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Research Article

Quantitative Analysis of Anisotropy of Mammary Ductal Epithelial Cells of Buffalo Mammary Gland

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Abstract

Growth of mammary gland is physical process and the physical parameters like coordination between individual cells decide growth characteristics of tissue. Here we described a method for automated measuring of average orientation and anisotropy of mammary epithelial cells (MEC) in tissue sections. We quantitatively analyzed anisotropy of MEC of terminal ends of mammary ducts in buffalo mammary glands. Images of histological sections from mammary cancer, non-lactating and prepubertal mammary glands of water buffalo were processed usingImageJ- image analysis software. Quantitative analysis of orientation angle and anisotropy of MEC was performed using Fibril Tool- a plug-in of Image J. Results indicated that although the orientation angles (ranges between -90 to +90) of MEC of growing end of mammary ducts are similar among mammary cancer, non-lactating and prepubertal glands, but anisotropy (value ranges from 0 to 1) of MEC varies between mammary cancer and prepubertal tissue (0.12 + 0.01 vs. 0.17 + 0.01; P = 0.007) indicating MEC in mammary cancer are least ordered (17.27% lower) than the prepubertal tissue. In other words, mammary cancer-have poor organization pattern of MEC for directional growth. This study provides a way to quantify average orientation and anisotropy of MEC in raw histological images and provided an *in situ* method of quantitative analysis of cell orientation for the growth.

Keywords: *Water buffalo; Mammary epithelial cell; Anisotropy; Orientation angle*

Abbreviations: MEC: Mammary epithelial cell; RGB: Red, green and blue; TDU: Terminal ductal unit; ROI: Region of interest; FNDC3B: Fibronectin type III domain containing 3B

Introduction

Disruption of cytoskeletal organization is the hallmark features of cancer cells. It affects mechanical properties of the cell by altering cell structure, orientation and ultimately their growth kinetics. Quantifying fibrillar orientation and anisotropy (orderly arrangements of cytoskeletal filaments) of cell might help in understanding key questions how cell changes their shape during active growth of vessels and mammary ducts in normal and cancerous tissues.

Automated methods for assessing tissue orientation have been developed for image processing for textural pattern to make use of image processing algorithm [1-3]. Further advent in this technology led to measurement of orientation in microscopic image in measuring disarray of myocardial tissue [4]. A recent development in imaging techniques and image analysis tools like Fibril Tool [5] which quantify orientation and anisotropy of fibrillary structures in raw images. Fibril Tool utilizes nematic tensor to quantify orientation of cytoskeletal filaments and provide a quantitative output of how well the fibers are aligned. Fibril Tool is plug-in of ImageJ that utilizes circular statistics to quantitate orientation angle and anisotropy. Orientation angle is calculated as circular average of tangent direction over the region of interest (ROI), and anisotropy score is calculated as circular variance of tangent direction. Anisotropy scores ranges from 0 for no order (isotropic array of cytoskeletal filaments) to 1 for perfectly ordered (parallel array of filaments).

Here we quantify cellular orientation and anisotropy of mammary epithelial cells (MEC) of mammary ducts from cancerous and normal tissue (non-lactating and prepubertal) of water buffalo. Fibril Tool is appropriate for intensity signal in histological sections (email communication with Dr. Arezki Boudaoud, inventor of Fibril Tool) to quantify fiber orientation within the cell, keeping in mind that ROI of tissue sections should be equivalent between thetypes of tissue compared. Fibril Tool is a robust method of image analysis and requires no image-preprocessing, sources of image (bright field, fluorescent, electron microscopic etc.). Raw images can also be used either in RBG or grey scale as the analysis only depends on nematic tensor computed on the basis of pixel intensity of the image [5].

Materials and Methods

Tissue collection

Mammary parenchyma from buffalo mammary glands (N = 15) were obtained from achieved samples. In brief, mammary tissues were collected from buffalo slaughter house and were fixed in 10% neutral buffer formalin overnight. The next day, tissues were processed as per standard procedure and dehydrated formalin fixed tissues were embedded in molten paraffin, A 4-5 µm think tissue sections were mounted on positively charged glass slides (Globe Scientific). Histomorphology of mammary tissue were evaluated using hematoxylin and eosin staining. A flow diagram explaining the whole procedure has been given (Figure 1). As animal tissues were collected from slaughter house, it required no Institutional ethical committee permission. However, permission to carry out such studies, have been obtained from the Head of the Institution.

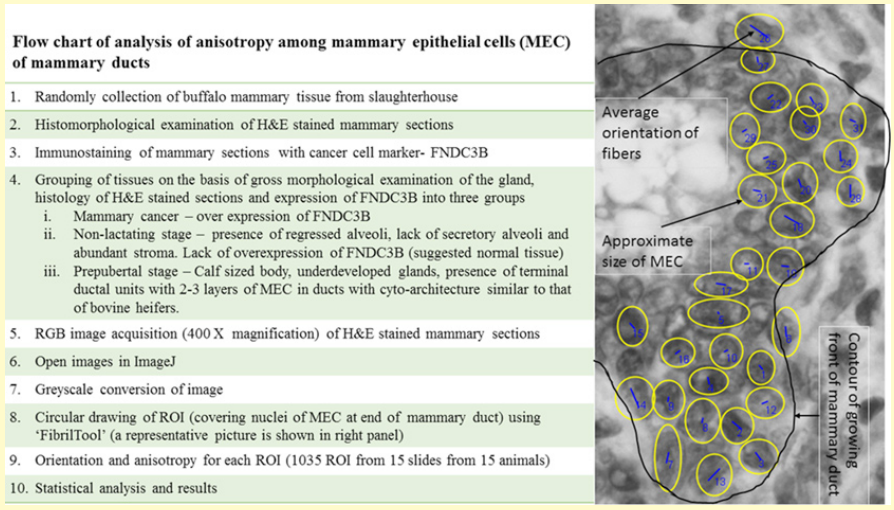

Figure 1: *Flow chart of the methodology for analyzing orientation and anisotropy of mammary epithelial cells with sample test image showing ROI and average anisotropy of each ROI.*

Image acquisition and anisotropy quantification of MEC

Greyscale image of mammary parenchymal tissue sections were obtained using Nikon 80i microscope with 40X objective (final magnification 400X) fitted with camera that were connected to computer. Images were acquired in *tiff format and converted with 8-bit greyscale resolution using Image J ver. 1.49 [5,6]. From 15 different animals (5 animals in each group), 1035 MEC (432 from cancer, 274 from non-lactating and 329 from prepubertal glands) from end of terminal ductal unit (TDU) were selected for analysis using Fibril Tool [5]. Selected MEC were analyzed for orientation angle and anisotropy and the data were saved in excel file for further analysis. Briefly, ImageJ were opened with double click of ImageJ icon and selected images were opened. RGB images were converted into 8-bit greyscale. From the grey scale image, MEC of TDU was selected as RIO. Sign '>>' from ImageJ toolbar were clicked to fetch 'stripped' Tool symbol. Since, it was a greyscale image, therefore for detection of fibril signal, default channel was selected as either red or blue or green

channel will produce the same results. Channel for drawing kept 'yes' as per default value that corresponds to the lines that is drawn automatically to represent average orientation and anisotropy of the fiber arrays. MECs were selected as ROI (yellow circular outline, Figure 1 right panel) and the areas were drawn using circular tool over the nuclei, avoiding cell periphery. A 1035 ROI (or MEC) were drawn manually each with average orientation (a blue color line segment in ROI, Figure 1; right panel) and anisotropy (proportional to the length of blue line segment. The data were exported in excel as output file containing file name, ROI number, x- and y-coordinate of ROI, average fiber orientation (value between -90 to +90) and anisotropy (a score between 0 to 1). A threshold of anisotropy 0.05, above which the data points were considered for analysis. Average orientation angle and anisotropy were calculated from five animals in each group and were analyzed.

Results and Discussion

Evaluation of normal/physiological stages of mammary tissue

Suspected cases of mammary cancer (n = 5) at the time of collectionwere not distinguished from non-lactating gland. It was evident after observation of histological sections by the presence of solidification of mammary ducts (arrows) (Figure 2A) and overexpression of FNDC3B, a marker of cancer cells [7,8], in the epithelial compartments (Figure 2B). Cyto-architecture of non-lactating mammary glands (n = 5) revealed no to minimal milk secretions in gross while collecting mammary tissue. Presence of regressed or poorly developed alveoli in histological sections marked the non-lactating stage. Expression of FNDC3B appeared to be in nuclear (arrow) and cytoplasmic compartment (arrow head) of MEC (Figure 2C). FNDC3B immunostaining of tissue was performed to checkits expression is normal or abnormal (overexpression). Prepubertal (4-6 months old; n= 5) buffalo mammary glands, in gross, had undeveloped glands with less parenchymal tissue and prominent fat pad. Histologically, prepubertal buffalo mammary glands revealed terminal ductal unit (TDU) as the distal tubular branching of mammary ducts having 2-3 layers of mammary epithelium (Figure 2D). Cyto architecture of prepubertal mammary gland wassimilar to that of heifers mammary glands as reported in the literature [9,10].

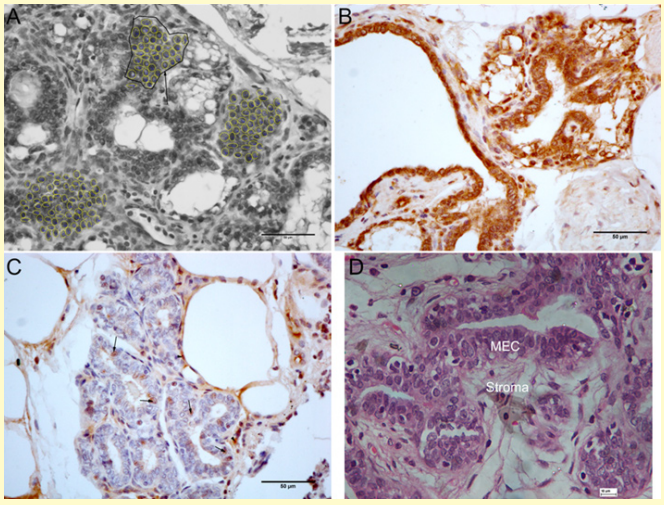

Figure 2: *Different types of buffalo mammary tissue used for the analysis of orientation angle and anisotropy. A- Representative Image of cancer tissue showing solidification of ducts (black irregular circle indicated by arrow) in grey scale. RGB and grey scale images give similar results. B- Over expression of FNDC4B, a cancer cell marker, in epithelial layers confirmed our idea that the observed mammary tissue is cancerous. C- Expression of FNDC3B in non-lactating mammary gland was normal and localized in the nuclei or cytoplasm of mammary epithelial cells. D- Prepubertal mammary gland showing cyto-organization of MEC (2-3 layers of cells) in terminal ductal unit. Scale bar A, B, C – 50 µm, D – 10 µm.*

Quantification of anisotropy and orientation of MEC

Distribution of growth anisotropy of MEC of mammary ducts of cancer, non-lactating and prepubertal glands follows the similar trend. The frequency of MEC with anisotropy value 0.2, remained highest in all three groups, namely cancer (45.0%), non-lactating (43.4%) and prepubertal glands (49.5%) (Figure 3A). Overall, anisotropy of MEC in cancer (0.12 + 0.01) decreased (P = 0.0007) in comparison to anisotropy of MEC in prepubertal glands (0.17 + 0.01) (Figure 3B). In contrast, no clear difference in terms of growth anisotropy could be observed in mammary ducts of non-lactating (0.14 + 0.02) and prepubertal glands. Mean orientation of each MEC (ROI) in each group of animals were calculated. Fibers orientation angles were measured within the range of -90 < 0 < + 90 and frequency of orientation angles in cancer, non-lactating and prepubertal mammary glands are presented in histogram (Figure 3C).

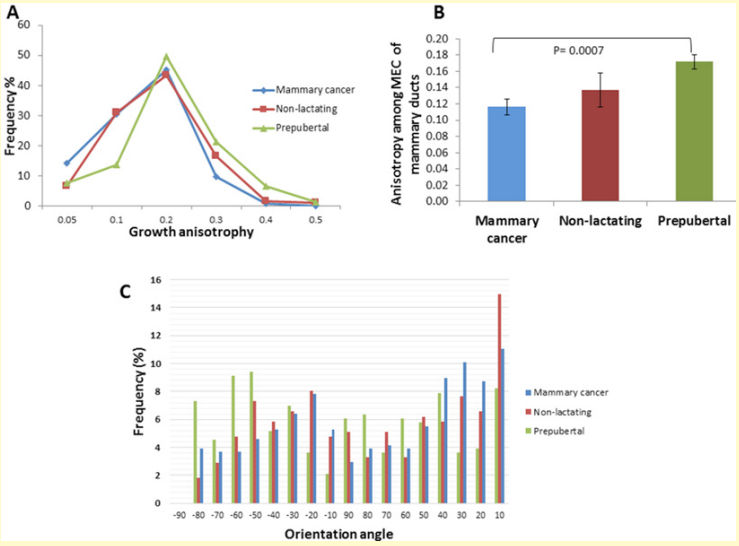

Figure 3: *Quantification of anisotropy and average orientation of MEC in mammary ducts of buffalo mammary glands. A - Frequency distribution of growth anisotropy of MEC in mammary cancer, non-lactating and prepubertal mammary glands. The maximum anisotropy score observed in MEC were close to 0.5 and frequency of anisotropy score 0.2 remained highest in all three groups. B- Average anisotropy of MEC located at the growing end of cancer tissue was lower (P = 0.007) than the anisotropy of MEC of prepubertal tissue. C – Histogram of frequency distribution of mean orientation angles of MEC in cancer (blue bars), non-lactating (red bars) and prepubertal (green bars) glands.*

We presented a method of automated measurement of fiber anisotropy and orientation in mammary ducts using Fibril Tool, a plug-in of image J- image analysis software. This method makes measurement of large number of MEC among the neighboring cells. Shape and size of MEC can be decided by the performer using various shaped tools to draw boundaries of MEC. Some areas of mammary tissues might contain large variation in fiber orientation and anisotropy like where mammary parenchyma makes gland cistern. We have collected mammary tissues from mid region of mammary parenchyma, we make no assumptions about the origin of tissue collected, and therefore variation in anisotropy of MEC located at the end of mammary ducts in cancer tissue is different from the prepubertal tissue and it is because of the disease condition. Although, the presence of disarray or variation in anisotropy alone is insufficient to determine if the tissue is from the cancer or prepubertal animals, we tested suspected cases of mammary cancer sample with cancer cell marker-FNDC3B. Over expression of FNDC3B in epithelial compartment confirmed such tissues of cancer in origin. Difference in anisotropy of mammary duct did not differ between cancer and non-lactating group and prepubertal and non-lactating group. The only difference in anisotropy was observed in cancer and prepubertal group. The reasons could be the differences in growth pattern of the

mammary ducts in prepubertal and cancerous glands. In prepubertal glands, mammary ducts are ramifying its branches in fat pad and growths of cells are faster. Likewise, when tissues turns out to be cancerous, growth of cells become faster. In non-lactating glands, regeneration of glands occurs instead active growth glandular tissue. Therefore, in non-lactating glands, growing ends of mammary ducts have limited growth.

Defects in cyto-architecture of cancer mammary gland

The boundaries between basal/myoepithelial (MyC) and luminal epithelial cells have been drawn between cancer tissue and prepubertal tissue to show that in cancer, orientation of plane of cell division is disturbed and cells are pushed towards apical side (yellow arrow) to fill the available space (lumen) and turned into solid structure (Figure 4A). In prepubertal mammary tissue, there were abundance of MyC at the basal layer of epithelium (Figure 4B, black arrows) and 1-2 layers of epithelial (either luminal or luminal and embedded) cells were seen with lumen inside the duct.

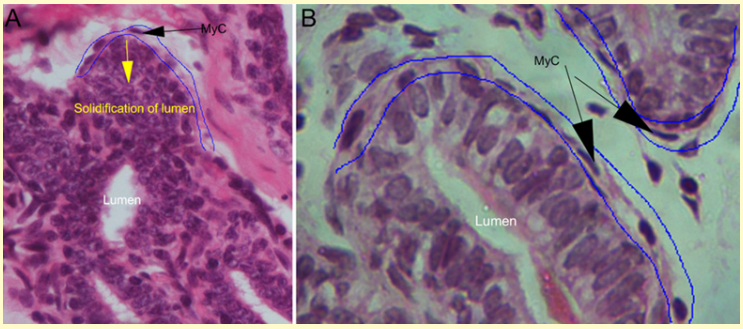

Figure 4: *Defects in cyto-architecture of mammary tissue in cancerous stage in comparison to prepubertal stage. Solidification of lumen and occurrence of more layers of polymorphic types of epithelial cells at the growing end of mammary duct (A) was the hallmark feature of mammary cancer. In prepubertal gland (B), presence of basal/myoepithelial cells and 1-2 layers of epithelial cells (mainly columnar type) were seen. Representative images were captured at 400X magnification and then regions of interest are cropped for vivid presentation.*

Normal cyto architecture of the prepubertal buffalo mammary gland is similar to that of bovine heifers. Prepubertal buffalo mammary gland consists of mammary ducts with distal ductular branching. In bovine, distal branching of mammary ducts has been referred as 'terminal ductal unit (TDU)' [11]. Similar to bovine mammary gland, we referred TDU as the the distal branching mammary duct in prepubertal buffalo. TDU consisted of single layer of basal/myoepithelial (MyC) cells and inner layer of luminal epithelial cells. Presence of 1-3 layers of epithelial cells are sandwiched between basal and luminal layer called embedded epithelial cells. In contrast, non-lactating buffalo mammary glands have one layer of MyC and inner layer of luminal epithelium.

We observed loss of MyC in mammary cancer (black arrow, spindle shaped nucleus). Loss of myoepithelial cells has been reported in the many breast carcinomas [12,13], as myoepithelial cells are considered as the guardian of the cell.

Conclusion

In this study, we were able to quantify the heterogeneity of fiber orientation and isotropy among neighboring MEC of mammary duct in mammary cancer, non-lactating and prepubertal mammary glands. We demonstrated that MEC at the growing end of mammary ducts in mammary cancer are less perfectlyordered (17.24% lower) than the mammary ducts of prepubertal glands. In prepubertal glands, MEC have better orientation of fibers for organized and directional growth of mammary ducts. This is the first report on quantification of fiberanisotropy and orientation of MEC in-situ among neighboring cells.

Conflict of Interest

Declare if any financial interest or any conflict of interest exists.

Acknowledgments

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