Comparative Analysis of Eosin-based Fluorescence Microscopy of Non-neoplastic Breast Tissue and Fibroadenoma

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Authors’ contributions
This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Fibroadenoma (FA) is one of the most frequently diagnosed benign neoplasm in women. Various researches have reported increased risk of breast cancer in females with FA. It stems from the proliferation of epithelial and stromal contents of the terminal duct lobular units (TDLU’S) of breast tissue, that are the primary sites for the histopathologic assessment which is the gold standard for the diagnosis of disease. However, this method is subjective and possess interobserver variability. Therefore, new quantitative methods are required to aid in diagnosis. Hence we evaluated fluorescence light intensity and its use in histopathologic evaluation.

Aim: The goal of this research was to compare and quantify red and green fluorescence light intensities of ductal cells and stroma of non-neoplastic breast tissue with fibroadenomatous tissue.

Method: A cross-sectional study was done in the Cell biology and histology lab of Ziauddin University. 44 slides of normal breast tissue and 44 slides of diagnosed fibroadenomatous tissue were taken from Dr Ziauddin Hospital, North Campus. Hematoxylin and eosin (H&E) staining of the
slides were done following standard protocols. On microscopic examination, the changes in light intensities of ductal cells and stroma of normal breast tissue and fibroadenoma were quantified using dual channel fluorescence microscopy using Nikon NIS imaging software.

**Results:** The results demonstrated statistically significant increase (p-value <0.05) in mean red (37.22±5.9) and green (22.47±6.6) light intensity of stroma in FA when compared with red (32.71±6.7) and green (17.01±4.3) light intensity of normal breast tissue. Whereas, R/G ratio for normal tissue was higher (1.95±0.11) than R/G for FA (1.74±0.37) with a p value of <0.05. Similarly, for ductal cells; statistically significant (p value <0.05) increase in mean red (38.86±5.4) and green (15.54±2.51) light intensity for FA was found when compared with red (29.62±1.89) and green (12.60±1.67) intensity of normal tissue. R/G ratio for FA (2.5±0.24) was compared to be higher than normal tissue (2.36±0.3) with a p value of <0.05.

**Conclusion:** The study suggests that fluorescence microscopy combined with quantitative assessment fluorescence light intensities may be a helpful tool for histomorphic evaluation of the breast tissue.

**Keywords:** Fluorescence microscopy; fibroadenoma; normal breast tissue; hematoxylin and eosin staining.

**ABBREVIATIONS**

- **FA:** Fibroadenoma
- **H&E:** Haematoxylin and eosin
- **TDLU:** Terminal Ductal Unit
- **SHG:** Second harmonic signals
- **DPX:** Dibutylphthalate Polystyrene Xylene

**1. INTRODUCTION**

Fibroadenoma is considered as the most prevalent benign neoplasm of breast in females. Although it can occur at any age, second decade of life has a peak incidence [1]. Ninety percent of the FA’s are unilateral and are located mostly at the upper outer quadrant [2]. Since fibroadenoma initiates from the concurrent proliferation of epithelial and stromal content of TDLU’S, they are often called as biphasic neoplasms [3]. Various researches have documented that females with fibroadenomas have higher risk of breast malignancy [4-6].

Excision of the mass surgically is the preferential treatment of fibroadenoma, which provides the pathological diagnosis of the tumor to determine the diagnostic accuracy of fibroadenoma [2]. However, it has been reported that pathological examination has a limitation of being a subjective process that does not use quantitative data for analysis [7-9]. Manual grading of a diseased tissue specimen performed by pathologists suffers through inter- and intra-observer variations [10]. It was revealed that the process of pathological diagnosis is subject to considerable variability; up to 25% [11]. Therefore, it could be benificial to search new methods that has a potential to aid in a histopathological assessment.

Optical imaging is a rapidly evolving field that encompasses several techniques that can be used in quantification of biologic tissue specimens. Eosin based fluorescence microscopy is an emerging method that has provided an objective assessment to aid in the diagnosis of several diseases [12]. This technique is able to perform fluorescent analysis of biopsies directly from eosin and hematoxylin (H&E) stained tissues. Fluorescence characteristics of protein–eosin complexes can demonstrate tissue changes as it progresses from normal to diseased condition. It uses a fluorescent light intensity source to excite tissue that in turn emits fluorescent light at various wavelengths i.e red and green [13]. These changes in light intensities can be reflected in a quantitative manner by fluorescence microscopy system [14]. Investigations have confirmed the utility of eosin-based fluorescence technique and have reported that fluorescence is a powerful tool to calculate changes in normal tissue and a diseased condition [15-17].

Gu et al. has stated that this technique has the ability to characterize the tissue, provide visual augmentation and differentiation of structures to aid standard light microscopy in the diagnosis of the disease [18]. However, to the best of our knowledge no data has been reported to have assessed the application of eosin-based fluorescence microscopy on breast tissue. Hence, in this article the quantitative potential of eosin-based fluorescence microscopy was
checked to aid in the diagnosis of normal breast tissue and FA.

2. METHODOLOGY

2.1 Study Design

It was a cross-sectional study, using non-probability consecutive sampling.

2.2 Study Area

The study was conducted at Cell biology and histopathology lab, Ziauddin University.

2.3 Population of Interest

44 pairs of unstained diagnosed non-neoplastic and fibroadenoma breast tissue slides were obtained from histopathology lab of Dr Ziauddin Hospital (North Campus).

2.4 Sample Collection

Tissue slides were obtained from the histopathology lab of Dr Ziauddin Hospital. Normal (non-) tissue was taken from the normal breast tissue area of mastectomy sections of patients who had undergone breast surgery and were histologically approved to be normal by a histopathologist. Whereas, FA sections were obtained from the fibroadenoma patients.

2.5 Sample Preparations

Tissue slides were stained with H&E following the standard protocol [19]. Chemicals utilized in this research were of high quality and research grade. Eosin and hematoxylin solutions were purchased from Carl Roth GmbH (Karlsruhe, Germany) catalogue #X883.1 and #T864.1 respectively. Mounting media (DPX), hydrogen peroxide and bovine serum albumin were bought from Riesledel-de Haen (Seelze, Germany). Xylene solution was used to deparaffinize the slides and then the slides were rehydrated using 100%, 90% and 70% graded alcohol. Slides were then stained using hematoxylin for 3 minutes and then rigorously washed with distilled water. Similarly, slides were stained with eosin for 30 seconds and then washed again. Then, 70%, 90% and 100% graded alcohol was used to dehydrate the slides and DPX mounting media was used to mount the slides.

2.6 Brightfield and Fluorescence Microscopy

The regions of interest in each slide (ductal cells of TDLU and stroma) was selected by a histopathologist using the subsequent strategy. Firstly, the microscope was set at a low magnification of 100x in order to observe the complete section on a slide. Then, histopathologist located the area of interest (ductal cells of TDLU and stroma) in each slide which showed characteristic features of normal breast tissue and FA. Secondly, the selected areas of interest were set at a higher magnification of 200x to attain high contrast fluorescence and brightfield images. Ts2R-FL inverted research microscope with colored camera was used to acquire images of H&E stained brightfield images of normal breast tissue and FA. Whereas, a dual channel (Texas Red and FITC optics in a single cube) fluorescence filter cube (wavelengths: emission 510–540 and 590–650 nm; excitation 458–505 and 555–585 nm & mirror 505–555 and 575–∞ nm) was used to acquire fluorescence images of H&E stained slides of the same field. Images were then processed in the computer and labelled accordingly. Finally, fluorescence images were analyzed and the light intensities of red and green fluorescence were measured and (R/G) ratio was calculated using, the Nikon, NIS Elements (Japan) imaging software for ductal cells and stroma of normal breast tissue and FA.

2.7 Statistical Analysis

SPSS version 20 was used to analyze the data. All quantifiable variables were represented as mean and ± standard deviation. A pooled t-test was applied to find the difference in light intensities between FA and normal breast tissue. In all analysis, a p-value of < 0.05 was considered to be statistically significant.

3. RESULTS

3.1 Fluorescence and Brightfield Images of Normal Breast Tissue

Fig. 1 represents a brightfield image of normal breast tissue (A) and the corresponding dual channel fluorescence image (B) of the same field at 200x magnification. Stroma surrounding the lobular structure is known as interlobular stroma (a) which is visible in the figure. It consists of dense fibrous connective tissue and an abundance of fat cells which makes up the adipose tissue. Whereas, intralobular stroma (b) can be seen within the lobule, which is composed of loose connective tissue. However, both the interlobular stroma and intralobular stroma of normal breast tissue are mainly composed of collagen bundles (red arrow) which...
generate strong fluorescence intensity. Hollow cavities visible in the figure are known as alveoli (c) which are lined by two layers of cells. Inner layer is composed of secretory epithelial cells (yellow arrow) and the outer layer is of contractile myoepithelial cells (blue arrow). A nest of alveoli is known as a lobule which is embedded in intralobular stroma and lined by a continuous basement membrane. However, some other alveoli are almost filled with epithelial cells, as the alveolar epithelium slightly increases.

3.2 Fluorescence and Brightfield Images of Fibroadenoma

The corresponding brightfield image (A) and the representative dual channel fluorescence image (B) of FA are shown in Fig. 2. The figure displays that ducts of FA have been compressed into a cleft like structure (yellow arrow) and are surrounded by a proliferated stroma (blue arrow).

Epithelial lining of the ducts are visible as narrow strands (a) in the stroma. However, the basement membrane (red arrow) is still intact. When compared with normal breast tissue, collagen fibres (b) around the ducts are found in abundance in FA, which may have quantitatively resulted in higher fluorescence intensities. Therefore, the significant alterations in light intensity exhibiting from stroma can be seen and quantified (Table 1 and 2).

3.3 Quantification of Red and Green Light Intensities of Normal Tissue and Fibroadenoma (Duct)

To further characterize the changes from normal to benign condition (fibroadenoma), changes in fluorescence light intensities of ductal cells and stroma were quantified. The quantitative results shown in Table 1 reveal that for ductal cells; mean red light intensity for normal tissue was (29.6±4.6) compared to FA (38.9±5.4). Whereas, mean green light intensity for normal tissue was (12.6±1.7) when compared to fibroadenoma (15.5±2.5). Therefore, R/G for normal tissue was calculated to be (2.36±0.3) and R/G for fibroadenoma was (2.5±0.24). The results show a statistically significant difference in fluorescence light intensity of FA when compared with normal tissue (p value <0.05) (Table 1). The results illustrates that fluorescence light intensity of ductal cells were increased in FA.

3.4 Quantification of Red and Green Light Intensities of Normal Tissue and Fibroadenoma (Stroma)

The quantitative results shown in Table 2 reveal that for stroma; mean red light intensity in normal tissue was found to be (32.71±6.7) when compared with fibroadenoma (37±5.9). Whereas, mean green light intensity of stroma also increased from normal (17±4.3) to fibroadenoma (22.47±6.6). Therefore, R/G for normal tissue was calculated to be (1.95±0.11) and R/G for fibroadenoma was (1.74±0.37). The results show a statistically significance difference in fluorescence light intensity of FA when compared with normal tissue (p value <0.05) (Table 2). The results illustrates that fluorescence light intensity of ductal cells were increased in Fibroadenoma.

4. DISCUSSION

Fibroadenoma is the most common benign neoplasm in young women which originates from terminal ductal lobular unit (TDLU). Morphologically, this grayish white firm rubbery neoplasm is composed of proliferating mesenchymal and epithelial structures. Several studies have reported fibroadenoma as an increased risk factor for breast cancer [5]. Moreover, the origination of malignancy within fibroadenomas has also been reported in literature [20]. Since the pathological classification of breast tissue diseases depends on a subjective criteria; therefore, there is a need to develop new techniques that has the quantitative potential to diagnose breast tissue diseases.

Table 1. Quantification of red and green light intensities of normal tissue and fibroadenoma (Duct)

| Parameters         | Study groups    | Mean Intensity | ±SD  | P value |
|--------------------|-----------------|----------------|------|---------|
| Red light intensity| Normal          | 29.6           | ±4.6 | <0.001* |
|                    | Fibroadenoma    | 38.9           | ±5.4 |         |
| Green light intensity| Normal          | 12.6           | ±1.7 | <0.001* |
|                    | Fibroadenoma    | 15.5           | ±2.5 |         |
| R/G light intensity| Normal          | 2.36           | ±0.3 | <0.001* |
|                    | Fibroadenoma    | 2.5            | ±0.24|         |

*Significance of the mean difference is at 0.05 level. Independent t-test was applied
Fig. 1. Brightfield image (A) represents (a) as interlobular stroma; (b) as intralobular stroma and (c) as alveoli. The corresponding dual-channel fluorescence image (B) of normal breast tissue at 200x represents blue arrow: myoepithelial cell; yellow arrow: epithelial cells; red arrow: stroma (collagen)

Fig. 2. Brightfield image of fibroadenoma (A) represents (a) as narrow ducts; (b) as stroma and red arrow as basement membrane. The corresponding dual-channel fluorescence image (B) of FA at 200x represents yellow arrow: compressed duct structure; Blue arrow: proliferated stroma (collagen fibres)

Table 2. Quantification of different light intensities of normal tissue and fibroadenoma (Stroma)

| Parameters       | Study groups  | Mean Intensity | ±SD  | P value |
|------------------|---------------|----------------|------|---------|
| Red light intensity | Normal        | 32.7           | ±6.7 | 0.001*  |
|                  | Fibroadenoma  | 37             | ±5.9 |         |
| Green light intensity | Normal       | 17             | ±4.3 | 0.001*  |
|                  | Fibroadenoma  | 22.47          | ±6.6 |         |
| R/G light intensity | Normal       | 1.95           | ±0.11|         |
|                  | Fibroadenoma  | 1.74           | ±0.37|         |

*Significance of the mean difference is at 0.05 level. Independent t-test was applied.
One such promising technique is eosin based fluorescence microscopy. Over decades ago, fluorescence characteristics of hematoxylin and eosin stained sections were described [21]. However, very little research has been done using fluorescence microscope to observe hematoxylin and eosin stained sections for assessing tissue microenvironment. Skin, teeth, kidney, cervix, spleen and placentae have been assessed in the past [14,17,22-25]. These researches have confirmed the reproducibility and utility of eosin-based fluorescence microscopy in order to quantify and characterize features that are challenging to visualize on brightfield microscope. Warram et al., claimed that this imaging method uses the fluorescent properties of cellular structures and can aid in the diagnosis of a disease when histopathology is inconclusive. This diagnostic method can distinguish normal tissue from abnormal tissue through quantitative analysis [26]. Furthermore, Ragazzi et al. stated that as normal cells becomes diseased, they undergo structural and metabolic changes that are reflected in the changes in fluorescence light intensities [27]. Even so, no studies have examined if this method can aid in the diagnosis of benign lesions (fibroadenoma) of breast.

In this study, unique fluorescent signatures were found in normal breast tissue and FA. The first indicator was the statistically significant increase in red and green fluorescence light intensity in ductal cells when we compared normal breast tissue with fibroadenoma which can be attributed to the fact that the ducts are irregular in architecture and are reduced to slits form in fibroadenoma condition. Secondly, the alteration of stromal morphology is also a significant feature of fibroadenoma. The stroma surrounding the ducts apparently increases and the collagen fibres are relatively randomly organized in FA which was indicated quantitatively through the increase in red and green light intensity of the stroma when compared with normal breast tissue [28]. Similar findings regarding the proliferated stroma of FA was documented [18]. Another study done via multiphoton microscopy showed that stroma generate strong SHG signals and is very sensitive to stromal composition, therefore it revealed significant alterations in the morphology and increase in stromal content [29]. From these findings, we infer that proliferation of stromal content in FA and changes in ductal morphology has an impact on red and green light intensities when a normal breast tissue is compared with FA.

5. CONCLUSION

This study quantitatively demonstrates differences in the red and green light intensities of ductal cells and stroma using fluorescence microscope between non neoplastic FA breast tissue. Use of this quantifiable eosin fluorescence technique on routine H&E-stained sections may uncover more valuable findings and improved morphological details. Significantly increased red and green light intensities were found when non neoplastic breast tissue was compared with fibroadenoma. The study concludes that fluorescence microscopy may be helpful as an additional histopathological diagnostic tool.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical clearance taken from institutional ethics committee and preserved by the author(s). Reference code: 3001220HSANA.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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