Application of Magnetic-Resonance-Spectroscopy-Based Metabolomics to the Fine-Needle Aspiration Diagnosis of Papillary Thyroid Carcinoma

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Key Words

Thyroid cancer • Diagnosis • Magnetic resonance spectroscopy • Metabolomics • Fine-needle aspiration biopsy

Abstract

Objective: This study explores the potential use of high-resolution magic angle spinning proton magnetic resonance spectroscopy as an ancillary diagnostic technique for papillary thyroid carcinoma in thyroid fine-needle aspiration biopsies. The method has already been shown to be effective in the classification of various other nonthyroid cancers. **Study Design:** Twenty-six samples (13 paired cytologic and histologic samples) from patients being evaluated for thyroid abnormalities at the Massachusetts General Hospital were spectroscopically analyzed, and included: papillary thyroid carcinomas ($n = 4$), follicular adenomas ($n = 4$), and normal thyroid samples ($n = 5$). Metabolic profiles were statistically generated based on the spectroscopy results, which were then correlated with the final cytologic and histologic diagnoses from the same samples to determine the diagnostic capacity of the profiles. **Results:** Principal component analysis of the tissue samples revealed statistically significant correlations among principal components and various cytologic and histologic features. Canonical score 1, calcu-

lated with principal components in correlation with analyzed pathologies, revealed the ability of the metabolic profile to differentiate all three examined histologic tissue groups (ANOVA, $p < 0.0001$). Applying coefficients of principal components and canonical scores obtained with tissue samples directly onto spectral results of cytology samples, the calculated canonical score 1 also revealed similar trends among the three fine-needle aspiration biopsy groups. In particular, the papillary thyroid carcinoma group exhibited significant differences from both the adenomatous and normal cytology groups ($p < 0.0170$). **Conclusions:** The results indicate the potential of high-resolution magic angle spinning proton magnetic resonance spectroscopy as an ancillary marker for papillary thyroid carcinoma in fine-needle aspiration biopsy specimens.

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Background

Over the past 3 decades, thyroid fine-needle aspiration biopsy (FNAB) has emerged as one of the most important clinical tools in the initial evaluation of thyroid nodules [1–3]. FNAB is considered a safe, cost-effective, and overall accurate technique [4, 5]. Among those thyroid nodules that are malignant, papillary thyroid carcinoma

(PTC) is the most common [6–8], and the accurate FNAB diagnosis of PTC is critical because of implications for the subsequent clinical management [9]. Efforts to further improve the efficacy of FNAB as a diagnostic test for PTC have shown some success through the application of molecular markers such as BRAF mutations, and RET-PTC gene rearrangements, as well as immunocytologic panels [10, 11]. Here, we present proton magnetic resonance spectroscopy ($^1\text{HMRS}$) as a potential additional ancillary study that may also be useful in the FNAB diagnosis of PTC.

During the development and progression of malignancies, in addition to histologic and cytologic changes, metabolic processes at the cellular level are also known to display alterations. These metabolic alterations can be measured and quantified using a technique known as magnetic resonance spectroscopy (MRS). The problem with the MRS method (a.k.a. NMR, for nuclear magnetic resonance), however, is that it was originally developed for measurements of aqueous solutions, and it cannot produce sufficient spectral resolutions to identify and quantify individual metabolites either in *in vivo* settings or in *ex vivo* measurements of intact tissues and cells. To address this problem, the use of an NMR method with magic angle spinning (MAS) in which samples are mechanically rotated at an angle of 54.7° (known as the ‘magic angle’) from the direction of the applied magnetic field was developed and termed the high-resolution (HR) MAS $^1\text{HMRS}$ technique. This HRMAS $^1\text{HMRS}$ method can be used for the direct measurement of metabolic alterations within unaltered biological samples [12, 13]. Thus, the HRMAS method can measure individual metabolites of intact tissue and cells, and at the same time it preserves the tissue intact for subsequent histopathologic (or cytopathologic) evaluations. This technique has been used to correlate metabolic alterations with pathological changes in tissues including oncological changes (a process known as MRS-based metabolomics) [14]. Since the development of HRMAS $^1\text{HMRS}$, our group and others have demonstrated the application of HRMAS in classifying and subclassifying tumors, detecting the presence of small levels of malignant cells, and gauging the aggressiveness of tumors [13].

In this study, we explore the feasibility of conducting HRMAS $^1\text{HMRS}$ measurements on unaltered thyroid FNABs and their corresponding histologic samples of PTC, follicular adenomas (FAs), and normal tissue. Here we present data showing that such measurements are possible, and they have the potential to be used as an ancillary diagnostic technique for PTC.

Materials and Methods

Sample Collection

This study was approved by the IRB of Partners Healthcare and the Massachusetts General Hospital. The authors take responsibility to maintain relevant documentation in this respect.

Surgically resected thyroid tissues were obtained in the frozen section laboratory retrospectively from patients with PTC ($n = 4$), FA ($n = 4$), and normal thyroid (tissue uninvolved by FA or PTC) ($n = 5$). The FNAB passes were performed using a 25-gauge needle on tissue specimens acquired in the frozen section laboratory at the Massachusetts General Hospital. Two alcohol-fixed smears stained with a modified H&E were prepared, and the remaining FNAB sample was used for spectral analysis. Histologic samples measuring $0.5 \times 0.5 \times 0.5$ cm were taken from the corresponding FNAB site. Both histologic and cytological samples for spectral analysis were placed in 1.5-ml cryovials without any treatment, snap frozen, and stored at -80°C for a duration of approximately 2–8 weeks prior to the spectroscopic measurements.

HRMAS $^1\text{HMRS}$

For the current study, a total of 26 samples (13 tissue-FNAB pairs) were analyzed. Spectral analysis was performed on a Bruker (Billerica, Mass., USA) AVANCE spectrometer operating at 600 MHz (14.1 T). Samples were placed in a 4-mm rotor with plastic inserts creating a spherical sample space of $\sim 10 \mu\text{l}$ located at the center of the detection coil. For tissue samples, approximately 10 mg were used, while for FNAB, $10 \mu\text{l}$ were injected into the rotor. Approximately $2.0 \mu\text{l}$ of D_2O was added into the rotor with the tissue or cytology sample in order to lock the magnetic field according to the measurement ^2H signal. All spectroscopy measurements were carried out at 4°C for metabolite preservation.

A repetition time of 5 s and 32 proton signal averages were used to acquire each spectrum. Spectra were collected with spinning rates of 3,600 Hz. A CPMG filter (100 ms) synchronized to sample rotation rates was included in the pulse sequence to reduce broad resonances associated with probe background, rotor, and/or macromolecules.

Spectroscopic data were processed with Nuts software (Acorn NMR, Inc., Livermore, Calif., USA). All free induction decays were subjected to routine spectral processing procedures, including 1-Hz line-broadening before Fourier transformation, baseline correction, and phase adjustments. The resonance intensities reported here represent integrals of curve fittings with Lorentzian-Gaussian line shapes normalized by the total spectral intensities measured between 0.5 and 4.5 ppm. All spectra were processed manually and objectively without knowledge of tissue pathological information. Following curve fittings of the entire 0.5- to 4.5-ppm region, 11 resonance regions that have universal appearances in every tissue and cyto-FNAB spectrum were selected for further statistical analyses.

Pathology

Following spectroscopic measurements, tissue samples were fixed in formalin, embedded in paraffin, and prepared for $5\text{-}\mu\text{m}$ H&E-stained sections. Sets of serial sections cut $150 \mu\text{m}$ apart were obtained from each sample. Cytological evaluation was conducted on smears prepared from the FNAB samples. Features were semiquantitatively evaluated microscopically for both histologic and cytology samples as the percent composition of tumor, fibrous tissue, inflammation, colloid, vasculature, normal follicular cells, and blood.

Table 1. Results of the microscopic evaluation for both tissue and FNAB samples

	Case	Tumor, n (%)	FV, n (%)	Infla, n (%)	Colloid, n (%)	N. Epi, n (%)	Blood, n (%)
PaCa	1	60 (95)	35 (2)	5 (0)	0 (0)	0 (0)	0 (3)
	2	40 (80)	40 (15)	0 (5)	20 (0)	0 (0)	0 (0)
	3	32 (85)	40 (10)	8 (0)	6 (0)	14 (0)	0 (5)
	4	23 (95)	62 (3)	0 (0)	13 (2)	0 (0)	2 (0)
FA	5	81 (n.c.)	13 (n.c.)	5 (n.c.)	1 (n.c.)	0 (n.c.)	0 (n.c.)
	6	38 (80)	37 (10)	5 (0)	20 (5)	0 (0)	0 (5)
	7	5 (70)	13 (0)	0 (0)	82 (15)	0 (0)	0 (15)
	8	75 (80)	20 (0)	0 (0)	5 (10)	0 (0)	0 (10)
Normal	9	0 (0)	15 (10)	8 (0)	44 (0)	33 (80)	0 (10)
	10	0 (0)	11 (10)	4 (0)	70 (40)	15 (50)	0 (0)
	11	0 (0)	10 (0)	0 (0)	70 (25)	20 (65)	0 (10)
	12	0 (0)	10 (0)	0 (0)	80 (70)	10 (20)	0 (10)
	13	0 (n.c.)	15 (n.c.)	10 (n.c.)	47 (n.c.)	28 (n.c.)	0 (n.c.)

PaCa = Papillary carcinoma; Tumor = papillary carcinoma and FA, respectively; FV = fibrovascular; Infla = inflammation; N. Epi = normal epithelia; n.c. = nonconclusive.

Statistical Analysis

The intensities of 11 resonance regions from 13 tissue spectra were subjected to principal component (PC) analysis (PCA). PCA identifies combinations (PCs) of these spectral intensities that may reflect distinct pathological processes if they exist in the set of the samples. A positive contribution of a certain intensity indicates elevation of the metabolite within the component (process), and a negative contribution suggests suppression. The PCs thus obtained from PCA of spectral intensities were then evaluated by analysis of variance (ANOVA) for their abilities to differentiate among three pathology groups (PTC, FA, and normal thyroid), and by linear regression analysis against tissue pathology measured from the same sample after spectral analysis. Pathology-relevant PCs and pathology features were then subjected to canonical analysis to identify canonical scores that could differentiate among the three pathology groups of tissue samples.

Using tissue samples as a training cohort, coefficients for PCs and canonical scores thus obtained with tissue samples were applied directly to FNAB spectral results to calculate the corresponding canonical scores for FNAB samples, as a testing cohort. The resulting FNAB canonical scores were evaluated with ANOVA for differentiating among three pathology groups (PTC, FA, and normal). FNAB samples and their correlations with tissue canonical scores were evaluated using a paired t test. Statistical analysis was conducted using JMP statistical discovery software (SAS, Cary, N.C., USA).

Results

Thirteen paired FNAB and tissue samples were obtained for this study. Results of the microscopic evaluation for both tissue and FNAB samples are listed in ta-

ble 1, and examples of paired spectra from the tissue and cytology samples for PTC, FA, and normal are presented in figure 1. PCs from PCA represent a unique combination of the metabolic variability in the samples and have the potential to indicate unique biochemical and/or pathological processes. Evaluations of correlations among PCs and underlying tissue pathologies revealed that PCs 1, 3, 5, and 6 may reflect various quantified pathological features with or close to statistically significant levels (table 2).

We conducted canonical analysis for tissue samples based on these four PCs and the volume percentages of the five quantified pathological features. ANOVA analysis of the resulting canonical scores 1 and 2 reveals the abilities of these scores to differentiate among the three pathology groups (fig. 2a, b). Canonical analysis of tissue spectroscopic results initiated a hypothesis-discovery process. These hypotheses, i.e. that specific linear combinations of metabolic intensities based on PCA and canonical analysis may reflect tissue pathology, are further tested by applying the coefficients from these tissue analyses to spectral results of FNAB samples.

Although the levels of statistical significance of the resulting FNAB canonical scores are less than those observed with histologic samples, they follow a similar trend (fig. 2a, b). Paired t test results of high statistical significance between tissue and FNAB canonical score 1 ($p < 0.0003$) were observed. FNAB canonical score 1 distinguishes PTC from benign samples (including both FA

Table 2. Correlations among PCs and underlying tissue pathologies

	PC 1	PC 3	PC 5	PC 6
Tumor		$r = 0.717$ $p < 0.006$		
Fibrovascular	$r = -0.496$ $p = 0.085$			
Inflammation				$r = -0.661$ $p < 0.014$
Colloid		$r = -0.716$ $p < 0.006$		
Benign epithelia			$r = 0.567$ $p < 0.044$	

and normal groups) with statistical significance ($p < 0.017$). Furthermore, the resulting FNAB canonical score 2 separates neoplastic (PTC and FA) from nonneoplastic with $p < 0.016$, while the distinction between FA and normal was slightly below the level considered statistically significant ($p = 0.062$).

Discussion

Previous studies have attempted to define noninvasive radiological means to aid in the management of thyroid nodules. Such studies have yielded intriguing results but have yet to be integrated into clinical practice. These investigations can be grouped by either their *in vivo* nature or their *ex vivo* nature. *In vivo* examinations are marred by several issues, not least among them being the financial burden of using whole-body MR scanners as a screening tool. Additional technical limitations of *in vivo* thyroid MR include magnetic susceptibility issues, movement of the tumor from swelling and breathing, and spectral contamination from adjacent fat. Such limitations mean that *in vivo* diagnostics can only identify large tumors [15]. Of more importance to the goal of defining tumors based on their biochemical and molecular properties, *in vivo* examinations identify only one to two critical metabolites (choline and creatine). These two metabolites (choline alone or the choline/creatine ratio) have known associations with cancer and can be up to 100% sensitive and 88.88% specific for detecting thyroid cancer; however, they do not subcategorize the form of thy-

Fig. 1. Paired spectra from the paired tissue and cytology samples for PTC, FA, and normal.

roid malignancy and, therefore, may not reduce the number of unnecessary surgeries for the disease, which is the overall aim of all MR studies [15, 16]. *Ex vivo* studies have been performed using MRS on both tissue and FNAB samples. These studies have also limited themselves to examinations of two metabolite regions (lipids at 1.7 ppm and amino acid residues at 0.9 ppm); the ratio of the metabolites (1.7/0.9) was 100% accurate in distinguishing normal from malignant tissues, and tumors labeled as benign could be grouped into those with a ratio indicat-

Fig. 2. Correlations of canonical scores 1 (a) and 2 (b) for differentiating among the three pathology groups. ANOVA analysis of tissue spectroscopy results in canonical scores 1 and 2 that can differentiate among the three pathology groups, with $p < 0.0001$ and $p < 0.0003$, for scores 1 and 2, respectively. The coefficients of these tissue scores formed by the specific linear combinations of metabolic intensities based on PCA and canonical analyses are applied to FNAB spectral results and tested for their abilities to

differentiate among different groups. FNAB canonical score 1 distinguishes PTC from benign samples (including both FA and normal groups) with $p < 0.017$, while FNAB canonical score 2 separates neoplastic (PTC and FA) from nonneoplastic with $p < 0.016$. The dashed lines in these plots link the data points between pairs of tissue and their corresponding FNAB samples. Brackets are used to indicate statistical relationships between different sets of samples. T = Tissue; C = cytology; PaCa = papillary carcinoma.

ing a truly benign status versus those that could have malignant potential [17–21]. Again, though, these investigations were based on limited metabolites and did not analyze the ability of this ratio to classify the type of thyroid malignancy present.

Metabolomic Profiles Provide a New Paradigm for Classifying Tumors

Our results indicate that metabolomic profiles are sensitive enough to delineate differences between normal cells, PTCs, and FAs at the molecular level in tissue and FNAB samples. The first aim of the study was to explore the feasibility of conducting HRMAS ^1H MRS measurements on unaltered thyroid FNAB specimens. We have found the analysis of both thyroid tissue and FNAB specimens to be straightforward, requiring no optimization deviating from analysis of other tissue and biological fluid samples. The HRMAS ^1H MRS method is ideal for studies of intact biological samples since, unlike ex vivo MRS of tissue extractions, it preserves the cellular architecture and allows for microscopic evaluation of the samples following spectroscopic measurements.

Our results were generated using a small finite amount of the sample (approximately 10 mg tissue and $\sim 10\ \mu\text{l}$ of FNAB); these are amounts that can easily be acquired at the time of biopsy without the patient incurring additional invasive procedures and, of equal importance, without the clinician losing an amount of sample that would interfere with their microscopic diagnosis. Of critical importance here is the demonstration that FNAB samples can be used for HRMAS ^1H MRS studies, regardless of the amount of cells contained in the sample. Visual examination of the FNAB samples revealed varying amounts of cells present, but due to the sensitivity of the instrument to detect small amounts of metabolites this difference in cellular concentration did not create problems in the analysis.

In this retrospective study of a limited number of samples from only three types of thyroid pathologies, we explore the potential utility of HRMAS MRS in differentiating these thyroid groups. Although we designed the study by using tissue samples as a training cohort and the FNAB samples as a testing cohort to discover the parameters for the metabolomic profiles, these cohorts produced from

the same patients are different from the true training and testing cohorts generated from different patient populations. Nevertheless, our findings demonstrate the potential to distinguish between samples with neoplastic versus normal cells and malignant versus benign cells among the three pathology types studied. Furthermore, larger studies using a greater range of tumors including variants of PTC, particularly the follicular variant of PTC, will be useful to validate these results and further refine the metabolomic profiles. In addition, when perfected for the evaluation of PTCs in FNAB samples, techniques such as HRMAS ¹HMRS could be applied to the 'AUS/FLUS' category of FNABs, many of which are microscopically sub-follicular variants of PTC.

Conclusions

Our findings demonstrate the potential of detecting and classifying thyroid tumors using FNAB metabolomic profiles generated by tissue measurements with

HRMAS ¹HMRS and quantitative histopathology. This method of studying unaltered samples provides biochemical information not currently attainable with contemporary diagnostic tools. Metabolomic profiles from tissue, particularly from FNAB cytology samples, have the potential to be used in conjunction with current diagnostics to help guide the clinical management of patients with thyroid nodules.

Disclosure Statement

The are no competing interest to declare by any of the authors.

Acknowledgements

The authors acknowledge partial support from NIH grants CA115746 (L.L.C.) and CA095624 (L.L.C), and from Massachusetts General Hospital and the A.A. Martinos Center for Biomedical Imaging.

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