human bladder urothelium cells or processed urine from healthy individuals should be used for a better mimicking of the sample and a more accurate characterisation. Analysis of specific mutations could also be performed *in situ* by FISH or after cell lysis to obtain DNA content. Recovery of isolated cells by reversing the flow or the creation of lateral channels for cell retrieval from independent rows would also be relevant for further functional characterisation of captured cells. Overall, these preliminary results are very exciting, with the proposed experiments contributing to a better understanding of the clinical significance of isolated cells and paving the way for early and non-invasive detection of bladder cancer using point-of-care microfluidic-based systems.

REFERENCES

1. Cancer Research UK. Bladder cancer incidence statistics. at <http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bladder-cancer/incidence#heading-Eight>
2. Sullivan, P. S., Chan, J. B., Levin, M. R. & Rao, J. Urine cytology and adjunct markers for detection and surveillance of bladder cancer. *Am. J. Transl. Res.* **2,** 412–40 (2010).
3. Smith, Z. L. & Guzzo, T. J. Urinary markers for bladder cancer. *F1000Prime Rep.* **5,** 21 (2013).
4. Chade, D. C., Shariat, S. F., Godoy, G., Meryn, S. & Dalbagni, G. Critical review of biomarkers for the early detection and surveillance of bladder cancer. *J. Mens. health* **6,** 368–382 (2009).
5. Chen, Y., Tu, J. J., Kao, J., Zhou, X. K. & Chen, Y.-T. Survivin as a useful adjunct marker for the grading of papillary urothelial carcinoma. *Arch. Pathol. Lab. Med.* **132,** 224–31 (2008).
6. Moussa, O. *et al.* Evaluation of survivin reverse transcriptase-polymerase chain reaction for noninvasive detection of bladder cancer. *J. Urol.* **175,** 2312–6 (2006).
7. Shariat, S. F. *et al.* Urine detection of survivin is a sensitive marker for the noninvasive diagnosis of bladder cancer. *J. Urol.* **171,** 626–30 (2004).
8. Craene, B. De & Berx, G. Regulatory networks defining EMT during cancer initiation and progression. *Nat. Rev. Cancer* **13,** 97–110 (2013).
9. Lamouille, S., Xu, J. & Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **15,** 178–96 (2014).
10. Knowles, M. A. & Hurst, C. D. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nat. Rev. Cancer* **15,** 25–41 (2014).
11. Margulis, V., Lotan, Y. & Shariat, S. F. Survivin: a promising biomarker for detection and prognosis of bladder cancer. *World J. Urol.* **26,** 59–65 (2008).
12. Krajcovic, M. *et al.* A non-genetic route to aneuploidy in human cancers. *Nat. Cell Biol.* **13,** 324–330 (2011).