Novel Image Analysis to Link Sub-Nuclear Distribution of Proteins with Cell Phenotype in Breast Neoplasia

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Abstract

The hypothesis driving this work is that the organization of nuclear proteins is intimately linked to the phenotype of cells and tissues. When cells arrest proliferation, undergo apoptosis or differentiate, the distribution of their nuclear proteins changes. Conversely, forced alteration of the distribution of nuclear proteins results in modified cell phenotype.

To quantitatively assess how nuclear organization is linked to mammary phenotype, we have developed imaging methods to measure the distribution of the fluorescently-stained nuclear mitotic apparatus (NuMA) protein in different phenotypes obtained using three-dimensional culture of human mammary epithelial cells. Automated image segmentation of DNA-stained nuclei was developed to isolate thousands of nuclei from three-dimensional confocal images. Prominent features of fluorescently-stained NuMA within each nucleus were detected using a novel local bright feature (LBF) analysis technique. The normalized spatial density of these bright features was then calculated as a function of the distance from the nuclear perimeter to its center. This radial distribution, termed the radial-LBF feature, was then calculated on a per nucleus basis. The results revealed marked changes in the distribution of the density of NuMA bright features as nonneoplastic cells underwent phenotypically normal acinar morphogenesis. In contrast, no reorganization of NuMA was detected during the formation of tumor nodules by malignant cells. This analysis also discriminated between proliferating non-neoplastic and proliferating malignant cells, suggesting that this method is capable of identifying alterations linked not only to the proliferation status but also to the malignant character of cells.

Based on these results, we have developed a spectral clustering analysis method that groups similar nuclei based on their NuMA radial-LBF features. We have applied these techniques to subdivide 2673 nuclei of nonneoplastic cells during the 10 day time course of acinar morphogenesis into four clusters. The results are very exciting because they indicate a clear correlation between the distribution of NuMA in the resulting clusters and the phenotype of the cells which corresponds to stepwise stages in the morphogenesis process (i.e., proliferation, growth-arrest, or full differentiation with complete polarity axis). Experiments are currently being expanded to include neoplastic cells in the cluster analysis and to apply the LBF analysis techniques to biopsy samples.

This work has furthered our basic understanding of alterations characteristic of malignant transformation by quantifying nuclear protein distribution. The fully automated image analysis techniques developed have defined molecular signatures of nonneoplastic and malignant breast phenotypes that have broad applications in molecular targeting, cancer imaging, screening and diagnosis of early stages of breast cancer.

Introduction

The HMT-3522 cancer progression series of human mammary epithelial cells (HMECs), cultured in 3-D laminin-rich extracellular matrix, constitutes a physiologically relevant model for studying the relationship between cellular organization and gene expression in normal and malignant cells (1,2-4,6). In such cultures, non-neoplastic HMT-3522 S1 HMECs (5) reproduce the formation of phenotypically normal, tissue-like glandular structures referred to as acini (6). Acinar morphogenesis proceeds by stepwise events including a proliferation stage from days 1 to 6 of culture, followed by growth arrest and the formation of the baso-apical polarity axis. Upon completion of acinar morphogenesis at day 10, S1 cells are organized into spherical and hollow structures delineated by a basement membrane at their basal pole, and a lumen at their apical pole. On the other hand, malignant T4-2 cells, which were derived from S1 cells (7), continue to proliferate, and form disorganized and invasive tumor-like nodules under the same culture conditions (8). The distribution of nuclear proteins including retinoblastoma protein Rb, splicing factor SRm160, and nuclear mitotic apparatus (NuMA) protein is remarkably different between S1 cells in the early stage of acinar morphogenesis and S1 cells in fully formed acini (1). NuMA is diffusely distributed within the nuclei of proliferating cells, but aggregates into foci of increasing size as cells arrest proliferation and complete acinar morphogenesis. Importantly, the distribution of NuMA in acinar S1 cells is similar to that observed in biopsies of normal breast tissue, indicating that the 3-D model of acini formation reproduces physiologically relevant features of NuMA organization.

Here, we report the use of confocal imaging to record the changes in the pattern of NuMA staining in HMECs expressing different phenotypes, and the development of an image analysis technique, the radial local bright feature (radial-LBF) analysis, to translate the visual observations of the complexity of NuMA staining into quantitative results. In this method, regions of local brightness in images of fluorescently-immunolabeled NuMA are isolated by an adaptive local bright feature technique (LBF analysis). The density of local bright features is then calculated within a set of concentric, volumetric terraces that subdivide the nucleus radially from its periphery to its center. The distribution of the bright features of NuMA can be represented by a simple graph, which permits an easy quantification of the changes in the spatial organization of this protein associated with different mammary phenotypes. The method relies on the delineation of individual nuclei, and in order to analyze thousands of nuclei in a short period of time, we have also developed a novel automated, three-dimensional segmentation technique. Using the radial-LBF analysis, we measured a striking reorganization of NuMA during acinar morphogenesis, while no such reorganization occurred during the formation of tumor-like nodules. Importantly, the radial-LBF analysis of NuMA distribution also permitted a clear discrimination also between proliferating non-neoplastic cells and proliferating malignant cells, which had not been achieved so far using other evaluation methods.

Methods

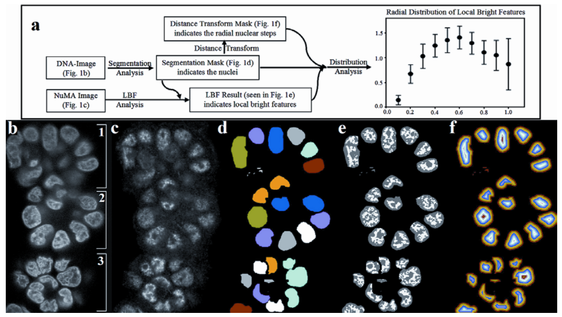


Figure 1.

Local Bright Feature (LBF) analysis of NuMA distribution from three-dimensional images. a. Flowchart of the imaging processing steps including a graph of the relative density of local bright features of NuMA in 77 nuclei from the three acini depicted in Fig 1b. The radial distributions of local bright features within each nucleus in the NuMA image are calculated by first generating a segmentation mask from the image of DAPI-stained DNA. The segmentation mask not only defines the extent of each nucleus, but it is also used to define a set of radial steps within each nuclear volume. The graph shows multi-overlay plots representing the relative density of NuMA bright features extracted by LBF analysis (ordinate) as a function of the relative distance from the perimeter (0.0) to the center (1.0) of the nuclei (abscissa). b-h.

Distribution density of the bright features of NuMA in acinar cells. HMT-3522 S1 HMECs were cultured in 3-D to induce acinar morphogenesis. Each panel corresponds to the application of the different steps of distribution analysis starting from the same original image. **b.** Fluorescence micrograph of DAPI-stained nuclei from a single optical section containing three acini [1;2;3]. **c.** Fluorescence micrograph of Texas-red immunolabeled NuMA from the optical section corresponding to the DAPI image shown in a. **d.** Segmentation mask derived from the DAPI-stained image showing a single slice of individually enumerated nuclei. **e.** Composite view of the local bright features (light gray) extracted by the LBF analysis overlaid with the segmentation mask (dark gray). **f.** Concentric terraces resulting from the application of the distance transform on the segmentation mask.

Results

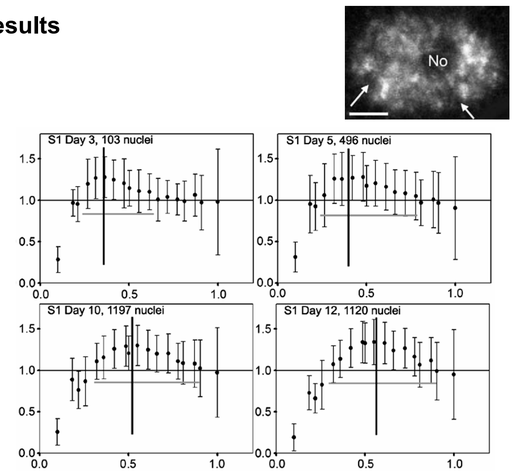
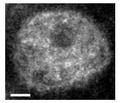


Figure 2.

Average relative density of local bright features of NuMA during acinar morphogenesis.
S1 cells were cultured in 3-D for 3, 5, 10 and 12 days. Plots represent the relative density of NuMA bright features extracted by LBF analysis (ordinate) of a population of nuclei as a function of the relative distance from the perimeter (0.0) to the center (1.0) of the nuclei (abscissa) for each time point. Vertical lines (black) represent the location of the peak of bright feature density in the nucleus. Horizontal lines (gray) represent the extent of nuclear volume with densities of bright features above the average. The number of days the cells were in culture and the number of nuclei analyzed are indicated above each corresponding graph. Bars represent the standard deviation. The micrograph at the top of the figure shows the typical NuMA organization in a single nonneoplastic S1 nucleus from an acinar.



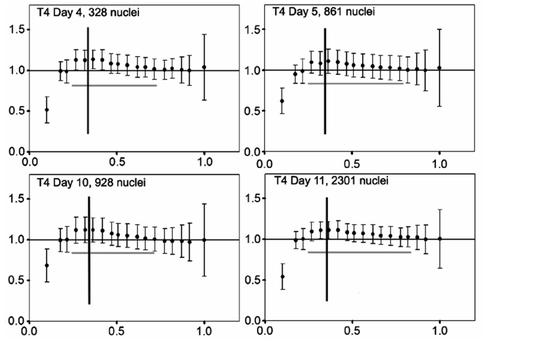


Figure 3.

Differences in the relative density of NuMA bright features between nonneoplastic and malignant cells.
T4-2 cells were cultured in 3-D for 4, 5, 10 and 11 days. Plots represent the relative density of NuMA bright features extracted by LBF analysis (ordinate) of a population of nuclei as a function of the relative distance from the perimeter (0.0) to the center (1.0) of the nuclei (abscissa) for each time point. Vertical lines (black) represent the location of the peak of bright feature density in the nucleus. Horizontal lines (gray) represent the extent of nuclear volume with densities of bright features above the average. The number of days the cells were in culture and the number of nuclei analyzed are indicated above each corresponding graph. Bars represent the standard deviation.
The micrograph at the top of the figure shows the typical NuMA organization in a single malignant T4 nucleus.

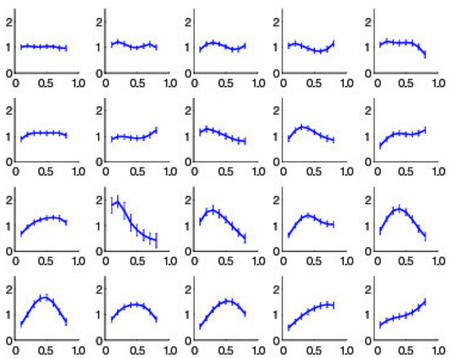


Figure 4.

Clustering analysis of the radial distribution of NuMA in nonneoplastic S1 and malignant T4 cells.
Our goal is to use the NuMA distribution results and create a statistical analysis technique capable of predicting the phenotype of individual cells based on their NuMA organization. The radial distribution of NuMA can be thought of as a "feature" of the nucleus. We have used cluster analysis to group the radial distribution of NuMA from 2673 nonneoplastic S1 nuclei and 4418 malignant T4 nuclei into 20 clusters. Figure 4 shows the means and standard deviation of the nuclear distribution or each of the 20 cluster, which have been arbitrarily numbered and ordered. The cluster number, not shown, is read from left to right, top to bottom.

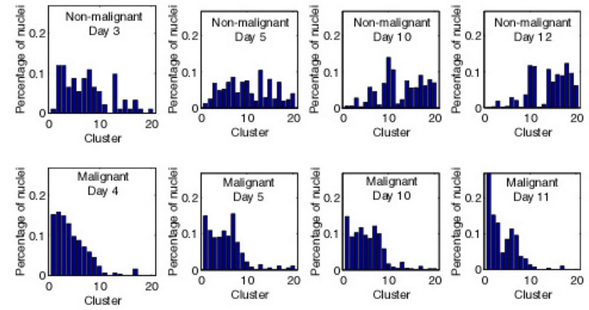


Figure 5.

Cluster histograms relate the nuclei in the 20 clusters to their known phenotype and generate a unique relational signature for each phenotype from which we can predict the phenotype of nuclei based on their NuMA organization.

Figure 5 shows the probability histogram of the number of nuclei of a particular phenotype as a function of the cluster number (1-20) for each of the 20 clusters. Each of the 8 possible phenotypes is written on the histograms. The histograms show that for nonneoplastic S1 cells, the radial distribution of NuMA favors clusters numbered less than 10 at Day 3 while favoring clusters numbered greater than 10 at day 12. Conversely, this histograms show that all malignant cells favor clustered numbered less than 10.

The phenotype of subsequent nuclei was then predicted by considering the NuMA organization of the nuclei in question along with its nearest neighbours. By using this scheme, we were able to predict the phenotype, the number of days in culture, of nonmalignant S1 cells with an accuracy of 74+-2%. We were able to predict if nuclei were nonmalignant or malignant with an accuracy of 95+-3%.

Conclusions

We have developed an image-based analysis to automatically quantify the nuclear distribution of proteins within individual cells, from 3D fluorescence images.

We have confirmed initial visual observations that reported nuclear reorganization of NuMA in cultured human mammary epithelial cells during acinar morphogenesis.

We report that NuMA organization is measurably different in malignant epithelial cells from nonneoplastic epithelial cells.

Using cluster analysis we can predict the phenotype of individual nuclei based on their measured NuMA distribution with high accuracy.

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