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Towards early detection of cervical cancer: Fractal dimension of AFM images of human cervical epithelial cells at different stages of progression to cancer

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Abstract

We used AFM HarmoniX modality to analyse the surface of individual human cervical epithelial cells at three stages of progression to cancer, normal, immortal (pre-malignant) and carcinoma cells. Primary cells from 6 normal strains, 6 cancer, and 6 immortalized lines (derived by plasmid DNA-HPV-16 transfection of cells from 6 healthy individuals) were tested. This cell model allowed for good control of the cell phenotype down to the single cell level, which is impractical to attain in clinical screening tests (*ex-vivo*). AFM maps of physical (nonspecific) adhesion are collected on fixed dried cells. We show that a surface parameter called fractal dimension can be used to segregate normal from both immortal pre-malignant and malignant cells with sensitivity and specificity of more than 99%. The reported method of analysis can be directly applied to cells collected in liquid cytology screening tests and identified as abnormal with regular optical methods to increase sensitivity.

Keywords

Cervical cancer; Early cancer detection; Novel detection methods; Atomic force microscopy; Pap smear/liquid cytology tests

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Introduction

Cervical cancer is the 2nd most frequent type of cancer among women worldwide with approximately 288,000 deaths per year¹; more than 12,000 women were diagnosed with this disease in 2012 in the US only. Morbidity and mortality of cancer can be substantially decreased if cancer is detected early.^{2–4} In particular, cytological screening tests of cervical cancer such as Pap smear^{2,4} and liquid-based cytology³ have helped to decrease mortality from cervical cancer by 70–80%.^{1,5} The main advantages of these tests are their simplicity and minimal invasiveness (the cells are obtained from the cervix using a combination of a spatula and brush). Despite the impressive success of these tests, their sensitivity (the percentage of “true-positive” cases that are detected) for detecting preinvasive cervical lesions is far from desirable, with mean sensitivity of only 55% (range 30–80%). The sensitivity of detection for invasive carcinoma is also not perfect, ranging from 55% to 80% in different studies.^{6,7} According to the American Cancer Society data, each year in the United States alone approximately 3.6 million cytological tests are classified as equivocal, out of which only 8% of women will have pre-invasive (high-grade squamous intraepithelial) lesions, and 0.4% will have carcinoma as found in further testing that involves invasive tissue biopsies. Specificity (true-negative) is relatively high, reaching 95%.⁸ Thus, more accurate tests may substantially decrease the cost and patient inconvenience of screening by eliminating additional steps of HPV DNA testing and colposcopy with biopsy.

Here we describe a potentially significant improvement of sensitivity and specificity of the liquid-based cytology screening tests by introducing a new imaging modality, atomic force microscopy (AFM). Although AFM is relatively slow, this modality can be used in combination with the existent standard optical tests to provide the answer on malignancy of suspicious cells (typically a few) identified with the optical tests.

A few AFM modes (modalities) which allow obtaining information not only about surface geometry but also about its physical properties are novel in biology,^{9,10} and in particular, in the study of the cell.^{11–14} Compared to optical microscopy, AFM has a substantially higher resolution. Compared to electron microscopy, AFM can collect images in three dimensions with a considerably superior vertical resolution (up to 0.01 nm). Importantly, preparation of samples is much faster for the AFM method than for electron microscopy. Finally, AFM can provide unique information on *physical* properties of cells, which might be potentially used to study diseases.¹⁵ AFM is broadly used to study cell morphology and mechanics. Malignantly transformed cells differ from normal cells in terms of cell growth, morphology, cell–cell interaction, organization of the cytoskeleton, and interactions with the extracellular matrix.¹⁶ Atomic force microscopy is theoretically capable of detecting most of these changes (see recent reviews^{15,17} and the references therein). Recently introduced scanning probe techniques such as Peak Force QNM¹⁸ and HarmoniX¹⁹ allow measurement of physical properties of cells (rigidity, adhesion, topography, viscoelastic energy losses, etc.) with the lateral resolution up to ~3–5 nm (the vertical resolution for topography is ~0.1 nm). Specifically, we show that the adhesion maps of the cell surface clearly discriminate between normal cells and a mixture of cancer or precancerous (immortal) cells.

Recently, we demonstrated that the surface of normal human cervical epithelial cells differs substantially from the surface of malignant cells by studying viable²⁰ and fixed^{21,22} cells with AFM as well as by using nonspecific (just physical) adhesion of fluorescent probes for the surface of viable cells.^{23–25} Although those results demonstrated the substantial changes of the cell surface when cells become malignant, it was done either on a large number of cells^{23–25} or without the study of intermediate steps of cancer progression^{20,21} which is the most valuable for early detection of cancer. In addition, statistical analysis of the changes has yet to be done. Both these studies are done in the present work.

Here we expand our method of fractal analysis introduced in²¹ to study the change of fractal behavior of the cell surface during cancer progression, from normal through immortal (pre-malignant) to carcinoma (malignant) cells. We demonstrate that fractal dimension, a parameter calculated from the AFM scans, can be used to detect both premalignant and malignant cells with sensitivity and specificity higher than 99%. 6 normal cell strains and 6 cancer cell lines analyzed in this work were directly derived from healthy and malignant cervical tissues of 6 healthy and 6 cancer patients, respectively. 6 immortalized cell lines were derived by transfection of normal cells collected from 6 healthy individuals with plasmid DNA containing the complete HPV-16 genome. The cell lines and strains may certainly be different from ex vivo cells obtained in the clinical screening tests. Sampling and laboratory errors are not reproduced as well. All that will be a subject of future clinical study. It should be noted that besides being a standard first step, the use of the cell model in this work allows for a high level of control of the cell phenotype down to the single cell level, which is impractical to attain on ex vivo cells obtained in the clinical screening tests.

It should be noted that the idea of using fractal geometry to detect cancer has been suggested previously.^{26–28} Fractals²⁹ are “self-similar” irregular curves or shapes that repeat their pattern when zoomed in or out. These complex disorderly patterns are typically formed under far-from-equilibrium conditions,³⁰ or emerge from chaos.³¹ Recently, a fractal structure of chromatin has been used to show how the cell’s nucleus holds molecules that manage nuclear DNA in the right location.³²

The possible connection between cancer and fractals is based on the presumed imbalance of various biochemical reactions which is typically associated with cancer. This could result in chaos and the appearance of fractal geometry of cancer. Tumor vasculature and antiangiogenesis demonstrated explicit fractal behavior,^{27,33} and cancer-specific fractal behavior of tumors at the *macroscale* was recently found when analyzing the tumor perimeters.³⁴ Similar analysis for the *micro- and submicron-scales* (done on one-dimensional perimeter of cross-sections of individual cell nuclei) did show different fractal dimension,³⁵ though it did not provide any noticeable improvement in identification of cancer cells compared to just a visual discrimination of neoplasia currently used by the pathologists.³⁶ The present report is the first that suggests that the fractal dimension can be treated as a new “physical marker” for identification of *individual* cervical cells at different stages of progression to cancer without tissue biopsy with sensitivity and specificity of more than 99%.

Methods

Cell culture

We used primary cultures of human cervical epithelial cells prepared from tissues collected from the transformation zone of cervix from 6 cancer patients and 6 healthy individuals. The cell isolation was performed by a two-stage enzymatic digestion using dispase to remove the epithelium and then trypsin to disperse the individual epithelial cells.³⁷ All human tissue was obtained from the Cooperative Human Tissue Network. Informed consent was obtained from patients according to the published guidelines.³⁸ Each tissue was digested for 16 hours at 4 °C in dispase. Then, the layer of epithelial cells was removed from the underlying connective tissue by scraping. The sheet of epithelial cells was cut into 1mm² pieces and digested in 0.25% trypsin at 37 °C for 10 minutes. Trypsin was neutralized by adding fetal bovine serum. The cells were collected by low speed centrifugation. Cultures consisting of 95% epithelial cells were maintained in keratinocyte serum-free medium (KSFM, Invitrogen, Carlsbad, CA) which prevents outgrowth of fibroblasts and other stromal cells.

Six immortalized (pre-malignant) cell lines were prepared here in two steps: 1) transfection of normal cervical cells with the complete HPV-16 genome, and 2) subsequent immortalization of the transfected cells. HPV genes were introduced into cultured cervical cells by transfection with plasmid DNA containing the complete HPV-16 genome in combination with the neomycin resistance gene.³⁹ Subsequently, medium was changed and cells grew for 24 hours before cultures were split 1:3. After 24 hours, transfected cells were selected by growth for 2 days in KSFM containing 200 µg/ml G418 and used immediately. Only immortalized cells survived after 60–150 population doublings (PDs). Normal cervical cells were used between 20 and 40 PDs, and carcinoma cell lines were used at 40–290 PDs. The slightly higher number of PDs for (pre)cancer cell lines avoids potential confusion because any normal cells (epithelial cells or stromal cells) that may contaminate the cancer culture dishes would die out by that number of PDs. Cancerous CXT-2, 3, 5, 6, 7, 8, precancerous CX-16-2, 16-4, 16-11, 16-12, 16-14, 16-15 cell lines and normal HCX-160, 265, 277, 278, 369, 372 strains were used here.

Cell fixation and preparation for AFM study

To mimic the processing of cells in the screening liquid cytology tests, cells were fixed and dried before imaging with AFM. To avoid drying artifacts, freeze-drying was used. These steps were done as follows. All cells cultured in 60 mm tissue culture dishes were used for experiments when the cells reached <50% confluency. The cells were then washed twice with 1X phosphate buffered saline (PBS), and then treated with 4 ml of Karnovsky's fixative overnight at 4 °C. After treatment, the cells were washed twice with 4 ml of 0.2M sodium phosphate buffer at an interval of 2 hours to remove excess Karnovsky's fixative and kept overnight at 4 °C. Finally, the cells were washed with 5 ml of DI water twice before freeze drying. (The cell samples thus prepared can be preserved for several weeks with DI water at 4 °C before freeze drying them.) After fixing, water was removed by freeze-drying (using Labconco Lyph-Lock 12 freeze dryer). After freeze-drying, cells were preserved in a dessicator (relative humidity <10%). The cells were imaged under AFM directly in culture

dishes within 30 minutes after removal from the dessicator. The dried samples can be preserved for at least several weeks in a desiccator at room temperature.

Atomic force microscopy

A Nanoscope™ Dimension 3100 (Bruker/Veeco, Inc., Santa Barbara, CA) atomic force microscopes with Nanoscope V controller were used in the present study. AFM cantilever holders for operation in air were employed. To measure the maps of cell adhesion, the HarmoniX mode of operation were utilized. Bruker/Veeco cantilevers for imaging in air were used (HarmoniX standard cantilevers for HarmoniX mode were used). This mode is capable of collecting physical information about the cells surface, including topology and adhesion. A ste-by-step data acquisition and analysis are described in the Supplementary Materials.

Calculation of the fractal dimension

Fractal analysis of AFM images/maps was done with the help of the Fourier analysis. Specifically, 2D Fourier magnitude $F(u, v)$ of the AFM images can be found as follows:

$$F(u, v) = \sum_{x=0}^{N_x-1} \sum_{y=0}^{N_y-1} z(x, y) \exp[-i2\pi(ux/N_x + vy/N_y)] / N_x N_y, \quad (1)$$

where $z(x, y)$ is the value of the image at point/pixel (x, y) , N_x , N_y are the number of pixels in the x , y directions. The magnitude was then treated in polar coordinates and averaged over the polar angles:

$$A(Q) = 1/\pi \int_0^\pi F(Q \cos \varphi, Q \sin \varphi) d\varphi. \quad (2)$$

A is a function of reciprocal space Q (inverse lateral size of the geometrical features on the AFM image, L). Linear behavior of $A(Q)$ in the log-log scale (or $A(Q) \sim Q^b$) is a defining feature of fractals. An important parameter of fractals is the fractal dimension α , which can be defined as $\alpha = 2 - b$. Such a definition of the fractal dimension gives $\alpha = 2$ for flat and $\alpha = 3$ for infinitely rough surfaces.

To calculate the Fourier spectra, Scanning Probe Image Processor (SPIP) software (Image Metrology A/S, Denmark) was utilized. The fractal dimension was found by fitting the obtained spectra with $A(Q) \sim Q^b$ function by using, e.g., Origin 9.0 software (OriginLabs, Inc.). An example of such processing is shown in the Supplementary Materials (Section 1, Step 4b, Figure S1).

When analyzing the magnitude behavior from the fractal point of view, strictly speaking, it is impossible to obtain the exact fractal behavior from the maps observed for any realistic surface. The power dependence of the magnitude which defines the fractal, rigorously speaking, should be observed in the entire geometrical (infinite) scale of Q . This is impractical because of natural limitations⁴⁰ due to the finite size of data and finite digitalization/pixelization of any image. It implies that the fractal geometry cannot be even

considered for the size of geometrical features $L (=Q^{-1})$ that are either greater than the size of the recorded image or smaller than the size of each pixel. For example, if the analyzed AFM images of $5 \times 5 \mu\text{m}^2$ is recorded with 256×256 pixels, the fractal behavior can be analyzed for L ranging between $\sim 5 \mu\text{m}$ and 20nm ($=5 \mu\text{m}/256$). Moreover, we noticed in,²¹ the magnitude of the adhesion maps of normal cells could be well approximated as $A(Q) \sim Q^b$ only for the range of $Q > 3 \mu\text{m}^{-1}$ (the scale of $L < 300 \text{nm}$). To be consistent, and because in future clinical tests the type of cell is unknown, we calculate the fractal dimension for $L < 300 \text{nm}$ ($Q > 3 \mu\text{m}^{-1}$) for all cell types. Therefore, rigorously speaking, the fractal dimension is then defined within these limits in this work.

Results

Representative examples of $10 \times 10 \mu\text{m}^2$ adhesion maps of normal, pre-malignant and malignant cells are shown in Figure 1. The height (topography) images of the same cell areas are shown for reference (no discrimination between different cell types was found in the height mode, see Figure 4 below). The peak force images are shown to visualize small features that are not clearly seen in the height scans. This is useful, for example, to avoid analyzing possible artifacts. For example, such an artifact is clearly seen in the middle right side of the peak force image of the normal cell (panel G). It is almost impossible to see this artifact in the adhesion (panel A) and topography/height (panel D) images. All images were recorded with 512×512 pixel resolution. Each pixel in those maps is a well-defined numerical value (brighter means higher value). One can see that normal cells look smoother in the adhesion. However, just the analysis of roughness parameters did not show any statistically noticeable segregation between normal and malignant/pre-malignant cells (not shown).

The adhesion maps were analysed for fractal geometry, as described in the Method section and the Supplementary Materials. The majority of values of fractal dimension were derived for $5 \times 5 \mu\text{m}^2$ zoomed areas of the maps exemplified in Figure 1. This allowed us to avoid counting obvious artifacts that sometimes occurred on $10 \times 10 \mu\text{m}^2$ areas (mostly due to the attached debris). In some cases when the number of artifacts was high, smaller zoomed areas (down to $2 \times 2 \mu\text{m}^2$) were analyzed. Typically, the variation of the fractal dimension between these zoomed areas was 3–4% within one cell. Robustness of the fractal dimension (weak or no dependence on the imaging parameters) was previously demonstrated in our previous work,²¹ and elaborated in the Supplementary Materials.

Figure 2, A shows histograms of distributions of fractal dimension derived from adhesion maps of 128 normal, 245 premalignant, and 170 malignant cells. The results of similar analysis done for the height images are shown in Figure 2, B. This latter analysis was done for only 50–70 cells of each type because it was a screening test; and since no noticeable discrimination between different cell phenotypes was observed, no more cells were analyzed. One can see a clear segregation between normal and pre-malignant/malignant cells when using the fractal dimension derived from the adhesion maps, Figure 2, A. The box statistical graphs are also shown in Figure 2. One can see from Figure 2 that there is a clear discrimination (at the confidence $P < 0.0001$) based on the adhesion maps. The analysis of the height images does not indicate any discriminating power.

To analyze potential clinical relevance, it is instructional to show fractal dimension for each human subject that were used in the cell derivation. This is shown in Figure 3. One can see that the fractal dimension is a clear separator for normal and pre/malignant cells for each of the human subject cell sources. By performing one-way ANOVA tests, one can see that the mean values of the fractal dimension of different types of cells are significantly different at the confidence level at least $P < 0.0001$.

While ANOVA tests are useful to demonstrate the statistically significant difference between the observed populations of cells, ROC (receiver operating characteristic) curves give a statistical analysis useful for potential cancer diagnostics because it visualizes sensitivity and specificity of the described method to detect cancer. Figure 4 shows ROC curves for discrimination of normal and pre-malignant (immortal) cells, normal and cancerous cells, immortal and cancer cells. Table 1 shows the appropriate statistical data for the ROC curves shown in Figure 4. One can see that sensitivity (true positive) and specificity (true negative) of the detection of both pre-malignant and malignant cells is more than 99%. At the same time, separation of pre-malignant and malignant cells can only be done with sensitivity and specificity of ~66% (likelihood ratio of 1.92, fractal dimension >2.418). However, if identification of cancer is more important, sensitivity to detect cancer (vs immortal) can be improved to 89% while keeping specificity of 50% (likelihood ratio of 4.5, fractal dimension >2.450).

Discussion

Fractal dimension as a potential biophysical marker of cancer cells

It is somewhat expected that pre-malignant cells have the fractal dimension in-between the normal and malignant. But it is rather non-trivial to see that pre-malignant cells have the fractal dimension almost entirely different from normal cells. This implies that the cell surface changes substantially at an early stage of progression towards cancer. Thus, it is conceivable to expect that the fractal dimension can be used as a physical “marker” for the detection of pre-malignant cells, i.e., for early detection of cervical cancer. It is worth noting that the change of surface properties of cervical epithelial cells at the stage of immortalization has been recently reported.²³ However, these results were found on thousands of cells simultaneously, and therefore, had less practical significance for future clinical use in which the analysis of few individual cells is needed. Our current work extends the value of previous results to the level of individual cells. This makes it practical for direct implementation in the analysis of cells obtained in the liquid cytology screening tests.

It is worth stressing that these values of sensitivity and specificity are derived based on the fractal dimension of *single* cells. Therefore, it is expected that only a few cells might be needed to make a conclusive measurement. Because of very high accuracy of segregation of normal and pre/cancer cells, the entire size of the sample needed for make a conclusive measurement will be defined by type I error (alpha, sampling and human error) in the power calculator. This error can only be defined in the actual clinical study.

Biological and physical reasons for the observed results

The physical changes happening on the cell surface during progression towards cancer is still mostly unknown. For example, a difference in cilia on the surface of cancer and normal cells has been found only recently.⁴¹ Similarly, we found that microvilli, microridges, and glycocalyx (collectively called cellular brush) were qualitatively substantially different on normal and cancer cervical cells.²⁰ Molecular brushes on living cells, composed of the glycocalyx layer, the pericellular molecular coating^{42,43} are known to be responsible for cell-cell interaction, immune response, cell migration, differentiation, and proliferation.^{44,45} It was shown that the size of the pericellular coating^{43,46} correlated with the degree of invasiveness of cancer (though it is unknown if just the size or possibly different molecular composition or both is the cause). Thus, while specific biochemical path responsible for the change of the cell brush is yet to be found, it is plausible to expect that the physical parameters of the cell surface change during progressions to cancer.

AFM is a high-resolution surface imaging technique. While the lateral resolution can be of the order of a few nanometers, the vertical special resolution can be as high as 0.01nm. Thus, it is conceivable that one can detect the changes of the cellular surface brush/pericellular coating by means of the AFM technique. Obviously, imaging of viable cells in their native environment may carry more information about cell properties than the study of dried fixed cells. However, because the liquid cytology tests operate with fixed and dried cells, the ability to discriminate malignancy based on the study of dried fixed cells allows for simple modification of the screening liquid cytology test. Secondly, it is impossible to obtain high resolution images of the surface of viable cells because the cellular brush is in constant Brownian motion. In addition, the brush can easily be disturbed by the imaging AFM probe when in aqueous medium. When dried, the cell surface has a well-defined border. This allows obtaining AFM images with higher resolution of surface features. The brush is obviously collapsed after drying. To avoid the drying artifacts, the freeze-drying approach was used.

Let us now try to understand why the fractal segregation of normal and pre/malignant cells is only seen when analyzing the maps of adhesion, but not the height images. It is plausible to expect that the lateral resolution of AFM is higher in the adhesion mode when image soft samples. This is because the lateral resolution is defined by the area of contact between the AFM probe and surface. In the height mode, the area can be substantially larger because it is defined by the deformation of the surface with the AFM probe. When dealing with mapping the adhesion, the area of contact is defined by the area of a bridge formed between the probe and surface at the moment of disconnection. For the most of materials, the area of the bridge is noticeably smaller than the area of the elastic contact. When the material is soft, the adhesion is not substantially larger than the load force, and the viscous part of the modulus is less than the elastic one, the difference in the area can be rather large. Thus, we can conceive the difference in fractal behavior between normal and pre/malignant cells was not observed in the height images because there was no sufficient lateral resolution in the height images currently recorded by AFM.

Let us demonstrate quantitatively that the adhesion data are much more sensitive to the underlined geometry than what can be imaged in the height data channel. The adhesion force

that occurs due to the contact interaction of the AFM probe with the cell surface can be estimated as⁴⁷ $F_{adh} = 2\pi W R_1 R_2 / (R_1 + R_2)$, where R_1 is the radius of the AFM probe, R_2 is the radius of curvature of the surface at the point of contact, W is the adhesion energy per unit area between a flat contact of two materials of the probe and surface. The modeling of the contact as interaction of two spheres is definitely an approximation. Nevertheless, it seems to be a reasonable one because we deal with fixed dried cells, and therefore, their deformation is small. We observed such deformations in the range of 1–5 nm (for typical adhesion forces of 1–15 nN). This is definitely smaller than the radius of the AFM probe (~10–15 nm) and the majority of the surface features (though one has to be careful with the surface features below 5 nm). W is usually defined by van der Waals interaction unless there is a specifically strong interaction between the AFM probe and cell surface. The latter could be easily seen through its specific behavior of the retraction force curves.⁴⁸ It is not observed in our case (not shown).

Processing the values of the adhesion force through the above equation, one can derive the radius of curvature of the surface features at each point of the image, $R_2 = F_{adh} R_1 / (2\pi R_1 W - F_{adh})$. An example of the derived radii at different (points) pixels of surface (along one line scanned with AFM) is shown in Figure 5, *A*, *B*. For comparison, the same figure shows the height information recorded in parallel. One can see that it is impossible to record the radii information in the height mode that would be comparable to the radii derived from the adhesion maps. A representative statistics of the radii of the surface R_2 derived from the collected adhesion maps are shown in Figure 5, *C* (one image of a cancer cells was processed). One can see that the most probable (mean) value of this radius is of the order of 1 nm. As was mentioned above, the formula used to derive this radius is valid only up to the radius of ~5 nm. The surface deformation (stretching) has to be taken into account for smaller radii. It is qualitatively clear that the stretching of the bridge between the AFM probe and the cell surface will lead to a decrease of the surface contact radius, i.e. the mean value of the surface features detected with AFM in adhesion maps is even smaller than 1 nm. To resolve such “adhesion” radii in the height mode, one would need to image cells with the lateral resolution of below single nanometers (the height resolution is not a concern), which is not practical to attain with AFM on such soft samples as fixed cells at present. Thus, we can conceive that the cancer and precancerous signatures found in the adhesion maps may be explained by the different surface geometry of cells at the submicron scale (the scale of calculation of the fractal dimension).

Conclusion statements

Here we report a novel AFM-based method of cell imaging that can be used to increase the accuracy of early detection of cervical cancer in the liquid cytology tests. Current screening tests require improvement for detection of cancer within just a few “suspicious” cells that are identified with standard optical microscopy. The limited number of cells analyzed makes the use of AFM, which is rather slow imaging technique, feasible. The analysis of AFM images/maps collected on human cervical epithelial cells was performed at three stages of progression towards cancer, from normal through immortal (pre-malignant) to carcinoma (malignant) cells. The primary cell cultures for normal and cancer cells were derived from healthy tissue and tumors, respectively. Premalignant cells were obtained from normal ones

by transfection the cells with plasmid DNA of HPV virus. The cell models in vitro were used because of good control of the cell type, which is impractical to attain in clinical screening tests (ex-vivo).

The fractal analysis of the AFM maps was performed on fixed dried cells imaged to obtain the maps of physical (nonspecific) adhesion between the AFM probe and cell surface. We demonstrated that a parameter of fractal geometry called fractal dimension could be used to segregate normal from both immortal pre-malignant and malignant cells with sensitivity and specificity of more than 99%. We observed a significant change in the fractal dimension when cells transformed from normal to pre-malignant or to malignant stages. Although the average value of the fractal dimension of pre-malignant and malignant cells was significantly different at the confidence level $P < 0.0001$, the sensitivity and specificity of separation of pre-malignant and malignant cells was just ~66% (though it could be improved to 89% if letting specificity to be decreased to 50%). To define the actual accuracy for the described method in clinical tests, a further clinical study is needed. Nevertheless, based on the reported results, it is conceivable to expect a considerable improvement in the accuracy of early detection of cervical cancer. As such, the fractal dimension could be tested as a new potential “physical marker” for identification of individual cervical cancer cells without biopsy.

We also presented evidence that the reason for the observed change in the cellular surface is presumably due to the physical changes in the pericellular brush, which was observed previously when cells become malignant. The biochemical pathway responsible for such a change is yet to be identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2015.04.012>.

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Figure 1. Representative examples of $10 \times 10 \mu\text{m}^2$ adhesion maps and height images of normal, pre-malignant, and malignant cells. The brighter means the higher value either of adhesion or height.

Figure 2.

(**A**) Fractal dimension analysis of the adhesion maps; fractal dimension was calculated on 128 normal, 245 premalignant, and 170 malignant cells. (**B**) Fractal dimension analysis of the height images of normal, pre-malignant, and malignant cells. Fractal dimension was calculated on 50–70 cells for each cell type. *** stands for the statistical significance at the confidence level $P = 0.0001$. Histograms (with the lognormal distribution curve) and box-plots are shown. The box body is 25–75%, whiskers show one standard deviation, the bar in the box stands for the average, and the square is for mean.

Figure 3.

Fractal dimension analysis of the adhesion maps of normal, pre-malignant, and malignant cells for each of 18 subjects of the cell sources. Fractal dimension was calculated on 128 normal, 245 premalignant, and 170 malignant cells. The average and one standard deviation are shown.

Figure 4. ROC curves for discrimination of normal and immortal (pre-malignant) cells, normal and cancerous (malignant) cells, immortal, and cancer cells.

Figure 5.

A representative line of data of (A) height. (B) shows the radii of curvature of the features on the cell surface derived using the adhesion channel; (C) a representative statistics of the radii of the surface R_2 derived from the collected adhesion maps.

Table 1

Statistical data of the ROC graphs shown in Figure 4.

Area under the ROC curve	Normal vs cancer	Normal vs immortal	Immortal vs cancer
Area	1.000	0.998	0.740
Std. Error	0.0	0.00137	0.0252
99% confidence interval	1.000–1.000	0.995–1.000	0.675–0.805
<i>P</i> value	<0.0001	<0.0001	<0.0001