Morphometric and Fractal Dimension Analysis Identifies Early Neoplastic Changes in Mammary Epithelium of MMTV-cNeu Mice

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Abstract. *Fractal dimension has emerged as a clinically useful tool in the diagnosis and management of breast cancer. The aim of the present study was to determine if fractal dimension can be applied for the analysis of a pre-clinical breast cancer mouse model, MMTV-cNeu. Using fractal dimension in conjunction with conventional morphometric measurements, the ductal epithelial networks of pubertal-stage MMTV-cNeu mice were quantitatively compared with those of wild-type mice. Significant alterations in ductal epithelial network growth and organization were detected during early neoplasia in MMTV-cNeu mice. Moreover, the left-side networks were significantly more affected relative to their wild-type counterparts than were the right-side networks, a finding that is consistent with elevated left-side tumor incidence reported for breast cancer patients. Taken together these results demonstrate that combined fractal dimension and morphometric analysis is an objective and sensitive approach to quantitatively identify ductal epithelial aberrancies that precede overt mammary carcinoma formation.*

Breast cancer is the most common type of cancer that occurs in women and is the second leading cause of women's cancer-related deaths (1). Diagnosis, prognostication, and therapeutic decisions in the management of breast cancer are guided by disease staging and other criteria, including hormone receptor expression, Her-2/Neu amplification, and histological tumor type (2, 3). While incorporation of these parameters has been useful in identifying patients who stand to benefit from targeted biological and endocrine therapies, the utility of histological tumor grading in assessing chemotherapeutic benefit has been shown to be relatively less predictive, in part due to its semi-quantitative nature (4). In an attempt to overcome this limitation, fractal dimension analysis has emerged as an alternative approach to assess tumor morphology for breast and other cancer types (5, 6).

Fractal dimension is a quantitative tool for objective measurement of complex structures that cannot be readily-described and quantified by application of Euclidian geometry. The ductal epithelial network of the mammary gland, the site where breast tumors originate, can be considered a fractal object and its topological dimension, or fractal dimension (D), is expressed by a non-integer number lying between the Euclidian integers 1 and 2 for a two-dimensional object. Computation of the fractal dimension allows for quantification of the complexity, or chaos, and space-filling properties associated with the structure of interest, *i.e.* the ductal epithelium. The greater the value of the fractal dimension of the object, the greater is its irregularity and complexity (chaos).

Fractal analysis has been applied to delineating the growth and complex architecture associated with a variety of tumors (7), including breast ductal carcinomas in images generated by optical coherence tomography (8), mammography (9, 10), magnetic resonance (11, 12), needle biopsy smears (13, 14) and histological methods (4, 15). It moreover has been used to distinguish benign from malignant tissues in resected specimens from breast-conserving surgeries (16, 17). Increased fractal dimension is significantly associated with

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Key Words: Mammary gland, MMTV-cNeu, fractal dimension, left-right asymmetry, neoplasia.

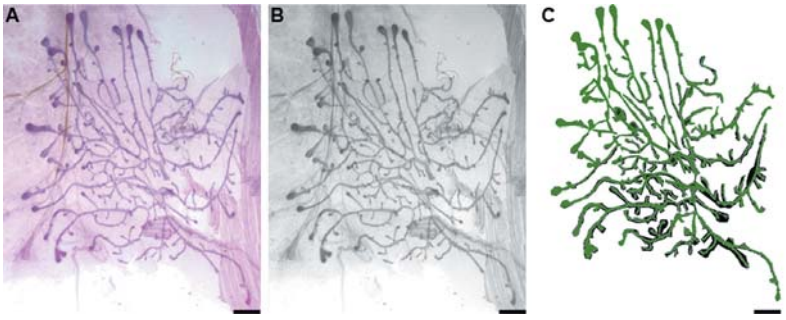

Figure 1. *Example of 8-bit monochrome image conversion. (A) A composite image of a carmine red-stained whole mount prepared from wild-type (WT) mouse TMG. (B) 8-bit gray scale image of TMG. (C) Manually traced and isolated TMG used for analysis with MetaMorph® image analysis software (in all figures scale bar=1 mm).*

higher tumor grade (*i.e.* loss of differentiated structure), larger tumor size, and positive lymph node status, all of which are indicators of a more aggressive disease (15, 18). Consistent with this, increased fractal dimension has been also shown to be significantly associated with lower disease-specific and overall survival of breast cancer patients (4).

Given the clinical utility of fractal dimension, we have investigated whether fractal analysis can be applied in morphological assessments in pre-clinical breast cancer mouse models. For the present study we conducted fractal and conventional morphometric analysis in a widely used breast cancer mouse model, MMTV-cNeu. MMTV-cNeu$^{Tg/+}$ mice overexpress the *ErbB2/Neu* oncogene, which-models Her-2+ breast cancer, and develop mammary tumors relatively rapidly, *i.e.* by approximately four months of age (19, 20). Using combined fractal dimension and morphometric analyses, we found that this approach detected quantitative changes in mammary ductal epithelial growth and complexity that preceded overt tumor formation. Moreover, when analyzed independently, our results showed that left-side mammary glands were more labile to oncogene-driven changes in ductal morphology compared to right-side glands, a difference that is consistent with elevated left-side tumor incidence that occurs in breast cancer patients (21). Together, these results indicate that fractal dimension analysis can be applied in conjunction with conventional morphometric measurements in a murine breast cancer model to quantify changes in the ductal epithelium that occur during early neoplasia. This combined methodological approach is highly sensitive and has provided the first documentation that lateralized morphological alterations initiate early in the neoplastic process.

Materials and Methods

Mice. All experiments were performed in accordance with the regulations of the Medical University of South Carolina Institutional Animal Care and Use Committee. FVB/N wild-type and FVB/N-TgN (MMTVNeu) 202Mul) mice were obtained from Taconic (Germantown, NY, USA) and JAX® Mice and Services (Bar Harbor, ME, USA). Wild-type and single-copy MMTV-cNeu$^{Tg/+}$ mice were used for all experiments and fed Harlan Teklad rodent diet 2918 and provided water *ad libitum*.

Histology and image collection. Carmine red stained whole mounts (22) prepared from #3 and #8 thoracic mammary glands of day-28 mice (21) were imaged on an Olympus SZX12 stereomicroscope equipped with a Spot camera. Overlapping images of each whole mount were processed into a single composite image with Adobe Photoshop®.

Image analysis. The color images of the mammary glands were converted to 8-bit monochrome images for image and fractal analysis. The mammary gland within an image was outlined and isolated from the background tissue and defined as a Region of Interest (ROI) (Figure 1). The isolated image of the mammary gland was thresholded using the set threshold sub-routine of MetaMorph Image analysis software (ver. 6.1). The area (A) and integrated optical density (IOD) of the ductal epithelial networks were measured using the integrated morphometry analysis sub-routine of MetaMorph. The fractal dimension (D), was determined by the box counting method using HarFA software (23) [http://www.fch.vutbr.cz/lectures/imagesci]) applied to the isolated image of the mammary gland using the same threshold values.

Integrated optical density (IOD). The IOD of the mammary gland ROI delineated by the thresholded boundaries is considered to be the "mass" of the ROI and a measurement of the total amount of labeled material in the region (24-28). The IOD of a selected region can be expressed as the weighted sum of the image histogram in which each term in the histogram is multiplied by the gray value it represents. When applied to thresholded boundaries the IOD is defined by the following expression:

$$\mathrm{IOD}(T1, T2) = \sum_{GV=T1}^{T2} H(GV) \times GV$$

Where the upper and lower thresholds defining the ROI in the histogram are given by T1 and T2. GV is the gray value of each pixel and H (GV) is the gray level histogram.

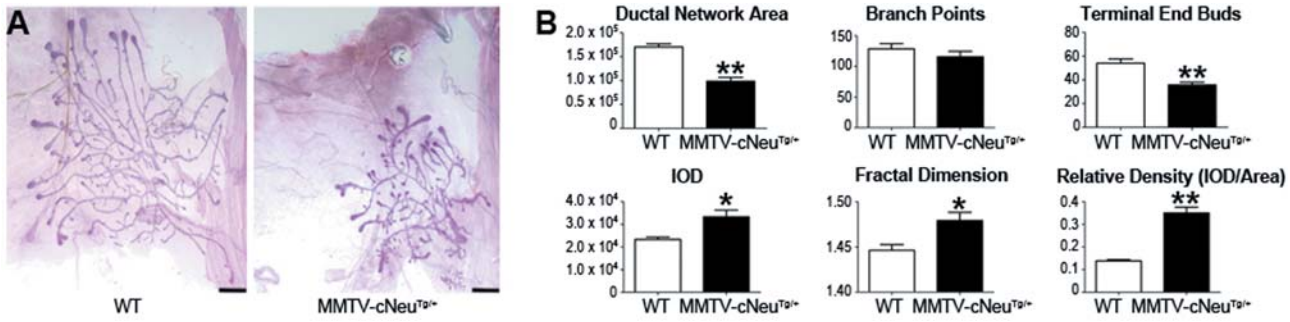

Figure 2. *Morphometric and fractal analysis of ductal networks of wild-type versus MMTV-cNeu mice. (A) Representative images of carmine red-stained TMGs from wild-type (WT) and MMTV-cNeu$^{Tg/+}$ mice. (B) Morphometric analysis of #3/8 TMGs of WT versus MMTV-cNeu$^{Tg/+}$ mice. Bars are representative of mean±SEM (WT n=16 MMTV-cNeu$^{Tg/+}$ n=26). Unpaired Student's t-test, *p<0.001, **p<0.0001.*

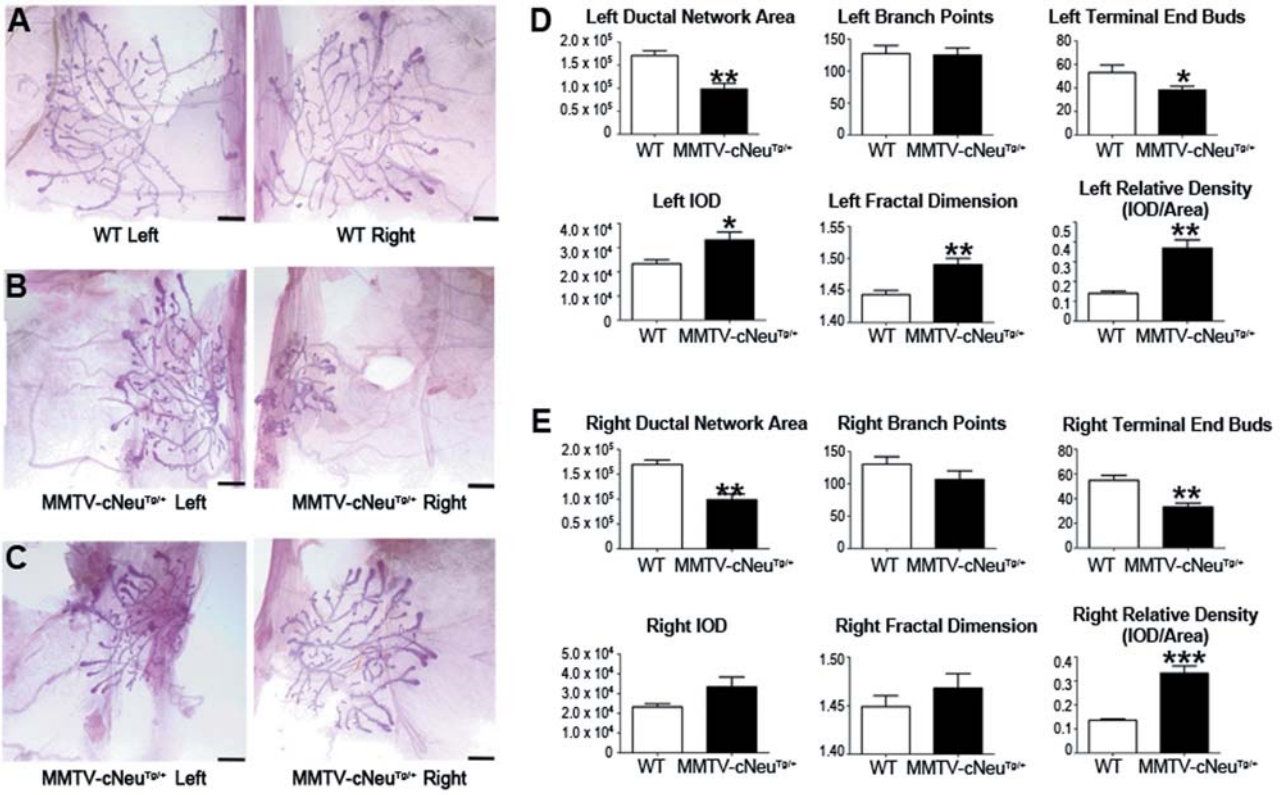

Figure 3. *Independent left- and right-side analysis of ductal networks in wild-type versus MMTV-cNeu mice. (A) Representative images of a carmine red stained pair of TMGs of wild-type (WT) mice. (B, C) Representative images of two different pairs of carmine red stained TMGs of MMTV-cNeu$^{Tg/+}$ mice. (D) Morphometric analysis of #3 left TMGs of WT versus MMTV-cNeu$^{Tg/+}$ mice. (E) Morphometric analysis of #8 right TMGs of WT versus MMTV-cNeu$^{Tg/+}$ mice. Bars are representative of mean±SEM (WT N=8 MMTV-cNeu$^{Tg/+}$ N= 13). Unpaired Student's t-test, *p<0.05, **p<0.001, ***p<0.0001.*

Application of the fractal dimension (D): The thoracic mammary glands in the wild type and MMTV-cNeu mice appear as irregular and complex objects composed of parts at different levels of resolution (ducts of different bore sizes) which are functionally and physiologically similar (self-similar) to the whole object. Under the conditions of these properties, the thoracic mammary glands can be considered fractal objects and their topological dimension, the fractal dimension (D), be expressed by a non-integer number lying between two Euclidian integer topological dimensions (29). The values of D characterizing the thoracic mammary glands are therefore fractional. Since the thoracic mammary gland is essentially a 2-dimensional object, the D values will lie between 1 and 2. As

the mammary gland becomes more complex and irregular, its D value becomes greater approaching 2. In applying fractal analysis, the D value of the mammary gland is determined by applying the box-counting method (29, 30). The box-counting method has been the most widely used and general model for applying fractal analysis to biological and non-biological systems. The box-counting method consists of a grid of boxes of size e superimposed over the image of the structure, and the number of boxes containing any part of the structure recorded as *N(e)*. A fractal object expresses a straight line when Log[*N(e)*] is plotted against Log(*1/e*). The box fractal dimension D can be determined from the slope of the regression line. That is: D= Log[*N(e)*]/Log(*1/e*). The D values of the thoracic mammary glands were determined using HarFA software (23) [http://www.fch.vutbr.cz/lectures/imagesci]. The HarFA software assigned mesh sizes of boxes with e values ranging from 2 to 207 pixels and 30 steps within this range were calculated to generate the Log[*N(e)*] *versus* Log(*1/e*) lines to determined .

Branch points and terminal end buds (TEBs). Branch points and TEBs were quantified by manual counting from the images.

Results

Whereas the mammary gland begins its development during embryonic mid-gestation stages, the majority of its growth and development takes place post-natally, with the first substantial expansion of the ductal epithelial network occurring during puberty. Genetic, hormonal, and environmental factors that perturb the ductal architecture during puberty or other periods of growth and morphogenesis also increase the risk of developing breast cancer later in life (31-33). Mammary ductal epithelial branching and elongation are driven by bifurcation of specialized invasive structures located at the ends of the rudimentary ducts, termed terminal end buds (TEBs) (34). Shorter (secondary) side branches also arise as lateral sprouts from trailing ducts, increasing the area of the ductal tree with each successive ovarian cycle (35). The pattern of mammary branching morphogenesis is non-stereotypical (*i.e.* it varies from individual to individual), and is controlled by paracrine-derived signals within the local microenvironment (35, 36).

In order to determine if changes in ductal epithelial growth and complexity can be identified during early neoplasia, several morphological and fractal parameters of ductal epithelial networks were quantified and compared between thoracic mammary glands (TMG) of pubertal-stage wild-type and MMTV-cNeu mice (Figure 2). We chose to focus specifically on TMGs for two reasons. Firstly, the vast majority of tumors in MMTV-cNeu mice develop in the thoracic glands compared to the cervical or inguinal glands; and secondly, based on differences amongst signaling pathways that regulate induction of the five pairs of mouse mammary glands, as well as their anterior-posterior anatomical locations, it appears that TMGs most closely model human breast development (21). Thus, we reasoned that if early neoplastic changes were present, they would be detectable in the TMGs.

Consistent with a previous report (37), we found that the area occupied by the ductal epithelial network in TMGs in wild-type mice is significantly greater than the area of the ductal epithelial network in TMGs in the MMTV-cNeu mice (Figure 2). This indicates that the ductal epithelial networks in TMGs in MMTV-cNeu mice are smaller, but not necessarily morphologically different from the TMGs present in the controls. To assess potential morphological differences, fractal dimension, branch points, TEBs, and IOD were quantified. Application of the fractal dimension (D) is a measure of disorder, or chaos, of the epithelial network. The D for MMTV-cNeu TMGs is significantly greater than D for the wild type TMGs (Figure 2). This indicates that the ductal epithelial networks of MMTV-cNeu TMGs are more complex and more space-filling despite smaller size (Area) than those in wild-type mice. Interestingly, the increase in D for MMTV-cNeu TMGs does not appear to be the result of an increase in branch points or TEBs, which are the same or decreased, respectively (Figure 2). This indicates that the increase in D of the MMTV-cNeu TMGs results from an overall lack of order of the entire network structure, suggesting that the ErbB2/Neu oncogene promotes a disorganized pattern during epithelial network development.

Although determination of D can quantify an object, a value of D does not uniquely specify a particular morphology. In other words, objects of vastly different morphology can have the same or similar fractal dimensions. To adequately describe the morphology of an object, an additional measurement in conjunction with D is required to provide a unique identifier, which quantifies the object. Ideally, such an additional measurement would be determinant of the structure or distribution of material within the thresholded boundary of the region of interest. In the present study, we applied the concept that the IOD is a measure of the mass of the ductal network within the ROI (24-28). Mass measurement deals with this distribution of material within the ROI and leads to the concept of relative density, here defined as IOD/Area. Thus, application of the term IOD/A provides additional information on the concept of mass density or relative density of the mammary ductal network (38). Taken together, these two measurements, D and IOD/A, improve the quantitative description and provide unique characterization and quantitation of the epithelial network morphology of MMTV-cNeu TMGs compared to WT TMGs.

As shown in Figure 2, the relative density of the TMG epithelial network, measured as the IOD/A, is significantly greater in the MMTV-cNeu mice compared to wild-type mice. This indicates that there is more physical material content (Carmine alum-stained epithelium) in the TMGs of MMTV-cNeu mice. Since the MMTV-cNeu TMG networks have smaller area with a larger D, the expression of the greater IOD/A suggests that the ductal walls may be thicker

and contain smaller lumens than the ducts in the wild-type controls. Taken together, these results indicate that the overexpression of the *ErbB2/Neu* oncogene results in delayed epithelial growth with an overall concomitant increase in chaos that is consistent with ductal hyperplasia.

Because epidemiological studies of breast cancer patients indicate that significantly more tumors arise in the left breast compared to the right (21), we next investigated whether the morphological defects in MMTV-cNeu mice were present to the same extent in both the left and right TMGs. Comparing the left TMG ductal networks in the MMTV-cNeu mice to those in the left TMGs of wild-type mice, indicated significant differences in all measurable parameters except branch points (Figure 3D). By contrast, the right-side ductal epithelial networks of MMTV-cNeu mice expressed a more normal morphometric pattern (Figure 3E). Whereas right side MMTV-cNeu networks had decreased area and number of terminal end buds, there was no difference in the D values of the right TMGs of the MMTV-cNeu mice compared to the right TMGs of controls. This indicates that although the right side TMGs of MMTV-cNeu mice are smaller, they nevertheless exhibit a normal degree of tissue organization and space-filling properties that are the same as wild-type controls. Additionally no difference was detected in the IOD of the right networks of the MMTV-cNeu mice relative to the right-side wild-type TMGs. However, the relative density (IOD/A) of the right side MMTV-cNeu TMGs was greater than relative density of wild-type right-side TMGs. Together, this suggests that ductal epithelium on the left side is more susceptible to ErbB2/Neu-mediated effects on ductal morphology than is the right side epithelium. Thus, fractal image analysis may be useful in defining tissue of risk (pre-neoplastic tissue or tissue initially undergoing neoplastic transformation) to cancer before appearance of the tumor.

Discussion

Our results demonstrate that when combined with conventional morphometric analysis, fractal dimension is a highly sensitive and quantitative tool by which one is able to evaluate and compare murine ductal epithelial growth and morphology. This combined approach facilitates precise morphological description which is independent of landmarks such as the lymph node (typically used in semi-quantitative inguinal gland analysis) and permits for inclusion of regions of epithelium that may otherwise be obscured by contaminating muscle tissue (which frequently occurs with cervical and thoracic gland dissections). Similar to its clinical utility, application of fractal dimension to the Her-2+ breast cancer mouse model demonstrates that fractal dimension can identify aberrations in tissue architecture that are not necessarily obvious nor easily appreciated by conventional, semi-quantitative image inspection. The objective nature of fractal analysis and the ease of use of this method position it as a tool that can be used to standardize morphological assessment of mammary epithelial growth and differentiation in both normal and neoplastic development. Because the combined image and fractal analysis used in the present study utilizes commercially available software and can be applied to archived specimens (*i.e.* coverslipped mammary whole mounts), this approach offers a means by which results may be reproducibly and quantitatively compared across existing mouse mammary models, as well as in breast cancer mouse models that may be developed in the future.

In addition to its diagnostic and therapeutic decision making utility for breast cancer patients, fractal dimension may also be useful to identify women at heightened risk for developing breast cancer. Fractal dimension analysis of mammographic images has been used for retrospective identification of hormone-associated changes in breast tissue linked with women who were later diagnosed with breast cancer (39). In another retrospective study, fractal dimension analysis detected architectural distortions that were present in screening mammograms taken on average 15 months prior to clinical breast cancer diagnosis (40, 41). In our study we found that fractal dimension can also be effectively used in a pre-clinical breast cancer mouse model to similarly detect changes in tissue organization that arise during early oncogenesis. By applying combined fractal/morphometric analysis to MMTV-cNeu mice, we found that numerous aberrances develop in the growth and branching pattern of ductal epithelium during early neoplasia, well in advance of appreciable tumor formation. A particularly intriguing finding was that in addition to overall decreased ductal network area and alterations in other morphological parameters, the epithelial networks of MMTV-cNeu mice showed more pronounced abnormalities in ductal epithelial network organization and complexity in the left-side glands than did the right-side glands. This finding suggests that MMTV-cNeu mice may be an appropriate model to investigate left-right differences in neoplastic development, an area that has yet to be addressed at the cellular or molecular level, despite the fact that epidemiological studies consistently find increased tumor incidence on the left side in breast cancer patients (21, 42).

In summary, the results of our study demonstrate that combined fractal and conventional morphometric analysis is an objective, quantitative method to document early neoplastic changes in ductal epithelial morphology occurring prior to mammary carcinoma development. The sensitivity of the current approach in a pre-clinical breast cancer mouse model yields results comparable to those in clinical studies of human breast cancer patients and offers opportunity for investigators to standardize analyses made across the numerous murine models that are currently in use in studies of both normal and neoplastic mammary gland development.

Acknowledgements

Supported by National Institutes of Health R21HD068993 (A.F.R.) and conducted in facilities supported, in part, by MUSC Hollings Cancer Center Support Grant P30CA138313.

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Received December 16, 2013
Revised January 9, 2014
Accepted January 13, 2014