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Modern methods to differentiate benign thyroid nodules from malignant ones

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Abstract: *Objective: The purpose of this study is to compare the capsular structure appearing in follicular adenoma and papillary thyroid carcinoma to differentiate the benign and malignant thyroid nodules.*
Methods: Second Harmonic Generation (SHG) Microscopy was used to image collagen distribution in the capsules of several types of nodules. The tissue fragments were formalin-fixed, paraffin-embedded but without H&E staining, with 4-5 microns thick sections. From the same sample, two consecutive sections were made. The first was colored with H&E and capsule images were acquired with an optical microscope. The second was not colored with H&E. On the uncolored sample, type I collagen from the capsule was visualized by SHG microscopy. In addition, a parameter was used to study the orientation of collagen fibers, obtained from the Fast Fourier Transform (FFT) analysis of 2D-images.
Results: SHG microscopy images were acquired to assess the collagen organization of tumoral capsular thyroid nodules previously diagnosed as benign or malignant by conventional H&E staining. Different degree of collagen fibers orientation was observed from the two kinds of capsules and quantified using FFT analysis.
Conclusion: These above described microscopy method can be used to distinguish between benign and malignant thyroid nodules, based on different degree of the capsular collagen fibers orientation.

Keywords*: follicular adenoma, papillary thyroid carcinoma, benign and malignant thyroid nodules, capsular collagen fibers*

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INTRODUCTION

The clinical and imagistic diagnosis of malignancy must be confirmed in pathology departments.

Although histopathology and cytopathology are the main tools for diagnosing cancer, there are some difficult cases that pathologists may encounter during routine diagnosis procedures, where the differentiation between a benign tumor proliferations from a malignant one is very difficult to achieve. In such situations complementary examination procedures are required (eg. serial sections from formalin-fixed, paraffin-embedded tissue blocks, mounted on glass slides and usual stained with H&E, immunohistochemistry, molecular studies, electron microscopy, etc.).

Another method which can be used in thyroid pathology for diagnostic purposes is Second Harmonic Generation (SHG) Microscopy.

SHG microscopy is a label-free laser scanning

microscopy technique that exploits a nonlinear optical effect – the generation of the second harmonic in samples illuminated by an intense laser field [1, 2]. SHG can occur only from materials that do not have a center of symmetry, thus analyzing absolute molecular structure in organized organic environments, such as collagen fibrils, microtubules and the myosin chain of muscle cells. SHG microscopy has been proven to be a useful tool in biomedical tissue imaging [2] because it can be used for imaging endogenous structures like type I collagen [1] as well as for the characterization of collagen organization [3-5] and has been recognized as a promising approach for the diagnostics of different cancer types [6-8], by detecting collagen structure modifications [7].

The thyroid gland is wrapped in a thin fibrous capsule, from which originate numerous fibrous septae that penetrate the thyroid parenchyma and divide it into incomplete lobules (the so-called thyromeres). Macroscopically, the thyroid capsule seems complete, but it is incomplete from the microscopic point of view, as it adheres tightly to adjacent structures (laryngeal thyroid cartilage and trachea) [9].

Nodular enlargement of the thyroid gland comprises those situations in which one or more nodules are present. This presence of nodular enlargement is the most common in the case of non-toxic nodules, also known as adenomatous goiter or colloidal goiter.

Polinodular goiter with different-sized follicles can be a clinical manifestation of a wide range of different pathologies and not the expression of a single disease [10].

The usual classification of neoplasms of follicular epithelial cells are in benign (adenomas) and malignant (carcinomas) tumor proliferation. The benign ones are called follicular adenomas [11], which are encapsulated, non-invasive tumors showing evidence of thyroid follicular cell proliferation without the specific nuclear characteristics of thyroid papillary carcinoma [12].

Papillary Thyroid Carcinoma (PTC) is a malignant epithelial tumor, usually invasive, showing evidence of abnormal proliferation of thyroid follicular cells with characteristic nuclear changes: irregular contours and nuclear pseudoinclusions, nuclear membrane irregularities, and chromatin marginalization. For a correct diagnosis of the classical PTC, the papillary fibrovascular axes, the invasive character or the characteristic cytological features are required. [12].

The encapsulated variant of PTC is a type of proliferation with typical cytological and architectural changes, entirely surrounded by a thin, fibrous capsule that may be intact or infiltrated only focally by the tumor. If the capsule is invaded by the proliferation of malignant tumors, we are dealing with an aggressive form of PTC.

The encapsulated variant represents about 10% of all cases of PTC and has an excellent prognosis. Differential diagnosis is made with follicular adenoma with papillary hyperplasia [12].

Over the years, diagnostic criteria for follicular and papillary thyroid carcinomas have changed, but the two names have been retained. There have been several variants of papillary carcinoma for which the primary diagnostic criterion is based on nuclear features [11].

Some thyroid encapsulated neoplasms may present diagnostic difficulties due to the fact that only nuclear changes are sufficient to justify and diagnose PTC but there is the uncertainty about the capsular or vascular invasion. Encapsulated follicular tumors lacking the PTC nuclear characteristics are difficult to differentiate. For example, follicular carcinoma (a malignant tumor) can be distinguished from follicular adenoma (a benign tumor) only by demonstrating invasive character in the nodule capsule or capsular vessels [12].

We started from the idea that SHG can be used to observe three-dimensional structures of a tissue sample without the need for staining.

In this way, this method helps us in the correct diagnosis of encapsulated tumor entities that require complementary examinations from the pathologist or in order to determine a possible capsular structural change before the onset of malignant transformation of a benign encapsulated tumor.

OBJECTIVE

The purpose of this study was to use the collagen structure in the nodule capsule to obtain information on the follicular adenoma capsule and encapsulated papillary thyroid carcinoma (PTC) capsule for differentiating between benign and malignant thyroid nodules. The study was based on optical microscopy images and SHG microscopy, with the latter used for the visualization of type I collagen. We also show that the FFT analysis of the SHG images can provide information about the collagen fiber orientation.

MATERIALS AND METHODS

A batch composed of encapsulated-PTC and follicular adenoma tissue from 24 sections tissue samples (12 for Encapsulated-PTC capsules and 12 for follicular adenoma capsules) was used for this study. Sections of 4-5 µm were made from each paraffin tissue block. They were mounted on glass blades and stained with Hematoxylin and Eosin (H&E) and visualized with a light field microscope (Leica DM3000). The tissue architecture of the normal, benign and malignant regions of interest and the capsule surrounding the nodules have been identified. Unstained pairs of the H&E stained tissue sections were used for SHG imaging for collagen organization assessment.

SHG imaging was performed using a Leica TCS-SP confocal laser scanning microscope modified for nonlinear optical imaging [13]. We have used a mode-locked Ti:Sapphire laser operating at 780 nm and with ~150 fs pulses. A 40X, 0.75 numerical aperture objective was used to focus the laser beam on the sample, while the SHG signals were collected in the forward direction through a condenser lens. The average power reaching the sample plane was kept less than 15 mW and no photodamage was observed on the samples.

SHG images were used to estimate the collagen fiber orientation by FFT analysis using ImageJ software. For an image containing aligned fibers, we expect an FFT image with higher values along the direction orthogonal to the direction of the fibers and with an elliptic intensity plot. On the other hand, for an image with randomly oriented fibers, the FFT image intensity plot should be close to a circle. The FFT power spectra images were first binarized and then fitted with an ellipse. A collagen orientation index was calculated based on the length of the minor (S) and major (L) axes of the ellipse: $N = 1 - S/L$ [14]. Collagen orientation can thus be represented by an index ranging from 0 (random fibers) to 1 (aligned fibers).

RESULTS

Optical microscopy images of H & E stained sections do not give us information about the ultrastructure of the studied tumoral nodules capsule. The capsule appears as a fibrous structure surrounding the tumoral nodule. It looks identical to the follicular adenoma nodule and to the Encapsulated-PTC nodule. So this optic method is limited in terms of capsule study.

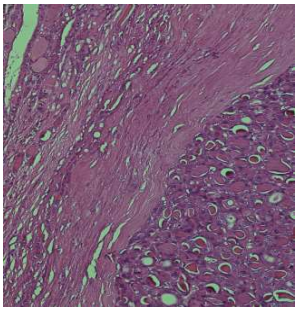
On the other side, the SHG helps to study the ultrastructural changes of the capsule. SHG highlights type I collagen in the capsule structure. So we can see if there are qualitative changes of this type of collagen from the two kinds of capsules. In addition, using FFT analysis of SHG images we can bring precious differentiation information of the two types of capsules: the follicular adenoma nodule or the Encapsulated-PTC nodule. We have noticed that in the Encapsulated-PTC nodule capsule there appears to be a collagen fiber organization as if they were trying to isolate malignant epithelial proliferation.

We acquired images with Second Harmonic Generation Microscopy which were used to assess the collagen orientation of tumoral capsular thyroid nodules previously diagnosed as benign or malignant by conventional H&E staining.

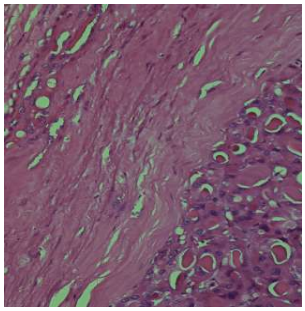
We compute the FFT spectra for the SHG images acquired for the considered pathologies. One can see that the average collagen orientation (N) computed from the FFT spectrum is higher for the follicular adenoma capsule ($N = 0.69$) than for the encapsulated-PTC capsule ($N = 0.4$). The FFT spectra were calculated for the entire images. We consider that if the spectra are computed for smaller regions of interest (ROIs), a distribution of values can be obtained, depending on the degree of alignment for the collagen fibers in the considered ROI. Averaging over a larger area, which is

also the case of the whole area analysis, it results in a lower N. Even though N was calculated on whole areas, the obtained results show a difference in collagen organization between the two capsules.

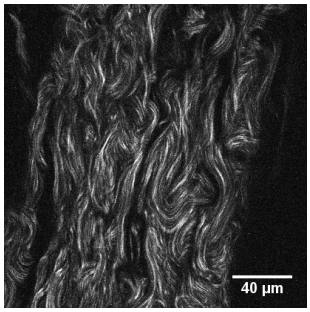
Figure 1. Follicular adenoma capsule



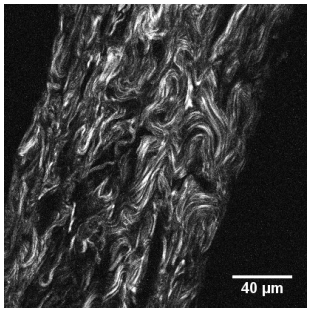
a). H&E 100x



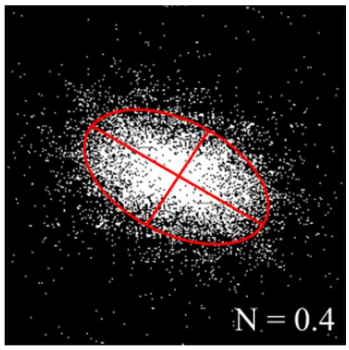
b). H&E 200x



c). SHG image 1

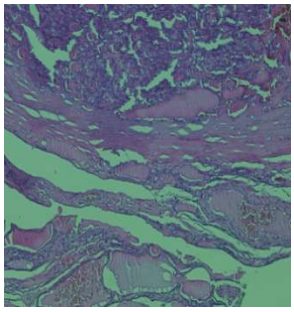


d). SHG image 2

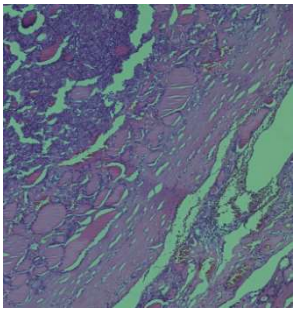


e). The FFT power spectra images - Follicular Adenoma Capsule

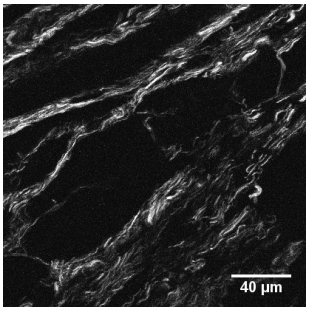
Figure 2. Encapsulated-PTC capsule



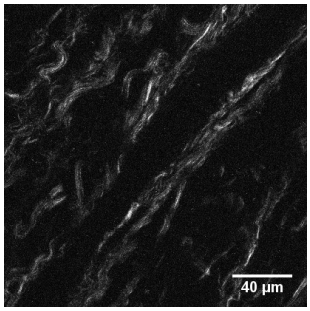
a). H&E 100x



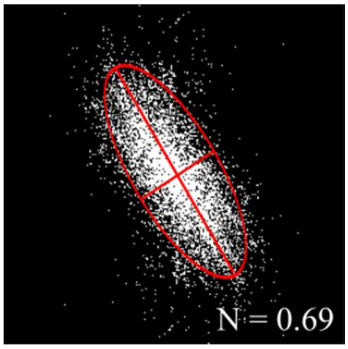
b). H&E 200x



c). SHG image 1



d). SHG image 2



e). The FFT power spectra images – Encapsulated-PTC Capsule

CONCLUSIONS

We found differences in the orientation of collagen fibers between the capsules surrounding benign nodules (follicular adenoma) and malignant ones (Encapsulated-PTC) by using SHG microscopy images and FFT analysis of these images.

Our research indicates that different collagen structures can be detected in the malignant nodules capsule (PTC) compared to the benign nodule capsule and the thyroid capsule.

Higher orientation was detected for the malignant capsule but that does not mean the collagen fibers are organized or that the malignant tissue organizes the fibers. We believe it is rather a defense mechanism that goes from the level of the thyroid adjacent stroma of tumoral nodules.

Future plans

The present study deals with the differential diagnosis between follicular adenoma versus encapsulated – PTC, since both are clear cases, thus making it easier to prove the validity of the theory regarding the reorganizing of the collagen fibers within the two types of nodules, as proven with SHG Microscopy.

Having completed that, the research may advance to analyzing less obvious situation:

1. Differential diagnostic between:
- Follicular adenoma versus follicular carcinoma;
- “Non-invasive follicular thyroid neoplasm with papillary-like nuclear features” (NIFTP) versus encapsulated papillary thyroid carcinoma with capsular invasion;
- Adenomatous nodule vs follicular adenoma.

2. Early detection of papillary carcinomas developed on follicular adenomas.

The SHG images can be developed as markers for use in clinical applications – a complementary method to early detection of suspected malignant nodules.

These above described microscopy methods help us distinguish between benign or malignant thyroid nodules, based on the capsular collagen organization.

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