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ORIGINAL ARTICLE

Characterization of thyroid tissue using infrared spectroscopy

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Abstract

Introduction: In recent years, the number of surgical procedures involving the thyroid gland has increased worldwide; however, no increase in mortality rate has been observed. The Fourier Transform Infrared (FTIR) spectroscopy technique presents evidence in the characterization of multiple tissues, such as the thyroid gland, with the advantage of being rapid and preserving the tissue analyzed. **Objectives:** Characterize both healthy and pathological thyroid tissues by FTIR spectroscopy. **Methods:** Patients were selected at the Head and Neck Surgery Service of the Hospital das Clínicas, Ribeirão Preto Medical School, University of Sao Paulo - USP, Ribeirão Preto-SP, Brazil, from 2014 to 2015. The sample consisted of 44 patients with reference for thyroidectomy. The analysis was performed by defining the areas of each band using the OriginPro 8.6.0 software. The band was then normalized to 1240 cm^{-1}. The mean area was calculated using the Student's *t*-test with $p<0.05$. After calculation of the means, the second-order derivative of the spectrum was evaluated to show the positions of each absorption band. **Results:** The infrared spectrum of each piece was obtained and expressed as a function of absorbance and wave numbers in the mean IR (4000-900 cm^{-1}). The present study demonstrated that, in the analysis of thyroid tissue by FTIR spectroscopy, it is possible to differentiate benign nodules from healthy tissue with significant difference in the area of the B-band between healthy tissue and goiter, which corresponds to 1452.90 cm^{-1} in healthy tissue (proteins and lipids) and 1069.80 cm^{-1} in goiter (DNA), as well as significant difference in width between normal thyroid tissue and carcinoma of the C band. **Conclusions:** FTIR spectroscopy is able to differentiate pathologically altered thyroid tissues from the thyroid gland compared with findings in healthy thyroid tissues. In patients with benign nodular disease of the thyroid gland, it is possible to differentiate healthy goiter tissue with statistical significance, as well as malignant nodules from healthy tissue through FTIR spectroscopy.

Keywords: thyroid neoplasms; spectroscopy; fourier transform infrared; goiter.

Introduction

Thyroid cancer comprises a group of tumors with peculiar characteristics, from tumors of low aggressive behavior and localized disease to advanced tumors with bleak prognosis. According to the National Cancer Institute (INCA), the estimated incidence of new cases of thyroid cancer in Brazil in 2016 was 1090 cases for males and 5870 cases for females[1]. Higher incidence of cases of thyroid cancer was found in the female gender (82% - 660 cases)[2] compared with that in the male gender (18% - 147 cases)[3,4]. Increased incidence of new cases of thyroid cancer has been observed worldwide, attributed both to development of diagnostic tools and greater access of the population to the health system.

Presenting good cost-benefit, fine-needle aspiration (FNA) cytology is considered the most accurate diagnostic method (95% accuracy in experienced hands); and is part of therapeutic decision algorithms in patients with solitary or multiple thyroid nodules[5]. Numerous classifications are used to describe the results of FNA cytology; the Bethesda classification was adopted in this study. This classification was designed to enable better communication between physicians and institutions for standardization of FNA cytology results, which are classified from I to VI according to cellular aspects. This classification stratifies the risk of malignancy in nodules characterized within this pattern as follows: for nodules with benign result, the risk of malignancy is less than 1%; for follicular lesion of unknown significance (FLUS), it ranges from 5 to 10%; for follicular neoplasia, between 20 and 30%; for suspected malignancy, from 50 to 75%; and for nodules with malignant results, the risk is 100%. Surgery is indicated for cases of suspected malignancy (Bethesda IV and V) or malignant lesions. For cases of follicular lesion of indeterminate significance, which correspond to 24% of the results, surgery is not always indicated, and should be based on the association with other variables[6].

A cancer biomarker is any structural or functional entity that can be objectively measured, such as genes, proteins, metabolites, intermediary activities and pathways, morphology, cytogenetic parameters, as well as any detectable physical characteristic or change associated with cancer in humans. Tumor biological markers are products of certain cells that can distinguish them from normal ones[7].

An ideal test should require little specimen manipulation, be non-invasive, highly sensitive, specific, and reproducible, as well as feasible so that costs of clinical testing can be reduced. Thus, it is imperative that a technology capable of defining the histopathological diagnosis without the need for intraoperative excisional biopsy be developed. A methodology aiming to perform an “optical biopsy” of tissues in thyroid surgeries could be very useful in enabling adequate surgical treatment in a single procedure, avoiding reoperations as well as unnecessary treatments. Since the mid-twentieth century, infrared (IR) spectroscopy coupled with microscopy has been recognized as a non-destructive method of high analytical capacity for application in cancer research.

The objective of the present study was to characterize thyroid gland tissues by Fourier Transform Infrared (FTIR) spectroscopy, analyze the ability of FTIR spectroscopy to differentiate pathological tissues from the thyroid gland from spectroscopic findings in healthy tissues, and assess the ability of FTIR spectroscopy to identify neoplasms of the thyroid gland, as well as differentiate malignant lesions from benign ones.

Methods

This study was approved by the Research Ethics Committee of the Hospital of Clinics, Ribeirão Preto Medical School, University of Sao Paulo (HCFMRP-USP) under protocol no. 14495/2012.

Samples from 44 patients of both genders, obtained by thyroidectomy at the HCFMRP-USP, were used in the study. The samples were submitted to FTIR spectroscopy and then sent to the Pathology Laboratory, where they were stained with hematoxylin-eosin. A Nicolet 380 (Nicolet, USA®) spectrometer was used for the IR analysis. Analysis was performed by defining the areas of each band using the OriginPro 8.6.0 (Origin Lab Corporation, Northampton, MA, USA) software. The band was then normalized to 1240 cm^{-1} (DNA, RNA, phospholipids). The mean of the areas was calculated using the Student's *t*-test with $p<0.05$. After calculation of the means, the second-order derivative of the spectrum was assessed to show the positions of each absorption band. The ratios were also analyzed. By means of this ratio, differentiation between benign, malignant and healthy tissue was performed through analysis of variance (one-way ANOVA) and the Tukey and Fisher tests. Next, the ratios between the B and C bands were compared using the A band. One-way ANOVA also applied to identify differences between the samples.

Each band was analyzed using the Student's *t*-test for the paired and independent samples and the Wilcoxon test for the paired samples and comparison of two independent samples. The paired tests were applied for comparison between healthy tissue and tumor (adenomas and carcinomas) and tests for independent samples were used for comparison between nodules (benign or carcinoma).

Results

The study sample was composed of 44 patients undergoing thyroidectomy, of both genders, aged 54 years on average. Data of the participants were distributed in groups according to the anatomopathological results (Tables 1 and 2).

For the patients with benign tumor, statistically significant difference was found in the area between normal tissue and that with benign tumor regarding the B band ($p=0.0418$). For the sample of patients with carcinoma, statistically

Table 1. Tumor distribution according to anatomopathological results obtained after spectroscopic analysis.

Anatomopathology	Patients
Colloid goiter	16
Follicular adenoma	3
Hashimoto's thyroiditis	4
Papillary carcinoma	19
Follicular carcinoma	2
Total	**44**

Table 2. Mean peak absorption of A, B and C bands with their respective standard deviation and biological association.

Band	Sample	Absorption peaks (cm^{-1}) + SD	Biological association
A	Goiter	3496.42 (21)	*cis*-ordered substructures
	Normal	1636.11 (1)	β configuration - Amide I
	Carcinoma	1639.47 (13)	β configuration - Amide I
B	Goiter	1069.80 (22)	DNA
	Normal	1452.90 (6)	Proteins and Lipids
	Carcinoma	1452.89 (6)	Proteins and Lipids
C	Goiter	741.36 (22)	DNA-RNA
	Normal	1239.82 (1)	DNA
	Carcinoma	1240.88 (5)	DNA

SD = standard deviation.

significant difference was observed between normal tissue and tissue with carcinoma for the C-band width (p=0.0238), with broader width in the tissue with carcinoma compared with that of the healthy tissue (Tables 3 and 4).

Subsequently, comparison between the pathological tissues (carcinoma *vs*. benign tumor) was conducted. No statistically significant difference was identified for any of the bands.

Analysis of the ratios was then performed. Calculation of ratios is mainly used in the differentiation of DNA, RNA, and amides[8]. In the present study, this method was adopted by selecting an absorption band as reference. For the calculation of proteins and lipids associated with the B and C bands (DNA, RNA, and phospholipids), the amide groups I and II (band A) were used as reference. The protein:DNA ratio was also assessed. No significant difference between benign and malignant nodules was observed by this method of analysis.

Subsequently, comparison between the pathological tissues (carcinoma × benign tumor) was conducted. Although the p-value for area A in the following table shows statistical significance, a difference cannot be assigned because, regarding the area, an analysis adopting mean ratios must be performed with a standard band of low interference, as described ahead.

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Table 3. Mean, median, and standard deviation of the variables area, position, and width in each band in patients with benign tumor in the two tissues collected, followed by the *p*-value of the Student's *t*-test and Wilcoxon test for paired data.

	Normal				Benign tumor				
Variable	**N**	**Mean**	**Median**	**SD**	**N**	**Mean**	**Median**	**SD**	***p*-value**
Area A	19	14.36	13.88	2.79	23	15.61	15.26	3.26	0.1219
Position A	18	1636.12	1635.42	1.37	23	1636.7	1636.87	2.06	0.5184
Width A	18	73.34	70.31	15.09	23	77.43	69.57	22.9	0.6397
Area B	18	0.73	0.67	0.31	22	0.9	0.87	0.27	0.0418
Position B	18	1452.9	1456.06	6.65	23	1452.73	1455.1	6.51	0.5876
Width B	18	56.14	55.41	17.62	23	63.4	69.78	21.16	0.4301
Area C	18	0.58	0.51	0.35	23	0.76	0.74	0.44	0.0806
Position C	18	1239.82	1239.82	1.37	23	1238.39	1240.07	8.01	0.6570
Width C	18	34.89	33.71	6.3	23	39.27	34.17	16.88	0.8650

SD = standard deviation.

Table 4. Mean, median, and standard deviation of the variables area, position, and width in each band in patients with carcinoma in the two tissues collected, followed by the *p*-value of the Student's *t*-test and Wilcoxon test for paired data.

	Normal				Carcinoma				
Variable	**N**	**Mean**	**Median**	**SD**	**N**	**Mean**	**Median**	**SD**	***p*-value**
Area A	17	13.9	13.95	2.09	19	13.53	13.4	3.18	0.6641
Position A	17	1640.72	1637.35	14.33	20	1639.47	1636.39	13.3	0.1413
Width A	17	77.78	70.12	30.33	20	75.81	69.45	28.18	0.3719
Area B	17	0.73	0.75	0.24	19	0.74	0.72	0.27	0.9187
Position B	18	1448.91	1455.34	18.1	19	1452.89	1456.06	6.82	0.8945
Width B	18	58.8	59.82	18.4	20	64.93	71.22	19.47	0.4513
Area C	17	0.47	0.39	0.36	19	0.57	0.49	0.31	0.2874
Position C	18	1241.38	1240.55	3.26	20	1240.89	1239.34	4.98	0.6017
Width C	16	31.89	32.38	4.11	18	37.51	36.26	7.26	0.0238

SD = standard deviation.

Discussion

Identification of thyroid nodules <1cm increased with the advent of ultrasound (US) and the greater access of the population to diagnostic tools, increasingly demanding highly accurate methods in cancer identification, avoiding overtreatment of the population, defined as "treatments conducted with solid bases and the patients' preference, but that will not assist with and/or change their clinical condition"[9]. There is controversy regarding the increased incidence of thyroid neoplasms. Analysis of the overdiagnosis of thyroid neoplasms, mainly after the 1980s, identified substantial change in the incidence curve

Table 5. Mean, median, and standard deviation of the variables area, position, and width in each band in the two patient samples (benign tumor and carcinoma), followed by the *p*-value of the Student's *t*-test and Mann-Whitney test for comparison of independent samples.

	Carcinoma				Benign tumor				
Variable	N	Mean	Median	SD	N	Mean	Median	SD	*p*-value
Area A	19	13.53	13.4	3.18	23	15.61	15.26	3.26	0.0441
Position A	20	1639.5	1636.39	13.3	23	1636.7	1636.87	2.06	1.0000
Width A	20	75.81	69.45	28.18	23	77.43	69.57	22.9	0.9903
Area B	19	0.74	0.72	0.27	22	0.9	0.87	0.27	0.0612
Position B	19	1452.9	1456.06	6.82	23	1452.7	1455.1	6.51	0.6866
Width B	20	64.93	71.22	19.47	23	63.4	69.78	21.16	0.7995
Area C	19	0.57	0.49	0.31	23	0.76	0.74	0.44	0.1178
Position C	20	1240.9	1239.34	4.98	23	1238.4	1240.07	8.01	0.9613
Width C	18	37.51	36.26	7.26	23	39.27	34.17	16.88	0.7641

SD = standard deviation.

Table 6. Analysis of variance (ANOVA) of the means of the B-band ratios in normal and pathological tissues with the number of analyses performed, mean ratios, and their standard deviation.

	N	Mean	Standard deviation
B-band ratio - normal	18	0.048	0.012
B-band ratio - benign	20	0.058	0.013
B-band ratio - normal	15	0.051	0.01
B-band ratio - malign	15	0.058	0.033

N= number of analyses performed.

estimated by age, with an increase in the incidence attributed to younger patients[10]. Utilizing data from the 1960s (prior to the introduction of US) as reference, the authors estimated a historical curve and verified exponential growth with age similar to that of most epithelial tumors. It was also observed that, in countries where a policy of attention focused on nodule detection was applied, the incidence was even higher without changes in the mortality rate. These data provide support for increased attention regarding reference for thyroidectomy. The study of new techniques in the differentiation of thyroid nodules can assist with selection of the patients who will undergo appropriate surgical treatment, avoiding overtreatment. In this study, FTIR spectroscopy was evaluated for differentiation of thyroid tissues.

A study using FTIR spectroscopy for detection of thyroid carcinoma showed difference between nodular goiter and papillary thyroid carcinoma at the peaks of 1640 cm^{-1} (amide I), 1240 cm^{-1} (DNA), and 1550 cm^{-1} (amide II)[11]. In contrast, in the present study, benign nodules were compared with carcinoma at 1640 cm^{-1} (p: 1.0), 1240 cm^{-1} (p: 0.9), and 1452 cm^{-1} (p: 0.6), and no significant differences were observed with respect to wave numbers, area, and mean diameter. Identification of thyroid tissues by FTIR spectroscopy was studied in intraoperative thyroidectomy for benign and malignant nodules[12]. The authors identified seven absorption peaks, with significant difference in the positions 1460 and 1400 cm^{-1} (lipids), 1240 cm^{-1}(nucleic acid), and 1160 cm^{-1} (carbohydrate). These parameters alone were not able to differentiate the two groups. Thus, a discriminant analysis was developed using the MATLAB software. The results indicated 84.4% sensitivity, 88% specificity, and 86.6% accuracy. In this scenario, auxiliary methods are used in band analysis. In the present survey, calculation of the inter-band ratios was used to differentiate the tissues, with the amide I and II regions as reference and the Student's *t*-test and Wilcoxon test for analysis. Other regions are used as parameter. In this study, DNA was used (1121 cm^{-1}), identifying higher intensity of absorbance in high-grade lymphomas[13].

According to the update of the Brazilian Consensus on Thyroid Nodule and Cancer, lobectomy is indicated in suspected <4 cm nodules with undetermined cytology, low risk at US, and low clinical suspicion[14]. This guideline also recommends that initial lobectomy be performed on classic papillary carcinoma <1 cm, unifocal, without lymph node involvement and extrathyroidal invasion. Completion of partial thyroidectomy can be discarded in papillary carcinoma - described previously, minimally invasive follicular carcinoma (<2cm), or follicular variant of encapsulated papillary carcinoma without lymphatic invasion and <2cm. In this sense, FTIR spectroscopy would be able to identify molecular changes consistent with benign or malignant thyroid nodules, adding information to whether an initial surgical approach to preservation of thyroid tissue could be attempted. This study demonstrated that, in the analysis of thyroid tissue by FTIR spectroscopy, it is possible to differentiate benign nodules from healthy tissue with significant difference in the B band area comprised between 1345 cm^{-1} and 1482 cm^{-1}, and malignant nodules from healthy tissues with significance between mean diameter at 1240 cm^{-1}.

The use of FTIR spectroscopy in the field of oncology encompasses research on different structures and tissues. A study on parathyroid and thyroid differentiation[15] demonstrated that, with the use of fluorescence in the proximal IR, it is possible to differentiate these tissues, with greater intensity of fluorescence of the parathyroid compared with that of the thyroid with p=5.91 x 10^{-14}. The advantage of optical methods is speed, which enables almost instant identification of the tissues, avoiding their removal for diagnostic interpretation. The spectroscopy system used during the surgeries consisted of a spectrometer coupled to a diode laser, a computer, and a fiber optic probe. A FTIR spectrometer was used in the operation room for detection of lymph node metastases from papillary carcinoma in lymph nodes, with procedure time of 2-3 minutes[16]. The authors found an increase in the intensity ratios of 1240 cm^{-1}, 3280 cm^{-1},1640 cm^{-1} and 1546 cm^{-1} (nucleic acid) and 1400 cm^{-1}(lipids) in metastatic lymph nodes. In the sample of this study, the band at 1240 cm^{-1}, which corresponds to DNA, presented difference in diameter between healthy tissue and carcinoma.

A study[17] verified differences between the ratio of normal and precancerous areas in the regions of 1130-1180 cm^{-1} and 1180-1260 cm^{-1}. In the present investigation, we used calculation of the ratios between areas with difference between goiter area and normal tissue and Student's *t*-test and Wilcoxon test with difference between malignant and normal tissues. A study conducted FTIR spectrometry on lung cells induced to epithelial-mesenchymal transformation[18]. Such alteration is associated with the potential of cellular metastatic dissemination. Induction occurred by the use of β-1 transformation factor, and it was confirmed by immunohistochemistry. The differentiated cells showed changes in protein concentration and nucleic acid turnover at 960-1250 cm^{-1} and 1400-1600 cm^{-1}, respectively. The study presented the particularity of analyzing the potential of spectroscopy in the identification of cells with potential for metastatic dissemination. Unmodified cells were used in the present study; therefore, future studies may be conducted on thyroid cells induced to differentiation.

The study of prostate tissues over generations was a subject of research[19]. The authors evaluated benign prostatic tissues treated by transurethral prostatectomy in patients of the same age group (60-69 years) using the techniques ATR-FTIR associated with Raman. Samples were selected from 1983 to 2013 and compared by multivariate principal component analysis followed by linear discriminant analysis (LDA), sequential progression algorithm, and genetic analysis followed by linear discriminant analysis (GALDA). After the spectroscopic analysis, the samples from the 1983-1984 and 2012-2013 were submitted to immunohistochemistry to assess the possible epigenetic alterations. By means of ATR-FTIR and Raman, they concluded that there was variation in the spectrum area containing DNA-RNA (1000-1490 cm^{-1}) between the generations. Possible related risk factors include dietary changes (alcohol, red meat, and vegetables) and metabolic syndrome (dyslipidemia, diabetes, hypertension, and low HDL levels). Thus, the study could list which lifestyle changes may have an impact on prostate cancer prevention. Spectroscopy, through the detection of changes at the molecular level, allows differentiation of different tissues over the years. In this way, it is also possible to use samples from tissue banks to evaluate changes in the epigenetic profile. In the present study, formalin tissues were used for spectroscopic analysis and no other method was applied in the discriminant analysis.

Tumors of the adrenal gland were analyzed by FTIR in tissues submitted to surgical resection. The method used applied microspectroscopy coupled to a spectroscope for differentiation between adenoma, hyperplasia, and pheochromocytomas. The authors found significant differences in the region between 2997 cm^{-1} and 2800 cm^{-1}, which correspond to lipids, and between 1770 cm^{-1} and 1485 cm^{-1}, assigned to amide bands and nucleic acids. Lipid levels were higher in adenoma and hyperplasia than in pheochromocytomas, which are richer in protein. The structural protein intensity ratios α and β (1654 cm^{-1} and 1631 cm^{-1}) were slightly increased in adenomas compared with those in pheochromocytoma and hyperplasia. The next step was to evaluate which structure presented the most significant difference. Multiple discriminant analysis using the Wilks λ partial F-tests was applied, noting that the lipid:protein ratio was the most significant parameter in the general discrimination of the lesions. No discriminant analysis was performed in the present study. This test may be applied in future studies, analyzing multiple types of thyroid lesions and which ratio of the spectrum region may have greater influence on its differentiation[20].

Conclusion

FTIR spectroscopy is able to differentiate pathologically altered thyroid tissues from the thyroid gland compared with findings in healthy thyroid tissues. We conclude that, in patients with benign nodular disease of the thyroid gland, it is possible to differentiate goiter with statistical significance, as well as malignant nodules from healthy tissue through FTIR spectroscopy.

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