Published in final edited form as:

Cytometry A. 2020 November ; 97(11): 1145–1155. doi:10.1002/cyto.a.24011.

Morphology, Motility, and Cytoskeletal Architecture of Breast Cancer Cells Depend on Keratin 19 and Substrate

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Abstract

Cancer cells gain motility through events that accompany modulation of cell shape and include altered expression of keratins. However, the role of keratins in change of cancer cell architecture is not well understood. Therefore, we ablated the expression of keratin 19 (K19) in breast cancer cells of the MDA-MB-231 cell line and found that cells lacking K19 become more elongated in culture, with morphological reversion toward the parental phenotype upon transduction of *KRT19*. Also, the number of actin stress fibers and focal adhesions were significantly reduced in *KRT19* knockout (KO) cells. The altered morphology of *KRT19* KO cells was then characterized quantitatively using digital holographic microscopy (DHM), which not only confirmed the phenotypic change of *KRT19* KO cells but also identified that the K19-dependent morphological change is dependent on the substrate type. A new quantitative method of single cell analysis from DHM, via average phase difference maps, facilitated evaluation of K19–substrate interactive effects on cell morphology. When plated on collagen substrate, *KRT19* KO cells were less elongated and resembled parental cells. Assessing single cell motility further showed that while *KRT19* KO cells moved faster than parental cells on a rigid surface, this increase in motility became abrogated when cells were plated on collagen. Overall, our study suggests that K19 inhibits cell motility by regulating cell shape in a substrate-dependent manner. Thus, this study provides a potential basis for the altered expression of keratins associated with change in cell shape and motility of cancer cells.

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Conceptualization: B.M.C. and C.B.R.; Conducting experiments: P.S., V.K.L., and T.N.; Result analysis: V.K.L., P.S., C.B.R., and B.M.C.; Writing-original draft preparation: P.S., V.K.L., B.M.C., and C.B.R.; Review and editing: V.K.L., P.S., C.B.R., and B.M.C.; Supervision: C.B.R. and B.M.C.; Funding acquisition: B.M.C. and C.B.R.

Additional Supporting Information may be found in the online version of this article.

Conflicts Of Interest

The authors declare no conflict of interest.

Key terms:

cancer; keratin; keratin 19; cytoskeleton; cell morphology; digital holographic microscopy; cell motility; substrate; collagen

THE keratin family of intermediate filament proteins is cytoskeletal proteins that play a critical role in maintaining mechanical stability of epithelial cells. There are 28 type I keratins and 26 type II keratins that become expressed in development-, context-, and tissue-specific manners. Heterodimerization between one type I and one type II keratins starts an assembly into 8–10 nm filaments (1,2). Once polymerized, keratin filaments form an elaborate cytoskeleton network inside the cell, strengthening cell-to-cell attachment through desmosomes and cell-to-substrate attachment through hemidesmosomes.

During tumor development, transformation of normal cells to metastatic cancer cells involves various changes at the genetic, epigenetic, and cellular levels (3,4). For example, changes at the cellular level include epithelial to mesenchymal transition (EMT) when epithelial cells lose their polarity and cell–cell adhesion (5). In the process, cell morphology changes from that of the epithelial to mesenchymal shape and cell motility increases. During this process, levels of proteins expressed in epithelial cells such as E-cadherin and keratins become lower whereas proteins expressed in mesenchymal cells such as snail and vimentin become more prevalent. Consistent with this, increased cell migration and invasion have been demonstrated following a loss of expression of keratins K8 and 18 in several cancer cell lines (6–9), as well as loss of K6 and all keratins in keratinocytes (10,11).

In seeming contrast to the role of keratins in EMT and the above-mentioned cell lines, higher expression of certain keratins has been associated with increased cell migration (12,13). For example, cell migration and invasion has been shown to require K14 in breast cancer (14), K17 in skin cancer cell line (15) and K80 in human colorectal carcinoma cell line (16). While cell signaling events involving CXCR3, Akt, and NF κ B have been implicated in these cell migration events requiring different keratins (12,13), the effects of individual keratins on cancer cell morphology and motility, and how single keratin genes and coordinated keratin gene regulation influence cell motility and ultimately tumor biology, are not clear.

In order to determine the effect of keratins in cancer cell morphology and cell motility, we decided to examine keratin 19 (K19). K19 is a smallest keratin at 40 kDa (17). Nevertheless, the primary structure of K19 includes a central rod domain that is highly conserved with other IF proteins and is critical for filament assembly (17,18). K19 is expressed in various simple and complex epithelia, but the normal function of this protein remains unclear. Still, K19 is highly expressed in several cancer types including breast cancer where its higher expression has been correlated with worse patient prognosis (19,20). Interestingly, shRNA-mediated knockdown and overexpression studies showed that K19 inhibits migration of breast cancer cells (21–23). However, the role of K19 in modulating cancer cell morphology and motility remains unclear.

Digital holographic microscopy (DHM) is a quantitative phase imaging technique that measures optical path length differences between cells and the surrounding environment, resulting in a phase delay of light transmitted through cells. This technique applied to live cells is noninvasive, label-free, and sensitive to minute alterations in cell shape and subcellular features (24,25) and movement (26,27). Parameter maps utilizing reconstructed optical phase contain information related to phase height and pixel intensity variation, which reveals fruitful information related to cell structure and function (28,29). Quantitative cell phase height maps recorded by DHM have been used to monitor and characterize alterations in cell phase height and shape parameters during the cell cycle related to division, apoptosis, and substrate adhesion (30–33). Moreover, these changes were related to morphological changes during cell motility (34,35).

Based on evidence of the role of the keratin cytoskeleton in influencing cancer cell behavior, it was hypothesized that loss of K19 would affect the highly motile breast cancer cell line, MDA-MB-231. Due to the presence of keratin in hemidesmosomes tethering cells to extracellular matrix, loss of K19 could interact with substrate to influence morphology and motility at the level of individual cells. The following objectives were used to test these hypotheses: determination of cytoskeletal architecture, phase volume, morphology, and functional motility of gene-edited K19 knockout and parental MDA-MB-231 cells on two substrates (glass and collagen) using multiple microscopy techniques including DHM, live cell video microscopy, Boyden chamber and scrape wound assays. It was found that a loss of K19 expression resulted in an elongated cell morphology and higher cell motility, and that these changes were dependent on the substrate type. These findings reveal an interaction between the extracellular milieu and keratin-dependent alteration of cancer cell morphology and motility.

MATERIALS AND METHODS

Cell Culture

The MDA-MB-231 cell line was a generous gift from Dr. Zaver Bhujwalla (Johns Hopkins School of Medicine, Baltimore, MD). Cells were grown in Dulbecco's modified essential medium (VWR Life Science, Radnor, PA) containing 10% fetal bovine growth serum (RMBIO, Missoula, MT), and 100 units/ml penicillin-100 µg/ml streptomycin (GE Healthcare, Logan, UT) at 37°C in 5% CO₂. MDA-MB-231 cells were authenticated to be 93% match of MDA-MB-231 (ATCC, HTB-26) cells using short-tandem repeat profiling service performed by ATCC (date performed: 12/28/18).

Plasmids and sgRNA for CRISPR and Generation of K19 KRT KO Cells

KRT19 KO cells were generated using the CRISPR/Cas9 system (36,37). The first exon of *KRT19* was targeted by cloning oligo-nucleotides 5' - CACCGCGAGGACACAAAGCGGGCGG-3' (forward) and 5' - AAACCCGCCCGCTTTGTGTCTCGc-3' (reverse complement) into pSpCas9 (BB)-2A-GFP vector (targeting sequence underlined). Sequence validated plasmid was transfected into MDA-MB-231 cells using continuum transfection reagents (Gemini Bio-Products, West Sacramento, CA) following the manufacturer's protocol. Fluorescence activated cell sorting

was then performed to isolate single cell clones. Clones that grew to form colonies were analyzed via western blotting and qRT-PCR to confirm ablation of K19 expression.

Cells Stably Expressing GFP-K19

Lentiviral supernatants were generated using the pLenti plasmids as described previously (37). GFP or GFP-K19 was cloned into pLenti CMV/TO hygro. Lentiviral supernatants, collected 24 h after transfection, were used to infect subconfluent MDA-MB-231 *KRT19* KO cells (KO1) in three sequential 4 h incubations in the presence of 4 µg/ml polybrene (Sigma-Aldrich, St Louis, MO). Transductants were selected in hygromycin (100 µg/ml), beginning 48 h after infection.

Antibodies

The following antibodies were obtained from commercial sources: mouse monoclonal (mAb) anti-tubulin (T9026) was from Sigma-Aldrich (St. Louis, MO); anti-K19 (A53-B/A2), anti-K5 (RCK103), anti-vimentin (V9) and anti-β-Actin (C4) were from Santa Cruz Biotechnology (Dallas, TX); mAb anti-Vinculin was from ThermoFisher Scientific (Hudson, NH); and mAb anti-K19 (ab52625) used for immunostaining was from Abcam (Cambridge, United Kingdom).

Western Blotting

Cells grown on tissue culture plates were washed with PBS and prepared in cold Triton lysis buffer (1% Triton X-100, 40 mM HEPES (pH 7.5), 120 mM sodium chloride, 1 mM EDTA, 1 mM phenyl methylsulfonyl fluoride, 10 mM sodium pyrophosphate, 1 µg/ml each of cymostatin, leupeptin and pepstatin, 10 µg/ml each of aprotinin and benzamidine, 2 µg/ml antipain, 1 mM sodium orthovanadate, 50 mM sodium fluoride) (37). Cell lysates prepared in Laemmli SDS-PAGE sample buffer were resolved by SDS-PAGE and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Immunoblotting was performed with the indicated antibodies, followed by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Sigma-Aldrich) and Amer-sham ECL Select Western Blotting Detection Reagent or Pierce ECL Western Blotting Substrate (Thermo Scientific, Hudson, NH). Signals were detected using ChemiDoc Touch Imager (Bio-Rad).

RNA Harvest, cDNA Synthesis, and qRT-PCR

RNA was harvested using Direct-Zol RNA MiniPrep Plus (Zymo Research, Irvine, CA) following the manufacturers' protocols. RNA was reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) using the manufacturer's protocol. qRT-PCR was performed on the first strand cDNA with the primers listed below and PerfeCTa® SYBR® Green FastMix®, ROX™ (Quanta bio, Beverly, MA) using the Applied Biosystems StepOne™ Real-Time PCR Systems. The following primers were obtained from the PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>): *KRT19*: 5' - AACGGCGAGCTAGAGGTGA-3' and 5' - GGATGGTCGTGTAGTAGTGGC-3'; *GAPDH*: 5' - AAGGTGAAGTCC GGAGTCAAC-3' and 5' - GGGGTCATTGATGGCAACAATA-3'; and *RPS18*: 5' - GCGGCGGAAATAGCCTTTG-3' and 5' - GATC ACACGTTCCACCTCATC-3'. The following program was used for all qRT-PCR reactions:

95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative quantifications or fold changes of the target mRNAs were calculated after normalization of cycle thresholds with respect to reference genes, *RPS18* and *GAPDH*, levels.

Cell Morphology

Forty-eight hours after seeding, cells were fixed using 50% methanol and stained using 0.1% crystal violet. Images of stained cells were taken with Olympus CK2 Inverted Trinocular Phase Tissue Culture Microscope (Olympus Optical Co., Japan) equipped with an AM Scope 3.7 digital camera (AmScope, Irvine, CA). ImageJ software was used to determine the area of cells.

Immunofluorescence Staining

For immunostaining of cells in culture, cells grown on glass coverslips (VWR Life Science) were washed in phosphate-buffered saline (PBS), fixed in fresh 4% paraformaldehyde in PBS, and permeabilized in 0.1% Triton X-100. Samples were blocked in 5% normal goat serum (NGS) in PBS for overnight before staining with primary antibodies diluted in blocking buffer for 16 h followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h. One microgram per milliliter of Hoechst 33342 and phalloidin (CytoPainter phalloidin-iFluors 488, Abcam) were used to stain nuclei and F-actin respectively, and coverslips were mounted on microscope slides with mounting media containing 1,4-diaza-bicyclo[2.2.2]octane (Electron Microscopy Sciences, Hatfield, PA). Labeled cells were visualized at RT by epifluorescence with an Olympus BX60 Fluorescence Microscope (OPELCO, Dulles, VA) using an UPlanFI 60X NA 1.3, phase 1, oil immersion objective (Olympus). Images were taken with an HQ2 CoolSnap digital camera (Roper Scientific, Germany) and Metamorph Imaging software (Molecular Devices, Sunny Vale, CA). ImageJ software version 1.51J (NIH, Bethesda, MD) was used to process images to compile figures and determine numbers of stress fibers and focal adhesions.

Collagen Preparation

Collagen hydrogels were prepared at a concentration of 4 mg/ml by using a stock type I collagen from rat tail tendon, at 8.86 mg/ml (Corning, Corning, NY). Stock collagen was mixed with 1 part of 10X phosphate buffered saline (PBS) (MilliporeSigma, Burlington, MA) supplemented with phenol red (Fisher Scientific, Hampton, NH) and the balance sterile de-ionized water. The solution was titrated to a pH of 7.4 by adding a few microliters of 1 M sodium hydroxide. Collagen pH level was indicated by a color shift from yellow to orangered. The final collagen solution was then added to 35-mm diameter glass-bottomed petri dish (Cellvis, Mountain View, CA). Dishes containing collagen were wrapped with parafilm to avoid evaporation and left inside an incubator for 30 min to polymerize at a temperature of 37°C.

DHM Setup and Imaging

A bi-telecentric digital holographic microscope (DHM) set up for vertically-oriented transmission imaging was used to capture quantitative phase height maps of cancer cells

(29). The DHM system utilized a HeNe laser with 633 nm central wavelength to generate a reference beam coherent with an object beam that passes through imaging objectives and a Petri dish, which were then combined at the detector to form digital holograms. The optical system had a lateral resolution of 1.2 μm based on the numerical aperture of the objective (0.30), but the hologram was recorded on the digital sensor at a scale of 0.18 $\mu\text{m}/\text{pixel}$. The holograms were captured by a 1.3 MP CMOS camera (Lumenera Corporation, Ontario, Canada). To reconstruct holograms and cancel the phase aberration, a Fresnel reconstruction and a principal component analysis (PCA) algorithms were used. Detailed information about the set up and reconstruction was published in (38). Cell height phase maps of two cancer cell lines MDA-MB-231 parental and *KRT19* knock-out (*KRT19* KO) cultured on glass and collagen were collected with sample size of $n = 155$, $n = 126$, $n = 156$, and $n = 163$, respectively. It is important to note that the phase height maps are calculated as $d_{PH} = OPL/n$, where OPL is the optical pathlength related to phase, ϕ , as $\phi = 2\pi(OPL)/\lambda$, with $\lambda = 633$ nm here, and $n = 1.381 - 1.3370 = 0.044$, the difference between the average real component of the refractive index of a cell and surrounding aqueous environment at the imaging wavelength (39).

Image Segmentation and Phase Parameters Quantification

Cell phase image segmentation and quantification of 11 phase parameters were implemented by using an in-house MATLAB code published in (33). Parameters from phase maps have been used previously to detect subtle morphological alterations of breast cancer cells with altered gene expression, associated with altered functional motile phenotypes (27). Besides shape parameters and statistics from the pixel histogram (mean, standard deviation, skew, kurtosis), texture features from the gray-level co-occurrence matrix were determined for 8-connected adjacent pixels (see Table S1). The following steps summarize segmentation and parameter measurement: (a) Detect background area in a phase image, (b) outline each individual cell to facilitate cell edge detection, (c) extract 11 parameters including phase textures and object shape. The average cell phase height, first-order pixel statistics of standard deviation, kurtosis, and skew, second order pixel texture statistics of contrast, correlation, homogeneity, and energy, and the shape parameters of area, eccentricity, and perimeter were calculated. The code allows users to save segmented cell phase height maps linked through filename to the phase parameters file, to facilitate comparison of quantitative data and image map representations of cells (33).

Selecting Representative Cells Using the Phase Signature Sum of Squared Deviations

The technique of selecting representative cells using a DHM phase map was reported in (35). The sum of squared deviations of all standardized phase parameters from their group means, for each cell, determines the similarity in phase profile of that cell compared to an average cell in the population. Therefore, a cell with the lowest sum of squared deviations (SSD) represents the most typical cell in terms of shape and phase signature for the entire population. The SSD of each cell was calculated as:

$$\text{SSD} = \sum_{i=1}^{11} (\phi_i - \bar{\phi}_i)^2, \quad (1)$$

where ϕ_i is the value of a parameter of a cell normalized to the maximum value of the parameter in a population, and $\bar{\phi}_i$ is the population's mean of that parameter.

Computing Difference Maps from DHM Cell Phase Height Maps

After segmentation, each of four experimental groups had $n = 126$ – 163 single cell phase height maps. In each group, segmented cell phase height maps were rotated and aligned along cell long axes in MATLAB. Averaged cell phase height maps of each group were computed from these aligned cell phase height maps, which were co-registered at the cell centroids and overlaid using MATLAB. Differences maps were generated by subtracting averaged maps from each other using ImageJ. Gray value distribution in difference maps were plotted to indicate differences among groups in terms of phase signals located at peripheral regions (left versus right) and front-end regions (leading edge versus trailing edge).

Scratch Closure Assay

Confluent monolayers of cells in a 24 well plate were scratched evenly down the middle of each well with a 200 μ l pipet tip. Cells were washed with PBS four times to remove any debris. Fresh medium was then added and cells were incubated in a 37°C CO₂ incubator (Thermo) for up to 8 h. Scratch area were imaged at 0, 2, 4, 6, and 8 h following the initial scratch. Bright-field microscopy images were captured using an AM Scope 3.7 digital camera (AmScope). Five random regions per well were used to determine the overall migration ability of cells. ImageJ software version 1.51J (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) was used to measure wound closure area at each time point. Image analysis was conducted in triplicate for all cell experiments.

Single Cell Motility Assay

2.5×10^3 cells were seeded on each 35 mm diameter tissue-culture polystyrene (TCPS) dish with or without type I collagen hydrogels (Sigma-Aldrich) polymerized at 4 mg/ml at 37°C. Cells were then incubated for 24 h in standard tissue culture conditions before cells were monitored over 24 h using the CytoSMART cell imager (Eindhoven, Netherlands).

MDA-MB-231 parental and *KRT19*KO cells were seeded on 35-mm diameter polystyrene (PS) treated dishes (CELLTREAT, Pepperell, MA) and 4 mg/ml collagen layer polymerized at 37°C were imaged by the CytoSMART™ 2 System (AACS-1003, Lonza, Switzerland) after 24 h culture to allow adhesion and elongation. Media was changed before the following 20 h time-lapse experiment, with images taken every 5 min. Each experimental group had three replicates (Petri dishes/time-lapse experiments). Images taken by CytoSMART™ were put into a stack in ImageJ creating time-lapse image sequences. A binary video of each experiment was processed by background subtraction, thresholding, removal of objects much smaller than cells, and hole filling. Each individual cell was tracked using an ImageJ plugin named WrmTrack (40) to get its average cell speed. Measurement of each individual cell was also visually inspected and compared to raw images to avoid tracking inaccuracies.

Boyden Chamber Assay

Boyden chamber assays were performed by seeding 50,000 cells of each cell line into a Boyden chamber (Greiner Bio-One, NC), placed in a 24 well-plate. First, 300 μ l media containing 1% serum was added in the upper chambers. After 6 h, media was removed and replaced by another 300 μ l free-serum media. Then, media at 600 μ l supplemented with 10% serum was filled in each well plate containing the chamber. After 24 h, media was removed from the chambers. The chambers were washed with 1X PBS, then fixed with 3.7% paraformaldehyde, permeabilized and stained with 1:1000 DAPI solution. Each chamber was imaged with an inverted fluorescent microscope (EXI-310, Accu-Scope, Commack, NY) under a 40X objective. Cells that passed through the membrane were measured by averaging the number of nuclei from four regions in one chamber and averaged again over nine replicate chambers per cell line.

Statistical Analyses

Statistical tests were performed using Systat version 13.1 (Systat Software, Chicago, IL). Significance differences among cell shape and phase parameters, and average speed were tested using ANOVA followed by post hoc pairwise comparisons with the Tukey's test. The significance level was set at $P < 0.05$.

Results

K19 Regulates the Shape and Size of MDA-MB-231 Cells

To study K19, we used the CRISPR/Cas9 system and generated clones of *KRT19* knockout (KO) cells in MDA-MB-231 breast cancer cell line (Fig. 1A). We used the CRISPR/Cas9 system to completely ablate K19 expression in the triple-negative subtype MDA-MB-231 cell line due to its highly migratory and metastatic properties. Examining two different clones of *KRT19* KO cells confirmed the absence of K19 expression while levels of other intermediate filament proteins K5 and vimentin remained unchanged (Fig. 1B). Depletion of K19 expression was also confirmed at the mRNA level as *KRT19* KO cells expressed significantly lower levels of *KRT19* mRNA (Fig. 1C). Examining *KRT19* KO cells under the microscope, we noticed that they exhibit significantly different cell morphology compared to parental MDA-MB-231 cells (Fig. 1D). Loss of K19 expression yielded cells with more elongated, spindled morphology. Change in cell shape accompanied decrease in cell areas of *KRT19* KO cells (Fig. 1E), suggesting that K19 plays a critical role in maintaining the cellular architecture of MDA-MB-231 cells. The requirement of K19 for normal shape of MDA-MB-231 cells was confirmed when re-expression of K19 through GFP-tagged K19 rescued the elongated, spindled morphology of *KRT19* KO cells (Fig. 1F).

K19 Is Required for the Proper Organization of Cytoskeletal Networks and Formation of Focal Adhesion

Since actin stress fibers and focal adhesions regulate cellular architecture (41,42), actin stress fibers and focal adhesions were examined in *KRT19* KO cells. Compared to the parental cells, number of cells containing actin stress fibers was significantly decreased in *KRT19* KO cells (Fig. 2A–E). Also, staining for focal adhesion marker vinculin showed

significant reductions in vinculin puncta in *KRT19* KO cells (Fig. 2F–J). Moreover, we also observed altered microtubule organization in *KRT19* KO cells as these cells exhibited decreased spreading of microtubule network throughout cells (Fig. S1). Taken all together, these data show the K19 ablation causes defects in cytoskeletal organizations that are critical to cell morphology.

Substrate-Dependent Regulation of Cell Morphology by K19

Since keratins assist in the cell–substrate interaction via hemidesmosomes, we decided to test different substrate conditions. To this end, we cultured cells either on glass, corresponding to a stiffer substrate, or on collagen, corresponding to a softer substrate (Fig. 3A). Also, DHM was used to measure various structural parameters of parental and *KRT19* KO cells. The morphology of MDA-MB-231 cells was quantitatively different between parental and *KRT19* KO cells on glass as expected from Figure 1, but the difference was not significant on collagen (Fig. 3). Representative phase height maps with the smallest sum of squared residuals indicating typical morphology from each group were introduced in Figure 3A. Parental cells were more rounded on glass but became elongated on soft collagen substrate. These representative maps also demonstrated that *KRT19* KO cells were more elongated than parental on glass, whereas the differences between the two genotypes were less noticeable on collagen (Fig. 3A). Substrate ($F = 7$), K19 ($F = 41$), and their interaction ($F = 30$) affected eccentricity, a measure of cells' circularity, while substrate ($F = 33$) and K19 ($F = 21$) affected cell area on phase height maps (ANOVAs, $P < 0.001$). Specifically, the eccentricity of *KRT19* KO cells was higher on glass (0.93 ± 0.01 versus 0.83 ± 0.01) but not on collagen (0.91 ± 0.01 versus 0.90 ± 0.01 ; Fig. 3B, post hoc test, $P < 0.001$). Additional texture parameters including contrast, correlation, energy, and homogeneity, describing how a single pixel intensity relates to its neighbors in a cell phase map, also showed substrate-specific differences between parental and *KRT19* KO cells (Tables S1–S3 and Fig. S2). The higher contrast but lower correlation, energy, and homogeneity of *KRT19* KO cells than parental cells on glass, differences that were erased on collagen, indicate a less uniform pixel-level texture of *KRT19* KO cells—more variation in intensity between neighboring pixels. However, the area of *KRT19* KO cells remained lower than parental on both glass (838 ± 24 versus $931 \pm 19 \mu\text{m}^2$) and collagen (741 ± 18 versus $817 \pm 18 \mu\text{m}^2$) substrates (Fig. 3B, post hoc test, $P < 0.01$). These data suggest that the elongated phenotype caused by the absence of K19 is dependent on the substrate type.

Morphological differences between parental and *KRT19* KO cells, modulated by adherence to different substrates, were also apparent from average phase difference maps (Figs. S3 and 4), indicating differences in optical phase within the cell borders. On glass, average phase height maps of parental minus *KRT19* KO cells that were co-aligned and co-oriented were positive (peaking at 1870–1900 nm) in lobes offset to the left and right of the centroid, indicating larger phase signal for parental cells in those locations (Fig. 4A, top row). The same maps were negative in lobes offset above (−1,070 nm minimum) and below (−830 nm minimum) the centroid, indicating larger phase signal for *KRT19* KO cells in these locations. In contrast, on collagen, the central region is negative/blue (−1,050 nm minimum) but the surrounding region was slightly positive/red (210 and 280 nm peaks at left and right of center) (Fig. 4A, bottom row). Comparing substrate effects, glass always produced a more

peaked phase height profile, for both parental (Fig. 4B, top row, -1,500 nm minimum) and *KRT19* KO (Fig. 4B, bottom row, -2,660 nm minimum) cells. When cells were cultured on collagen, parental cells lost more phase height from left and right sides of the cell (peaking at 1640 nm versus ~0 nm), whereas *KRT19* KO cells lost more phase height from leading and trailing edge of the elongated cells (peaking at 920 nm versus 140 nm). Altogether, these data suggest an internal redistribution of cell phase height dependent on K19 and substrate adhesion, and that elongated shape of cells became independent of K19 on collagen.

Substrate-Dependent Inhibition of Cell Motility by K19

Altered cell morphology is linked to different degrees and modes of cell motility (43,44). Therefore, we decided to assess the impact of substrate variation on cell motility of *KRT19* KO cells. Previous studies suggested that K19 hinders breast cancer cell migration (21–23). Indeed, a wound closure assay to test collective cell migration (Fig. S4) and Boyden chamber assay to test single cell migration (Fig. S5) showed that *KRT19* KO cells migrate faster than parental cells, confirming that K19 negatively affects cell movement on the rigid substrate.

To see if lack of morphological difference on collagen substrate would exert an effect on cell motility, we plated cells either on a tissue culture polystyrene with or without collagen matrix (Fig. 5). Then, cells were recorded live for 24 h and movement of individual cells were traced (Videos S1–S4). Cell speed was affected by K19 status ($F=33$) and substrate ($F=38$; 2-factor ANOVA, $P<0.001$). Specifically, *KRT19* KO cells moved faster than parental cells on polystyrene (0.62 ± 0.03 $\mu\text{m}/\text{min}$ versus 0.36 ± 0.03 $\mu\text{m}/\text{min}$, $P<0.001$), an effect lost on collagen (0.35 ± 0.03 $\mu\text{m}/\text{min}$ versus 0.27 ± 0.02 $\mu\text{m}/\text{min}$, $P=0.22$). Therefore, these results show that increased motility of *KRT19* KO cells are substrate-dependent and suggests that inhibition of cell motility by K19 is associated with its regulation of cell morphology.

DISCUSSION

Although studies have shown the potential role of K19 as a regulator of signaling pathways for cell migration (21–23), the mechanics of how K19 affects cancer cell morphology at the cellular level remained unknown. Contributing factors no doubt include the heterogeneous expression of keratins in cancer, both in keratin type and expression level, and the context-dependent functions of keratins whereby the same keratin protein seemingly performs opposing functions under different settings (13,45). Moreover, unlike normal epithelia where cells within a specific tissue exhibit relatively uniform morphology with a similar molecular makeup, the heterogeneity of cancer cells and their varying phenotypes (46) also present major challenges in identifying the exact role of keratins in mechanics of cancer cells. Nevertheless, keratins play a major role in cell-to-cell and cell-to-substrate adhesion through desmosomes and hemidesmosomes, whose breakdown and remodeling are critical for the phenotypic plasticity and migration of cancer cells (21,22). Indeed, the expression of keratins is lost in cancer during EMT, as cells undergo a dramatic shift in morphology and migratory potential (12).

We have shown previously that MDA-MB-231 cells become more elongated on collagen substrates compared to glass (33). Biochemical and biomechanical properties of different extracellular matrices affect the interplay between cell adhesion and contractility to determine cancer cell plasticity and cell motility (47,48). In light of this, it is notable that *KRT19* KO cells failed to become more elongated on collagen compared to glass and the difference in eccentricity of parental and *KRT19* KO cells were abrogated on collagen (Fig. 3). Similarly, we observed increased cell motility of *KRT19* KO cells on a rigid substrate as indicated by the speed of cell movement (Fig. 5). An increase in stroma stiffness and reorganization of stromal network into thicker bundles correlate with an increased metastasis (49). Therefore, K19 may act as a part of the mechanosensory machinery that regulates morphology and inhibits motility until cells encounter a favorable extracellular environment to initiate metastasis. Still, as evident from opposing functions of different keratins on cell morphology and migration (12,13,50), different keratins may regulate cells' interaction with their microenvironment in different manners. This may be true especially for cancer cell types with varying strengths of cell-to-cell adhesions and/or keratin levels. On this subject, cell shape of keratin-null mouse keratinocytes differed from *wild type* more prominently on a soft substrate (51) and keratin null keratinocytes did not display altered organizations of actin stress fibers and microtubules on a rigid substrate (10,51). Also, keratin expressed in basal epithelial cells, K14, promotes breast cancer cells to collectively invade the surrounding microenvironment for metastasis in part using mechanical cues via collagen receptor DDR2 (14,52). Dissecting the exact mechanism underlying the regulatory function of keratin on cell morphology and motility and how substrate stiffness is involved in such process will need to be addressed in the future.

Interestingly, *KRT19* KO cells showed more motility than parental cells on a rigid substrate despite fewer focal adhesions and actin stress fibers. Although focal adhesions and associated stress fibers play important roles in mechanotransduction and cell migration, cell migration is a complex process with multiple types of cell migrations not involving focal adhesions (43,44). In fact, stable focal adhesions and stress fibers can inhibit cell motility due to the burden of reorganizing such structures (42). Indeed, a recent study in skin keratinocytes has shown that K6 inhibits keratinocyte migration by regulating cell–cell and cell–substrate adhesion (53). Similarly, K19 containing keratin filaments may stabilize mechanical elements to inhibit cell migration in conjunction with other cytoskeletal proteins (54–57) on a rigid substrate as evident from a loss of actin stress fibers and focal adhesion in *KRT19* KO cells. Nevertheless, parameters such as cell area were affected by the absence of K19 on both collagen and glass. As knockdown of K8 and K18 in rat hepatoma cells (58) and loss of all keratins in mouse keratinocytes (51) also showed decreased cell area independent of substrate stiffness, some changes observed in *KRT19* KO cells may be due to functions universal to keratins. In this regard, nonmechanical functions of keratin, which include modulating cell growth through Akt/mTOR signaling pathway, may also be involved (59). These observations illustrate that various factors including those intrinsic and extrinsic to cells are at play for keratins to regulate cell mechanics.

Substrate and K19 status affected the phase height profile and texture of MDA-MB-231 cells, suggestive of substrate–K19 interactions affecting intracellular structures as well as cell shape. We suggest that average phase difference maps clarify trends in phase height

from cells' central bulk while avoiding confusion due to variability in cell shape by skewing the periphery toward zero. Average difference maps revealed phase volumetrically wider, shorter parental than *KRT19* KO cells on glass but not on collagen (Fig. 4A), and opposite effects of volumetrically widening parental cells on glass, but volumetrically lengthening *KRT19* KO cells on glass (Fig. 4B). Consistent with shifts in cells' internal volume, *KRT19* KO cells lost widely-distributed actin fiber and microtubule networks, as well as anchoring focal adhesions (Figs. 2 and 3), underpinning the synergy of cytoskeletal elements in defining cells' shape and internal structure. Altered phase height profiles in the central bulk of cells and the clumped appearance of cytoskeletal elements from immunofluorescence micrographs reinforce trends in phase height texture of *KRT19* KO cells on glass. Higher contrast, but lower correlation, energy, and homogeneity (Fig. S2) are interpretable as less uniformity of adjacent pixel intensities in phase height maps of *KRT19* KO cells. These trends in phase height texture would be expected from clumped intracellular material not able to be effectively distributed by the disrupted, altered cytoskeleton. Such texture parameters, originally developed to assess features of satellite imagery (60), were also shown to contribute to cancer cell classification by machine learning (33,35), though more work is needed to determine the sensitivity of texture features to phase height contributions of specific subcellular structures. The pixel-level phase information from DHM also makes possible the tracking of phenotypic shifts along the epithelial–mesenchymal axis (61). The sensitivity of digital holographic microscopy to cells' geometric height and density-driven internal spatial fluctuations in refractive index, demonstrated here and elsewhere (27,62) lead to the utility of this imaging approach in assessing cell-wide phenotypic and morphological alterations.

Conclusions

Overall, our study provides the role of K19 on cancer cell morphology and motility using immunofluorescence microscopy, DHM and live cell microscopy. The mechanical function of keratins in epithelial cells is well established and keratins are highly expressed in cancer (8,45,63). However, factors such as heterogeneity and plasticity of cancer cells make studying the role of these epithelial markers in cancer challenging as mentioned above. Still, based on the accumulating evidence of the association between their altered expression and cancer metastasis (12), it is highly likely that these cytoskeleton proteins play a major role in regulating cancer cell morphology and motility. Our findings suggest a role for keratin in the regulation of cancer cell phenotype to inhibit motility in a substrate-dependent fashion. Therefore, this study motivates the similar study of additional keratins whose levels are deregulated in cancer and may provide an important clue about the effect of the altered molecular makeup on cancer cells migration. Quantitative assessment of cell phase maps from digital holographic microscopy are quite useful in defining specific morphological features associated with altered gene expression, both in the bulk phase volume as well as cell shape.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all members of the Chung and Raub labs for their support. We also thank Dr. Greg Miller of the Department of Chemistry and Drs. Pamela Tuma and Venigallo Rao of the Department of Biology at The Catholic University of America for sharing their instruments.

Grant sponsor:

National Institutes of Health, Grant numberR03EB28017, Grant numberR15CA2113071

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Figure 1.

Loss of K19 in MDA-MB-231 cells results in an elongated cell morphology. **(A)** Schematic depicting generation of *KRT19*KO cells. *KRT19* gene with its six exons (orange) and corresponding K19 protein domains (dark blue) are shown. K19 protein domains include a central rod domain (coil1A, linker 1 (L1), coil 1B, L12, and coil 2) of ~310 amino acids with α -helical conformation flanked by nonhelical head and tail domains. Region of *KRT19* (146–165 nt) targeted by gRNA to ablate *KRT19* expression using the CRISPR/Cas9 system is shown in red. **(B)** Whole cell lysates of parental (P) control and two different clones (KO1 and KO2) of *KRT19*KO cell lines were harvested, and immunoblotting was performed with antibodies against the indicated proteins. **(C)** qRT-PCR performed showing mRNA levels of K19 in indicated cells. * $P < 0.001$. Data from three experimental repeats normalized to the

parental control are shown as mean $\pm$ SEM. **(D)** Bright field images after crystal violet staining were taken, and representative images are shown. Bar, 100 μ m. **(E)** Difference in cell area determined using ImageJ software. Data from at least four experimental repeats are shown as mean $\pm$ SEM. Differences are not statistically significant unless denoted by * $P < 0.05$; ** $P < 1 \times 10^{-4}$. **(F)** *KRT19* KO cells stably expressing GFP vector or GFP-K19 are shown. Nuclei are shown in blue. Bar, 20 μ m.

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Figure 2.

Loss of stress fibers and focal adhesions in *KRT19* KO cells. Immunofluorescence images of F-actin and K19 or F-actin alone for parental (**A & B**) and KO1 (**C & D**) cells are shown. Nuclei are shown in blue and stress fibers are indicated by arrows. Bar, 20 μ m. (**E**) Number of cells showing stress fibers were counted using ImageJ software. Immunofluorescence images of vinculin (green) and K19 (red) or vinculin alone for parental (**F & G**) and KO1 (**H & I**) cells are shown. Nuclei are shown in blue and focal adhesions as marked by punctate vinculin staining are indicated by arrows. Bar, 20 μ m. (**J**) Number of cells showing focal adhesions were counted using imageJ software. Data from at least four experimental repeats are shown as mean $\pm$ SEM. * $P < 1 \times 10^{-5}$.

Figure 3.

Parental and *KRT19* KO cell morphology are different, modulated by type of adherent substrate. (A) Phase height maps (optical path length divided by an assumed index of refraction mismatch), d , of MDA-MB-231 cells were collected by digital holographic microscopy. Representative cells with the smallest sum of squared deviations (SSD) of 17 phase parameters from the group average were collected for (clockwise from top left) parental cells on glass and collagen hydrogels, and *KRT19* KO cells on collagen hydrogels and glass. Values of SSD for each phase map are indicated in the lower right of each map. A color bar indicating height and scale bar (20 μm) is shown. Average (B) eccentricity and (C) projected area of both parental and *KRT19* KO on both substrates. Error bars represent standard deviations. Horizontal bars with asterisks (*) represent statistically significant differences by ANOVA and post hoc Tukey tests ($P < 0.05$).

Figure 4.

Average phase difference maps for four comparisons between groups of cells. (A) The K19 status and (B) adherent substrate are listed in the average phase height maps. Bar, 10 μm . Average phase difference map profiles from (C) top (leading edge, LE) to bottom (trailing edge, TE), and (D) left to right through the centroid are presented. Profile maps correspond to average phase and phase difference maps from the same row, with 0 representing the phase difference map centroid horizontal and vertical location.

Figure 5.

K19-dependent inhibition of cell motility depends on substrate stiffness. Cell motility of P and KRT19 KO cells cultured on tissue treated polystyrene (PS) dish with or without 4 mg/ml collagen. Average speed (µm/min) of individual cells was measured using the ImageJ software. Data from three experimental repeats are shown as mean $\pm$ SEM. Differences are not statistically significant unless denoted by * $P < 0.05$.