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Nanomechanical biomarkers of single circulating tumor cells for detection of castration resistant prostate cancer

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Abstract

BACKGROUND—Emerging evidence shows that nanomechanical phenotypes of circulating tumor cells (CTC) could become potential biomarkers for metastatic castration resistant prostate cancer (mCRPC).

METHODS—To determine the nanomechanical phenotype of CTCs we applied atomic force microscopy (AFM) employing the PeakForce quantitative nanomechanical (QNM) imaging. We assessed biophysical parameters (elasticity, deformation and adhesion) of 130 CTCs isolated from blood samples from 5 castration sensitive (CS) and 12 castration resistant prostate cancer (CRPCa) patients.

RESULTS—We found that CTCs from CRPCa patients are 3 times softer, 3 times more deformable and 7 times more adhesive than counterparts from CSPCa patients. Both non-supervised hierarchical clustering and principle component analysis show that three combined nanomechanical parameters could constitute a valuable set to distinguish between CSPCa and CRPCa.

CONCLUSIONS—Our study indicates that nanomechanical phenotypes of CTCs may serve as novel and effective biomarkers for mCRPC.

Keywords

biophysical biomarkers; single-cell analysis; elasticity; adhesion; deformability

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INTRODUCTION

About 30,000 men in USA succumb to fatal metastatic castration-resistant prostate cancer (mCRPC) with 16–18 month median survival after castration resistance is detected [1]. After years of research effort, mCRPC still lacks reliable early biomarkers for detection although biochemical recurrence with prostate specific antigen (PSA) elevation is a standard clinical marker. A portion (~65–70%) of prostate cancer (PCa) patients under castration therapy may not lead to aggressive progression but discrimination between fatal and indolent groups has been elusive even with combined Gleason scores, radiography and other biomarkers [2]. Misdiagnosis and overtreatment have been major concerns in clinical treatments for PCa patients [2]. There is an urgent need for early predictors that are valuable for detection for this aggressive mCRPC and patient treatment decisions.

As single-cell analyses reveal, cancer cells are highly heterogeneous [3]. Their heterogeneity is displayed as distinct cellular nanomechanical and nanochemical phenotypes in addition to genomic, epigenetic and transcriptional diversities. With cancer progression cells undergo substantial changes in cytoskeleton architecture, intracellular transport, and redistribution of adhesion molecules on cell membrane that lead to functional and morphological changes [4]. Structure-function studies point that the phenotypic changes and the acquisition of cancerous activities: replication and metastatic motility are closely related. Therefore, modifications of cytoarchitectural properties in cancer cells are so functionally crucial that they have emerged as new potential biomarkers for cancer detection, stratification, prognosis and prediction [5,6].

Two lines of evidence indicate that biophysical features of circulating tumor cells (CTCs) may be potential biomarkers for mCRPC. Firstly, enumeration of CTCs is superior to PSA in predicting survival of mCRPC patients [7]. CTCs are shed into blood stream from tumors during metastasis. Analysis of CTCs isolated from blood samples as “liquid biopsy” can provide a repeatable evaluation of patient’s pathological status with minimal invasiveness. The second line of evidence comes from morphological switches during epithelial-mesenchymal transition (EMT) and the molecular analyses in advanced CTCs [8–10]. During metastasis, EMT is crucial in the tumor cell morphogenesis: the apical-basal epithelial phenotype turns into the spindle-shaped mesenchymal counterpart for subsequent invasion [9]. A subset of CTCs from advanced PCa lose EpCAM or cytokeratin expression and instead exhibit features of EMT [11] with gene markers specific for mesenchymal and stem-like cells [10–12]. The mesenchymal gene markers have been shown to correlate with poor prognosis and acquired resistance to chemotherapy [9]. Moreover, our previous single-cell study indicates that incremental expression of EMT-related genes in CTCs are associated with mCRPC [13]. Taken together, the data implicate that EMT-related nanomechanical attributes of CTCs may shed new light on mCRPC and lead to the discovery of new biomarkers.

Reliable mapping of mechanical phenotypes of cancer cells was impossible until the inventions of new biophysical techniques such as atomic force microscopy (AFM), cytoindentations, magnetic twisting cytometry, optical tweezers and micropipette aspiration [14]. Among these, force-distance-based AFM is one of the most versatile and is

utilized for determination of biomechanical parameters such as elasticity (softness), deformation and adhesion. The probe of atomic force microscope can map single-cell nanomechanical features with nanometer precision and piconewton sensitivity [15]. AFM-based topographical assessment has differentiated nonmetastatic mesothelial cells and metastatic non-small lung, breast and pancreatic malignant cells from cancer patients [4,5]. Consequently, AFM could serve as a reliable biophysical tool with clinical applications.

To determine the nanomechanical phenotype of CTCs we used AFM equipped with the newest PeakForce (PF) quantitative nanomechanical (QNM) capabilities. The PF-QNM method enables collection of multiple parameters during a single scan of a live cell with unsurpassed resolution and accuracy. In our previous work we have shown with conventional force AFM that CTCs are at least as soft as cultured PCa cells [13]. Following this encouraging observation, we expanded the study to include a diverse set of PCa patients with metastatic disease. Moreover, we used a hyphenated technique interfacing AFM with an epifluorescent microscope to confirm CTCs identity and to study CTCs in an even more comprehensive way. We performed AFM imaging directly on the porous membrane used for CTC isolation thus simplifying and shortening the nanomechanical mapping procedure. Here we report that nanomechanical properties of CTCs isolated from prostate cancer patients could constitute a valuable set of parameters useful to distinguish between castration sensitive and resistant diseases. Specifically, using AFM mapping we examined the nanomechanical phenotype of CTCs isolated from 17 patients and found that castration resistant (CR) CTCs are about 3 times softer, 3 times easier to deform, and 7 times more adhesive than castration sensitive (CS) CTCs.

MATERIALS AND METHODS

Patients

The blood samples were collected from 17 patients who were consented according to the protocol approved at the UTHSCSA. The pathological status of 17 patients, including 5 castration sensitive and 12 castration resistant patients are presented in the Table 1.

CTC Isolation

The CTCs isolation and identification were carried out according to the method described previously with modifications (Fig. 1A) [13]. Blood of patients (~10 ml) was drawn in K₂EDTA tubes and mixed immediately and stored on ice before processing. *ScreenCell*® CC filtration kits (ScreenCell, Westford, MA) were used to separate potential CTCs according to manufacturer's protocol. Patient blood (~8 ml) was mixed with 1.3 ml *ScreenCell*® LC dilution buffer in a 15 ml sterile conical tube and incubated at RT for 2 min and then filtered through *ScreenCell* filters and washed using 1.5 ml PBS. The CTCs were immunostained with anti-CD45 conjugated with PE (BD Bioscience, San Jose, CA), anti-EpCAM-FITC (StemCell Technologies, Vancouver, Canada) and 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in DMEM+ 5% FBS for negative and positive selection of CTCs. Excess of antibodies was washed out using 50 µl PBS. The CTCs on the filters were kept in DMEM+ 5% FBS and subject to immediate AFM analysis after the staining.

Analysis of Nanomechanical Phenotype of CTCs

The cells captured on a filter were imaged directly on the filter without any additional processing or immobilization. The filter was mounted on a bottom of a 55 mm Petri dish and kept covered except during the AFM imaging. The cells were overlaid with 10 μ L of DMEM medium. Additional 10 μ L of the medium was supplemented after each hr of imaging or sooner if needed. Cells were observed for up to 3 hrs without morphological signs of loss of their viability. Cells were imaged using a Nanoscope Catalyst AFM (Bruker, Billerica, MA) mounted on a Nikon Ti inverted epifluorescent microscope. Before AFM imaging, light and fluorescent images were collected for each cell. The nanomechanical phenotype data for each individual cell were captured using PeakForce Quantitative Nanomechanical Mapping (QNM) AFM (Bruker, Billerica, MA) or a 3 $\times$ 3 array of single point measurements. The latter approach was used when the total number of available cells would preclude their imaging in a reasonable time within the viability window. Cell mapping allowed capturing and analyzing cell elasticity, deformation, adhesion, energy dissipation, surface roughness, and topography in the same scan. The last two parameters were not available using the single point measurements. The time of a single cell mapping was about 12 to 15 min, with squares from 30 $\times$ 30 to 50 $\times$ 50 μ m scanned with a resolution from 64 $\times$ 64 to 256 $\times$ 256 pixels (x, number of points per line by y, number of lines). SCANASYST-AIR (Bruker, Billerica, MA) probes were used for imaging. Nanomechanical parameters were calculated with Nanoscope Analysis software v.4.1 using retrace images. The measurements of the elastic modulus followed the rules published by Sokolov (2013) assuming a high heterogeneity of cell surface properties (brush and rigidity). Additionally, we included adhesion forces in the analysis. Calculations were performed based on the Sneddon model that approximates the mechanics of conical tip interactions with an object.

In our current studies we tested nanomechanical properties of CTCs as a biomarker that could help distinguish between CS and CR PCa cases. We defined the nanomechanical phenotype as a set of uncorrelated physical properties of an intact, live cell that includes cell elasticity, deformation, and adhesion. Elasticity is a property of a material that characterizes its capacity to resist deformation and measures rigidity of a cell. The elastic deformation is fully reversible and excludes cell membrane penetration with a probe or cell destruction. Elasticity is expressed in units of pressure (Pascals, Pa) as the Young's modulus. Higher values of the Young's modulus correspond to more rigid (less elastic) objects. Deformation is presented in units of length and assesses the depth of a cell indentation at a selected point by a preset force. Deformation includes elastic (reversible) and non-elastic (nonreversible; plastic) components, without cell fracturing. Deeper deformation means stronger capability of a cell to be distorted. Adhesion is measured in units of force (Newtons) and quantifies capability of a cell to cling to another material. Its mechanism involves mechanical, chemical, and electrostatic interactions between different materials. Larger values of adhesion correspond to a stronger hold between materials.

Statistical Analysis

For each cell we calculated mean values of Young's modulus, deformation, and adhesion when their distribution followed the normal spread. In the remaining cases we applied the mode instead. We excluded from our mapping the most peripheral, thin parts of a cell to

avoid interference from the support. Next, the data for each patient were averaged and presented in the form of box charts. We tested association between each of the parameters using Statistica and OriginPro 9.1 software (OriginLab, Northampton, MA). After data scaling, principal component analysis was used to further explore the main source of data variance. Then, we attempted to group the patients based on nanomechanical phenotype of their CTCs using classification trees. Finally, we attempted to classify the patients based on nanomechanical phenotype of their CTCs using hierarchical cluster analysis. Cutoff values for each component of the nanomechanical phenotype that distinguishes between CS and CR patients were calculated based on the canonical discriminant analysis.

RESULTS

Isolation and Immunostaining of CTCs

In the course of our study we collected blood samples from 17 patients diagnosed with metastatic PCa in that 5 patients were castration sensitive and 12 castration resistant (Table 1). The pathological status, PSA, CTC counts and CTCs analyzed by AFM for each patient are also shown.

The CTCs were isolated and immunostained with antibodies against CD45 and EpCAM (Fig. 1A). The CTCs were CD-45 negative (Fig. 1B). After several washes with medium or PBS, the majority of CD45⁺ blood cells were removed through the filtration. Residual CD45⁺ blood cells were rarely seen and CTCs retained were pure. CTCs stained with DAPI in nuclei implicated poor integrity of cell membrane of dying cells. These cells were excluded from further analysis.

Mapping Nanomechanical Parameters of CTCs Using AFM

Retained CTCs on the filters were subject to AFM analysis and nanomechanical parameter data collection. The AFM directly collects images of CTCs as shown in Fig. 2A. The light microscopy image (left) presents an AFM cantilever (black triangle) with a perpendicular tip (not visible) approaching a CTC on the filter. The enlarged and false colored AFM image of the CTC (right) is collected in a Peak Force Error channel with cell relief projected on a cell shape (height representation). The cell relief image reflects approximation of microtopography of a cell surface with a contribution from underlying cytoskeleton elements. The combined images collected from these two channels delineate the edges of cells.

The AFM probe-cell interactions as well as probe position-force relationship during cell surface mapping are illustrated in a scheme and a graph, respectively (Fig. 2B). The cell pair represents different properties corresponding to two CTCs (014 and 017) (Fig. 2C). The specific color maps of height (in 3D representation), peak force error (relief), elasticity (Young's modulus), adhesion, and deformation and bar graphs on the top row illustrate the mechanical differences between the cells for each parameter. The cell no. 014 was smoother, less rigid, but more sticky and deformable than the cell no. 017.

Limited Prediction Power of Individual Nanomechanical Parameters for CRPCa

From this set of CTCs (Table 1) we collected AFM data for 130 cells including complete images for 43 cells. The recorded Young's modulus was ranged from about 30 Pa (the softest cells) to more than 15kPa (the most rigid cells). In turn, deformation extended from 10 nm to 2 μ m and adhesion from 10 pN to 2 nN (the stickiest cells). Next, we set out to test if the mechanical properties of CTCs isolated from each patient associate with androgen resistance of their PCa. Fig. 3 shows distribution histograms of the mechanical parameters of cells for each group of patients. Numerical data with statistical significance of differences are summarized in Table 2. We noticed that distribution of the Young's modulus of cells in castration resistant prostate cancer (CRPCa) patients is clearly unimodal comparing to castration sensitive prostate cancer (CSPCa) patients (Fig. 3A). Variance of the modulus was also much narrower for the CRPCa cases. Moreover, on average CTCs of CSPCa patients were 3 times more rigid than CRPCa patients. In contrast, distribution of deformation was close to unimodal in both groups with a larger variance observed among the CR patients (Fig. 3C). CTCs from CRPCa patients were about 3 times more deformable than from the CSPCa. Finally, distribution of CTCs adhesion was similar for both the groups, but less clearly defined (Fig. 3B). Nevertheless, CTCs from the CRPCa patients were 5 times more adhesive than from the CSPCa.

When we plotted the data individually for each patient (Fig. 4), we noticed that the Young's modulus decreased monotonously from CSPCa to CRPCa patients (Fig. 4A). The lower rigidity was followed by a decrease of elasticity variance. Deformability increased in the opposite direction showing the highest values and variance among the CRPCa patients (Fig. 4B). The same pattern was repeated by adhesion data where the stickiest cells were identified in the CRPCa patients. These CTCs also showed the highest variance and a broad data range.

Combined Nanomechanical Parameters of CTC Discriminate CRPCa from CSPCa

A closer inspection of the individual components of the nanomechanical phenotype indicated that none of them alone could reliably distinguish between the patient groups. This status could be explained in part by a limited number of the available cells for imaging and their high nanomechanical variance in many patients. Therefore, we tested if the components are associated with each other. The substantial correlation was found between elasticity and deformation and weaker between elasticity and adhesion (Fig. 4D,E). Since it was obvious that for each component CSPCa patients occupied a distinct and relatively separate position on these graphs, we applied principal component analysis to establish which component of the mechanical phenotype plays a major role in the distinction between CS and CR PCa. As shown in Fig. 4G, elasticity is responsible for about 59% of the observed variance, whereas deformation has 25%, and adhesion 16% contribution, respectively.

Encouraged by these results we decided to check if we could classify all the patients into two distinct castration sensitivity related groups solely based on the mechanical phenotypes of their CTCs. For this purpose we used hierarchical cluster analysis. The results presented in Fig. 4H show an excellent clustering of CSPCa patients separate from the CRPCa

patients, with an exception of one patient, who based on the results of medical examination was classified as a border case. Finally, discriminant analysis allowed us to establish the cutoff values that could help to univocally classify an individual patient to one of the PCa groups. We postulate that if a collection of CTCs on average exhibits the Young's modulus below 2.91 kPa, deformation larger than 412 nm and adhesion stronger than 120 pN then such a patient should be classified as CR. Both PSA values ($t=0.472$) and CTC counts ($t=0.256$) of patients show no statistical correlations with their responses to castration therapy. Although pathological status of patients 7 and 18 showed borderline responses to castration therapy at the time of blood draws (Table 1), the nanomechanical parameters of CTCs collected from these patients revealed that they were castration resistant and were consistent in the later follow-ups. Patient 10 also displayed borderline castration resistance to the therapy at the time of blood draw, however, biophysical properties of CTCs showed mixed results with high elasticity and the low ends of stickiness and deformability. Interestingly, the later follow-ups of the patients did show significant responses to therapy.

DISCUSSION

The available blood based biomarkers have had enormous impact on early prostate cancer detection and treatment. They are broadly classified as molecular (e.g. PSA, proteomic fingerprinting, microRNAs) [16] and cell based markers such as CTCs [13,17]. Here we attempted to apply an AFM technology to introduce a nanomechanical phenotype of CTCs as a marker that may help to overcome sensitivity and specificity limitations of currently used tests.

Enumeration of CTCs has prognostic and predictive value in several cancers since metastasis formation is invasion and dissemination provided by CTCs [18,19]. Here we extend the value of CTCs into a more specific and potentially stronger marker of the advanced PCa using comprehensive evaluation of mechanical properties of a single cell. Indeed, our data for the first time show the potential power of nanomechanical phenotype to address diversity of CTCs properties that can be applied to determine disease burden in PCa patients. The three combined mechanical biomarkers of single CTCs are sensitive enough to predict later responses to castration therapy during follow-ups for patients with borderline responses at the time of first assessment.

The mechanical properties of CTCs collected with AFM align well with attributes established before for cultured cancer cells [20] and cells isolated from biopsies [21]. In construction of the mechanical phenotype we uniquely incorporated elasticity, adhesion and deformation of CTCs. The findings support our hypothesis that the selected physical properties of single cells can be efficiently applied to supplement or even validate cancer diagnosis.

A cell shape is maintained by a complex interplay of membrane properties, cytoskeleton architecture, and cytoplasm composition [5,22]. Although a cell shape can be challenged by a broad range of forces, to a certain extent it can be preserved due to mechanical capabilities of the cell. The capacity of a cell to reversibly withstand actions of the forces defines its elasticity. On the other hand, a depth to which a cell can distort under a particular force

without breaking the membrane continuity defines the cell deformation. All cells sense and physically interact with their environment also by directly sticking to other objects. This property is regulated by composition and content distribution of cell membrane and is defined as cell adhesion. We focused on these three fundamental physical properties to test if they could specifically mark a state of the disease such as prostate cancer.

The elastic properties of CTCs isolated from a peripheral blood of prostate cancer patients followed the range of rigidity found in cultured prostate cancer cell lines. The increased elasticity or the loss of cellular rigidity expressed as lower values of the Young's modulus is commonly detected in cultured cancer cells and biopsies from cancer patients [20,23]. This observation is in stark contrast with a physician's experience, where the presence of tumors is based on palpation of harder tissues. This paradox is associated with relative stiffening of the peripheral tumor stroma in turn resulting from accumulation of matrix deposition. However, this spatial organization of a tumor obscures the high elasticity of creating it individual cells.

From the parameters included in our nanomechanical phenotype only elasticity has been studied intensely as a potential marker of cancer progression. Scanning cells directly on the CTC-capturing filter without transferring them to a dish or a slide is an important difference with all previous studies, making a direct comparison of elasticity values difficult. Fortunately, the QNM mapping applied to PC3 cells freshly seeded on a filter gave results comparable with elasticity of the cells cultured in a dish (not shown), thus validating our approach. Moreover, we used conical tips to avoid problems with spherical tips that tend to stick to and even damage very adhesive cells. Application of the conical tips enforced the use of the Sneddon model instead of the more commonly utilized Hertz model. We collected comprehensive elasticity maps for each cell, whereas all previous data are based on either single point or a small array of point measurements often restricted to the nucleus area. Our approach helps to avoid many possible artifacts or lost information associated with limited probing of cells.

In general, with the same force load applied, cancerous cells appear softer than the more rigid normal cells. In our studies we compared rigidity of potentially cancerous cells and show that their Young's modulus differed widely even in an individual patient. Interestingly, as average elasticity of CTCs in a single patient increases, the variance of the Young's modulus decreases. We suspect that the population of CTCs becomes less diverse in terms of elasticity with the disease progression. The increased softness of cells could result from a morphological and molecular transition of CTCs into EMT-like cells, a known malignant invasion in castration-resistant patients. Molecularly, cell elasticity is a result of the unique association of cell membrane and cytoskeleton architecture, composition of cytoplasm, and particular distribution of organelles. Elasticity not only reflects but also amplifies changes in membrane make-up, cytoskeleton integrity, and ionic equilibrium of malignant cells. The major molecular factor contributing to the changes in elasticity is reorganization of actin[24] and cytokeratin filaments [25], and underlying vimentin fibers [26]. We conclude that elasticity remains the most important mechanical property of CTCs but alone it is not sufficient to classify patients into CS and CR categories.

Deformation is commonly associated with elasticity and often the terms are used interchangeably [27]. However, deformation is not intensive property of matter as is elasticity. As an extensive property, the deformation may at least in part depend on a cell height or its shape. Moreover, a deformable material may not necessarily be highly elastic. In general, deformation includes both elastic and non-elastic components allowing for a more permanent change in cell shape after probing it with an AFM tip. Even taking into account the above reservations, it seems plausible that exceptionally invasive CTCs of CRPCa patients are also very deformable as they aggressively extravasate into a new tissue. Taken together, joint application of elasticity and deformation significantly improves the patient classification.

Adhesiveness has been less explored as a parameter that could characterize mechanical properties of cells [23]. However, the recent studies on prostate cancer cultured cells indicate that inclusion of cell adhesiveness substantially improves power of the nanomechanical phenotype to predict metastatic potential [28]. Majority of studies on cell adhesion focuses on cell-cell or cell-substrate interactions [23,28,29]. They indicate that more metastatic cells are less adhesive. Our data suggest that CTCs acquired from patients manifesting more advanced disease were on average more adhesive. This discrepancy could be explained by the fact that we had opportunity to analyze the entire population of CTCs representing cells from the whole spectrum of invasiveness: including these that just become mobilized by EMT and those that are ready to seed new sites. We suspect that the latter cells should exhibit increased adherence to succeed in the colonization. On the other hand, our AFM technique specifically tests forces involved in tip-cell interactions, which may not necessarily mirror total complexity of the adhesive potential of a cell. To address this limitation we plan to use a potentially more accurate adhesion mapping with AFM tips with antibodies specifically recognizing adhesive surface antigens.

Our studies open a possibility to apply the nanomechanical phenotype of CTCs to identify patients with early advanced disease for their timely aggressive treatment. To reach this goal, we plan to expand the physical phenotypes to include cell morphometry and roughness of cell surface. In particular, morphometry of nucleus is established as discriminating factor between PCa patients facing different likelihood of disease progression [30]. Roughness as a parameter independent of cells morphometry is associated with nano- and micro structuring of membrane surface. It measures spatial organization of protein and lipid components of membranes and responds early to changes in physiological state of a cell [31]. Supplementing the proposed nanomechanical phenotype with these parameters should result in even more detailed and accurate classification of PCa patients. Moreover, our method could be applied in other cancers with CTCs. Finally, the relatively short time required for CTC isolation and AFM mapping in our procedure allows for downstream analysis of the same cells including genetic profiling or high content fluorescent staining.

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Figure 1.

Isolation of CTCs from patient blood samples and immunostaining of CTCs. **A:** Schematic illustration of CTC isolation, immunostaining and analysis using ScreenCell filtration kits, antibodies and AFM. **B:** Immunostaining of CTCs shows that two CTCs are EpCAM positive and CD45 negative. CTCs on *ScreenCell*® filters were stained with anti-EpCAM-FITC (green) and anti-CD45-PE (red). PC: phase contrast.

Figure 2.

Atomic force microscopy based measurement of nanomechanical phenotype of CTCs. **A:** The same CTCs retained on a filter was identified with a light microscope (left) and then imaged with QNM AFM (right). An AFM cantilever is visible as a black triangle on the top of the image. The AFM tip is mounted perpendicularly to the cantilever and pointing toward a viewer. The openings of filter pores appear as grey rings. The false colored AFM scan shows the cell rendered in a peak force error channel (relief) overlaid over the height image (topography). The color scale from black to white represents height from 0 to 10 μm. A white bar corresponds to 10 μm. **B:** Schematic illustration of nanomechanical parameters analysis and corresponding plots. Cartoon on the left illustrates our hypothesis stating that there is an association between the physical phenotype of CTC (shown in blue) and the cancer

prognosis. A red AFM probe scans a cell at a constant preset force creating nanomechanical maps that can distinguish between cells that have a high metastatic potential (they are very soft, deformable and sticky) from relatively benign cells (they are more rigid, less deformable but less adhesive). A plot on the right describes a relation between a tip-cell distance (x axis) and force applied to the cell (obtained from the degree of cantilever deflection, y axis). The blue trace corresponds to tip approach to the cell and the red trace represents withdraw of the tip from a cell. Elasticity (Young's modulus) is calculated from a tangent of linear part of the curve when the tip is still in contact with the cell. Adhesion is calculated from a negative dip of the curve when the tip attempts to free itself from the cell's grip. Deformation is computed as a depth to which the tip deforms the cells. The withdraw part of the curve is used in these calculations. C, The maps of very distinct nanomechanical properties of two CTCs isolated from a single patient are compared. Top row of bar graphs presents numerical data extracted from the maps shown in two middle rows. The bottom row shows corresponding color scales used in mapping of each collected parameter. Black color marks lower height, rigidity, adhesion and deformation whereas red represents taller object, more rigid, sticky and deformable. Numerical data for cell 014 are marked in red and for cell 017 in green. Images obtained in five channels are reported in columns: height (3D rendering), peak force error (relief), elasticity (Young's modulus), adhesion and deformation. The cell 014 exhibits smoother surface (lower roughness), higher elasticity, higher deformability and stronger adhesiveness than the cell 017.

Figure 3.

Frequency histograms show distinct distributions of nanomechanical properties deformation of CTCs for CS and CR patients. **A**, Elasticity of CTCs in CS patients is broadly spread up to 15 kPa with a majority of cases centered between 0.5 and 7 kPa. In contrast, CTCs from CR patients were generally softer with a main elasticity peak at 0.5 kPa and secondary peak at 3 kPa. **B**, Distribution of adhesion was poorly structured but it clearly pointed at a larger proportion of less sticky cells in CS patients. **C**, CTCs from CS patients were not easy to deform since the depth of tip travel for the majority of cells was recorded below 200 nm. In contrast, the distribution of CTCs deformability for CR patients was normal centered around 500 nm. CS: castration sensitive; CR: castration resistant.

Figure 4.

Combined nanomechanical properties of CTCs separate a group of CS from a group of CR patients. **A**, Elasticity of CTCs from CS patients drops from up to 15 kPa to less than 0.1 kPa for CR patients. **B**, Cells' adhesion at the same time increases from less than 100 nN to almost 2pN. **C**, Deformation of these cells increases from 50 nm in CS patients to more than 1.5 μ m in CR patients. **D**, Elasticity and **E**, deformation are strongly associated, whereas association of elasticity and adhesion is much weaker. **F**, Association between deformation and adhesion was not detected. **G**, Principal component analysis indicates that elasticity is

responsible for about 59% of the observed variance, whereas deformation has 25%, and adhesion 16% contribution, respectively. **H**, Hierarchical cluster analysis performed on all the components of nanomechanical phenotype clearly separates a group of CS from a group of CR patients. A group average with Euclidean distance was applied during the analysis. The data (**A–B**) are subject to student *t*-test and $p < 0.05$ is considered as significant. Adh: adhesion; Def: deformation; CS: castration sensitive; CR: castration resistant; YM: Young modulus.

Table 1

Patient pathological status and CTCs enumeration

Patient number	Metastatic site	Castrate	PSA (ng/ml)	Tumor grade (Gleason score)	Blood volume (ml)	CTC	CTC/ml	CTC for AFM analysis
1	Bone, lymph nodes and liver	Resistant	208.08	4+5	8	21	2.6	12
2	Bone	Resistant	21.32	4+5	15	35	2.3	7
3	Bone, lymph nodes, adrenal and liver	Resistant	2368.31	4+5	15	6	0.4	6
4	Bone	Sensitive	<0.1	4+4	15	9	0.6	9
5	Bone	Sensitive	6.83	NA	6.5	11	1.7	11
6	Bone and lymph nodes	Sensitive	132.5	5+4	8	6	0.8	2
7	Pelvic mass and lung	Resistant*	3.61	3+3	8	51	6.4	10
8	Lymph nodes	Resistant	76.48	4+3	8	NA	NA	9
9	Bone	Sensitive	3.27	4+4	8	17	2.1	5
10	Biochemical failure	Resistant*	3.62	4+4	8	8	1.0	7
11	Bone	Resistant	28.01	4+3	9	33	0.8	6
12	Biochemical failure	Sensitive	40.77	4+5	8	33	4.1	7
13	Bone and liver (BRCA mutation)	Resistant	<0.1	NA	8	48	6.0	9
14	Bone	Resistant	111.84	3+4	10	60	6.0	10
15	Bone and lymph nodes	Resistant	91.85	5+4	8	28	3.5	10
16	Bone and lymph nodes	Resistant*	206.64	5+4	8	24	3.0	8
17	Bone and lymph nodes	Resistant	20.12	NA	8	18	2.3	9

* : borderline

NA: no data available

Table 2

Nanomechanical properties of CTCs isolated from individual patients

Young Modulus [kPa]	Young Modulus [kPa]	SD	Adhesion [pN]	SD	Deformation [nm]	SD
1	1.80	2.49	582	369	557	468
2	2.46	2.17	795	722	429	166
3	1.08	1.44	329	251	610	598
4	3.27	2.00	76	50	344	359
5	3.97	4.47	364	557	349	368
6	8.60	6.36	55	35	91	101
7	1.94	2.28	496	394	938	448
8	1.92	1.25	1037	723	358	190
9	7.44	4.28	95	55	159	120
10	2.01	1.50	365	239	699	266
11	1.10	0.83	162	133	1287	647
12	7.62	7.71	63	30	209	282
13	0.29	0.40	287	264	702	299
14	1.32	1.94	274	267	605	403
15	1.48	2.31	635	560	837	650
16	1.52	1.66	1151	1022	809	640
17	2.08	3.02	519	676	979	852